The hallmarks of cancer
A long non-coding RNA point of view

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With the advent of next generation sequencing methods and progress in transcriptome analysis, it became obvious that the human genome contains much more than just protein-coding genes. In fact, up to 70% of our genome is transcribed into RNA that does not serve as templates for proteins. In this review, we focus on the emerging roles of these long non-coding RNAs (lncRNAs) in the field of tumor biology. Long ncRNAs were found to be deregulated in several human cancers and show tissue-specific expression. Functional studies revealed a broad spectrum of mechanisms applied by lncRNAs such as HOTAIR, MALAT1, ANRIL or lincRNA-p21 to fulfill their functions. Here, we link the cellular processes influenced by long ncRNAs to the hallmarks of cancer and therefore provide an ncRNA point-of-view on tumor biology. This should stimulate new research directions and therapeutic options considering long ncRNAs as novel prognostic markers and therapeutic targets.

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Introduction

In 2001, the human genome sequencing consortium released its final draft of the human genome.[1] Based on this work, the complete human transcriptome could be identified and characterized in recent years mainly due to the development of two new techniques: deep sequencing and DNA tiling arrays. These methods revolutionized our view of genome organization and content as they revealed an unexpected finding: a much larger part of the human genome is pervasively transcribed into RNA than previously assumed. It is estimated that up to 70% of the genome is transcribed but only up to 2% of the human genome serve as blueprints for proteins.[2-6] RNA molecules that lack protein-coding potential are collectively referred to as non-coding RNAs (ncRNAs) and well-known ncRNAs are classical "housekeeping" RNAs, such as tRNAs (tRNAs), rRNAs (rRNAs), small nuclear RNAs (snRNAs) and small nucleolar RNAs (snoRNAs), which are constitutively expressed and play critical roles in protein biosynthesis.

Long ncRNAs: What, Where and Why?

According to their size, ncRNAs are subdivided into two groups: small ncRNAs (< 200 nt) and long ncRNAs. In recent years, small ncRNAs like microRNAs (miRNAs), small interfering RNAs (siRNAs) or PIWI-interacting RNAs (piRNAs) received most attention and especially miRNAs were shown to play many important roles in cancer.[7-9] However, it has become increasingly clear that mammalian genomes encode also numerous long ncRNAs, defined as endogenous cellular RNAs of more than 200 nucleotides in length that lack an open reading frame of significant length (less than 100 amino acids).[10-13] Therefore, long ncRNAs (lncRNAs) constitute a very heterogeneous group of RNA molecules that allows them to cover a broad spectrum of molecular and cellular functions by implementing different modes of action.

Originally, lncRNAs were discovered via large-scale sequencing of full-length cDNA libraries in the mouse.[14] Other names such as large RNA, macroRNA and long intergenic ncRNA (lincRNA) are also used to refer to these. LncRNAs often overlap with or are interspersed between coding and non-coding transcripts.[4,15,16] From a genetic point of view long, ncRNAs fall into one or more of five broad categories: (1) sense or (2) antisense, when overlapping one or more exons of another transcript on the same or opposite strand, respectively; (3) bidirectional, when the expression of it and a neighboring coding transcript on the opposite strand is initiated in close genomic proximity, (4) intronic, when derived from an intron of a second transcript; or (5) intergenic, when it lies as an independent unit within the genomic interval between two genes.[12]

Several studies were conducted to identify lncRNAs in the human genome.[17-24] A recent study identified 5,446 lncRNA genes in the human genome and combined them with long ncRNAs from four published sources to derive 6,736 long ncRNA genes.[25] This study also investigated the protein-coding capacity of known genes overlapping with lncRNAs and revealed that 62% of known genes with "hypothetical protein" names lacked protein-coding capacity and this might even further enlarge the catalog of human lncRNAs.

For the vast majority of these recently discovered lncRNAs, the cellular function needs to be elucidated. For each individual molecule, it needs to be established whether it executes important functions or whether it just represents "transcriptional noise" or background transcription. Very convincing arguments that support the idea of functional relevance are conservation and regulation. In fact, some lncRNAs show clear evolutionary conservation or strict regulation, implying that they are of functional importance.[26-29] In addition, some transcripts are derived from ultra-conserved genomic regions (UCR) and these T-UCRs can be altered in human cancer.[30,31]

Also, long ncRNAs are often expressed in a disease-, tissue- or developmental stage-specific manner making these molecules attractive therapeutic targets and pointing toward specific functions for lncRNAs in development and diseases.[32-34] Nevertheless, our knowledge of how lncRNAs can act in the cell and which roles they might play in diseases, e.g., cancer is still very limited. However, recently it became obvious that lncRNAs play an important role in regulating gene expression at various levels, including chromatin modification, transcription and post-transcriptional processing.[16,35] For example, the lncRNAs Xist (X inactive-specific transcript) or HOTAIR (HOX Antisense Intergenic RNA) interact with chromatin remodeling complexes to induce heterochromatin formation in specific genomic loci leading to reduced target gene expression.[23,36-38] Long ncRNAs can also function by regulating transcription through a variety of mechanisms that include interaction with RNA binding proteins, acting as a co-activator of transcription factors, or repressing a major promoter of their target gene.[39-41] In addition to chromatin modification and transcriptional regulation, long ncRNAs can modulate gene expression at the post-transcriptional level or splicing level.[42-44] In **Figure 1** we provide an overview about lncRNA functions. However, this is only a snapshot of our current knowledge and more functional insights might be obtained in the future. Consequently, if we want to fully understand the complex mechanisms underlying malignant processes, e.g., carcinogenesis, metastasis and drug resistance, we need to consider this family of regulatory transcripts that add a new layer of complexity to biology.

Hallmarks of Cancer: The Basics

Cancer is one of the leading causes of death worldwide and accounted for 7.6 million (13% of all deaths) in 2008.[45] In the US, for example, lifetime probability of developing cancer is ~44% for men or ~38% for women, respectively.[46] Determining the causes of cancer is a complex issue, but well-known risk factors are alcohol and tobacco abuse, infections, radiation, obesity and a lack of physical activity.

Although "cancer" comprises a heterogeneous group of diseases, one characteristic and unifying feature is the creation of abnormal cells that grow beyond their natural boundaries. In 2000, Hanahan and Weinberg proposed six hallmarks of cancer that all together form the fundamental principle of this malignant transformation.[47] Since tumor formation is a multistep process, normal cells evolve progressively to the neoplastic stage and along their way they acquire particular capacities that enable them to become tumorigenic. These basic hallmark capabilities, distinct and supplementary, are: (1) sustaining proliferative signaling; (2) evading growth suppressors; (3) enabling replicative immortality; (4) activating invasion and metastasis; (5) inducing angiogenesis and (6) resisting cell death. Over the last decade, remarkable progress was made in the field of cancer research which led to a better understanding of these hallmark capabilities, but also led to modifications and, ultimately, expansions of the original concept.[48]

In this review, we would like to further expand our thinking of the causes and consequences of cancer by introducing long ncRNAs into cancer biology. Thus, we will first summarize the conceptual basis of each hallmark and, second, highlight latest findings that connect lncRNAs with these fundamental capacities. Moreover, we will point out new areas of cancer research by including lncRNAs into basic scientific questions. Finally, we
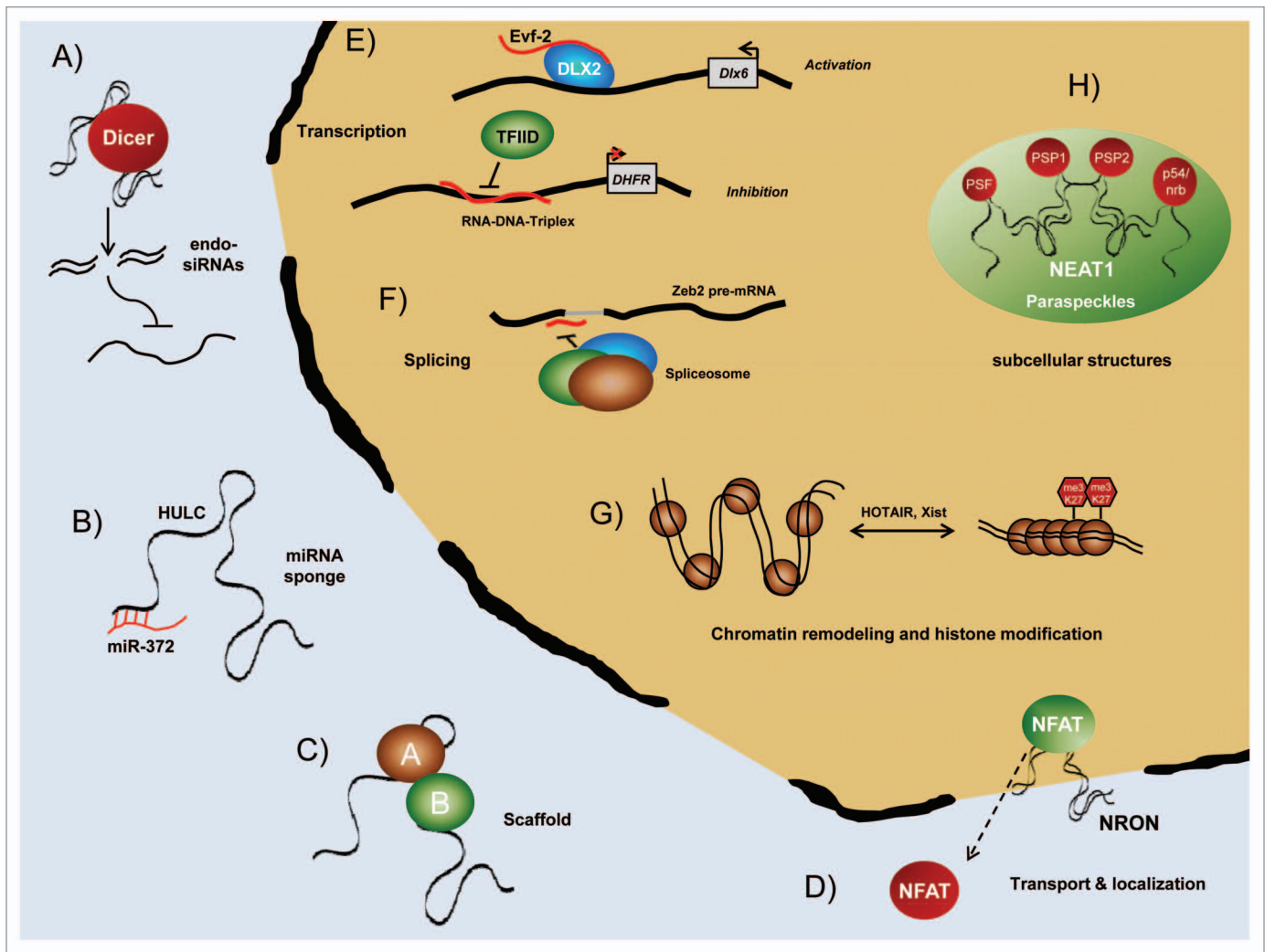
Figure 1. Cellular functions of long ncRNAs. LncRNAs can act in diverse ways in the cell. In general, they can regulate gene expression, influence protein localization (D) and are important for the formation of cellular substructures or protein complexes, where they fulfill scaffolding functions (C; H).[199] Regulating gene expression is one of the best studied functions of lncRNA and multiple mechanisms are applied by lncRNAs. (A) LncRNAs could be processed into small, single- or double-stranded RNAs that could act as endo-siRNAs targeting other RNAs, which subsequently leads to target degradation. (B) LncRNAs can act as "miRNA sponge" and sequester miRNAs to inactivate these small regulatory RNAs. This influences the expression of miRNA target genes.[200] (D) The interaction of lncRNAs with proteins can modulate protein activity and localization. For example, the lncRNA NRON (non-coding repressor of NFAT) binds to the cellular transcription factor NFAT (nuclear factor of activated T cells). This regulates nuclear-cytoplasmic trafficking of NFAT and finally leading to an repression of NFAT target gene expression.[201] (E) Furthermore, lncRNA regulate gene transcription via recruiting transcription factors to their target gene promoters, therefore activating gene expression.[39] However, they can also block binding of general transcription factors, potentially via formation of RNA-DNA-Triplexes.[40] (F) LncRNAs contribute to transcriptome complexity, as they can regulate alternative splicing of pre-mRNAs.[42] (G) The balance between transcriptional active euchromatin and silent heterochromatin is controlled by lncRNAs. They can interact with chromatin remodeling complexes and induce local or global changes in chromatin packaging.[23,202]

will introduce new therapeutic concepts that focus on lncRNAs as treatment targets.

Hallmarks of Cancer: Adding Long ncRNAs

Sustaining proliferative signaling. One of the most prominent characteristics of a cancer cell is its ability to proliferate constantly and in the absence of external stimuli. Normal cells carefully manage the production of growth promoting or inhibiting factors to ensure a tight control of cell number, tissue architecture and function. In contrast, tumor cells show deregulated signaling cascades that enable them to be more or less independent of proliferation signals, which results in unlimited growth. To achieve this independency, tumor cells acquired the capability to sustain proliferative signaling in multiple ways: (1) They produce their own growth factors and the corresponding receptor molecules themselves resulting in autocrine stimulation. (2) They might also produce paracrine signals to stimulate normal, tumor-associated cells (tumor stroma) which in turn produce various growth factors to support the cancer cells. (3) Moreover, growth factor receptor levels could be elevated or the receptor signaling cascade could be altered, making cancer cells hyperresponsive. (4) Last

but not least, cancer cells could become completely independent from exogenous growth factors, because of constitutive activation of downstream signaling pathways or the disruption of negative-feedback mechanisms.

With this in mind, we can now ask the question: Which roles do long ncRNAs play here? A first answer to this arises from hormone signaling, especially sex steroid hormone signaling. Estrogen, progesterone and androgen are well-known hormones targeting female mammary glands, ovary and uterus or the male testis and prostate gland. They exert their functions in these tissues through the specific interaction with their intracellular receptors, the estrogen receptor (ER), the progesterone receptor (PR) and the androgen receptor (AR). This regulates target gene expression, as these receptors function as transcription factors and the abnormal expression or function of these receptors has been implicated in tumors of reproductive organs in both, males and females.[49] As for many other transcription factors, the activity of these receptors is influenced by additional factors, so called coactivators or corepressors. While coactivators enhance the transcriptional activity, corepressors block their activity resulting in a complex interplay underlying coordinated gene expression. Changes in the expression level or pattern of these coactivators and corepressors can affect transcriptional activity of the steroid hormones and subsequently cause disorders of their target tissues.[50-52]

A unique coactivator for the steroid receptors PR, ER, GR (glucocorticoid receptor) and AR is the steroid receptor RNA activator (SRA). SRA acts as an ncRNA.[53] It is part of an RNA-protein complex containing also SRC-1 and it fulfills its trans-activation function through the AF-1 domain of the nuclear receptors. SRA expression can be detected in normal and malignant human mammary tissues. Interestingly, elevated levels of SRA are found in breast tumors and the increased SRA levels might contribute to the altered ER/PR action that occurs during breast tumorigenesis.[54] However, recent progress in this field has revealed a more complex situation: the *SRA1* gene might not only act as an ncRNA but also produces a protein that acts as a coactivator or corepressor, as well.[55,56] Alternative splicing balances the ratio of non-coding and coding transcripts derived from the *SRA1* gene.[57] This balance of transcripts not only characterizes specific tumor phenotypes but might also be involved in breast tumorigenesis and tumor progression by regulating the expression of specific genes.[58] This duality of RNA transcripts and the concept of coding and non-coding functions add another level of complexity and should be considered to gain deeper insights into complex regulatory circuits. In line with this concept, a recent report presented a novel, coding-independent function for the p53 mRNA.[59] Usually, the E3 ubiquitin ligase Mdm2 is a negative regulator of p53 protein expression. However, Mdm2 bound to p53 mRNA shows a different activity: it promotes p53 expression following genotoxic stress. This is achieved because the p53 mRNA binding to Mdm2 controls Mdm2 SUMOylation and nuclear trafficking and the accumulation of Mdm2 in nucleoli. This plays an important role in p53's capacity to respond to DNA damage.[60,61] These two examples emphasize the importance of being open-minded and reflect

RNA as a multi-functional molecule and not only an intermediate for protein synthesis.

In addition to SRA, there are several other long ncRNAs recently discovered which have a role in cell proliferation. In an exemplary study, Prensner et al. applied RNA-Seq technology and identified 121 differentially expressed long ncRNAs in prostate cancer whose expression patterns distinguished benign, localized and metastatic prostate cancer.[22] Moreover, they characterized one long ncRNA, PCAT-1 (prostate cancer associated transcript 1), in more detail. PCAT-1 was highly upregulated in a subset of metastatic and high-grade localized prostate cancers. To further explore the functional role of this novel ncRNA, over-expression and knockdown experiments were performed, which resulted in a modest increase in cell proliferation in case of stable overexpression and consistently a reduced proliferation rate (25–50%) after siRNA-mediated depletion. Gene expression profiling after knockdown of PCAT-1 in LNCaP cells identified 255 genes upregulated and 115 genes downregulated by the loss of PCAT-1. Gene ontology analysis of the upregulated genes showed enrichment for gene sets associated with mitosis and cell cycle, whereas the downregulated genes had no significant associations. Taken together, these results suggest that PCAT-1 functions as transcriptional repressor for a subset of genes and thereby might contribute to prostate cancer progression.

A further example of an lncRNA impacting cell proliferation is introduced by a novel role for the well-known small nuclear RNA 7SK, also known as RN7SK. A key function of this ncRNAs is the regulation of transcription elongation via binding to the positive transcription elongation factor b (P-TEFb) which abolishes its positive effect on RNA Polymerase II transcription elongation.[62,63] Now, HMGA1, a transcription factor and chromatin regulator, was identified as a novel 7SK interaction partner.[64] HMGA1 (high mobility group AT-hook 1) itself shows high expression levels in both, embryonic and transformed neoplastic cells.[65,66] In this recent study, 7SK RNA was shown to interact with HMGA1 and compete with its binding to DNA. This, in turn, has an impact on HMGA1 target gene expression affecting also growth-related genes. This again shows the diverse mechanistic functions of lncRNAs and underlines the need to develop new methods to identify and analyze these transcripts in more detail.

Finally, a recent study identified 216 putative long ncRNAs derived from promoter regions of cell cycle genes.[67] Many of these transcripts showed periodic expression during the cell cycle and an altered expression in human cancers. Their expression is regulated by specific oncogenic stimuli, stem cell differentiation or DNA damage and future work will elucidate their molecular functions and their role in cancer cell proliferation.

Taken together, the newly discovered long ncRNAs are more and more recognized as active molecules instead of "transcriptional noise" and evidence is accumulating that some of them have critical roles in carcinogenesis by influencing tumor cell proliferation.

Evading growth suppressors. A highly complementary hallmark capability for sustaining proliferative signaling in cancer cells is the ability to evade growth suppression. Several tumor

suppressive protein-coding genes that operate in diverse ways to inhibit cellular growth and proliferation had been discovered, e.g., *Tp53*, *PTEN* or *RB*. Activation of these tumor suppressor genes depends on external or internal stimuli and can either lead to cell cycle arrest or might induce senescence and even apoptosis in cells. Therefore, tumor cells must find a way to prevent their activation or expression. One mechanism how cancer cells deal with this is the complete loss of the tumor suppressor gene or the accumulation of mutations that render this gene inactive. In fact, more than 50% of human tumors contain a mutation or deletion of the *Tp53* gene and people who inherited only one functional copy of the *Tp53* gene will most likely develop tumors in early adulthood, a disease known as Li-Fraumeni syndrome.[68] Alternatively, tumor suppressor genes could also bind to other proteins expressed in tumor cells and therefore become inactivated or rapidly degraded. One such example is given by the human papilloma virus (HPV) oncogenes E6 and E7, which are preferentially expressed in human cervical cancers. They interact with Tp53 and RB, which leads to their inactivation.[69] Also, Mdm2 as mentioned earlier is the predominant E3 ubiquitin ligase of *Tp53* and therefore induces its degradation via the proteasome.[70]

In addition to these mechanisms, cancer cells have developed alternative ways to inhibit tumor suppressor functions with the help of long ncRNAs. For instance, five human ncRNA fragments interact with the tumor suppressor PSF.[71] The PSF protein represses transcription of proto-oncogenes via binding to their regulatory regions. It contains a DNA-binding domain and two RNA-binding domains that bind VL30-1 RNA in mice, which leads to the release of PSF from a repressed proto-oncogene and activates transcription of the latter ones.[72-74] However, VL30-1 RNA is not encoded in the human genome and Li et al. aimed at identifying human RNA counterparts that interact with PSF protein.[71] In their screen, they found five RNA fragments associated with PSF and releasing it from the human proto-oncogene GAGE6 regulatory region resulting in an activation of GAGE6 expression. Overexpression of these RNA fragments in a human melanoma cell line led to an enhanced tumorigenic phenotype. In an additional experiment, the relative amounts of the PSF-binding RNAs were determined in two human fibroblast cell lines and nine human tumor cells. Each tumor cell line expressed higher levels of PSF-binding RNAs compared with normal human fibroblasts. The pattern of expression differed among the tumor lines, suggesting that different groups of PSF-binding RNAs contribute to the tumorigenicity of each tumor cell line. Interestingly, the amount of PSF mRNA was not changed in eight out of nine tumor cell lines compared with fibroblasts, suggesting that not a decrease of PSF protein contributes to the tumorigenicity of human tumor cells but rather the increased expression of its interacting RNAs. Future work should therefore focus on protein-RNA interactions and reveal their molecular consequences.

A completely different mode of action is executed by the long ncRNA ANRIL (antisense non-coding RNA in the INK4 locus) to block the activity of tumor suppressor genes.[75] Instead of competing with DNA for binding to the repressor protein,

ANRIL interacts with SUZ12 (suppressor of zeste 12 homolog), a subunit of the polycomb repression complex 2 (PRC2) and recruits the complex to repress the expression of p15 (INK4B), a well-known tumor suppressor gene. Moreover, the depletion of ANRIL increases the expression of p15(INK4B) and inhibits cellular proliferation. A similar study identified CBX7 (chromobox homolog 7), a subunit of the polycomb repressive complex 1 (PRC1) as molecular interaction partner of ANRIL. This results in the recruitment of PRC1 to the p16(INK4A)/p14(ARF) locus and subsequent silencing of this gene locus by H3K27-trimethylation.[76] Both, CBX7 and ANRIL show elevated levels in human prostate cancer corroborating the importance of this interaction for tumor development. In line with these findings, a recent study from Jeannie T. Lee's lab identified > 9,000 PRC2-interacting RNAs via RIP-Seq (RNA immunoprecipitation and sequencing) technology in embryonic stem cells making it very likely that more and more genes will be identified that are regulated by the ncRNA-mediated recruitment of PRC2 and it will be interesting to see how this relates to carcinogenesis.[38] However, an unanswered question so far is how ncRNAs can specifically recruit these silencing machineries to only a subset or even only one specific gene.

In addition to these active oncogenic functions of long ncRNAs there are also tumor suppressor functions assigned to them. One very famous example is the ncRNA GAS5 (Growth Arrest-Specific 5). It was originally identified based on its increased levels in growth-arrested mouse NIH3T3 fibroblasts.[77] Conversely, GAS5 expression is strongly reduced in actively growing leukemia cells or NIH3T3 cells and in turn increases after density-induced cell cycle arrest.[78] The human GAS5 gene is transcribed from chromosome 1q25.1 and is alternatively spliced. Its exons contain a small and poorly conserved open reading frame that does not encode a functional protein.[79,80] GAS5 is a host gene for multiple snoRNAs, which are located in the introns and may mediate important biological activities.[81] In contrast to SRA, GAS5 functions as a "riborepressor": the ncRNA interacts with the DNA binding domain of the glucocorticoid receptors, thus competing with the glucocorticoid response elements in the genome for binding to these receptors. This suppresses the induction of several responsive genes including cellular inhibitor of apoptosis 2 (cIAP2) and ultimately sensitizes cells to apoptosis.[82] Moreover, GAS5 expression induces growth arrest and apoptosis independently of other stimuli in some prostate and breast cancer cell lines.[83] Interestingly, the inhibition of mammalian Target Of Rapamycin (mTOR) pathway via Rapamycin depends on GAS5. The mTOR pathway plays a critical role in control of cell growth and regulates cellular protein synthesis and proliferation.[84,85] Downregulation of GAS5 by RNA interference protects both leukemic and primary human T cells from the anti-proliferative effect of Rapamycin, suggesting that GAS5 might—directly or indirectly—be required.[86] How do cancer cells cope with this tumor-suppressive ncRNA? Breast cancers show a significantly lower GAS5 expression compared with normal breast epithelial tissues.[83] In addition, genetic aberrations of the GAS5 locus have been found in many types of tumors including melanoma, breast and prostate cancers but their functional significance still needs to be established.[87-89]

However, GAS5 is not the only ncRNA with growth-suppressive functions. John Rinn and colleagues identified several novel ncRNAs by asking how the transcription factor Tp53 can do both, activate and repress gene expression.[28] One answer to this question was given by the discovery of lincRNA-p21. This ncRNA is a direct p53 target gene residing next to the *p21* (Cdkn1a) gene on mouse chromosome 17. Its expression is activated upon DNA damage in different tumor models. LincRNA-p21 associates with hnRNP K, a well-known RNA binding protein and hnRNP K acts as a transcriptional repressor. LincRNA-p21 mediates the binding of hnRNP K to its target genes, which finally leads to gene silencing and the induction of apoptosis. However, this study was conducted in mice and although lincRNA-p21 seems to be conserved and is also induced in human fibroblasts after DNA damage induction, it needs to be determined, whether a similar mechanism can be found in normal human cells. Moreover, it will be interesting to see how human cancers regulate this tumor suppressive ncRNA, especially in Tp53-positive cancers that show an intact Tp53 response.

Interestingly, the expression of the tumor suppressor p21 is regulated by at least one other non-coding RNA transcript. Kevin Morris and coworkers found that transcriptional activation of *p21* gene depends on the post-transcriptional silencing of a p21-specific antisense transcript, which functions in Argonaute 1-mediated transcriptional control of *p21* mRNA expression.[90] In human cells, this bidirectional transcription could be an endogenous gene regulatory mechanism whereby an antisense RNA directs epigenetic regulatory complexes to a sense promoter, resulting in RNA-directed epigenetic gene regulation. The epigenetic silencing of tumor suppressor genes, such as p21, may be the result of an imbalance in bidirectional transcription levels and this imbalance allows the antisense RNA to direct silent state epigenetic marks to the sense promoter, resulting in stable transcriptional gene silencing.

In summary, these examples clearly demonstrate a role for long ncRNAs in evading growth suppressors and tumor cells must find ways to circumvent both, the activation of protein and non-protein tumor suppressor genes.

Enabling replicative immortality. In line with the first two hallmarks of cancer and closely related to proliferation is the third trait of cancer: unlimited replicative potential. In contrast to normal cells that are able to pass through only a limited number of cell division cycles, tumor cells show nearly unlimited replication. In normal cells, replication potential is limited due to the appearance of either senescence or crisis with the latter one finally ending in cell death. The chromosome ends, the telomeres, are crucial for this replication limit.[91,92] In vertebrates, these sequences are composed of multiple repeats of the hexanucleotide "TTA GGG" and protect the ends of chromosomes from end-to-end fusions.[93] However, in normal cells, these telomeric repeats shorten after each cell division and therefore the length of telomeric DNA dictates the number of cell division cycles.[94] The loss of these protective ends finally leads to crisis. Tumor cells have found two ways to circumvent the loss of telomeres: (I) About 90% of all human cancers express a specialized enzyme, called telomerase, which is able to add telomeric repeats to the end of the chromosomes. (II) The remaining 10% of all tumor cells employs alternative lengthening of telomeres (ALT), a nonconservative telomere lengthening pathway involving the transfer of telomere tandem repeats between sister chromatids.[95-98] The exact mechanism of the ALT pathway still needs to be uncovered. However, it could be shown that ALT cells produce abundant t-circles, possible products of intratelomeric recombination and t-loop resolution providing mechanistic insights.[99,100]

Interestingly, the major pathway involving telomerase critically depends on an ncRNA. The telomerase holoenzyme consists of a protein component, a reverse transcriptase named TERT (Telomerase Reverse Transcriptase) and an RNA primer, also known as TERC (Telomerase RNA Component) or TR (Telomerase RNA).[101] The telomeric RNAs are highly divergent between different species, varying in both size and sequence composition, from: 150 nt in ciliates and: 450 nt in vertebrates up to: 930–1,300 nt in the budding yeast. Telomere synthesis involves TERT-catalyzed reverse transcription of a small template region within TERC making it essential for immortalization. Not astonishing, the *TERC* gene is amplified in several human cancers, thus making it a potential target for therapeutic inhibitors of telomerase activity in cancer cells.[102-104] For a more detailed discussion on TERC structure and function please see refer to Theimer and Feigon.[105] For a more comprehensive summary on telomerase structure and function the interested reader is referred to Wyatt, West and Beattie.[106]

TERC is not the only RNA associated with telomeres. Recently, a group of long ncRNAs was discovered and named TERRA (telomeric repeat-containing RNA).[107-109] TERRA transcripts are derived from several subtelomeric loci. Telomere transcription is an evolutionarily conserved phenomenon in eukaryotic cells suggesting functional importance.[110] TERRA localizes to telomeres and is involved in telomeric heterochromatin formation.[111] TERRA is thought to be a negative regulator of telomerase, which may act globally or at individual telomeres as a direct inhibitor of the telomerase enzyme.[112] HnRNP A1 is a protein interaction partner of TERRA and together with POT1 (protection of telomeres 1), they act in concert to displace RPA (replication protein A) from telomeric ssDNA after DNA replication to promote telomere capping and preserve genomic integrity.[113] Reduction of TERRA transcription is necessary for telomerase-mediated telomere lengthening which may link TERRA to cancer. Telomerase-positive cancer cells with high levels of subtelomeric methylation display low levels of TERRA compared with matched ALT-positive cancer cells or normal cells.[114] Moreover, when cell extracts are incubated with an excess of synthetic RNA oligonucleotides mimicking TERRA, telomerase activity is inhibited.[109] However, a deregulation, silencing or mutation of TERRA in human cancer remains to be discovered. For a more detailed overview about TERRA the interested reader is referred to Caslini.[110]

Taken together, both introduced ncRNAs, TERC together with TERT forming a functional ribozyme and TERRA, which comprises a group of ncRNA transcripts, demonstrate once more the broad biological importance of long ncRNAs—here as regulators of genome stability and replication.

Activating invasion and metastasis. The fourth hallmark capability, the ability to invade and form distant metastases, is a highly challenging one and underlies a plethora of complex interactions and regulatory mechanisms. It was noted decades ago that epithelial carcinomas of higher grade show a more invasive phenotype and distant metastases and most patients die from these metastases and not from the primary tumor. Often, cancer cells undergo morphological alterations and change their cell-cell or cell-matrix interactions. All this enables them to successfully pass through the first steps of the multistep process of invasion and metastasis. This invasion-metastasis cascade comprises multiple biological changes that allow cancer cells to invade into healthy tissues, followed by intravasation into blood and lymphatic vessels.[115,116] During their transit through the lymphatic system and blood circulation, cancer cells must escape immune surveillance and show anchorage-independent growth and survival. Next, cancer cells must extravasate from the vessels into their target tissues to form micrometastases and eventually later on a secondary tumor. Much progress was made over the last years and interesting concepts, e.g., the regulation of invasion by the developmental regulatory program known as "epithelial-mesenchymal transition" (EMT) were put forward.[117-119] Also, several important factors of this cascade had been identified. For example, E-cadherin (CDH1) is a key cell-to-cell adhesion molecule and helps to assemble epithelial cell layers. An increased E-cadherin expression therefore inhibits invasion and metastasis formation. E-cadherin expression itself is regulated in multiple ways and involves also a natural antisense transcript (NAT) that regulates expression of Zeb2, a transcriptional repressor of E-cadherin.[42] Not surprisingly, E-cadherin is frequently downregulated or inactivated in human carcinomas.[120,121] In addition to E-cadherin, several other adhesion molecules that either mediate cell-to-cell or cell-to-matrix interactions are altered in some aggressive carcinomas. On the other hand, N-Cadherin (CDH2), which is found in migrating neurons and mesenchymal cells, is often upregulated in many invasive tumors. Besides these alterations within cancer cells, it becomes more and more obvious that also crosstalk between cancer cells and tumor stroma cells is required for this hallmark capability.[122-125] Also, cells of the immune system contribute to this capability by supplying, e.g., matrix-degrading enzymes and in that way support invasive tumor cell behavior.[123,126,127] One of the major tasks for the future is to identify a "metastatic signature" of genes that support the invasion of tumor cells and facilitate the formation of distant metastases in specific tissues.

As depicted earlier, some protein factors with important functions could already be identified. In addition, early work by Ji and Diederichs et al. established a role for long ncRNAs in metastasis formation. They identified MALAT1 (Metastasis-Associated Lung Adenocarcinoma Transcript 1, MALAT-1), also later referred to as NEAT2 (Nuclear-Enriched Abundant Transcript 2) as a prognostic marker for metastasis and patient survival in non-small cell lung cancer (NSCLC).[128] This ncRNA is extremely abundant in many human cell types and is highly conserved across several species underscoring its functional importance.[44,129] Moreover, the 8 kb long MALAT1 transcript can be processed into a highly conserved tRNA-like small cytoplasmic RNA of 61 nucleotides that is broadly expressed in human tissues.[130] However, the function of this so-called mascRNA is so far unknown.

MALAT1 is retained in the nucleus and specifically localizes to nuclear speckles.[29] These structures play a role in pre-mRNA processing and recently MALAT1 has been shown to regulate alternative splicing of pre-mRNAs by modulating the levels of active serine/arginine splicing factors.[44] These factors regulate tissue—or cell-type specific alternative splicing in a concentration—and phosphorylation-dependent manner. Depletion of MALAT1 alters the processing of a subset of pre-mRNAs, which play important roles in cancer biology, e.g., Tissue Factor or Endoglin.[131] This supports the hypothesis that MALAT1 could be a regulator of post-transcriptional RNA processing or modification. However, a recent study from the Rosenfeld lab indicates additional functions for MALAT1 in the nucleus.[132] Here, MALAT1 was shown to interact with the unmethylated form of CBX4 and this controls relocation of growth-control genes between polycomb bodies and interchromatin granules, places of silent or active gene expression respectively. Therefore, the exact mechanism of MALAT1 function is still a mystery and it seems not unlikely that this lncRNA might fulfill cell type- or tissue-specific functions.

MALAT1 expression can be found in many healthy organs with the highest levels of expression in pancreas and lung.[128] In several human cancers including lung cancer, uterine endometrial stromal sarcoma, cervical cancer and hepatocellular carcinoma (HCC), MALAT1 is upregulated.[128,133-135] In addition, it is significantly associated with metastasis in NSCLC patients. This association with metastasis is stage- and histology-specific. Therefore, MALAT1 can serve as an independent prognostic parameter for patient survival in early stage lung adenocarcinoma.[128] A recent study shed light onto the role of the metastasis marker MALAT1 as a potentially active player in the metastatic process. MALAT1 promotes cell motility of lung cancer cells through transcriptional or post-transcriptional regulation of motility-related genes.[136] Additionally, MALAT1 supports proliferation and invasion of cervical cancer cells and knockdown of MALAT1 in CaSki cells led to an upregulation of caspase-8 and -3 and Bax and the downregulation of Bcl-2 and Bcl-x_L.[133] Thus, both studies find a plethora of potential MALAT1 functions linked to proliferation, apoptosis, migration or gene regulation and future studies will have to unravel the specificity of these effects. Since both studies were performed with individual siRNAs only and lack rescue experiments, further investigations are necessary to ensure the specificity of the observed effects and to corroborate the functional importance of MALAT1 in carcinogenesis or metastasis. For this reason, we have recently developed a novel gene knockout strategy based on the stable bi-allelic integration of RNA destabilizing elements into the human genome with the help of so called Zinc Finger Nucleases.[129] This method yielded a highly specific and more than 1,000-fold reduction of MALAT1 expression in human A549 lung cancer cells and will allow deeper analysis of MALAT1 function in lung cancer and its role in migration and metastasis in a clean loss-of-function model.

A second long ncRNA involved in cancer metastasis is known as HOTAIR (HOX Antisense Intergenic RNA). It was discovered by the lab of Howard Chang as a 2.2 kb long ncRNA transcribed in antisense direction from the *HOXC* gene cluster.[23] The same study also revealed that HOTAIR functions in trans by interacting and recruiting the polycomb repressive complex 2 (PRC2) to the *HOXD* locus which leads to transcriptional silencing across 40 kb. Later, HOTAIR was found to interact with a second histone modification complex, the LSD1/CoREST/REST complex, which coordinates targeting of PRC2 and LSD1 to chromatin for coupled histone H3K27 methylation and K4 demethylation.[37] Given its important role in the epigenetic regulation of gene expression, it is not surprising that HOTAIR is deregulated in different types of cancer.[36,137-139] In human breast cancer, HOTAIR expression is increased in primary tumors and metastases and its expression level in primary tumors positively correlates with metastasis and poor outcome. Overexpression of HOTAIR in epithelial cancer cells alters H3K27 methylation via PRC2 and therefore alters target gene expression. This leads to increased cancer invasiveness and metastases. On the other hand, HOTAIR depletion inhibits cancer invasiveness.[36] In HCC, HOTAIR levels are increased compared with non-cancerous tissues and for those HCC patients, who received a liver transplantation, high HOTAIR expression levels are an independent prognostic marker for HCC recurrence and shorter survival.[137] Similar to breast cancer, HOTAIR depletion in liver cancer cells reduces cell invasion and cell viability. Moreover, HOTAIR might be a potential biomarker for the existence of lymph node metastasis in HCC.[140] In addition, HOTAIR suppression sensitizes cancer cells to tumor necrosis factorα induced apoptosis and renders them more sensitive to the chemotherapeutic agents cisplatin and doxorubicin.[137]

These two examples indicate already the functional importance of long ncRNAs for the activation of invasion and metastasis formation. Hence, future studies should foster the identification of such transcripts, e.g., in the context of epithelial-to-mesenchymal transition. LncRNAs could be important regulated genes during EMT or act as possible regulators for other EMT-relevant genes. In addition, ncRNAs could be involved in the complex interplay of tumor cells with the tumor stroma or might also influence inflammatory cell behavior. HOTAIR and MALAT1 could regulate a broad subset of genes involved in cancer cell invasion and metastasis. So, these lncRNAs are on the one hand part of the "metastatic signature" of gene expression and on the other hand they contribute to shaping it.

Inducing angiogenesis. When cancer cells grow and proliferate, tumor mass and size increases. This process would be limited by the natural diffusion limit of oxygen and nutrients, if tumor cells would not acquire the fifth trait: the ability to induce angiogenesis. The formation of new blood vessels, induced by tumor cells, secures not only a supply with nutrients and oxygen, but allows tumors to dispose their metabolic (toxic) wastes and enter the hematogenous metastatic process, as well. The processes of vasculogenesis and angiogenesis are usually restricted to embryonic development, but can become re-activated under specific conditions in adults. For example, angiogenesis is an important process in wound healing and female reproductive cycling. An "angiogenic switch" is also turned on during tumor progression, which leads to continuous sprouting of new vessels that help to sustain tumor growth.[141] To achieve this, tumor cells induce pro-angiogenic factors or block antiangiogenic signals.[142] Some of these angiogenic regulators are signaling proteins that bind to activating or inhibiting receptors present on the surface of endothelial cells.[143] One well-known activator of angiogenesis is VEGF-A (vascular endothelial growth factor-A), a secreted protein whose expression can be induced by hypoxia or oncogenic signals.[144] Its effect could be counterbalanced by Thrombospondin-1, which also binds to transmembrane receptors displayed by endothelial cells.[145] Thus, the expression of these factors needs to be tightly controlled and it is noteworthy that oncogenes like Ras and Myc contribute to proliferative signaling and angiogenesis. Therefore, it will be interesting to see, whether long ncRNAs with a role in cellular proliferation, as discussed above, will also play a role in tumor angiogenesis.

That long ncRNAs can also have functions in regulating the angiogenic process is becoming more and more evident. Nearly a decade ago, it was shown that αHIF, a natural antisense transcript (NAT) complementary to the 3' untranslated region of the hypoxia inducible factorα (HIF1α), negatively regulates the expression of HIF1α, a critical regulator of angiogenesis.[146,147] Overexpression of αHIF triggers HIF1α mRNA decay and HIF1α and αHIF constitute a negative feedback loop.[148] Interestingly, αHIF transcripts can be detected in several human tissues and cancers and αHIF is a marker for poor prognosis in breast cancer.[146,147,149]

However, αHIF is not the only antisense RNA associated with angiogenesis. Another NAT was discovered when studying the transcriptional unit of the human endothelial nitric-oxide synthase (eNOS). The transcript was termed sONE or NOS3AS and it regulates the expression of eNOS in a post-transcriptional manner under normoxia and hypoxic conditions.[150,151] In contrast to eNOS, sONE expression is detectable in a variety of cell types except endothelial cells. In tumors, protumorigenic agents, such as estrogen, induce eNOS expression and several cancer treatment methods influence eNOS expression and activity.[152] For a more detailed overview about eNOS function in cancer biology, please refer to Ying and Hofseth.[153] At the moment it is a matter of debate, whether sONE really acts as RNA, because also a protein product derived from this RNA has recently been described.[154]

Finally, a very recent study identified an NAT for tyrosine kinase containing immunoglobulin and epidermal growth factor homology domain-1 (tie-1), tie-1AS.[155] This long ncRNA is conserved in zebrafish, mouse and humans where it selectively binds to tie-1 mRNA in vivo and regulates tie-1 transcript levels, resulting in specific defects in endothelial cell contact junctions in vivo and in vitro. In addition, the ratio of tie-1 vs. tie-1AS is altered in human vascular-related disease states and it would be interesting to know if and how cancer cells can manipulate this ratio to achieve proper blood vessel formation.

The presented examples directly implicate long ncRNA-mediated post-transcriptional regulation of gene expression as a fundamental control mechanism for physiological processes, such

as vascular development and show their importance for cancer development. Future work should focus on the identification of novel long ncRNAs regulated by or being involved in the "angiogenic switch" of cancer cells. Most important questions are: (1) Can HIF1α, the major transcriptional regulator under hypoxic conditions, actively regulate long ncRNA expression? (2) Are lncRNAs involved in recruiting this transcription factor to its target genes? (3) Which signaling cascades can regulate lncRNA expression in endothelial or tumor cells, e.g., is VEGF signaling involved? (4) Can lncRNAs act as second messengers, i.e., can they function in a cytokine-like way to activate or repress endothelial cells or other cells involved in tumor angiogenesis like immune cells? (5) Are long ncRNAs involved in signal transduction pathway relevant for blood vessel formation?

Finding answers to these and similar questions will broaden our view on the complex interplay between tumor cells and supporting tumor stroma and could provide new anti-angiogenic treatment options.

Resisting cell death. The dream of immortality—for cancer cells it can come true, if they acquire the last hallmark capability: resisting cell death. Three major pathways can lead to cell death and their activation must be carefully controlled by tumor cells. The first mechanism leading to controlled cell death is apoptosis. Apoptosis can be induced by various external as well as internal stimuli and several studies have shown how highly malignant cancers can attenuate apoptosis and become therapy resistant.[156,157] Induction of DNA damage, e.g., by chemotherapeutic agents (cisplatinum, etoposide, etc.,) is one way to trigger apoptosis via the Tp53 (p53) pathway. Tp53 induces the expression of pro-apoptotic proteins Noxa and Puma (p53-upregulated modulator of apoptosis) leading to cell death. As mentioned earlier, 50% of all human cancers have either lost the *Tp53* gene or show mutations.[68] This makes them more resistant to such cellular stresses. Alternatively, tumors show an increased expression of survival factors or anti-apoptotic regulators like Bcl-2 and Bcl-x$_L$. A second mechanism leading to controlled cell death is autophagy. This mechanism usually operates at low levels in cells, but it can be activated by certain kinds of cellular stress, e.g., nutrient deficiency.[158] Autophagy can be seen as a recycling program that allows cells to break down their organelles and to use the degradation products to fuel biosynthesis pathways or use for energy production. Several regulatory components and effector proteins are known and the interested reader might refer to Levine and Kroemer and Mizushima.[158,159] Interestingly, autophagy can have both, beneficial effects for cancer cells or block carcinogenesis. For example, mice lacking the Beclin-1 gene, a critical factor for autophagy induction, show increased susceptibility to cancer. The same holds true for other components of the autophagy machinery.[158,160] On the other hand, severely stressed cells shrink via autophagy to a state of reversible dormancy and this survival response might be the reason for cancer recurrence after therapy.[160] The last mode of cell death is constituted by necrosis. Although often referred to as "uncontrolled" cell death, more and more evidence is accumulating that also necrosis is a controlled process rather than an undirected way to die.[161,162] Similar to autophagy, necrosis can do both—eliminate cancer cells or

promote their expansion. Necrotic cells can attract proinflammatory cells, which in turn can activate angiogenesis, cancer cell proliferation and invasiveness.[161,163] Thus, more studies are necessary to fully understand the double-edged nature of this process and how it can be manipulated to achieve a beneficial effect for the patient.

Maybe long ncRNAs can provide some insights here, as they were already shown to influence cell death decisions. Over a decade ago, PCGEM1 (Prostate-specific transcript 1) was identified as a prostate tissue-specific and prostate cancer-associated long ncRNA.[164] Later studies provided some insights into its function. They ruled out a role for this ncRNA in cell proliferation and colony formation and established a function for this ncRNA in apoptosis inhibition after doxorubicin treatment of prostate cancer cells.[33,165] The anti-apoptotic effect might result from a delayed induction of p53 and p21(Waf1/Cip1) after PCGEM1 overexpression in LNCaP cells. In addition, cleaved caspase 7 and cleaved PARP levels were strongly reduced after doxorubicin treatment in PCGEM1 transfected cells compared to normal cells. This PCGEM1-associated delay in apoptotic responses seems to be androgen-dependent, as androgen-independent variants of LNCaP cells did not exhibit this delay.

However, PCGEM1 is not the only long ncRNA with anti-apoptotic functions. A more global approach using differential display identified genes conveying drug resistance to cancer cells.[166] This led to the discovery of CUDR (cancer upregulated drug resistant), an ncRNA that confers resistance to doxorubicin and etoposide as well as drug-induced apoptosis in squamous carcinoma cells A431. One possible explanation for this function might be the observed downregulation of effector caspase 3 after CUDR overexpression.

A study from George Calin and Carlo Croce indicates that more long ncRNAs can be discovered that play a role in cell death control and that this might be an evolutionary conserved function.[31] Their study identified several hundred transcripts derived from ultraconserved regions (T-UCRs), which are consistently altered at the genomic level in human leukemias and carcinomas. More importantly, they examined the biologic effect of the ncRNA uc.73A(P), a significant upregulated T-UCR in colon cancer. Depletion of this ncRNA resulted in reduced cellular proliferation of COLO-320 cells and an increase in sub-G1 cells, suggesting higher apoptosis rates. Thus, uc.73A(P) could act as an oncogene by increasing the number of malignant cells as a consequence of reduced apoptosis.

Another study shows that long ncRNAs have a plethora of functions in tumorigenesis including apoptosis prevention.[167] Here, several long ncRNA, which are differentially expressed in melanoma cell lines in comparison to melanocytes and keratinocytes had been discovered. One of these long ncRNAs, SPRY4-IT1, is derived from an intron of the *SPRY4* gene. SPRY4-IT1 is predominantly localized in the cytoplasm of melanoma cells, and SPRY4-IT1 knockdown results in defects in cell growth, differentiation and higher rates of apoptosis in melanoma cell lines.

Finally, DNA damage can induce five long ncRNAs from the *p21* promoter, and one of these was named PANDA (P21 associated ncRNA DNA damage activated).[67] Further experiments

Table 1. LncRNAs and the hallmarks of cancer

Cancer Hallmark	LncRNA	Mode of action	Reference
I. sustaining proliferative signaling	SRA	Transcriptional co-activator	53, 54, 58
	PCAT-1	Regulating gene expression	22
	RN7SK	Regulating transcription	62–64
	ncRNAs derived from cell cycle gene promoters	Unknown	67
	KRASP1	miRNA sponge	203
	PR antisense	Regulating gene expression	204
II. Evading growth suppressors	PSF-interacting RNA	Modulating protein activity	71
	ANRIL	Chromatin remodeling	75, 76
	GAS5	Competitor	77–83, 86–89
	lincRNA-p21	Transcriptional co-repressor	28
	E2F4 antisense	Regulating gene expression	205
III. Enabling replicative immortality	TERC	RNA primer	101–103, 105
	TERRA	Enzymatic inhibitor	107–114
IV. Activating invasion and metastasis	MALAT1	Modulating protein activity; sensor; scaffold	29, 44, 128–136
	HOTAIR	Chromatin remodeling	23, 36, 137
	HULC	miRNA sponge	173, 174, 200
	BC200	Translational modulator	206, 207
V. Inducing angiogenesis	αHIF	RNA decay	146–149
	sONE/NOS3AS	RNA decay	150, 151, 154
	tie-1AS	RNA decay	155
	ncR-uPAR	Regulating gene expression	208
VI. Resisting cell death	PCGEM1	Regulating gene expression	33, 164, 165
	CUDR	Regulating gene expression	166
	uc.73A(P)	Unknown	31
	SPRY4-IT1	Unknown	167
	PANDA	Modulating protein activity	67
	LUST	RNA-Splicing	209
	PINC	unknown	210

showed that PANDA acts in trans via interaction with the transcription factor NF-YA and limits the expression of proapoptotic genes. Consequently, PANDA depletion markedly sensitized human fibroblasts to apoptosis by doxorubicin. In contrast, DNA damage can also lead to the activation of cis-acting ncRNAs. Wang et al. showed that ncRNAs induced by DNA damage derive from regulatory regions of the human *CCND1* promoter and bind to TLS (translocated in liposarcoma) protein.[41] TLS in turn inhibits cyclin D1 expression via interaction with and inhibition of CBP (CREB-binding protein) and p300.

Future studies, conducted to understand cell death regulation, will have to include long ncRNAs into the analyses to achieve a complete overview about activators and inhibitors of this important cancer hallmark. Especially, the growing fields of autophagy and necrosis research are promising areas to unravel further functions for long ncRNAs in healthy and malignant states.

All in all, the presented studies strongly emphasize the functional importance of long ncRNAs and provide first mechanistic insights how long ncRNAs can contribute to the hallmark capacities of cancer cells. A complete list with the herein described lncRNAs and additional examples can be found in **Table 1**. An extensive list of lncRNAs with a connection to cancer is provided in an overview article from Spizzo et al.[168]

Next, we want to provide a brief overview on what is needed for a better understanding of long ncRNA biology, including their discovery and functional analysis.

Long ncRNAs: Future Challenges

Long ncRNAs have proven to be important regulators in health and disease. However, only individual examples have been functionally studied in detail so far and many important questions remain to be addressed. Biochemical analyses are needed to explore the functions and mechanisms of action of this novel class of biomolecules. This is where long ncRNAs raise a lot of challenges due to their mechanistic heterogeneity that is just beginning to emerge from the first discoveries in this field. However, a good way to start with are classical loss-of-function or gain-of-function studies using RNA interference to knockdown long ncRNAs, genetic loss-of-function models or overexpression. Subsequent analyses of tumor-relevant phenotypes such as proliferation, migration, cell viability or apoptosis could provide first insights into ncRNA functions. However, RNA interference-mediated loss-of-function studies should always be scrutinized to exclude frequent off-target effects and a validation of the phenotype specificity in rescue experiments reversing the phenotype by overexpression of the targeted gene should always be included. Furthermore, studying the localization of lncRNA can also provide valuable insights into its function. Establishing the cellular fraction, in which the lncRNA is normally present, can guide further analyses on the molecular function.

To unravel ncRNA mechanisms at the molecular level, identification of protein interaction partners of individual lncRNAs is informative and necessary to fully understand the mechanistic

process accomplished by the ncRNA-based on the assumption that most ncRNAs will function in ribonucleoprotein complexes. A direct way to identify these proteins would be the application of RNA affinity purification, which uses the ncRNA as bait to fish out all putative important protein interaction partners. This could also be expanded to identifying ncRNA-interacting RNA or DNA molecules.[169] These methods are of special interest as lncRNAs have been shown to play important regulatory roles and can function at the level of chromatin. To determine where lncRNAs bind to chromatin, two methods, called ChIRP and CHART, have been developed recently using complementary oligonucleotides to pull down lncRNAs associated with chromatin.[170,171] Alternatively, RNA immunoprecipitation-sequencing (RIP-Seq) or PAR-CLiP (Photoactivatable-Ribonucleoside-Enhanced Crosslinking and Immunoprecipitation) represent complementary approaches to study RNA-protein interactions.[172] Here, all RNAs that bind to a specific protein of interest are pulled down to identify substrate, target or regulator RNAs—either coding or non-coding. Expanding RIP-based analysis to long ncRNAs will help to create an experimentally documented RNA-protein interactome atlas, including both coding and non-coding transcripts. Such atlas can be a helpful guide for in-depth studies on the functions of each long ncRNA.[11]

Besides their molecular analysis, long ncRNA expression patterns should be further analyzed with genome-wide approaches, especially to discover these molecules in a wide variety of human diseases, e.g., cancers. This could be achieved by microarray-based profiling, deep sequencing or RNA-Seq followed by careful and detailed validation of the expression and sequencing data by qRT-PCR (quantitative polymerase chain reaction), Northern Blotting and Rapid amplification of cDNA ends techniques.

Long ncRNAs specifically expressed or silenced in human cancers could play an important role in these cancer entities and therefore might represent novel therapeutic target genes. Thus, in the last paragraph we will introduce several novel approaches in cancer therapy that target RNA molecules or make use of their tumor-specific expression. In addition, we will outline promising concepts to interfere with ncRNA function or expression, which could become beneficial in cancer therapy.

Fighting Cancer: Are ncRNAs a New Answer?

Currently, cancer therapy is greatly hampered by many difficulties, e.g., specific targeting of cancer cells without interfering with normal tissue function, specific delivery of antitumor drugs, and in case of carcinoma of unknown primary, exact characterization of the malignancy. Here, long ncRNAs could offer a number of advantages, both as diagnostic and prognostic markers but also as novel specific therapeutic targets. The latter, of course, first requires detailed knowledge about the tumor-specific ncRNA function and its requirement for essential cancer cell properties.

For instance, the long ncRNA HULC (highly upregulated in liver cancer) is a liver-specific RNA that is highly expressed in primary liver tumors and hepatic metastases of colorectal carcinoma, but is not found in primary colon cancers or in non-liver metastases.[173,174] PCGEM1, PCA3 (Prostate cancer gene 3) or PRNCR1 (prostate cancer non-coding RNA 1) are three long ncRNAs exclusively associated with prostate cancer.[175-177] This makes them interesting candidates for tumor- and tissue-specific treatments and their expression can be used to identify unknown primary tumors. Moreover, long ncRNAs can also be used to differentiate between subtypes of the same cancer.[22,36] As ncRNAs can be easily detected from minute amounts, e.g., in biological fluids like blood and urine, using qRT-PCR amplification, they are of great diagnostic value. For example, HULC can be easily detected in the blood of HCC patients using qRT-PCR.[174] In addition, a quantitative PCA3 urine test with the potential for general use in clinical settings was developed; the Progensa™ PCA3 urine test. This specific test can help patients who had a first negative prostate biopsy to avoid unnecessary repeated biopsies.[178] Furthermore, lncRNA expression can correlate with patient response to chemotherapy and therefore can be seen as predictive marker. For example, the expression of the long ncRNA Xist strongly associates with the disease-free survival of Taxol-treated cancer patients.[179] LncRNAs are also powerful predictors of patient outcome, e.g., MALAT1 can serve as an independent prognostic parameter for patient survival in early-stage lung adenocarcinoma.[128] The same is true for HOTAIR whose expression positively correlates with metastasis and poor outcome in primary breast tumors, gastrointestinal, hepatocellular and colorectal cancers.[36,137,139,140]

These few examples of long ncRNAs show already the great value of these newly discovered transcripts and it is highly likely that many other long ncRNA markers will be discovered in the near future. Hence, currently generated cancer genome data can only be fully exploited if also the non-coding content of the human cancer genome is studied in great detail—after all, it constitutes the large majority of the genomic information! The more we learn about long ncRNA expression patterns in different types of cancer—as well as in healthy cells—the higher the chances for an improved diagnosis and better prognosis will be.

Finally, long ncRNAs are interesting targets in cancer therapy and especially their cancer- and tissue-specific expression could be a major advantage over other therapeutic options. In fact, antitumor strategies that focus on RNA as target molecule are currently under development.

For example, ribonucleases, small proteins that can enter cells by endocytosis, translocate to the cyctoplasm where they evade mammalian protein nuclease inhibitors and degrade RNA. This can lead to cell death and can be used as anticancer therapy. One of these ribonucleases called Onconase, an amphibian nuclease, even reached clinical trials.[180,181] Onconase is very stable, less catalytically efficient but more cytotoxic than most RNase A homologs. It targets tRNA, rRNA, mRNA as well as miRNAs and therefore shows cytostatic and cytotoxic effects.[182-186] Treatment of cancer cell lines with Onconase leads to suppression of cell cycle progression, predominantly through G_1, followed by apoptosis or cell senescence and Onconase even shows anticancer properties in animal models.[187-189] Onconase sensitizes cells to a variety of anticancer modalities, suggesting its application as an adjunct to chemotherapy or radiotherapy.[190-193]

In addition to this general approach that targets all cellular RNAs, there are currently new strategies under development that make use of the cancer- or tissue-specific expression of long ncRNAs to reduce the risk of affecting normal tissues during genetic treatment. For example, the expression of the lncRNA H19 is increased in a wide range of human cancers. A plasmid, BC-819 (DTA-H19), has been developed to make use of this tumor-specific expression of H19. The plasmid carries the gene for the A subunit of diphtheria toxin under the regulation of the H19 promoter. Intratumoral injections of this plasmid induce the expression of high levels of diphtheria toxin specifically in the tumor resulting in a reduction of tumor size in human trials. Recent studies have yielded encouraging results in a broad range of carcinomas including NSCLC, colon, bladder, pancreatic and ovarian cancers.[194-198]

Alternatively, one could also think about manipulating the expression of specific tumor-suppressive long ncRNAs that were discussed earlier:

GAS5 expression induces growth arrest and apoptosis independently of other stimuli in some prostate and breast cancer cell lines.[83] Therefore, finding a way to induce GAS5 expression in tumors or designing a vector that would induce the expression of GAS5 when injected into the tumor might provide an attractive therapeutic approach. The same holds true for TERRA, a negative regulator of telomerase.[114] A potential therapeutic strategy in telomerase-positive cancer cells would be to enhance TERRA expression or to administer synthetic TERRA mimics.

A supplementary approach would target oncogenic long ncRNAs that show an increased expression in cancer. Here, one could block their activity via multiple ways: (1) One could try to decrease their expression. This is not a trivial task and silencing of long ncRNA expression via siRNAs can be complicated, possibly because of the extensive secondary structure or intracellular localization. Therefore, our lab has developed a specific approach that highly efficiently silences lncRNA expression via genomic integration of RNA destabilizing elements.[129] This approach could be of special interest for blood-borne diseases where one could modulate the patient's genome in hematopoietic stem cells and use these "corrected" cells for gene therapy. (2) One could block molecular interactions and therefore functionally silence lncRNAs. This could be achieved via application of either small molecule inhibitors that mask the binding site in protein inter-action partners or antagonistic oligonucleotides that bind to the ncRNA and therefore hinder proteins from binding. (3) As long ncRNA function is most likely attributed to their secondary structure, one could develop methods to efficiently disrupt it. Again, small molecule inhibitors could be developed, e.g., via systematic evolution of ligands by exponential enrichment, which bind to lncRNAs and change their secondary structure or mimic their secondary structure and compete for their binding partners. Also, antagonistic oligonucleotides could target a specific region of the ncRNA and block its correct folding. (4) Once we have understood how ncRNAs can specifically recruit chromatin-remodeling complexes to specific genes, we could make use of this by creating artificial ncRNAs with pre-designed targeting specificities. With this, one could silence driving oncogenes, e.g., Ras or Myc at the genomic level in cancer cells.

However, before we can make use of these new therapeutic options many more functional and structural studies are necessary to fully understand long ncRNA biology—even for individual examples. At the moment, we are just taking our first steps on the road to understanding the role of long ncRNAs in cancer. But as we move forward, we will discover new ncRNAs and find out more about their importance in cancer, which will inevitably help us to design better therapeutic agents. Given the exponentially growing number of newly discovered long ncRNAs, many great discoveries may be expected in this field strongly encouraging basic science as well as clinical research in this exciting field.

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No potential conflicts of interest were disclosed.

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