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Review

Reactive Transcription and LncRNA Function: An Evidence-Tiered Framework for Stress, Inflammation and Cancer

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Abstract

Background: Long non-coding RNAs (lncRNAs) are among the most frequently reported changes in stress, inflammation and cancer transcriptomes, yet whether an induced lncRNA is a driver, a regulator or a by-product of altered transcription is rarely tested. Two positions dominate the literature: that most lncRNAs are transcriptional noise, and that most are functional. Both rest on real data, and neither tells an investigator what to conclude about a specific lncRNA in a specific dataset. **Scope and approach:** We use "reactive transcription" as an operational null category and not as a new mechanism: lncRNA output that is accounted for by upstream inputs and by known features of RNA polymerase II transcription, termination and decay. We review the mechanisms by which non-functional transcripts can accumulate when cells are stressed: pervasive initiation, incomplete termination, altered decay and neighbor spillover. We then examine the evidence that stress-responsive lncRNAs can nonetheless be essential, using p53-, nuclear factor κB (NF-κB), hypoxia- and endoplasmic reticulum (ER) stress-responsive loci, cancer genomics and genome-scale CRISPR (clustered regularly interspaced short palindromic repeats) screens as test cases. The genomics section includes an audit of the claim that indels in NEAT1 and MALAT1 reflect a transcription-associated mutational process and not selection. **Main argument:** Induction under stress is an expected property of both functional and non-functional transcripts and therefore carries little discriminating information. We propose a four-tier evidence ladder (Tier 1, responds; Tier 2, locus-dependent; Tier 3, product-dependent; Tier 4, mechanism), record physiological relevance as a separate qualifier, and classify perturbation designs by the carrier they can implicate: the DNA element, the act of transcription, the RNA molecule or an encoded peptide. **Translation and disease:** We apply the same logic to lncRNA-embedded open

Academic Editor: Ivana De la Serna

Received: 28 October 2026
Revised: 18 November 2026
Accepted: 21 November 2026
Published: 27 November 2026

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reading frames (ORFs) and separate four levels of evidence: ribosome association, translation, peptide detection and peptide-dependent function. Stress can remodel translation independently of transcript abundance, and in CRISPR screens only a minority of tested ORFs scored for the phenotype assayed, which is not an estimate of how many are functional. We treat infection and neuropsychiatric disease as hypotheses for which direct data are still sparse. **Conclusions:** The framework is a decision aid for individual loci, not a claim about the whole class. A reactive lncRNA can be a useful biomarker without being a driver or a target, and each claim should be worded at the tier of evidence it has reached.

Keywords: long non-coding RNA; pervasive transcription; cellular stress; inflammation; cancer; CRISPR screens; cis-regulation; evidence framework

1. Introduction

Protein-coding sequence makes up less than 2% of the human genome [1], but a far larger share is transcribed. The ENCODE project reported that roughly three quarters of the genome is covered by transcripts, depending on the read-depth and cell-line thresholds applied [2]. How much of that signal reflects biological function was contested from the start [3,4], and the dispute has shaped how long non-coding RNAs (lncRNAs; RNAs longer than 200 nucleotides that have little or no capacity to encode protein) are studied.

The current debate has three well-developed positions (Table 1). Palazzo and Lee argued that function must be demonstrated rather than assumed for non-coding transcripts [5]. Palazzo and Koonin later proposed that most produced RNA is junk in the evolutionary sense, but that junk transcripts are the raw material from which functional lncRNAs arise by non-adaptive routes such as constructive neutral evolution [6]. Ponting and Haerty reviewed lncRNAs from a genome-wide perspective and concluded that most transcript models reflect noise or minor regulatory roles, leaving relatively few lncRNAs that contribute centrally to development, physiology or behavior [7]. The opposing consensus statement by Mattick and colleagues emphasizes that lncRNAs evolve faster than protein-coding sequence, are cell-type specific, and regulate differentiation, development and other physiological processes [8]. A third body of work asks a different question: when a lncRNA locus matters, is it the RNA molecule, the act of transcription, or the DNA element at the locus that does the work [9–11]?

Table 1. Positions in the lncRNA function debate and where this review sits.

Position	Representative Sources	What Supports It	Main Limit
Most lncRNA transcripts are non-functional by-products	Palazzo and Koonin [6]; Ponting and Haerty [7]	Weak selective constraint; low abundance; rapid turnover	Does not say which loci are the exceptions; constraint tests have limited power for lineage-specific regulatory function
lncRNAs are broadly functional	Mattick et al. [8]	Many loci with phenotypes; modular structure; cell-type specificity	Evidence comes mostly from selected examples; assays often cannot separate RNA from DNA effects
Many lncRNA loci act through DNA elements or	Engreitz et al. [9]; Gil and Ulitsky [10]; Núñez-Martínez and Recillas-Targa [11]	Promoter, splicing and enhancer manipulations at individual loci	Categories overlap; most loci have not been dissected

transcription, not the mature RNA			
This review: induction under stress is weak evidence either way; function must be established locus by locus	Sections 3–7	Mechanistic prediction of reactive output (Section 3) plus functional counter-examples (Sections 4 and 6)	Framework is qualitative; several mechanistic assumptions still need direct tests

This review addresses a narrower problem than the general debate. Stress, inflammation and cancer are conditions in which transcription is globally perturbed: transcription factors are activated, chromatin opens, copy number changes and cell composition shifts. In these settings, the observation that a lncRNA is "differentially expressed" is expected for functional and non-functional transcripts alike. We use "reactive transcription" to mean output that changes because the transcriptional environment changed, without implying that the product is inert. The term parallels the passenger-mutation concept in cancer genomics, in which changes that accompany a process are distinguished from those that drive it [12]; we use it as an analogy and not as a claim of equivalence.

Operational definition: "Reactive transcription" is not proposed as a distinct biochemical mechanism. It names a null hypothesis for an observed change in lncRNA output: the change is accounted for by upstream inputs (activity of the locus's own promoter or of a neighboring promoter or enhancer, shared transcription-factor inputs, termination capacity, nuclear decay, copy number and cell composition), and removing the locus, its transcription or its RNA does not change the phenotype under the inducing condition. The null is useful only if it can fail, so it carries three predictions. First, the change should scale with the activity of the nearest active promoter or shared input, and nascent-RNA profiling should place it at initiation or elongation as that input predicts (Section 3.1). Second, if termination or decay capacity is the cause, transcripts that share the vulnerability should shift together, and acute depletion of the responsible factor should mimic or occlude the stress effect (Sections 3.2 and 3.3). Third, perturbing the locus should leave the response and neighboring-gene expression unchanged in the inducing condition (Section 7). Findings that challenge the null are loci whose perturbation changes the response only under stress, such as PURPL (Section 4.2), and loci that act through a defined product, such as Pvt1b (Section 4.2). Findings consistent with it are the shared transcription-factor inputs of lncRNAs with no known role in Zip14-deficient mice (Section 3.4), the poor overlap between differentially expressed and required lncRNAs in endoderm differentiation (Section 6.3) and the transcription-associated indel footprint at NEAT1 and MALAT1 (Section 6.1). None of these settles the status of an individual lncRNA, which is why the category is paired with the evidence ladder.

Relation to earlier work: This review does not claim a new transcriptional mechanism. Pervasive transcription, divergent initiation, Integrator-dependent termination and nuclear RNA surveillance are each established (Section 3), cis-acting lncRNA mechanisms and the separation of locus, transcription and RNA effects have been reviewed (Section 7), and stress-responsive lncRNAs have been cataloged (Section 4). To our knowledge, what has not been done is to turn these mechanisms into explicit, falsifiable predictions for a stress-induced lncRNA, to test those predictions against loci that are reactive and functional at once, and to attach a wording rule to each level of evidence so that a study can be classified before it is interpreted. That combination, and not any single mechanism, is the contribution.

The review makes five contributions. First, it lists the mechanisms that can generate reactive lncRNA output and states what each predicts and how it can be tested (Section 3). Second, it maps the stress programs that move lncRNAs (p53 and DNA damage, NF-

κB and inflammation, hypoxia, and ER, oxidative and metabolic stress) and examines stress-responsive lncRNAs that are both reactive and demonstrably functional, so that the framework is tested against its counter-examples (Sections 4 and 6). Third, it proposes a tiered evidence ladder that separates reactive expression, locus dependence, product dependence and mechanism, records physiological relevance as a separate qualifier, and classifies common perturbation designs by the carriers they can implicate (Section 7). Fourth, it applies the same reasoning to translation of lncRNA-embedded open reading frames (ORFs), separating ribosome association, translation, peptide detection and peptide-dependent function, and covering stress-regulated translation, CRISPR translatome screens and tumor antigens (Section 8). Fifth, it applies the ladder to infection and neuropsychiatric disease, where we state hypotheses rather than conclusions (Section 9). We do not argue that lncRNAs are generally non-functional, and we do not argue that induction can be ignored.

2. The Scale and Quality of the LncRNA Catalog

Any statement about "most lncRNAs" depends on the catalog behind it, and catalogs differ substantially. GENCODE reports that new long-read data have produced a dramatic expansion and reconfiguration of its lncRNA catalog, with roughly 25% of human and mouse genes modified in a two-year interval [13]. A capture long-read sequencing effort added 17,931 novel human genes and 140,268 novel transcripts to GENCODE, and the authors report that the new models show evolutionary constraint, well-formed promoters and links to phenotype-associated variants [14]. CHESS 3, built from nearly 10,000 RNA-seq experiments across 54 body sites and filtered with machine learning for noise, contains 41,356 genes, of which 19,839 are protein-coding [15]. The RNA Atlas extended coverage to non-polyadenylated and small RNAs that earlier compendia had missed [16]. Resources that aggregate more permissively list roughly 95,000 lncRNA genes (NONCODE v7, LncBook 2.0) [17,18].

These numbers are not in conflict, because they answer different questions. Inclusion rules for expression level, splicing, boundaries and filtering of low-confidence models determine the denominator. A claim such as "X% of lncRNAs are conserved" or "Y% are functional" therefore cannot be compared across studies unless the catalog and filter are stated.

Two features of the annotation problem bear directly on the reactive-transcription question. First, boundaries: A reanalysis of public RNA-seq data from five human cell lines and several subcellular fractions attributed about 80% of the transcripts from unannotated intergenic regions to "fuzzy" transcription, in which existing genes are transcribed beyond their annotated ends. Most of the rest arose from putative lncRNA loci that are seldom spliced, and these transcripts were typically removed quickly, by XRN2 on chromatin or by the exosome off it [19]. This extends an older exchange in which van Bakel and colleagues argued that most so-called dark-matter transcripts derive from known genes [20] and Clark and colleagues defended the reality of pervasive transcription [21]. The newer data suggest that both are partly right: much intergenic signal is gene-associated, and a distinct, unstable residue remains. Second, assembly: The T2T-CHM13 reference resolves regions that were absent from earlier assemblies and changes variant calls in them [22], so catalogs built on earlier references may under-represent repeat-rich loci where lncRNAs are common.

The practical consequence is that a stress-induced "new lncRNA" should be checked first against gene boundaries, since a share of such calls will be extended or alternative-terminated forms of neighboring genes rather than independent transcription units.

3. Mechanisms That Generate Reactive Transcription

The mechanistic case for reactive lncRNA output rests on how RNA polymerase II (Pol II) behaves outside protein-coding genes. The mechanisms below are summarized in Figure 1. Each mechanism is stated with its prediction and with the experiment that would test it. Where the evidence is indirect we say so. For each route we separate what is established (the machinery generates or restricts non-coding output under the conditions tested) from what is hypothesized (stress alters that machinery enough to change lncRNA output). Section 3.5 summarizes the status of each route.

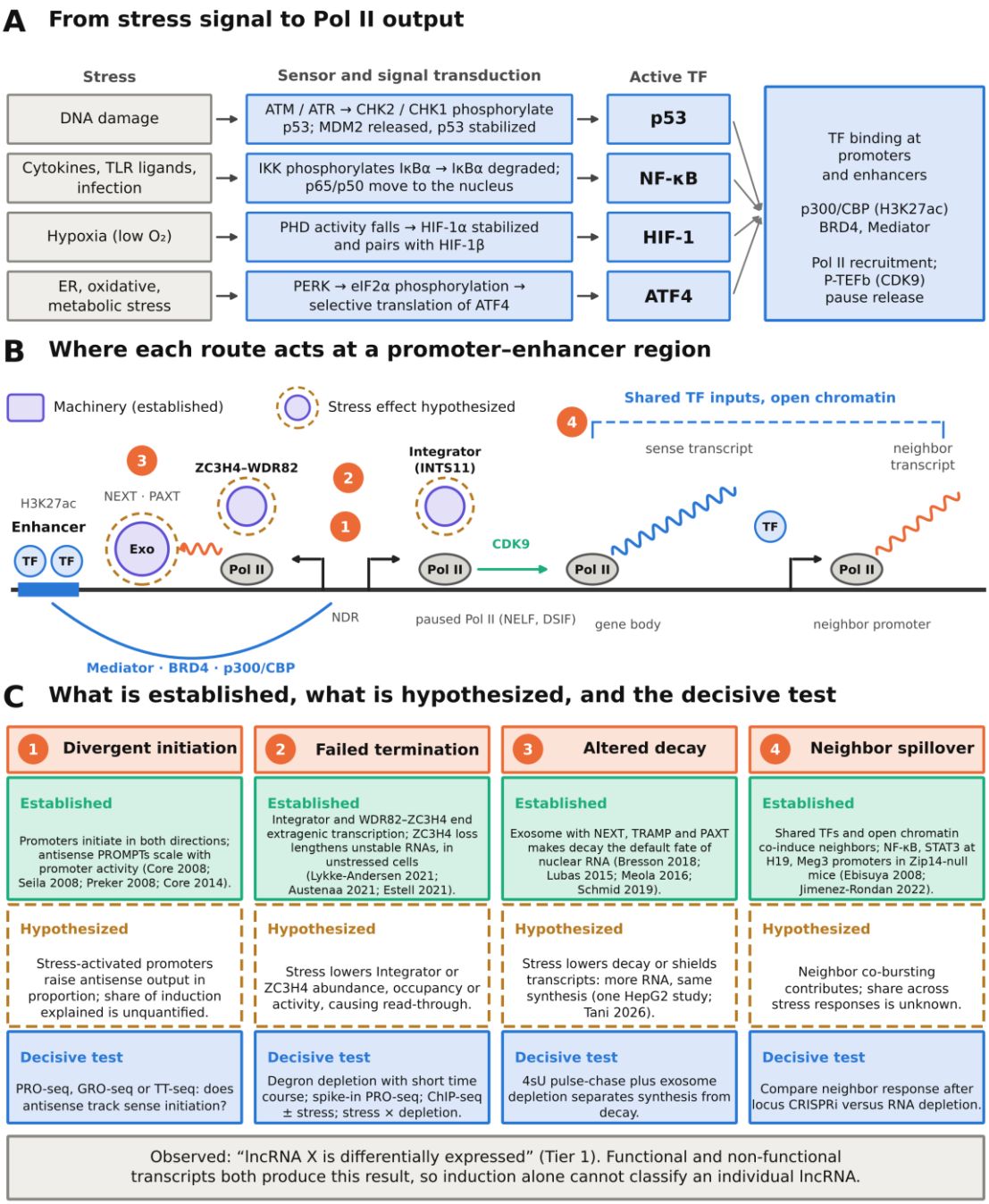


Figure 1. Stress produces reactive lncRNA output through four routes that do not require function. (**A**) Stress signals act through sensors (ATM/ATR and CHK1/CHK2, IKK, PHD enzymes, PERK) and transcription factors (p53, NF-κB, HIF-1, ATF4) that recruit co-activators (p300/CBP, BRD4, Mediator) and release paused Pol II through P-TEFb (CDK9). (**B**) Schematic of a promoter–enhancer region showing where each route acts: (1) divergent initiation, (2) termination by Integrator (INTS11)

and ZC3H4–WDR82, (3) nuclear decay by the exosome with NEXT and PAXT, and (4) neighbor spillover through shared transcription-factor inputs and open chromatin. Dashed amber rings mark steps at which an effect of stress is hypothesized and has not been shown. (**C**) For each route, what is established (green), what is hypothesized (dashed amber) and the decisive test (blue). Depletion of Integrator or ZC3H4 shows that the machinery restricts non-coding output, whereas stress-dependent loss of this function has not been shown. Induction alone cannot assign a transcript to the functional or the reactive class (Tier 1). Sources for panel C: route 1, [23–26]; route 2, [27–29]; route 3, [30–34]; route 4, [35,36].

3.1. Pervasive Initiation and Divergent Transcription

Mammalian promoters initiate transcription in both directions. Divergent transcription at active promoters produces short, unstable antisense RNAs, called promoter upstream transcripts (PROMPTs) or upstream antisense RNAs (uaRNAs), in addition to the sense transcript [23–25]. Nascent-RNA profiling shows that promoters and enhancers share a common initiation architecture [26]. Because the antisense output scales with promoter activity, a gene activated by stress is expected to raise its associated non-coding output without any dedicated regulation of the latter. This prediction is testable with nascent-RNA methods (global run-on sequencing, GRO-seq; precision run-on sequencing, PRO-seq; transient transcriptome sequencing, TT-seq), which separate synthesis from stability.

3.2. Termination and Its Failure

Non-coding transcription is restrained by dedicated termination machinery. The Integrator complex acts genome-wide, largely independent of sequence, near the start of transcription units, and has been proposed to provide quality control of non-productive transcription [27]. Poorly spliced first exons of enhancer-derived and promoter-antisense lncRNAs engage a termination checkpoint that depends on WDR82 and ZC3H4 [28], and loss of ZC3H4 raises and lengthens several hundred unstable transcripts, antisense RNAs and super-enhancer transcripts in particular [29]. These findings establish that termination actively suppresses extragenic transcription. They imply that conditions which weaken termination would raise lncRNA output, but this implication is a hypothesis. We are not aware of direct evidence that physiological stress saturates Integrator or WDR82–ZC3H4 in a way that explains lncRNA induction, and the test needs care in design and interpretation, as described next.

Interpreting depletion experiments: Two statements must be kept apart. "The factor normally restricts non-coding output" is supported by depletion in unstressed cells: when Integrator or ZC3H4 is removed, unstable transcripts rise and lengthen [27–29]. "Stress disrupts the factor" is a separate claim that needs its own evidence, namely lower abundance, occupancy or activity of the factor at the affected loci under stress. The first does not imply the second. Depletion designs also carry confounds. Sustained loss of Integrator or ZC3H4 alters the expression of many protein-coding genes, including transcription factors and cell-cycle regulators, so a lncRNA change measured days after depletion can be secondary to altered cell state. Both factors act at protein-coding genes as well as non-coding ones, depletion can reduce viability or arrest the cell cycle, and the stressor and the depletion may each change global transcription, so normalization choices matter. Finally, steady-state RNA-seq cannot separate increased synthesis from increased stability.

A design that separates a direct, stress-dependent termination defect from secondary effects would combine five elements. (1) Acute depletion with a degron system and sampling at short intervals (minutes to a few hours) that precede secondary changes, with the full time course shown and not a single end point. (2) Nascent-RNA profiling with

spike-in normalization (PRO-seq or TT-seq), analyzed for read-through beyond annotated 3′ ends and for increased polymerase density downstream of termination sites, which are the signature of a termination defect and are distinct from increased initiation. (3) Complementary measurements of dynamics and stability, by metabolic-labeling pulse-chase (for example with 4-thiouridine) and by depletion of an exosome component, to separate synthesis from decay. (4) Occupancy of the factor at the affected loci, by chromatin immunoprecipitation sequencing (ChIP-seq) or cleavage under targets and release using nuclease (CUT&RUN), with and without stress. (5) A factorial design, stress by depletion, with discriminating predictions: if stress acts through the factor, the stress effect on the affected transcripts should be reduced or occluded in depleted cells and the read-through pattern should resemble that of depletion; if the two act independently, the effects should be additive and the read-through patterns different. Without such data we treat termination failure as a candidate explanation and not as an established cause of stress-induced lncRNA output.

3.3. Nuclear RNA Decay as the Default Fate

Nuclear RNAs are surveyed by the exosome and its adaptors nuclear exosome targeting (NEXT), Trf4/5–Air1/2–Mtr4 polyadenylation (TRAMP) and poly(A) tail exosome targeting (PAXT), and decay has been argued to be the default fate of newly made nuclear RNA, with export and stabilization requiring specific features [30–33]. Most unstable lncRNAs are removed this way. If decay capacity falls, or if specific transcripts are sequestered away from nucleases, apparent abundance rises even when synthesis is unchanged. Tani reviews this idea for short-lived lncRNAs (half-life under 4 h) and argues that they could act as kinetic sensors of stress [34]. The same review stresses the limits of current data: much of the evidence for stress-induced changes in nuclease activity comes from a single study in HepG2 cells without independent replication, and abundance alone cannot say whether a change arises from altered synthesis or altered half-life [34]. Genome-wide measurements of RNA flow across chromatin, nucleus and cytoplasm, which resolve release, export and decay rates, now make this distinction feasible for lncRNAs [35,36]. Ribosome association adds a second route to the same ambiguity: it can stabilize a lncRNA or, conversely, expose it to translation-coupled decay of the nonsense-mediated type [37]. A change in a lncRNA's translation status under stress can therefore change its steady-state level with no change in its synthesis, a point that links this section to Section 8.

3.4. Neighbor Spillover and Shared Inputs

Transcription of one gene can raise expression of its neighbors through shared chromatin accessibility and local polymerase concentration. This was first described as ripples of expression around active genes [38]. Two lncRNA–gene pairs that respond to the same transcription factor or to the same open domain will correlate whether or not either regulates the other. Shared transcription-factor inputs are documented for lncRNAs directly: in mice lacking the zinc transporter Zip14, NF-κB and STAT3 binding rose at the promoters of H19, Meg3 and U90926, and H19, Meg3, Bvht, Pvt1 and Neat1 were among the most highly expressed lncRNAs in that dataset [39]. Similarly, MALAT1 expression was deregulated in miR-34a knockout cells, which shows that the level of a lncRNA often described as constitutively abundant moves with changes in an upstream p53-network regulator [40]. Neither observation shows that the lncRNA contributes to the phenotype. Co-variation is therefore consistent with cis-regulation by the lncRNA but does not demonstrate it. We note that the literature cited in favor of direct co-bursting of neighboring promoters is thinner than the literature on single-gene bursting [41], so we

treat co-bursting as a possible contributor and not as an established main cause of lncRNA–gene correlation.

3.5. What the Mechanisms Do and Do Not Show

These mechanisms show that some lncRNA signal is predicted by promoter activity, termination capacity and decay kinetics rather than by regulatory intent. They do not show that any given induced lncRNA belongs to that category. Section 4 turns to loci that are reactive in exactly these ways and are nevertheless required for the response. The status of each route is therefore as follows. Divergent initiation that scales with promoter activity, and nuclear decay as a default fate, are established as general properties of transcription [23–26,30–33]; their quantitative contribution to any particular stress response is untested. That termination machinery restricts non-coding output when depleted is established [27–29]; that stress impairs this machinery is a hypothesis (Section 3.2). Neighbor spillover and shared transcription-factor inputs are documented for specific loci [38,39] but have not been quantified across stress responses.

4. Reactive Is Not the Same as Non-Functional: Stress, P53, Nf-Kb and Hypoxia

The mechanisms in Section 3 predict that stress will raise some lncRNA output for reasons unrelated to function. The converse also holds: many lncRNAs that are induced or repressed by stress are required for the response. This section first maps the stress programs that move lncRNAs (Section 4.1) and then asks which of the resulting loci have evidence that goes beyond correlation (Section 4.2).

4.1. Stress Programs and the LncRNAs They Move

Long non-coding RNAs make up a significant share of stress-responsive genes, and the stress literature is organized around a few upstream programs [42] (Figure 2). We summarize four of them and note for each what the published evidence does and does not establish.

p53 and DNA damage: The p53 network contains lncRNAs that act upstream and downstream of p53, and reviews describe them as fine-tuning the tumor-surveillance program [43,44]. A pan-cancer analysis of transcriptome responses to diverse stresses identified a core set directly transactivated by p53 across stresses and cell types, linked to growth suppression and patient survival, and one member arrested cells in G2 [45], and GUARDIN is a p53-responsive example [42]. Loss of LINC01021 increased the number of significantly regulated mRNAs about 1.8-fold and changed the splicing of ARHGAP12, HSF2 and LYN, which places it as a modulator of a subset of p53 targets and not as a master switch [46]. For DNA-damage responses specifically, NEAT1 paraspeckles have been proposed as a structural scaffold [47]. These examples show that p53-responsive lncRNAs exist and some matter. They do not show that the p53-responsive set is functional as a whole, and the loci with demonstrated effects (Table 2) are few relative to the number reported as induced.

NF-κB and inflammation: BORG is induced by NF-κB after doxorubicin [42], and reviews catalog lncRNAs that shape NF-κB-driven macrophage inflammation [48]. Conserved lncRNAs also take part in NF-κB signaling during aging [49]. The Zip14 data in Section 3.4 show the other side of the same coin: the same transcription-factor inputs that drive functional inflammatory lncRNAs also drive lncRNAs with no known role [39].

Hypoxia: Hypoxia-inducible factor 1α (HIF-1α) and lncRNAs interact in both directions, since lncRNAs are HIF targets and also modulate HIF activity [50]. Under hypoxia, lncRNA-LET is repressed through histone deacetylase 3 and is linked to hypoxia-

driven metastasis [51]. The review literature on hypoxic lncRNAs is large, but many entries rest on expression changes and knockdown in a single cell line, which are Tier 1 or weak Tier 2 evidence in the terms of Section 7.

ER, oxidative and metabolic stress: ER stress regulates lncRNA expression in cancer [52], and lncRNAs and microRNAs have been proposed as regulators of resistance to oxidative, metabolic and genotoxic stress [53]. NBR2 and HAND2-AS1 respond to energy stress, H19 acts in a context-dependent way and NEAT1 contributes to genotoxic-stress responses in myeloma [42]. For oxidative stress, the data on short-lived lncRNAs come from one HepG2 study, and no study has yet examined whether oxidized lncRNAs are degraded preferentially, protected or left alone [34].

What these programs do not settle is classification. In each program the pattern in the sources we reviewed is the same: many lncRNAs are listed as responding, a few have been perturbed at the locus under the inducing condition, and fewer still have been separated into DNA, transcription, RNA and peptide effects. Figure 2 therefore lists, for each program, what the cited evidence leaves open; that assessment is ours and is not a conclusion reported by the original studies.

Table 2. Stress-responsive lncRNAs with experimental evidence of dependence.

lncRNA	Context	Strongest Design Reported	What It Does Not Settle	Tier Reached (Qualifier)
lincRNA-p21	p53 activation	Genetic models that separate DNA elements, transcription and mature RNA; production of the RNA through conserved regions in exon 1 promotes cis-activation of the neighboring gene, whereas accumulation of mature lincRNA-p21 is dispensable [54]. Earlier in vivo work identified functional cis-regulatory DNA elements at the locus [55]	Dependence is on the locus and nascent transcription, not demonstrably on the mature RNA	Tier 3 (transcription-dependent)
Pvt1b	p53; DNA damage; oncogenic signaling	Pvt1b RNA production is necessary and sufficient to suppress Myc transcription in cis; its loss accelerates tumor growth in a lung cancer mouse model [56]	Isoform-specific and cis-acting; says little about other PVT1 isoforms	Tier 3 (RNA-dependent, in cis); in vivo
DINO	p53 signaling	Dino-null mice develop more malignant tumors than controls, with a tumor spectrum distinct from p53-null mice [57]	Germline knockout; does not distinguish locus from RNA	Tier 2; in vivo (germline knockout)
PURPL	p53 activation	CRISPR interference (CRISPRi) depletion changed 7 genes in untreated liver cancer cells but about 800 genes after p53 activation [58]	Function is visible only under stress; baseline data would have missed it	Tier 2 (condition-dependent)
NORAD	DNA damage; irradiation	Knockdown aggravated cell-cycle arrest, apoptosis and	Knockdown-based; DNA versus RNA effects untested	Tier 2 (knockdown)

		senescence in irradiated retinal pigment epithelial cells and in a mouse model [59]		
NEAT1	Cellular stress	Neat1-null mice react inadequately to physiological stress (hyperlocomotion, panic escape) without neuronal loss or overt neuroinflammation [60]; stress and immune roles reviewed in [61]	Knockout removes the locus; the RNA-specific contribution is not isolated	Tier 2; in vivo (knockout)
MALAT1	Lung adenocarcinoma	CRISPR activation of MALAT1/Malat1 alone enabled metastatic progression in a K-ras/p53 mouse model, through CCL2-mediated macrophage recruitment [62]	Gain of function at elevated levels; does not show that physiological MALAT1 variation drives metastasis	Tier 2 (gain of function); in vivo
lincRNA-Cox2	Macrophage inflammatory signaling	Mediates both activation and repression of immune response genes [63]; promotes late inflammatory gene transcription through SWI/SNF-mediated chromatin remodeling [64]	Mechanistic claims depend on cell type and stimulus	Tier 2 (mechanism context-dependent)
NEAT1 (IL8 induction)	Immune stimulation	NEAT1-dependent relocation of SFPQ from the IL8 promoter to paraspeckles mediates IL8 expression [65]	Single target gene	Tier 2, partner identified; carrier not separated
LOUP	Macrophage differentiation; Toll-like receptor 4 (TLR4)–NF-κB signaling	CRISPR interference (CRISPRi) screens in monocytic cells identified LOUP as a multifunctional locus regulating differentiation and inflammatory signaling [66]	Locus-level result	Tier 2
lncRNA-LET	Hypoxia	HDAC3-mediated repression of lncRNA-LET contributes to hypoxia-driven metastasis [51]	Example of a stress-repressed lncRNA with a reported causal role	Tier 2 (as reported)

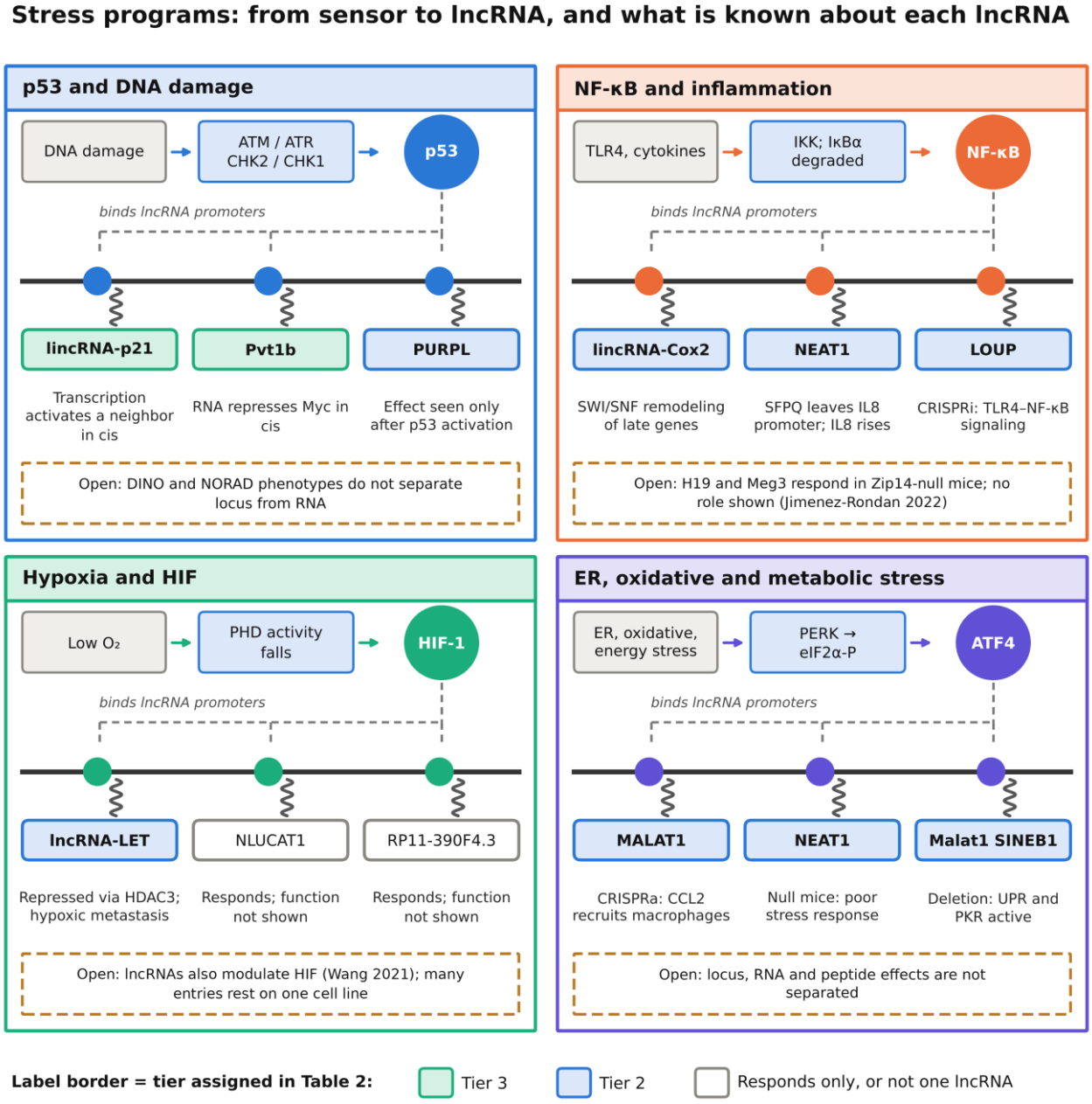


Figure 2. Stress programs, the lncRNAs they move and what is known about each. In each of four panels the upstream chain (stress, sensor, transcription factor) leads to transcription factor binding at lncRNA promoters. Each lncRNA label shows the evidence tier assigned in Table 2 (green, Tier 3; blue, Tier 2; gray, responsive only or no single lncRNA identified), with a one-line summary of the documented interaction. The dashed amber box in each panel states what the evidence does not settle; these notes reflect our reading of the cited studies and are not conclusions drawn by their authors. Listing a lncRNA under a stress is a Tier 1 statement. Studies cited in the panels: Zip14-null mice [36]; lncRNAs and HIF [50].

4.2. Stress-Responsive LncRNAs with Evidence of Dependence

Table 2 summarizes loci for which the strongest published evidence goes beyond correlation. The table is illustrative and not exhaustive, and the fourth column records what each experiment does not show. The last column gives the tier that we assign from

the design in the third column (Section 7), with physiological-relevance qualifiers in parentheses; the assignment is ours and not a conclusion of the original authors.

Four points follow from these cases.

Responsiveness is a property of many functional lncRNAs. As Section 4.1 describes, p53-responsive and NF-κB-responsive lncRNAs include loci with demonstrated roles, and CARINH is an interferon-induced, conserved lncRNA implicated in antiviral defense, inflammatory disease and cancer [67]. If reactive output were a signature of non-function, this literature would not exist.

Function can be conditional. PURPL had almost no effect on the transcriptome in unstressed cells but altered about 800 genes once p53 was activated [58]. A comparison of baseline against stressed expression would classify PURPL by its induction, whereas the biologically relevant fact is that its effect depends on the stress. This is an argument for testing function under the condition in which the lncRNA is induced.

Dependence on the locus is not dependence on the RNA. For lincRNA-p21, the transcription event through exon 1, but not accumulation of the mature RNA, is needed for cis-activation [54]. This is consistent with a regulatory role that is real but not carried by the RNA product, and it is the reason Section 7 separates DNA-element, act-of-transcription and RNA-molecule effects.

Disrupting a stress-buffering lncRNA can also be informative about stress itself. Deleting the SINEB1 element of Malat1 gave diffuse nuclear speckles and cytoplasmic Malat1, activated the unfolded-protein response and protein kinase R (PKR), caused DNA damage and apoptosis and mislocalized TDP-43 [68]. In CD8 T cells, Malat1 inhibits miR-15/16 to support cytotoxic T-cell activation and memory formation [69]. These are domain-level or cell-type-specific results that show a lncRNA matters under stress, but they do not by themselves identify whether the RNA, the locus or a peptide (Section 8) carries the effect. They are Tier 2 for the perturbation used and Tier 3 only if the perturbation preserves the other candidates.

The reactive-transcription model is therefore not contradicted by these loci. Its mechanistic predictions (Section 3) concern what is expected for transcripts that carry no function. What it cannot do is classify an individual induced transcript, because induction occurs for both kinds. Reactive output can still be informative as a readout of cell state even when it is not causal, a point we return to in Section 10.

5. Conservation and Evolution

Conservation is the most common argument in both directions of the debate. Chromatin-state maps revealed over a thousand large intergenic transcripts that are strongly conserved across mammals [70], which was an early argument for function. Comparative transcriptomics later showed that lncRNA repertoires turn over rapidly and that only a minority of lncRNA families have detectable orthologs in distant species [71,72]. The same data are read differently by each side: rapid turnover is evidence for weak constraint on most loci [6,7], and it is also what lineage-specific function would look like.

Four lines of evidence, summarized in Table 3, show why sequence conservation alone is an unreliable guide.

Function can persist without sequence similarity. Zebrafish and mammalian lincRNAs with little sequence similarity can have conserved roles in embryonic development [73]. NEAT1 provides a clear case. Orthologs identified across 545 mammals lack sequence similarity to one another while retaining synteny, G-quadruplexes, protein-binding motifs and self-complementary regions, whereas MALAT1 function seems to

depend on conserved primary sequence under purifying selection [74]. The two stress-associated nuclear lncRNAs therefore follow different evolutionary rules.

Synteny and modules reveal constraint that alignment misses. An orthology workflow combining synteny with genome-alignment conservation, applied to 13 vertebrates with 18,859 human lncRNAs as reference, yielded about 200 putative orthologs per species on average under strict criteria and up to roughly 5000 under permissive ones; more than 450 human lncRNAs had strictly defined counterparts in two or more of the other species [75]. A separate comparison of human lncRNA exons found 340 pairs with similar sequence or structure embedded in different contexts, grouped into 106 clusters that are conserved across great apes and show purifying selection [76]. Conserved paralogous lncRNAs, such as HOXA-AS3 and HOXB-AS3, regulate neighboring HOX genes in cis and are found in several vertebrates [77]. Syntenic lncRNA loci are largely near protein-coding genes and carry DNA regulatory features, so the constrained unit may be the locus and not the transcript [78].

Structure conservation is rarely demonstrated. The R-scape test found a lack of evidence for conserved secondary structure in the lncRNAs it examined [79], and a helix-level reanalysis reinforced that result for a selection of lncRNAs while exposing an artifact of building alignments from covariation [80]. This supports caution about structure-based claims. It does not show that conserved structure is absent from lncRNAs generally, because few have been tested.

Non-conserved does not mean irrelevant. Some human de novo genes arose from neutrally evolving lncRNA loci, and a few have since taken on roles in brain development [81]. In genome-wide association data, non-conserved lincRNAs are linked to cardiometabolic traits about as often as conserved ones [82]. Chen and colleagues also found distinct classes of mammalian lncRNAs with different evolutionary behavior, which argues against treating lncRNAs as a single population [83].

Table 3. How to read conservation evidence for a stress-responsive lncRNA.

Evidence	Supports	Does Not Exclude
Sequence conserved across mammals or vertebrates	Constraint on the transcript, a splice site or an embedded element	Constraint on an overlapping DNA element or neighboring gene
Syntenic locus without sequence similarity	Constraint on the locus, possibly a DNA regulatory element	An RNA-based function with rapid sequence turnover
Conserved secondary structure (covariation)	Selection on the RNA fold	Function without structure (few lncRNAs have been tested)
No detectable conservation	Lineage-specific origin or no constraint	Recent, lineage-specific function

For the reactive-transcription question, the practical conclusion is asymmetric. Strong conservation of sequence, structure or synteny is positive evidence of selection and should lead to the locus being prioritized. Absence of conservation is uninformative about function and is better treated as a feature of young or fast-evolving loci. The earlier claim that lncRNAs generally evolve without constraint is not supported by the evidence reviewed here.

6. Cancer: Copy-Number Effects, Driver Evidence and Functional Screens

Cancer genomes are the setting in which the reactive-transcription question is most concrete, because copy-number change, altered chromatin and altered cell composition all shift lncRNA signal. Two kinds of evidence bear on it: genetic evidence from tumor genomes, and functional evidence from perturbation screens.

6.1. Genetic Evidence

A lncRNA that lies inside a recurrently amplified region will be overexpressed with the amplicon, and an association between its expression and a tumor phenotype should be adjusted for copy number before it is read as evidence of a driver role. This is the cancer-genomics form of the concern raised in Section 3. A second confounder is mutational: transcription itself shapes the local mutation spectrum, so recurrent mutations in a highly transcribed lncRNA can reflect expression and not selection.

The tumor-genome evidence suggests that non-coding driver mutations exist but are a minority. In the Pan-Cancer Analysis of Whole Genomes (PCAWG), only 785 of 5913 driver point mutations (13%) fell in non-coding positions [84], and a review of non-coding drivers describes the emerging landscape as one in which such drivers are relatively infrequent [85].

The case of NEAT1 and MALAT1 is the clearest test of whether mutations in a highly expressed lncRNA reflect selection or transcription, and the primary analysis answers it directly. In the PCAWG driver-discovery analysis of 2658 whole genomes, several significant non-coding elements (the ALB 3′ UTR, NEAT1, MALAT1 and MIR122) were hit by many indels [86]. The authors concluded that "the indels in MALAT1, NEAT1, ALB and MIR122 are not driver events" and that they "are the result of a transcription-associated mutational process" [86]. Three observations support this reading. First, indels in these genes were strongly enriched in events of 2 to 5 bp (Fisher's $p < 6.8 \times 10^{-5}$). Second, mutations in ALB, NEAT1 and MALAT1 were not associated with changes in gene expression. Third, the same process affects at least 18 additional genes in different tumor types, most of them highly expressed and tissue-specific [86]. The authors add that the previously reported oncogenic effects of altered MALAT1 or NEAT1 expression may be unrelated to these mutations [86].

The audit therefore supports the claim, with one qualification that matters for this review. The analysis concludes about the mutations and not about the genes: it shows that the recurrent indels are a footprint of transcription, not that NEAT1 or MALAT1 lack function. Independent functional evidence comes from other layers, for example the mouse transposon-mutagenesis hits in orthologs of NEAT1 and LINC-PINT [87] and the perturbation studies in Table 2. Reactive output of this kind leaves a footprint in the genome, and recurrence of mutations in a highly expressed lncRNA is not by itself evidence of a driver. This is the mutation-level counterpart of the Tier 1 limitation.

There is also evidence in the other direction. The Cancer LncRNA Census (CLC) compiled 122 GENCODE lncRNAs with strong functional or genetic evidence of a causal role in cancer; these genes were overrepresented among drivers inferred from somatic mutation patterns and among mouse transposon-mutagenesis hits. The mouse screens produced 10 tumor-causing mutations in orthologs of 8 lncRNAs, including LINC-PINT and NEAT1 [87]. The second edition (CLC2) lists 492 cancer lncRNAs and reports that germline variants, somatic mutations and expression changes affect them in ways consistent with their inferred roles [88]. These sets are small relative to the catalog (Section 2) but are defined by strict evidence criteria, so they set a lower bound on the number of lncRNAs with cancer roles and not an estimate of it.

6.2. The Pvt1–myc Locus as a Worked Example

PVT1 sits next to MYC and is frequently co-amplified with it, which makes it an obvious candidate for hitchhiking. The literature does not support a single reading. Tseng and colleagues reported that cancer cells with MYC copy-number gain depend on PVT1 [89]. Cho and colleagues showed that the PVT1 promoter acts as a tumor-suppressive DNA boundary element that limits MYC promoter activation [90]. A p53-dependent isoform, Pvt1b, suppresses Myc in cis through its RNA, and its loss accelerates tumor

growth in a mouse lung cancer model [56]. The same locus therefore contains an amplified region, a DNA regulatory element and an RNA isoform with a distinct function. We present PVT1 as an open question of attribution, which is the main purpose of the framework in Section 7.

6.3. Functional Screens

Genome-scale perturbation screens test dependence directly (Table 4). CRISPRi of 16,401 lncRNA loci across seven cell lines identified 499 loci (about 3%) whose targeting reduced growth, and 89% of these did so in only one cell type [91]. This number describes importance for the phenotype tested under the conditions tested; it is not a prevalence of function, and a locus that is negative at baseline is not thereby non-functional. A locus that is not expressed in a given line cannot score in that line, which alone can make a hit look cell-type specific. A CRISPRi screen of the lncRNA loci active during endoderm differentiation found dozens that the process required, yet most of the differentially expressed lncRNAs were not among them [92]. This last observation supports a core point of the reactive-transcription view: expression change is a poor predictor of dependence. Screens in KRAS-mutant non-small-cell lung cancer mapped lncRNAs across several cancer hallmarks and found vulnerabilities that antisense oligonucleotides (ASOs) could target in two- and three-dimensional models [93]. CRISPR activation (CRISPRa) screens in breast cancer cells, both genome-wide and targeted, found lncRNAs that alter the response to palbociclib; lncRNAs that regulated proliferation more strongly tended to confer less sensitivity to CDK4/6 inhibition [94].

Most recently, transcriptome-wide screens with the RNA-targeting CRISPR-associated protein Cas13 in five cell lines identified a core of 15 shared essential lncRNAs and more than 700 context-specific ones, and most of these acted without involving the nearest protein-coding gene [95]. A pooled Cas13d screen of 844 breast-cancer-associated lncRNAs likewise scored proliferation, and one hit, KILR, was followed up as a tumor suppressor that sequesters the replication protein RPA1 [96]. Both studies rank loci by a single growth phenotype and depend on guide design, so their hits are prioritizations that need independent validation (Section 7.2). Figure 3 sets the scale of these results against the size of the catalog.

Table 4. Screening evidence and how it bears on reactive transcription.

Study	Design	Main Result	Reading
Liu et al. 2017 [91]	CRISPRi, 16,401 lncRNA loci, 7 cell lines	499 loci required for growth; 89% in one cell type only	Minority of tested loci scored for growth; context-dependent; not a measure of functional prevalence
hESC differentiation screen [92]	CRISPRi, loci expressed in endoderm differentiation	Dozens required; most differentially expressed lncRNAs not required	Expression change poorly predicts dependence
Esposito et al. 2022 [93]	CRISPR pipeline, KRAS-mutant NSCLC	Hallmark-specific vulnerabilities, targetable by ASOs	Dependence can be therapeutically actionable
Wang et al. 2026 [94]	CRISPRa, breast cancer cells; 815-line drug-response data	lncRNAs modulating palbociclib response	Gain-of-function adds a view beyond essentiality
Liang et al. 2026 [95]	Cas13, 5 cell lines	15 shared and more than 700 context-specific essential lncRNAs; mostly independent of neighboring genes	Essential set is substantial but context-specific

Taken together, the genetic and screening evidence supports two statements that are both true. Most lncRNA loci tested did not score for the phenotype and condition assayed, and a substantial number, in the hundreds across cell contexts, did. Both statements are conditional on the screen. A locus that did not score did so for one phenotype, in one set of cell lines and at one level of perturbation, which is not evidence that it is non-functional. Conversely, the proportion of loci that scored cannot be used as an estimate of how many lncRNAs are functional, and it is not comparable between screens that differ in library, cell panel, perturbation method and phenotype (Figure 3). Screen-positive loci still require the dissection described in Section 7.

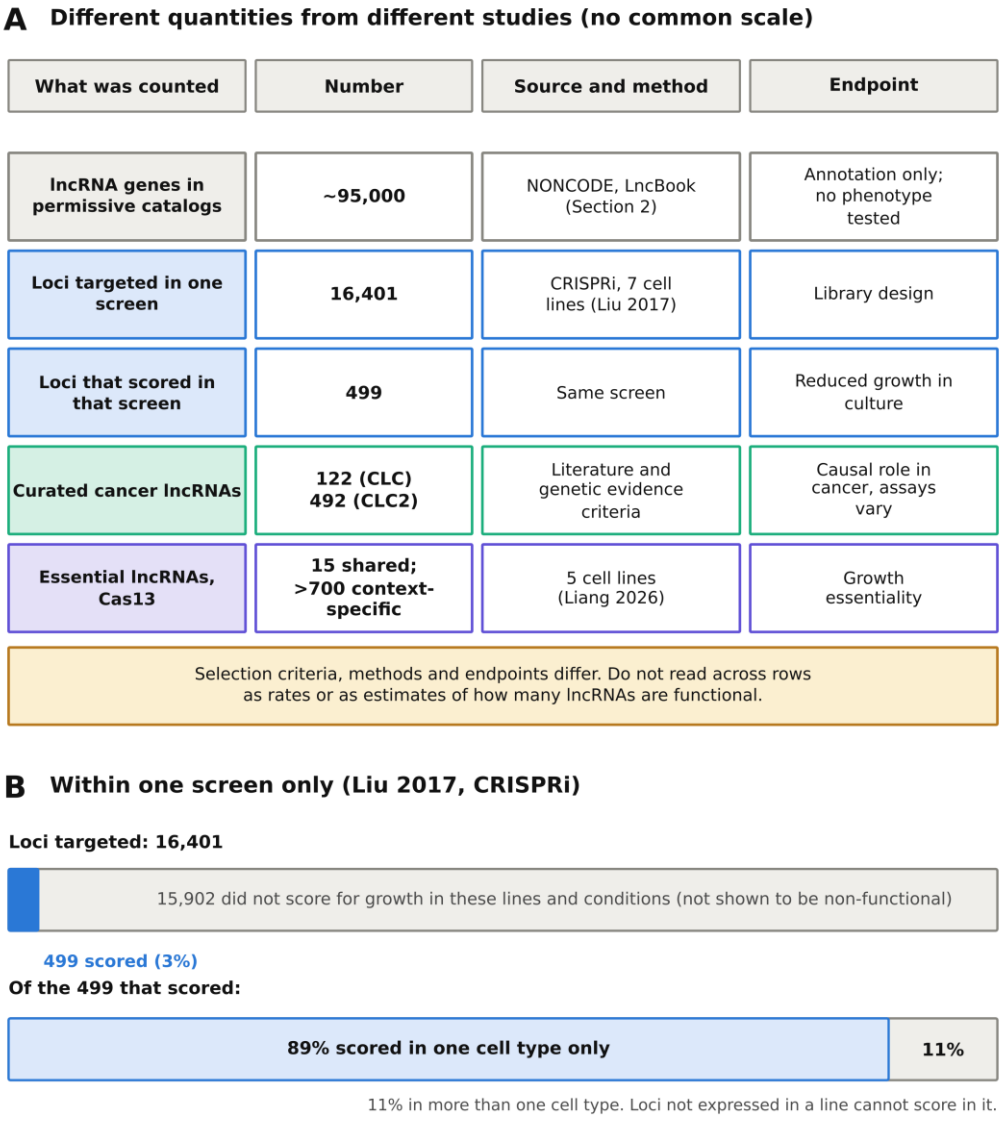


Figure 3. Quantities reported by different lncRNA catalogs and screens. (**A**) Catalog size, loci targeted, loci scoring in one CRISPRi screen, curated cancer lncRNA sets (Cancer LncRNA Census, CLC, and its second edition, CLC2) and essential lncRNAs in Cas13 screens, listed with the source, method and endpoint of each. The numbers differ in selection criteria, methods and endpoints and are shown without a common scale; they are not rates and are not estimates of how many lncRNAs are functional. (**B**) Within a single screen, 499 of 16,401 targeted loci scored for reduced growth, and 89% of those scored in one cell type only. Sources are given in Sections 2 and 6.3 and in Table 4. The CRISPRi screen is Liu 2017 [91] and the Cas13 screens are Liang 2026 [95].

Proliferation as a readout: Most pooled screens score growth or viability, because cell number is a robust, scalable readout of guide depletion. Growth is only one cancer-relevant phenotype. A lncRNA that is dispensable for proliferation in two-dimensional culture can still be required for migration, invasion, metastatic colonization, survival under drug or microenvironmental stress, or therapeutic resistance. Examples in this review run in both directions: CRISPRa of MALAT1 alone enabled metastatic behavior in lung adenocarcinoma (Table 2), the function of PURPL appeared only after p53 activation [58], and CRISPRa screens identified lncRNAs that altered the response to palbociclib [94]. Proliferation assays also have limits of their own: they do not distinguish cytostatic from cytotoxic effects, they depend on medium, density and duration, and a change in proliferation confounds assays of migration or invasion unless it is controlled for. Claims about metastasis or resistance therefore need phenotype-specific assays, for example transwell or three-dimensional invasion assays with proliferation controls, dose–response measurements of drug sensitivity and in vivo colonization, and the screens in Table 4 should be read as testing the phenotype they assayed. A negative proliferation screen is not a negative result for these other phenotypes.

7. DNA Element, Act of Transcription or RNA Product: An Evidence Ladder

Sections 3–6 point to the same practical problem: a lncRNA can respond to stress, be required at its locus and still not act through its RNA. Núñez-Martínez and Recillas-Targa separate two RNA-independent contributions of a lncRNA locus, transcription itself and the regulatory activity of the underlying DNA, and they review the experiments that separate them from RNA-based mechanisms [11]. Engreitz and colleagues showed for individual loci that promoters, transcription and splicing can regulate neighboring genes locally, and Gil and Ulitsky reviewed how cis-acting lncRNAs fit this picture [9,10]. We build on that work by arranging the questions into tiers, so that a claim can be worded at the tier it has reached (Figure 4 and Table 5).

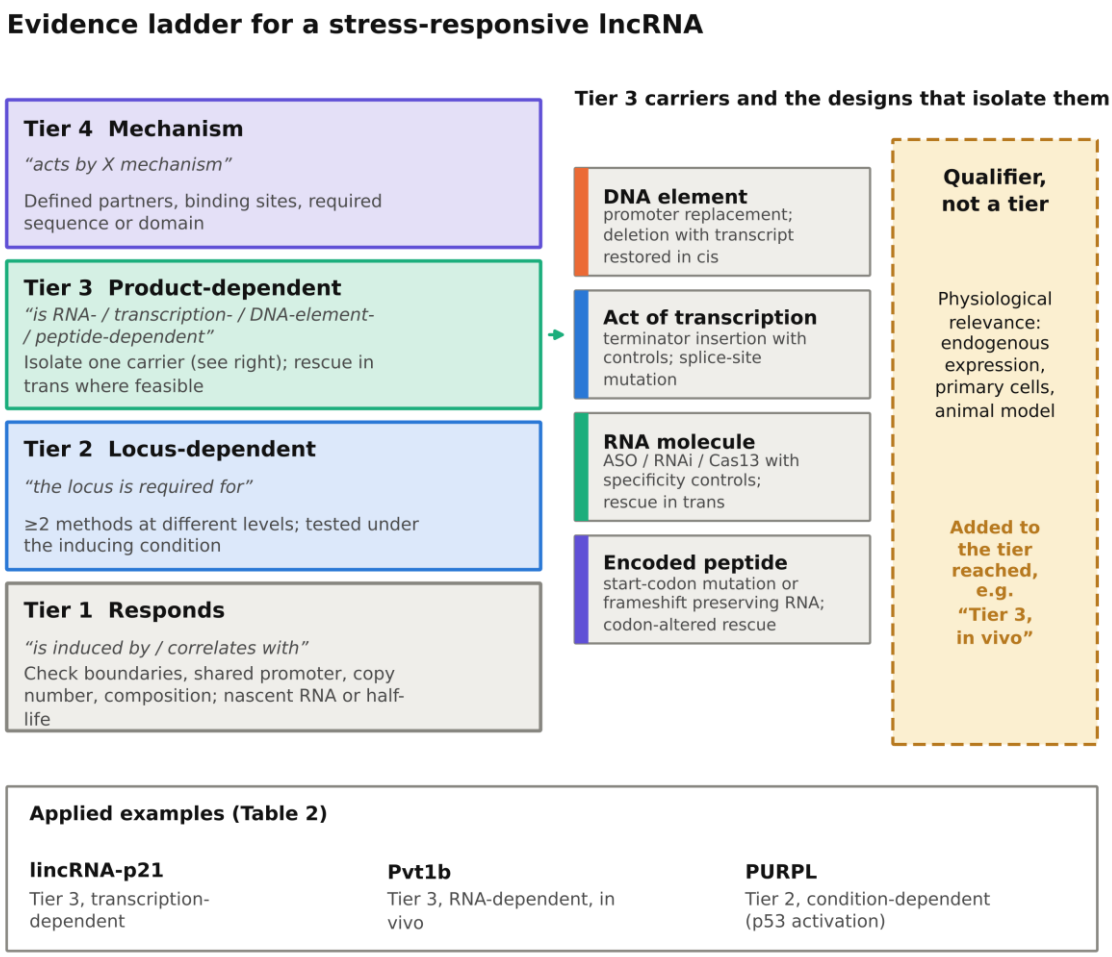


Figure 4. Evidence ladder for a stress-responsive lncRNA. Each tier lists the wording the evidence supports and the minimum evidence required. Tier 3 requires that one carrier be isolated: the DNA element, the act of transcription, the RNA molecule or an encoded peptide; the designs that isolate each are listed (Table 6). Physiological relevance is a qualifier appended to the tier reached and is not part of Tier 4. Examples are taken from Table 2.

Table 5. Evidence ladder for a stress-responsive lncRNA.

Tier	Question	Minimum Evidence	Claim That Is Allowed	Common Pitfalls
1. Responds	Does the transcript change with the stimulus, and is this change not explained by artifact?	Reproducible change in an annotated, independent transcription unit; check of gene boundaries, bidirectional promoter, copy number and cell composition; nascent-RNA or half-life data when synthesis and stability must be separated	"is induced by/correlates with"	Extended forms of neighboring genes; shared promoter; amplicon co-gain; tissue-composition shifts
2. Locus-dependent	Is the locus required for a phenotype or for neighboring gene expression?	Perturbation of the locus with at least two independent methods;	"the locus is required for"	CRISPRi spreading to a bidirectional neighbor; off-target RNA interference (RNAi) or

		test under the stimulus that induces it		antisense effects; conditional function missed at baseline
3. Product-dependent	Which carrier is responsible: the DNA element, the act of transcription, the RNA molecule or an encoded peptide?	Separation of promoter DNA, transcription, splicing, mature RNA and, where an ORF exists, peptide (Table 6); the carrier that was isolated is stated; rescue in trans where feasible	"is RNA-dependent", "is transcription-dependent", "is DNA-element-dependent" or "is peptide-dependent", according to the carrier isolated	Treating a deletion phenotype as an RNA phenotype; trans rescue that fails because the RNA acts only in cis
4. Mechanism	Which partners and steps mediate the effect?	Defined interactors and binding sites; sequence or domain requirements; consistent loss- and gain-of-function results	"acts by X mechanism in Y context"	Over-expression artifacts; mechanism shown in one cell line only
Qualifier: physiological relevance	Does the effect occur at endogenous expression levels, in primary cells or in vivo?	Endogenous or near-endogenous expression; primary cells, an animal model or patient-derived material	Appended to the tier reached, for example "Tier 3, in vivo"	Treating an in vivo phenotype as a mechanism; overexpression in vivo

Table 6. Perturbation designs, the carriers they can implicate and the tier each supports on its own.

Design	**What It Changes**	**Carriers It Can Implicate**	**Highest Tier It Supports Alone**	**What Would Sharpen It**
Promoter or transcription-start-site deletion	Removes promoter DNA, transcription and RNA	DNA element, transcription, RNA, peptide	Tier 2	Restoring the transcript in cis from a different promoter; neighbor expression reported
CRISPRi (catalytically inactive Cas9 fused to a KRAB repressor)	Silences promoter chromatin and transcription; can spread to a neighboring promoter	DNA element or chromatin, transcription, RNA	Tier 2	Neighbor expression checked; agreement with an RNA-level method
Terminator or polyadenylation-signal insertion	Truncates the transcript and keeps promoter DNA	Separates promoter DNA from downstream RNA; transcription is partly retained	Tier 2, or Tier 3 with controls	Insertions at several positions; control insertion; truncated sequence supplied in trans
RNA depletion (ASO, RNAi, Cas13)	Lowers RNA; locus largely intact	RNA, or a peptide encoded by it	Tier 2; Tier 3 (RNA) with specificity controls	Several independent guides or oligonucleotides; rescue; concordance with CRISPRi
Re-expression in trans (cDNA rescue)	Restores the RNA product in a knockout or depleted cell	RNA, or a peptide encoded by it	Tier 3 if rescue occurs; a failure is uninformative for cis-acting RNAs	Codon-altered and start-codon-mutant variants

Splice-site or exon mutation that preserves transcription	Alters RNA processing; transcription continues	Separates the act of transcription from the mature RNA	Tier 3	RNA abundance and localization measured
Start-codon mutation or frameshift that preserves RNA; codon-altered rescue	Prevents translation without lowering RNA	Separates peptide from RNA	Tier 3 (peptide)	RNA level verified; peptide detected

7.1. Why the Tiers Matter

Tier 1 is the stage at which the reactive-transcription mechanisms of Section 3 apply. A lncRNA that cannot pass Tier 1 should not be interpreted further, and one that passes it has only been shown to respond. Several widely used designs stop at Tier 2. Deleting or silencing a lncRNA promoter removes DNA regulatory elements and transcription at the same time as the RNA, so the result does not discriminate among them. This is the point at which the lincRNA-p21 data are instructive: genetic models that disrupted DNA elements, transcription and mature RNA accumulation separately showed that transcription through conserved exon 1 supports cis-activation, and that mature RNA accumulation is dispensable [54].

Tier 3 is where claims about cis-regulation are most often over-stated. Statistical frameworks for cis-regulation can now be applied at scale. TransCistor, in the form published by Dhaka and colleagues, tests cis-regulatory activity at a controlled false-discovery rate and found cis-activity for a minority of the 268 human and 134 mouse lncRNAs examined, while recovering established cis-acting lncRNAs such as XIST [97]. That result argues against assuming cis-regulation from proximity and correlation, and it also shows that real cis-acting lncRNAs can be detected when the test is applied.

Classifying designs: To keep the tiers consistent, we assign a study the highest tier whose minimum evidence it meets in a single experimental system, and we do not add evidence across systems. Table 6 shows how common designs map to the carriers they can implicate. Promoter or transcription-start-site deletion removes the DNA element, the act of transcription and the RNA together and supports Tier 2 only. Promoter silencing by CRISPRi supports Tier 2 for the same reason. Insertion of a polyadenylation signal or other terminator downstream of the promoter keeps the promoter DNA while truncating the transcript, so it separates the DNA element from the downstream RNA but does not separate the act of transcription from the RNA unless the truncated sequence is supplied in trans. RNA depletion leaves the locus intact and therefore addresses the RNA, but it supports a Tier 3 RNA claim only if the effect is specific and, for RNAs that can act in trans, rescued by re-expression. Splice-site or exon mutations that preserve transcription separate the act of transcription from the mature RNA. A start-codon mutation or frameshift that preserves the RNA, with rescue by a codon-altered ORF, separates peptide from RNA [98,99]. A Tier 3 claim is worded according to the carrier that was isolated: DNA-element-dependent, transcription-dependent, RNA-dependent or peptide-dependent.

Mechanism and physiological relevance: These are different questions. Tier 4 asks which partners and steps carry the effect (defined interactors, binding sites, and the sequence or domain required). Physiological relevance asks whether the effect occurs at endogenous expression levels, in primary cells or in an animal model. A mechanism can be worked out in an overexpression system with no evidence that it operates in vivo, and an in vivo phenotype can exist without any mechanism. We therefore record physiological relevance as a qualifier attached to the tier reached (for example, "Tier 3, in vivo") and not as part of Tier 4. On this scheme the mouse tumor model of Pvt1b [56] adds an in vivo qualifier to a Tier 3 claim and does not by itself raise it to Tier 4.

7.2. Methodological Caveats at Tier 2

Tier 2 evidence depends on the perturbation method. RNA interference can have off-target effects on gene expression [100] and small interfering RNA (siRNA) can trigger innate immune responses independently of the target sequence [101], which is a particular concern in studies of inflammatory phenotypes. CRISPR interference can repress a bidirectional neighbor together with the intended lncRNA promoter, and an antisense oligonucleotide acts on the RNA but leaves the locus intact. Kopp and Mendell, and Bassett and colleagues, discuss these issues and the need for complementary approaches [102,103]. For that reason a Tier 2 claim should use at least two methods that act at different levels (DNA, transcription, RNA) and should be tested under the stimulus that induces the lncRNA, as the PURPL data illustrate [58]. We do not claim that RNAi-based findings in the literature generally fail to replicate with CRISPR; the available evidence supports caution and triangulation, not a general discount.

Method-specific limits: Each common method has a characteristic failure mode (Table 6). CRISPRi, which uses a catalytically inactive Cas9 (dCas9) fused to a Krüppel-associated box (KRAB) repressor, acts on chromatin at the promoter. It therefore silences transcription of the locus and any DNA regulatory activity at once, can spread to a neighboring promoter that lies close to the target, and cannot show that the RNA is required. RNAi acts mainly on mature RNA in the cytoplasm and is less effective against nuclear-retained lncRNAs; its off-target effects arise through partial complementarity of the seed region, and double-stranded RNA can trigger innate immune responses [100, 101]. Gapmer ASOs recruit RNase H1 and act on nuclear RNA, but they depend on target accessibility, can hybridize to unintended transcripts, can be toxic at higher doses and act on nascent as well as mature RNA, so an effect on the locus rather than on the RNA product is not fully excluded [102,103]. Cas13-based knockdown targets RNA with a programmable guide, but guide-dependent off-target effects are a recognized concern, so hits from pooled Cas13 screens, such as those in [95,96], are best confirmed with independent guides and an orthogonal method. No single method establishes RNA dependence.

Combining independent strategies: The methods fail in different ways, so agreement between them is informative and disagreement is also informative. If CRISPRi of the promoter and an RNA-level method (ASO, RNAi or Cas13) give the same phenotype, and re-expression of the RNA in trans restores it, the data are consistent with an RNA-dependent effect; cis-acting RNAs will not be rescued in trans, and a failed rescue is then uninformative. If CRISPRi or a promoter deletion gives the phenotype but RNA depletion does not, the effect more likely resides in the DNA element or in the act of transcription, and this should be tested with promoter-replacement or premature-termination designs. If only one method gives a phenotype, the result stays at Tier 2 and is treated as method-dependent until a second, independent method agrees. Phenotypes should be measured under the inducing condition, and the expression of the neighboring gene should be reported for each perturbation.

7.3. Applying the Ladder

For lincRNA-p21, genetic models separated the DNA elements, the act of transcription and the mature RNA, and assigned cis-activation to transcription through exon 1 and not to the accumulated RNA, so the evidence reaches Tier 3 as a transcription-dependent claim [54,55]. For Pvt1b, the evidence reaches Tier 3 for suppression of Myc in cis through the RNA, with an in vivo qualifier from a mouse lung tumor model [56]; no Tier 4 claim is made here. For PURPL, the evidence reaches Tier 2 under p53 activation, with no data at baseline that would have predicted it [58]. For most lncRNAs reported as "upregulated" in a disease dataset, the evidence is Tier 1 or less, and the claim should be

worded accordingly. The ladder is therefore a way of stating what has been shown, not a verdict on whether a lncRNA is a driver.

8. Translation and Micropeptides: The Same Question at the Protein Level

8.1. Scale, Annotation and Proteomic Support

The label "non-coding" is an annotation decision and not a measurement of whether a transcript is translated. Ribosome profiling (Ribo-seq) has identified thousands of translated open reading frames outside canonical coding sequences, including on transcripts annotated as lncRNAs. Chen and colleagues reported 3455 non-canonical coding sequences, 818 of them on lncRNA transcripts [104]. A community effort to standardize annotation cataloged 7264 Ribo-seq ORFs, of which 609 had mass-spectrometry support [105]. Profiling of 11 primary human cells and tissues identified 7767 high-confidence small ORFs, with 603 small ORF-encoded peptides detected in mass-spectrometry data [106]. GENCODE now integrates Ribo-seq and proteomics evidence to refine its annotation of translated sequences [13]. Estimates of how many lncRNAs are translated depend on definitions: one analysis suggested that about 40% of lncRNAs may be translated, with small ORFs of median length about 44 amino acids [107], whereas a sequence-based survey found small ORFs in roughly 20% of about 36,000 lncRNA genes [108]. Reviews now describe this as a "ghost proteome" whose detection is limited by search space and filtering [109].

The proteomic evidence is much thinner than the ribosome-profiling evidence (Figure 5A). An N-terminal proteogenomics study in a HEK293T cytosolic extract detected 2896 unique protein N termini, only 19 of which indicated novel proteins, and concluded that confident reports of novel proteins demand strict filtering and manual curation [110]. Mass-spectrometry support covers about 8.4% of the Ribo-seq ORFs in the consortium catalog [105] and about 7.8% of the high-confidence small ORFs in the primary-tissue map [106]. The denominators differ between studies, so the percentages show an order of magnitude and not a ranking. Translation and a stable, functional protein product are therefore different claims.

Reviews of non-canonical ORFs describe how a few well-studied examples show essential roles in diverse processes, while most remain uncharacterized [111].

Four levels of evidence: Claims about lncRNA-embedded ORFs should be worded at the level the data reach (Table 7). Ribosome association means that an RNA co-sediments with, or is crosslinked to, ribosomes. It does not show that translation occurred, because ribosomes can bind non-coding RNAs without productive elongation, and association can itself shorten or lengthen the RNA's half-life [37]. Translation is supported when ribosome profiling shows in-frame, triplet-periodic footprints with a defined start site, or when a reporter shows frame-dependent output; this shows that the ORF is decoded and not that a stable product accumulates. Detection means that the product itself was observed, by mass spectrometry with spectra matched to a synthetic peptide, by an epitope-tagged knock-in or by a validated antibody. Peptide-dependent function requires a phenotype that is lost when translation is prevented without disturbing the RNA, for example by a start-codon mutation or frameshift that preserves RNA abundance, and that is restored by a codon-altered ORF. A phenotype after knockout or CRISPR disruption of the ORF region is not sufficient, because the same edit also changes the DNA element and the RNA.

Absence at one level: Failure to detect a peptide by mass spectrometry does not mean that the ORF is untranslated: small, hydrophobic, low-abundance or rapidly degraded peptides are poorly captured by standard workflows and by search databases

that omit non-canonical ORFs [109,110]. We therefore read the 8.4% and 7.8% mass-spectrometry support figures above as the share detected in each dataset and not as estimates of the translated fraction. In the other direction, a Ribo-seq signal does not mean that a functional peptide exists (Section 8.4).

Terminology: We use "small ORF" for an ORF encoding fewer than 100 amino acids, "peptide" for the product of any translated ORF, and "micropeptide" only where the product has been detected, as stated. "Microprotein" is used only where the cited authors use it.

Table 7. Levels of evidence for translation and function of lncRNA-embedded open reading frames.

Level	Typical Evidence	What It Supports	What It Does Not Show
1. Ribosome association	Polysome fractionation; ribosome co-purification; footprints without frame or start-site support	The RNA is physically near ribosomes	Decoding of the ORF, a product or a function; association can change RNA stability
2. Translation	Ribo-seq with triplet periodicity and a defined start site; frame-dependent reporter	The ORF is decoded under the condition tested	A stable product or a function
3. Detection	Mass spectrometry with spectra matched to a synthetic peptide; tagged knock-in; validated antibody	A stable product exists in the sample	Function; absence of a mass-spectrometry signal is uninformative
4. Peptide-dependent function	Start-codon mutation or frameshift that preserves RNA; rescue with a codon-altered ORF	The phenotype requires translation of the ORF	Mechanism (Tier 4); physiological relevance; stress dependence unless tested under stress

8.2. Stress Remodels Translation Independently of Transcript Abundance

Stress can alter translation more than it alters transcript levels, and the two layers need not agree. In HEK293T cells exposed to 4 Gy X-rays, translation was transiently inhibited and 362 genes changed at the transcript level, against 975 at the translation level. Of the translated small ORFs detected, 601 were in lncRNAs, and for the RP11-6N17.4 small ORF a peptide was produced from a transfected construct and was abolished by mutating its start codon; endogenous detection and function were not reported. Bypass of upstream ORFs drove translation of MAPKBP1 and DDIT3 [112]. Three consequences follow for the reactive-transcription argument. RNA-seq cannot say whether a lncRNA-embedded ORF is translated more or less under stress. A transcript that appears unchanged can be translated differently. And, as noted in Section 3.3, the ribosome association that translation implies can itself raise or lower the RNA's half-life [37].

A neuronal example links the two levels. The lncRNA Malat1, usually described as a nuclear speckle component, is trafficked to the cytoplasm in neurons as a localized mRNA that encodes a small peptide (M1), and this is enhanced by KCl-induced synaptic stimulation [113]. The finding is consistent with the cytoplasmic Malat1 seen after SINEB1 deletion [68]. It is a clear instance in which a stimulus changes where a lncRNA is and whether it is translated, and it means that a phenotype attributed to Malat1 RNA under stimulation has a peptide as a candidate explanation.

8.3. Functional Screens of Non-Canonical Open Reading Frames

CRISPR screens now test dependence on non-canonical ORFs directly (Table 8). Prensner and colleagues tested 553 candidates drawn from published non-canonical ORF

datasets. Where an ORF had a phenotype, CRISPR tiling and start-codon mutagenesis showed that translation, and not an RNA-based mechanism, was responsible; GREP1, for example, encodes a secreted protein that is abundant in breast cancer [98]. A screen of the unconventional translatome in cancer cells found 56 ORFs targeted by two or more depleted sgRNAs, 49 of which were valid hits and 25 were upregulated in colon adenocarcinoma; SMIMP, encoded by a primate-specific lncRNA, failed to rescue when its start codon was mutated, and knockout reduced xenograft tumor growth [99]. A companion screen identified GT3-INCP, encoded by LINC00992, in estrogen-receptor-positive breast cancer, where the peptide interacts with GATA3 and a start-codon mutant failed to rescue the effect of depleting its RNA [114]. In medulloblastoma, 2019 non-canonical ORFs were targeted in seven lines, of which 387 (19.2%; the authors report 21.1%) were essential in at least one line and 121 in at least two; translation of the upstream ORF (uORF) was the decisive feature, as start-codon base editing showed, and the ASNSD1 uORF was a worked example [115]. In adipocytes, 1695 small ORFs were targeted, yielding 109 proliferation hits and 38 lipid-droplet hits, including a microprotein from the lncRNA 923011K14Rik [116]. Functional genomics has also flagged more than a thousand human microproteins needed for normal cell proliferation, although, as the authors acknowledge, separating a microprotein's role from that of its DNA, its RNA and the translation process itself remains difficult [117]. Non-cancer examples exist, such as the micropeptide encoded by LINC01013 that activates fibroblasts [118].

Two cautions apply when reading these screens (Figure 5B). First, the readouts and denominators differ: the medulloblastoma figure [115] is the share of targeted ORFs essential in at least one line (387 of the 2019 targeted, or 19.2%; the authors report 21.1%, which does not correspond to their stated count of targeted ORFs, and we use the value computed from the stated counts), whereas the 6.4% obtained by dividing the 109 proliferation hits by the 1695 small ORFs targeted in adipocytes [116] is our calculation and is not a figure reported by those authors. The two numbers should not be compared as rates. Second, a minority of tested ORFs scored in any screen. This is compatible with pervasive translation of mostly non-functional ORFs but does not demonstrate it, because absence of a phenotype reflects the phenotype, cell lines and perturbation used, and the screens are not comparable as rates. The screens show that some non-canonical ORFs matter. They do not show how many are induced or required under stress, because none of the studies above was designed to test dependence under a stress condition.

Established and proposed roles: Established micropeptides and proposed stress-associated roles should be kept apart. Peptide-dependent function, shown by start-codon mutation and, where reported, by failed rescue with the mutant, has been established for GREP1 [98], SMIMP [99], GT3-INCP [114] and the ASNSD1 uORF [115], in settings that were not designed to test stress dependence. For GT3-INCP, the endogenous peptide was detected by mass spectrometry and antibody in estrogen-receptor-positive breast cancer cells, a start-codon mutant failed to rescue the growth defect after depletion of its RNA, the wild-type ORF rescued tumor formation in an orthotopic xenograft model, and the peptide interacts with GATA3 [114]; these rescues rely on overexpression. By contrast, the stress-associated cases remain proposals. The RP11-6N17.4 small ORF showed rising ribosome density after X-ray irradiation, and a peptide was produced from a transfected construct and lost when the start codon was mutated; endogenous detection and function were not reported [112]. Translation of cytoplasmic Malat1 into the M1 peptide is enhanced by synaptic stimulation, and a peptide-based explanation for Malat1 phenotypes is a hypothesis [113]. The lncRNA-derived antigens in hepatocellular carcinoma show that peptides are made and presented and not that they act [119,120]. No study reviewed here shows peptide-dependent function of a lncRNA-embedded ORF under the stress that induces it.

Table 8. CRISPR screens of non-canonical open reading frames and what each shows.

Study	Scope	Result	What It Does Not Show
Prensner et al. [98]	553 candidate non-canonical ORFs; CRISPR tiling and start-codon mutagenesis	Effects required translation and not an RNA-mediated mechanism; GREP1 encodes a secreted protein	Whether the peptides act under stress; most candidates had no effect
Zheng et al., Nat Struct Mol Biol [99]	Unconventional translatome in cancer cells; 56 ORFs with ≥2 depleted sgRNAs	49 valid hits, 25 upregulated in colon adenocarcinoma; SMIMP start-codon mutant failed rescue; knockout reduced xenograft growth	Mechanism and stress dependence for most hits
Zheng et al., J Clin Invest [114]	Screen of cryptic lncRNA-encoded ORFs	GT3-INCP (LINC00992): endogenous peptide detected; start-codon mutant failed to rescue; xenograft rescue; interacts with GATA3 in estrogen-receptor-positive breast cancer	Dependence under stress; rescue relies on overexpression; generality across tumors
Hofman et al. [115]	2019 non-canonical ORFs in seven medulloblastoma lines	387 of 2019 (19.2%; reported as 21.1%) essential in ≥1 line, 121 in ≥2 lines; uORF translation critical	Stress-conditioned dependence; readout is survival in culture
Pai et al. [116]	1695 small ORFs in adipocytes	109 proliferation hits and 38 lipid-droplet hits; microprotein from lncRNA 923011K14Rik	Cross-screen comparability; stress-conditioned dependence

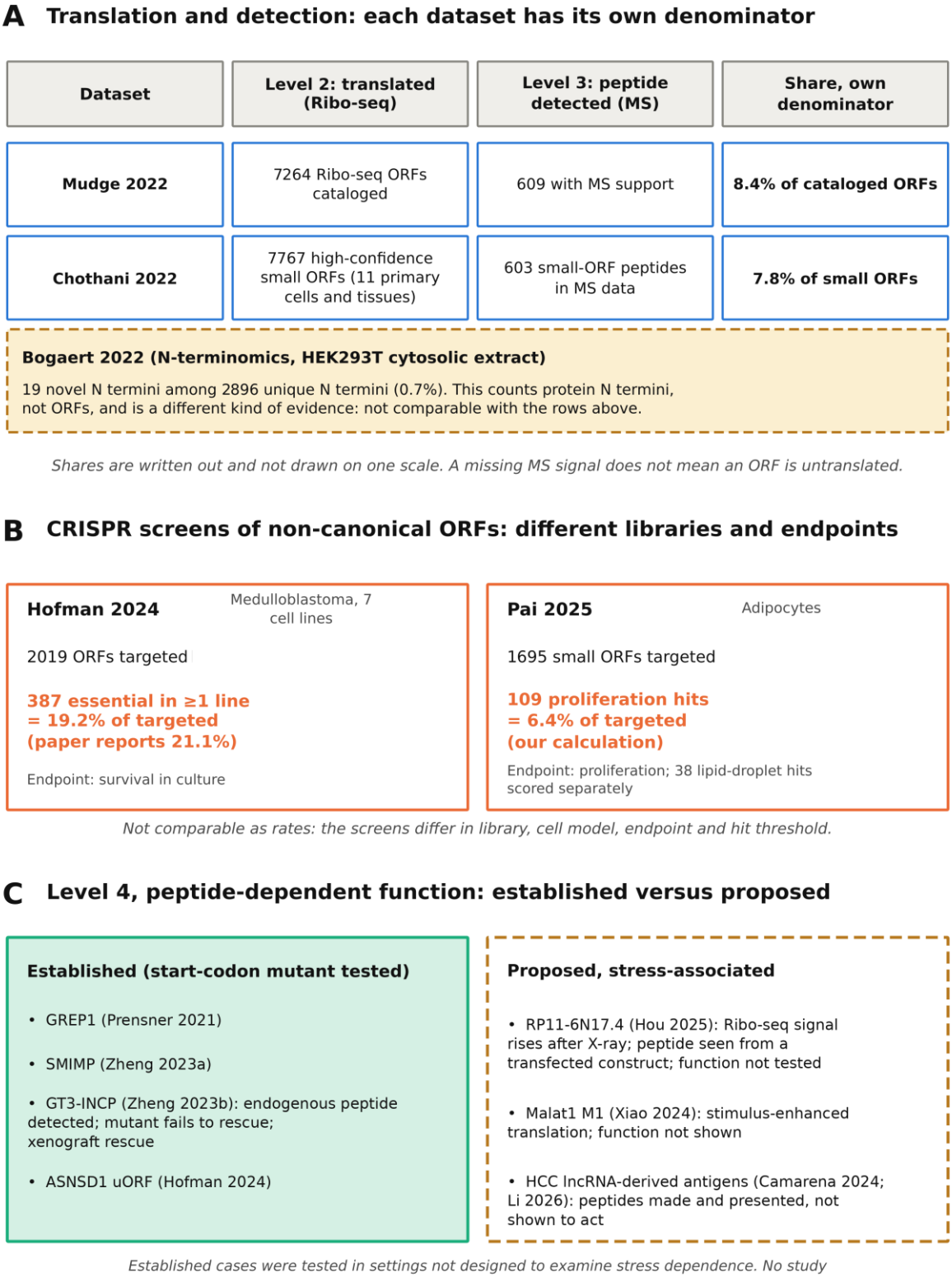


Figure 5. Evidence for translation and function of non-canonical open reading frames. (**A**) Ribosome-profiling (Ribo-seq) and mass-spectrometry (MS) counts from three datasets, each with its own denominator: 609 of 7264 Ribo-seq ORFs (8.4%), 603 of 7767 small ORFs (7.8%) and 19 novel N termini among 2896 (0.7%; a count of N termini and not of ORFs). The values are not comparable and are not estimates of the translated fraction. (**B**) Two CRISPR screens: 387 of 2019 targeted ORFs essential in at least one medulloblastoma line (19.2%; the source reports 21.1%) and 109 proliferation hits among 1695 targeted small ORFs in adipocytes (6.4%; our calculation, not reported by the

original authors). Libraries, cell models and endpoints differ, so these values are not rates to be compared. (**C**) ORFs with peptide-dependent function shown by start-codon mutation, compared with stress-associated proposals for which function has not been shown. Sources for panel A: Mudge 2022 [105], Chothani 2022 [106] and Bogaert 2022 [110]; for panel B: Hofman 2024 [115] and Pai 2025 [116]; for panel C: GREP1 [98], SMIMP [99], GT3-INCP [114], ASNSD1 uORF [115], RP11-6N17.4 [112], Malat1 M1 [113] and HCC lncRNA-derived antigens [119,120].

8.4. Evolution, Proteome Triage and Tumor Antigens

The reactive-transcription logic applies to translation as well. Translation is pervasive outside canonical coding regions and is reported to be especially prominent in aging, neurodegeneration and cancer. Most of the resulting polypeptides are likely non-functional. Non-coding sequences and the genetic code bias translation products toward hydrophobic C-terminal tails that are recognized by the BAG6 triage complex and degraded or sent to membranes, whereas canonical proteins have evolved to avoid such tails. Even so, translation of non-coding sequence is a prerequisite for the emergence of new coding sequences [121], and translated small ORFs in lncRNAs are a plausible substrate for the evolution of new proteins [107]. A lncRNA-embedded ORF that is translated under stress is therefore a candidate and not a conclusion, in the same way as a lncRNA that is induced under stress.

Antigen studies show the same pattern from the immunological side. In a meta-cohort of 117 hepatocellular carcinoma tumors, about 40% of tumor-specific antigens derived from non-canonical ORFs, including ORFs on 33 tumor-specific lncRNAs [119]. A proteogenomic study identified 104 novel lncRNA-derived peptides in hepatocellular carcinoma, of which 46 were tumor-specific and 13 prognostic, with a gain of 0.085 in AUC when added to a prognostic model [120]. Reviews of the field summarize the same trend [122]. These results support translation of non-canonical ORFs in tumors and their immunological visibility. Because antigen presentation requires only that a peptide be made and processed, they do not show that the peptide or the lncRNA has a function, and they therefore sit at the Tier 1 level for the purposes of Section 7.

8.5. Implications for the Evidence Ladder

For the evidence ladder, translation adds a question at Tier 3: Is the effect carried by the RNA or by an encoded peptide? The designs that answer it are those used in the studies above. Start-codon mutations and frameshifts that preserve the RNA address the question directly [98,99], rescue with a codon-altered ORF distinguishes peptide from RNA, and peptide detection by mass spectrometry or a tagged knock-in confirms that a stable product exists [110]. For stress-responsive lncRNAs, a peptide hypothesis is also a possible explanation for phenotypes attributed to the RNA, and it should be tested before a purely RNA-based mechanism is claimed. Where a peptide is shown to carry the effect, the claim should be worded as peptide-dependent. An ORF-disruption phenotype, or ribosome association or a Ribo-seq signal alone, should not be worded as peptide-dependent (Table 7).

9. Infection and Neuropsychiatric Disease: Hypotheses and Tests

The reactive-transcription framework was first formulated for cancer, and extending it to infection and neuropsychiatric disease requires more caution, because lncRNA-specific data in these areas are less complete. We therefore state what is known, what is predicted and how each prediction could be tested (Table 9). These are hypotheses and not conclusions.

Table 9. Hypotheses for infection and neuropsychiatric disease, with discriminating tests.

Hypothesis	Prediction	Discriminating Test	Would Count Against
Interferon-linked lncRNAs in infection largely reflect bystander and composition effects	Induction tracks interferon-stimulated gene programs and cell-type proportions	Single-cell RNA-seq separating infected and bystander cells; deconvolution of bulk data	Induction persists within a defined cell type after adjusting for interferon signaling
Activity-dependent lncRNAs report neuronal activity history	Levels follow stimulation and decay with activity; knockdown has little effect on the activity-dependent program	Stimulation time courses with nascent-RNA labeling; locus perturbation measuring downstream programs	Loss of the lncRNA alters the downstream program
Bulk-tissue changes in disease reflect cell or synapse loss	Differences shrink after normalizing for cell composition	Single-nucleus RNA-seq of matched post-mortem tissue	Differences remain within a cell type
A lncRNA in a disease-associated locus mediates risk	Perturbing the lncRNA, and not only the locus, changes a disease-relevant phenotype	Tier 2 and Tier 3 tests of the ladder in Table 5	Locus effect persists after RNA-level perturbation

9.1. Infection and Inflammation

Infection triggers innate immune sensing and cytokine programs that reshape host transcription. This is well described for malaria, dengue and leishmaniasis [123–125], but the published descriptions concern protein-coding responses and immune signaling, and direct data on host lncRNAs in these diseases are sparse. What is established comes from other settings and cuts both ways. Interferon-induced lncRNAs such as CARINH have been implicated in antiviral defense, inflammatory disease and cancer [67], NEAT1 relocates SFPQ from the IL8 promoter to paraspeckles to promote IL8 expression after immune stimulation [65], and lincRNA-Cox2 acts as both activator and repressor of immune response genes [63]. These loci are induced by infection or immune activation and are functional, so induction in infected tissue cannot be used to classify a lncRNA as a by-product.

What the framework adds is a set of design requirements. In bulk tissue from patients, changes in lncRNA abundance can arise from altered cell composition, for example expansion of neutrophils or lymphocytes, as well as from regulation within a cell type. Bystander cells exposed to interferon induce interferon-stimulated programs without being infected, so infected-versus-uninfected comparisons need to be resolved by cell. Both issues can be addressed with single-cell data and deconvolution before any lncRNA is called a regulator.

9.2. Neuropsychiatric and Neurodegenerative Disease

Neuronal gene expression is activity-dependent, which creates two plausible routes to reactive lncRNA output. First, lncRNA levels may track neuronal activity history. We use the analogy of a tachometer, a readout of the engine's speed and not a control on it. This analogy is ours and is a hypothesis, not an established finding. Second, bulk-tissue measurements can change with the loss of synapses or neurons; synaptic elimination has been implicated in schizophrenia [126], and transcripts enriched at synapses would then appear reduced in bulk samples.

Published work shows that the functional reading is also plausible. Gomafu (MIAT) responds to neuronal activation and participates in the alternative splicing associated with schizophrenia [127]. BACE1-AS is elevated in Alzheimer's disease and exerts rapid feed-forward control over beta-secretase [128]. Neat1-null mice show altered adaptive behavioral responses to stress without overt neuroinflammation [60]. A review of non-

coding RNAs in neurodegeneration argues that several have essential roles [129]. For these loci, activity-dependent regulation and a causal role are not exclusive. Genome-wide association studies of schizophrenia identify many non-coding loci [130], but association with a locus does not identify which transcript at that locus matters.

There is also a boundary on the spillover view. The lncRNA 17A, which is upregulated in inflammatory states and alters splicing of a potassium-channel-interacting protein, is transcribed by RNA polymerase III [131], so models based on RNA polymerase II promiscuity cannot explain every disease-associated lncRNA.

10. A Practical Framework and Implications for Biomarkers and Targets

10.1. A Reporting Checklist

The tiers in Table 5 translate into a short checklist that authors, reviewers and editors can apply to a study of a stress- or disease-associated lncRNA.

Identity. State the catalog and filter used and check gene boundaries against neighboring genes and alternative termination (Section 2).

Synthesis versus stability. Where the claim depends on a change in abundance, report nascent-RNA or half-life data, or state that the question is open (Section 3).

Confounders. Adjust for copy number, shared promoters or enhancers, and cell composition (Sections 3 and 6).

Perturbation. Use two methods that act at different levels, and test under the inducing condition (Section 7.2).

Attribution. Say whether the effect was assigned to the DNA element, the act of transcription, the RNA or an encoded peptide, and by which experiment (Sections 7 and 8).

Wording. Describe the result at the tier it reached. "Is induced by" is a Tier 1 claim and "drives" or "regulates" requires Tier 3 or higher.

10.2. Intended Use Determines the Tier Required

Reactive output is often informative about cell state even when it is not causal. Short-lived lncRNAs have been proposed as kinetic sensors of cellular stress, with the important qualification that abundance alone does not identify the mechanism [34]. A biomarker therefore needs a different level of evidence from a therapeutic target (Table 10). The clinical precedent is PCA3, a urinary lncRNA assay for prostate cancer, which functions as a diagnostic marker irrespective of whether the RNA is causal [132]. General reviews note that many lncRNAs have been proposed as cancer biomarkers, but the quality of validation varies widely [133].

Table 10. Evidence required for different uses of a stress-responsive lncRNA.

Intended Use	Minimum Tier	Additional Requirements
Biomarker of cell state or disease	Tier 1	Specificity against other stresses and diseases; stability in the sample type; analytical validation in independent cohorts; adjustment for cell composition
Prognostic or predictive marker	Tier 1	Independent validation; added value over existing markers
Mechanistic explanation of a phenotype	Tier 3	Perturbation at more than one level; attribution to DNA, transcription, RNA or peptide

Therapeutic target	Tier 3, preferably Tier 4, plus a physiological-relevance qualifier	Physiological expression levels; in vivo model; tolerability; delivery of the antisense or editing agent

This separation resolves an apparent tension in the literature. A lncRNA that is reactive and has no causal role can still be a good biomarker, while a lncRNA that makes a good biomarker is not thereby a target. The tension arises only when the same dataset is used to support both uses without the corresponding evidence. Figure 6 summarizes the decision path from an induced lncRNA to a claim.

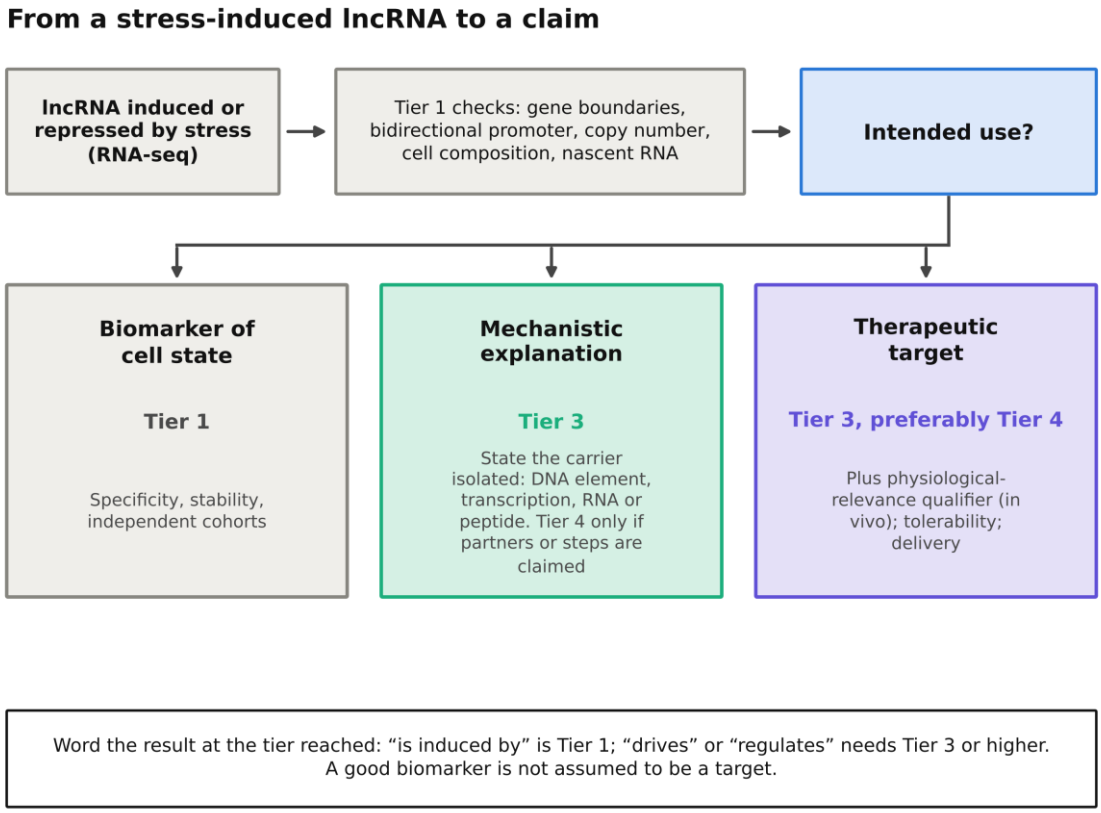


Figure 6. From an induced lncRNA to a claim. Tier 1 checks (identity, synthesis versus stability, confounders) precede the intended use, which sets the minimum tier: biomarker (Tier 1), mechanistic explanation (Tier 3, with the carrier stated) and therapeutic target (Tier 3, preferably Tier 4, with a physiological-relevance qualifier). The wording of the claim should match the tier reached.

11. Limitations, Open Questions and Conclusions

11.1. Limitations

This is a narrative review and not a systematic one. The studies discussed were selected to test the framework against its strongest counter-examples and are not a complete census of the literature. The tiers are qualitative, and the placement of a given study depends on judgment about what its experiments show. Many of the functional examples come from cell lines and mice, and their relevance to human disease is not established. The mechanistic argument in Section 3 is built from well-characterized features of Pol II transcription and RNA decay, but we found no direct evidence that stress saturates Integrator, WDR82–ZC3H4 or the exosome to an extent that explains lncRNA induction. This is a hypothesis with a stated test and is not a finding. The infection and neuropsychiatric sections are hypotheses, because direct lncRNA data in those settings

are limited. Finally, large pooled screens depend on library design, guide filtering, cell panel and phenotype, so a screen hit is a prioritization and not a verdict, and screen-derived proportions are not comparable across studies (Sections 6.3 and 8.3) [95,96]. Several sources for the stress programs in Section 4.1 are reviews, and where a particular locus matters to a conclusion the primary study should be consulted.

11.2. Open Questions

Does stress measurably change termination or decay capacity, and does that change explain lncRNA induction? Nascent-RNA and half-life profiling with and without Integrator or ZC3H4 depletion would answer this.

What fraction of stress-responsive lncRNAs that are required at the locus act through the RNA, through transcription or through DNA? Systematic application of Tier 3 designs is needed, since few loci have been dissected.

- How many lncRNA-embedded ORFs yield stable, functional peptides under stress, as opposed to being translated and rapidly degraded, and does their translation or ribosome association change under the stresses that induce the lncRNA?
- Which lncRNAs are essential under conditions other than proliferation in culture, such as stress, inflammation and differentiation?

11.3. Conclusions

Induction by stress, inflammation or oncogenic signaling is expected for many non-functional transcripts, because their output follows promoter activity, termination capacity and decay kinetics. It is also characteristic of lncRNAs that are required for the response. Responsiveness therefore cannot classify an individual lncRNA as a by-product or as a regulator. Function is better established locus by locus, under the inducing condition, with perturbations that separate DNA, transcription, RNA and peptide effects. The evidence ladder in Table 5 provides a way to word a claim at the tier it has reached, and the use-based requirements in Table 10 show why a reactive lncRNA can be a useful biomarker without being a driver or a target. The framework accepts that functional lncRNAs exist, and it does not require that most lncRNAs be functional. It asks that each claim carry the evidence that matches its wording. The same discipline applies to encoded peptides: a lncRNA-embedded open reading frame that is translated under stress is a candidate, and in screens only a minority of tested open reading frames scored for the phenotype assayed, which is not an estimate of how many are functional.

Author Contributions: Conceptualization, Y.M.B., M.S.I. and A.M.M.; data curation, all authors; validation, E.S.M. and M.S.I.; writing—original draft, all authors; writing—review and editing, all authors; supervision, Y.M.B. and M.S.I.; project administration, Y.M.B. and M.S.I. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: All data extracted and analyzed during this study are available within the paper.

Acknowledgments: The figures were first drafted with Google NotebookLM and were redrawn for this revision in Python (matplotlib) with the help of an AI assistant (Claude "Sonnet 5.5", Anthropic), from scientific content and source materials provided by the authors. The authors reviewed and verified the figures for scientific accuracy and take full responsibility for the final content.

Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

4sU	4-thiouridine
ASO	antisense oligonucleotide
AUC	area under the curve
Cas13	CRISPR-associated protein 13
ChIP-seq	chromatin immunoprecipitation sequencing
CLC/CLC2	Cancer LncRNA Census/second edition
CRISPR	clustered regularly interspaced short palindromic repeats
CRISPRa/CRISPRi	CRISPR activation/interference
CUT&RUN	cleavage under targets and release using nuclease
dCas9	catalytically inactive Cas9
ER	endoplasmic reticulum
GRO-seq	global run-on sequencing
HIF-1α	hypoxia-inducible factor 1α
KRAB	Krüppel-associated box
lncRNA	long non-coding RNA
NEXT	nuclear exosome targeting
NF-κB	nuclear factor κB
ORF/uORF	open reading frame/upstream ORF
PAXT	poly(A) tail exosome targeting
PCAWG	Pan-Cancer Analysis of Whole Genomes
PKR	protein kinase R
Pol II	RNA polymerase II
PRO-seq	precision run-on sequencing
PROMPT	promoter upstream transcript
Ribo-seq	ribosome profiling
RNAi	RNA interference
siRNA	small interfering RNA
TLR4	Toll-like receptor 4
TRAMP	Trf4/5–Air1/2–Mtr4 polyadenylation
TT-seq	transient transcriptome sequencing
uaRNA	upstream antisense RNA

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