

# Dealing with Pervasive Transcription

Torben Heick Jensen,<sup>1,\*</sup> Alain Jacquier,<sup>2,\*</sup> and Domenico Libri<sup>3,4,\*</sup>

<sup>1</sup>Centre for mRNP Biogenesis and Metabolism, Department of Molecular Biology and Genetics, Aarhus University, 8000 Aarhus C., Denmark

<sup>2</sup>Institut Pasteur, CNRS-UMR 3525, Unité de Génétique des Interactions Macromoléculaires, 75015 Paris, France

<sup>3</sup>CNRS-UPR 3404 Centre de Génétique Moléculaire, 91190 Gif-Sur-Yvette, France

<sup>4</sup>Present address: CNRS-UMR7592 Institut Jacques Monod, 75013 Paris, France

\*Correspondence: [thj@mb.au.dk](mailto:thj@mb.au.dk) (T.H.J.), [jacquier@pasteur.fr](mailto:jacquier@pasteur.fr) (A.J.), [libri@cgm.cnrs-gif.fr](mailto:libri@cgm.cnrs-gif.fr) (D.L.)  
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Eukaryotic genomes are pervasively transcribed. However, it is unclear how many newly found RNAs have functions and how many are byproducts of functional, or spurious, transcription events. Cells control the accumulation of many opportunistic transcripts by limiting their synthesis and by provoking their early transcription termination and decay. In this review, we use *S. cerevisiae* and mammalian cells as models to discuss the circumstances by which pervasive transcripts are produced and turned over. This ultimately relates to the likelihood, and potential mechanism, of molecular function.

## Introduction

With historic attention on protein-coding genes, many ncRNAs have led a life in the dark. Recently, the appreciation of a high number of ncRNA loci by high-throughput technologies has created renewed interest and urged questions about the “usefulness” of such transcription activity. The term “pervasive transcription” refers to the generation of a large ensemble of different RNAs distinct from those that encode protein and those with established functions like tRNAs, rRNAs, snRNAs, and snoRNAs. Thus, in a sense, pervasive transcripts are defined by default, the term “pervasive” primarily referring to their widespread nature. This could suggest that these RNAs are “ill-defined,” representing a random spread of transcriptional noise from which specific entities would be difficult to isolate. However, many pervasive transcripts are as well defined as their traditional counterparts even though they are often devoid of any known function. Here we focus on *S. cerevisiae* and mammalian genomes and describe the mechanisms underlying the generation and the control of pervasive transcription.

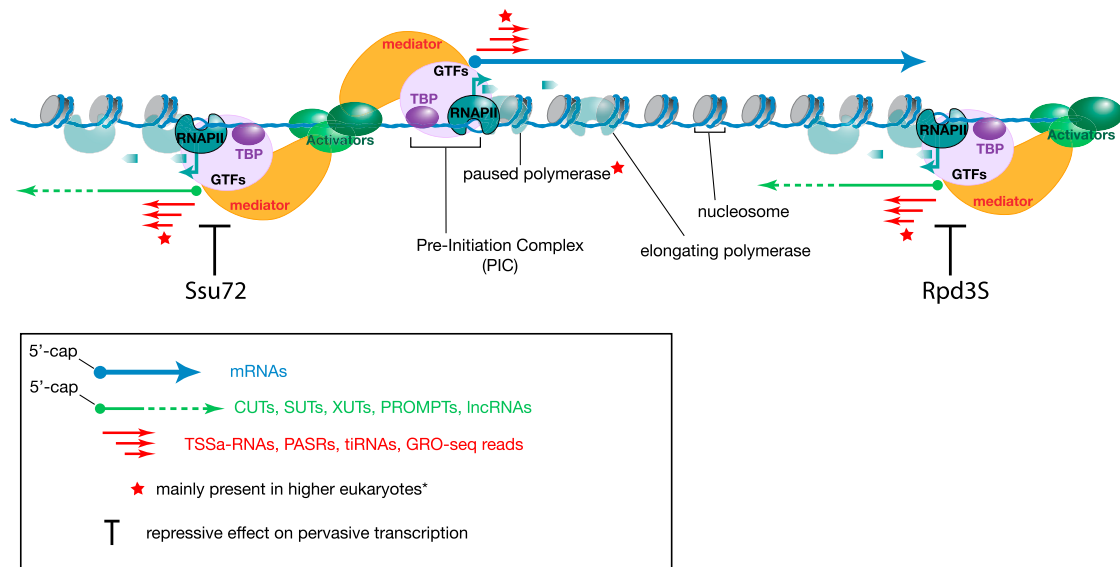
## Origins of Pervasive Transcription

### In the Yeast *Saccharomyces cerevisiae*

In *S. cerevisiae*, unconventional transcripts were discovered when two chromatin remodeling factors were mutated. These factors, Spt6p and Spt16p, are required to re-establish a correct nucleosome organization behind elongating RNA polymerase II (RNAPII), and their inactivation provokes spurious intragenic transcription from cryptic promoters within gene bodies (Kaplan et al., 2003). Soon after, the existence of pervasive transcription was also revealed by transcriptome analysis of strains defective in certain RNA degradation pathways. The absence of Rrp6p, a nuclear-specific catalytic subunit of the *S. cerevisiae* 3'-5' exoribonucleolytic and endoribonucleolytic RNA exosome, exposed a layer of “hidden transcription” that generates so-called “cryptic unstable transcripts” (CUTs), which are normally not allowed to accumulate to detectable levels in wild-type cells (Davis and Ares, 2006; Houalla et al., 2006; Wyers et al., 2005). During a subsequent genome-wide characterization of CUTs (Neil et al., 2009; Xu et al., 2009), pervasive transcripts were discovered

that were less sensitive to Rrp6p activity and therefore named “stable unannotated transcripts” (SUTs). Loss of another ribonuclease, the cytoplasmic 5'-3' exoribonuclease Xrn1p, revealed yet another class of RNA, the so-called “Xrn1-sensitive unstable transcripts” (XUTs; van Dijk et al., 2011). Based on their supposedly different pathways of transcription termination and RNA turnover, CUTs, SUTs, and XUTs form distinct classes. However, these are blurred with a transcript from one class often sharing origin with one from another. For example, a part of the transcription initiation events producing CUTs may escape the early transcription termination-coupled nuclear degradation mechanism of these RNAs (see below) and instead terminate further downstream near a polyadenylation (pA) site. The resulting “elongated CUTs” (eCUTs, Marquardt et al., 2011) are then retained in the nucleus or exported to the cytoplasm and, depending on how efficiently and by which pathway they are degraded, they can instead be classified as XUTs or SUTs. In fact, in the absence of Xrn1p, SUTs and CUTs are found on average to be stabilized 7.9- and 3.6-fold, respectively (van Dijk et al., 2011). These relationships are grounded in the general circumstance that in the absence, or via the exhaustion, of one RNA degradation pathway, transcripts are simply turned over by another. Thus, new transcript classes will expectedly be revealed as other decay factors are manipulated, either alone or in combination with others. Indeed, inactivation of the nuclear 5'-3' RNA exonuclease Rat1p has revealed “telomeric repeat-containing RNAs” (TERRAs) involved in telomere-length homeostasis (Luke et al., 2008).

Although pervasive transcripts in *S. cerevisiae* were initially defined via the pathways mediating their suppression, we now begin to also understand the mechanisms governing their production. CUTs, SUTs, and XUTs are all transcribed by RNAPII. CUTs and SUTs almost exclusively originate from nucleosome-depleted regions (NDRs) at the 5' and 3' ends of genes (Figure 1). Strikingly, CUTs and SUTs often appear to result from divergent transcription from gene promoters, suggesting that most promoters are intrinsically bidirectional and that their apparent polarity is often due to the instability of one of the divergent transcripts. As discussed below, this concept is conserved in mammalian cells. In eukaryotes, transcription initiation requires



**Figure 1. ncRNAs Associated with mRNA Transcription Units**

Schematic representation of a generalized *S. cerevisiae*/mammalian mRNA transcription unit with the organization of transcription factors and associated ncRNAs. Upstream antisense ncRNA production arises from a distinct PIC with the same composition as the one driving mRNA transcription (Rhee and Pugh, 2012; Venters and Pugh 2013). See text for details. Note that Ssu72 and Rpd3S are not the only factors modulating pervasive transcription at the level of chromatin. They are emphasized here because they are proposed to distinguish the 3' asncRNAs arising from divergent promoters (suppressed by Ssu72) from the "free-standing" ones (more sensitive to Rpd3S).

the assembly of an RNAPII-recruiting "preinitiation complex" (PIC) upstream of the transcription start site (TSS). Evidence indicates that divergent transcripts emanate from separate PICs within the same NDR. Mutating the TATA box at an mRNA-producing PIC site not only repressed mRNA production but also enhanced divergent transcription, indicating that separate PICs compete for a common pool of general transcription factors (GTFs) (Jacquier, 2009; Neil et al., 2009). Consistently, high-resolution genome-wide studies of GTF occupancies have demonstrated that, as for divergent mRNA-mRNA producing genes, divergent CUT-mRNA pairs employ independent PICs (Figure 1) (Rhee and Pugh, 2012). However, despite competition for GTFs, mRNAs and their associated divergent transcripts tend to be coregulated, implying that they are controlled by the same transcriptional activators (Neil et al., 2009; Xu et al., 2009). CUT transcription may also run in the same direction as mRNA transcription and depend on the same core promoter. Such organization can be exploited for regulatory purposes, as in the case of genes of the nucleotide biosynthesis pathway, or when the generation of CUT-like transcripts via early transcription termination attenuates expression of mRNAs (see below). A recent study suggests that such RNAs may constitute an abundant class (Tuck and Tollervey, 2013).

Because of the high gene density in *S. cerevisiae*, an NDR 5' to a gene is in 50% of cases also 3' to an upstream convergent gene. In such a configuration, divergent transcription away from the downstream promoter will correspond to pervasive transcription from the 3' end of, and antisense into, the preceding gene. In addition, antisense ncRNAs (asncRNAs) may emanate from 3' NDRs independently of a downstream divergent promoter (Murray et al., 2012). Interestingly, these two distinct

origins of 3' asncRNAs display different sensitivities to mechanisms that suppress pervasive transcription initiation. Transcription from 3' NDRs, unrelated to divergent mRNA promoters, is generally repressed by the Rpd3S histone H4 deacetylase complex (Churchman and Weissman, 2011; Tan-Wong et al., 2012), which presumably tightens their "loose" nucleosome structure. Instead, repression of divergent transcription from mRNA promoters requires the RNAPII-CTD phosphatase Ssu72p, the activity of which promotes gene looping between 5' and 3' ends of some loci, indicating that three-dimensional gene structure contributes to promoter polarity (Tan-Wong et al., 2012). In the case of canonical CUTs, 3' antisense transcription usually terminates in due time as to not interfere with transcription of the upstream, sense mRNA. However, transcription events that evade early termination, such as those producing SUTs and XUTs, may reach the mRNA promoter and interfere with gene expression.

It is worth noting that, similar to mRNAs, expression profiles of ncRNAs vary with external stimuli. Although part of this is likely to be indirectly due to overall changes in protein coding-gene activities, it may also imply a widespread role of ncRNA transcription in gene regulation. A striking example of this has been found by studying yeast sporulation, which induces a profoundly modified profile of ncRNA with the induction of the so-called "meiotic-specific noncoding transcripts" (MUTs; Lardenois et al., 2011). Some of these noncoding transcription events, induced in response to nutrient deprivation, negatively regulate genes that are only expressed in diploid cells to induce gametogenesis (van Werven et al., 2012).

#### **In Mammalian Cells**

Although earlier reports had already noted transcription of intergenic regions (e.g., Ashe et al., 1997), it is from studies of

mammalian transcriptomes that the term “pervasive transcription” first originated (Birney et al., 2007; Carninci et al., 2005; Kapranov et al., 2007; Katayama et al., 2005). Large-scale analyses, like those of the human ENCODE project (Djebali et al., 2012), showed that a combined total of ~75% of the genome is transcribed within 15 human cell lines analyzed. Primary transcripts from protein-coding genes explain just ~25% of genomic output (~1.5% of this being exonic RNA) but are an order of magnitude more abundant than ncRNA. Such high-genome activity implies significant overlap among transcripts (albeit not necessarily in the same tissue).

In animal cells, ncRNAs are generally categorized as short or long. While the definition varies somewhat between studies, long ncRNAs (lncRNAs) are commonly defined as being >100–200 nt. An emerging feature for this type of pervasive transcripts is that a significant fraction is, in one way or another, associated with transcription of mRNAs, including lncRNAs originating from protein-coding gene promoter or enhancer regions. The short RNA category includes the functionally well-characterized miRNAs. These are generally associated with specific RNP complexes, with the small RNAs providing substrate specificity. We do not consider such RNAs to be “pervasive,” and these are thus beyond the scope of this review. Yet, there are plenty of RNAs <200 nt that do not associate specifically with known proteins and whose function, if any, remains elusive. Their first description came from pioneering studies using tiling microarrays, where they were found originating from both the beginnings and ends of protein-coding genes (Kapranov et al., 2007). Consequently, they were named “promoter-associated small RNAs” (PASRs) and “terminator-associated small RNAs” (TASRs), respectively. Small RNAs, reminiscent of PASRs, were subsequently characterized by RNA sequencing (RNA-seq) and named “transcription start site-associated” (TSSa)-RNAs (Seila et al., 2008) or tiny (ti)-RNAs (Taft et al., 2009). A recent size analysis and alignment of these small transcripts relative to gene TSSs suggested that they represent nascent RNA degradation products that remain associated with, and protected by, TSS-proximally stalled RNAPII (Valen et al., 2011). As determined, e.g., by the sequencing of global run-on transcripts (GRO-seq; Core et al., 2008), stalled, or poised, RNAPII is found at the majority of mammalian promoters. Strikingly, transcription from these predominantly CpG-rich NDRs occurs bidirectionally from separate PICs, generating TSSa-RNAs, overlapping with RNAPII GRO- and ChIP-seq tags, in a divergent and virtually symmetrical pattern (Core et al., 2008; Ntini et al., 2013; Seila et al., 2008; Sigova et al., 2013; Venters and Pugh, 2013) (Figure 1). However, while both transcription directions contain peaks of “active” histone modifications, such as histone H3 acetylation and H3K4 trimethylation, chromatin modifications associated with transcription elongation are only robustly observed in the direction of mRNA production (Seila et al., 2008). This is because the fraction of divergent transcription that ignores/escapes RNAPII stalling is still rapidly terminated due to the presence of transcription-terminating pA signals in the upstream region of divergent transcription (Almada et al., 2013; Ntini et al., 2013). In contrast, mRNA-producing downstream regions contain a lower pA site density and are instead enriched for U1 snRNP binding sites, which are known to suppress pA site

utilization (Ashe et al., 1995; Kaida et al., 2010). In addition, divergent transcripts, called promoter upstream transcripts (PROMPTs) or upstream antisense RNAs (uaRNAs), are targets of the nuclear exosome (Flynn et al., 2011; Ntini et al., 2013; Preker et al., 2008) and therefore reminiscent of *S. cerevisiae* CUTs, whereas more stable mammalian lncRNAs may be compared to SUTs or XUTs. Parenthetically, the absence of TSSa RNAs in *S. cerevisiae* probably reflects only low levels of poised RNAPII at the 5' end of genes in this species (Rhee and Pugh, 2012).

A class of transcripts not found in yeast are the lncRNAs associated with enhancers. These so-called enhancer RNAs (eRNAs) (Kim et al., 2010; De Santa et al., 2010) represent an estimated 19% of lncRNAs in human ESCs (Sigova et al., 2013). This estimate likely underreports eRNAs, because these are also rapidly degraded by the exosome (Andersson et al., unpublished data) and therefore fall below the radar of many transcriptome experiments. Interestingly, eRNA transcription is often bidirectional and associated with both RNAPII stalling and the production of small TSSa RNAs highly resonant with transcription initiation profiles of protein-coding gene promoters (Venters and Pugh, 2013). Moreover, the pA and U1 snRNP binding-site content around enhancers resembles that of PROMPT regions, suggesting that DNA/RNA sequence coupled to the activity of cellular ribonucleases exerts a major impact in the “pruning” of mammalian transcriptomes. This includes the production of the class of long intergenic (or long intervening) noncoding RNAs (lincRNAs), that contains lncRNAs clearly separate from protein-coding genes and have received much attention due to the successful functional characterization of some of its members (Rinn and Chang, 2012; Ulitsky and Bartel, 2013). lincRNAs are often defined to be multi-exonic. However, as single exonic lincRNAs also exist and as the short-lived versions are hard to distinguish from eRNAs, present lincRNA counts are associated with considerable uncertainty. Moreover, lincRNAs often overlap PROMPTs (Sigova et al., 2013) and with repetitive parts of the genome that are expressed (Djebali et al., 2012; Kelley and Rinn, 2012), further complicating a clear division of transcripts.

### The Cellular Toolbox to Tame Pervasive Transcription

Although pervasive transcription certainly serves some function, most is likely due to leaky transcription initiation. Uncontrolled transcription needs to be contained because it can disrupt cellular events, such as transcription itself, or the maintenance of genome stability. Moreover, excessive nonfunctional RNA levels might be toxic if the RNAs are inappropriately translated, interact with complementary DNA sequences, or sequester limiting RNA-binding factors.

#### Suppressing Pervasive Transcription at the Level of Its Initiation

The amount of information sufficient to initiate transcription is low, and TSSs readily appear when the control exerted by chromatin is naturally leaky or loosened artificially, for example by mutation. Several factors ensure that transcription does not start within genes, where “protective” nucleosomes must be systematically evicted by traveling RNAPs. These factors include chromatin remodelers, such as Spt6p and the FACT complex (Kaplan et al., 2003), as well as proteins that modify histones and enable their association with the template DNA. As mentioned above, a

key complex here is the Rpd3S histone deacetylase (HDAC) (Figure 1), which is recruited by methylated histone H3K36, a mark characteristic of coding regions, and established by the RNAPII CTD Ser2P-dependent methyltransferase Set2p (Carozza et al., 2005; Keogh et al., 2005). Rpd3p deacetylates histone H4 to suppress spurious intragenic transcription initiation. Analogous mechanisms of suppression occur in human cells, but they do not involve H4 deacetylation. Like in yeast, methylated histone H3K36 functions as a recruitment platform, bringing in both the FACT chromatin remodeling complex (Carvalho et al., 2013) and the demethylase, KDM5B (Xie et al., 2011). Both suppress internal transcription initiation. KDM5B is proposed to do so by removing H3K4 methylation, restricting this mark to gene promoters. Consistently, depletion of KDM5B leads to spurious internal initiation and increased levels of H3K4 methylation in coding regions (Xie et al., 2011).

Like the NDR of a gene promoter allowing the assembly of transcription initiation complexes, the generally low nucleosome occupancy of nongenic regions exposes many cryptic initiation signals. In *S. cerevisiae*, the ATP-dependent chromatin remodeling factor Isw2p functions by “pushing” nucleosomes away from coding regions, restricting nongenic NDR sizes. Deletion of the *ISW2* gene therefore results in the genome-wide increase of spurious transcripts (Whitehouse et al., 2007; Yadon et al., 2010), presumably because broadened NDRs allow additional access to transcription promoting signals.

#### **Suppressing Pervasive Transcripts by Termination-Coupled RNA Decay**

In the many cases where suppression of cryptic initiation fails, transcription termination enforces control over pervasive transcription. In *S. cerevisiae*, transcription of CUTs is terminated quickly after initiation by an essential pathway that depends on the so-called Nrd1p-Nab3p-Sen1p (NNS) complex (Arigo et al., 2006a; Thiebaut et al., 2006). A salient feature here is that transcription termination is directly coupled to transcript degradation, coordinating the control of inappropriate transcription with the rapid elimination of its unwanted RNA byproducts (Arigo et al., 2006a; Thiebaut et al., 2006; Vasiljeva and Buratowski, 2006). Although initially discovered because of its involvement in the generation of functional and long-lived small nuclear (sn) and nucleolar (sno) RNAs (Steinmetz et al., 2001), the NNS complex is probably even more devoted to the control of pervasive transcription (P. Cramer, personal communication). The two RNA binding proteins Nrd1p and Nab3p recognize short termination signals within the nascent RNA (Figure 2A, top; Creamer et al., 2011; Porrua et al., 2012; Wlotzka et al., 2011). Nrd1p also interacts with the RNAPII CTD, a property thought to be important for NNS termination activity (Gudipati et al., 2008; Kubicek et al., 2012; Vasiljeva et al., 2008a), perhaps by recruiting the Sen1p RNA helicase to trigger the dissociation of the transcription elongation complex from the DNA template (Porrua and Libri, 2013). The NNS complex also physically associates with the nuclear RNA exosome and the “Trf4p, Air2p, Mtr4p polyadenylation” (TRAMP) activator complex (Vasiljeva and Buratowski, 2006), which is involved in the degradation of CUTs (Figure 2A, bottom). The interaction of TRAMP/exosome with the NNS complex is believed to couple NNS-directed termination and RNA decay.

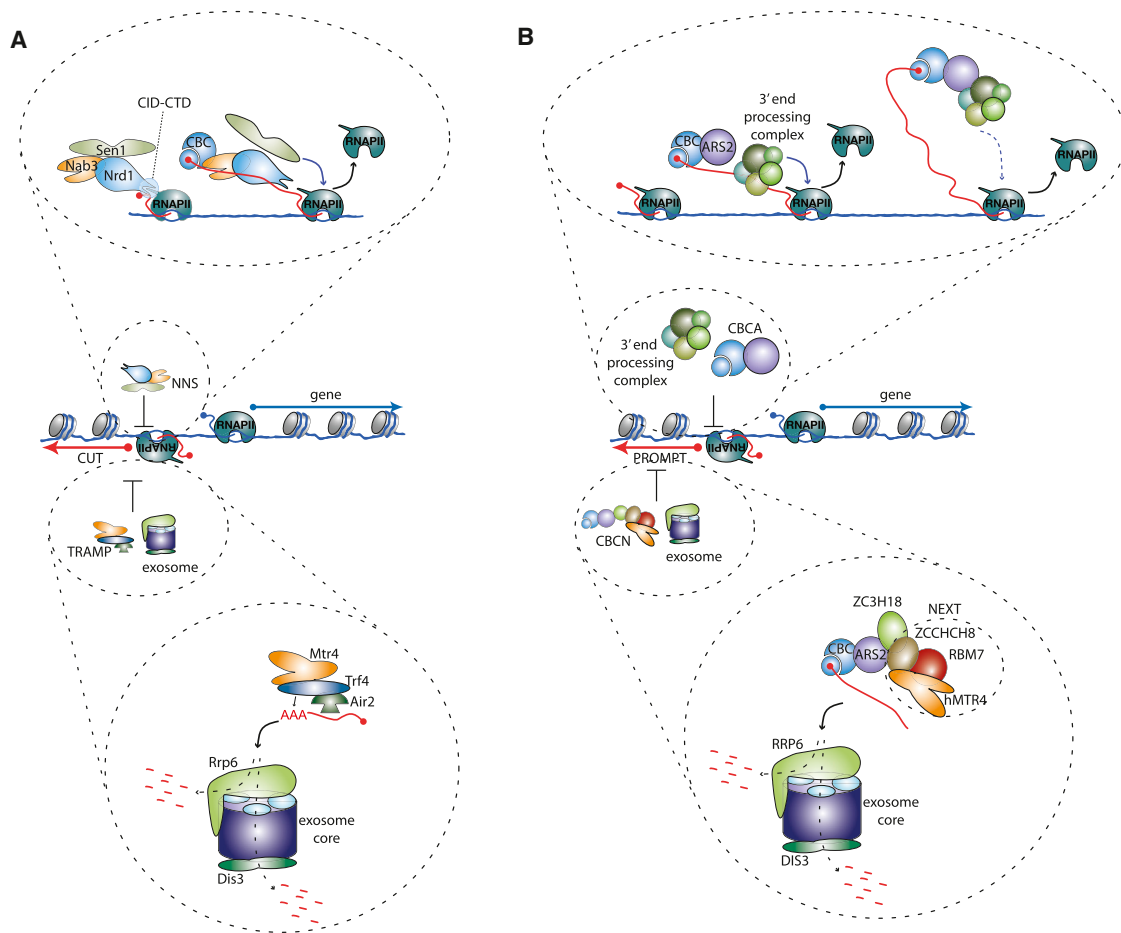
*S. cerevisiae* has an additional transcription termination pathway, which utilizes the cleavage polyadenylation factor (CPF) as well as cleavage factors I (CFI) and II (CFII). This pathway is used to generate mRNAs that are generally exported to the cytoplasm for translation. An important feature discriminating the fate of stable mRNAs from unstable CUTs is therefore their different transcription termination pathways. Choice of the utilized mechanism critically depends on the position of the termination signals relative to the RNAPII TSS, and this “position effect” often predominates over the nature of the actual terminator. NNS-dependent termination preferentially occurs at TSS-proximal (<1 kb) sites and is relatively ineffective at recognizing downstream termination sites (Gudipati et al., 2008; Kopcewicz et al., 2007; Porrua et al., 2012; Steinmetz et al., 2006a). The phosphorylation status of the RNAPII CTD and its readout by termination factors are presumably involved in orchestrating the position effect: CTD repeats phosphorylated at Ser5 residues, occurring early in transcription, favor an interaction with Nrd1p of the NNS pathway, whereas Ser2 phosphorylation, occurring later in transcription, promotes the recruitment of the CPF-CFI/II complex. An impact of Ser7 phosphorylation on NNS termination has also been suggested (Kim et al., 2011; Mayer et al., 2010).

The TSS-terminator distance is also a key element in the suppression of human uaRNAs/PROMPTs (Ntini et al., 2013; Almada et al., 2013). No Nrd1p and Nab3p homologs have been described in humans, and it is unknown whether the Sen1p homolog, Senataxin, functions in the transcription termination of lncRNA. Instead, the presence of conventional AAUAAA hexamer-containing pA sites at noncanonical TSS-proximal positions triggers transcription termination and rapid exosomal degradation of these transcripts (Figure 2B). Thus, transcription termination normally associated with stable RNA production provokes degradation when it occurs too early in the transcription process. This suggests that eukaryotic cells are well adapted to preferentially eliminate short transcripts, which are more likely made from spurious transcription events. Although the factors employed may not be strongly conserved between *S. cerevisiae* and mammals, one commonality is the exploitation of transcription termination systems that directly couple to RNA decay activities. Mammalian systems appear to utilize the RNA 5' cap to direct such termination-induced turnover (Andersen et al., 2013; Hallais et al., 2013). This is achieved by a physical link between the nuclear cap binding complex (CBC) and the nuclear exosome targeting (NEXT) (Andersen et al., 2013). Importantly, the CBC also functions in transcription termination of a variety of short transcripts, including PROMPTs. A key factor here is ARS2, a protein that not only connects the CBC to NEXT but also links to transcription termination (Figure 2B). Analogously, the *S. cerevisiae* NNS complex also associates with both the exosome and the CBC (Vasiljeva and Buratowski, 2006). How exactly these connections between the CBC-bound 5' cap and the 3' end-targeting exosome translate into suppression of spurious transcription awaits further experimental detail.

#### **Suppressing Pervasive Transcripts Posttranscriptionally**

Transcription of SUTs, and most likely also XUTs, is terminated by the CPF-CFI/II pathway, circumventing transcription-coupled turnover. Yet SUTs remain sensitive to the nuclear exosome





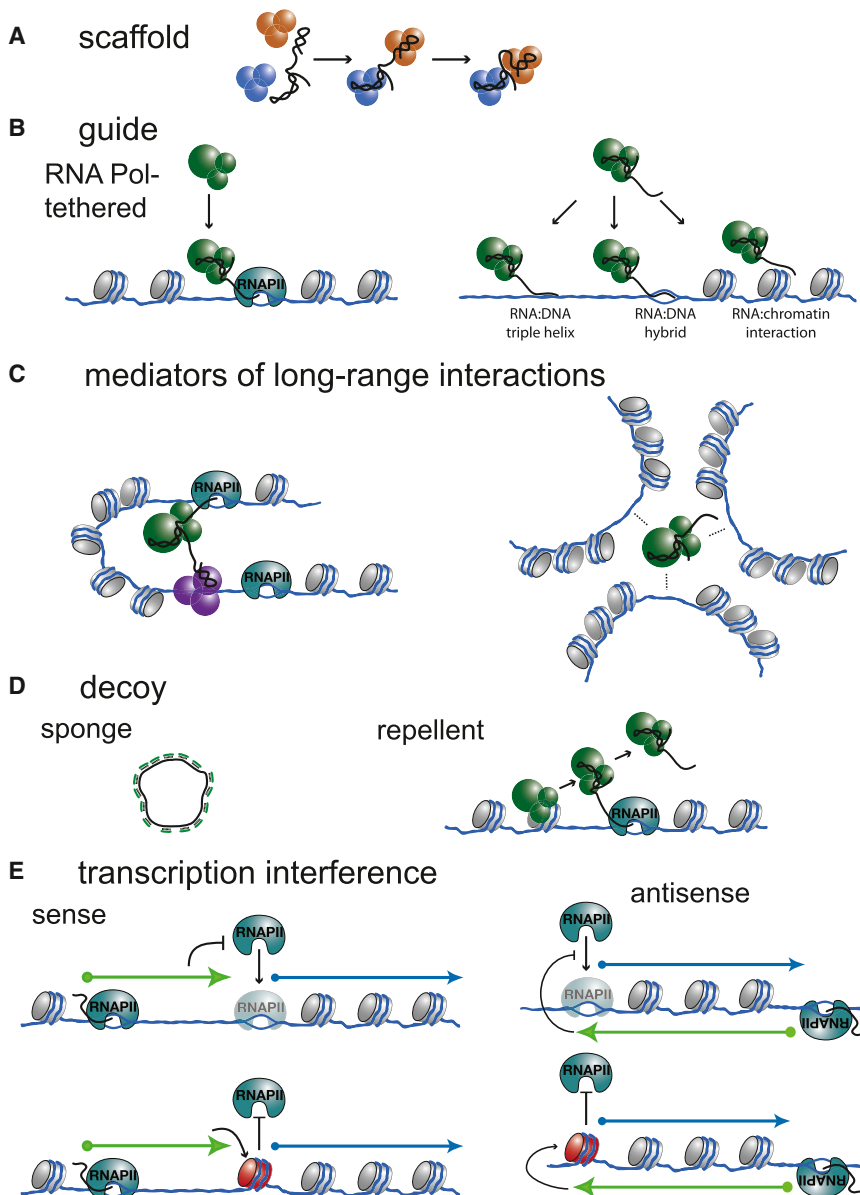
**Figure 2. Control of Pervasive Transcription by Termination-Coupled Degradation**

Schematic representation of cellular transcription termination (upper parts) and RNA degradation (lower parts) pathways, controlling pervasive transcription in *S. cerevisiae* (A) and human (B) cells. Transcription termination by the NNS- and the CBC-ARS2-3' end-processing complexes are illustrated for CUTs (bottom left) and PROMPTs (bottom right), respectively. See text for details. Both systems function most efficiently at shorter distances from TSSs. This is probably due to the phosphorylation status of the RNAPII CTD (not shown here) and possibly also the proximity of the cap (green filled circles). CID, CTD interaction domain. Transcription-coupled degradation pathways are illustrated for CUTs (bottom left) and PROMPTs (bottom right), including the usage of the conserved RNA exosome complex to degrade RNA processively (via Dis3p/DIS3) or distributively (via Rrp6p/RRP6). CUTs are polyadenylated by the TRAMP4 complex, which stimulates degradation by the exosome. PROMPTs are targeted to exosomal degradation by the nuclear exosome targeting (NEXT) complex, which includes the RNA-binding protein RBM7; the Zinc knuckle protein ZCCHC8; and hMTR4, the human homolog of *S. cerevisiae* Mtr4p (Lubas et al., 2011). The CBC-ARS2 (CBCA) complex connects together with the uncharacterized ZC3H18 protein to the NEXT complex, forming CBC-NEXT (CBCN) and creating a link from the 5' cap to the 3' end-targeting RNA exosome.

(Gudipati et al., 2012a), suggesting the existence of other instability determinants for these transcripts. As discussed above, XUTs are efficiently degraded in the cytoplasm by Xrn1p (van Dijk et al., 2011). SUTs and XUTs therefore exemplify the existence of a second line of degradative control of pervasive transcripts that occurs at later steps, possibly tapping into the normal life cycle of mRNA. In human cells, a lncRNA degradation mechanism that is seemingly not associated with transcription termination, has also been described (Beaulieu et al., 2012). This pathway, which is conserved in *S. pombe* (Lemay et al., 2010; Lemieux et al., 2011), depends on the recognition of the pA tail of these RNAs by the nuclear pA-binding protein, PABPN1, which in turn recruits the exosome. Given the redundancy of RNA turnover pathways, it is likely that additional post-transcriptional decay pathways await discovery.

### Lessons from Studies on Pervasive Transcription How Much ncRNA Is Functionally Relevant?

Since new RNA discovery has been spurred by technological rather than genetic advances, functional interrogation has lagged behind transcript annotation. Proper mutational studies are difficult because most lncRNAs are not conserved. RNA interference methods are also challenging, e.g., because it is hard to silence nuclear chromatin-associated molecules. Finally, it is not straightforward experimentally to discriminate between a direct function of the mature lncRNA in question, its nascent precursor, or the transcription process producing it. Perhaps this is why functional mechanisms have only been described for relatively few human lncRNAs (for recent reviews, see Guttmann and Rinn, 2012; Hu et al., 2012; Lee, 2012; Ulitsky and Bartel, 2013; Wang and Chang, 2011). Molecular tasks of lncRNAs



**Figure 3. Suggested Functions of ncRNAs or Their Transcription**

Proposed roles of nuclear lncRNAs (A–D) and their noncoding transcription events (E). See text for details. Note that of the three depicted possible interactions shown in (B), an RNA:DNA triple helix interactions has been demonstrated (Schmitz et al., 2010). (D) An lncRNA sponge is depicted as a circle, which was recently demonstrated to sequester miRNAs efficiently due to its stable nature and the presence of multiple miRNA-binding sites (Hansen et al., 2013) (Memczak et al., 2013). (E) Transcription interference mechanisms may occur via direct obstruction by RNAPII (upper part) or via transcription-mediated induction of repressive chromatin (lower part).

newly detected RNA may harbor, examples are discussed below in which a short half-life may still be compatible with function.

The best-described function of lncRNAs is perhaps that in gene regulation at the level of chromatin. Here, a common theme is the ability of lncRNA to engage with chromatin modifying complexes, such as the Polycomb repressive complex 2 (PRC2), as exemplified by the long studied *Xist* lncRNA, which establishes the phenomenon of X chromosome inactivation (Lee, 2012). However, a reported wealth of suggested PRC2-lncRNA interactions awaits further functional characterization (Guttman and Rinn, 2012; Khalil et al., 2009; Zhao et al., 2010), including a thorough sorting of direct and indirect recruitment mechanisms (Brockdorff, 2013). Apart from their capacity to form flexible entities scaffolding higher-order RNP structure, RNAs are in principle uniquely suited as transcriptional regulators due to their natural tethering to transcription sites. Hence, it has been suggested that

include, but are not restricted to, (1) scaffolds for RNP formation; (2) devices to guide modifying complexes to their molecular targets; (3) mediators of long-range chromatin interactions; and (4) decoys, sponges that sequester other RNAs/proteins, or so-called “repellents,” that facilitate the titration of protein or RNA away from their sites of action (Figures 3A–3D). Moreover, many small RNAs are hosted within larger molecules, and lncRNAs may often simply constitute leftover material derived from the passenger RNA excision process. The generally short half-lives of lncRNAs are often used as an argument to contradict any functional impact. Presently, it is therefore anyone’s guess how much of this plentiful biological material is functionally relevant, and final proof will have to come from careful examination of individual examples. While cellular abundance is certainly a valid criterion when considering the kind of function a given

lncRNAs may have a 5’ end module assembling relevant chromatin complexes, while its 3’ end anchors to the transcribing RNA polymerase establishing regional specificity (Lee, 2012). Interestingly, this model accommodates labile lncRNAs, as these would rapidly be removed after leaving the chromatin template, hereby ensuring site-specificity of the process. It could also explain the relatively few reports existing on *trans*-acting lncRNAs, where target recognition determinants remain a challenge to decipher. In addition to a role as transient recruiters of chromatin modifying complexes, short-lived lncRNAs may also serve as “repellents” to evict factors from chromatin (Figure 3D, right). This has been demonstrated for the *Jpx* lncRNA, which titrates the repressive CTCF protein away from the *Xist* promoter, enabling its transcriptional activation (Sun et al., 2013). The concept has also been shown in *S. pombe*,

where RNA expressed from heterochromatic regions competes with methylated histone H3K9 for binding to the chromodomain of Swi6 (HP1 in human), explaining the dynamic nature of Swi6's interaction with chromatin and how its spreading can be prevented (Keller et al., 2012, 2013). Swi6, in turn, is suggested to target the heterochromatic RNA for rapid degradation off of chromatin, keeping soluble RNA levels low. An interesting mechanistic difference between the eviction of CTCF and Swi6 is that while Jpx RNA binds CTCF with sequence specificity, any RNA will bind the hinge region of Swi6 and cause a conformational change of the chromodomain, resulting in loss of affinity for H3K9-methylated nucleosomes.

A lncRNA subgroup that has recently received ample attention is the eRNAs (Kim et al., 2010; De Santa et al., 2010). In fact, enhancers may constitute the most commonly transcribed noncoding elements in higher eukaryotes (Djebali et al., 2012). However, as pointed out earlier, their overlap with other lncRNA is presently fuzzy (Kowalczyk et al., 2012). Enhancer activity changes during differentiation, and in many ways enhancers are similar to promoters (i.e., by binding GTFs and RNAPII) (Hnisz et al., 2013). One model suggests that RNAPII loaded at enhancers scans the DNA template (while producing eRNA) for available promoters (Dean, 2006). Another posits that genomic sequences between enhancers and promoters loop out to allow their physical contact (Calo and Wysocka, 2013), enhancing promoter access to PIC components and RNAPII, possibly via its relocation to a nuclear environment that is favorable for transcription. As promoter-enhancer loops also accommodate long-range interaction, sometimes between different chromosomes, this can presumably also occur without RNAPII scanning (Sanyal et al., 2012). While enhancer transcription may serve to maintain an available NDR as well as to attract components important for chromatin looping (Kaikkonen et al., 2013), it has been less clear whether eRNAs themselves contribute to function. Interestingly, however, recent papers suggest that eRNAs, together with their protein partners, may bridge promoter-enhancer connections, serving a structural role in establishing or maintaining these links (Figure 3C) (Lai et al., 2013; Lam et al., 2013; Li et al., 2013; Melo et al., 2013; Mousavi et al., 2013). The generality of these observations, as well as the mechanisms involved in creating the initial contact mediated, or exploited, by these low-copy RNA molecules, await further investigation.

#### Functional Transcription Events

Contrary to higher eukaryotes, only circumstantial evidence for a regulatory function of pervasive transcripts in *S. cerevisiae* is available. Although direct effects of the nascent RNA are difficult to rule out, most reported instances describe a noncoding transcription event running through an mRNA-gene promoter and preventing, or delaying, the activation of that promoter (Bumgarner et al., 2009, 2012; Castelnuovo et al., 2013; Hongay et al., 2006; Houseley et al., 2008; Martens et al., 2004, 2005; Pinskaya et al., 2009; van Werven et al., 2012). This was first demonstrated by the *SRG1-SER3* regulon, where sense transcription from the upstream noncoding *SRG1* locus invades the downstream *SER3* promoter and prevents its activation in the presence of serine (Figure 3E, left image) (Martens et al., 2004, 2005). At low-serine conditions, *SRG1* transcription is off, allowing *SER3* to be expressed. Transcription interference

regulation also occurs at the *IME4* gene, required for the initiation of meiosis. In this case antisense transcription, overlapping the *IME4* promoter, prevents *IME4* activation in haploid cells (Figure 3E, right image) (Hongay et al., 2006). In another interesting example, the *STE11* gene is regulated by a complex interplay of upstream concurrent sense and antisense transcription events, where a negative *STE11* regulator (*ICR1*) is itself regulated by transcriptional interference from another noncoding gene (*PWR1*) (Bumgarner et al., 2009, 2012). The overall notion of crossregulation by noncoding transcriptional interference was generalized by Steinmetz and colleagues, who took a genome-wide approach to report numerous such cases (Xu et al., 2011). Noncoding transcription can cause interference in several possible ways. A common theme is the establishment of a chromatin state that represses transcription initiation (but not elongation) at promoters that depend on chromatin remodelers/modifiers for activity (Figure 3E, lower parts). The factors required for such repression are often also involved in preventing intragenic transcription. At the *SRG1-SER3* regulon, *SRG1* transcription increases nucleosome occupancy at the *SER3* promoter, reducing the NDR required for activation (Hainer et al., 2011). This transcriptional repression relies on the chromatin remodeling activities FACT and Spt6p/Spn1p. Similarly, noncoding transcriptional repression of the *IME1* master regulator of meiosis utilizes the establishment of repressive methylation marks, H3K4me2 and H3K36me, deposited by the Set1p and Set2p methyltransferases. These marks, in turn, recruit the HDAC complexes SET3C and RPD3C(S) (Kim and Buratowski, 2009; Kim et al., 2012; van Werven et al., 2012). A similar mechanism, involving histone methylation and deacetylation, has been described for the attenuation of the *GAL1-GAL10* locus by cryptic transcription (Houseley et al., 2008; Pinskaya et al., 2009). Transcription interference could in principle also occur without the addition of repressive marks, but directly by obstructing transcriptional activators from binding (Figure 3E, upper parts). However, considering the low frequency with which most *S. cerevisiae* genes are transcribed and the generally low level of regulatory transcription as measured by single molecule RNA fluorescent in situ hybridization (FISH) assays (Bumgarner et al., 2012; van Werven et al., 2012), it is hard to imagine that the mere eviction of *trans*-activators by RNAPII would result in a stable repressive state.

Regulatory noncoding transcription, that does not elicit interference, also occurs in *S. cerevisiae*. For instance, transcription of the *URA2* and *IMD2* genes may start at two sets of different TSSs, with only the more downstream one generating functional transcript (Kuehner and Brow, 2008; Thiebaut et al., 2008). When expression of the gene is required, transcription initiation switches from the upstream to the downstream TSS, thus skipping termination and degradation signals and allowing expression of a functional mRNA. In these cases, "on-off" gene transitions translate into the regulated selection of TSSs, and the ncRNA produced is a byproduct of such regulation. Early transcription termination events, generating CUT-like transcripts overlapping the 5' region of an mRNA, can also negatively regulate gene expression (Arigo et al., 2006b; Steinmetz et al., 2006b; Kim and Levin, 2011). In this case, the produced ncRNA is again not part of the regulation mechanism.

The elusive nature of ncRNA function in *S. cerevisiae* is highlighted by the *PHO84* gene. Here, an unstable asncRNA was suggested to trigger chromatin modification and histone deacetylation of the *PHO84* gene promoter (Camblong et al., 2007, 2009). The *PHO84* asncRNA accumulates upon lowering Rrp6p activity, conditions under which expression of the sense *PHO84* is strongly repressed. *Trans*-expression of the *PHO84* asncRNA could repress the *PHO84* sense transcript, presumably by associating with the *PHO84* locus, suggesting a role for the RNA (Camblong et al., 2009). However, single-molecule RNA FISH analyses recently revealed that sense and antisense *PHO84* RNA never coexist at the *PHO84* locus; *PHO84* asncRNA is efficiently exported to the cytoplasm (Castelnuovo et al., 2013). In a revised model, Rrp6p suppresses *PHO84* silencing, directly or indirectly, by influencing early (NNS-dependent) termination of *PHO84* antisense transcription, preventing transcribing RNAPII from reaching the *PHO84* promoter. Thus, while the expression of the *PHO84* asncRNA in *trans* inhibits *PHO84* gene activity, this inhibition does not require the Hda1/2/3 complex and is therefore unlikely to occur via the same mechanism (Camblong et al., 2009). This finding begs the question of whether other gene silencing examples observed upon depletion of *S. cerevisiae* RNA decay factors are indeed mediated by the upregulated ncRNA molecules or are triggered by alternative mechanisms. In one example, stabilization of the short RTL asncRNA by deletion of the *XRN1* gene was proposed to regulate *TY1* transposition by directly decreasing expression of the sense *TY1* RNA (Berretta et al., 2008). How the cytoplasmic stabilization of RTL asRNA leads to a regulatory nuclear role remains to be clarified. The same challenge applies to explain the suggested repressive roles of a set of newly identified XUTs on their cognate sense genes (van Dijk et al., 2011). Finally, in addition to regulating the expression of nearby genes, cryptic transcription and its establishment of chromatin domains has also been implicated in maintaining rDNA copy number (Houseley et al., 2007; Vasiljeva et al., 2008b). However, the mechanism underlying repression of recombination between repeats and whether the ncRNA has a role remain unclear (Houseley et al., 2007).

Perhaps direct lncRNA-mediated regulation simply plays a larger role in metazoan nuclei, as claimed by their suggested regulatory effects on cellular differentiation and specification programs (Guttman et al., 2011). Indeed, no PRC2 homologs exist in *S. cerevisiae* cells and upstream activating sequences are, due to the highly compact genome, usually positioned no more than a few hundred base pairs away from the regulated gene. Still, although suggestions of lncRNA-mediated functions have dominated reports from higher eukaryotic model systems, other studies propose that noncoding transcription may play an equally important role. For example, it was recently demonstrated that transcription of the *Airm* (antisense *Igf2r* RNA non-coding) locus, and not its lncRNA product, governs the paternal imprinting of the *Igf2r* gene (Latos et al., 2012). By gradually truncating the *Airm* transcription event via the insertion of transcription-terminating pA site cassettes, it was shown that early transcription termination prevents *Igf2r* silencing. Conversely, positioning the *Airm* promoter in close proximity (700 bp) to the *Igf2f* promoter maintained silencing, which was also obtained when “*Airm* RNA information” was omitted altogether. Another

recent example involves the highly expressed metastasis associated lung adenocarcinoma transcript 1 (*MALAT1*) ncRNA, previously suggested to be involved in nuclear speckle formation (Tripathi et al., 2010). Remarkably, however, in a mouse loss-of-function model, omission of the *MALAT1* promoter indicated that *MALAT1* RNA is dispensable for this process (Zhang et al., 2012). Instead, the expression of neighboring genes was mildly upregulated, suggesting that *MALAT1* transcription may exert a regional repressive effect. Consistently, *MALAT1* locus positioning and high transcription activity are conserved, whereas *MALAT1* primary RNA sequence is not. Like the *PHO84* asncRNA from *S. cerevisiae*, *MALAT1* therefore constitutes an example in which the monitored steady-state localization of an lncRNA does not readily reveal its mechanism of action.

### Conclusions

Cells with both relatively small (*S. cerevisiae*) and large (mammalian) genomes tolerate pervasive transcription remarkably well. Although a first line of nucleosome defense is established to prevent inappropriate RNA polymerase initiation, an equally important protection downstream of initiation relies on the widespread use of transcription termination pathways and the massive employment of RNA decay activities to dampen the accumulation of unwanted transcripts. Such strategy to invest resources in cleaning up may at first glance seem wasteful. However, many of the machineries, used to suppress unwanted products, are not earmarked to this task but rather “reused” from processing and turnover reactions of the more conventional pool of cellular RNA. They are also enrolled for specific regulation mechanisms, as exemplified by the NNS-TRAMP-exosome termination-degradation pathway, which is directly involved in the downregulation of the *NRD1* gene itself (Arigo et al., 2006b), of the ribosomal protein gene *RPL9B* (Gudipati et al., 2012b), and probably of many other genes (Tuck and Tollervey, 2013). Moreover, a broad expression of genomes is likely to hold advantages over an investment in fail-safe transcription initiation control. Clearly, it offers the huge potential of regulatory transcription, for example through the mechanism of transcription interference, for which functional examples conceivably have been evolved from a plethora of originally neutral neighboring and/or overlapping transcription events. Moreover, flexible transcription initiation may also leave more room for malleable regulation. In particular, refraining large genomic regions from being completely silenced may prevent the formation of too tightly compacted chromatin domains, which would otherwise be hard to reopen. Finally, pervasive RNAs, or their transcription, may aid the 3D shaping of chromosomes into active and repressive domains.

Another likely advantage of pervasive transcription is the existence of a large mass of unstable transcripts, whose levels are easily modified, offering the ability of regulation at the level of RNA turnover; i.e., alteration of the activity of a degradation machinery might be sensed due to an ncRNA increase, resulting in factor titration and the generation of complex effects on cellular physiology. Pervasive transcription also exposes the transcriptome to selective pressure, which provides a repertoire of raw material for natural selection at the level of RNA. Although this is still quite speculative, the wealth of transcripts arising from



enhancers, promoters, and other regions of open chromatin may constitute ideal precursors for the emergence of new RNA genes. Critical steps in evolving longer RNAs with higher steady-state levels could involve changes in pA- and U1-site sequences, creating a pA/U1 axis compatible with stable RNA production (Almada et al., 2013; Ntini et al., 2013). In interesting support for such a scenario, Almada and colleagues found evidence for an evolutionary age-dependent progressive gain of U1 sites and a concomitant loss of pA sites within the TSS-proximal regions of mouse genes (Almada et al., 2013), implying a selection against promoter-proximal transcription termination over time. A similar trend was observed upstream of mouse promoters, suggesting that a subset of these regions could move toward more robust expression. Longer transcription units and/or RNAs are more “dangerous” to the cell, as they are more likely to interfere with neighboring regions; however, for the same reason, they have larger regulatory potential. Longer RNAs, or the events producing them, might therefore represent a step in the evolution of novel function from the “primordial soup” of ubiquitous cryptic transcription.

All organisms carry a significant number of so-called “orphan” genes, i.e., protein-coding sequences without recognizable homologs in distantly related species. A prevailing model was that orphan genes arose by a duplication-divergence mechanism (Domazet-Lošo and Tautz, 2003). Indeed, the alternative “de novo gene emergence,” without initial raw material in the form of a duplicated gene, seemed unlikely because it would imply the simultaneous acquisition of both transcriptional and translational capacities for a single sequence. The discovery of pervasive transcription has indeed made the possibility for de novo evolution of genes a more likely hypothesis (Carvunis et al., 2012; Siepel, 2009; Tautz and Domazet-Lošo, 2011). Yet, it remains an ongoing challenge to decipher which of the many pervasive transcription events are simply tolerated because they are not harmful, and which are evolutionary maintained because they are regulatory useful.

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