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Although studies of micro-RNA have dominated the fields of molecular and cellular biology in recent years, a surprisingly wide array of cellular functions is also associated with large noncoding RNA (lncRNA). This growing family of diverse regulatory RNAs now includes at least six members (Table 1): steroid receptor activator RNA (SRA), 7SK small nuclear RNA, 6S RNA, the small noncoding RNA polymerase III transcript called B2 RNA, heat shock RNA-1 (HSR1), and Evf-2 noncoding RNA. SRA appears to be an important co-regulator of nuclear receptors (NRs) (*1*). 7SK, together with the HEXIM-1 protein (a protein whose expression is increased in vascular smooth muscle cells in response to hexamethylene bisacetamide), is involved in repression of the activity of transcription elongation factor b (P-TEFb) protein kinase and transcription mediated by RNA polymerase II (*2*). B2 and 6S repress transcription via direct binding to RNA polymerase II and bacterial RNA polymerase during stress, respectively (*3*, *4*). HSR1 cooperates with translation elongation factor eEF1A to activate heat shock transcription factor 1 (HSF1) (*5*). Each of these lncRNAs requires cooperation with a protein partner to exert its effect on transcription, either directly (B2 and 6S) or indirectly (SRA, HSR1, and 7SK). Two recent reports describing SLIRP, an SRA-interacting NR co-repressor (*6*), and Evf-2, a lncRNA involved in the transcription of homeodomain-containing proteins Dlx-5 and Dlx-6 (*7*), add to our understanding of the processes controlled by lncRNA.

lncRNA	Organism	Function	Protein partner	Refs.
SRA	Mammals	NR co-activator	SRC-1, SLIRP, SHARP, SKIP, and others	(*1*)
Evf-2	Mouse	Activator of DLX-5/6 enhancer	Dlx-2	(*7*)
7SK	Human cell lines	Suppressor of P-TEFb (general transcription inhibition)	HEXIM-1	(*2*)
B2	Mouse	General transcription inhibitor	RNA polymerase II	(*3*)
6S	Bacteria	General transcription inhibitor	Bacterial RNA polymerase	(*4*)
HSR1	Mammals	Activator of HSF1 (master regulator of heat shock genes)	eEF1A	(*5*)

Table 1. Large noncoding RNAs and their roles in transcription regulation.

NRs are a diverse family of proteins that interact with cognate ligands in soluble form. NR signaling plays an important role in tumorigenesis, especially in hormone-dependent cancers (*8*). SRA was originally described as a transcriptional coactivator for the steroid hormone receptor. It was identified as an RNA component of the steroid receptor coactivator–1 (SRC-1) complex (*1*). RNA-binding DEAD box proteins p68 and p72 act as estrogen receptor (ER)–α coactivators through the N-terminal activation function–1 domain (AF-1) of the ER (*9*). SRA also enhances transcriptional activity of ER-α, but not ER-β , through its AF-1 domain, in a manner that is independent of other protein activators (*10*). Activation is mediated by mitogen-activated protein kinase (MAPK) and requires serine-118 of the ER-α AF-1 domain. The expression of SRA is increased in many human tumors. However, transgenic mice expressing increased amounts of human SRA, although displaying increased proliferation and differentiation in SRA-expressing tissue, do not show malignancies (*11*). Thus, overexpression of SRA itself is insufficient to induce tumorigenesis. The co-repressor SHARP [SMRT (silencing mediator for retinoid and thyroid hormone receptors) and HDAC1(histone deacetylase-1)–associated repressor protein] binds SRA and represses its function in activating ER-dependent transcription, suggesting that SRA/protein interactions are important in modulating NR activity and signaling (*12*).

Hatchell *et al.* (*6*) identified SLIRP (SRA stem-loop interacting RNA-binding protein) as a NR co-repressor. They used STR7, an 89-nucleotide stem-loop SRA structure that augments ER activation (*13*), as bait to find specific SRA-interacting proteins in nuclear extracts of various cell lines. The results of RNA electromobility shift essay and ultraviolet cross-linking studies indicate that nuclear proteins bind SRA STR7 in vitro. A yeast three-hybrid screen was used to isolate a cDNA clone that encodes a 109–amino acid protein that consists almost entirely of an RNA recognition motif (RRM). The RRM is one of the most abundant protein domains in eukaryotes and is present in an estimated 2% of all gene products. This, combined with the extreme conservation of the motif, points to its ancient origin and fundamental importance. Interestingly, the high sequence similarity between SLIRP and SHARP implies that both proteins may act through the same target domain of SRA; that is, STR7. The expression pattern of SLIRP varies between different cell lines and tissues, and in some cells, protein abundance differs dramatically from that of the corresponding mRNA. Immunohistochemistry data suggest that SLIRP is more abundant in some tumor cells than in normal tissue.

The authors show in vivo association between SLIRP and SRA in several cancer cell lines, using an immunoprecipitation RT-PCR (reverse transcriptase–polymerase chain reaction) technique. Depletion of SLIRP by small interfering RNA (siRNA) resulted in increased amounts of SRA copurifying with SRC-1, another in vivo interaction target of SRA. This implies that a balance may exist between SRA complexes that contain either SRC-1 or SLIRP and that this balance may affect the co-regulating properties of SRA. The function of SLIRP as a repressor of SRA-mediated NR coactivation was demonstrated in a series of

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extensive cotransfection experiments. Cotransfection of SLIRP with various NR reporters and SRA resulted in the repression of 17β-estradiol (E2)-, glucocorticoid-, androgen-, thyroid-, and vitamin D receptor–induced transcription. Conversely, cells in which SLIRP expression was reduced exhibited a 10-fold increase in dexametazone (Dex)–responsive promoter activation. Taken together, these data strongly suggest that SLIRP is a general co-repressor of the NR superfamily. Colocalization of the SLIRP gene with that encoding another NR co-repressor, SKIP, both located on human Chr14q24.3, raised the intriguing possibility that two factors are coordinately regulated and act together in ER signaling. This, however, was not the case, as SKIP and SLIRP appeared to have opposite effects on ER signaling. The SLIRP-mediated repression was abrogated by mutations in the RRM domain. Similarly, mutating SRA STR7, but preserving the overall structure, decreased SRA-dependent activation by 70% and completely prevented SLIRP from functioning as a co-repressor. These data provide strong support for the idea that there is direct in vivo interaction between SLIRP and STR7.

Hatchell *et al*. used chromatin immunoprecipitation (ChIP) assays to show that SLIRP, but not HuD (another RRM-containing RNA-binding protein), was recruited to E2- and Dex-responsive promoters. This recruitment appeared to be dependent on SRA, because SRA siRNA lowered the amount of SLIRP recruited to the Dex-responsive promoter. Moreover, SLIRP seems to be responsible for the recruitment of the NR co-repressor NCoR to E2 promoters. NCoR is a broad-spectrum NR promoter repressor. Thus, it appears that SLIRP plays a dual role in the repression of NR promoters. First, it competes for SRA binding with SRC-1 (*14*) and other SRA-binding NR co-modulators. Second, SLIRP recruits NCoR to NR promoters.

Surprisingly, SLIRP was found to be predominantly mitochondrial, which is consistent with the presence of an N-terminal mitochondria localization signal in the SLIRP sequence. The mitochondrial localization was critical for the co-repressor function of SLIRP. These findings correlate with increased expression of SLIRP in energy-demanding tissues such as skeletal muscles, heart, and liver. It is not clear whether SLIRP interacts with specific mitochondrial RNA targets other than SRA. Combined with the SLIRP-mediated repression of peroxisome proliferator–activated receptor γ (PPARδ)–mediated signaling, these results indicate the possible involvement of SLIRP in the regulation of lipid and energy metabolism.

In another striking example of trans-acting lncRNA, Kohtz's group has identified an alternatively spliced form of Evf-1 noncoding RNA, which they called Evf-2 (*7*). Evf-1 was originally found as a downstream target of the patterning protein Sonic hedgehog (*15*). Sonic hedgehog homolog (SHH) is one of three proteins of the mammalian hedgehog protein family (*16*). It is an extensively studied ligand of the hedgehog signaling pathway, which regulates vertebrate organogenesis. Recently, Kohtz and Fishell (*15*) demonstrated that Evf-1 is a 2.7-kb polyadenylated ncRNA, originating upstream of the mouse gene encoding Dlx-6, whose expression correlates closely with that of Dlx-6. Vertebrate Dlx proteins belong to the homeodomain protein family and play a crucial role in neuronal differentiation and migration (*17*). They are also involved in craniofacial and limb patterning during development. Dlx genes are found in two-gene clusters divided by a conserved intergenic enhancer in the loci encoding the Dlx-5 and Dlx-6 genes and the Dlx-1 and Dlx-2 loci (*18*). These enhancers, called ei and eii, belong to ultraconserved noncoding sequences found in vertebrates, featuring an unusually high sequence similarity between the enhancers as found in humans, rodents, and zebrafish (*7*, *19*). Evf-2 is transcribed from ei, and the difference from Evf-1 results from an alternative transcription initiation site, alternative splicing of exon 3, and alternative polyadenylation. The two forms share a ~2.5-kb region of identity and differ in their 3′ and 5′ sequences. The expression of Evf-1 and -2 (Evf-1/2) and Dlx-5 and -6 (Dlx5/6) follows a similar pattern, as might be expected from their overlapping genomic organization. Conservation of exon 2, which is unique to Evf-2, suggests a functional role for the Evf-2 splice variant. Studies by Feng *et al*. revealed that Evf-2 and Dlx-2 cooperate to activate both the ei and eii Dlx-5/6 enhancer. There was no apparent synergy between the actions of the ei and eii enhancers, and both were targeted by Evf-2 and Dlx-2, with the activity of ei being stronger than that of eii.

Functional Evf-2 is a single-stranded RNA. Neither antisense Evf-2 nor a double-stranded form of Evf-2 can efficiently support activation of the Dlx-5/6 enhancer. siRNA directed against Evf-2 diminished trancriptional activation in a dose-dependent manner and caused nearly complete degradation of Evf-2. The minimal functional domain of Evf-2, retaining 85% of full-length activity, was mapped to nucleotides 1 to 395. This minimal region contains one open reading frame encoding a conserved 19–amino acid peptide. Complexes between Evf-2 and Dlx-2 were detected by immunoprecipitation followed by RT-PCR in both cell lines and embryonic nuclear extracts. In single cells prepared from the ventral telencephalon of an embryonic rat on day 12.5 of development, Evf-2 and Dlx-2 colocalized in two spots in the nucleus. Taken together, these data convincingly demonstrate that the Evf-2 transcription-enhancing activity requires single-stranded sense RNA. The specificity of the process is determined by Dlx-5/6 target DNA, Dlx homeodomain protein, and cell type. The authors propose that Evf-2 RNA belongs to a new class of lncRNA, which they call transcription-regulating ultraconserved ncRNA (trucRNA).

Obviously, there are similarities between the specificity and co-regulator functions of the two lncRNAs described in these studies. Both SRA and Evf-2 require the cooperation of a specific protein partner to exert their trans-regulating effect. Other examples of lncRNA regulating transcription in a similar way include 7SK and B2. Both, however, are transcriptional repressors (Table 1). Hatchell *et al*. demonstrated that the mode of action of SRA is actually dependent on the specific protein-binding structure in RNA. The presence of SLIRP effectively changes the state of the NR promoter to a repressed one, whereas its removal results in a switch of the promoter to the active state. Another lncRNA co-activator (HSR1), which requires cooperation of a protein partner, eEF1A, to activate HSF1, the master transcription factor for heat shock and other stress-related genes, was described recently (*5*). Despite apparent differences in the mechanism (HSR1 and eEF1A facilitate and mediate the trimerization of HSF1 but not its transactivation capacity), the involvement of lncRNA in transcriptional regulation seems to be a much more general phenomenon than previously appreciated. One of the possible mechanisms of action of such RNAs may be to provide a scaffold for the assembly of functional transcription regulation complexes, or to stabilize such complexes, or both. Evf-2 is the first example of an lncRNA that is developmentally regulated and specifically affects the transcription

of homeodomain proteins. Other ultraconserved noncoding regions and enhancers may be similarly transcribed to generate regulatory lncRNA, given the abundance of ultraconserved noncoding regions in the vertebrate genome (*20*). Future studies should concentrate on these regions to identify functional transcripts originating from enhancer or conserved noncoding elements that play transcriptional regulatory roles in various developmental and signaling pathways.

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