In vivo-applied functional RNAs as tools in proteomics and genomics research

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Non-natural, functional RNA molecules, such as short interfering (si) RNAs, aptazymes, maxizymes and intramers, allow modulation of gene function at the mRNA or protein level. This review discusses recent advances made in the expression and application of these functional RNAs and illustrates how engineered, intracellularly active RNAs can serve as promising tools for understanding the function of genes and their protein products or as potential therapeutic agents.

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Selective modulation of genes and gene products in the context of the living cell is of utmost importance for elucidating biological function of individual genes, proteins and protein networks. Owing to the massive amount of data accumulated through various genome-wide sequencing projects, there is an urgent need to develop novel tools for the discovery and validation of drug targets, and to open avenues for newer concepts in drug therapy and research. Recent developments indicate that functional, synthetic RNA molecules, such as siRNAs, protein-binding RNA motifs (aptamers) and catalytic RNAs (ribozymes, maxizymes and aptazymes [1]), can serve as effective and highly specific tools in functional genomics and proteomics. Although synthetic versions of functional RNAs have proved immensely useful, considerable efforts have also been invested into developing systems that allow endogenous expression to assist in the intracellular application of these molecules. Such endeavours are expected to provide entry into rapid loss-of-function phenotypic analysis of genes and proteins in living cells or transgenic animal models, specific targeting of disease-related transcripts, and might lead to innovative approaches in gene therapy.

Genetic knockdown technologies, such as antisense, ribozymes and RNA interference (RNAi), permit inactivation of genes without directly manipulating the gene of interest [2–4]. Alternatively, intracellularly expressed RNA aptamers (intramers) have been used to modulate gene function at the protein level. Recent advances achieved in intracellular expression and selected applications of functional RNA molecules are addressed in this review.

Intracellularly expressed catalytic nucleic acids

Ribozymes and deoxyribozymes have been used successfully to suppress the expression of therapeutically relevant genes in various cells, tissues and organisms, to analyse the function of genes in eukaryotic cells, and in gene repair. Extensive studies have proved that ribozymes are well suited for these purposes, although certain problems, such as cleavage efficiency, *in vivo* stability, cleavage-site selection and target accessibility, remain to be optimized [4–6]. A target site on an mRNA is often rendered inaccessible to ribozymes owing to complex secondary and tertiary nucleic acid structures. To overcome this limitation, Taira and colleagues have engineered RNA–protein hybrid ribozymes that can access sites on target RNA, independently of its folding [7]. Hammerhead ribozymes (HHR), embedded within the 3′-terminus of human gene encoding $tRNA^{Val}$, were used as active constructs to enable RNA polymerase III-dependent RNA expression. This HHR–tRNA construct was further appended at the 3′-end to a cytoplasmic RNA transport signal – the constitutive transport element (CTE) from simian type D retroviral RNA. Strong interaction of this element with intracellular RNA helicases enables RNA-binding, -sliding and unwinding activities of helicase-bound ribozyme hybrids at the target mRNA. The rationale for this design relies on the guidance provided by helicase in resolving complex RNA structural features, followed by steering ribozyme to the mRNA cleavage site. CTE–ribozyme hybrids were targeted against the TAR region of HIV-1 long-terminal repeat and mRNA encoding mouse procaspase CPP32, structures that are otherwise difficult to resolve for effective ribozyme cleavage. The CTE–ribozyme constructs were expressed under the control of RNA polymerase III, after the transfection of mouse NIH3T3 cells with appropriate expression plasmids. Inhibition of LTR-driven luciferase activity and quantification of procaspase-3 levels revealed that CTE–ribozyme constructs were considerably more active and efficient in targeting mRNA cleavage than was the expressed ribozyme alone. As the observed activity was not dictated by increased expression or stability of ribozyme transcript, the authors concluded that enhanced gene inactivation was owing to the unwinding activity of helicases that are recruited by the transport element. Yokoyama and coworkers also used a similar HHR expression system, based on the gene encoding $tRNA^{Val}$, to probe the roles of p300 and cAMP-response element binding protein-binding proteins as transcriptional coactivators in

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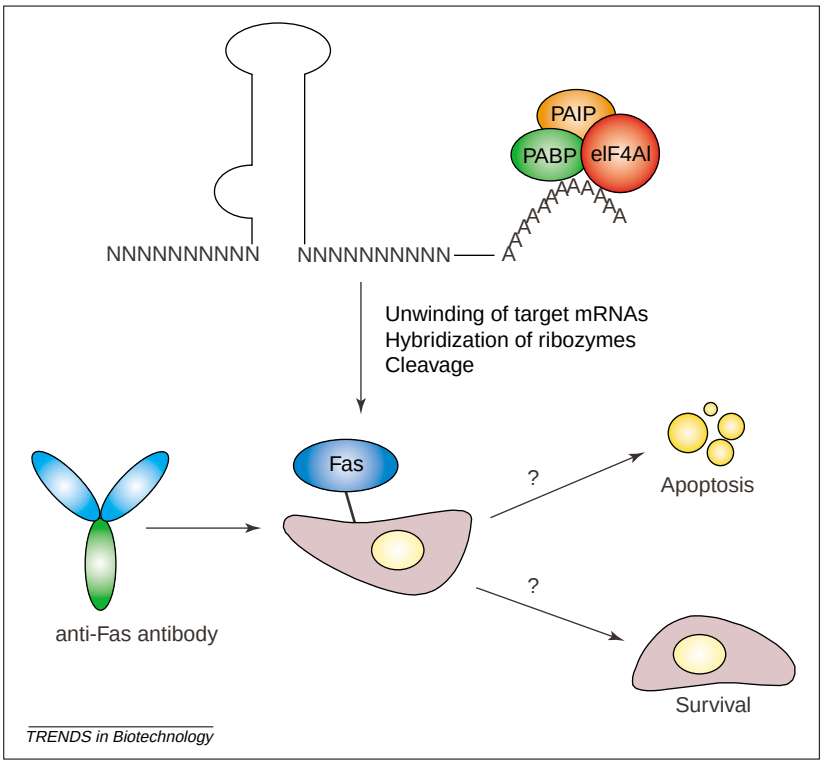

Fig. 1. Recruitment of RNA helicase through poly(A)-connected ribozyme libraries for mRNA target accessibility. The question marks indicate that depending on the mRNA targeted by the ribozyme, cells either survive or die from apoptosis. Abbreviations: PABP, poly(A)-binding protein; PAIP, PABP-interacting protein-1.

retinoic acid-induced differentiation, cell-cycle exit and apoptosis of embryonal carcinoma cells [8].

The concept of RNA helicase-associated ribozymes was extended to identify genes that function in the Fas-mediated and tumor necrosis factor-α (TNF-α)-mediated apoptosis pathways [9,10]. This was achieved by substituting mRNA-binding arms of the ribozyme for randomized sequences, and using a polyadenosine [poly(A)] motif for recruiting helicase eIF4AI to the ribozyme via interactions associated with poly(A)-binding protein (PABP) and the PABP-interacting protein-1 (PAIP). In one study on PABP [9], a transient expression plasmid expressing helicase-associated ribozyme libraries was applied to identify genes involved in Fas-mediated apoptosis (Fig. 1). This analysis was performed in HeLa-Fas cells using mRNA of the proapoptotic factor, Fas-associated death domain (FADD) protein, as the target. In this study, ten nucleotides in each ribozyme-binding arm were randomized and introduced into cells by transfection with retroviral vectors that encode for randomized ribozyme-poly(A) hybrid libraries. Interestingly, four genes known to be involved in the Fas-mediated apoptosis pathway (FADD itself and the caspases 3, 8 and 9) were among 119 identified positive clones. Special mention must be made regarding judicious use of the poly(A) motif, which offered two major benefits: recruitment of an endogenous RNA helicase and highly efficient colocalization with target mRNA.

Hybrid ribozyme libraries, containing randomized substrate-binding arms, were stably introduced into human breast carcinoma cultured cells (MCF-7) using a retroviral expression system. Infected cells were treated with recombinant TNF-α and cycloheximide to potentiate TNF-α-mediated apoptosis. Surviving clones were collected after 72 h and genomic DNAs containing ribozymes were isolated, PCR-amplified with ribozyme-specific primers, and the resulting ribozyme sequences were determined. Cognate target gene sequences of various ribozymes were located and identified by database searches by this methodology. Consequently, several genes including TNF receptor 1-associated death domain (TRADD), caspases and others with proapoptotic function in TNF-α-induced apoptosis were identified. In addition, up to 30 new target genes showed significantly reduced apoptosis after treatment with TNF-α. Importantly, it was demonstrated that poly(A)-linked ribozyme pools led to fewer false positives, thus validating the claim that such hybrids are more effective for gene function studies than are ribozymes that are not poly(A) linked. It is currently not evident whether the use of CTE or poly(A) modification is equivalent or whether one is superior than the other. However, the poly(A)-connected ribozymes have the advantage of recruiting eIF4AI, a helicase utilized in general translation, thereby potentially increasing the efficiency of colocalization of these ribozymes with the target mRNA.

Expression of siRNAs

siRNAs are RNA duplexes of 21–23 nucleotides that can inhibit expression of mammalian genes with complementary sequences by RNAi [11–13]. Within a relatively short time-span, several research groups have independently constructed vector systems that allow stable intracellular expression of siRNAs. Such results have propelled development of an extremely powerful method for rapid analysis of loss-of-function phenotypes in a variety of mammalian cells. Moreover, it is also possible to envisage a futuristic approach for the generation of transgenic animals.

Brummelkamp *et al.* [14] have developed an expression system called pSUPER (suppression of endogenous RNA) to generate endogenous siRNAs in mammalian MCF-7 cells for the suppression of the *CDH1* gene required for the activation of the anaphase-promoting complex. This vector encodes RNAs transcribed under the control of polymerase-III H1-RNA promoter, which folds back into a strategically designed stem-loop structure, leading to expression of siRNAs after further intracellular processing. Three constructs, differing in loop length, were evaluated and interestingly, an endogenously expressed hairpin closed by a nine nucleotide loop resulted in 90% reduction in the expression of CDH1 protein, similar to the results obtained with synthetic siRNA 21-nucleotide duplexes. It was shown that pSUPER vector could efficiently mediate stable suppression of gene expression over long periods, without any detectable cellular toxicity.

Several independent studies also constructed cassettes for siRNA expression of fold-back stem-loop structures, under the control of U6 promoter [15–18]). mRNA encoding human lamin A/C was targeted by Paul *et al.* [15] and a cassette coding for 19 base-pair

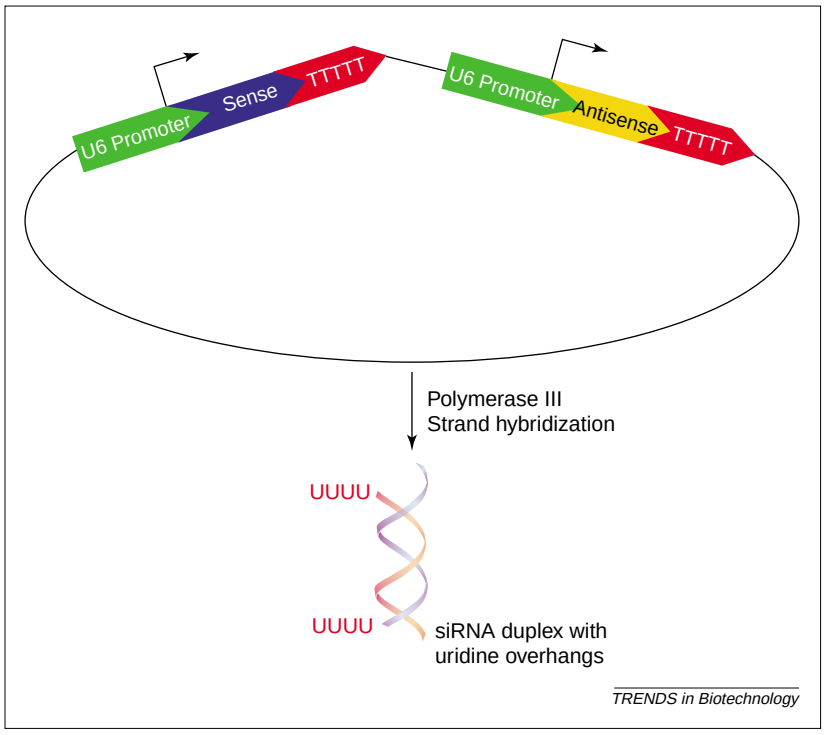

Fig. 2. Intracellular expression of siRNAs under control of U6 promoter.

helical siRNA stem, containing a 3′-UUUU overhang at the end of antisense strand acting as a terminator sequence, was expressed in HeLa cells. RNA sense- and antisense strands were connected by a stable, highly structured UUCG tetraloop, thereby enabling synthesis and efficient *in vivo* annealing of double stranded siRNA. The level of lamin A/C expression was directly visualized in cells using anti-lamin A/C antibodies and the percentage of lamin A/C in transfected versus non-transfected cells was quantified. Comparable reductions in lamin A/C levels were observed for both anti-lamin siRNA and its hairpin modification.

The expression of modified siRNA duplexes containing an inherent continuous stem-loop structure was chosen to facilitate annealing of two strands and to circumvent the probability of inefficient siRNA production. High-yielding endogenous expression of siRNAs as independent strands has been achieved under the control of a U6 promoter [19,20]. In contrast to other systems, these RNA expression cassettes used two independent U6 promoters – for sense- and antisense strands – in which a short stretch of uridine residues terminated the synthesized RNA sequence (Fig. 2). In one report, siRNA targeted mRNA encoding the fusion of HIV-1 Rev and enhanced green fluorescent protein [19]. Two sites on the fused *rev* gene were tested for siRNA accessibility. Both sites were found to be active and afforded specific potent inhibition of HIV-1 replication. Miyagishi and Taira [20] also reported a similar strategy for mammalian siRNA expression system, and RNAi-aided silencing of an endogenous gene coding β-catenin was demonstrated. Based on the results discussed here, it is difficult to predict whether the hairpin- or two-strand methodology used for siRNA expression are equivalent to one another or whether one of the two is advantageous for certain applications.

McManus *et al.* [21] have established an intracellular expression system for hairpin micro RNA under the control of polymerase-III H1-RNA promoter, targeted towards CD8α silencing. This vector was transiently expressed along with CD4- and CD8α-expression vectors in HeLa cells, and effects of gene knockdown were compared. The engineered vector having H1 promoter-silenced CD8α to a level comparable to silencing by exogenously added siRNA targeted towards the same 3′-untranslated region of CD8α. The effect was highly specific because CD4 expression remained unaffected and control plasmids lacking promoter sequence failed to exhibit CD8α silencing.

These studies confirm that intracellular siRNA expression is a generally applicable technique for RNA interference and reliably inhibits expression of targeted genes. Stable expression of siRNAs will not only enable functional analysis of cellular genes that are difficult to treat with exogenous siRNAs (e.g. human T-cells), but also serve as a starting point for novel gene-based therapeutic applications to treat persistent viral infections [18]. Two more methods for stable expression of siRNA in mammalian cells have been reported in the literature, increasing the repertoire of technologies that involved RNA interference [22,23].

Direct targeting of intracellular proteins by RNA intramers

The approaches discussed so far alter the genetic information of an organism and, in most cases, the expression pattern of selected genes. Although being extremely useful for assessing gene function, these methods do not usually indicate whether a target qualifies for inhibition by antagonistic mechanisms. It is also difficult to elucidate which domain of the protein is important for its function using an siRNA approach. In other words, genetic knockdown approaches alone are insufficient for comprehensive validation of a protein target. Conclusions regarding these questions require direct recognition and inhibition of a protein target by an inhibitory molecule. Therefore, approaches delivering additional information about the function of a protein by inactivating it directly in its natural compartment are becoming increasingly important. Moreover, methods that allow rapid identification of specific inhibitors for such targets are urgently required to meet increasing demand for validation of hundreds of potential targets in the post-genomics era.

Several criteria need to be fulfilled before such methods can be used extensively. Important requirements include: routine applicability; performance independent of the target; ready automation; compatibility with molecules that cover nearly unlimited shape-space and which can be unambiguously identified within complex mixtures; and finally, applicability in an intracellular context. *In vitro* selection of functional nucleic acids, which fulfils these criteria, allows rapid selection of aptamers, specific binders and potential protein

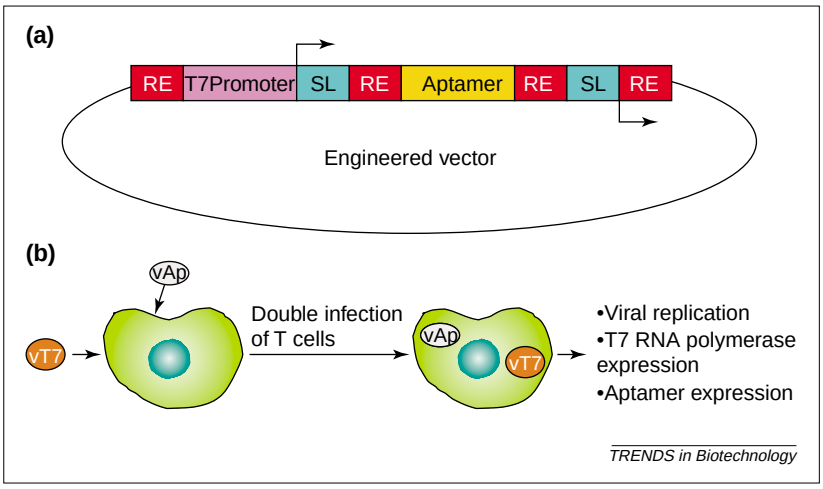

Fig. 3. Intramer expression system based on double infection. (a) Engineered vector for aptamer expression under the control of T7 promoter. (b) Infection by recombinant vaccinia virus. Abbreviations: RE, restriction sites; SL, stabilizing stem-loop structures; vT7, T7 RNA polymerase-expressing transgenic vaccinia virus; vAp, Aptamer-encoding transgenic vaccinia virus.

inhibitors, which are easily isolated from randomized nucleic acid libraries containing up to 10^{15} different molecules. Aptamers can also be expressed inside cells and they can alter cell phenotypes by modulating the biological function of the targeted protein. Such intracellularly expressed functional aptamers are referred to as 'intramers'. Intramers permit precise investigation of specific molecules, for example specific discrimination of homologous proteins [24]. These nucleic acids are as efficient as are monoclonal antibodies in probing functional characteristics such as binding affinity and specificity.

Intramers have been applied for inhibiting HIV-1 Rev protein function. When expressed under the control of RNA polymerase III-dependent expression vectors, intracellular anti-Rev aptamers effectively inhibited HIV-1 production in cell cultures [25]. Similarly, Konopka *et al.* [26] demonstrated reduced HIV-1 replication in infected HeLa cells that expressed anti-Rev DNA aptamer under the control of human cytomegalovirus or Rous sarcoma virus promoters.

These functional probes also inhibit endogenous proteins in the nucleus and in the cytoplasm. Thomas *et al.* [27] constitutively expressed an aptamer that was selected to bind yeast RNA polymerase II, under the control of RNA polymerase III promoter. A cell growth defect similar to that observed in yeast mutant displaying a reduced level of RNA polymerase II obtained with this aptamer. Interestingly, this defect was solely observed in intramer-expressing cells but not in the cells that expressed non-binding negative RNA controls. Shi *et al.* [28] showed that a pentameric aptamer directed against B52 [a member of *Drosophila melanogaster* serine/arginine (SR)-protein family] could be inserted into the polytene chromosome, resulting in aptamer-expressing transgenic flies. Similar to the lethal phenotype in B52-knockout flies, expressed multimeric anti-B52 intramer led to a 50% reduction in the development of adult transgenic flies. By contrast, overexpression of B52 can result in certain morphological defects, such as absence of salivary glands or missing bristles in adult animals. Interestingly, effects resulting from overexpression of B52 could be successfully reversed in the presence of pentameric intramer, indicating their role as *in vivo* antagonists or inhibitors of B52.

Recently, Blind *et al.* [29] reported intramer-mediated modulation of cytoplasmic membrane-protein domain function leading to highly specific cellular effects. RNA aptamers recognizing the cytoplasmic domain of the β_2-subunit of the human $\alpha_L\beta_2$-integrin were selected. These heterodimeric transmembrane proteins mediate adhesion of leukocytes to the endothelial cells in immune and inflammatory responses. Cytoplasmic domains of integrin α and β chains are also thought to be involved in the transmission of signals from inside the cell across the plasma membrane to the surface, thus activating an extracellular domain of $\alpha_L\beta_2$-integrin for binding to intercellular adhesion molecule 1 (ICAM-1) [30]. Corresponding intramers were expressed under the control of T7 RNA polymerase promoter in leukocyte cytoplasm, using an intramer expression system based on recombinant vaccinia virus. Transcription of intramers occurred exclusively in the cytoplasm because vaccinia viruses replicate exclusively in this compartment. Double infection with two recombinant viruses, one carrying intramer-encoding DNA and the other encoding T7 RNA polymerase, led to high cytoplasmic levels of expressed intramers (Fig. 3). These constructs specifically inhibited $\alpha_L\beta_2$-integrin-mediated, phorbol ester-stimulated cell adhesion to immobilized ICAM-1. This study established intramers as powerful tools for modulation of cytoplasmic membrane-protein domain function, leading to highly specific cellular effects.

A similar technology was applied by Mayer *et al.* [31] to target the Sec-7 domain of cytohesin 1, a cytoplasmic signalling molecule. This domain functions as a small guanine nucleotide exchange factor (GEF) on ADP-ribosylation factor (ARF) GTPases, and has implications in the control of vesicle transport and cell adhesion [32]. Selected aptamer specifically inhibited GDP–GTP exchange on ARF-1, thus preventing its activation *in vitro*. When expressed in T-cell cytoplasm, anti-cytohesin-1/Sec-7 intramers specifically led to a cell-spreading deficiency accompanied by a dramatic reorganization of F-actin distribution when the cells adhered to fibronectin. The observation of a similar effect, following dominant-negative expression of a GEF-deficient mutant of cytohesin-1, was a strong indication that this effect results from inhibitory activity of the intramer. In short, efficient combinatorial selection of an inhibitor, combined with an active intracellular expression in an appropriate cellular compartment, can be applied for direct investigation of individual protein function.

With the availability of several other intracellular RNA expression systems, it should now be possible to express intramers similar to siRNAs or ribozymes. A potential problem encountered with these RNA products is the direction and location in the cytoplasm, depending on whether cytoplasmic proteins or inner plasma membrane proteins are to be targeted. One way to circumvent this problem is to fuse aptamers to

RNA sequences that mimick nuclear export signals. Hamm *et al.* [33,34] and Grimm *et al.* [35], have described such RNA motifs, and their application in RNA selection has also been described. Alternatively, it was recently shown that the terminal stem of the nuclear export motif of adenoviral VA1 RNA is sufficient for its export [36]. Gwizdek *et al.* have defined a short minihelix motif that can also serve as a *cis*-acting cytoplasmic RNA-localization signal.

siRNA and helicase-recruiting technologies provide a more simple and viable rationale for the design of the targeting agent than does aptamer (intramer) technology. Although aptamers can now be obtained more easily and within shorter time-frames by automated selection techniques, they still require characterization by classical biochemical methods to determine whether or not they act as inhibitors. However, intramer technology can provide valuable information about the proteome and therefore, it is an important complement to siRNA and helicase-recruiting technologies for target validation and drug discovery processes.

Outlook

This review illustrates recent advances in intracellular expression of functional RNA molecules, and possible applications in genomics and proteomics research and in the treatment of diseases. The siRNA technology is already being applied in many laboratories to assess the roles of genes by loss-of-function phenotype analyses. Ribozymes also offer high potential, but are complicated in their design and applications. However, as discussed, engineered ribozymes with randomized hybridizing arms allow *de novo* identification of genes that are involved in certain key cellular functions, such as apoptosis. These applications have not yet been achieved with siRNAs, although there is good reason to expect that this will soon be accomplished.

Finally, intracellularly applied aptamers can be used as direct biochemical tools for obtaining specific inhibitors targeting a gene product within the context of its natural cellular expression environment and in an appropriate cellular compartment, providing information that cannot be generally obtained by downregulating expression of a certain gene alone. However, it remains to be shown that intramers can target a wide variety of proteins inside cells. Moreover, it would be desirable to design generally applicable systems that allow expression of intramers under the control of inducible endogenous promoters to ascertain that the intramer is reliably colocalized with the target. Thus, all the nucleic acid-based technologies described in this review complement each other and offer versatile approaches for elucidation of gene and protein function.

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