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Tethered Function Assays as Tools to Elucidate the Molecular Roles of RNA-Binding Proteins

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Abstract

Dynamic regulation of RNA molecules is critical to the survival and development of cells. Messenger RNAs are transcribed in the nucleus as intron-containing pre-mRNAs and bound by RNA-binding proteins, which control their fate by regulating RNA stability, splicing, polyadenylation, translation, and cellular localization. Most RBPs have distinct mRNA-binding and functional domains; thus, the function of an RBP can be studied independently of RNA-binding by artificially recruiting the RBP to a reporter RNA and then measuring the effect of RBP recruitment on reporter splicing, stability, translational efficiency, or intracellular trafficking. These tethered function assays therefore do not require prior knowledge of the RBP's endogenous RNA targets or its binding sites within these RNAs. Here, we provide an overview of the experimental strategy and the strengths and limitations of common tethering systems. We illustrate specific examples of the application of the assay in elucidating the function of various classes of RBPs. We also discuss how classic tethering assay approaches and insights gained from them have been empowered by more recent technological advances, including efficient genome editing and high-throughput RNA-sequencing.

Keywords

Tethered function assays; MS2 coat protein; Lambda N; BoxB; Nucleocytoplasmic transport; Subcellular localization; Translation; RNA stability

1 Introduction

Since the development of tethered function assays in the Wickens lab, where they were first applied to establish the independence of the poly(A) binding protein's RNA-binding activity on its mRNA stabilization activity [1], they have been used to investigate the roles of RBPs in all aspects of RNA metabolism. They have been used to assign specific roles to putative RBPs, dissect the function of individual RBPs within large complexes and separate functional domains of multi-domain RBPs. Because of the ease with which RBPs can be tethered to different sites on the reporter, the assay has also been invaluable in probing the dependence of RBP function on site-specific recruitment to target RNAs. Although tethered function assays have been used predominantly for RBPs, in principle the protein of interest itself does not need to have RNA-binding activity, since the artificial tether accomplishes recruitment. This allows the study of protein complexes that are recruited by RNA-binding proteins. Several well-characterized RNA-binding moieties and the cognate RNA elements they bind have been adapted as the tethers in these assays, and a handful are frequently employed.

Tethered function assays have three practical advantages over alternative methods to investigate RBP function [2, 3]. First, they are more straightforward to set up, carry out and interpret than approaches that rely on genetic manipulation, gene expression knockdowns or overexpression. Tethered function assays allow studies of essential RBP genes, do not require genetically tractable cell types, and circumvent pleiotropic effects of RBP depletion or over-expression on cellular homeostasis that may influence the target gene(s) under study. Second, RBPs can be studied without knowing its endogenous RNA targets. Instead, the effect of RBP binding is investigated with reporter assays that produce robust and straightforward readouts. Specialized reporter constructs enable studying the effect of the RBP of interest RNA in a particular RNA processing step. Third, as RNA metabolism is highly coupled, RBPs that affect a processing step that lies upstream of the one under study often influence the latter. In depletion or over-expression studies, these indirect effect may confound results. However, since these assays depend on artificial reporter constructs, careful experimental controls, independent confirmation of results and follow-up studies are required for meaningful interpretations.

Here, we describe how to design and carry out these assays, provide the strength and limitations of common tethering systems that have been developed to date and illustrate their utility with examples from the literature.

2 Designing and Performing Tethered Function Assays and Interpreting Their Results

Here we describe practical considerations of tethered function assays. For more detailed methods, we refer to two excellent protocol articles for specific application of tethering assays in studying RNA degradation [4] and nonsense mediated RNA decay and translational initiation [5].

2.1 Constructs

A tethered function assay comprises two components. First, a reporter DNA construct is designed which, when expressed in cells, is transcribed into an mRNA that may encode a functional protein (for example GFP, luciferase, or LacZ), depending on the application. The mRNA product also contains an RNA structural element that is recognized by an exogenous RNA-binding moiety. Second, an effector construct encodes the RBP (or RBP domain) fused to this moiety, such that the tagged RBP, when co-expressed, is recruited to the reporter mRNA by binding to the RNA element with strong affinity and specificity. As will be discussed later in this chapter, these RNA structural elements invariably are RNA stem-loop structures—so-called ‘hairpins’, most commonly from bacteriophage genomic RNAs, which are recognized by the phage coat proteins as their cognate RNA-binding moieties.

Most commonly, following ectopic expression of both constructs in cells, the effect of RBP tethering on target mRNA metabolism is determined by standard molecular biology techniques. For example, the effects of the RBP on reporter mRNA stability or splicing might be evaluated by reverse transcription PCR or northern blot [2, 3], while changes in RNA subcellular localization might be identified by fluorescence in situ hybridization (FISH) [6]. Assays using *in vitro* transcribed mRNA reporters and purified recombinant RBP fusions, both added to cell-free extracts competent for the RNA processing step under study, have also been reported [7, 8]. Lastly, for mRNA localization studies, the transcript of interest has been tagged by inserting recruitment tethers into the 3′ UTR of endogenous genes via homologous recombination in mouse [9]. Figure 3.1 shows an example of a tethered function assay using the bacteriophage MS2 hairpin structure (see below) placed in the 3′ untranslated region (UTR) of a reporter.

2.2 Position and Number of the Tethering Sites

The most crucial factor in the design of these assays is the position of the hairpin in the reporter construct. This decision is usually guided by a hypothesis or some prior knowledge regarding the function of the RBP of interest when bound to natural transcripts. Tethering of RBPs to different regions of the transcript may reveal region-specific RBP functions, with different effects on reporter expression. For example, while tethering of the RBP Staufen (STAU1) to the 3′ UTR of a reporter led to its degradation, 5′ UTR tethering promoted translation [10, 11]. The former observation reflects Staufen’s role as an inducer of nonsense-mediated RNA decay (NMD) via recruitment of UPF1, while the latter may be a consequence of Staufen’s function as an enhancer of translational initiation of structure-repressed transcripts [10, 12].

Of all regions of a transcript, the 3′ UTR arguably contains the greatest density of regulatory elements. The spatial positioning of an RBP binding site in the 3′ UTR is usually not critical for RBP function, and most tethering studies of RNA turnover, transport and localization have used tethers placed in the 3′ UTR [2, 3]. In contrast, an exploration of RBP function in translational initiation will insert the tether in the 5′ UTR [13, 14] or in the intervening sequence between the two coding portions of a bicistronic construct [5, 15]. And as we will see later in this chapter, the functional outcome of splicing factor recruitment to pre-mRNA processing is exquisitely sensitive to the precise binding site relative to the

spliced exon; here, the hairpin tether is placed in introns and exons at various distances from the splice junctions.

Although a single hairpin is often sufficient for productive RBP recruitment in cells, the increase in stability of the interaction afforded by cooperative binding of phage coat proteins bound to two hairpins of the MS2 phage (see below) was required for biochemical purification of bound complexes [16]. For RBPs functioning in RNA degradation (including NMD), a commensurate relationship between the number of tethers and the magnitude of the decrease in reporter signal was found [17–20]. In contrast, translational stimulation was reported to increase with the number of hairpins recruiting the germ cell-specific DAZL protein to the reporter 3' UTR [21], while this was not observed for the histone mRNA-specific 3' UTR binding protein, SLBP; here a single tethering site sufficed for maximal stimulation [22]. Thus, there is a consensus that multiple hairpins are generally advantageous and may maximize the number of RBPs recruited, thus increasing signal-to-noise and the likelihood of observing an effect on the reporter. Most studies have employed several (three to six) hairpins in a tandem array, but as many as 24 have been used in single-molecule mRNA localization studies [9]. However, long arrays of hairpins significantly lengthen the target gene region (for example, the 3' UTR or an intron) of the reporter beyond its natural size; such highly engineered constructs may no longer be recognized and processed by cellular machinery that needs to act on the reporter prior or subsequent to binding of the RBP of interest. For example, an analysis of RBP function in deadenylation-mediated mRNA degradation requires the reporter mRNA to be properly cleaved and adenylated before RBP action. Pilot experiments, using tethered RBPs with known effect on the particular aspect of mRNA metabolism under investigation, in conjunction with reporters containing different hairpin numbers, will identify optimal conditions.

2.3 Limitations, Controls, and Interpretation

Tethered function assays are not suitable for all RBPs. The RNA-binding sites of helicases and nucleases largely overlap with the active sites of these enzymes, so their 'RNA-binding' and 'functional' domains are not readily separable. In some cases, the RBP must bind the mRNA at a specific site or in a specific geometry, or be able to move freely along the RNA substrate or cycle in and out of a complex; here, artificial tethering may fail to properly localize the RBP. RBPs often require additional components to form a functional effector complex and these cofactors may not be expressed in the cell type studied. Even if expressed, they may fail to productively assemble on the reporter, for example if RBP binding to its natural RNA target induces a conformational change in the RBP that is required for their recruitment [23, 24]. And finally, the RNA-binding moiety, the RBP, or the hairpin may be occluded or misfolded in these artificial constructs; these challenges can be addressed in part by exploring different RBP-tag fusion constructs (for example, N- vs. C-terminal fusion and different linker sequences between the RBP and the tag) or tethering systems, and placing the hairpin at different locations on the reporter. Thus, in general, only positive results of tethering assays are meaningful.

Standard negative controls include expression of (1) the untagged RBP along with the reporter, (2) the RNA-binding moiety alone along with the reporter, and (3) of a reporter that

either lacks the hairpin or, preferably, contains a binding-incompetent hairpin mutant in the presence of the tagged RBP. These controls ensure that the observed effect on reporter mRNA metabolism is indeed due to RBP recruitment, rather than due to overexpression of the RBP or the RNA-binding moiety in the cell, or the presence of a hairpin in the reporter *per se*. Tethering hairpins are relatively weak RNA secondary structures which nevertheless may interfere with RNA processing or translation, particularly when placed in the 5' UTR or the coding sequence (CDS). A careful tethering study will quantify this effect by comparing the activity of the untagged reporter to that of a reporter harboring (a) binding-incompetent mutant hairpin(s), before employing the functional tethering reporter with the same number of functional tethers [14]. For tethering assays at the 5' UTR, steric hindrance of translational initiation by the presence of a protein, presumably independent of its identity, near the cap has been suggested, as increasing the distance between the cap and the tethering site relieved the observed translational inhibition [25].

Finally, as is the case with all experiments involving ectopic expression of reporter and effector constructs, their relative and absolute expression levels need to be carefully titrated so as to maximize specific reporter signal, while minimizing the risk of artifactual results stemming from their (over-) expression in the cell.

2.4 Follow-Up and Validation

Tethering assays rely on engineered and therefore artificial RNAs and proteins; insights gained from them are therefore only meaningful when confirmed by alternative approaches. As we will see later in this chapter, where we describe examples of tethering assay applications, these specific approaches depend on the biological question; however, they almost always involve investigation of RBP function in the context of its natural RNA targets. If endogenous targets are unknown, cross-linking immunoprecipitation followed by RNA sequencing (CLIP-seq) [26, 27] in relevant cell types might be performed to identify them (see also van Nostrand *et al.*, Chap. 1, this Volume). Knockdown of the RBP of interest, followed by global transcriptome analyses or more specialized phenotypic readouts, will provide clues to the role of the RBP in cellular homeostasis. Finally, biochemical purification of the recruited protein complexes might be used to reveal additional components of the effector complex of the RBP [16].

3 Tethering Systems

Although the bacteriophage MS2 system is the most widely used tethering system [16], several other methods (primarily of bacteriophage origin) are available. In the next section, we will discuss the most commonly used systems in detail, together with their advantages and drawbacks. Table 3.1 presents a summary of these tethering systems along with their characteristics.

3.1 MS2

RNA bacteriophages are small viruses with an icosahedral shell that are capable of infecting bacteria. Their single-stranded RNA genome is roughly 3500 nucleotides (nt) long and encodes structural proteins as well as proteins for viral maturation, replication and the lysis

of their bacterial host. These phages were initially isolated from *E. coli*, but are also found in other species of bacteria. The role of the coat protein is twofold: (1) it is the major structural protein of the viral particles and (2) it acts as a translational repressor of the viral replicase. During the late stages of *E. coli* infection by the bacteriophage, translation of the replicase gene is repressed due to binding of the MS2 coat protein to specific sequence elements in the replicase mRNA. This interaction has proven in both its affinity and specificity to be ideally suited for the tethering system.

The popularity of the bacteriophage MS2 (or the closely related R17) tethering system can largely be credited to its physical and functional characteristics: (1) the MS2 coat protein (MCP) is relatively small (14 kDa, 129 amino acids) thus reducing its potential to interfere with the function of the fused RBP, (2) it binds its 21 nt hairpin (Fig. 3.2a) with high affinity ($K_d = 10^{-9}$ – 10^{-8} M) and selectivity, limiting potential off-target binding, and (3) the MS2 hairpin-MCP interaction is well-characterized [16, 28, 36]. In addition, mutations in both the MS2 hairpin and the coat protein have been identified that increase the interaction affinity. A single U to C substitution in the loop increases binding affinity by 50-fold over wild type (Fig. 3.2b) [36–39]; this mutant is commonly used in tethering assays. Conversely, a mutant lacking the single-stranded ('bulged') adenosine within the stem essentially abolishes binding activity [36, 40, 41] and is sometimes used as a negative (recruitment-deficient) control in tethering assays [7]. A V29I mutation in the MCP modestly increases binding strength to the hairpin (by ~7.5-fold), albeit with an apparent commensurate decrease in specificity [42].

MCPs bind the hairpin as pre-formed dimers, thus recruiting two copies of the fused RBP of interest to the tethering site. Bound coat protein dimers interact cooperatively with one another when tandem arrays of hairpins are present [43]. These properties maximize RBP occupancy but may prevent tight control of recruitment. At concentrations above ~1 μ M, MCP dimers assemble into stable capsid-like structures that are not in equilibrium with soluble dimers and do not bind RNA, thereby decreasing the apparent affinity at high capsid concentrations [40]. Given that even moderately abundant endogenous proteins have intracellular concentrations in the millimolar range [44], it is surprising that capsid formation does not appear to be a problem in tethering assays, where the MCP-RBP fusion protein is usually over-expressed. Mutation screens have identified MCP mutants that dramatically increase the threshold concentration for capsid formation [42, 45–47], some without affecting the RNA-binding specificity or affinity; however, these are not widely used in tethering assays.

3.2 λ N

The bacteriophage λ is the most well-characterized of the lambdoid phages, a family of bacterial DNA viruses. Upon infection, phage promoters are sequentially active, and regulation is mediated by the synthesis of anti-termination proteins. The transcriptional repressor *cro* and the anti-terminator *N* are expressed from the pR and pL promoters, respectively. In the absence of anti-terminator *N*, transcription is stopped at the terminator sequences downstream of the *N* and *cro* genes (Fig. 3.3a). To activate the anti-termination function of *N* and enable the expression of the delayed early genes of the bacteriophage, *N*

must bind RNA polymerase at the nutL and nutR sites to facilitate read-through at the terminator sequences (Fig. 3.3b).

Both the nutR and nutL regions contain two conserved sequence elements of which only the boxB element forms a 15 nt hairpin to which protein *N* binds (Fig. 3.2b) [48]. Upon binding of *N* to the boxB element of nutR and nutL, additional bacteriophage and host proteins are recruited to achieve expression of the downstream genes. Since its first application for RBP tethering [49], the λ *N*/boxB system has gained popularity due to the extremely small size of the *N* protein (12.2 kDa), which has been suggested to reduce the risk of interfering with the function of the fused RBP [19]. Of the 107 amino acids that constitute the *N* coat protein, the 22 residues of the RNA-binding domain are crucial for RNA recognition [29]. A synthetic peptide consisting of these amino acids binds the boxB element with affinity and specificity similar to those of its full-length counterpart ($K_d = 10^{-9}$ – 10^{-8} M) [50, 51]. Additionally, a triple mutant of the peptide (M1G D2N Q4R) with a ~70-fold increased affinity at physiological monovalent cation concentration has been designed [43] but it is unclear to what extent specificity is maintained. In contrast to the MCP, the λ *N* protein binds its RNA element in a 1:1 stoichiometry [52], and therefore non-cooperatively, which may be advantageous for experiments requiring tight control of target occupancy. Although direct comparisons have not been done, it appears that the λ *N*/boxB system performs very similar to the MCP/MS2 system.

3.3 PP7

PP7 is a single-stranded RNA bacteriophage of *Pseudomonas aeruginosa* and is a distant relative of the MS2 and Q β bacteriophages (see below). Similar to MS2, the PP7 coat protein is a translational repressor of the viral replicase gene. The PP7 coat protein binds its hairpin (Fig. 3.2c) with a K_d of $\sim 10^{-9}$ M [30], in line with other phage-derived tethering systems; however, the sequence homologies between the PP7 and MS2 coat proteins and their RNA hairpins are very limited [53]. Although the PP7 system has been used on its own in standard tethering assays [54], its strength lies in its compatibility with the MS2 system: the PP7 and MS2 coat proteins discriminate against the respective non-cognate hairpin with ~1000-fold specificity [40, 53]. This orthogonality of the PP7 and MS2 systems has allowed real-time imaging of allele-specific transcription dynamics in yeast. In a diploid yeast strain, one allele of *MDN1* was tagged with 24 MS2 hairpins in the 3' UTR, the other with 24 PP7 hairpins. Transcripts produced from the two loci were distinguished using MCP and PP7 fusions of two different fluorescent proteins [55]. Similarly, fluorescence complementation at the site of recruitment of two halves of a single fluorescent protein, tagged with MS2 and PP7 coat protein moieties, allowed for reduction in background fluorescence of unbound probe in live-cell RNA localization studies [56].

3.4 Iron Responsive Protein (IRP)

Iron is essential for life. It is primarily involved in the synthesis of heme and hemoglobin in erythroid cells of most vertebrates [57], enabling transport of oxygen throughout the body. Both proteins are involved in the uptake (transferrin receptor) and storage (ferritin) of iron and are translated from transcripts containing one or more iron responsive elements (IREs) in either of their UTRs. These elements consist of roughly 30 nucleotides that are highly

conserved in vertebrates, some insects and many bacteria, and form stem-loops. A consensus sequence and structure for IREs has emerged and comprises a 6-nt loop motif (5'-CAGWGH-3', where W = A or U and H = A, C or U) that sits atop a 5-nt loop-proximal stem, which itself is separated from the distal stem by an asymmetrical bulge that contains an unpaired C (Fig. 3.2e) [58]. In iron-starved cells, IREs are bound by proteins IRP1 and IRP2, which results in translational repression for IREs located in the 5' UTR or RNA stabilization for IREs located in the 3' UTR. The mammalian transferrin receptor 1 (Tfr1) mRNA contains five such IREs in its 3' UTR, while both the ferritin heavy (H) and light (L) chain transcripts each contain a single IRE in their 5' UTRs. Iron starvation thus results in (1) the stabilization of the otherwise unstable transferrin receptor mRNA and (2) the translational inhibition of both chains of the ferritin protein messages [59]. This causes increased uptake of iron and also minimizes the sequestration of iron into ferritin (for a review, see [60]). Upon repletion of iron, the transferrin receptor mRNA is degraded and iron is stored by ferritin (Fig. 3.4).

The iron response protein (IRP) tethering system was first used by the Hentze lab [49]. Similarly to PP7, it is not widely used but its compatibility with other tethering systems has enabled recruitment of two different RBPs to two different locations within the same reporter. Gehring *et al.* [19] used such a dual reporter to show that a reduction in reporter mRNA levels, observed when λN -fused UPF3B was tethered to its 3' UTR via five boxB sites, depended on translation – an observation that would support UPF3Bb—triggered NMD of the message. To this end, they incorporated a single IRE in the 5' UTR, which allowed recruitment of *endogenous* IRPs. Inhibition of translation by iron depletion in cultured cells harboring the reporter restored transcript levels, as expected for an NMD target. In addition to its suitability in such duplex (or multiplex) tethering assays, the IRP system's advantage may lie in the higher affinity of the IRP proteins for its hairpin ($K_d = 10^{-12}$ – 10^{-11} M) compared to phage coat protein systems. Although not tested, this high affinity may aid in biochemical purification of co-recruited proteins. On the other hand, the IRPs are large; fusing a bulky protein to an RBP might alter its conformation, or hinder RNA target or protein cofactor binding. A total of 12 mammalian mRNAs have been reported to contain IREs, most coding for proteins with known roles in iron metabolism [59], and binding of the to these mRNAs may alter cellular gene expression and thereby confound results.

3.5 Q β , GA, Tat/TAR, and U1A

Here, we briefly draw attention to four alternative tethering systems that have not been extensively utilized in the field; they possess unique and useful features that may prove advantageous for certain experimental systems or when multiplexing of tethering systems is required.

Q β and GA—These are derived from coat proteins of the eponymous bacteriophages which, although only distantly related to MS2, show extensive structural similarity as well as common features for recognition of their cognate hairpins [61, 62, 63]. Two mutants of the Q β coat protein, D91N and Q65H, have shown a higher affinity for its hairpin (Fig. 3.2g) than wild-type but they also bind the MS2 hairpin more efficiently [64]. On the other hand,

the MCP has a 100-fold greater affinity for its own hairpin than for the GA-associated hairpin (Fig. 3.2h). In contrast, the GA protein binds its own and the MS2 hairpin with the same affinity [65]. Given the high degree of similarity between the MS2, Q β and GA systems, the use of MS2 is preferred because it is better characterized.

Tat/TAR—One of the few non-bacteriophage tethering systems is derived from the bovine immunodeficiency virus (BIV). Only 17 amino acids from the BIV Tat (transactivator of transcription) protein are necessary to bind the 28-base BIV TAR (trans activator response) element (Fig. 3.2d) with high affinity ($K_d = 10^{-9}$ – 10^{-8} M) [66]. Wakiyama and colleagues [67] provided proof-of-principle for the utility of this system by showing that tethering to the 3' UTR of Tat-fused TNRC6B (a member of the GW182 family of proteins functioning in microRNA-mediated gene repression) via four TAR elements effected a magnitude of reduction in reporter activity similar to that seen with λ N-fused TNRC6B and a 5x boxB reporter. No other groups have since used it for tethering proteins to a reporter, likely due to the widespread use of better characterized tethering systems.

U1A—The N-terminal 100 amino acids, containing one of two RNA recognition motifs (RRM), of the human U1 small nuclear ribonucleoprotein (snRNP)-specific protein U1A (also known as SNRPA), binds with high specificity and affinity to a ~25 nt hairpin structure (Fig. 3.2f) in U1 snRNA, called U1A hpII (Fig. 3.2f). Its 5'-AUUGCAC-3' single-stranded loop sequence is critical for binding [68]. Because yeast U1 snRNA lacks this hairpin [69], the RNA-protein interaction has mainly been employed in yeast for tracking subcellular localization of RNA transcripts and studying mRNA processing [70–73].

4 Applications of Tethered Function Assays

In what follows, we highlight select publications that illustrate the versatility of tethered function assays, as they have been used in virtually all areas of RNA processing and RBP research. As we have seen, the development of tethered fluorescent proteins probes for tracking RNA subcellular localization is an active area of investigation, and we provide additional examples this extension of canonical tethered function assays.

4.1 mRNA Stability

Poly(A) binding protein: functional analysis of an essential gene—A tethering assay was first developed by Coller *et al.* [1] to study the role of the poly(A)-binding protein (Pab1p) in yeast. Previous studies had shown that *PAB1* is an essential gene and that lethality of a *pab1* deletion could be suppressed by mutations in mRNA decay-related genes, suggesting that Pab1p protects mRNAs from degradation. However, since *PAB1* is essential, genetic studies could not be used to investigate whether mRNA stabilization simply required Pab1p's presence on mRNAs, or whether this function was dependent on Pab1p's interaction with the poly(A) tail. With yeast strains expressing non-essential reporters under transcriptional pulse-chase conditions, Coller *et al.* [1] showed that while Pab1p, when fused to an MCP dimer fusion and recruited to reporters via two MS2 hairpins, stabilized both a deadenylated reporter mRNA and one that cannot be adenylated (Fig. 3.5), it did not affect the reporter deadenylation rates [1]. Thus, Pab1p stabilized mRNAs

independent of their poly(A) tail, suggesting a very simple model: the poly(A) tail serves as a binding site for Pab1p molecules; deadenylation removes these Pab1p sites and upon departure of the last Pab1p molecule, RNA degradation is triggered. Using a reporter with a stem-loop structure in the 5' UTR that rendered it translation-incompetent, the authors also showed that mRNA stabilization by Pab1p requires some aspect of translation. It is now well established that the mRNA 3' end processing machinery and translational initiation complexes are physically and functionally linked [74].

Exon junction complex proteins: linking nonsense-mediated RNA decay to splicing—One of the most important mRNA quality control checkpoints in the cell is the nonsense-mediated mRNA decay pathway (NMD). In a manner that is dependent on prior splicing, exon-exon junctions are marked by deposition of the exon junction complexes (EJCs) [75–77], which allow cells to distinguish legitimate stop codons from premature termination codons (PTCs) (Fig. 3.6a). A termination codon located more than 55 nt upstream of the last exon-exon junction is sensed as premature, subjecting the mRNA to NMD. Lykke-Andersen *et al.* [18] used a human β -globin mRNA tethering reporter to validate candidate human homologs of three yeast proteins, termed Up frameshift (Upf) proteins 1–3, which had been identified through genetic screens as suppressors of a frameshift mutation [78]. MS2-based tethering of the candidates to the reporter 3' UTR circumvented the requirement for prior splicing. Using this system, the authors then asked which of seven proteins that had been reported as members of a multiprotein post-splicing complex were required for linking NMD to splicing via binding to Upf(s) (Fig. 3.6b) [79]. The RBPs RNPS1 and, to a lesser extent, Y14 (RBM8A) triggered NMD when tethered to the reporter 3' UTR (Fig. 3.6c) and both interacted with Upf proteins in co-IP assays. It is now known that Y14 is part of the heterotrimeric core EJC, while RNPS1 is a member of the EJC-peripheral ASAP effector complex, which links the EJC to mRNA quality control pathways and the translational machinery [80].

YTHDF2: Understanding the mechanism of RNA destabilization by an RNA modification reader protein—Adenosine N6 methylation (m^6A) has recently been identified as an additional layer of posttranscriptional gene regulation. Members of the YTH domain family (YTHDF) of RNA-binding proteins recognize m^6A in RNAs and have been identified as m^6A binding proteins by affinity chromatography. Wang *et al.* [81] used λ /boxB-based tethering assays to investigate the outcome of forced recruitment of a YTHDF2 construct lacking its C-terminal RNA-binding domain, to a reporter mRNA containing five boxB hairpins in its 3' UTR. They observed a reduced steady-state level and shorter poly(A) tail of the YTHDF2-bound reporter. These findings, along with results from knockdown and immunocytochemistry experiments led them to propose that YTHDF2 targets m^6A -containing mRNAs to sites of RNA decay, such as processing bodies.

4.2 Translation

eIF4F complex: function of core translation initiation factors—Translation initiation is the rate-limiting step in translation and thus most translational regulation occurs at this stage. In cap-dependent translation, the cap-binding complex eIF4F, consisting of eIF4E, -G and -A, recruits a preassembled 43S ribosomal preinitiation complex (PIC), which

then scans the mRNA 5' end for a start codon. It is now known that eIF4E recognizes the cap and binds to eIF4G, which in turn recruits the PIC via interacting with eIF3, a component of the PIC [74]. Tethered function assays proved invaluable in dissecting the roles of the eIF4F complex subunits. In a study that pioneered the use of both λ N/boxB and IRP/IRE tethering systems, the Hentze lab showed that forcible recruitment, to the intercistronic space of a bicistronic reporter, of an eIF4GI mutant lacking the eIF4E binding domain supported translation of the downstream cistron [49]. Similar results were obtained with an eIF4E mutant lacking the cap binding domain, but not with eIF4E lacking the eIF4G binding domain or with full-length eIF4A [49]. These studies showed that eIF4G and eIF4E are sufficient for translational initiation in the absence of cap binding. The failure of tethered eIF4A to promote translation was attributed to the fact that this helicase might require flexible association with the RNA, which tethering—trivially—prevents. As described above, this finding underscores the limitations of tethered function assays for helicases.

AGO2: mechanism of microRNA-mediated gene silencing—microRNAs (miRs) and endogenous siRNAs are important regulators of transcription and translation. Both classes are found in RNA-protein complexes at their target mRNAs with GW182 and Argonaute (Ago) proteins at their core [82], which recruit additional factors to form the RNA-induced silencing complex (RISC) [83]. In a series of tethered function assays using a λ N/boxB-based luciferase reporter, the Filipowicz and Izauralde labs elucidated the roles of RISC and accessory components in gene silencing and shed light on its molecular mechanism [17, 84, 85] (Fig. 3.7a). Tethering of AGO1, AGO2, AGO4 and GW182—but not HIWI (PIWIL1), a Paz and PIWI domains protein like Ago proteins—to the 3' UTR of a luciferase reporter via the λ N/boxB system reduced luciferase activity in a manner dependent on the number of boxB elements (Fig. 3.7b), but not their position within the 3' UTR. Upon siRNA-mediated depletion of GW182, but not of AGO2, repression of an AGO1-tethered reporter was partially relieved. These simple assays provided fundamental insights into mechanism of miRISC-mediated silencing and provided the groundwork for many later in findings from global analyses, such as the additive effect of multiple miRs bound to target mRNAs, the functional redundancy of AGO1 and AGO2, and the importance of GW182, which is now known to interact with PABP and deadenylase complexes. They also provided the first insights into the contribution of both mRNA degradation and translational inhibition to miR-mediated gene silencing.

TYF: molecular mechanism of PER translational stimulation—Tethered function assays have played a central role in appreciating posttranscriptional mechanisms in controlling of circadian rhythms. The product of the *Drosophila* gene 24 (TYF) was identified in a mutation screen to be necessary for robust behavioral rhythms in pacemaker neurons. Lim et al. [8] observed that the core circadian clock protein PERIOD (PER) depended on the expression of TYF, which had no known functional domains. To understand if TYF acts transcriptionally or post-transcriptionally, they employed tethering assays in transfected *Drosophila* cells and, notably, in translation-competent *Drosophila* cell-free extracts. Here, a recombinant MCP-tagged C-terminal fragment of TYF robustly upregulated translation of an *in vitro* transcribed luciferase mRNA reporter harboring six MS2 hairpins in its 3' UTR. Co-IP of PABP and eIF4E with TYF and polysome profiling

studies further corroborated a role for TYF in promoting translation [86]. Co-IP and mass-spectrometry studies further identified ATAXIN-2 (ATX2) as a TYF interactor; tethered ATX2 stimulated reporter activity similar to TYF. These results revealed a central role for ATX2, an RNA-binding protein whose human homolog is implicated in neurodegenerative disease, in controlling circadian timing at the posttranscriptional level (see also Benegiamo *et al.*, Chap. 5, this Volume).

4.3 Pre-mRNA Splicing

SR proteins: functional dissection of RBP domains in cell-free extracts—

Graveley and Maniatis [7] were the first to report the use of tethering assays to study splicing and used them to dissect the roles of individual RBP domains. Serine/arginine (SR) proteins are essential components of the intronic splicing machinery. They are bipartite RBPs with one or two RRM, critical for RNA-binding [87, 88], and one arginine/serine-rich (RS) domain necessary for SR protein function [89–93]. They regulate constitutive splicing by interacting with components of the basic splicing machinery but are also involved in the regulation of alternative splicing events. Splicing enhancers located downstream of the regulated intron can be bound by SR proteins, which enhances splicing of the upstream intron [87, 94]. If placed downstream of an intron, SR binding sites can function as splicing enhancers by recruiting basic components of the splicing machinery. In order to test whether the ‘general’ and ‘regulatory’ functions of SR proteins can be uncoupled, Graveley and Maniatis [7] constructed three *in vitro* transcribed mRNA reporters consisting of enhancer-dependent introns with a single MS2 hairpin replacing the downstream splicing enhancer (Fig. 3.8a). Using recombinant proteins consisting of the RS (Arg/Ser-rich) domains of the SR proteins SF2/AF (SFRS1), SC35 (SRSF2), and 9G8 (SRSF7) and the MCP, alternative splicing was observed in HeLa cell nuclear extracts (which contain endogenous SR proteins), indicating that the splicing enhancer function of RS domains is separable from their RNA-binding domains. However, these hybrid proteins were not able to substitute for endogenous SR proteins; in extracts lacking the latter, purified SR proteins were required to observe splicing. The authors thereby showed that the functions of SR proteins in the basic splicing reaction are separable from their role as splicing enhancers [7].

TRA2: investigation of context-dependence of splicing factor binding—

In addition to their role as splicing enhancers, certain SR proteins and related factors can act as splicing repressors. For example, the SR-related protein TRA2 binds an intron (termed M1) within its own pre-mRNA and promotes M1 retention [95]. In order to investigate the requirements for splicing repression by TRA2 in cell-free extracts, Shen and Mattox [96] tethered TRA2 to two different locations within an M1 intron reporter and found that both caused intron retention, while exonic positioning downstream of the 3′ splice site supported splicing activation. TRA2 domain analyses using these reporters showed that the C-terminal RS domain (RS2) was sufficient to cause activation (in exonic placement), while repression (in intronic placement) required an intact RRM. These results indicated that TRA2’s functions in splicing are context-dependent and likely mediated by recruitment of distinct effector complexes by separable regions of TRA2.

RBFOX1: identification RBP-recruited effector complexes—Sun *et al.* [97]

investigated the mechanism of RBFOX1-mediated splicing activation and repression by tethering this alternative splicing factor to different sites on a reporter minigene. Members of the RBFOX family of proteins control exon inclusion and exclusion in a position-dependent fashion: they promote exon inclusion when bound downstream of an alternative exon, and exon exclusion when bound upstream [98, 99]. In order to understand the molecular mechanism underlying this context-dependent regulation, Sun *et al.* [97] tethered domains of the RBFOX1 protein to a single MS2 hairpin located in introns downstream and upstream of alternative exon 7 of the *SMN2* gene, which is devoid of natural RBFOX binding sites in this region (Fig. 3.8b). While the Ala/Tyr/Gly-rich C-terminal domain of RBFOX1 was sufficient for exon inclusion in the downstream configuration, both the C-terminal domain and the central RNA RRM domain were required for exon skipping in the upstream configuration. Co-immunoprecipitation (co-IP) studies using the C-terminal domain as bait identified several proteins, including the RBP hnRNP H1 and the signalling protein TFG. siRNA-mediated depletion of either protein reduced exon inclusion and exclusion of endogenous RBFOX targets, confirming their role in RBFOX-mediated control of alternative splicing. This study highlights the utility of tethering assays in shedding light on the identities of effector complexes recruited by RBPs.

4.4 RNA Transport and Localization

She2p: control of *ASH1* mRNA trafficking—One of the best examples illustrating the use of tethering systems in the study of mRNA localization is the yeast *ASH1* mRNA, whose CDS and 3' UTR contain a total of four *cis*-acting localization elements. During late anaphase, *ASH1* mRNA is localized to the bud tip and sorted to the nucleus of the daughter cell, where its protein product inhibits mating type switching [100, 101]. Genetic studies identified five genes, *SHE1-5*, essential for Ash1p sorting, three of which were known or hypothesized to encode proteins involved in organization of the actin cytoskeleton (*SHE4* and *SHE5/BNI1*) or encode a type V myosin (*SHE1/MYO4*). Long et al. [102] investigated the role of the remaining two She proteins. Tethering She3p in a *she2* deletion strain was sufficient to induce localization to the bud tip, as judged by fluorescence *in situ* hybridization (FISH). Since electrophoretic gel mobility shift assays showed that She2p has binding activity to one of the localization elements, and She2p and She3p interacted in a yeast two-hybrid assay, the authors proposed that She2p directly binds to the *ASH1* transcript's localization elements factors and associates with a She3p/Myo4p complex to transport *ASH1* mRNA along actin filaments. A schematic overview of this system can be found in Fig. 3.9a, b.

***ASH1* and β -actin mRNAs: monitoring cytoplasmic transport of RNAs—**An

adaptation that naturally follows from tethered function assays is the use of forcibly recruited fluorescent proteins to visualize the transport and identify the subcellular localization of tagged RNAs (Fig. 3.9c). In contrast to FISH, this approach allows real time (live-cell) imaging of RNAs and facile detection of their co-localization with proteins. FISH requires fixed (dead) cells and it is often difficult to identify conditions under which both probe hybridization to the RNA and antibody binding to (a) protein(s) of interest are efficient and specific. *ASH1* mRNA was the first RNA to be visualized [103]: GFP fused to

a high-affinity mutant of the MCP was used to track *ASH1* mRNA tagged with six repeats of the MS2 hairpin. The chimeras were expressed from plasmids in yeast strains deleted for either one of the five *SHE* genes, which allowed the dissection of the roles of the She proteins in formation of the *ASH1* mRNA transport complex, its spatial dynamics within the cell, and its anchoring at the bud tip upon arrival. Advances in fluorescence imaging and image analysis have since allowed the visualization of single RNA molecules, perhaps culminating in the ability to track individual transcripts expressed from their endogenous locus in mammalian cells. Lionnet et al. [9] isolated primary embryonic hippocampal neurons from a mouse strain engineered to express β -actin mRNA tagged with 24 MS2 hairpins from both alleles. Expression of a fusion protein consisting of yellow fluorescent protein (YFP), a nuclear localization signal, and the MCP from a lentiviral vector allowed to follow the motions of single β -actin containing particles. The use of nuclear localized YFP coat protein fusion protein and the large number of MS2 hairpins were required to overcome the fluorescence background from free (non-RNA bound) YFP fusion protein in the cytoplasm. Certainly, a future challenge will be the application of this approach to less abundant transcripts, and the study of mRNA transport dynamics in living organisms.

Monitoring nucleocytoplasmic transport of RNAs—A similar approach allows monitoring the nucleocytoplasmic transport of single mRNP complexes. Mor *et al.* [104] generated inducible gene constructs encoding fusions between a green or blue fluorescent protein and portions of the human dystrophin gene. The constructs also specified 24 MS2 hairpins in the 3' UTR of the chimeric transcripts. Upon stable expression in mammalian cells, transcripts were detected by transient expression of a plasmid encoding an MCP fused to a red fluorescent protein. In this manner, both the transcript and its protein product could be detected simultaneously and in real time. These experiments showed that mRNP transport to the nuclear pore complex occurs on the minute time scale, consistent with passive one-dimensional diffusion along chromatin channels. In contrast, export is rapid (within 0.5 s), indicating active transport, and is not rate-limiting. This study has enabled significant insights into fundamental aspects of nucleocytoplasmic transport of a model mRNA, which can now be extended to the investigation of the function of endogenous genetic elements, such as sequence motifs in UTRs and introns, in this process.

5 Conclusions and Outlook

Tethered function assays are conceptually simple experiments for rapid functional determination of the effect of a specific RBP on the metabolism of a reporter mRNA. As we have seen, they have been instrumental in all areas of RNA research, where they continue to be employed for the functional dissection of RBP domains and the protein complexes they recruit, and for understanding how the function of an RBP depends on where in a target RNA it is recruited. Of course, validation of tethering assay results by other means is always necessary since the method relies on artificial reporters, which are often ectopically over-expressed from minimal constructs lacking gene features deemed non-essential for the readout of interest. This reductionist approach has nevertheless been useful because it dissects RNA processing events and thus allows the study of the impact of a given RBP on a single event in isolation. In reality, however, RNA processing events are highly

interdependent, and so the absence of an RBP effect in a tethering assay for a given process does not necessarily exclude a role for that RBP in this process. For example, the deposition of EJCs, which requires splicing, stimulates translation [105], so the intronless reporters typically used for interrogating RBP effects on translation will not identify RBPs that may affect translation via modulating EJC function. Tagging of genes at their endogenous genomic loci at scale, now facilitated by CRISPR/Cas9 genome editing technology, will enable tethering assays that are sensitive to such ‘reach-through’ effects of RBPs, while at the same time approximating expression levels and processing pathways of natural mRNAs.

More recently, the standard tethering method has been adapted for live-cell tracking of RNAs; although other systems are being developed [106], it is the only approach widely used for real-time imaging of RNAs at the single-molecule level. Here, a superior next-generation approach would have to combine the sensitivity of detection afforded by tandem arrays of hairpins, while simultaneously overcoming the need for such tags in the first place. A related application in which tethering tags may feature very prominently in the future—again facilitated by CRISPR/Cas9—is their use as affinity tags for identification of proteins and nucleic acids in complex cellular RNP structures. Many complex macromolecular assemblies in the cell—including nuclear bodies such as Cajal bodies, speckles and paraspeckles [107]—harbor hallmark RNA molecules that may be harnessed, when tagged with boxB or MS2 hairpin tethers, as baits for the biochemical isolation of interacting protein components, RNAs, and chromatin domains, via affinity purification on λ N peptide or MCP columns. An analogous approach termed RNA affinity purification (RAP) used immobilized oligonucleotides that hybridize to Malat1, a non-coding RNA specific to nuclear speckles, and identified RNAs and chromatin associated with these bodies [108, 109]. A tethering method in place of an antisense approach may allow purification under more physiological or more stringent conditions, or at higher yields.

As more and more RBPs are appreciated as playing central roles in disease-related cellular processes [110], interest in elucidating RBP function and targets is increasing accordingly (see also Hattori *et al.*, Chap. 7; Bejar, Chap. 9; and Fan and Leung, Chap. 11; all this Volume). Tethering assays will facilitate identification of these functions, while CLIP-seq approaches [26, 27] will provide the means to elucidate RBP mRNA targets. The combination of both approaches will help uncover if and how the lifecycles of specific RNAs are perturbed when their bound RBPs carry disease-relevant mutations. Clearly, such an approach would require parallel functional interrogation of these RBPs with a battery of tethering reporters—both published and newly developed—that inform on different aspects of RNA metabolism. The ability to conduct tethering assays at scale will also elucidate the effect of specific combinations of RBPs, particularly those with known opposing roles in a given process. Such multiplex assays are already possible because several tethering systems, for example the λ N/boxB and MCP/MS2 systems, are orthogonal. It is clear that tethering assays will remain at center stage in RBP research, empowered by genome editing to create physiologically relevant reporters, and by RNA-seq methods to validate their findings.

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Fig. 3.1.

A tethered function assay of an RBP that is tethered to the 3' UTR. The recruitment of the RBP to the reporter mRNA is artificial via the interaction of the hairpin and hairpin-binding moiety fused to the RBP of interest. The biological effect of binding can be studied by standard molecular biology techniques. In this example, an MS2 tethering system is shown, but alternatives exist

Sequences of all mRNA hairpins discussed in this chapter. **(a)** Wild type (*left*) and the high affinity mutant (*right*) of the MS2 hairpin. **(b)** The 15 nt boxB elements of the λN system. Both versions have been used for tethering and show a similar affinity for the λN protein despite the single nucleotide difference. **(c)** and **(d)** The PP7 and TAR hairpins, respectively. **(e)** A canonical structure of the iron-responsive element (IRE). N denotes base pairs whose nucleotide identities are not critical for IRP binding. **(f, g, h)** The hairpins of the U1A, Q β and GA phages, respectively

Fig. 3.3.

λN anti-terminator function. (a) The expression of the immediate early genes *N* and *cro*. The RNA polymerase is released from the DNA at both terminator sequences T_L and T_R . (b) Read-through of the terminators occurs when the *N*-protein associates with the RNA polymerase at the *nutL* and *nutR* sites. This results in the extended transcription into the delayed early genes of bacteriophage λ . P_L and P_R promoter *left* and *right*, *nutL* and *nutR* binding sites for the anti-terminator, T_L and T_R *left* and *right* terminator sequences

Fig. 3.4.

The iron metabolism in humans. **(a)** In iron starved cells, the transferrin receptor mRNA is bound by IRP 1 and/or 2 proteins, resulting in the stabilization of the mRNA and thus expression of the receptor. The ferritin (responsible for storing iron) mRNA is also bound by IRP1 and/or 2 and is subsequently repressed by translational inhibition. **(b)** In iron depleted cells, the situation is reversed: iron taken up by the cell is stored in ferritin stores, while the transferrin receptor expression is inhibited by mRNA degradation

Fig. 3.5.

Overview of the mRNA stability experiments performed by Collier *et al.* [1] (a) By tethering Pab1p to an unstable reporter gene, an increase in stability was observed. (b) This example is a good illustration of how controls should be included in a tethering assay. In their study, Collier *et al.* used both tether controls and reporter controls. The tether controls evaluate the effect of the tether on the reporter; this is determined by tethering either an MS2 alone or an MS2 protein fused to a protein of similar size as Pab1p (Sxl in this case). The reporter controls determine whether the observed increase in mRNA stability is the result of the tethering of the protein. This can be achieved by using a reporter mRNA without a tether hairpin and/or a reporter with the hairpin in the antisense orientation. Adapted from [1]

Fig. 3.6.

The tethered function assay used for studying NMD. **(a)** Schematic overview of the NMD mechanism. The EJC complex (consisting of eIF4AIII, Y14, MAGOH and BTZ and Upf3) recognizes the exon-exon junction between exon 1 and exon 2. Next, the SURF complex (consisting of PIKK (not shown), SMG1, Upf1, eRF1 and eRF3) is recruited to the premature termination codon (PTC) and initiates transcript degradation. SMG5, SMG6 and SMG7 are not shown in this figure. **(b)** The top construct of the panel shows the wild type situation: 2 exons with a stop codon downstream resulting in a stable mRNA. The bottom construct illustrates what happens if a PTC is present. Upstream of the exon-exon junction, at least 5 proteins bind the mRNA as a complex and trigger NMD. **(c)** Tethering each individual protein downstream of a wild type stop codon, to the 3' UTR mimics NMD and therefore, mRNA stabilization by each individual protein of the complex can be studied. Protein RNPS1 and to a lesser extent Y14 elicit NMD, while the other three proteins (DEK, REF and SRm160) do not alter mRNA stability. From [79]

Fig. 3.7. Studying mRNA translational inhibition by Ago protein via a tethering assay. **(a)** Downstream of a reporter gene, 1–5 BoxB elements are inserted. By fusing the 22 N-terminal amino acids of the λ N protein to the Ago2 protein, Ago2 can be tethered to the reporter and inhibit translation. **(b)** This graph illustrates that increasing the number of boxB elements in the 3' UTR of an mRNA proportionally decreases translation. From [17]

Fig. 3.8.

Overview of two different tethering approaches for studying splicing events. **(a)** The reporter construct contains two exons flanking an enhancer-dependent intron. Downstream of exon 2, an MS2 hairpin replaces the endogenous enhancer sequence. The RS domains of three different SR proteins are tethered to the reporter, resulting in the splicing of the intron. From [7]. **(b)** The *SMN2* reporter construct used by Sun *et al.* Exons 6 and 8 of the *SMN2* locus are flanking the alternative exon 7 (the alternative exon in the figure). The hairpin is located in the intron downstream of the alternative exon. Tethering RBFOX proteins to the reporter resulted in the retention of the alternative exon. From [97]

Fig. 3.9. mRNA localization studies by tethered function assay. **(a)** The *cis*-acting element straddling the CDS and 3' UTR of the *ASH1* gene is bound by She2p, which recruits She3p and She1p to the complex. She1p binds to the actin filament and localizes the mRNA to the bud tip. **(b)** The experiment performed to demonstrate that She2p is the natural tether to the *ASH1* mRNA. When She3p is tethered to the reporter, it causes localization of the mRNA in the absence of She2p. **(c)** MCP-GFP fusions can be used to study the localization of an MS2-tagged mRNA of interest in the cell. Of course, other tethering systems can be used for this approach. From [103]

Table 3.1

Key characteristics of hairpins and hairpin binding moieties used in tethering assays

Name	Minimal protein size (aa)	Dimer/monomer	Hairpin size (nt)	Dissociation constant (K_d) ^a (Ref.)
MS2	129	Dimer	21	10^{-9} – 10^{-8} M [28]
Δ N	22	Monomer	15	10^{-9} – 10^{-8} M [29]
PP7	127	Dimer	25	$\sim 10^{-9}$ M [30]
TAT/Tar	17	Monomer	28	10^{-9} – 10^{-8} M [31]
IRP	IRP1 889	Monomer	30	10^{-12} – 10^{-11} M [32]
	IRP2 964			
U1A	102	Monomer	29	10^{-11} – 10^{-10} M [33]
Q β	129	Dimer	20	10^{-9} – 10^{-8} M [34]
GA	129	Dimer	23	10^{-9} – 10^{-8} M [35]

aa amino acids, *nt* nucleotides, K_d equilibrium dissociation constant^aFor the wild-type interaction