RNA Labeling

International Edition: DOI: 10.1002/anie.201604107
German Edition: DOI: 10.1002/ange.201604107

Modifying the 5′-Cap for Click Reactions of Eukaryotic mRNA and To Tune Translation Efficiency in Living Cells

*Josephin M. Holstein, Lea Anhäuser, and Andrea Rentmeister**

Abstract: *The 5′-cap is a hallmark of eukaryotic mRNAs and plays fundamental roles in RNA metabolism, ranging from quality control to export and translation. Modifying the 5′-cap may thus enable modulation of the underlying processes and investigation or tuning of several biological functions. A straightforward approach is presented for the efficient production of a range of N7-modified caps based on the highly promiscuous methyltransferase Ecm1. We show that these, as well as N^2-modified 5′-caps, can be used to tune translation of the respective mRNAs both in vitro and in cells. Appropriate modifications allow subsequent bioorthogonal chemistry, as demonstrated by intracellular live-cell labeling of a target mRNA. The efficient and versatile N7 manipulation of the mRNA cap makes mRNAs amenable to both modulation of their biological function and intracellular labeling, and represents a valuable addition to the chemical biology toolbox.*

Eukaryotic messenger RNA (mRNA) has emerged as a valuable alternative to DNA-based vectors for exogenous protein expression in eukaryotic cells. Benefits include almost immediate translation and typically higher transfection efficiencies than when using DNA because delivery to the cytoplasm is sufficient. Although low stability of mRNA compared to DNA can be an issue, the fact that mRNA does not permanently alter the genome of the cell is now considered advantageous for therapeutic applications.[1] Consequently, approaches to tune the translation efficiency and stability of specific transcripts are attracting increasing interest.[2] One crucial element for translation initiation of mRNAs is the interaction of the 5′-cap with the eukaryotic translation initiation factor eIF4E, and alterations of the 5′-cap can directly affect translation.[3] In addition to their general importance for translation, mRNAs can be asymmetrically distributed in eukaryotic cells, and in several cases even locally translated.[4] However, the molecular details of mechanisms such as active transport leading to subcellular localization are poorly understood.[5] To investigate these dynamic processes, methods for labeling mRNAs in living cells are required. Ideally, the mRNA of interest should be altered as little as possible, which makes two-step chemoenzymatic approaches[6] an attractive alternative to the currently more widespread use of fluorescently labeled RNA-binding proteins (reviewed in Ref. [7]). To date, click reactions of RNA are typically performed in fixed cells, except for one report where the tetrazine ligation of a short synthetic RNA was performed in living cells.[8]

We show for the first time the manipulation of a specific eukaryotic mRNA in living cells. The translation efficiency of the mRNA can be tuned without altering its sequence and without the use of RNA-binding proteins, simply through enzymatic attachment of small functional groups at either the *N*7 position of the purine ring or the exocyclic amino function (N^2) of the cap structure. These modifications can be used for click chemistry in living cells and thus allow both labeling of the mRNA and tuning of its biological function in living cells (Figure 1).

We previously reported a chemoenzymatic approach to label in vitro produced and capped RNAs at the N^2 position of the 5′-cap using a variant of the trimethylguanosine synthase from *Giardia lamblia* (GlaTgs2-V34A).[9] However, the activity of this methyltransferase requires prior methylation of the *N*7 atom, and even engineered variants showed compromised activity with larger *S*-adenosyl methionine (AdoMet) analogues, thus limiting the labeling yield to approximately 30%.[9b,c] Searching for a more straightforward and efficient approach, we noted Ecm1, a cap (guanine *N*7) methyltransferase from the microsporidian parasite *Encephalitozoon cuniculi*, the active site of which is located in a cleft rather than a binding pocket (Figure S1 in the Supporting Information).[10] Based on the crystal structure, we anticipated that Ecm1 might be highly promiscuous regarding the cosubstrate because large substituents at the S atom should be able to point out of this cleft (see Figure S1 in the Supporting Information). Indeed, we found that recombinantly expressed Ecm1 was not only able to transfer a methyl group (Figure S2) to *N*7 of the minimal substrate GpppA (**1**) as previously described,[10a] but also to install allyl-, pentenynyl, vinylbenzyl-, or azidobutenyl groups from the respective AdoMet analogues (**2b**–**e**), which were synthesized as previously described.[9c,11] Products **3a**–**e** were confirmed by reversed-phase HPLC and mass spectrometry (Figure 2 and Figures S3, S4). Strikingly, Ecm1 was able to efficiently and almost quantitatively convert even the sterically demanding substrates **2d** and **2e** (Figure 2C and Figure S3, based on HPLC analysis), thus distinguishing it from GlaTgs-V34A, which also shows substrate promiscuity but with markedly lower activity on **2d** and **2e** (Figure 2D). The pronounced substrate promiscuity enables fast and efficient conversion of

[*] J. M. Holstein, L. Anhäuser, Prof. Dr. A. Rentmeister
University of Muenster, Department of Chemistry
Institute of Biochemistry
Wilhelm-Klemm-Strasse 2, 48149 Muenster (Germany)
E-mail: a.rentmeister@uni-muenster.de
Prof. Dr. A. Rentmeister
Cells-in-Motion Cluster of Excellence (EXC 1003—CiM)
University of Muenster (Germany)

Supporting information for this article can be found under: http://dx.doi.org/10.1002/anie.201604107.

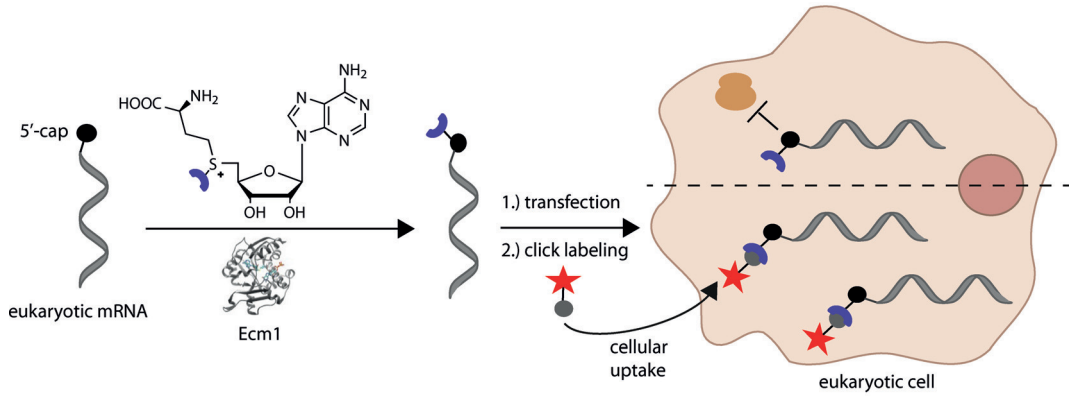

Figure 1. The concept of tuning translation and labeling mRNA in living cells through modification of the 5′-cap. The 5′-cap of the mRNA is modified at position *N*7 using methyltransferase Ecm1 from *Encephalitozoon cuniculi*, which is able to efficiently transfer bulky side chains from AdoMet analogues.

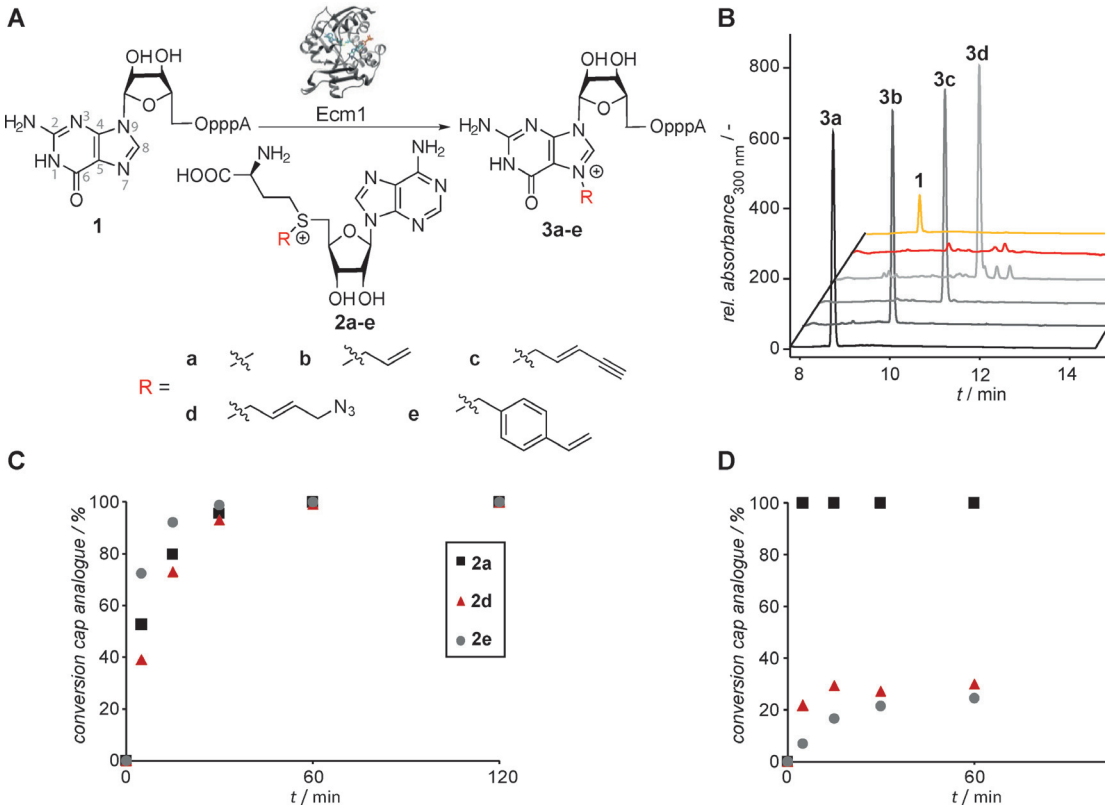

Figure 2. Enzymatic modification of the cap analogue GpppA (**1**) at *N*7 using Ecm1. A) Ecm1 catalyzes the transfer of methyl (a), allyl (b), pentenynyl (c), 4-azidobut-2-enyl (d), or 4-vinylbenzyl (e) residues to the *N*7 atom of the cap analogue **1**. B) HPLC analysis of representative modification reactions. In each case 1 mM **1**, 5 mol% Ecm1, and a 1–3-fold molar excess of the corresponding AdoMet analogue (**2a–e**) were used. Absorbance at 300 nm, which is indicative of *N*7-modified cap analogues, was monitored. Unmodified **1** shows only weak absorbance at that wavelength. The time offset is 2%. C, D) Time-course measurements for Ecm1- (C) and GlaTgs2-V34A- (D) catalyzed reactions using **2a**, **2d**, or **2e** at 5 mol% enzyme. Data show average values of two independent measurements from different protein preparations. Gray numbers indicate purine ring numbering.

a range of AdoMet analogues and thus makes Ecm1 an ideal enzyme for producing modified 5′-caps.

To ensure that our approach is suitable for longer RNAs, we produced a 106 nt RNA through in vitro transcription and used the *Vaccinia* capping system (but omitting AdoMet **2a**) for 5′-guanylation to give the GpppRNA. This GpppRNA was used for *N*7-azido modification with Ecm1 and **2d**. To facilitate analysis, modified RNA was reacted in a strain-promoted azide–alkyne cycloaddition (SPAAC) with DBCO-SRB.[12] Both the SPAAC reaction and the inverse-electron-

demand Diels–Alder (IEDDA)[13] reaction were first established on the *N*7-modified caps **3d** and **3e**, respectively. Click reactions of **3d** with DBCO-SRB or Alkyne-conjugated Megastokes Dye, as well as **3e** with tetrazine-BDP, yielded the expected products, as confirmed by mass spectrometry and in-gel fluorescence analysis (Figure S6, S7).

The *N*7-azido modified 106 nt RNA was reacted with DBCO-SRB under the established conditions and analyzed after gel electrophoresis by in-gel fluorescence. In the case of the *N*7-modified RNA, a strong fluorescent band at the correct length was detected (using a green LED and a 520/35 nm excitation filter, as well as a 609/54 nm green emission filter), thus indicating that labeling was successful and did not cause significant RNA degradation. As expected, the control RNA without a cap did not fluoresce at that wavelength when subjected to the same treatment (Figure 3B and Figure S21). Similarly, modification of this model RNA with a 4-vinylbenzyl residue (also at *N*7 of the cap) allowed efficient RNA labeling through IEDDA with tetrazine-BDP (Figure S8). These data demonstrate that different modifications attached to the *N*7 position of capped RNAs can be used for site-specific labeling in subsequent click reactions.

Methylation at position *N*7 is crucial for efficient translation, and the *N*7-methylated cap is specifically and tightly bound by the eukaryotic translation initiation factor eIF4E.[3d] Alterations at this position are therefore expected to interfere with translation as reported for ethyl and benzyl groups in vitro.[14] To assess the effect of a panel of modifications, including bioorthogonal groups, on translation, we constructed two reporter mRNAs, namely a firefly luciferase (FLuc) mRNA and an eGFP mRNA, which are approximately 1000–2000 nt long, including 5′- and 3′-UTRs as well as a poly(A) tail (Figures S9–S11). The *N*7-modified cap analogues were incorporated into these reporter mRNAs by in vitro T7 transcription.[15] As a negative control, RNA capped with ApppG (which cannot undergo cap-dependent translation) was produced. Non-capped mRNAs were digested with RNA 5′-polyphosphatase in combination with the exoribonuclease Xrn1 (Figure S12).

Translation analysis in vitro revealed that replacing the *N*7 methyl group with larger residues, including vinylbenzyl and azidobutenyl groups, in most cases abrogated translation (Figure 3C) as expected. The *N*7-benzyl group, however, reduced the translation efficiency only moderately, thus suggesting that bulkiness of the *N*7 substituent is not the only factor governing interaction with eIF4E. Our observation is in line with a previously proposed π interaction with W166 of eIF4E[14] and suggests that even substituents at *N*7 may be used to tune translation efficiency. Single substitutions at the N^2 position had a more subtle effect and allowed gradual reduction of the translation efficiency (Figure 3D).

To ensure that the effect of cap modifications on translation is biologically relevant, we also tested the translation of differently capped eGFP mRNAs in living cells. HeLa cells transfected with $m_2^{7,3'\text{-O}}$GpppG-[an "anti-reverse" cap analogue (ARCA)] or N^2-allyl-modified eGFP mRNA developed green fluorescence, whereas *N*7-allyl-modified or *N*7-azidobut-2-enyl-modified eGFP mRNA or the negative control with an ApppG cap did not result in a significant signal over background in confocal laser scanning microscopy images (Figure 4 and Figure S13). The results were confirmed by flow cytometry data for ARCA-, *N*7-azidobut-2-enyl- or ApppG-capped eGFP mRNAs (Figure S15). These experiments suggest that translation efficiencies obtained in vitro reflect the actual translation in a eukaryotic cell and rule out alternative translation initiation mechanisms that might outperform the effect of cap modifications on the 5′-cap in living cells. Our results indicate that some enzymatic modifications of the eukaryotic 5′-cap are better tolerated than others and that these modifications can be used to tune the translation efficiency of mRNAs.

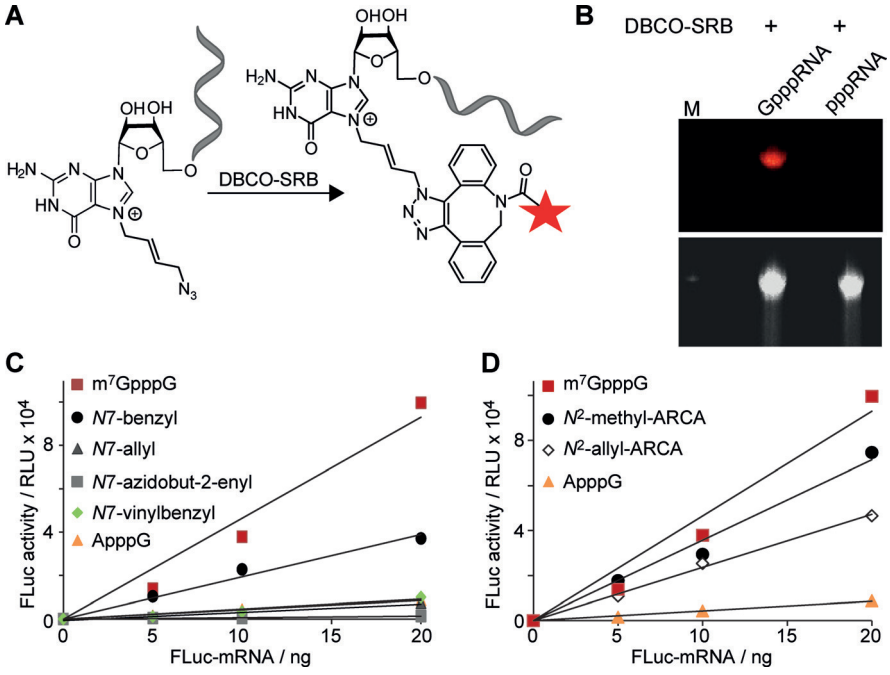

Figure 3. Labeling of *N*7-azidobut-2-enyl-modified RNA through SPAAC and translation efficiency of differently capped FLuc mRNAs. A) In vitro produced and 5′-capped RNA was reacted with Ecm1 and the AdoMet analogue **2d** for *N*7 modification, followed by click labeling with DBCO-SRB. B) Samples of 106-nt RNAs with and without *N*7-modified cap were reacted with DBCO-SRB and analyzed by in-gel fluorescence (10% denat. polyacrylamide gel). Upper panel shows SRB fluorescence (using green LED with a 520/35 nm excitation filter and a 609/54 nm green emission filter), lower panel shows ethidium bromide (EtBr) staining. M: RiboRuler Low-Range RNA Ladder. C, D) Translation efficiency of FLuc mRNAs with *N*7- (C) or N^2- (D) modified 5′-caps. The RNAs were translated for 60 min in rabbit reticulocyte lysate and luciferase activity was measured in quadruplicate (RLU: relative light units). Data points show averages of 2–4 independent experiments. DBCO = dibenzocyclooctyne, SRB = sulforhodamine B.

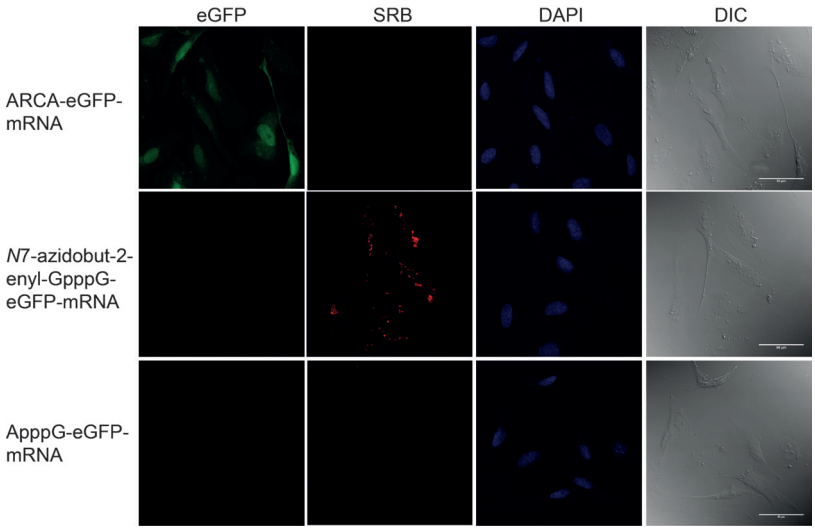

Figure 4. Bioorthogonal fluorescence labeling of eGFP mRNA in living HeLa cells. HeLa cells were transfected with *N*7-azidobut-2-enyl-GpppG-, ARCA-, or ApppG-modified eGFP mRNA, followed by incubation with DBCO-SRB and washing. For imaging, the cells were fixed after the in-cell click reaction. Panels show eGFP fluorescence, SRB fluorescence, DAPI fluorescence and DIC from left to right. Scale bars are 50 μm. DAPI = 4′,6-diamidino-2-phenylindole, a DNA stain, DIC = differential interference contrast.

In addition to tuning translation, some non-natural cap modifications can be used for subsequent bioorthogonal reactions, potentially even for mRNA labeling in living cells. Intracellular labeling of a designated target mRNA that does not contain alterations of its nucleotide sequence would enable directly investigation of the subcellular localization and trafficking of this mRNA at a given time point. To date, covalent labeling of RNA in mammalian cells has been achieved after random incorporation of modified nucleotides into nascent transcripts or through enzymatic modification of RNA tags appended to the mRNA of interest or synthetic siRNAs.[6,8] However, except for the tetrazine ligation reported by Pyka et al.[8] the click reaction itself has only been performed after fixation of the cells.[6] We have shown previously and above that both N^2 and *N*7 cap-modified model mRNAs can be reacted in various click reactions, including the bioorthogonal SPAAC and IEDDA reactions.[9a–c,16] We now set out to achieve intracellular labeling of cap-modified but otherwise unaltered eGFP mRNA in living mammalian cells based on the SPAAC reaction that has been successfully applied to label other classes of biomolecules on the cell surface of model organisms and even inside living cells.[17]

To this end, we transfected HeLa cells with these cap-modified mRNAs, followed by the addition of DBCO-SRB. For imaging on a confocal laser scanning microscope, cells were fixed with paraformaldehyde after the in-cell click reaction. For the *N*7-azido-modified mRNA, red fluorescent dots were detected, whereas mRNAs without azido modification (ARCA and ApppG caps) did not show significant red fluorescence. This indicates that the SPAAC reaction of an mRNA was successful in living cells and that excess SRB dye efficiently diffuses out (Figure 4). Quantitative RT-PCR measurements confirmed that eGFP mRNA was present at all time points at higher concentrations than the highly abundant β-actin mRNA (Figure S16). As expected, the ARCA-modified eGFP mRNA yielded a green fluorescent signal, thus confirming eGFP production. This indicates that at least a fraction of the mRNAs is available in the cytoplasm and is translated after transfection.

In conclusion, we have developed an enzymatic approach that enables tuning of the translation efficiency and intracellular labeling of a specific mRNA in living cells. We have shown that the methyltransferase Ecm1 shows remarkable promiscuity and allows almost uncompromised transfer of sterically demanding side chains from various AdoMet analogues. This efficient transfer enables for the first time easy and straightforward enzymatic production of long translatable cap-modified mRNAs as exemplified for eGFP and luciferase mRNAs, which are approximately 1000–2000 nt long. Tuning translation through cap modification allows study of the functions of a target mRNA beyond serving as a template for translation. In combination with bioorthogonal click chemistry, we provide a tool for intracellular labeling of a target mRNA, which will be valuable for studying subcellular localization during dynamic processes such as outgrowth of polarized cells or development.

Acknowledgments

A.R. gratefully acknowledges financial support from the Emmy Noether Programme of the Deutsche Forschungsgemeinschaft (RE 2796/2-1) and the Fonds der Chemischen Industrie (Dozentenstipendium). We thank Prof. Dr. Karl-Heinz Klempnauer for providing the plasmid containing the firefly luciferase gene. We thank Sabine Hüwel for excellent technical assistance. This work was supported by the Deutsche Forschungsgemeinschaft, DFG EXC 1003 Cells in Motion—Cluster of Excellence, Münster, Germany. J.M.H. and L.A. thank the Fonds der Chemischen Industrie for doctoral fellowships.

Keywords: bioorthogonal chemistry · Ecm1 · mRNA · RNA labeling · translation

How to cite: *Angew. Chem. Int. Ed.* **2016**, *55*, 10899–10903
Angew. Chem. **2016**, *128*, 11059–11063

[1] A. N. Kuhn, T. Beissert, P. Simon, B. Vallazza, J. Buck, B. P. Davies, O. Tuereci, U. Sahin, *Curr. Gene Ther.* **2012**, *12*, 347–361.

[2] a) A. N. Kuhn, M. Diken, S. Kreiter, A. Selmi, J. Kowalska, J. Jemielity, E. Darzynkiewicz, C. Huber, O. Tureci, U. Sahin, *Gene Ther.* **2010**, *17*, 961–971; b) J. Kowalska, A. Wypijewska del Nogal, Z. M. Darzynkiewicz, J. Buck, C. Nicola, A. N. Kuhn, M. Lukaszewicz, J. Zuberek, M. Strenkowska, M. Ziemniak, M.

Maciejczyk, E. Bojarska, R. E. Rhoads, E. Darzynkiewicz, U. Sahin, J. Jemielity, *Nucleic Acids Res.* **2014**, *42*, 10245 – 10264.
[3] a) Z. Wieczorek, A. Niedzwiecka-Kornas, L. Chlebicka, M. Jankowska, K. Kiraga, J. Stepinski, M. Dadlez, R. Drabent, E. Darzynkiewicz, R. Stolarski, *Z. Naturforsch. C* **1999**, *54*, 278 – 284; b) E. Darzynkiewicz, J. Stepinski, I. Ekiel, Y. Jin, D. Haber, T. Sijuwade, S. M. Tahara, *Nucleic Acids Res.* **1988**, *16*, 8953 – 8962; c) E. Darzynkiewicz, I. Ekiel, S. M. Tahara, L. S. Seliger, A. J. Shatkin, *Biochemistry* **1985**, *24*, 1701 – 1707; d) J. Marcotrigiano, A. C. Gingras, N. Sonenberg, S. K. Burley, *Cell* **1997**, *89*, 951 – 961.
[4] K. C. Martin, A. Ephrussi, *Cell* **2009**, *136*, 719 – 730.
[5] R. J. Weatheritt, T. J. Gibson, M. M. Babu, *Nat. Struct. Mol. Biol.* **2014**, *21*, 833 – 839.
[6] a) F. H. Li, J. S. Dong, X. S. Hu, W. M. Gong, J. S. Li, J. Shen, H. F. Tian, J. Y. Wang, *Angew. Chem. Int. Ed.* **2015**, *54*, 4597 – 4602; *Angew. Chem.* **2015**, *127*, 4680 – 4685; b) S. C. Alexander, K. N. Busby, C. M. Cole, C. Y. Zhou, N. K. Devaraj, *J. Am. Chem. Soc.* **2015**, *137*, 12756 – 12759; c) A. A. Sawant, A. A. Tanpure, P. P. Mukherjee, S. Athavale, A. Kelkar, S. Galande, S. G. Srivatsan, *Nucleic Acids Res.* **2016**, *44*, e16.
[7] a) A. K. Rath, A. Rentmeister, *Curr. Opin. Biotechnol.* **2015**, *31*, 42 – 49; b) D. Schulz, A. Rentmeister, *ChemBioChem* **2014**, *15*, 2342 – 2347.
[8] A. M. Pyka, C. Domnick, F. Braun, S. Kath-Schorr, *Bioconjugate Chem.* **2014**, *25*, 1438 – 1443.
[9] a) D. Schulz, J. M. Holstein, A. Rentmeister, *Angew. Chem. Int. Ed.* **2013**, *52*, 7874 – 7878; *Angew. Chem.* **2013**, *125*, 8028 – 8032; b) J. M. Holstein, D. Schulz, A. Rentmeister, *Chem. Commun.* **2014**, *50*, 4478 – 4481; c) J. M. Holstein, D. Stummer, A. Rentmeister, *Chem. Sci.* **2015**, *6*, 1362 – 1369; d) J. M. Holstein, D. Stummer, A. Rentmeister, *Protein Eng. Des. Sel.* **2015**, *28*, 179 – 186.
[10] a) S. Hausmann, S. S. Zheng, C. Fabrega, S. W. Schneller, C. D. Lima, S. Shuman, *J. Biol. Chem.* **2005**, *280*, 20404 – 20412; b) C. Fabrega, S. Hausmann, V. Shen, S. Shuman, C. D. Lima, *Mol. Cell* **2004**, *13*, 77 – 89.
[11] a) C. Dalhoff, G. Lukinavicius, S. Klimasauakas, E. Weinhold, *Nat. Protoc.* **2006**, *1*, 1879 – 1886; b) C. Dalhoff, G. Lukinavicius, S. Klimasauskas, E. Weinhold, *Nat. Chem. Biol.* **2006**, *2*, 31 – 32; c) W. Peters, S. Willnow, M. Duisken, H. Kleine, T. Macherey, K. E. Duncan, D. W. Litchfield, B. Luscher, E. Weinhold, *Angew. Chem. Int. Ed.* **2010**, *49*, 5170 – 5173; *Angew. Chem.* **2010**, *122*, 5296 – 5299.
[12] a) N. J. Agard, J. A. Prescher, C. R. Bertozzi, *J. Am. Chem. Soc.* **2004**, *126*, 15046 – 15047; b) M. F. Debets, S. S. van Berkel, S. Schoffelen, F. P. Rutjes, J. C. van Hest, F. L. van Delft, *Chem. Commun.* **2010**, *46*, 97 – 99.
[13] M. L. Blackman, M. Royzen, J. M. Fox, *J. Am. Chem. Soc.* **2008**, *130*, 13518 – 13519.
[14] E. Darzynkiewicz, J. Stepinski, I. Ekiel, C. Goyer, N. Sonenberg, A. Temeriusz, Y. Jin, T. Sijuwade, D. Haber, S. M. Tahara, *Biochemistry* **1989**, *28*, 4771 – 4778.
[15] "Enzymatic RNA Synthesis using Bacteriophage T7 RNA Polymerase": H. Gruegelsiepe, A. Schön, L. A. Kirsebom, R. K. Hartmann, in *Handbook of RNA Biochemistry* (Eds.: R. K. Hartmann, A. Bindereif, A. Schön, E. Westhof), Wiley-VCH, Weinheim, **2008**, pp. 3 – 21.
[16] a) F. Muttach, A. Rentmeister, *Angew. Chem. Int. Ed.* **2016**, *55*, 1917 – 1920; *Angew. Chem.* **2016**, *128*, 1951 – 1954; b) D. Stummer, C. Herrmann, A. Rentmeister, *ChemistryOpen* **2015**, *4*, 295 – 301.
[17] a) S. T. Laughlin, J. M. Baskin, S. L. Amacher, C. R. Bertozzi, *Science* **2008**, *320*, 664 – 667; b) S. T. Laughlin, C. R. Bertozzi, *ACS Chem. Biol.* **2009**, *4*, 1068 – 1072; c) P. V. Chang, J. A. Prescher, E. M. Sletten, J. M. Baskin, I. A. Miller, N. J. Agard, A. Lo, C. R. Bertozzi, *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 1821 – 1826; d) H. E. Murrey, J. C. Judkins, C. W. Am Ende, T. E. Ballard, Y. Fang, K. Riccardi, L. Di, E. R. Guilmette, J. W. Schwartz, J. M. Fox, D. S. Johnson, *J. Am. Chem. Soc.* **2015**, *137*, 11461 – 11475.

Received: April 27, 2016
Revised: June 21, 2016
Published online: August 11, 2016

