Available online at www.sciencedirect.com

ScienceDirect

Current Opinion in Biotechnology

Genetic switches based on nucleic acid strand displacement

Tianhe Wang, Henning Hellmer and Friedrich C Simmel

Toehold-mediated strand displacement (TMSD) is an isothermal switching process that enables the sequence-programmable and reversible conversion of DNA or RNA strands between single- and double-stranded conformations or other secondary structures. TMSD processes have already found widespread application in DNA nanotechnology, where they are used to drive DNA-based molecular devices or for the realization of synthetic biochemical computing circuits. Recently, researchers have started to employ TMSD also for the control of RNA-based gene regulatory processes *in vivo*, in particular in the context of synthetic riboregulators and conditional guide RNAs for CRISPR/Cas. Here, we provide a review over recent developments in this emerging field and discuss the opportunities and challenges for such systems in *in vivo* applications.

Address
Physics of Synthetic Biological Systems – E14, Physics Department and ZNN, Technische Universität München, Am Coulombwall 4a, 85748 Garching, Germany

Corresponding author: Simmel, Friedrich C (simmel@tum.de)

Current Opinion in Biotechnology 2023, **79**:102867

This review comes from a themed issue on **Systems Biology**

Edited by **Howard Salis**

For complete overview of the section, please refer to the article collection, "Systems Biology (2023)"

Available online 17 December 2022

https://doi.org/10.1016/j.copbio.2022.102867

Introduction

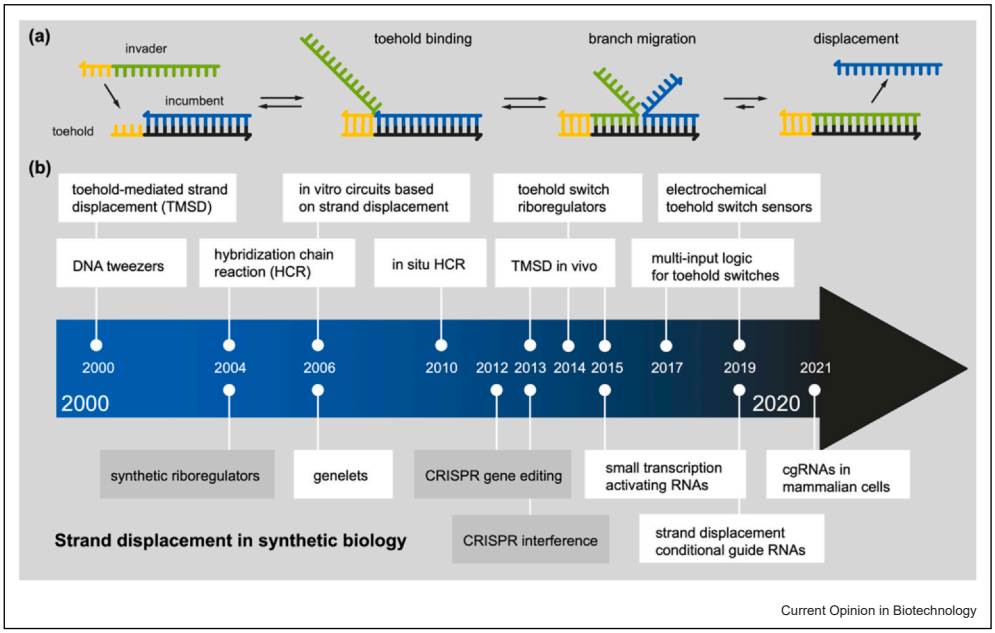
Nucleic acid strand displacement (SD) reactions involve the competition of multiple DNA or RNA molecules for binding to a complementary strand. Strand displacement was initially studied in the context of DNA recombination reactions in bacteriophages [1,2], and later extensively utilized in DNA nanotechnology [3]. A prototypical example is given by a three-way branch migration process, in which two strands of the same sequence attempt to bind to their complement. When a base pair between the 'incumbent' strand and the complement breaks, the 'invader' can make an additional base pair, and vice versa, resulting in the stochastic migration of the branch point along the complement strand. SD processes can be biased in one direction by adding a single-stranded overhang to the complement (called 'toehold'), which makes the binding of the invader thermodynamically more favorable than that of the incumbent strand (Figure 1a). Toehold-mediated strand displacement (TMSD) was applied for the actuation of DNA constructs such as 'DNA tweezers' [4], followed by a wide range of other 'non-biological' applications in nanophysics, molecular computing, or molecular robotics.

As nucleic acids are the carriers of biological information, however, it seems natural to seek for applications of TMSD reactions also in a biological context. An early step in this direction was the conception of the hybridization chain reaction (HCR) [5], which is an enzyme-free amplification scheme used for the detection of nucleic acid sequences. HCR is based on two hairpins, which have complementary sequences, but whose hybridization with each other is kinetically inhibited. Invasion of one of the hairpins by an initiator strand activates hybridization and triggers a chain reaction of hybridization events. Next to applications 'in the test tube', HCR was shown to be useful as an amplification scheme coupled to fluorescence *in situ* hybridization in fixed cells [6]. Other types of TMSD reactions that take biological inputs have resulted in sensors and logic circuits that respond to micro-RNA (miRNA) patterns or detect single-nucleotide polymorphisms [3]. While most of these applications were developed in an *in vitro* context, over the past years, an increasing number of nucleic acid devices has been demonstrated that implement TMSD-based switching in the context of gene regulation *in cellulo*.

Synthetic riboregulators

Naturally occurring RNA-based gene regulation processes often involve RNA conformational changes, and thus appear to be naturally compatible with TMSD. For instance, translational riboswitches contain an aptamer sequence in the 5′ untranslated region (UTR) of mRNA molecules, to which a small metabolite can bind. Binding leads to a refolding of the riboswitch, which either releases an initially sequestered ribosome-binding site (RBS), or hides it within an RNA duplex. Thus, binding of the metabolite leads to either activation or deactivation of translation of the mRNA molecule [7].

Figure 1



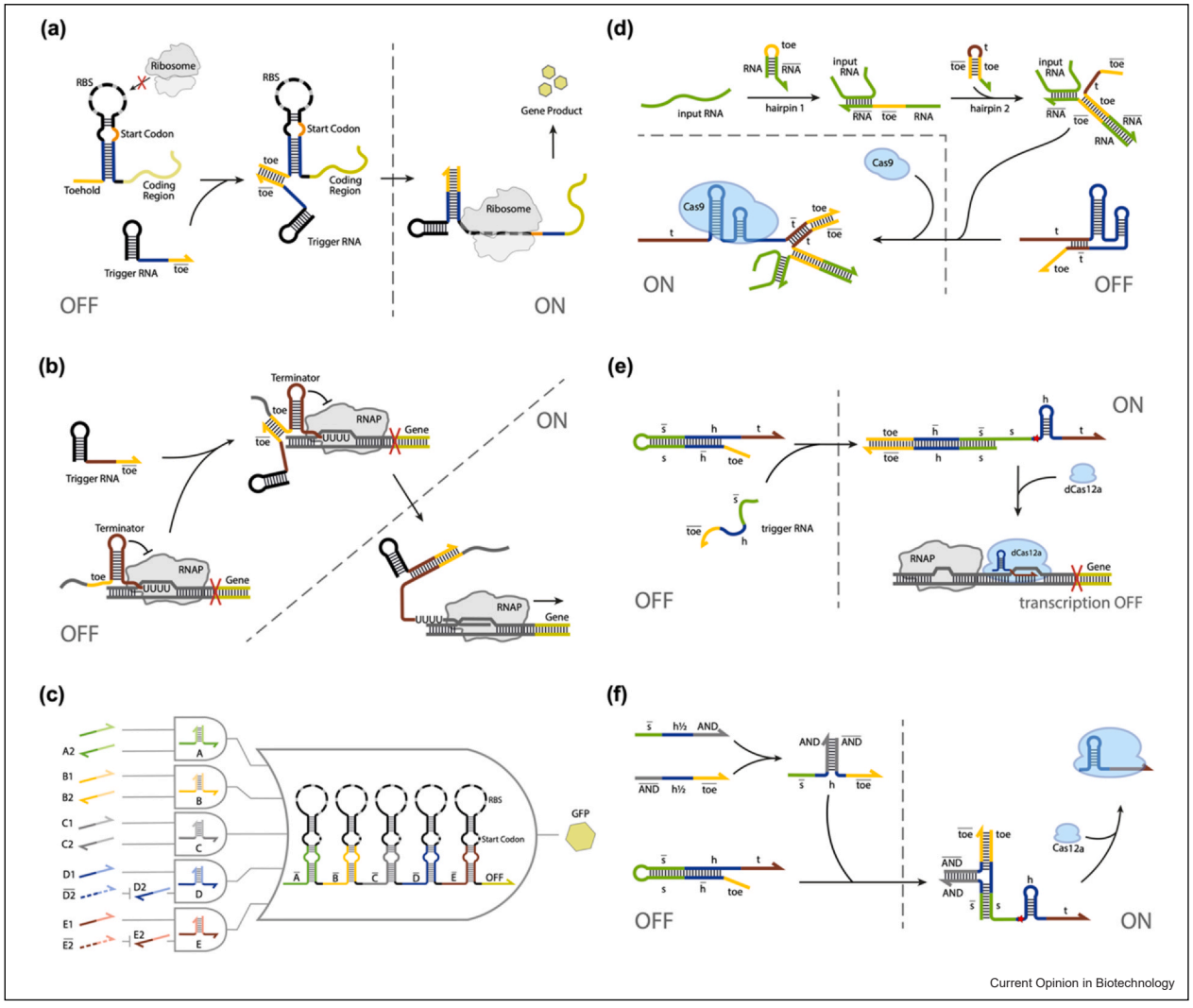
TMSD and synthetic biology. **(a)** A schematic representation of a typical TMSD process involving an invader, an incumbent, and a complementary strand. Binding of the invader to the toehold region triggers a three-way branch migration process that ultimately leads to the displacement of the incumbent. **(b)** A timeline of milestones for dynamic DNA nanotechnology in relation to applications in synthetic biology (references in the text). The development of synthetic riboregulators and CRISPR techniques (gray) is also noted as they are relevant in this context.

One of the first examples of a synthetic riboregulator controlling the expression of a protein in *E. coli* comprised of a 'cis-repressing' RNA motif [8], which sequestered the RBS within the stem of a hairpin structure in the 5′-UTR. Translation could be activated by a 'trans-acting' trigger RNA molecule, which was designed to bind to the cis-repressing RNA's hairpin, breaking the secondary structure and thus releasing the RBS. A genuine *in vivo* application of TMSD was next provided by the so-called 'toehold switch' [9] (Figure 2a), which was based on the concept of the cis-repressor. Differing from the original design, the toehold switch was equipped with an unpaired 14-nt toehold region at the 5′-end of the regulatory hairpin, while RBS and start codon were placed within a hairpin loop and a bulge in the stem, respectively. The toehold switch is activated by a trigger RNA, which is complementary to the toehold and most of the stem sequence. Binding of the trigger to the toehold promotes invasion of the hairpin stem, which exposes the RBS and the start codon and thus enables translation initiation. Importantly, the toehold switch design allows to choose the sequence of the trigger RNA without any constraints and can thus be adapted to arbitrary RNA inputs. When using RNA triggers transcribed from high copy number plasmids in *E. coli*, the toehold switches showed ON/OFF ratios of up to several-hundred fold.

Several design variations of the toehold switches were developed recently, among them, toehold switch repressors [10] and riboregulators that merged toehold switching with the design principles of naturally occurring riboswitches [11]. Synthetic riboregulators can also be designed to control transcription processes via switchable termination. The so-called small transcription-activating RNAs, modulate the formation of an intrinsic terminator structure at the 5-UTR of an mRNA transcript, leading to transcriptional activation by almost 100-fold (Figure 2b) [12].

The adaptability of the toehold switches to arbitrary inputs has been utilized for the creation of biosensors that were operated in cell-free gene expression systems. Toehold switches were applied in low-cost diagnostics for the detection of RNA viruses such as Ebola, Zika, and SARS-CoV-2 [16–18], and were recently coupled to an electrochemical readout [19]. By balancing the energies of binding of cognate and mismatched trigger strands, single-nucleotide-specific programmable riboregulators were realized [20]. The modularity of their design combined with the free choice of trigger sequences was also utilized to create toehold switches with complex input logic. For instance, several toehold switches can be connected together to create multi-input AND, OR, or NOR logic (Figure 2c) [10,13,21],

Figure 2



RNA SD in riboregulators and conditional CRISPR systems. **(a)** In a toehold switch, RBS and start codon are sequestered in a loop and bulge, respectively. TMSD by a trigger RNA opens the toehold switch hairpin, releases the RBS, and thus activates translation [9]. **(b)** Transcriptional regulation can be exerted by influencing the formation of a terminator hairpin. Disruption of the terminator by the trigger RNA allows transcriptional elongation to proceed [12]. **(c)** Multiple toehold switches can be coupled in series (shown here) or in parallel to enable control of gene expression via multi-input logic [13]. **(d)** TMSD can be used to activate or deactivate CRISPR/Cas processes. In the image, the handle of a Cas9 guide RNA is blocked by a secondary structure, but can be released by an RNA complex containing an appropriate toehold. The reaction of the input RNA with hairpins 1 and 2 serves to decouple input sequence from CRISPR target (spacer) sequence [14]. **(e)** Activation of guide RNAs for Cas12a can make use of the RNA-processing function of Cas12a, which cleaves off the 5′-sequence upstream of the handle (red dot) [15]. **(f)** Input logic can be easily realized by assembling the trigger from components. In the image shown, the green–blue and the blue–yellow sequence domains alone cannot invade the conditional guide RNA. When they hybridize together, they can activate the CRISPR/Cas12a process, resulting in 'AND gate' input logic [15].

transcriptional and translational switching can be combined to result in AND [22] or NOR logic [11], and also more complex functions have been demonstrated [23].

Application of toehold-mediated strand displacement to CRISPR/Cas

While riboregulators rely solely on nucleic acids, other powerful regulatory processes such as CRISPR/Cas or RNA interference (RNAi) are based on ribonucleoprotein complexes. CRISPR/Cas systems utilize RNA-dependent nucleases such as Cas9 or Cas12a, which are directed to their cleavage site on a DNA duplex by guide RNA molecules (gRNAs), which contain a spacer sequence (binding to the targeted DNA sequence) and a handle sequence, whose secondary structure is recognized by the Cas protein. Influencing the handle's secondary structure or its accessibility can thus be used to control the CRISPR/Cas processes. Cas-derived, noncleaving

effectors can be applied for gRNA-controlled transcriptional repression or activation (termed CRISPR interference (CRISPRi) [24] or activation [25]), base- and prime-editing [26], and many others. Introduction of ligand-binding aptamers into the gRNAs has been shown to render CRISPR processes controllable by the presence of small-molecule ligands [27,28].

A variety of conditional CRISPR systems based on TMSD-switchable gRNAs have been demonstrated to work *in vivo* since 2019 [15,29–31]. To this end, guide RNAs were augmented with effector sequences containing a toehold region that folds back onto the gRNA and thus prevents binding of the Cas protein (Figure 2d,e). Displacement of the effector by a trigger RNA restores the handle structure and thus activates the CRISPR/Cas process. Depending on the Cas protein, the processes differ in detail. For instance, gRNAs for Cas9 are extended at the 3′-end, while gRNAs for Cas12a are elongated at the 5′-end. Further, in contrast to Cas9, Cas12a has an RNAse activity that cleaves off the 5′-sequence after binding to the handle (Figure 2e).

When operated as transcriptional repressors in *E. coli* (CRISPRi with catalytically inactive proteins dCas9 and dCas12a), these systems provide up to 50-fold repression or activation [15] and allow orthogonality between multiple gRNAs [29,30,32]. Optimized designs have resulted in improved gRNA processing, efficiency, and ON/OFF ratios [33,34]. gRNAs can also be hybridized to additional blocking strands, which can be removed by TMSD to restore activity [35]. More versatile TMSD-based conditional guide RNAs have made use of engineered Cas9 and gRNA variants, and have been demonstrated to function both in bacterial and human cells [31].

Among the challenges of implementing TMSD with naturally occurring RNA molecules such as gRNAs are the sequence constraints imposed by them, as often part of the natural sequence or its complement have to be used in the trigger RNAs. In order to address this issue, several methods were developed to decouple input and gRNA sequences [14,30,32]. In particular, the RNA-processing function of dCas12 was used to remove any sequence context from the gRNAs, which was used to implement logic gates as well as orthogonal switching without sequence constraints [15,32,36]. The use of designed nonrepetitive gRNA handles [37] as targets for trigger strands might provide an alternative approach to alleviate such constraints for Cas9.

TMSD was also integrated with microRNA or ribozyme action, and even implemented in mammalian cells [36]. We note that TMSD was also already applied to control RNAi in mammalian cells, where functional siRNAs were assembled by branch migration from precursor duplexes that had been transfected into the cells [38,39].

The design–build–test–learn cycle for strand displacement systems

Among the major advantages of applying TMSD to RNA regulation is its sequence programmability, which enables a comparatively simple and fast design–build–test–learn (DBTL) cycle (Figure 3). While it is currently still very difficult to design proteins and protein–protein interactions *de novo*, and typically cumbersome to implement and debug them in the lab, these steps are relatively straightforward for nucleic acid-based structures and interactions.

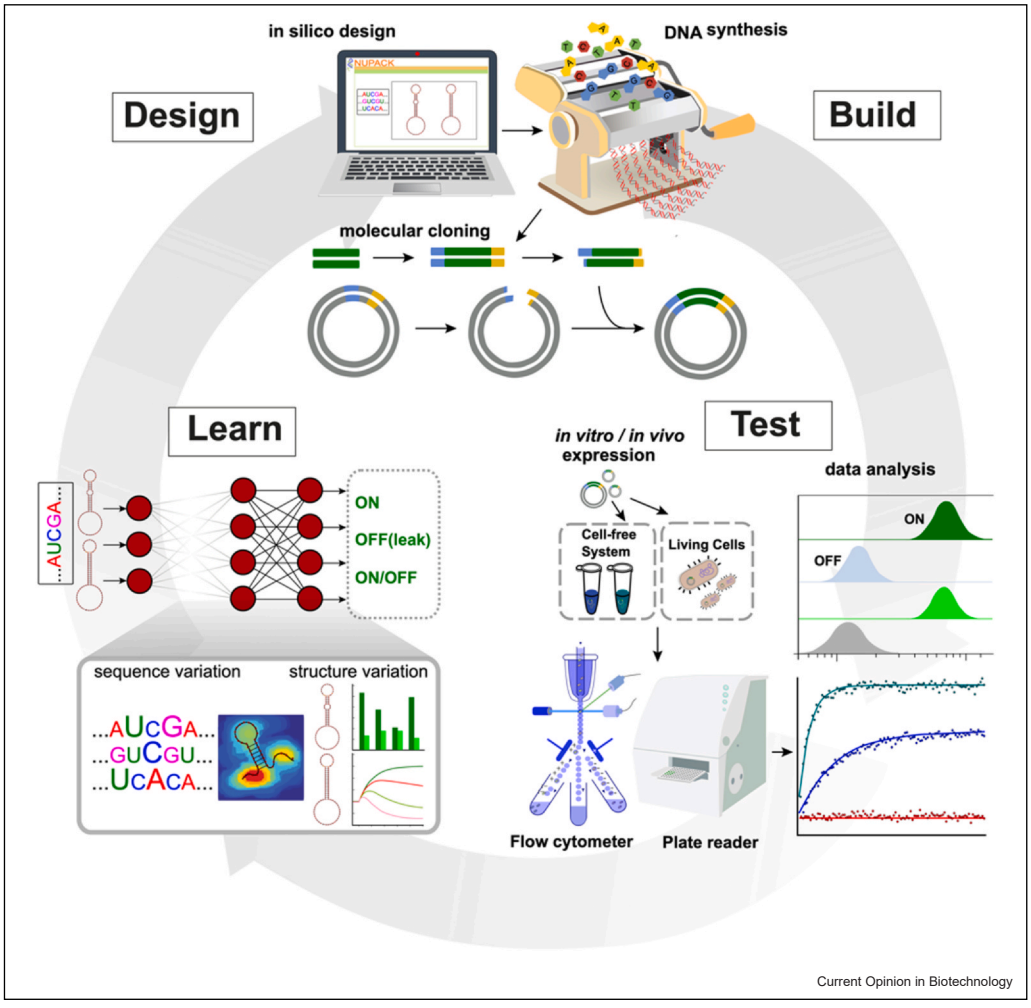
'Design' is greatly facilitated by software tools for the prediction of nucleic acid structures and thermodynamics such as NUPACK [40,41], ViennaRNA [42], or Mfold [43], or also for the translation rate of an RBS [44]. Other tools such as Multistrand [45] and OxDNA [46] allow to analyze the kinetics of SD processes at different levels of coarse-graining. In the 'build' phase, oligo sequences are synthesized and assembled into gene templates [47], which can be converted into plasmids using standard cloning techniques. In the 'test' phase, genetic switches and circuits can be assessed in cell-free, bacterial, or eukaryotic expression systems. The performance of the systems is typically monitored via the expression of fluorescent proteins, aptamers, or enzymatic processes, which makes use of lab equipment such as microplate readers, flow cytometry, or droplet microfluidics.

In many cases, the sequence-function relationship turns out to be more complex than anticipated by software-supported 'rational design', and screening of alternative variants may become necessary. In these cases, it is relatively straightforward to generate sequence libraries and assess their performance using high-throughput methodology. Successful library members can then be identified by sequencing. Expression profiles and their corresponding sequences or secondary structures can be used to 'learn' improved design rules and implement them in the DBTL cycle. For instance, based on the characterization of a library of more than 90 000 toehold switch riboregulators, deep neural network approaches were recently used to predict the performance of toehold switches, and also to gain biological insight into salient sequence and structural features [48,49].

Challenges

Despite recent examples of successful implementation of TMSD processes for gene regulation, several challenges remain, which refer to sequence constraints and a lack of performance *in vivo*. As mentioned, sequence constraints imposed by biological sequences can be circumvented by smart sequence or structure design, or they may implicitly be accounted for by the utilization of machine-learning approaches.

Figure 3



RNA-based gene regulatory switches can be developed in a straightforward DBTL cycle. RNA structure *Design* is guided by powerful computational tools. The designed sequences can be readily synthesized using modern oligo synthesis and gene assembly methodology (*Build*). Various platforms for the high-throughput characterization (*Test*) of regulatory function of RNA components, both *in vivo* and in cell-free expression systems, exist, for example, flow cytometry, plate readers, and sequencing. Information gained in these experiments can be used to train machine-learning models (*Learn*) that in turn will allow to further inform and refine the computational design. Machine-learning pipelines for toehold switches have been demonstrated in Ref. [48,49].
Learn panel adapted from Angenent-Mari et al. [48].

One of the major problems, however, is the *in vivo* efficacy of the RNA hybridization and TMSD reactions themselves. As a second-order reaction, binding of an input RNA to a toehold depends on the concentration of the species and the accessibility of the sequences. *In vivo* TMSD reactions are thus strongly affected by the (in-)stability of RNA, interactions with other cellular components, subcellular localization, and the low concentrations of endogenous transcripts. *In vivo* stability of RNA can be improved by adding RNA hairpins, RNA triplex-forming structures [50], and other secondary structural elements [51,52], or utilizing RNA-stabilizing proteins such as, for example, Hfq in bacteria [53]. Interactions with other RNA strands might be considered by computationally screening the transcriptome, or — in the absence of detailed knowledge on intracellular RNA structure and RNA–protein interactions — by machine-learning-assisted approaches [54]. Compartmentalization plays an important role as it may either prevent interactions between separate components, or speed them up by colocalization. While nuclear localization in eukaryotic cells can be controlled by the choice of the promoter, emerging concepts such as liquid–liquid-phase separation in RNA–protein droplets are currently underexplored [55]. Finally, the low concentration of endogenous RNAs as potential inputs for TMSD circuits might be one of the most difficult problems to tackle, and so far, only few convincing examples have

demonstrated the use of sRNA or miRNA as inputs [14,36,56]. In the case of miRNAs or gRNAs, the RNA molecules are protected from degradation within their ribonucleoprotein complexes. In other cases, such as mRNA, other measures will have to be taken, for example, colocalizing transcripts with the input stage of the circuits to promote hybridization, or by developing *in vivo* signal-amplifying circuits that reliably detect the rarely occurring hybridization events. In this context, RNA-guided RNA editing processes by adenosine deamines acting on RNA have been recently successfully utilized to detect very low RNA copy numbers [57,58].

Conclusion

Over the past years, it has been demonstrated that one of the key processes of dynamic nucleic acid nanotechnology — TMSD — can be successfully employed *in vivo*, which has resulted in novel gene regulatory mechanisms for synthetic biology. The realization of toehold switch riboregulators and related systems has shown great promise for biosensing and biocomputing applications, and SD-switchable guide RNAs can be used to render CRISPR processes dependent on RNA inputs. Among the major advantages of processes that rely mainly on RNA–RNA interactions is their sequence programmability, ease of experimental implementation, and the resulting efficient DBTL cycle. Furthermore, sequence-defined interactions provide a rational and potentially scalable way to connect components into larger circuits and networks. Many challenges remain that currently prevent a more widespread utilization of SD processes *in vivo*. Next to the sequence constraints imposed by the environment, these refer to the control of degradation, localization, and efficient hybridization of RNA species inside cells. We anticipate that an improved understanding of intracellular RNA structure, localization, and interactions, supported by mechanistic modeling and machine-learning approaches, will ultimately make SD a powerful and programmable concept for the realization of *in vivo* biocomputing circuits.

Funding

This work was supported by the Deutsche Forschungsgemeinschaft (grant no. DFG SI761/5–1).

CRediT authorship contribution statement

Tianhe Wang: Conceptualization, Visualization, Writing – original draft; **Henning Helmer:** Conceptualization, Visualization, Writing – original draft; **Friedrich C. Simmel:** Supervision, Conceptualization, Visualization, Writing – original draft, Writing – review & editing.

Data availability

No data were used for the research described in the article.

Conflict of interest statement

The authors declare no conflict of interest.

Acknowledgements

We gratefully acknowledge support by the Deutsche Forschungsgemeinschaft.

References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Lee CS, Davis RW, Davidson N: **A physical study by electron microscopy of the terminally repetitious, circularly permuted DNA from the coliphage particles of *Escherichia coli* 15**. *J Mol Biol* 1970, **48**:1-22.

2. Broker TR, Lehman IR: **Branched DNA molecules — Intermediates in T4 recombination**. *J Mol Biol* 1971, **60**:131-149.

3. Simmel FC, Yurke B, Singh HR: **Principles and applications of nucleic acid strand displacement reactions**. *Chem Rev* 2019, **119**:6326-6369.

4. Yurke B, Turberfield AJ, Mills AP, Simmel FC, Neumann JL: **A DNA-fuelled molecular machine made of DNA**. *Nature* 2000, **406**:605-608.

5. Dirks RM, Pierce NA: **Triggered amplification by hybridization chain reaction**. *Proc Natl Acad Sci USA* 2004, **101**:15275-15278.

6. Choi HM, Beck VA, Pierce NA: **Next-generation *in situ* hybridization chain reaction: higher gain, lower cost, greater durability**. *ACS Nano* 2014, **8**:4284-4294.

7. Tucker BJ, Breaker RR: **Riboswitches as versatile gene control elements**. *Curr Opin Struct Biol* 2005, **15**:342-348.

8. Isaacs FJ, Dwyer DJ, Ding C, Pervouchine DD, Cantor CR, Collins JJ: **Engineered riboregulators enable post-transcriptional control of gene expression**. *Nat Biotechnol* 2004, **22**:841-847.

9. Green AA, Silver PA, Collins JJ, Yin P: **Toehold switches: *de-novo*-designed regulators of gene expression**. *Cell* 2014, **159**:925-939.

10. •• Kim J, Zhou Y, Carlson PD, Teichmann M, Chaudhary S, Simmel FC, Silver PA, Collins JJ, Lucks JB, Yin P, *et al.*: ***De novo*-designed translation-repressing riboregulators for multi-input cellular logic**. *Nat Chem Biol* 2019, **15**:1173-1182.
This paper shows gene regulation logic with RNA-based translational repressors in *E.coli*.

11. Wang T, Simmel FC: **Riboswitch-inspired toehold riboregulators for gene regulation in *Escherichia coli***. *Nucleic Acids Res* 2022, **50**:4784-4798.

12. Chappell J, Takahashi MK, Lucks JB: **Creating small transcription activating RNAs**. *Nat Chem Biol* 2015, **11**:214-220.

13. •• Green AA, Kim J, Ma D, Silver PA, Collins JJ, Yin P: **Complex cellular logic computation using ribocomputing devices**. *Nature* 2017, **548**:117-121.
In this paper, multi-input logic gates with toehold siwtch translational activators are demonstrated.

14. Lin J, Liu Y, Lai P, Ye H, Xu L: **Conditional guide RNA through two intermediate hairpins for programmable CRISPR/Cas9 function: building regulatory connections between endogenous RNA expressions**. *Nucleic Acids Res* 2020, **48**:11773-11784.

15. •• Oesinghaus L, Simmel FC: **Switching the activity of Cas12a using guide RNA strand displacement circuits**. *Nat Commun* 2019, **10**:2092.
This paper shows toehold-mediated switching of guide RNAs for CRISPR/Cas12a *in vitro* and in bacteria

16. Pardee K, Green AA, Ferrante T, Cameron DE, DaleyKeyser A, Yin P, Collins JJ: **Paper-based synthetic gene networks**. *Cell* 2014, **159**:940-954.

17. Pardee K, Green AA, Takahashi MK, Braff D, Lambert G, Lee JW, Ferrante T, Ma D, Donghia N, Fan M, *et al*.: **Rapid, low-cost detection of Zika virus using programmable biomolecular components**. *Cell* 2016, **165**:1255-1266.

18. Carr AR, Dopp JL, Wu K, Mousavi PS, Jo YR, McNeley CE, Lynch ZT, Pardee K, Green AA, Reuel NF: **Toward Mail-in-Sensors for SARS-CoV-2 detection: interfacing gel switch resonators with cell-free toehold switches**. *ACS Sens* 2022, **7**:806-815.

19. Mousavi PS, Smith SJ, Chen JB, Karlikow M, Tinafar A, Robinson C, Liu W, Ma D, Green AA, Kelley SO, *et al*.: **A multiplexed, electrochemical interface for gene-circuit-based sensors**. *Nat Chem* 2019, **12**:48-55.

20. Hong F, Ma D, Wu K, Mina LA, Luiten RC, Liu Y, Yan H, Green AA: **Precise and programmable detection of mutations using ultraspecific riboregulators**. *Cell* 2020, **180**:1018-1032.e1016.

21. Ma D, Li Y, Wu K, Yan Z, Tang AA, Chaudhary S, Ticktin ZM, Alcantar-Fernandez J, Moreno-Camacho JL, Campos-Romero A, *et al*.: **Multi-arm RNA junctions encoding molecular logic unconstrained by input sequence for versatile cell-free diagnostics**. *Nat Biomed Eng* 2022, **6**:298-309.

22. Lehr FX, Hanst M, Vogel M, Kremer J, Goringer HU, Suess B, Koeppl H: **Cell-free prototyping of AND-logic gates based on heterogeneous RNA activators**. *ACS Synth Biol* 2019, **8**:2163-2173.

23. Falgenhauer E, Muckl A, Schwarz-Schilling M, Simmel FC: **Transcriptional interference in toehold switch-based RNA circuits**. *ACS Synth Biol* 2022, **11**:1735-1745.

24. Qi Lei S, Larson Matthew H, Gilbert Luke A, Doudna Jennifer A, Weissman Jonathan S, Arkin Adam P, Lim Wendell A: **Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression**. *Cell* 2013, **152**:1173-1183.

25. Kiani S, Chavez A, Tuttle M, Hall RN, Chari R, Ter-Ovanesyan D, Qian J, Pruitt BW, Beal J, Vora S, *et al*.: **Cas9 gRNA engineering for genome editing, activation and repression**. *Nat Methods* 2015, **12**:1051-1054.

26. Anzalone AV, Koblan LW, Liu DR: **Genome editing with CRISPR–Cas nucleases, base editors, transposases and prime editors**. *Nat Biotechnol* 2020, **38**:824-844.

27. Kundert K, Lucas JE, Watters KE, Fellmann C, Ng AH, Heineike BM, Fitzsimmons CM, Oakes BL, Qu J, Prasad N, *et al*.: **Controlling CRISPR-Cas9 with ligand-activated and ligand-deactivated sgRNAs**. *Nat Commun* 2019, **10**:2127.

28. Iwasaki RS, Ozdilek BA, Garst AD, Choudhury A, Batey RT: **Small molecule regulated sgRNAs enable control of genome editing in *E. coli* by Cas9**. *Nat Commun* 2020, **11**:1394.

29. •• Siu KH, Chen W: **Riboregulated toehold-gated gRNA for programmable CRISPR-Cas9 function**. *Nat Chem Biol* 2019, **15**:217-220.
This paper shows toehold-mediated switching of guide RNAs for CRISPR/Cas9.

30. Li Y, Teng X, Zhang K, Deng R, Li J: **RNA strand displacement responsive CRISPR/Cas9 system for mRNA sensing**. *Anal Chem* 2019, **91**:3989-3996.

31. •• Hanewich-Hollatz MH, Chen Z, Hochrein LM, Huang J, Pierce NA: **Conditional guide RNAs: programmable conditional regulation of CRISPR/Cas function in bacterial and mammalian cells via dynamic RNA nanotechnology**. *ACS Cent Sci* 2019, **5**:1241-1249.
This paper demonstrates gene activation with switchable guide RNAs for Cas9 in bacterial and mammalian cells.

32. Collins SP, Rostain W, Liao C, Beisel CL: **Sequence-independent RNA sensing and DNA targeting by a split domain CRISPR-Cas12a gRNA switch**. *Nucleic Acids Res* 2021, **49**:2985-2999.

33. Hunt VM, Chen W: **A microRNA-gated thgRNA platform for multiplexed activation of gene expression in mammalian cells**. *Chem Commun* 2022, **58**:6215-6218.

34. Hunt VM, Chen W: **Deciphering the design rules of toehold-gated sgRNA for conditional activation of gene expression and protein degradation in mammalian cells**. *ACS Synth Biol* 2022, **11**:397-405.

35. Hao Y, Li J, Li Q, Zhang L, Shi J, Zhang X, Aldalbahi A, Wang L, Fan C, Wang F: **Programmable live-cell CRISPR imaging with toehold-switch-mediated strand displacement**. *Angew Chem Int Ed Engl* 2020, **59**:20612-20618.

36. •• Oesinghaus L, Simmel FC: **Controlling gene expression in mammalian cells using multiplexed conditional guide RNAs for Cas12a**. *Angew Chem Int Ed* 2021, **60**:23894-23902.
This paper demonstrates gene activation with switchable guide RNAs for Cas12a in mammalian cells.

37. Reis AC, Halper SM, Vezeau GE, Cetnar DP, Hossain A, Clauer PR, Salis HM: **Simultaneous repression of multiple bacterial genes using nonrepetitive extra-long sgRNA arrays**. *Nat Biotechnol* 2019, **37**:1294-1301.

38. Afonin KA, Viard M, Martins AN, Lockett SJ, Maciag AE, Freed EO, Heldman E, Jaeger L, Blumenthal R, Shapiro BA: **Activation of different split functionalities on re-association of RNA-DNA hybrids**. *Nat Nanotechnol* 2013, **8**:296-304.

39. Groves B, Chen YJ, Zurla C, Pochekailov S, Kirschman JL, Santangelo PJ, Seelig G: **Computing in mammalian cells with nucleic acid strand exchange**. *Nat Nanotechnol* 2016, **11**:287-294.

40. Dirks RM, Pierce NA: **A partition function algorithm for nucleic acid secondary structure including pseudoknots**. *J Comput Chem* 2003, **24**:1664-1677.

41. Zadeh JN, Steenberg CD, Bois JS, Wolfe BR, Pierce MB, Khan AR, Dirks RM, Pierce NA: **NUPACK: analysis and design of nucleic acid systems**. *J Comput Chem* 2010, **32**:170-173.

42. Lorenz R, Bernhart SH, Honer Zu Siederdissen C, Tafer H, Flamm C, Stadler PF, Hofacker IL: **ViennaRNA Package 2.0**. *Algorithms Mol Biol* 2011, **6**:26.

43. Zuker M: **Mfold web server for nucleic acid folding and hybridization prediction**. *Nucleic Acids Res* 2003, **31**:3406-3415.

44. Salis HM, Mirsky EA, Voigt CA: **Automated design of synthetic ribosome binding sites to control protein expression**. *Nat Biotechnol* 2009, **27**:946-950.

45. Schaeffer JM, Thachuk C, Winfree E: **Stochastic simulation of the kinetics of multiple interacting nucleic acid strands**. International Workshop on DNA-Based Computers. Springer; 2015:194-211.

46. Poppleton E, Romero R, Mallya A, Rovigatti L, Sulc P: **OxDNA.org: a public webserver for coarse-grained simulations of DNA and RNA nanostructures**. *Nucleic Acids Res* 2021, **49**:W491-W498.

47. Kosuri S, Church GM: **Large-scale *de novo* DNA synthesis: technologies and applications**. *Nat Methods* 2014, **11**:499-507.

48. • Angenent-Mari NM, Garruss AS, Soenksen LR, Church G, Collins JJ: **A deep learning approach to programmable RNA switches**. *Nat Commun* 2020, **11**:5057.
These two papers establish deep learning-based pipelines for the investigation and improvement of synthetic riboregulators.

49. • Valeri JA, Collins KM, Ramesh P, Alcantar MA, Lepe BA, Lu TK, Camacho DM: **Sequence-to-function deep learning frameworks for engineered riboregulators**. *Nat Commun* 2020, **11**:5058.
These two papers establish deep learning-based pipelines for the investigation and improvement of synthetic riboregulators.

50. Wilusz JE, JnBaptiste CK, Lu LY, Kuhn C-D, Joshua-Tor L, Sharp PA: **A triple helix stabilizes the 3′ ends of long noncoding RNAs that lack poly(A) tails**. *Genes Dev* 2012, **26**:2392-2407.

51. Zhang Q, Ma D, Wu F, Standage-Beier K, Chen X, Wu K, Green AA, Wang X: **Predictable control of RNA lifetime using engineered degradation-tuning RNAs**. *Nat Chem Biol* 2021, **17**:828-836.

52. Cetnar DP, Salis HM: **Systematic quantification of sequence and structural determinants controlling mRNA stability in bacterial operons**. *ACS Synth Biol* 2021, **10**:318-332.

53. Updegrove TB, Zhang A, Storz G: **Hfq: the flexible RNA matchmaker**. *Curr Opin Microbiol* 2016, **30**:133-138.

54. Mihailovic MK, Vazquez-Anderson J, Li Y, Fry V, Vimalathas P, Herrera D, Lease RA, Powell WB, Contreras LM: **High-throughput *in vivo* mapping of RNA accessible interfaces to identify functional sRNA binding sites**. *Nat Commun* 2018, **9**:4084.

55. Lyon AS, Peeples WB, Rosen MK: **A framework for understanding the functions of biomolecular condensates across scales**. *Nat Rev Mol Cell Biol* 2021, **22**:215-235.

56. •• Lin J, Wang W-J, Wang Y, Liu Y, Xu L: **Building endogenous gene connections through RNA self-assembly controlled CRISPR/Cas9 function**. *J Am Chem Soc* 2021, **143**:19834-19843.

This paper shows the use of endogenous RNA species for conditional CRISPR/Cas9 processes.

57. Jiang K, Koob J, Chen XD, Krajeski RN, Zhang Y, Volf V, Zhou W, Sgrizzi SR, Villiger L, Gootenberg JS, *et al*.: **Programmable eukaryotic protein synthesis with RNA sensors by harnessing ADAR**. *Nat Biotechnol* 2022,1-10, https://doi.org/10.1038/s41587-022-01534-5

58. Kaseniit KE, Katz N, Kolber NS, Call CC, Wengier DL, Cody WB, Sattely ES, Gao XJ: **Modular, programmable RNA sensing using ADAR editing in living cells**. *Nat Biotechnol* 2022,1-6, https://doi.org/10.1038/s41587-022-01493-x