Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

HHS Public Access

Author manuscript

DNA RNA Nanotechnol. Author manuscript; available in PMC 2021 July 23.

Published in final edited form as:

DNA RNA Nanotechnol. 2016 January ; 3(1): 11–13. doi:10.1515/rnan-2016-0002.

RNA Toehold Interactions Initiate Conditional Gene Silencing

Paul Zakrevsky,

Gene Regulation and Chromosome Biology Lab, Center for Cancer Research, National Cancer Institute, Frederick, MD 21702

Eckart Bindewald,

Basic Science Program, Leidos Biomedical Research, Frederick National Lab for Cancer Research, Frederick, MD 21702

Bruce A Shapiro

Gene Regulation and Chromosome Biology Lab, Center for Cancer Research, National Cancer Institute, Frederick, MD 21702

RNA interference (RNAi) is a powerful approach for the knockdown of target gene expression. Not long after its initial discovery, RNAi garnered interest as a potential therapeutic tool to combat cancer and disease. For RNAi based therapy to be effective, it is desirable that the RNAi response be induced only in a specified cellular context where the resulting gene silencing would be advantageous. To fulfill this criterion, RNAi substrates can be linked to a diagnostic component, enabling them to distinguish diseased cells from normal tissue. Aptamers that specifically bind a particular cell-surface protein have frequently been linked to siRNAs and larger RNA nanoparticles to restrict RNAi substrate uptake to particular target cells [1-3]. More recently, researchers have been exploring the use of single-stranded toeholds and branch migration to induce formation of RNAi-activating RNA duplexes in a conditional fashion upon recognition of specified nucleic acid triggers [4-6]. Two new articles published by Bruce Shapiro and colleagues in *Nano Letters* explore the extent to which RNA toehold interactions can be used to prompt conditional RNAi-mediated gene silencing [7,8].

Previous work demonstrated that splitting a functional RNA duplex into two initially inert RNA/DNA hybrid duplexes allows for conditional formation of an RNA duplex when both hybrids are present (Figure 1, left). The newly formed RNA duplex is functional as it acts as a substrate for Dicer and initiates the RNA interference pathway. Now, Afonin *et al*. show in their recent publication that single-stranded (ss) RNA, rather than ssDNA, is preferential for use as the toehold within cognate hybrid duplexes, providing several benefits compared to previous cognate hybrid designs. From the perspective of thermodynamics, the use of RNA toeholds is advantageous as it greatly reduces the length of the single-stranded ends required to unzip the hybrids and generate the functional RNA element. From a design perspective,

This work is licensed under the Creative Commons Attribution-NonCommercial-NoDerivs 3.0 License. http://creativecommons.org/licenses/by-nc-nd/4.0/

Corresponding author: Bruce A Shapiro, Gene Regulation and Chromosome Biology Lab, Center for Cancer Research, National Cancer Institute, Frederick, MD 21702, shapirbr@mail.nih.gov.

Competing Financial Interests: The authors declare that they have no competing financial interests.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

because RNA is used as the toehold strand, the toehold sequence can inherently be part of the functional Dicer substrate RNA, or other potential RNA moiety, reducing the size and minimizing the design constraints of the resulting hybrid duplexes. Conditional hybrids that contain ssRNA toeholds also prove advantageous for incorporation into more complex RNA nanoparticles. The authors demonstrate that RNA nanorings functionalized with RNA-toeholded hybrids exhibit increased yields from enzymatic co-transcriptional synthesis, as well as reduced overall nanoparticle size, compared to nanorings functionalized with DNA-toeholded hybrid duplexes.

In the second publication from Shapiro and colleagues, Bindewald *et al.* present a novel two-stranded RNA switch that releases a small hairpin RNA (shRNA) upon recognition of a specific cellular mRNA (Figure 1, right). The RNA switch initially exists in an inactive state, with the shRNA strand sequestered in an inactive fold through formation of an inter-strand pseudoknot structure. Interaction of the switch's ssRNA toehold with a complementary "trigger" RNA unzips the switch and leads to a conformational rearrangement. This conformational change produces a functional shRNA structure able to act as a Dicer substrate and activate the RNAi pathway for knockdown of target gene expression. As a proof of concept, the authors show that the RNA switch is able to silence eGPF expression in response to the presence of connective tissue growth factor (CTGF) mRNA in human breast cancer cells. Notably, any combination of mRNA triggers and silencing targets can theoretically be accommodated by this switch design, as there is no overlap between the sequence region that encodes the functional shRNA stem and the region that interacts with the RNA trigger.

The prediction of base pairings within nucleic acid nanostructures is a computational task that has a variety of challenges. Central to the design of the conditional hybrid duplexes and the two-stranded RNA switch is HyperFold, a newly developed secondary structure prediction algorithm described in detail in the article from Bindewald *et al.* Because the self-assembly of RNA complexes is an interplay of several RNA strands, the computational approach involves predicting the folding properties of all possible combinations of strand complexes. Also, the general problem of finding the lowest free energy base pairing is difficult for computational algorithms. If one cannot formulate any constraints that the base pairing of the minimum free energy structure must adhere to, then a computer algorithm cannot do much better then to search through essentially all possible combinations. Because the number of possible base pair combinations grows exponentially with sequence length, searching through all possible base pair combinations quickly becomes prohibitive for nucleic acid structures possessing long strands and pseudoknotted structures. HyperFold solves this problem using a tunable heuristic that depends on a single parameter. At one parameter setting the search is essentially a complete enumeration of all possible combinations of helices that have a certain minimum length. For a different parameter choice, the algorithm uses the faster method of simply placing the next most-stable helix. By default the search utilizes a "middle-ground" between those two extremes. The resulting search strategy is a tunable approach for predicting the interactions between multiple RNA and DNA strands with possibly complex "knotted" structures.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

These recent advancements in RNA nano-design and conditional RNAi activation represent noteworthy steps forward in RNA nanotechnology towards potential therapeutic applications. However, many significant hurdles still remain. While each of the approaches above present intrinsic benefits in their design and are able to activate the RNAi pathway in a controllable fashion, both approaches exhibit inherent flaws unique to each design. Split-function nucleic acid hybrids show prolonged persistence in the blood *in vivo* [4], but require interaction between two exogenous cognate hybrids for RNAi activation after cellular entry. This allows RNAi activation to be temporally controlled by the administration of the second hybrid, however, this mode of activation does not allow for gene silencing in response to a cellular RNA marker. While the two-stranded RNA switch is activated through interaction with an endogenously expressed transcript, the authors note that nuclease susceptibility may be problematic and likely to result in non-triggered RNAi activation. Interestingly for these two platforms, the weakness of one approach is a significant strength of the other. Although initially conceived and presented as distinct entities, the underlying design principles of these constructs, as well as other areas of RNA nanotechnology, do not need to remain mutually exclusive.

Previous research efforts have seen the incorporation of multiple RNA functional modules within RNA nanoparticles, resulting in the generation of singular entities that harbor multiple distinct functionalities [9, 10]. This same approach could also be applied to the modular functional elements themselves. Through combining the advantageous design characteristics exhibited by multiple individual nano-constructs (such as RNA/DNA cognate hybrids and RNA switches), functional modules with increased robustness and improved function are likely possible. By coupling these improved functional moieties with other aspects of nucleic acid nanotechnology, one can ultimately envision nuclease resistant, multi-faceted nucleic acid nanoparticles that employ multiple modes of diagnostic action and conditional functions. While there is still significant ground left to cover, the approaches for conditional gene knockdown presented recently in *Nano Letters* lay out the foundation for feasible, diagnostic-linked RNAi therapies in the future.

Acknowledgment:

This work has been funded in whole or in part with Federal funds from the Frederick National Laboratory for Cancer Research, National Institutes of Health, under Contract No. HHSN261200800001E. This research was supported [in part] by the Intramural Research Program of the NIH, National Cancer Institute, Center for Cancer Research. The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. Government.

References

[1]. McNamara JO, et al. (2006) Cell type-specific delivery of siRNAs with aptamer-siRNA chimeras. Nat Biotechnol 24(8):1005–1015. [PubMed: 16823371]

[2]. Zhou J, et al. (2013) Functional in vivo delivery of multiplexed anti-HIV-1 siRNAs via a chemically synthesized aptamer with a sticky bridge. Mol Ther 21(1):192–200. [PubMed: 23164935]

[3]. Shu D, et al. (2015) Systemic Delivery of Anti-miRNA for Suppression of Triple Negative Breast Cancer Utilizing RNA Nanotechnology. ACS Nano 9(10):9731–9740. [PubMed: 26387848]

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

[4]. Afonin KA, et al. (2013) Activation of different split functionalities on re-association of RNA-DNA hybrids. Nat Nanotechnol 8(4):296–304. [PubMed: 23542902]

[5]. Hochrein LM, Schwarzkopf M, Shahgholi M, Yin P, & Pierce NA (2013) Conditional Dicer substrate formation via shape and sequence transduction with small conditional RNAs. J Am Chem Soc 135(46):17322–17330. [PubMed: 24219616]

[6]. Afonin KA, et al. (2014) Co-transcriptional production of RNA-DNA hybrids for simultaneous release of multiple split functionalities. Nucleic Acids Res 42(3):2085–2097. [PubMed: 24194608]

[7]. Afonin KA, et al. (2016) The Use of Minimal RNA Toeholds to Trigger the Activation of Multiple Functionalities. Nano Lett 16(3):1746–1753. [PubMed: 26926382]

[8]. Bindewald E, et al. (2016) Multistrand Structure Prediction of Nucleic Acid Assemblies and Design of RNA Switches. Nano Lett 16(3):1726–1735. [PubMed: 26926528]

[9]. Shu D, Shu Y, Haque F, Abdelmawla S, & Guo P (2011) Thermodynamically stable RNA three-way junction for constructing multifunctional nanoparticles for delivery of therapeutics. Nat Nanotechnol 6(10):658–667. [PubMed: 21909084]

[10]. Afonin KA, et al. (2014) Multifunctional RNA nanoparticles. Nano Lett 14(10):5662–5671. [PubMed: 25267559]

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

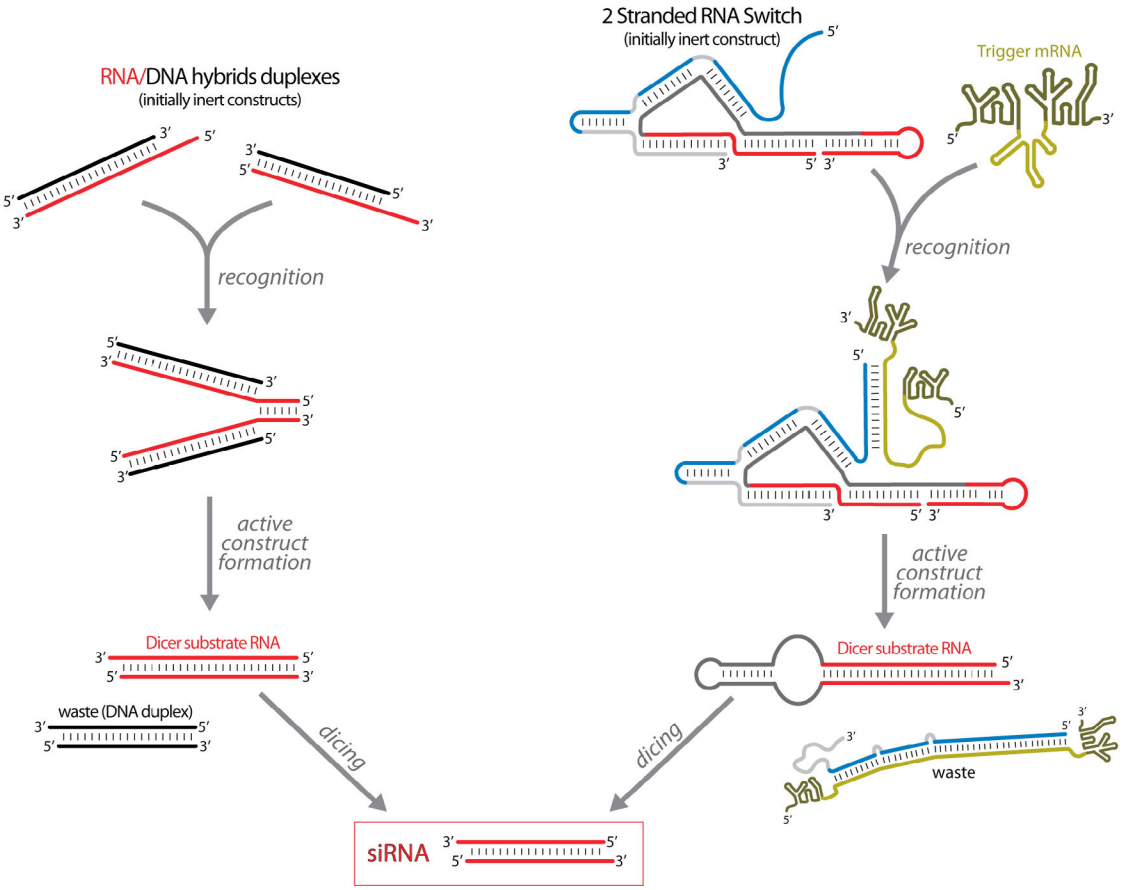

Figure 1:

Strategies that make use of RNA toehold interactions to initiate conditional release of Dicer substrate RNAs. (Left) A pair of RNA/DNA hybrid duplexes undergo strand exchange upon recognition and interaction of their single-stranded RNA toeholds. (Right) A two-stranded RNA switch undergoes conformational rearrangement if its RNA toehold interacts with a specific trigger mRNA. Each approach generates a Dicer substrate RNA upon conditional activation, which ultimately results in RNAi mediated gene silencing.