

Synthetic life

(continuation of „The molecular origins of life” SoSe 2022)



NaturalNews.com

WiSe 2022/23

Zbigniew Pianowski

Overview of the course

artificial ribozymes and aptamers for efficient catalysis and recognition (SELEX, DNAzymes, foldamers);

unnatural base pairing – expansion of the genetic alphabet;

Artificial genetic polymers and oligonucleotide analogues (XNA);

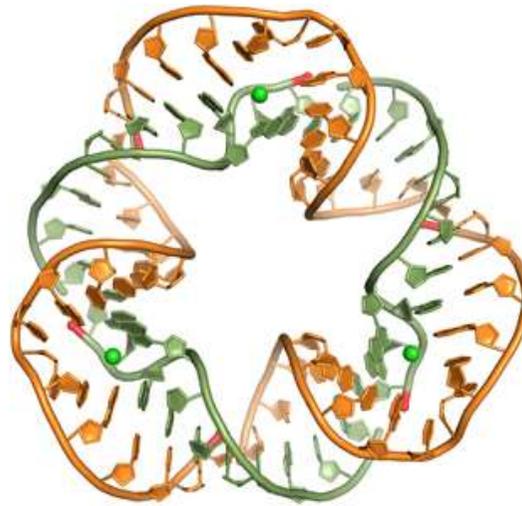
biosynthetic incorporation of **unnatural aminoacids (UAAs)** into proteins;

enzyme engineering – production of enzymes with unknown or unnatural properties, *ab initio* protein design, directed evolution, theozymes;

Artificial lipid vesicles as models for protocell multiplication;

design of artificial organisms

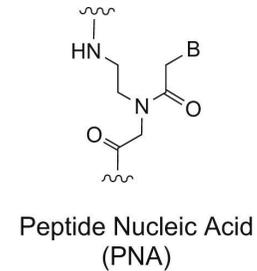
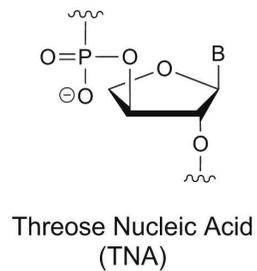
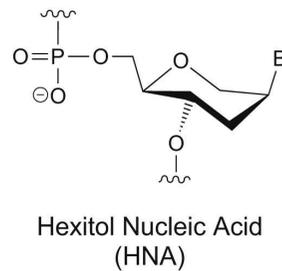
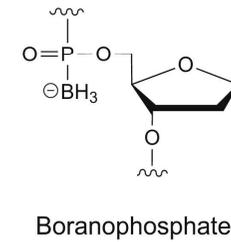
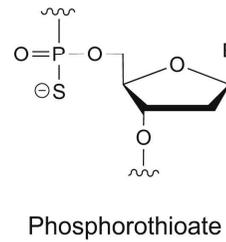
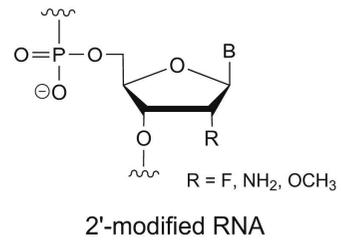
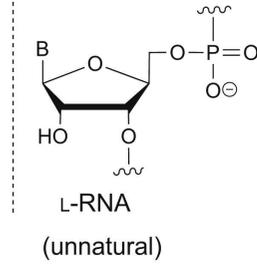
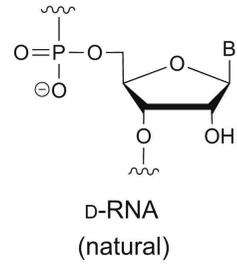
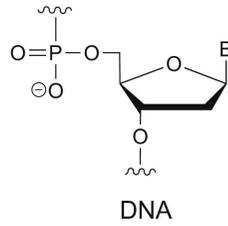
CHAPTER 1



OLIGONUCLEOTIDES

Part 3 – noncanonical backbone

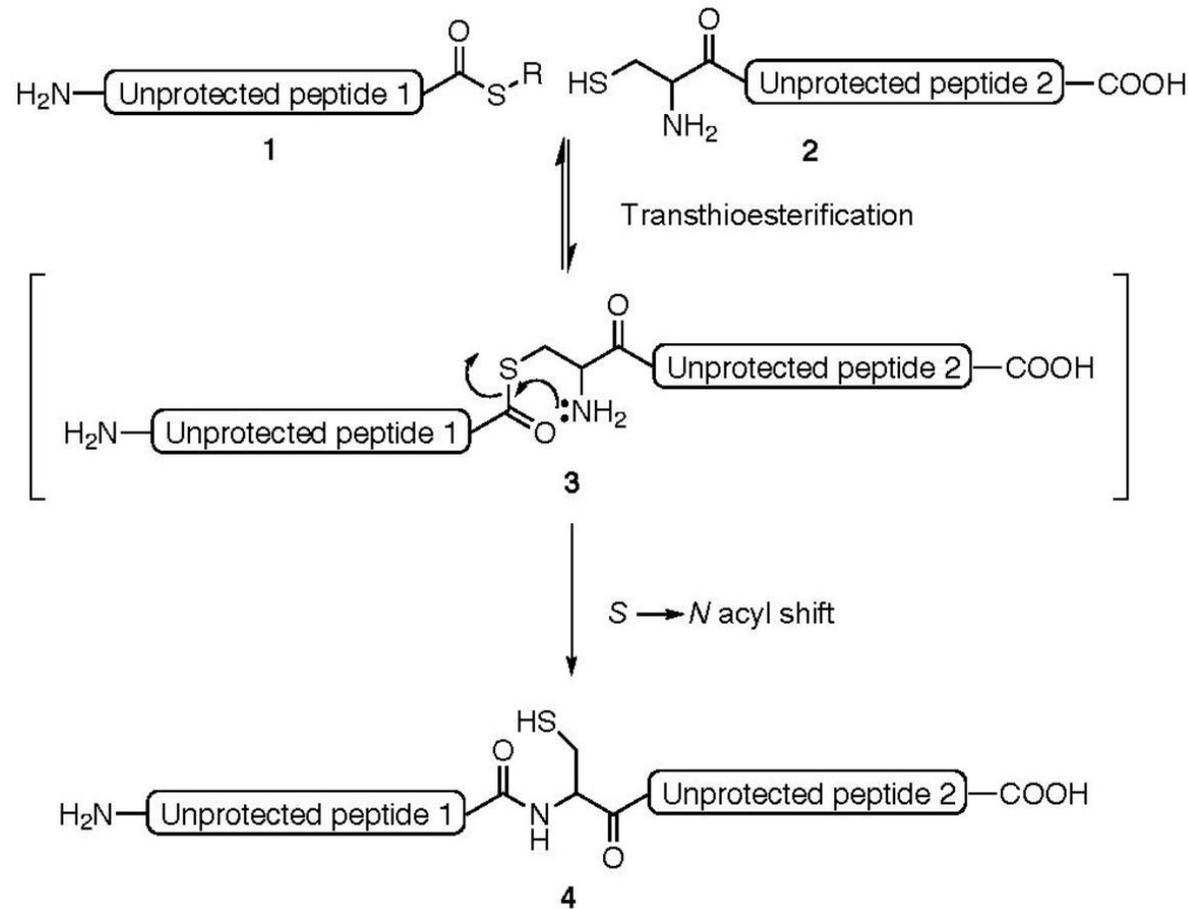
Artificial genetic polymers



Native chemical ligation

Native chemical ligation or **NCL** is an important extension of the chemical ligation field, a concept for constructing a large polypeptide formed by the assembling of two or more unprotected peptides segments. Especially, NCL is the most powerful ligation method for synthesizing proteins (native or modified) of moderate size (i.e., small proteins < 200 AA).

Originated from
Intein splicing

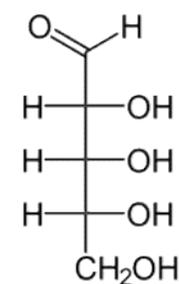
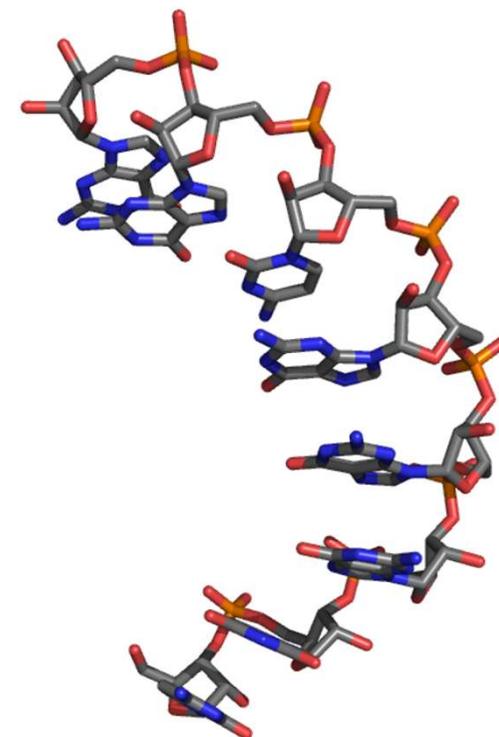


Spiegelmers: L-RNA

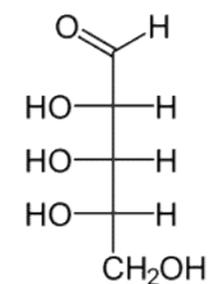
Aptamers (from the Latin aptus – fit, and Greek meros – part) are oligonucleotide or peptide molecules that **bind to a specific target molecule**. Aptamers are usually created by selecting them from a large random sequence pool, but natural aptamers also exist in riboswitches.

An **L-ribonucleic acid aptamer** (L-RNA aptamer, trade name **Spiegelmer** – from German Spiegel "mirror" – by Noxxon Pharma) is an RNA-like molecule built from L-ribose units. It is an artificial oligonucleotide named for being a mirror image of natural oligonucleotides.

L-RNA aptamers are a form of aptamers. Due to their L-nucleotides, they are highly resistant to degradation by nucleases. **Spiegelmers** are considered potential drugs and are currently being tested in clinical trials.

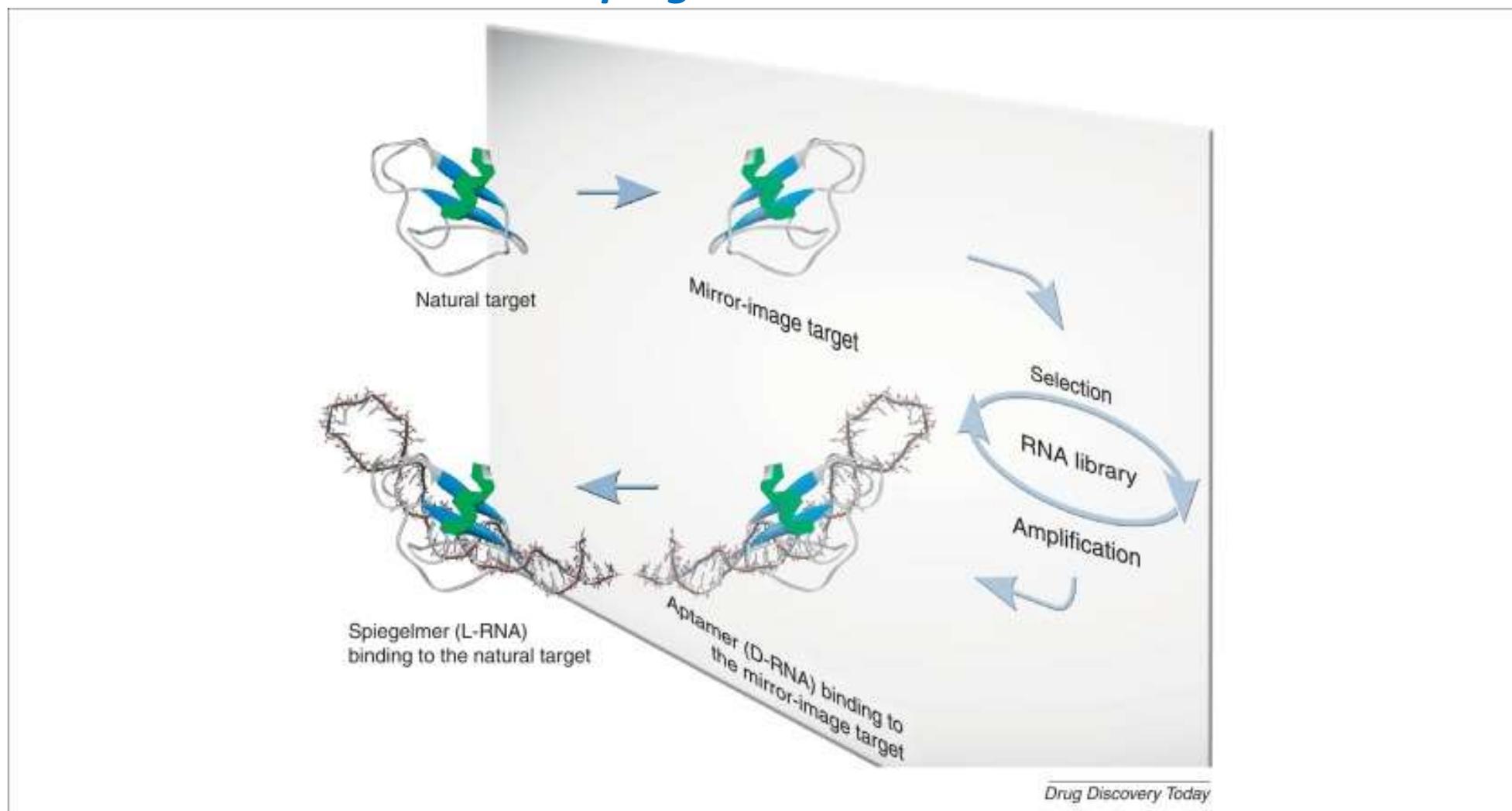


D-Ribose



L-Ribose

Spiegelmers: L-RNA

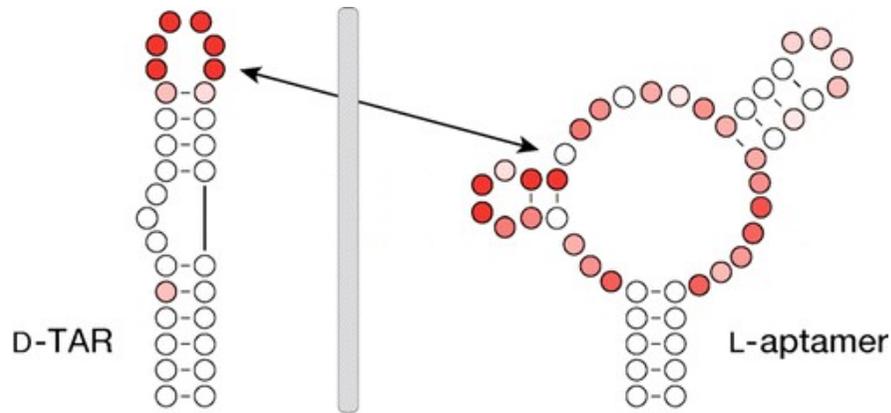


A. Vater, S. Klussmann, *Drug Discovery Today* **2015**, *20*, 147-155

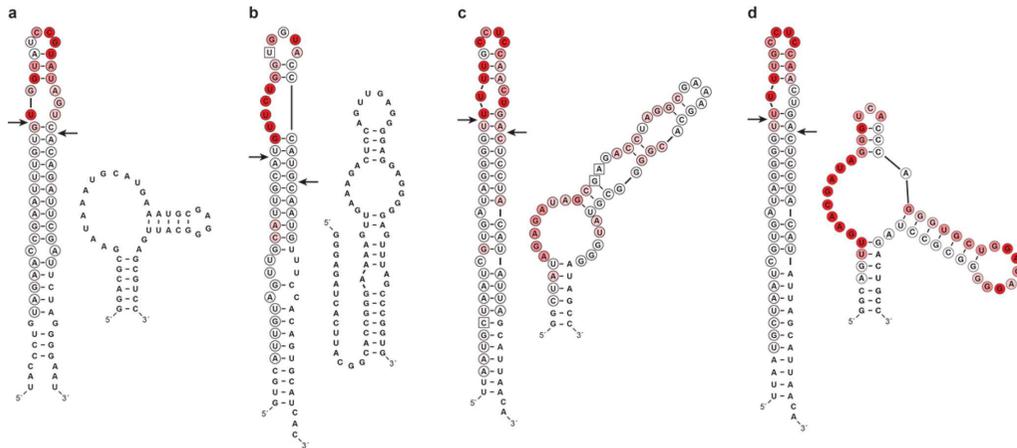
B.E. Young, N. Kundu, J.T. Szczepanski, *Chem. Eur.J.* **2019**, *25*, 7981–7990

Spiegelmers: L-RNA

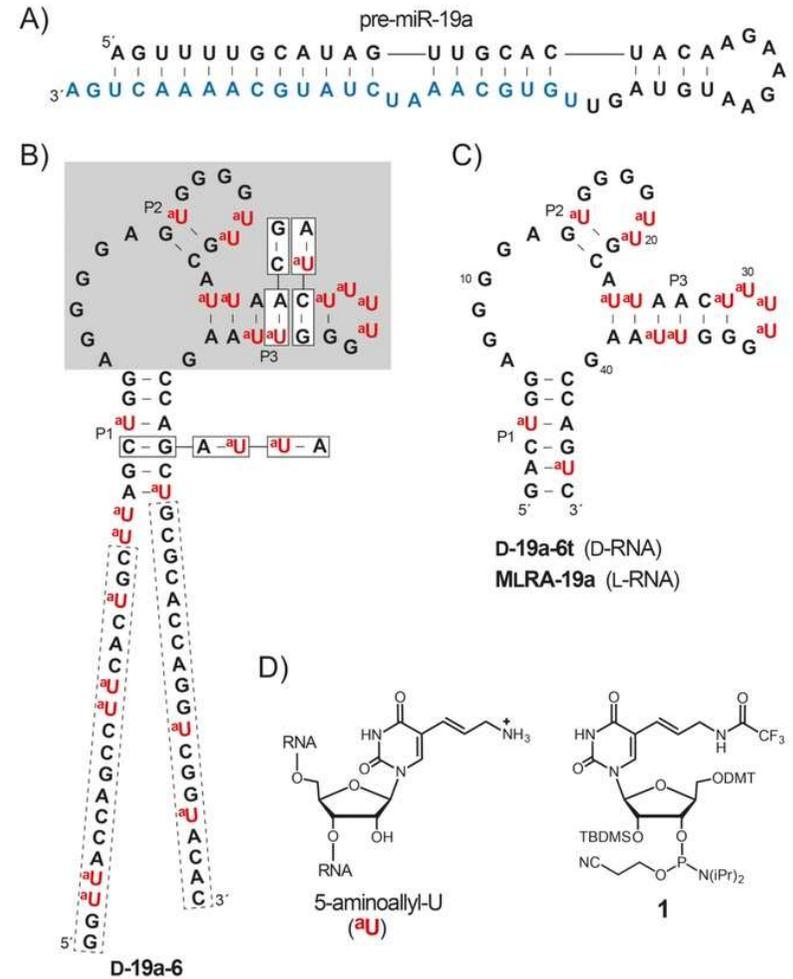
Aptamer selection against RNA with natural D-chirality



Spiegelmers/"AptamiRs" - a new class of miR inhibitors.



Spiegelmers with modified nucleotides

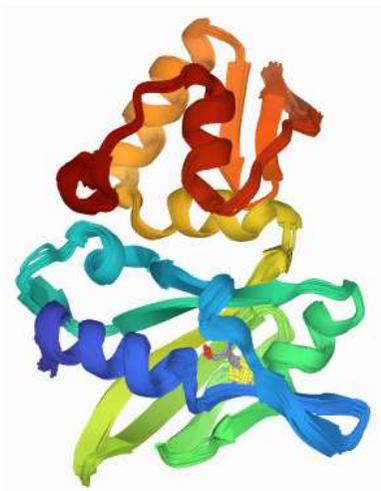


J.T. Szczepanski, G.F. Joyce, *J. Am. Chem. Soc.* **2013**, *135*, 36, 13290–13293

A.M. Kabza, J.T. Szczepanski, *ChemBioChem.* **2017**, *18*, 1824-1827

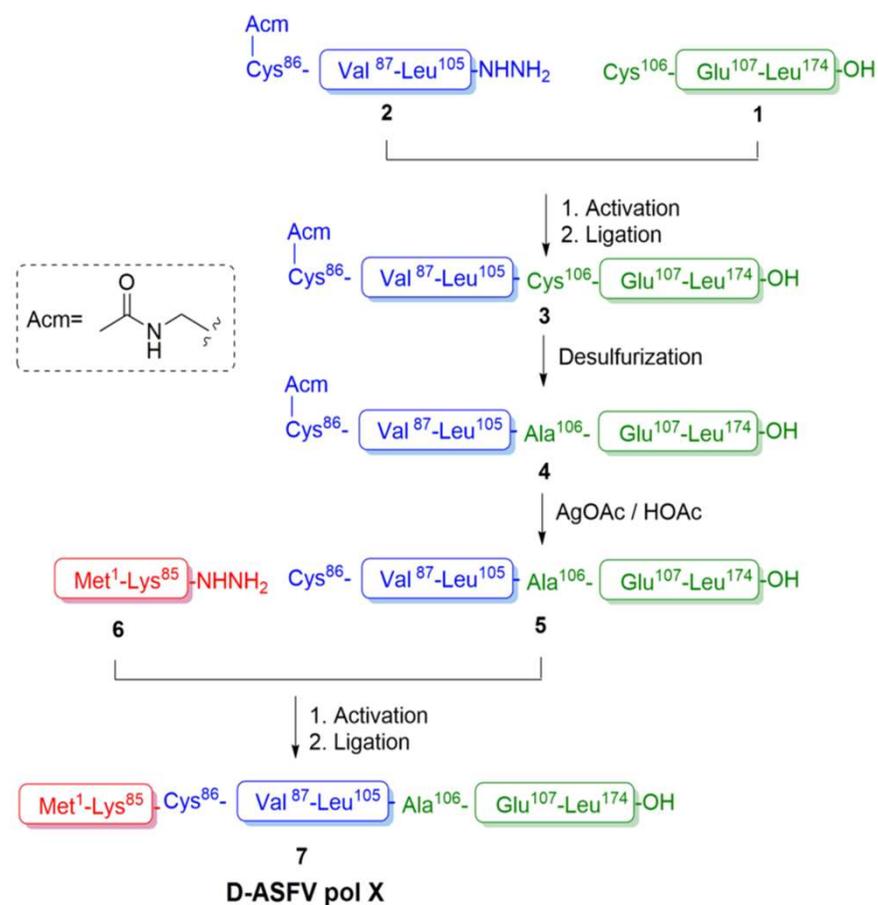
Processing of the mirrored genetic information

Synthesis of a mirrored 832-residue Taq polymerase or the 604-residue Klenow Fragment from *D*-aminoacids is still beyond reach with the current methods. However, progress in long peptide synthesis enabled solid-support synthesis followed by fragment ligation (NCL) of the mirror image configuration of polymerase X from African swine fever virus (**ASFV**), the shortest known polymerase (174 amino acids).



PDB: 1JQR

	10	20	30	40	50
MLTLIQGKKI	VNHLRSRLAF	EYNGQLIKIL	SKNIVAVGSL	RREEKMLNDV	
60	70	80	90	100	
DLIIIVPEKK	LLKHVLPNIR	IKGLSFSVKV	CGERKCVLFI	EWEKKTYQLD	
110	120	130	140	150	
LFTALAEKPK	YAIFHFTGPV	SYLIRIRAAL	KKKNYKLNQY	GLFKNQTLVP	
160	170				
LKITTEKELI	KELGFTYRIP	KKRL			



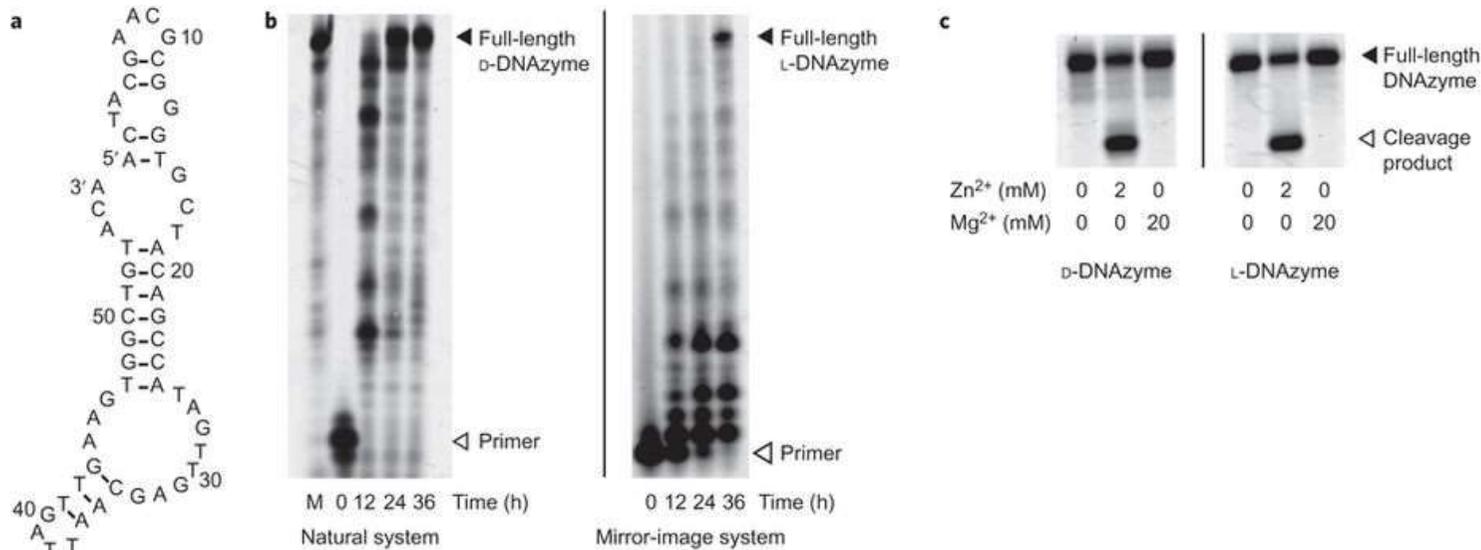
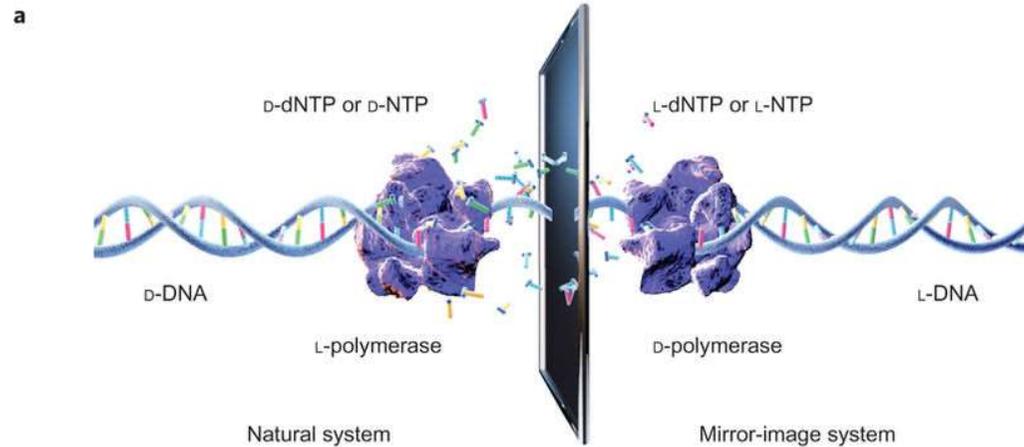
Showalter A.K. et al. *Nat Struct Biol* **2001**, *8*, 942-946

Z. Wang, W. Xu, L. Liu, T. F. Zhu *Nature Chem.* **2016**, *8*, 698-704

L-DNA polymerase

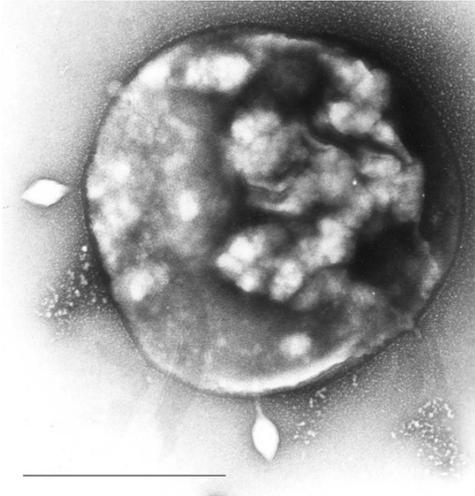
D-ASFV Pol X elongated an L-DNA primer with L-dNTPs - a functional 56-mer L-DNAzyme was made within 36 hours.

This poses an important proof of concept, however, polymerase X is a thermo-labile repair enzyme and its catalytic activity does not meet the requirements for a standard PCR



Z. Wang, W. Xu, L. Liu, T. F. Zhu *Nature Chem.* **2016**, *8*, 698-704

A thermostable L-DNA-polymerase

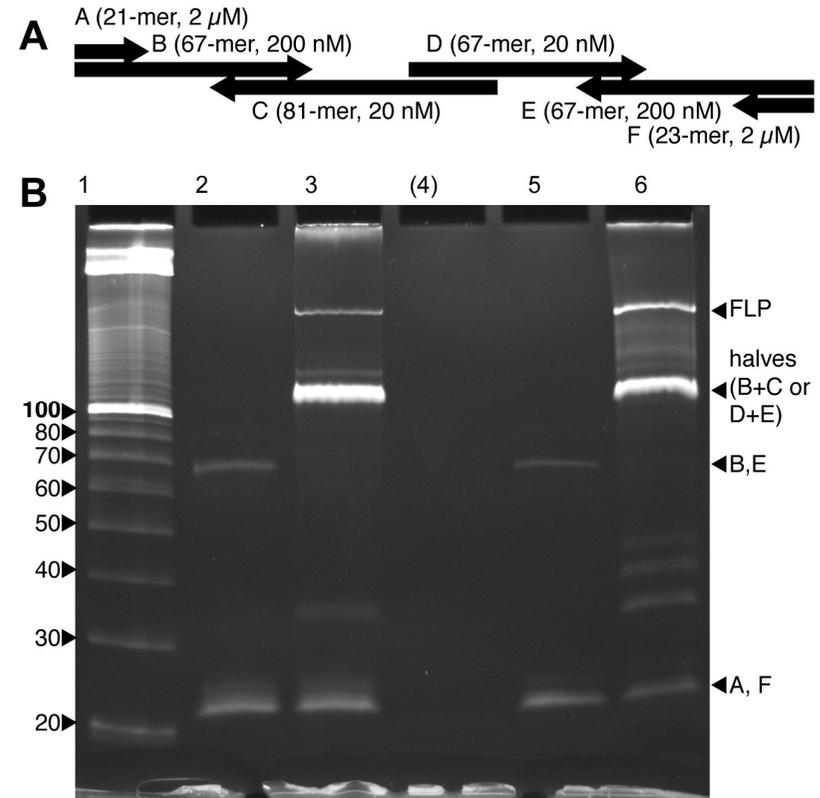


Cell of *Sulfolobus* infected by virus STSV1 observed under microscopy. Two spindle-shaped viruses were being released from the host cell.

A thermostable mirror-image polymerase **D-Dpo4-3C** (352 aa) is able to amplify L-DNA in a classical PCR reaction and can be used to assemble an L-DNA gene from L-DNA oligonucleotides.

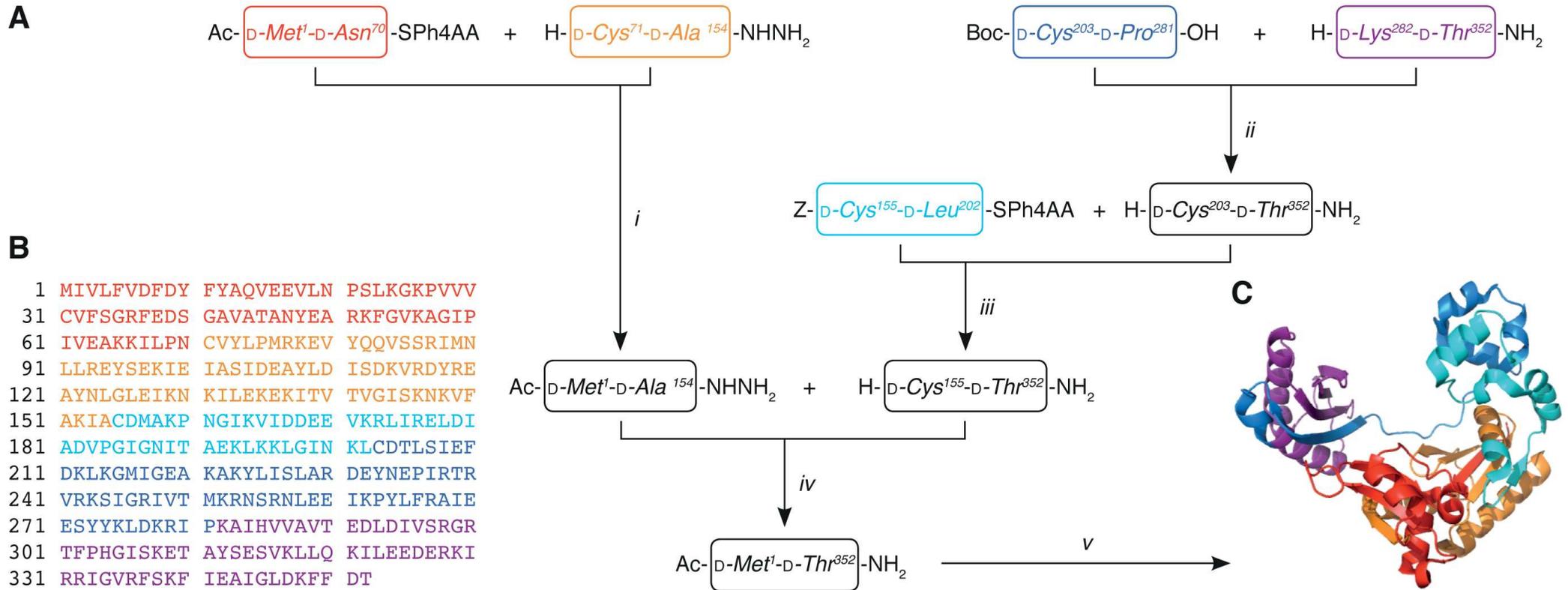
Tyr12Ala or Tyr12Ser → DNA-dependent **RNA polymerase**

Assembly of a mirror-image gene



A. Pech, S. Klussmann *et al.* *Nucl. Acid Res.* **2017**, 45, 3997-4005

A thermostable L-DNA-polymerase

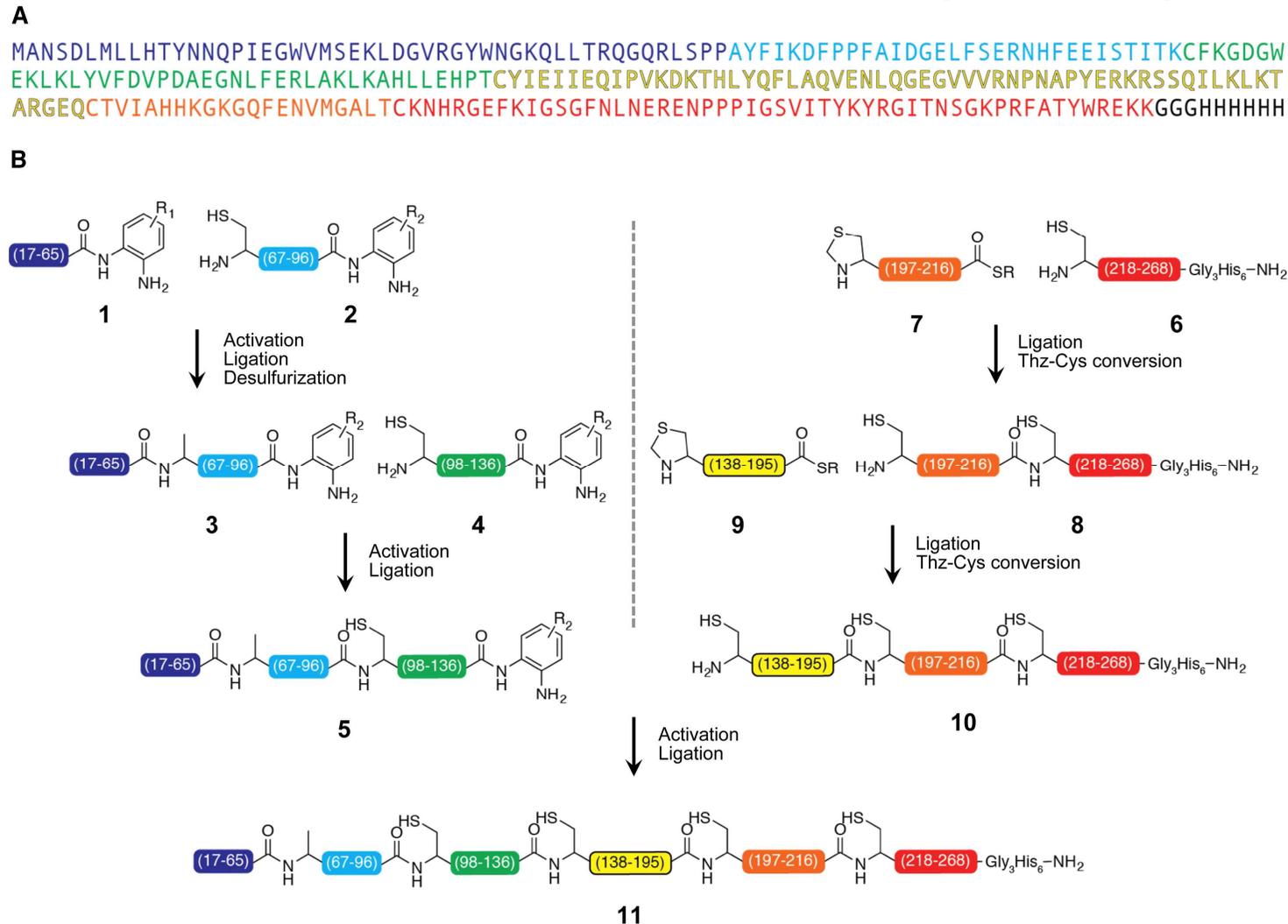


A. Pech, S. Klussmann *et al.* *Nucl. Acid Res.* **2017**, *45*, 3997-4005

Mirror-image DNA ligase

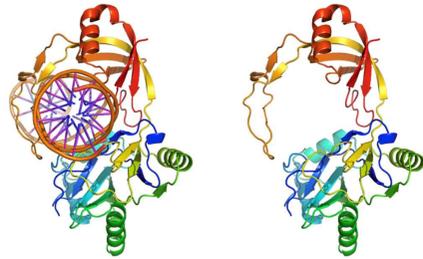
A functional DNA-ligase in the D-enantiomeric conformation has been synthesized. It exhibited DNA ligation activity on chirally inverted nucleic acids in L-conformation, but not acting on natural substrates and with natural co-factors. The ligase was based on the known structure of the *Paramecium bursaria* chlorella virus DNA-ligase and the homologous but shorter DNA-ligase of *Haemophilus influenzae*. The structure and the activity of the mirror-image ligase were characterized, documenting its enantiospecific functionality.

J. Weidmann *et al.*
Cell Chemical Biology **2019** 26(5), 645-651.e3



Mirror-image DNA ligase

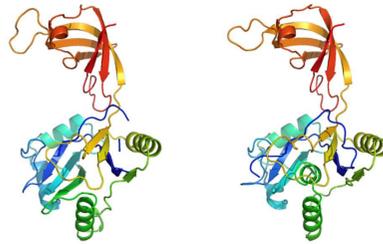
PBCV-1 DNA ligase 2Q2T
actual structure



full structure

DNA removed

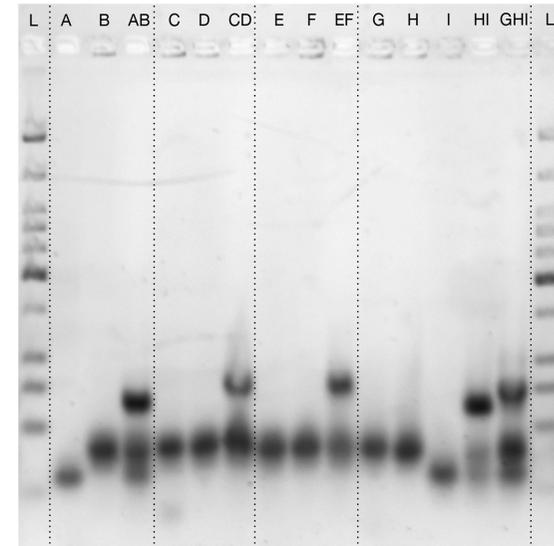
Model of LigA (268 aa)



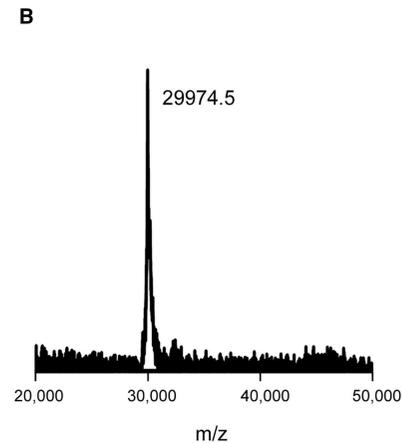
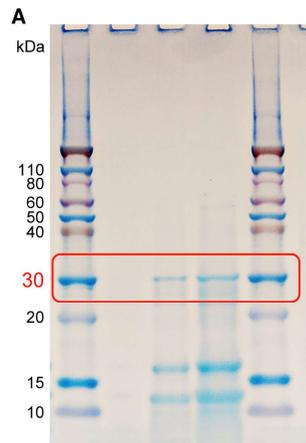
Phyre

SWISS-MODEL

Prediction of the LigA structure



**Enzymatic Ligation of Gene Fragments
Made of Synthetic L-DNA**

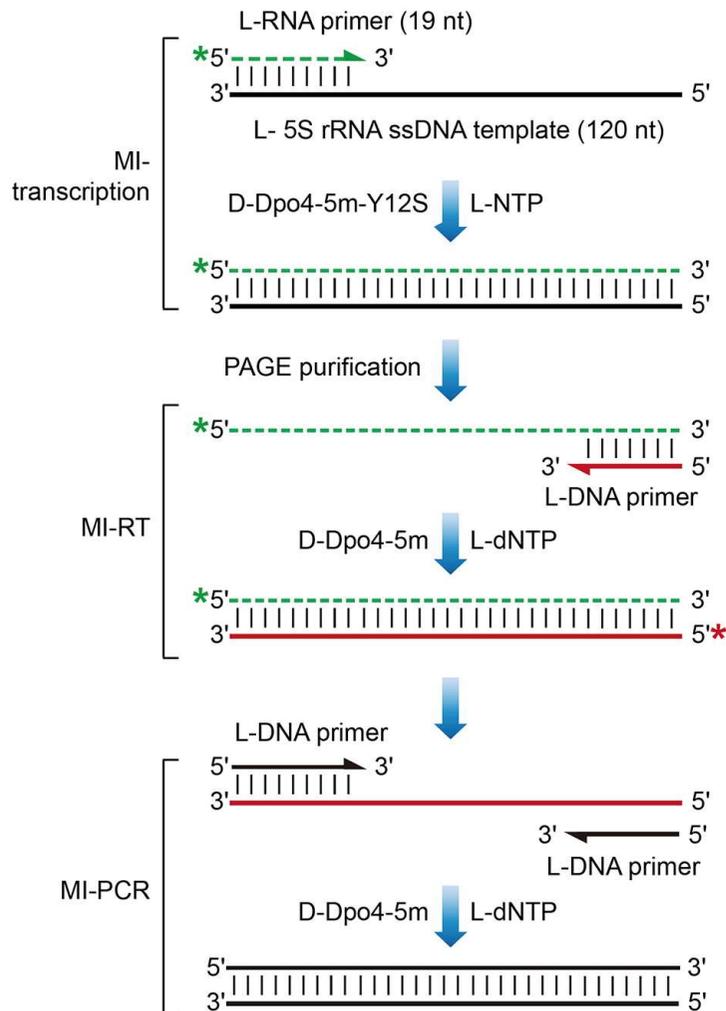


D-Protein LigA: (A) The product of the final chemical ligation after 12 h. The two peptides **5** and **10** and the final, full-length protein of about 30 kDa can be seen. (B) MALDI-TOF mass spectrum of LigA. Measured: 29,974.5 Da theoretical: 29,966.1 Da.

J. Weidmann *et al.*
Cell Chemical Biology **2019** 26(5), 645-651.e3

Mirror-image transcription, reverse transcription and amplification

A

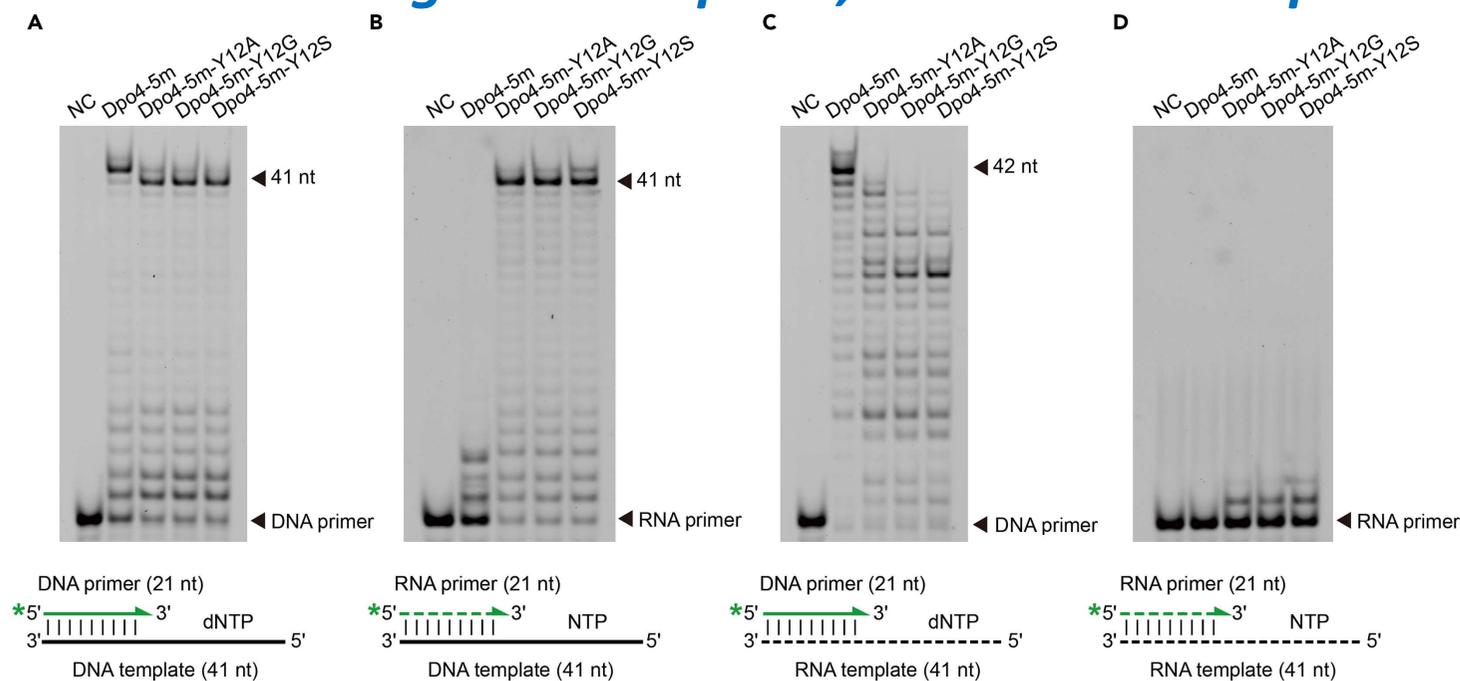


The transcription of a mirror-image gene into L-RNA, as well as reverse transcription of L-RNA into L-DNA by synthetic D-polymerases, based on designed mutants of Dpo4, have been demonstrated. The efficient mirror-image transcription system may enable enzymatic preparation of L-RNA molecules to further enable clinical applications of nuclease-resistant aptamer biosensors and drugs or studies on mirror-image or cross-chiral ribozymes and aptamers. The enzymatically transcribed **L-5S rRNA** shown in this study could be used as one component in a future effort to assemble a mirror-image ribosome, a step toward the realization of mirror-image life

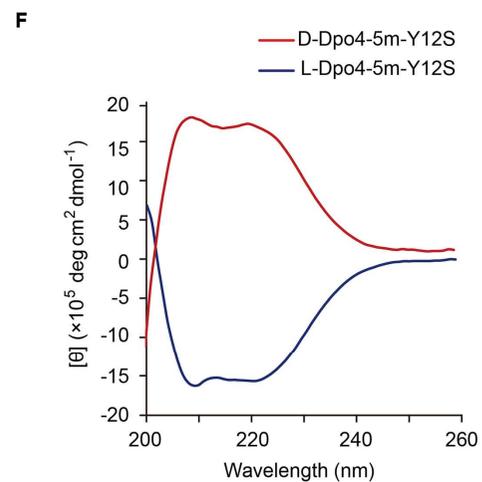
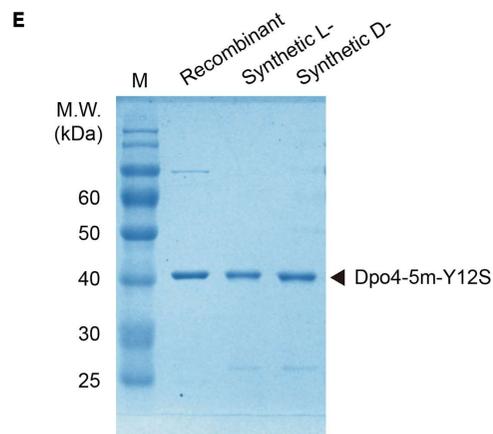
***Sulfolobus solfataricus* P2 DNA polymerase IV (Dpo4)**

M.Wang *et al. Chem.* **2019**, *5* (4), 848-857

Mirror-image transcription, reverse transcription and amplification

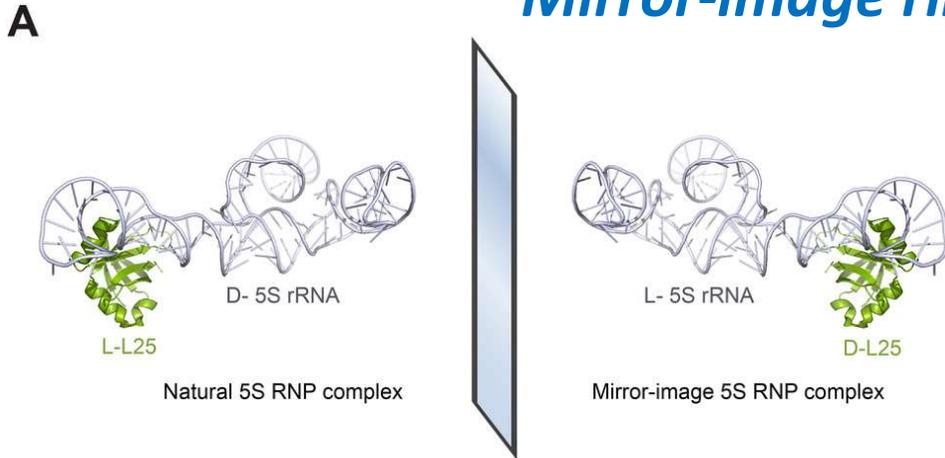


Sulfolobus solfataricus P2
DNA polymerase IV (Dpo4)



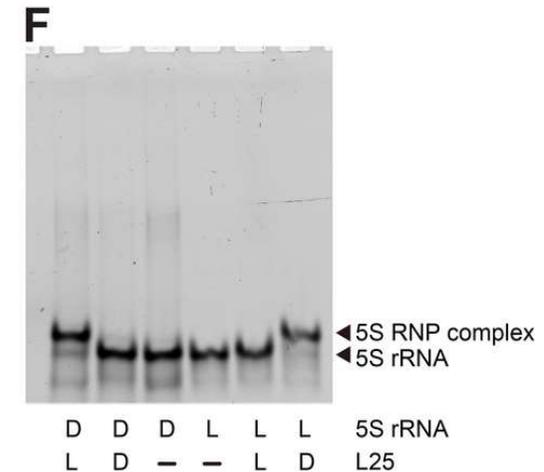
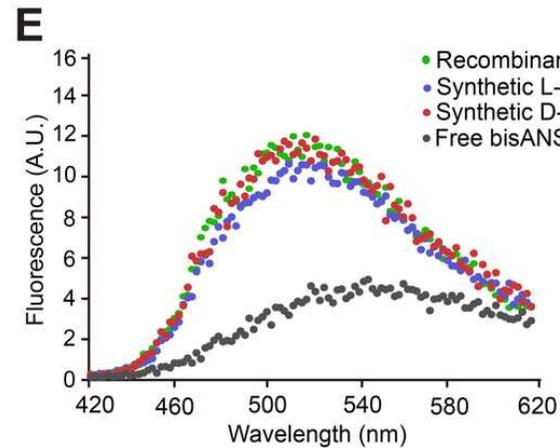
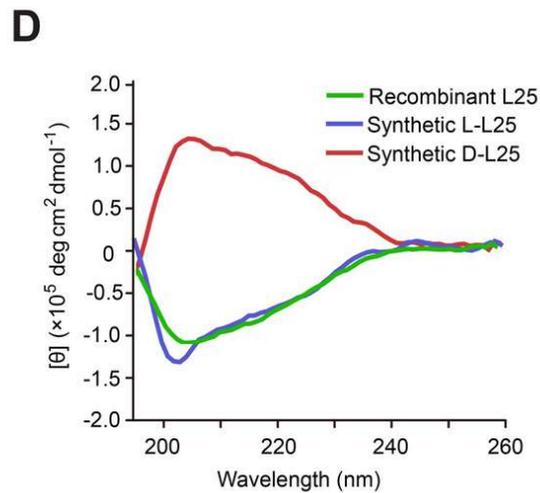
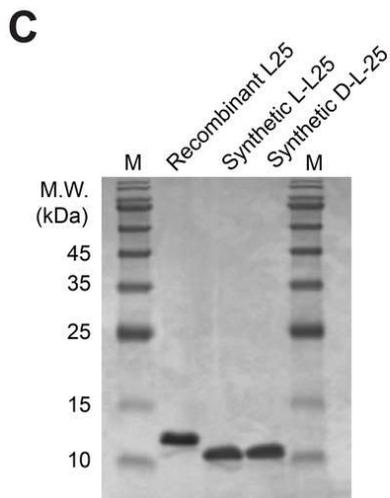
M.Wang et al. Chem. 2019, 5 (4), 848-857

Mirror-image ribonucleoprotein complex



Currently, the biggest challenge in establishing a mirror-image version of the central dogma is to build a **mirror-image ribosome-based translation machine**.

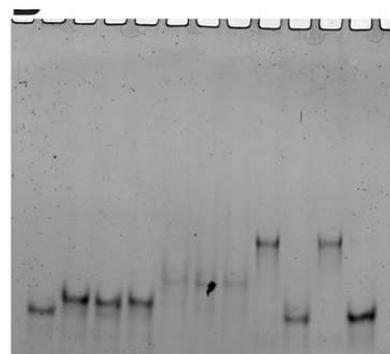
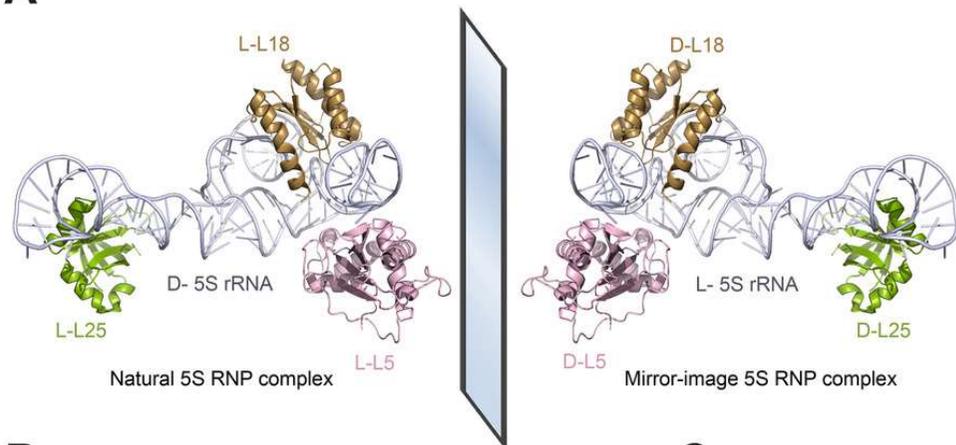
The natural and mirror-image versions of three ribosomal proteins (L5, L18, and L25) in the large subunit of the *Escherichia coli* ribosome have been chemically synthesized. The synthetic mirror-image proteins can fold *in vitro* and assemble with enzymatically transcribed mirror-image 5S ribosomal RNA into ribonucleoprotein complexes.



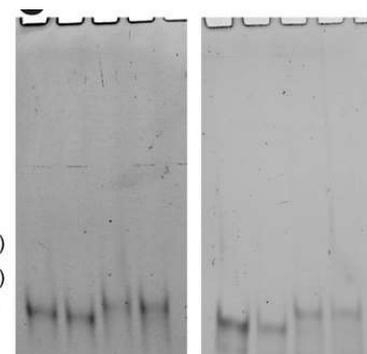
J.J. Ling *et al.*, *Angew. Chem. Int. Ed.* **2020**, *59* (9), 3724-3731

Mirror-image ribonucleoprotein complex

A

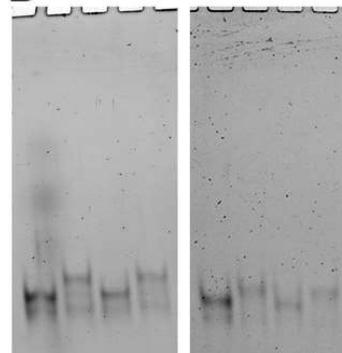


D	D	D	D	D	D	D	D	L	L	L	5S rRNA
-	L	-	-	L	L	-	L	L	D	-	Synthetic L5
-	-	L	-	L	-	L	L	L	D	-	Synthetic L18
-	-	-	L	-	L	L	L	L	D	-	Synthetic L25



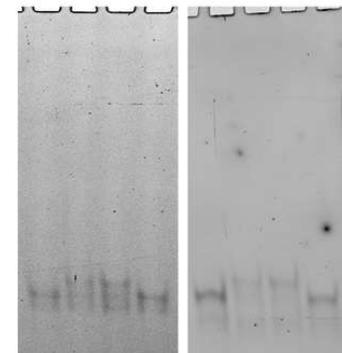
D	D	D	D	L	L	L	L	5S rRNA
D	D	D	D	L	L	L	L	L5-probe
-	L	-	-	-	D	-	-	Synthetic L5
-	-	L	-	-	-	D	-	Synthetic L18
-	-	-	L	-	-	-	D	Synthetic L25

D



D	D	D	D	L	L	L	L	5S rRNA
D	D	D	D	L	L	L	L	L18-probe
-	L	-	-	-	D	-	-	Synthetic L5
-	-	L	-	-	-	D	-	Synthetic L18
-	-	-	L	-	-	-	D	Synthetic L25

E



D	D	D	D	L	L	L	L	5S rRNA
D	D	D	D	L	L	L	L	L25-probe
-	L	-	-	-	D	-	-	Synthetic L5
-	-	L	-	-	-	D	-	Synthetic L18
-	-	-	L	-	-	-	D	Synthetic L25

In addition, the RNA–protein interactions are chiral-specific in that the mirror-image ribosomal proteins do not bind with natural 5S ribosomal RNA and vice versa. The synthesis and assembly of mirror-image 5S ribonucleoprotein complexes are important steps towards building a functional mirror-image ribosome.

However, the large subunit of bacterial ribosome alone is composed of a 5S RNA subunit (120 nucleotides), a 23S RNA subunit (2900 nucleotides) and 31 proteins...

Phage display

Phage display is a laboratory technique for the study of protein–protein, protein–peptide, and protein–DNA interactions that uses bacteriophages (viruses that infect bacteria) to connect proteins with the genetic information that encodes them. A gene encoding a protein of interest is inserted into a phage coat protein gene, causing the phage to "display" the protein on its outside while containing the gene for the protein on its inside, resulting in a connection between genotype and phenotype. These displaying phages can then be screened against other proteins, peptides or DNA sequences, in order to detect interaction between the displayed protein and those other molecules. In this way, large libraries of proteins can be screened and amplified in a process called in vitro selection, which is analogous to natural selection.

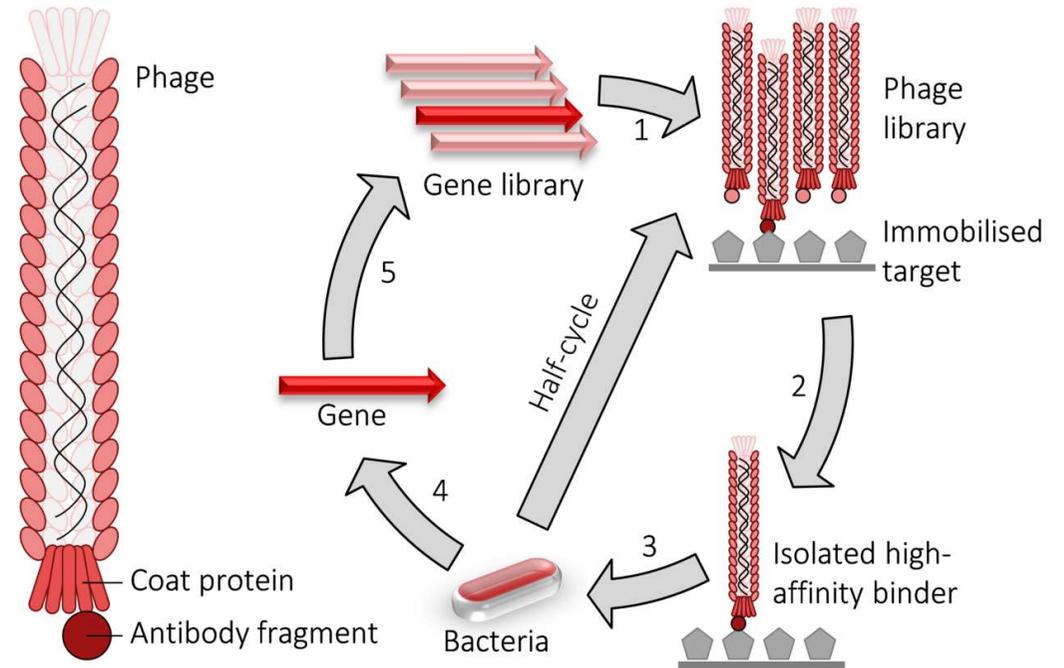
The most common bacteriophages used in phage display are M13 and fd filamentous phage, T4, T7, and λ phage.

G.P. Smith *Science* **1985**, 228 (4705), 1315–1317

J. Scott, G.P. Smith *Science* **1990**, 249 (4967), 386–390

J.W. Kehoe, B.K. Kay *Chem. Rev.* **2005**, 105 (11), 4056–72

Nobel prize in chemistry 2018



Phage display cycle.

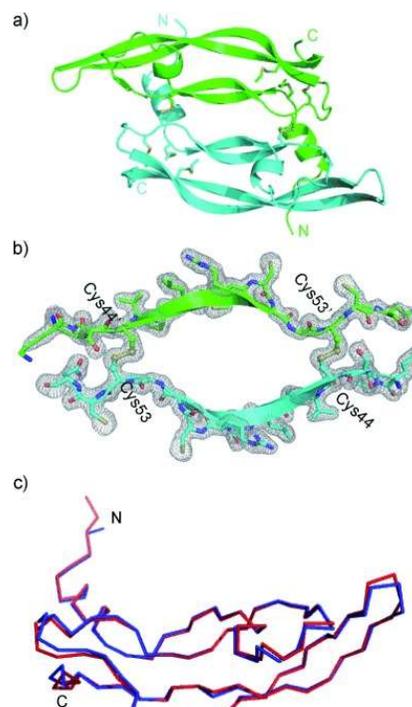
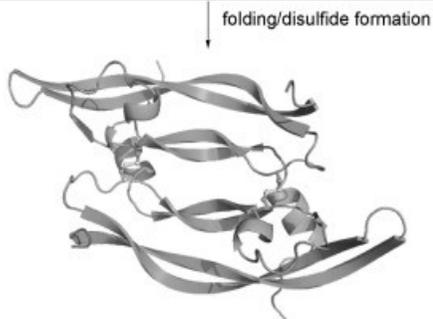
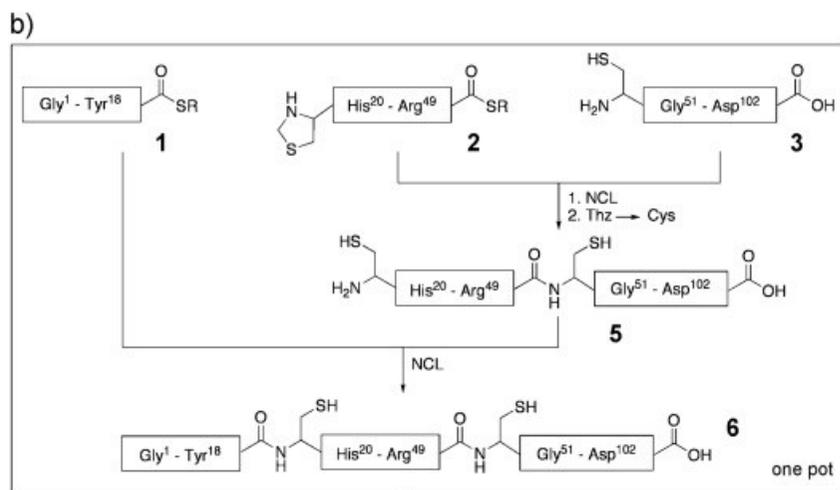
Thomas Shafiee - Own work, CC BY 4.0

- 1) fusion proteins for a viral coat protein + the gene to be evolved (typically an antibody fragment) are expressed in bacteriophage.**
- 2) the library of phage are washed over an immobilised target.**
- 3) the remaining high-affinity binders are used to infect bacteria.**
- 4) the genes encoding the high-affinity binders are isolated.**
- 5) those genes may have random mutations introduced and used to perform another round of evolution. The selection and amplification steps can be performed multiple times at greater stringency to isolate higher-affinity binders.**

D-proteins: almost ideal therapeutic agents

Polypeptides composed entirely of *D*-amino acids (*D*-proteins) inherently have *in vivo* properties that are proposed to be near-optimal for a large molecule therapeutic agent. Specifically, *D*-proteins are resistant to degradation by proteases and are anticipated to be nonimmunogenic. Furthermore, *D*-proteins are manufactured chemically and can be engineered to have other desirable properties, such as improved stability, affinity, and pharmacokinetics.

a) GQNHHEVVKF¹⁰ MDVYQRSYCH²⁰ PIETLVDIFQ³⁰ EYPDEIEYIF⁴⁰
 KPSCVPLMRC⁵⁰ GGCCNDEGLE⁶⁰ CVPTEESNIT⁷⁰ MQIMRIKPHQ⁸⁰
 GQHIGEMSFL⁹⁰ QHNKCECRPK¹⁰⁰ KD¹⁰²



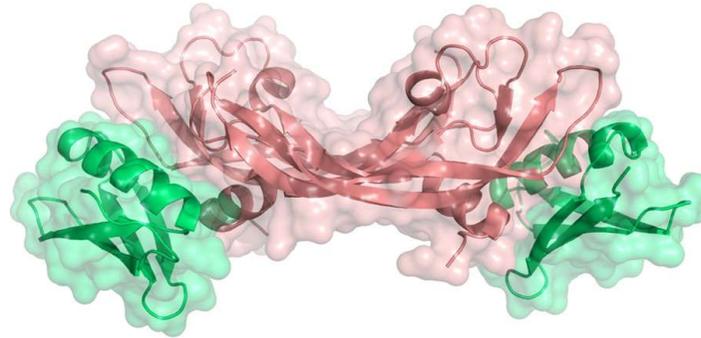
The 204-residue covalent-dimer **vascular endothelial growth factor (VEGF)** with full mitogenic activity was prepared from three unprotected peptide segments by one-pot native chemical ligations. The covalent structure of the synthetic **VEGF** was confirmed by precise mass measurement, and the three-dimensional structure of the synthetic protein was determined by high-resolution X-ray crystallography.

VEGF is a signal protein produced by cells that stimulates the formation of blood vessels. Cancers that can express **VEGF** are able to grow and metastasize. Overexpression of **VEGF** can cause vascular disease in the retina of the eye and other parts of the body.

L-VEGF: K. Mandal, S. Kent *Angew. Chem., Int. Ed.* **2011**, *50*(35), 8029-8033

D-VEGF: K. Mandal, S. Kent *et al. PNAS* **2012**, *109* (37), 14779-14784

D-proteins: almost ideal therapeutic agents



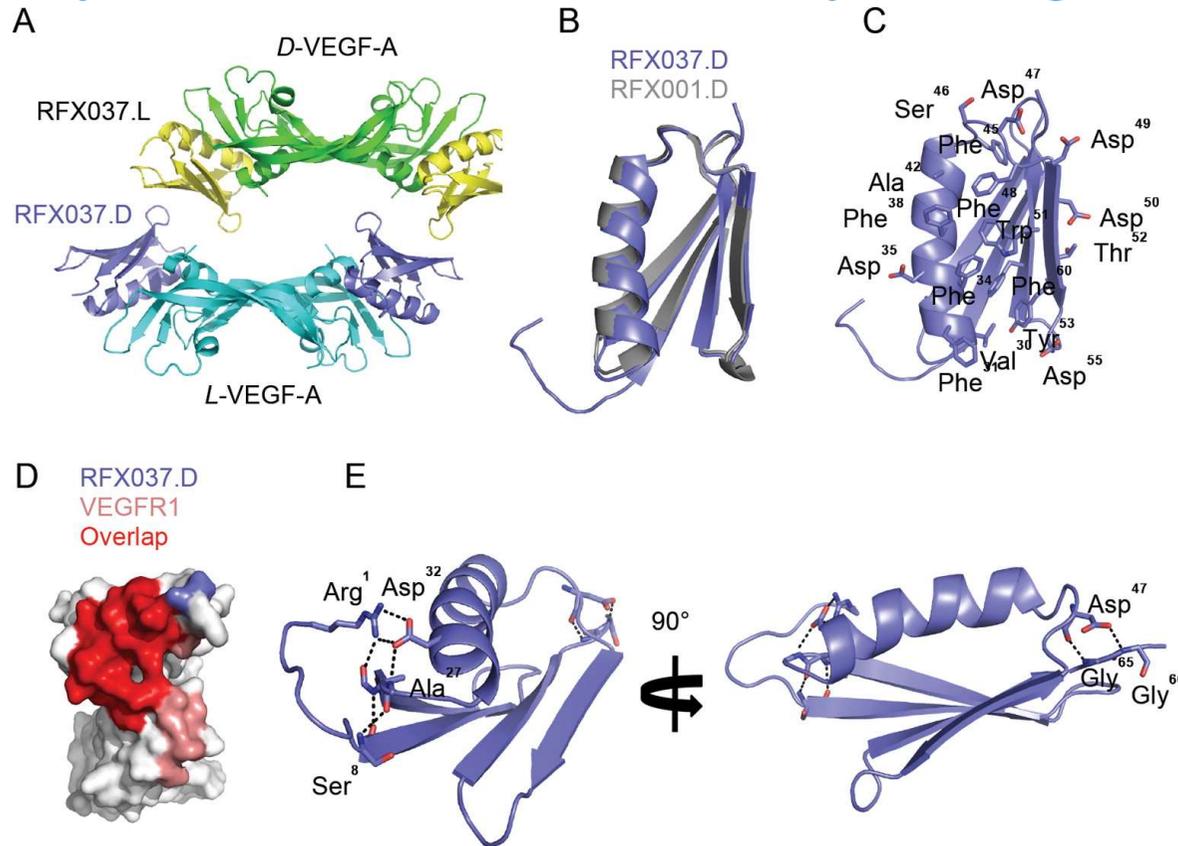
RFX037.D is a *D*-protein antagonist of natural vascular endothelial growth factor A (VEGF-A) that inhibited binding to its receptor, with extreme thermal stability ($T_m > 95$ °C) and high affinity for VEGF-A ($K_d = 6$ nM). It was identified using a combination of total chemical protein synthesis and mirror image phage display of proteins.

Comparison of the two enantiomeric forms of RFX037 revealed that the *D*-protein is more stable in mouse, monkey, and human plasma and has a longer half-life *in vivo* in mice. Significantly, RFX037.D was nonimmunogenic in mice, whereas the *L*-enantiomer generated a strong immune response. These results confirm the potential utility of synthetic *D*-proteins as alternatives to therapeutic antibodies.

T.N.M. Schumacher, P. Kim *et al.* *Science* **1996**, *271* (5257), 1854-1857

M. Uppalapati, S. Kent *et al.*, *ACS Chem. Biol.* **2016**, *11*, 1058-1065

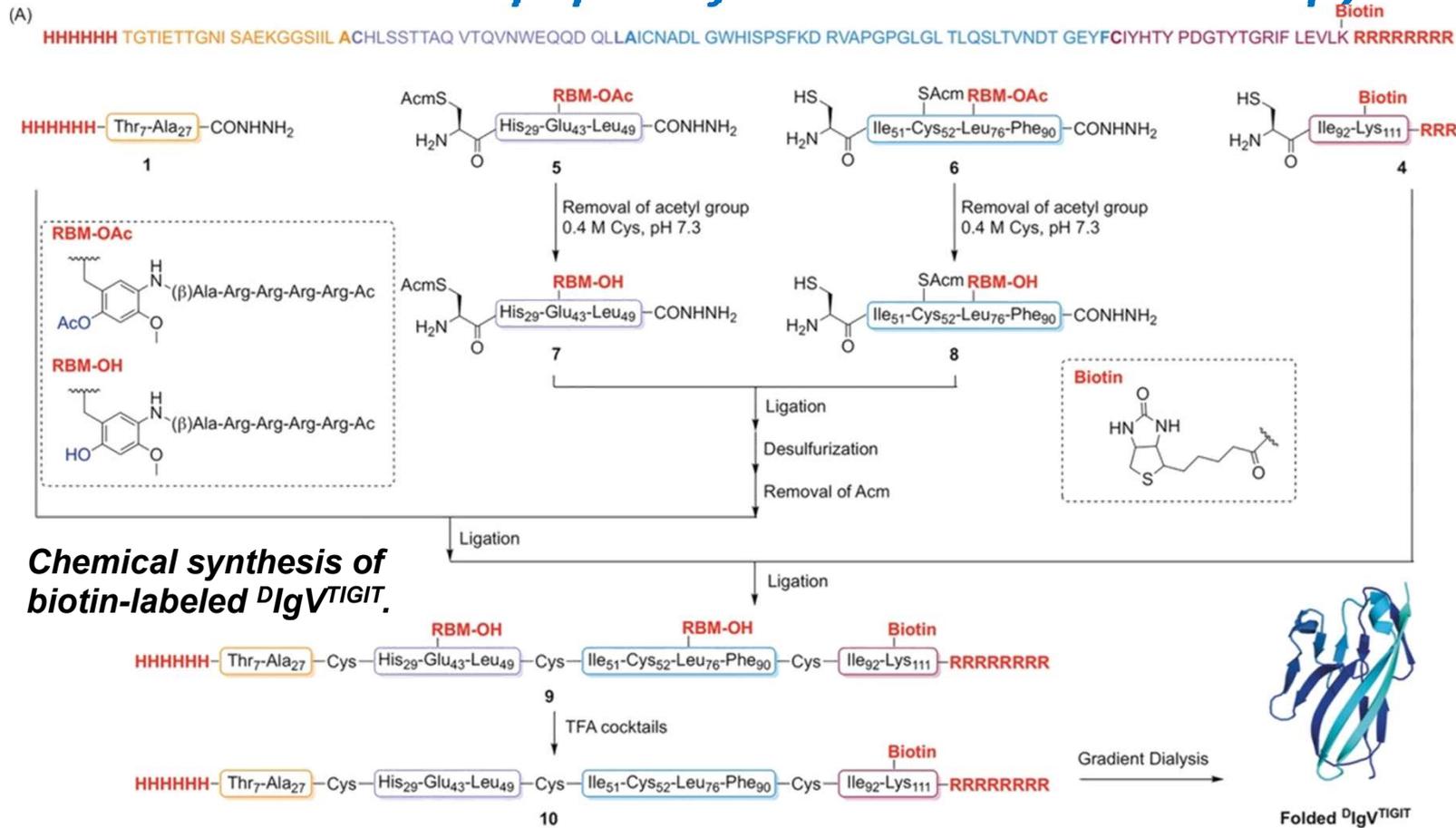
D-proteins: almost ideal therapeutic agents



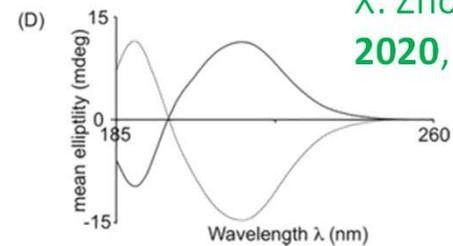
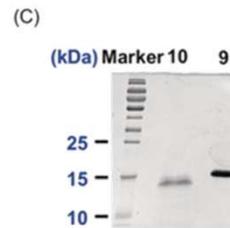
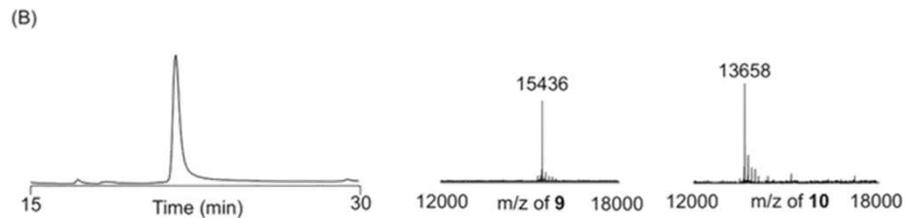
X-ray crystal structure of RFX037:VEGF-A heterochiral protein complex. (A) Two RFX037.L molecules (yellow) bound to one d-VEGF-A homodimer (green) and two RFX037.D molecules (blue) bound to one l-VEGF-A homodimer (cyan). (B) Superposition of RFX037.D (blue) and RFX001.D (gray, rcsb accession 4GLS). (C) RFX037.D side chains (shown as sticks) that contact l-VEGF-A. (D) The contact surfaces of l-VEGF-A to RFX037.D (blue), VEGFR1 (salmon), or both (red). (E) Hydrogen bond networks formed by intramolecular polar contacts originated from additional N- and C-terminal residues in RFX037.D.

M. Uppalapati, S. Kent *et al.*, ACS Chem. Biol. 2016, 11, 1058-1065

D-peptide for cancer immunotherapy



An immune checkpoint TIGIT is a novel target in cancer immunotherapy of anti-PD-1 resistant tumors. Here, mirror-image phage display bio-panning was performed using the *d*-enantiomer of TIGIT synthesized by hydrazide-based native chemical ligation.

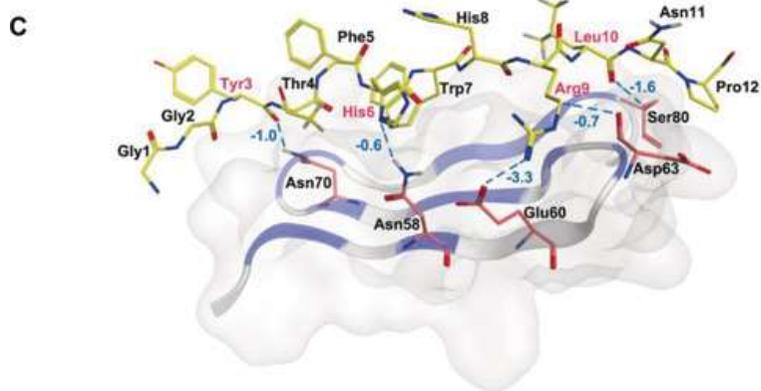
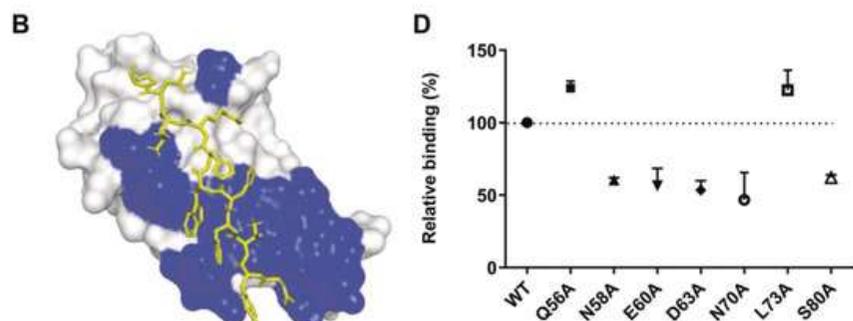


X. Zhou et al., *Angew. Chem. Int. Ed.* **2020**, *59* (35), 15114-15118

D-peptide for cancer immunotherapy

A

Name	Sequence	Frequency (n/34)	K _D (μM)	Relative Blocking
^D TBP-1	LTPHKHHKHLHA	14	2.79 ± 0.51	1
^D TBP-3	GGYTFHWRLNP	3	5.60 ± 2.56	16.2
^D TBP-6	AGMHVHYNWNHL	2	N/A	N/A
^D TBP-13	HGVALHIRLHAG	2	N/A	N/A
^D TBP-17	SAIHFHHPRWKP	2	48.3 ± 11.2	4.8
^D TBP-5	GNLTLHMHRSPS	1	6.75 ± 1.07	3.6
^D TBP-3S	NRHPWGLYGHTF	—	NB	1.6



The negative signaling pathway mediated by immune checkpoints (such as PD-1/PD-L1) leads to the exhaustion of immune cells and immune escape of cancer. However, the therapeutic effects of PD-1/PD-L1 blockade are limited (<30 % response rates), and adaptive resistance is often observed. TIGIT is a novel immune checkpoint molecule expressed on Nk and T cells, which competes with costimulatory receptor CD226 for the shared ligand PVR to deliver immunosuppressing signals and a potential therapeutic target.

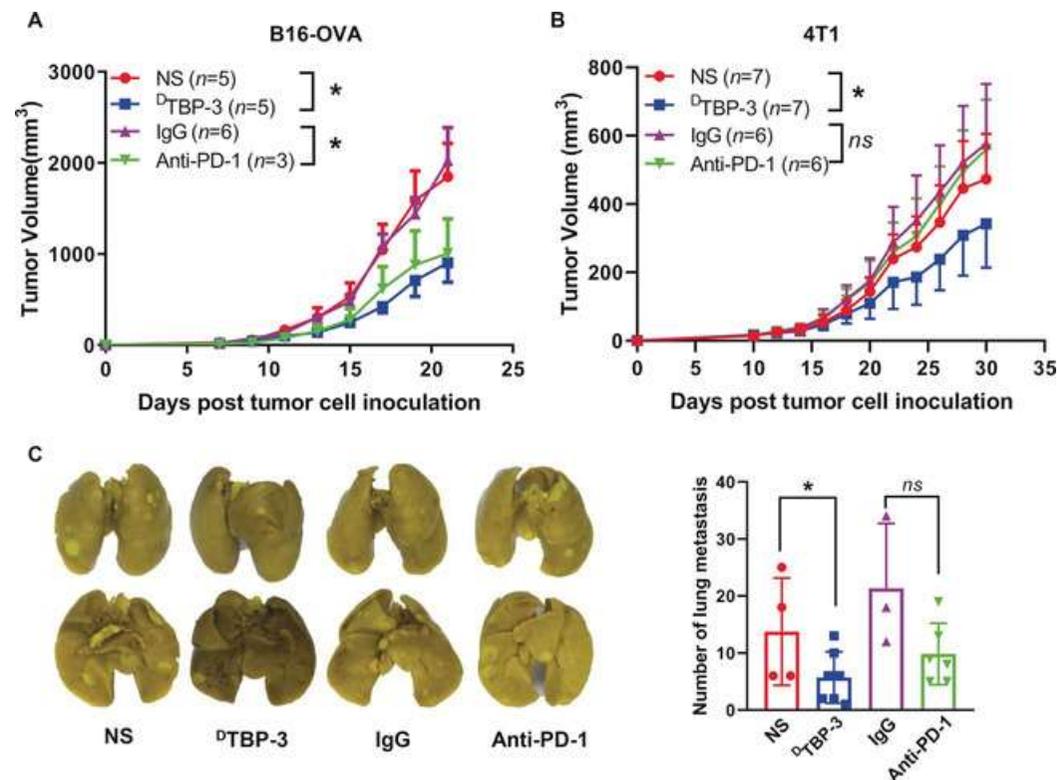
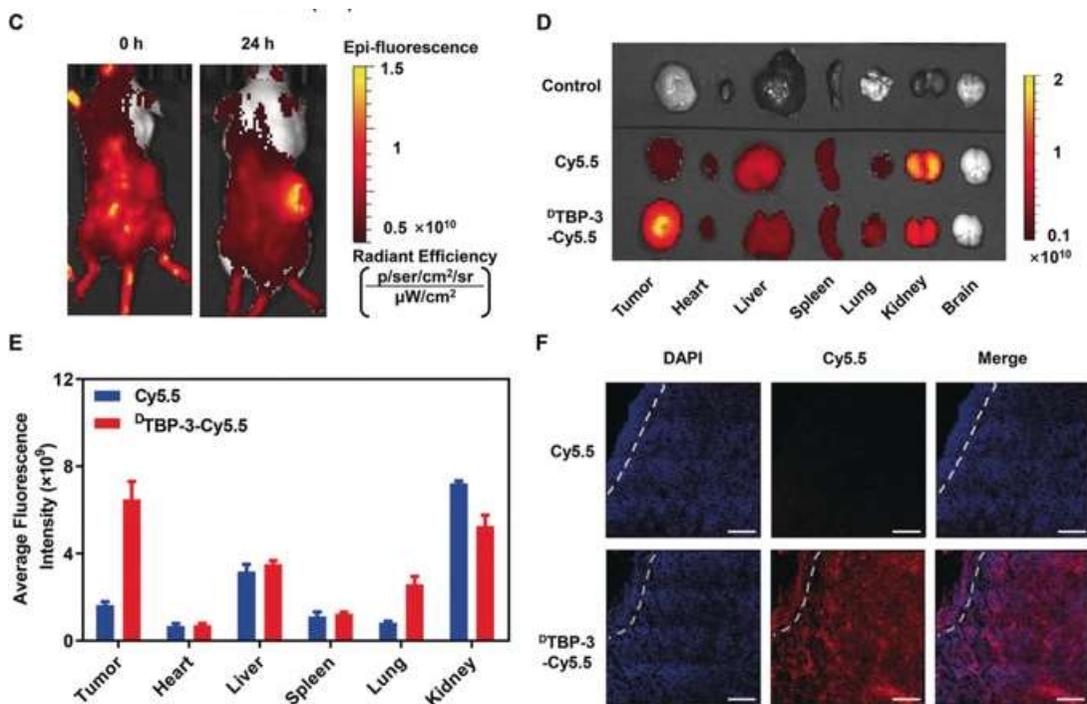
A biotin labeled *D*-enantiomer of the immunoglobulin variable domain of TIGIT (^DIgV^{TIGIT}-biotin) was synthesized by hydrazide-based native chemical ligation (NCL) and removable backbone modification (RBM) strategy.

Using the mirror-image phage display technique, a *D*-peptide ^DTBP-3 was identified, which could occupy the binding interface and effectively block the interaction of TIGIT with its ligand PVR. ^DTBP-3 showed proteolytic resistance, tumor tissue penetrating ability, and could inhibit tumor growth and metastasis in anti-PD-1 resistant tumor model.

X. Zhou et al., *Angew. Chem. Int. Ed.* **2020**, *59* (35), 15114-15118

D-peptide for cancer immunotherapy

^DTBP-3 is the first D-peptide targeting TIGIT, which could serve as a potential candidate for cancer immunotherapy.

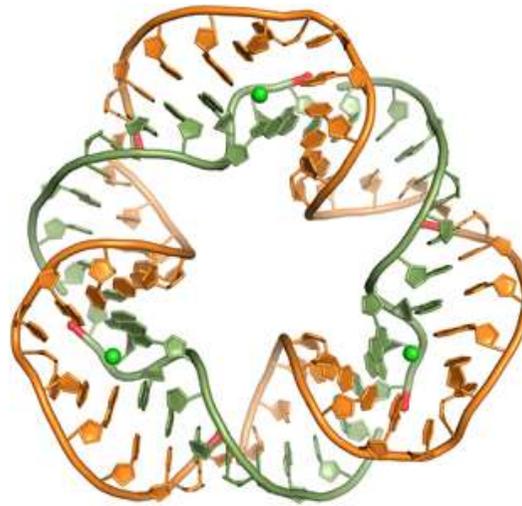


C) Near-infrared fluorescence imaging of CT26 tumor-bearing mice injected (i.v.) with Cy5.5 and ^DTBP-3-Cy5.5. D) Representative imaging and E) average fluorescent intensity (n=3) of tissues 24 h post injection. F) Representative fluorescence microscopy images of sectioned tumors. Scale bar=200 μ m.

The effects of ^DTBP-3 on anti-PD-1 responsive and resistant tumor models. Tumor growth curves of B16-OVA (A) or 4T1 (B). C) Representative images and statistics of lung metastases loci in 4T1 tumor model (*P<0.05).

X. Zhou et al., *Angew. Chem. Int. Ed.* **2020**, *59* (35), 15114-15118

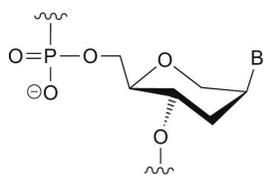
CHAPTER 1



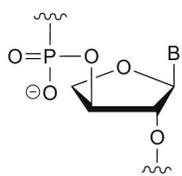
OLIGONUCLEOTIDES

Part 3 – noncanonical backbone – Xeno Nucleic Acids

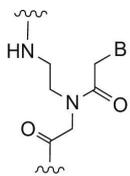
XNA – Xeno Nucleic Acids



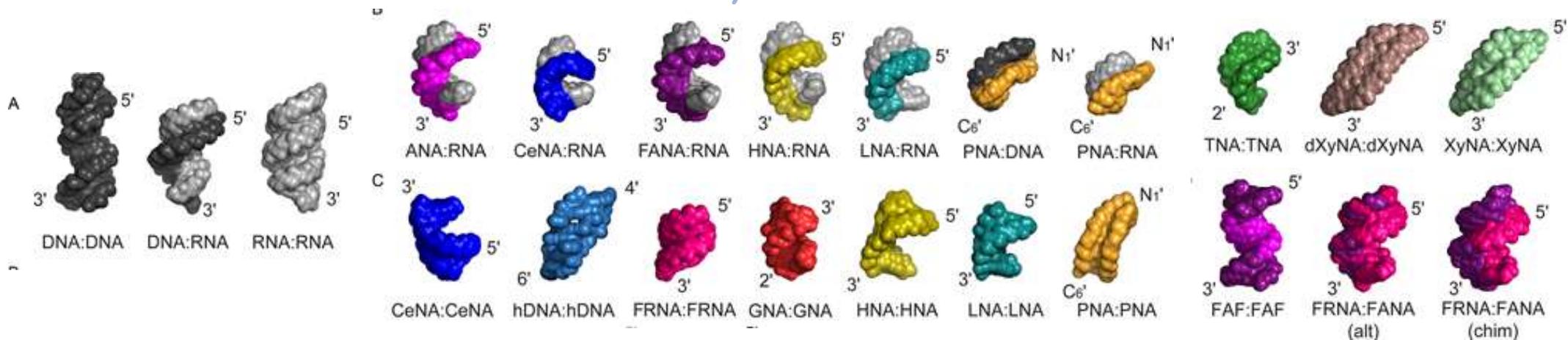
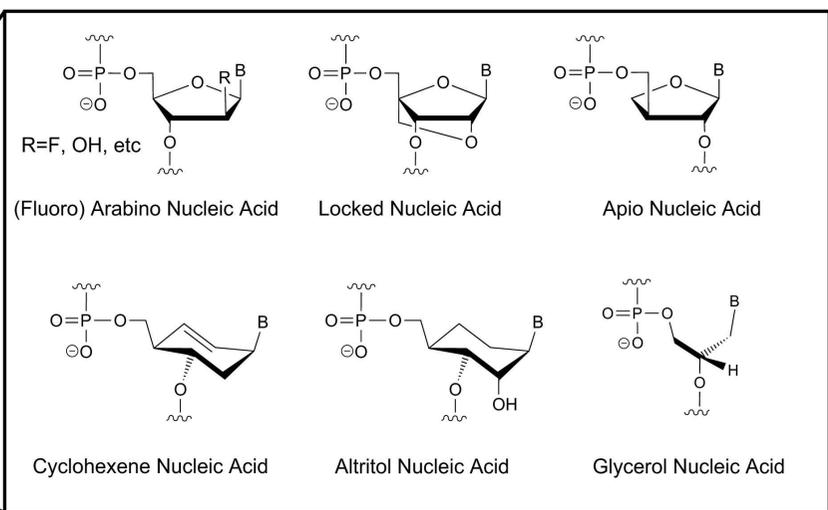
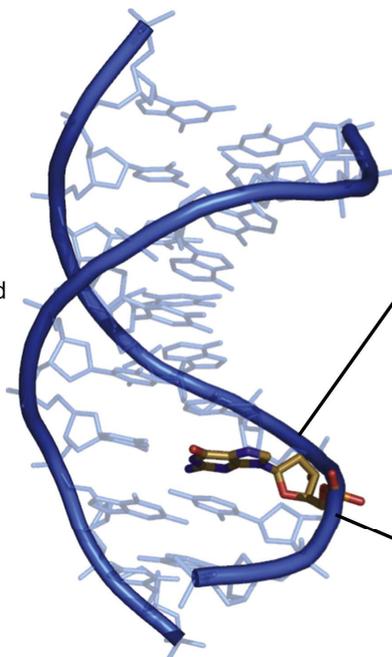
Hexitol Nucleic Acid (HNA)



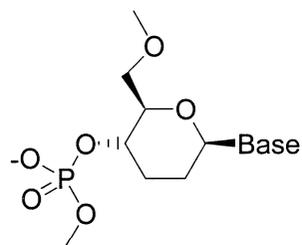
Threose Nucleic Acid (TNA)



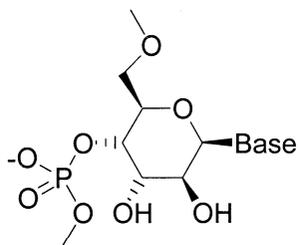
Peptide Nucleic Acid (PNA)



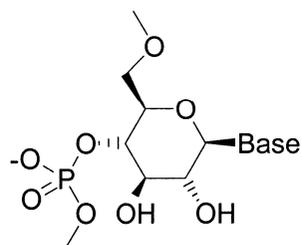
Overview of XNA



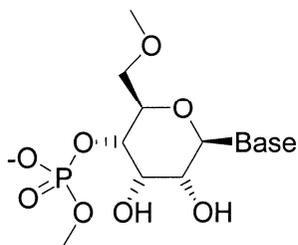
Homo-DNA
β-D, 4' → 6'



Altopyranosyl-NA
β-D, 4' → 6'

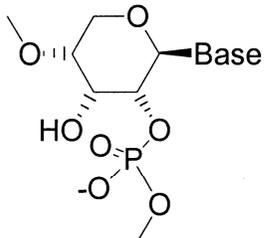


Glucopyranosyl-NA
β-D, 4' → 6'

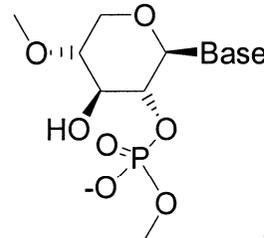


Allopyranosyl-NA
β-D, 4' → 6'

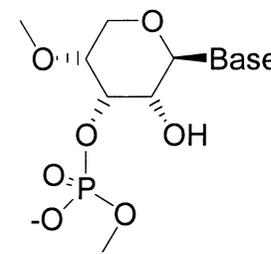
The 2' → 4' linked pentopyranosyl family



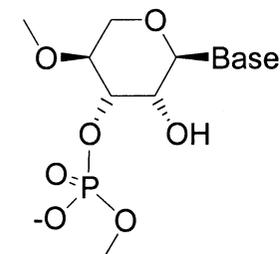
β-D-Ribopyranosyl-NA
(pRNA)



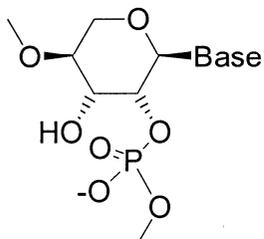
β-D-Xylopyranosyl-NA



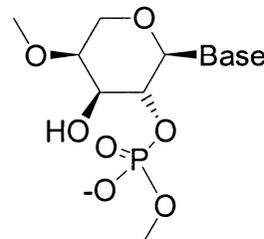
β-D-Ribopyranosyl-NA
3' → 4'



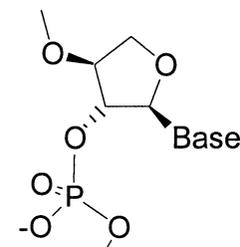
α-L-Lyxopyranosyl-NA
3' → 4'



α-L-Lyxopyranosyl-NA



α-L-Arabinopyranosyl-NA

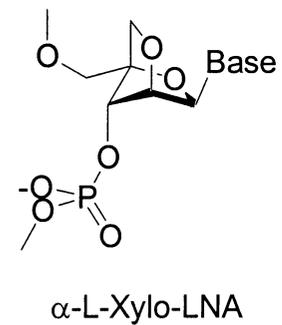
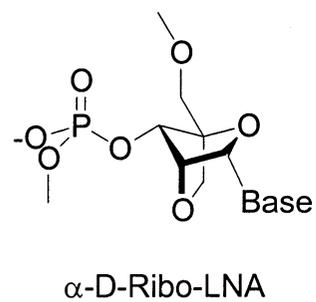
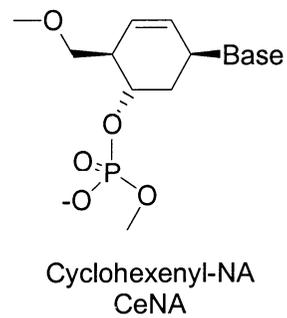
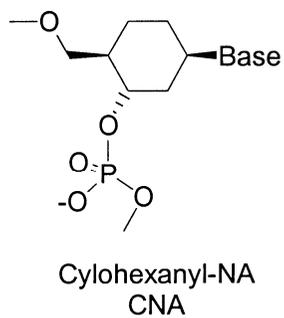
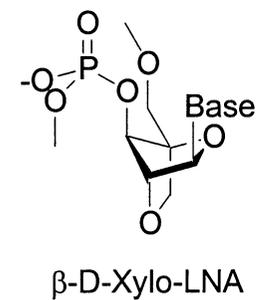
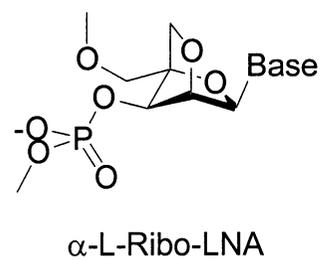
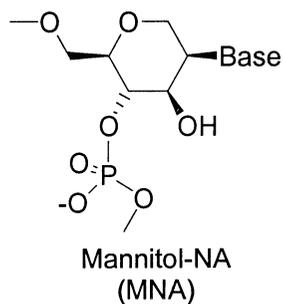
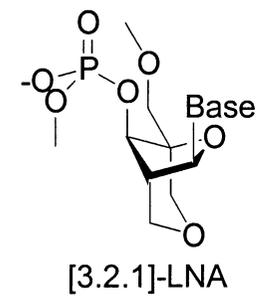
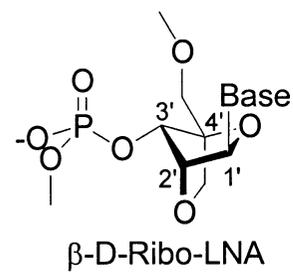
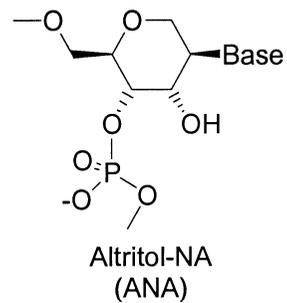
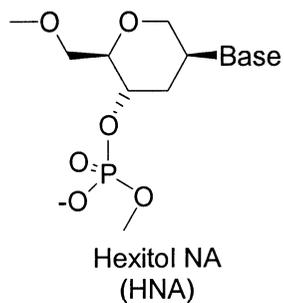


α-L-Threofuranosyl-NA
TNA

J. Hunziker, H. J. Roth, M. Bohringer, A. Giger, U. Diederichsen, M. Gobel, R. Krishnan, B. Jaun, C. Leumann and A. Eschenmoser, *Helv. Chim. Acta*, **1993**, *76*, 259–352

Review on the oligonucleotide modifications:
A. Eschenmoser *Angew. Chem., Int. Ed.* **2011**, *50*, 12412–12472
C. J. Leumann, *Bioorg. Med. Chem.*, **2002**, *10*, 841–854

Overview of XNA



XNA – Xeno Nucleic Acids

XNA - synthetic alternative to DNA and RNA as information-storing biopolymers that differs in the sugar backbone.

- at least 6 XNAs can store and retrieve genetic information
- Ongoing research to create synthetic polymerases to transform XNA →

Xenobiology

- (XNA) as information carriers, expanded genetic code and, incorporation of non-proteinogenic amino acids into proteins
- the **origin of life**: *Primordial soup* → (XNA →) RNA → RNA(+DNA)+Proteins
- development of industrial production systems with novel capabilities (pathogen resistance, biopolymer engineering)
- „genetic firewall” – excludes the risk of contaminating currently existing organisms (horizontal gene transfer)

The **long-term goal** - a cell that stores its genetic information on XNA, with different base pairs, using non-canonical amino acids and an altered genetic code.

So far cells have been constructed that incorporate only one or two of these features

XNA – Xeno Nucleic Acids

XNA are not recognized by natural polymerases.

One of the major challenges is to find or create novel types of polymerases that will be able to replicate these new-to-nature constructs. The method of polymerase evolution and design successfully led to the storage and recovery of genetic information (of less than 100bp length) from six alternative genetic polymers based on simple nucleic acid architectures not found in nature.

XNA aptamers, which bind their targets with high affinity and specificity, were also selected, demonstrating that beyond *heredity*, specific XNAs have the capacity for *Darwinian evolution* and *folding into defined structures*.

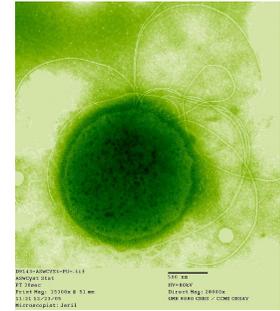
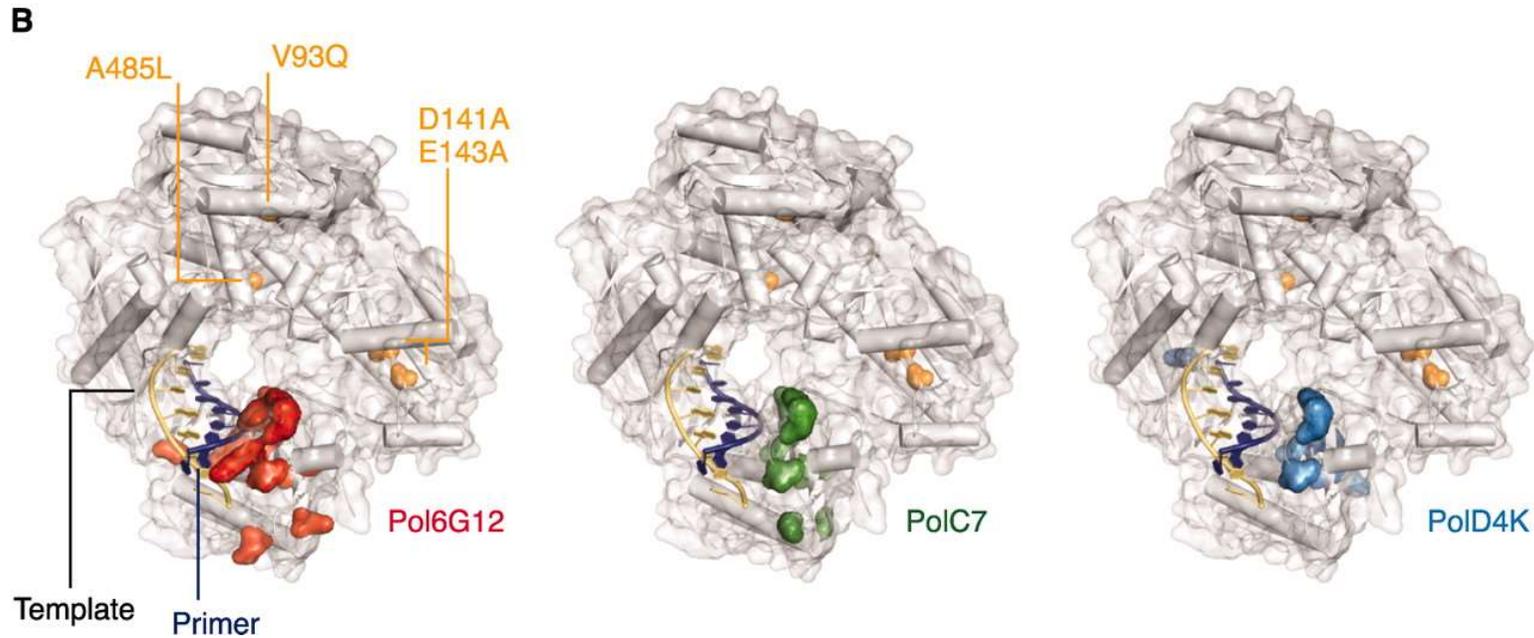
Thus, heredity and evolution, two hallmarks of life, are not limited to DNA and RNA but are likely to be emergent properties of polymers capable of information storage.

Engineering XNA polymerases

TgoT, a variant of the replicative polymerase of *Thermococcus gorgonarius*

A

	402	404	588	590	608	611	653	682	703	710	729	731
TgoT	YLD	..	FVT	..	LEIV	..	YEVPPPEKLVIIYEQITRDLKDYKATGPHVAV	..	VLKGS	GRI	..	AEY
Pol6G12	YLD	..	F AT	..	L KMV	..	YEVPPPEQLVIY QPITKQL HDY RARGPHVSV	..	V PKGS	GRI	..	AGY
PolC7	YLD	..	FVT	..	LEIV	..	YQVPP QQLAIYQPITRALQ DYK AKGPHVAV	..	VLKGS	GKI	..	AEY
PolD4K	Y PD	..	FVT	..	LEIV	..	YEVPT QHLVIHKQITRALN DYK AI GPHVAV	..	VLKGS	GRI	..	AEY



Thermococcus gorgonarius
(Angels Tapias)

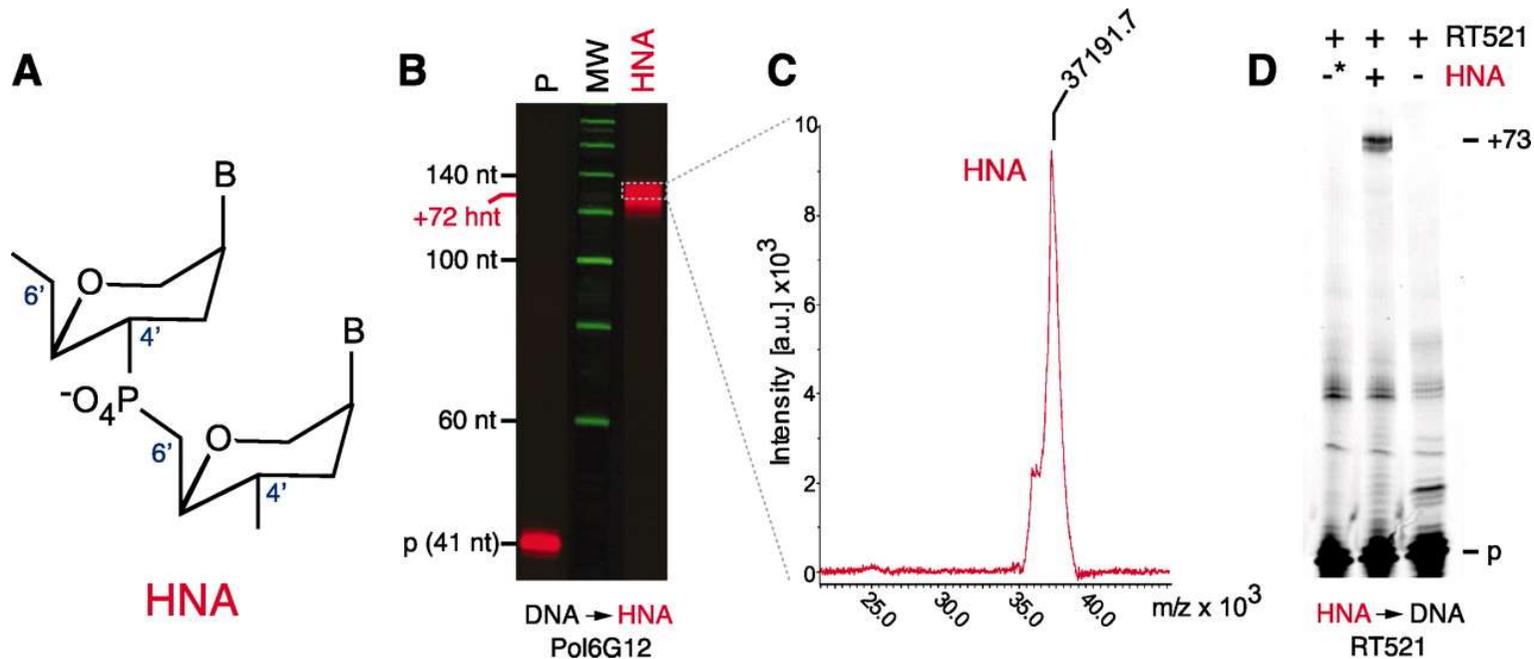
(A) Sequence alignments showing mutations from wtTgo in polymerases Pol6G12 (red), PolC7 (green), and PolD4K (blue).

(B) Mutations are mapped on the structure of Pfu (PDB: 4AIL).

Yellow - template; dark blue - primer; orange - mutations present in the parent polymerase TgoT

P. Herdewijn, P. Holliger, *et al. Science* **2012**, *336*, 341-344

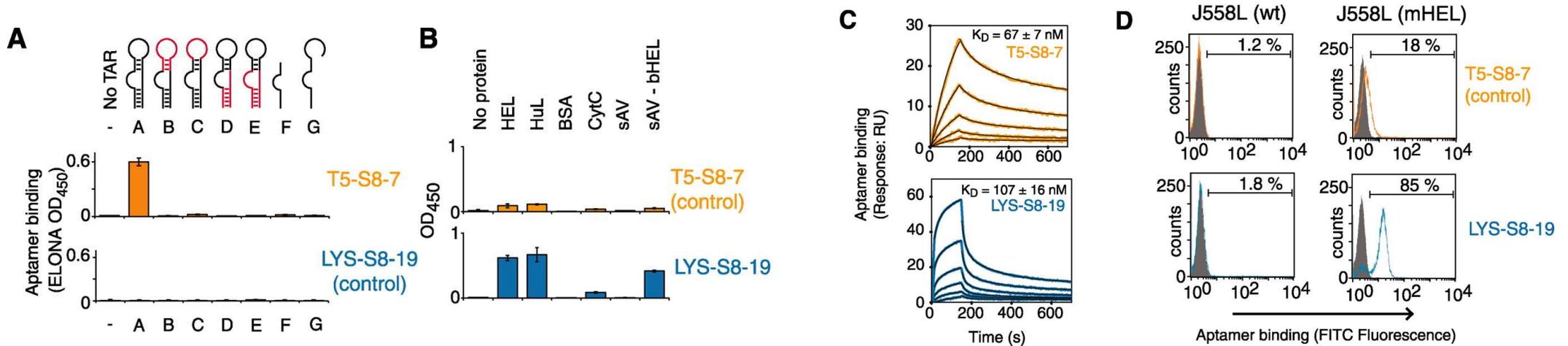
HNA synthesis



Pol6G12 extends the primer (p) incorporating 72 hNTPs against template T1 to generate a full-length hybrid molecule with a 37,215-dalton expected molecular mass.

HNA reverse transcription (DNA synthesis from an HNA template). Polymerase-synthesized HNA (from template YtHNA4) is used as template by RT521 for HNA-RT

HNA aptamers



Characterization of HNA aptamers. Anti-TAR aptamer T5-S8-7 and anti-HEL aptamer LYS-S8-19.

(A and B) Aptamer binding specificity against TAR variants (red, sequence randomized but with base-pairing patterns maintained) and different protein antigens (human lysozyme, HuL; cytochrome C, CytC; streptavidin, sAV; biotinylated-HEL bound to streptavidin, sAV-bHEL). OD, optical density.

(C) Affinity measurements of aptamer binding by SPR. RU, response units.

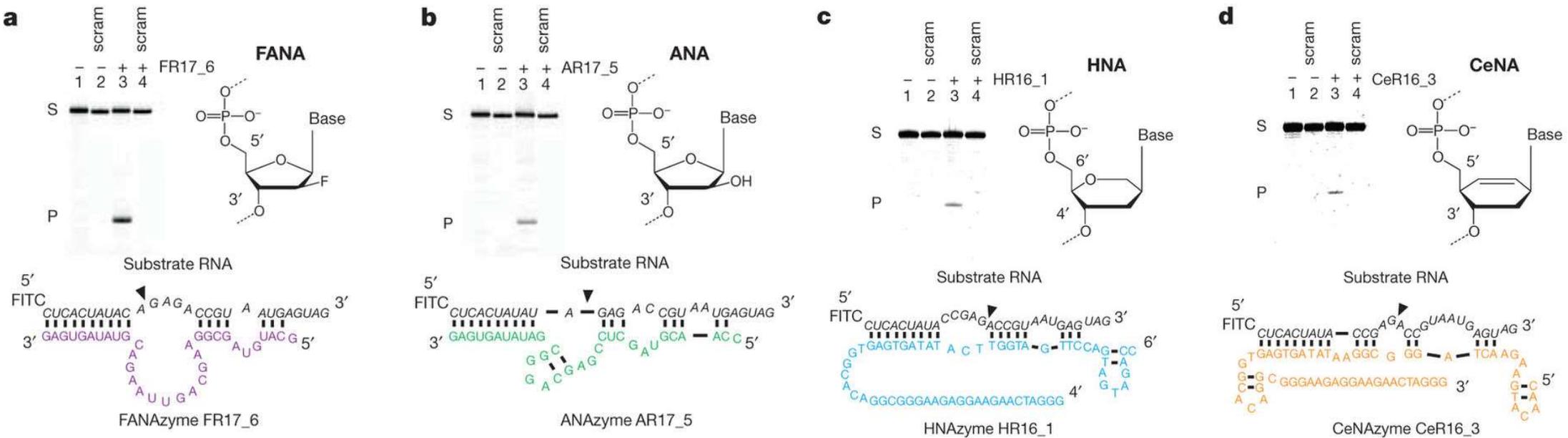
(D) FACS analysis of fluorescein isothiocyanate (FITC)-labeled aptamers binding to plasmacytoma line J558L with and without expression of membrane-bound HEL (mHEL). wt, wild type.

XNA – Xeno Nucleic Acids

XNA – complementarity to DNA, also used as genetic catalysts.

FANA, HNA, CeNA and ANA - cleave RNA (*XNAzymes*).

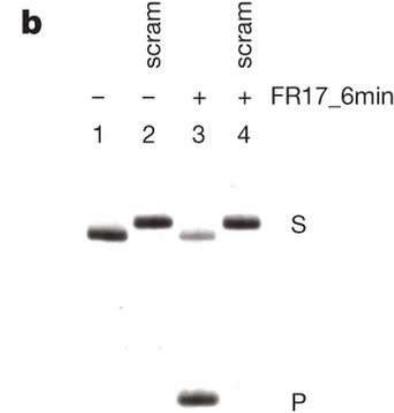
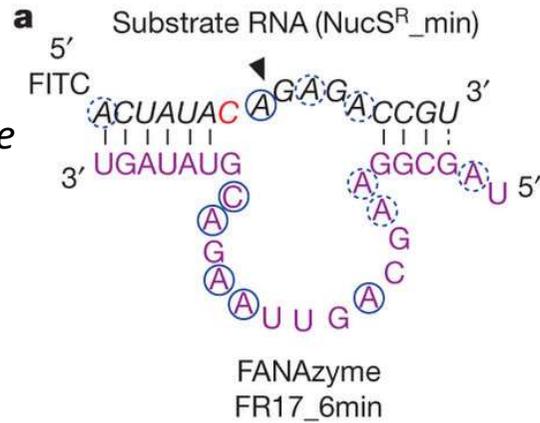
FANA XNAzymes can also ligate DNA, RNA and XNA substrates.



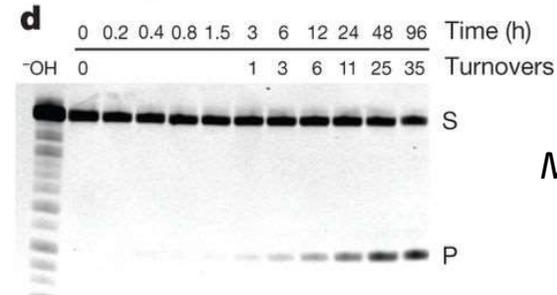
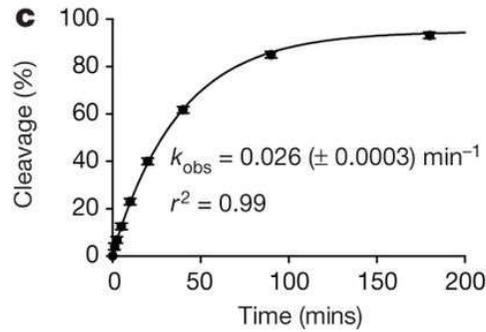
P. Herdewijn, P. Holliger, *et al.* *Nature* **2015**, *518*, 427-430

Chemical synthesis yields an active RNA endonuclease XNAzyme

Secondary FANAzyme structure



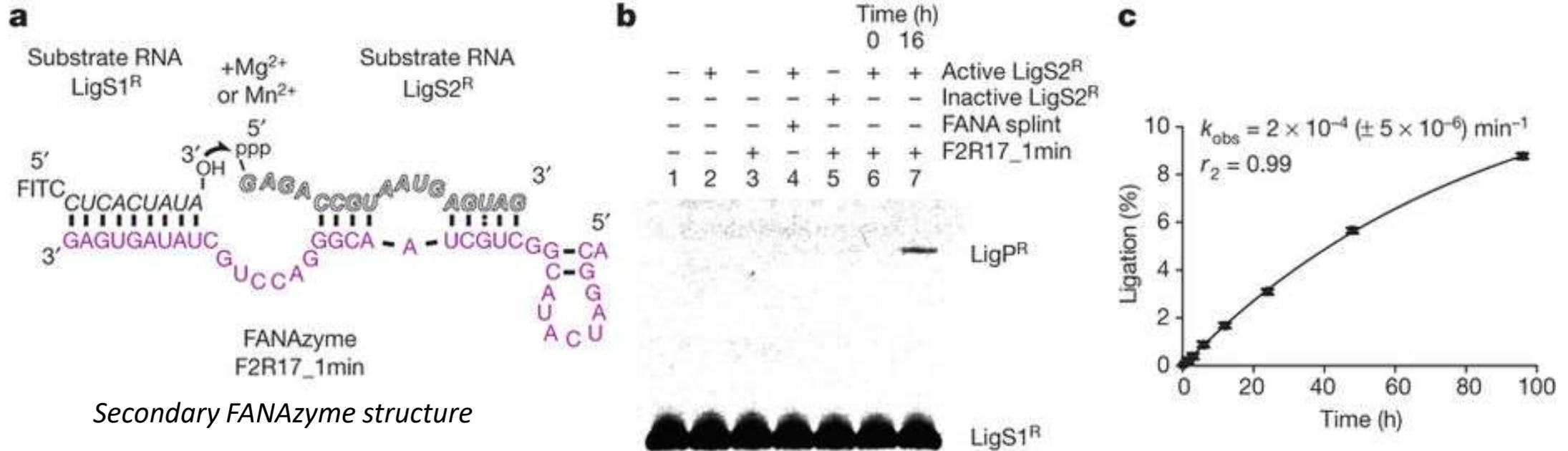
l. 1 and 3 - matching RNA
l. 2 and 4- scrambled RNA



Multiple turnovers

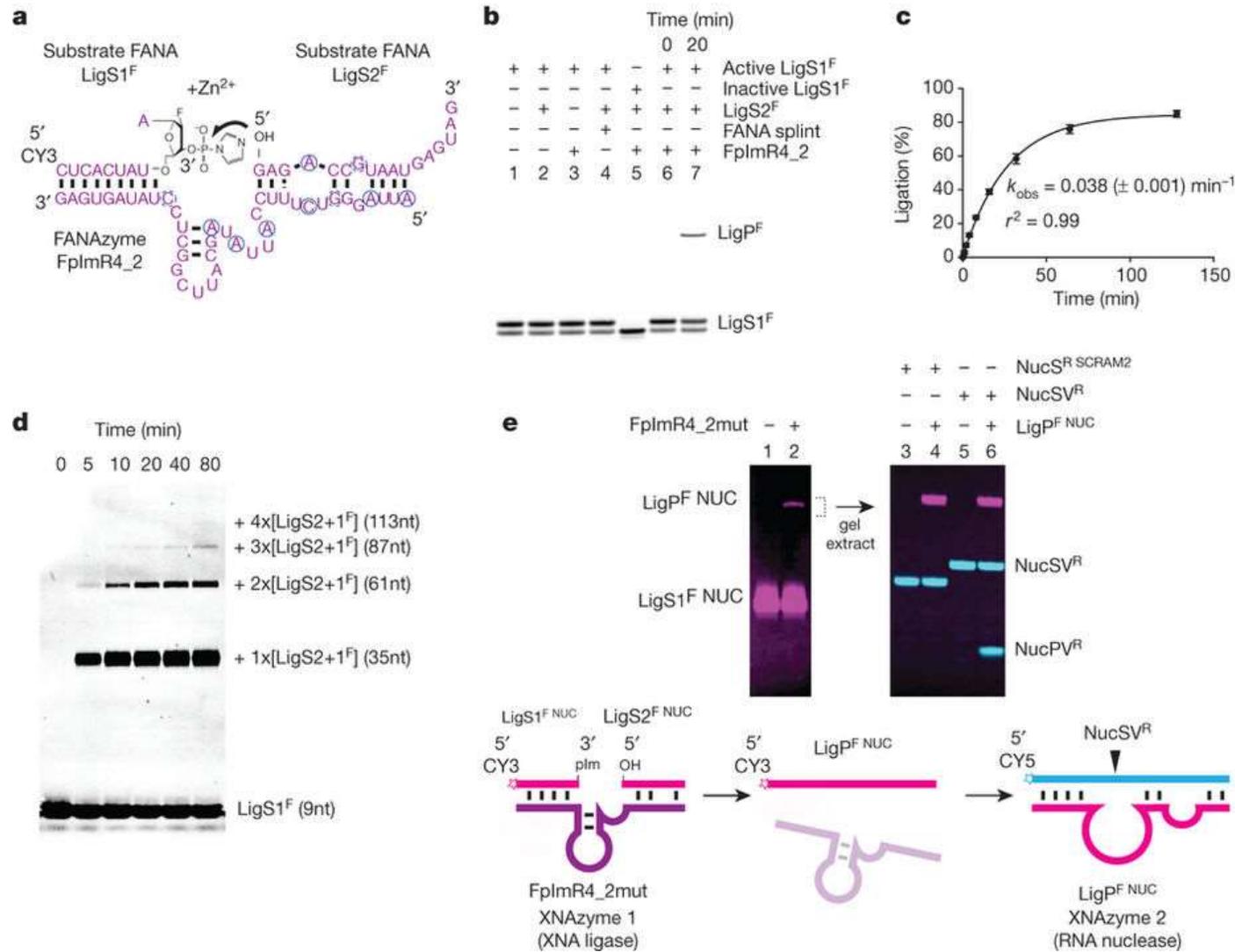
An RNA ligase XNAzyme (FANA)

FANA XNAzymes can also ligate DNA, RNA and XNA substrates.



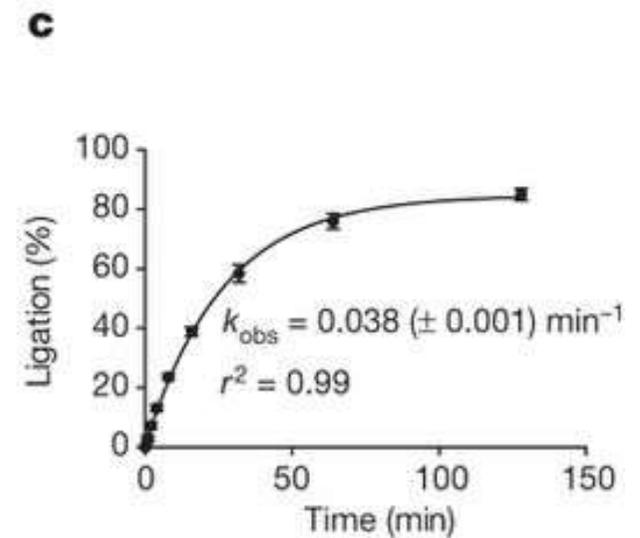
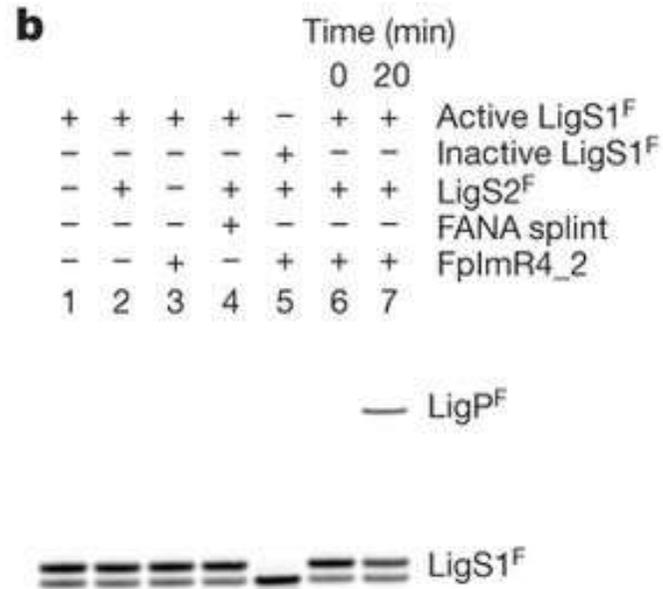
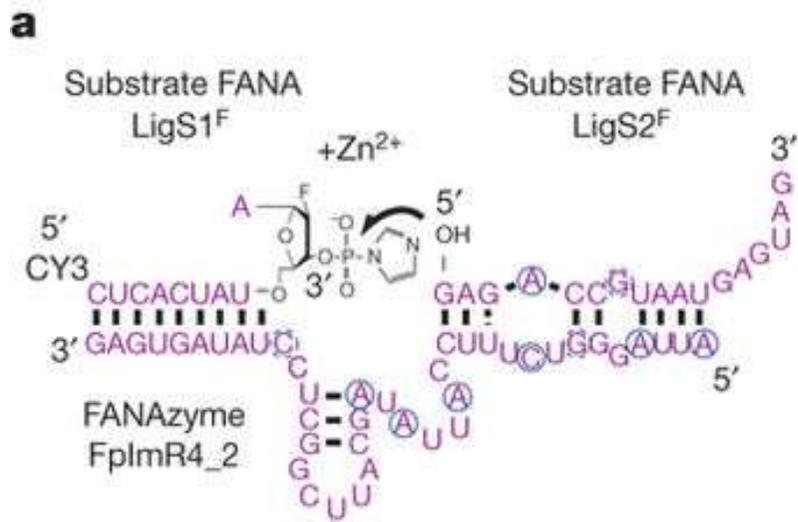
Pre-steady state trimolecular reaction rate (k_{obs}) at 25 °C (n = 3; error bars, s.d.).

XNA-XNA ligase XNAzyme (FANA): catalysis without natural nucleic acids

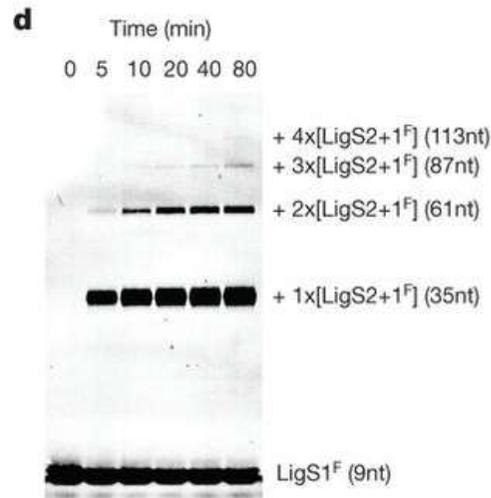


P. Herdewijn, P. Holliger, *et al.* *Nature* **2015**, *518*, 427-430

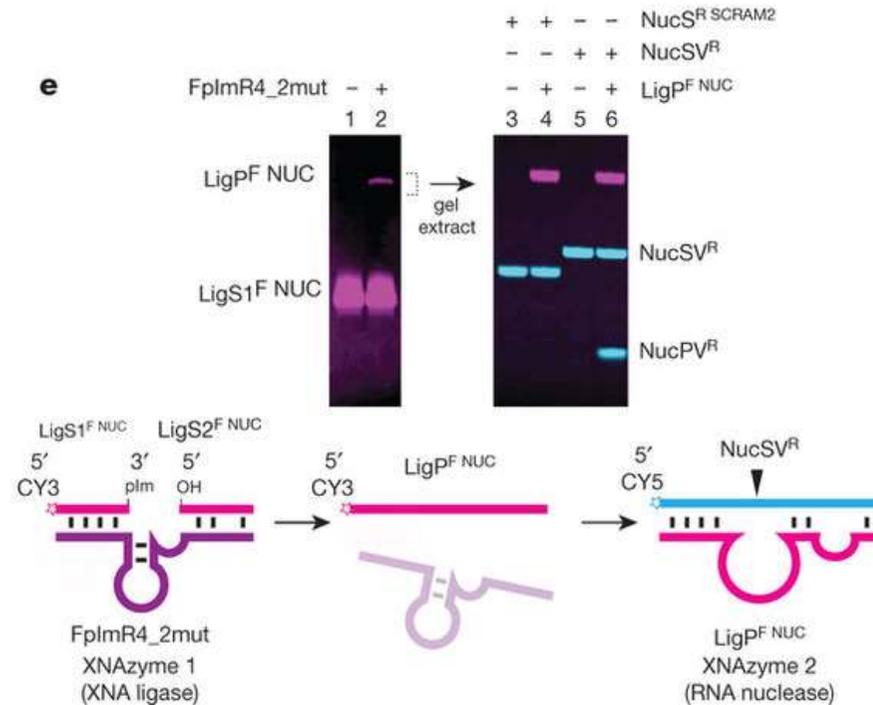
XNA–XNA ligase XNAzyme (FANA): catalysis without natural nucleic acids



XNA-XNA ligase XNAzyme (FANA): catalysis without natural nucleic acids



FplmR4_2-catalysed oligomerization of XNA (FANA) substrates



XNAzyme-catalysed assembly of an active XNAzyme. A variant XNA ligase (FplmR4_2mut) catalyses ligation (lane 2) of FANA substrates LigS1F NUC and LigS2F NUC. The product (LigPF NUC) is a variant of XNAzyme FR17_6 min (Fig. 2), which cleaves RNA substrate NucSVR (lanes 5 and 6), but not scrambled RNA (NucSR SCRAM2)(lanes 3 and 4).

Table 1 Polymerase-mediated synthesis of XNAs

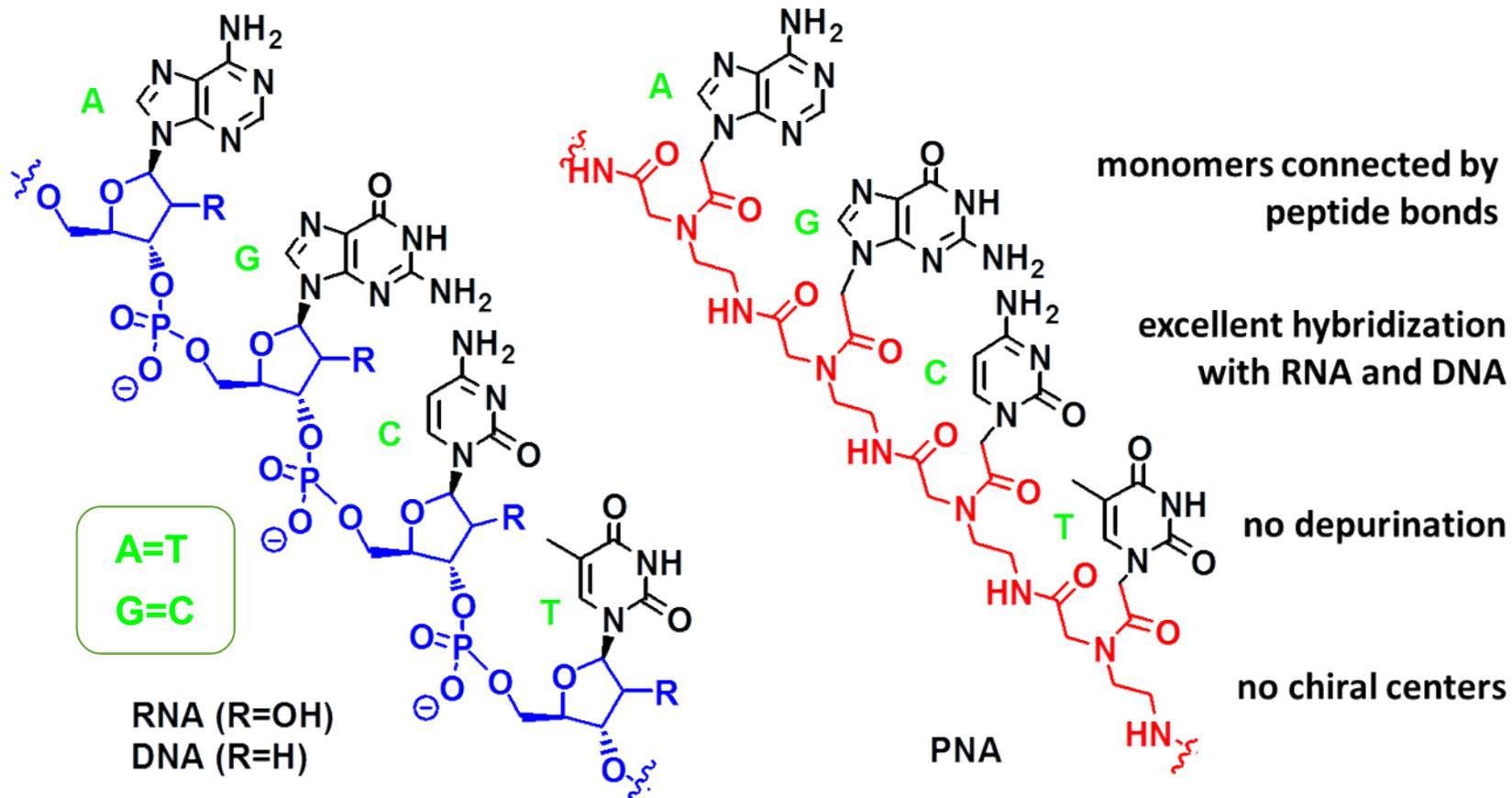
Pol Family	Polymerase	Novel Activity				
Pol A	Taq Tth Pol θ	2'F RNA 2'OMe RNA 2'-azido RNA	Pol X	(D-aa) ASFV pol	L-DNA L-RNA	
Pol B	Tgo KOD 9 ^o N Pfu phi29	CeNA LNA phNA HNA FANA CyDNA 2'F RNA ANA TNA 2'azido RNA tPhoNA	RNAP	T7 RNAP Syn5	2'F RNA 2'OMe RNA Ds-Pa UBP	
Pol Y	(D-aa) Dpo4	L-DNA	RT	HIV-RT	pyDAD-puADA UBP	

Table 2 FDA-approved nucleic acid therapeutics as of February 2020

Drug name (trade name)	Target	Modifications	Mechanism	Indication	Approval
Fomivirsen (Vitravene)	mRNA of the CMV immediate-early (IE)-2 protein	PS	ASO (translation blocking)	Cytomegalovirus retinitis (CMV)	FDA (1998) and EMA (1999) approved. FDA (2001) and EMA (2002) withdrawn
Pegaptanib (Macugen)	Vascular endothelial growth factor (VEGF165)	2'F, 2'OMe, PEG conjugate	Aptamer	Neovascular (wet) age-related macular degeneration	FDA approved (2004)
Mipomersen (Kynamro)	Apolipoprotein B-100 mRNA	2'MOE, PS, 5mC	ASO (RNase H)	Homozygous familial hypercholesterolemia	FDA approved (2013)
Eteplirsen (Exondys 51)	Exon 51 in dystrophin mRNA	PMO	ASO (splicing modulation)	Duchenne muscular dystrophy	FDA approved (2016)
Nusinersen (Spinraza)	Survival of motor neuron 2 (SMN2) pre-mRNA	2'MOE, PS, 5mC	ASO (splicing modulation)	Spinal muscular atrophy	FDA (2016) and EMA (2017) approved
Patisiran (Onpattro)	Transthyretin (TTR) mRNA	2'OMe	siRNA	Hereditary transthyretin-mediated amyloidosis	FDA and EMA approved (2018)
Inotersen (Tegsedi)	Transthyretin (TTR) mRNA	2'MOE, PS, 5mC	ASO (RNase H)	Hereditary transthyretin-mediated amyloidosis	FDA and EMA approved (2018)
Volanesorsen (Waylivra)	Apolipoprotein C ₃ (apo-CIII) mRNA	2'MOE, PS, 5mC	ASO (RNase H)	Familial chylomicronemia syndrome	EMA approved (2019)
Givosiran (Givlaari)	Aminolevulinatase synthase 1 (ALAS1) mRNA	PS, 2'F, 2'OMe, GalNAc conjugate	siRNA	Acute hepatic porphyria	FDA approved (2019)
Golodirsen (Vyondys 53)	Exon 53 in dystrophin mRNA	PMO	ASO (splicing modulation)	Duchenne muscular dystrophy	FDA approved (2019)

K. Duffy, S. Arangundy-Franklin, P. Holliger *BMC Biology*, 2020, 18, Art.# 112

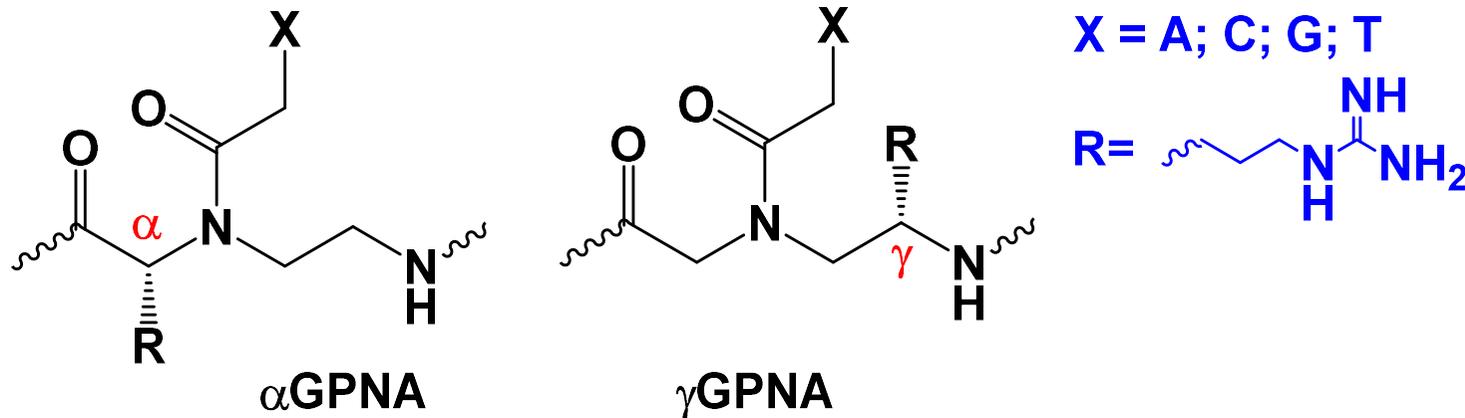
Peptidonucleic acids – functional DNA analogues



PNA – stable *ex vivo*, the backbone detected in cyanobacteria

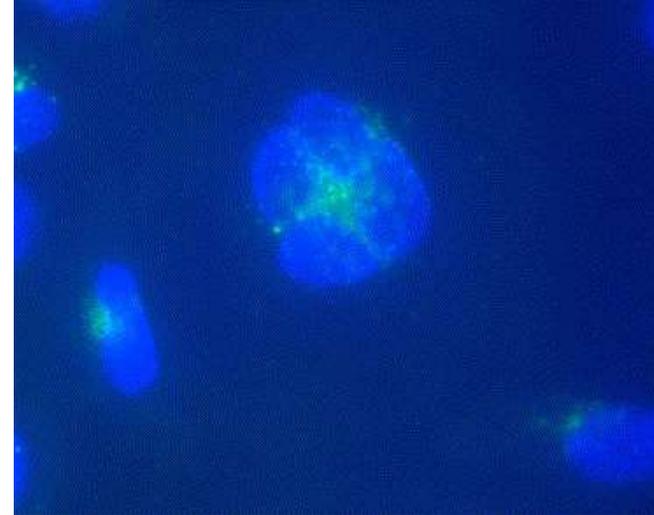
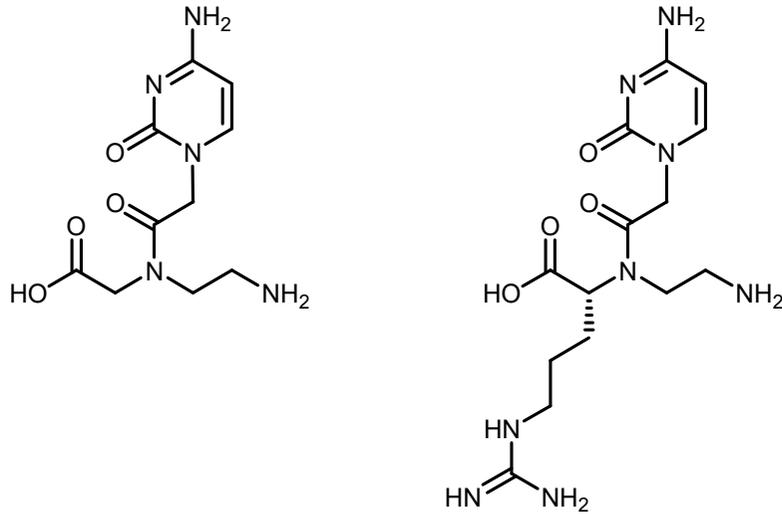
Applications: antigene, antisense agents; fluorescent DNA probes (FISH), anticancer, antiviral, antibacterial, antiparasitic agents; diagnostics, mol. biology

Structural modifications of the PNA - α GPNA, γ GPNA



- GPNA: Alkylguanidinium residues (Arg side chains)
- enhanced water solubility
- **cell permeability** (analogous to oligoarginine CPPs)
- α position \leftarrow *D*-arginine
- γ position \leftarrow *L*-arginine

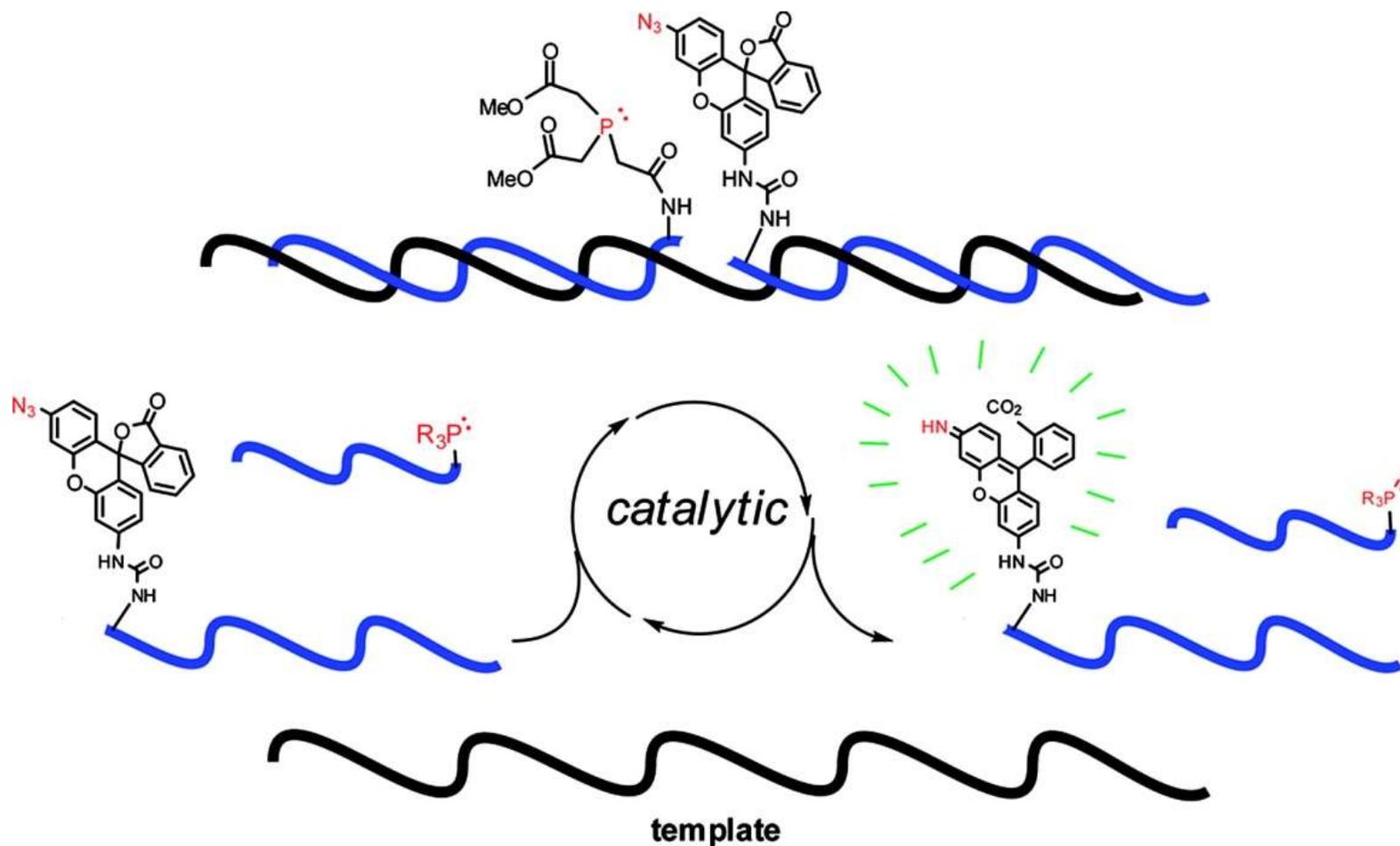
Cell-penetrating α GPNA



HeLa cells incubated with 1 μ M GPNA (FITC-^DCC^DAC^DCT^DCT^DGC^DCA^DAC^DGG^DGT-NH₂) for 16 h, Fixed, stained with DAPI. Nuclei (blue), GPNA (green).

P. Zhou, A. Dragulescu-Andrasi, B. Bhattacharya, H. O'Keefe, P. Vatta,
J. J. Hyldig-Nielsen and D. H. Ly *Bioorg. Med. Chem. Lett.* **2006**, *16*, 4931
A. Dragulescu-Andrasi, S. Rapireddy, G. He, B. Bhattacharya, J. J. Hyldig-Nielsen,
B. G. Zon, and D. H. Ly *J. Am. Chem. Soc.* **2006**, *128*, 16104

Cell-penetrating α GPNA for *in vivo* catalytic oligonucleotide sensing

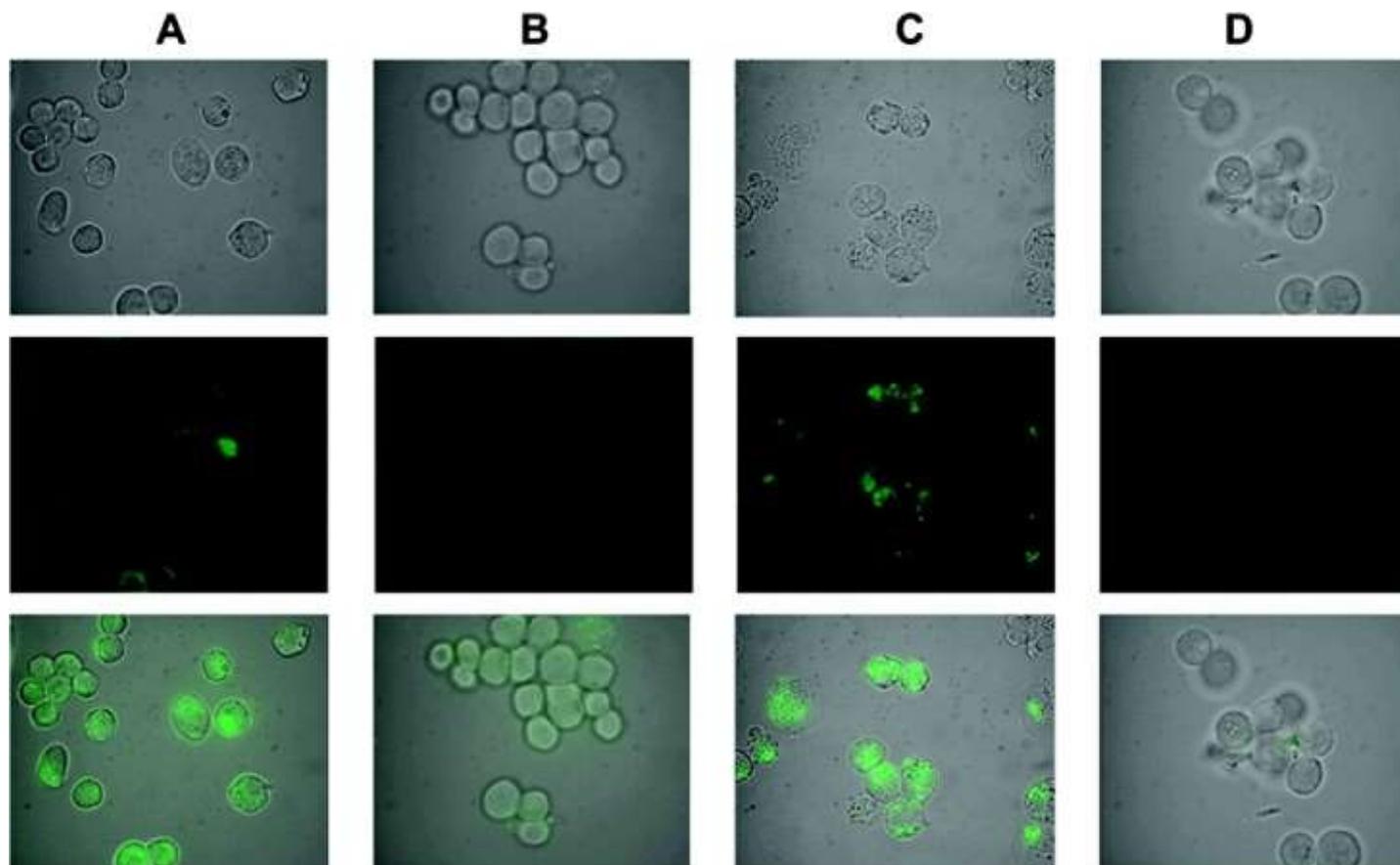


Z. Pianowski, N. Winssinger *Chem. Comm.* **2007**, 37, 3820-3822

Z. Pianowski et al. *J. Am. Chem. Soc.* **2009**, 131, 6492-6497

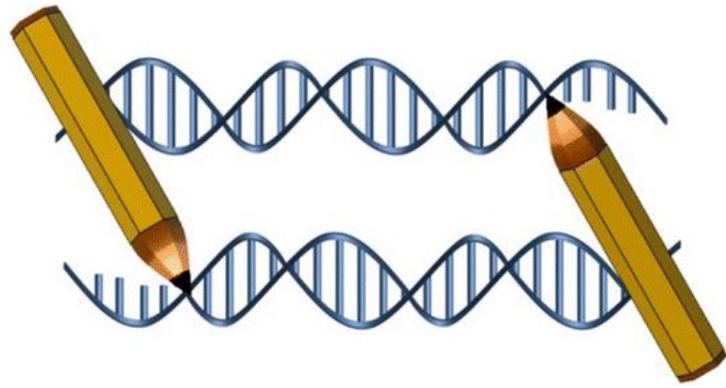
Cell-penetrating α GPNA for *in vivo* catalytic oligonucleotide sensing

Inside living cells

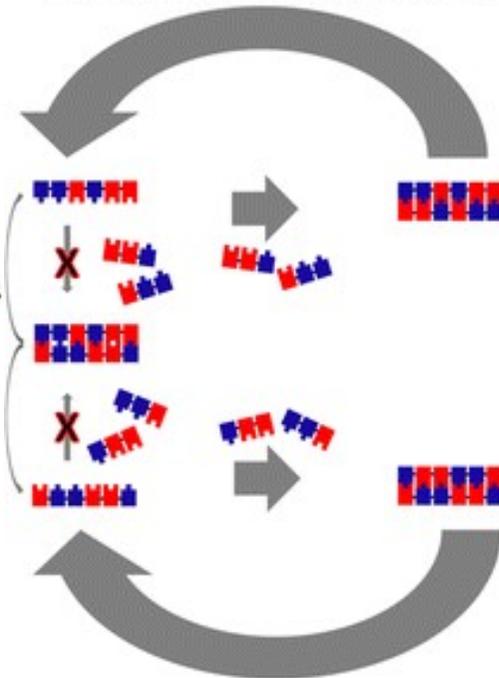


A, B – controls (+/-) **C** – matching PNA **D** – mismatched PNA

Abiotic self-replication

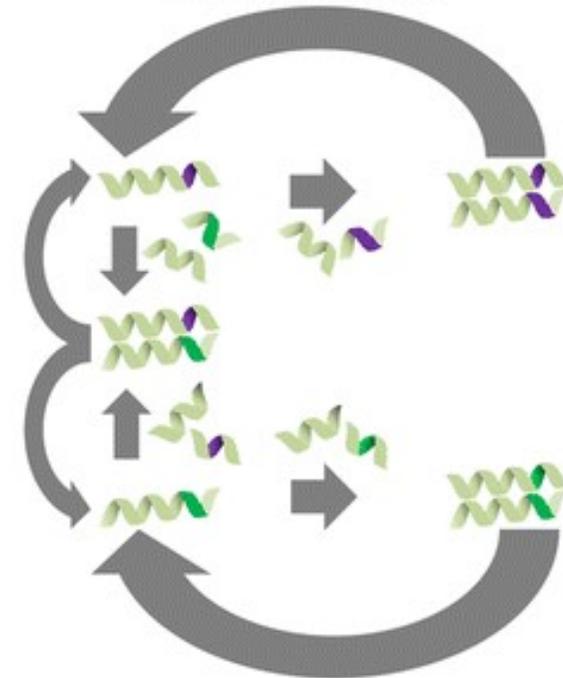


A. Darwinian/nucleic acid replicator



Cross-replication
disfavored

B. Peptide replicator

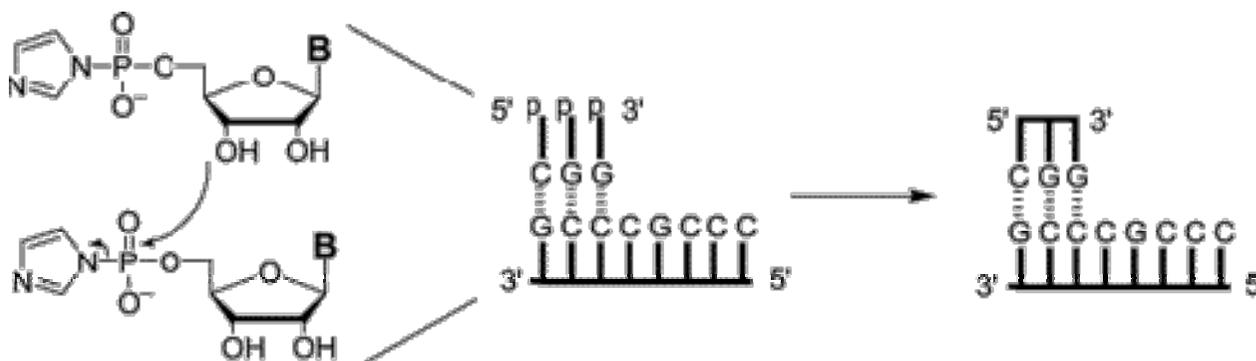


Cross-replication
allowed

(A) For nucleic acids replicators, templating is based on base pairing, so the formation of a mutant template is rare. Once formed, the mutant replicator forms a competing replication cycle. (B) For a peptide replicator, templating is less exact, so the formation of a mutant template is common. The mutant template can catalyze formation of mutant progeny or parental progeny, and the two species form a mutualistic network.

Meyer AJ, Ellefson JW, Ellington AD. *Acc Chem Res.* 2012 45(12):2097-2105.

Nonenzymatic templated nucleic acid synthesis – monomer/short oligomer



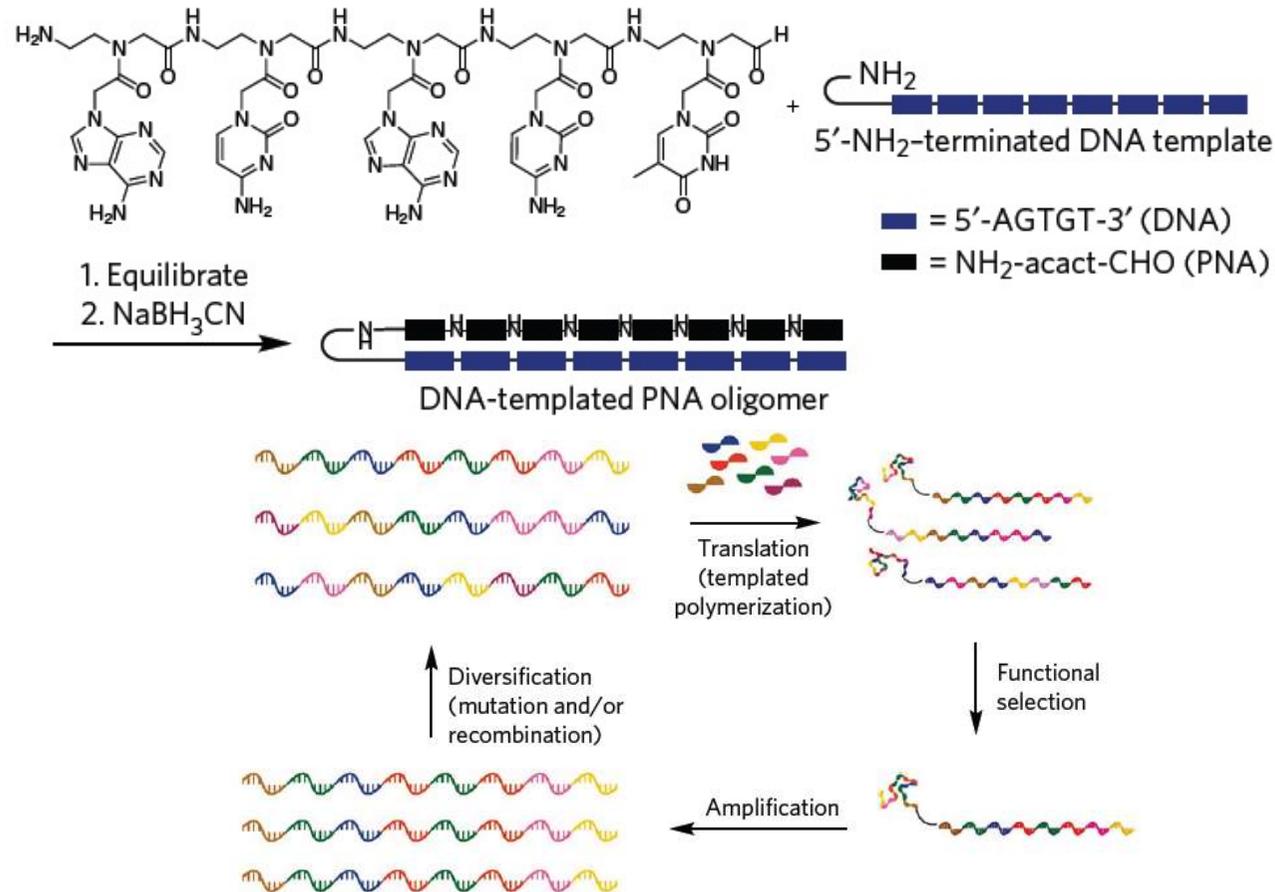
Problems:

- very slow reactions
- limited range of templates (mostly C-rich)
- poor regioselectivity (2'-5' linkages, predominant in some cases)
- 3'-aminonucleotides perform better, but undergo intramolecular cyclizations as side reaction

Lohrmann, R.; Orgel, L. E. *Tetrahedron* **1978**, *34*, 853

A. Silverman, E. Kool *Chem. Rev.* **2006**, *106*, 3775

Templated nucleic acid synthesis – short oligomer coupling

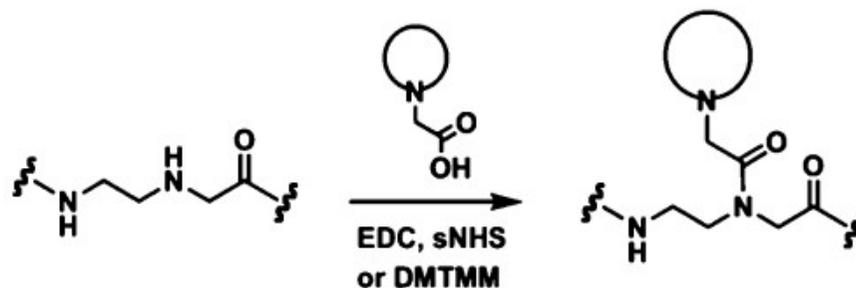
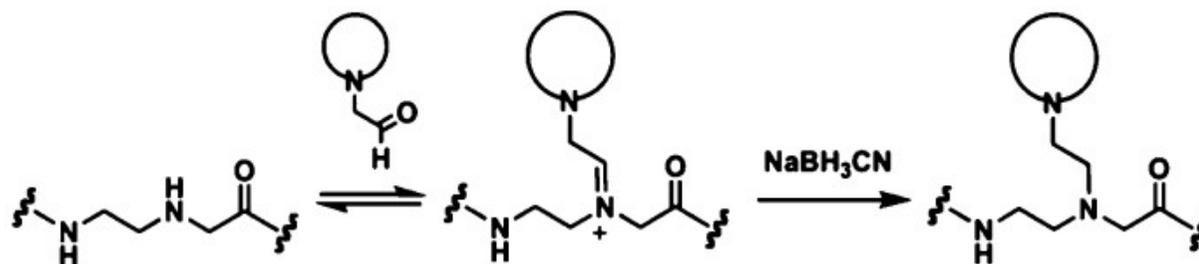
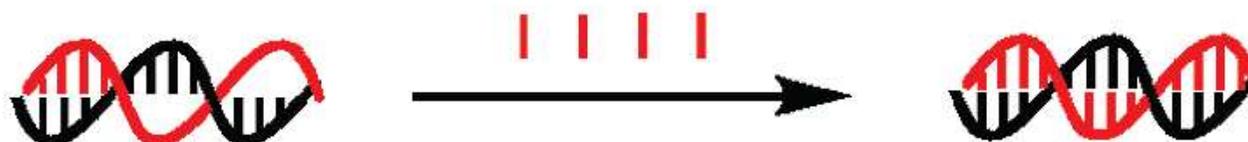


Limitations:

- slightly distorted backbone (amine instead of amide backbone every 5 bases)
- only carefully designed pentamers work – limiting the diversity for functional selection

Brudno Y, Birnbaum ME, Kleiner RE, Liu DR. *Nature Chem. Biol.* **2010**, *6*, 148-155.

Templated nucleic acid synthesis – base filling



Advantages:

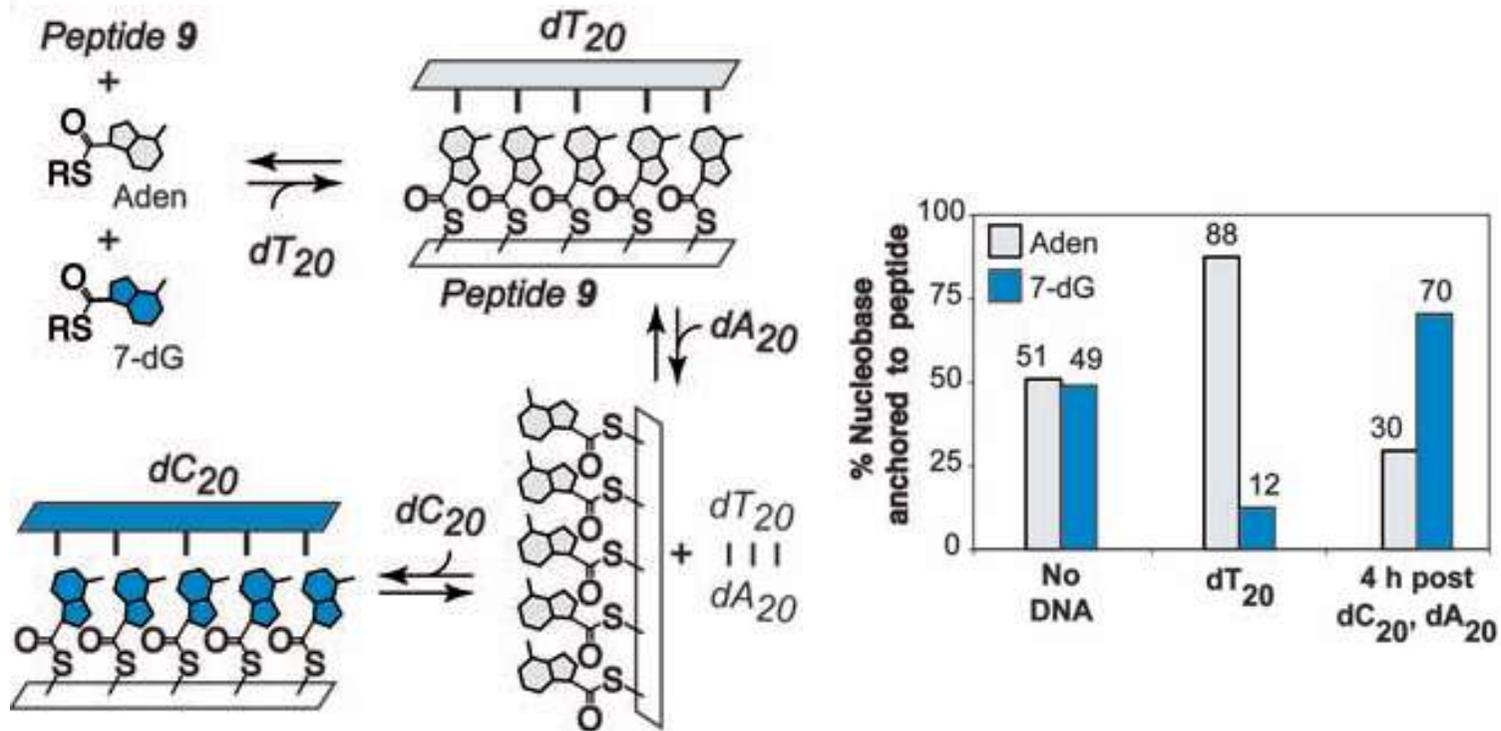
- no cross-reactivity
- selectivity increased by proximity of the reaction to the hybridization site

Limitations:

- single or double abasic sites (most efficient inside of the chain)
- Aldehydes give better yields and accuracy, but worse hybridization of the product

Heemstra JM, Liu DR. *J. Am. Chem. Soc.* **2009**, *131*, 11347-11349.

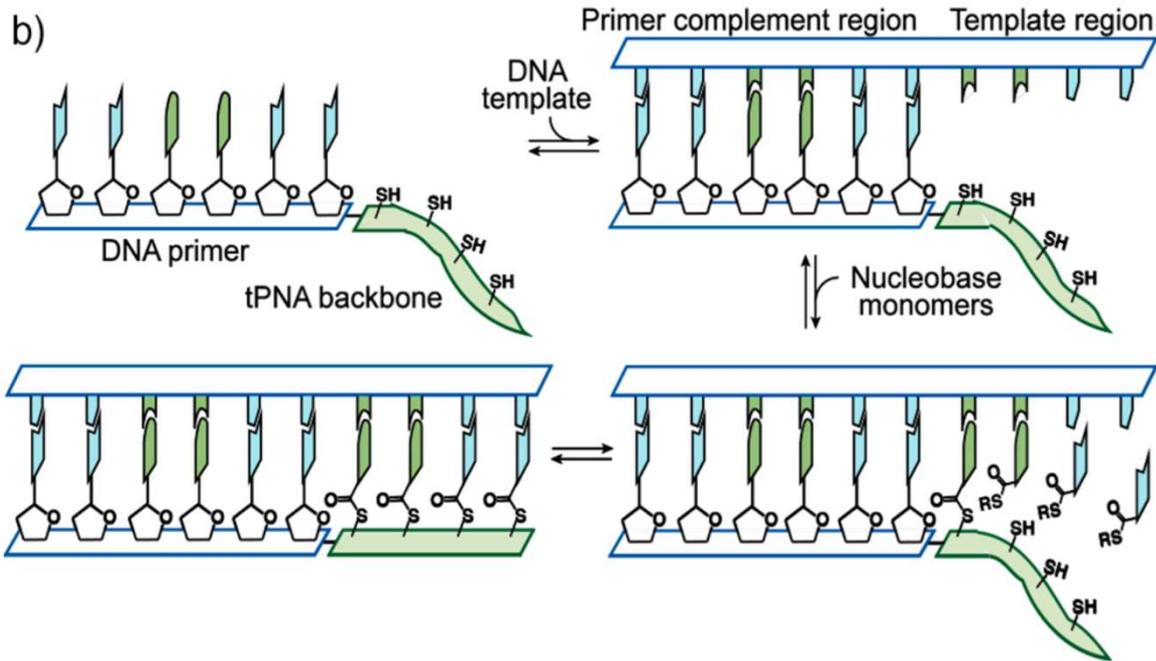
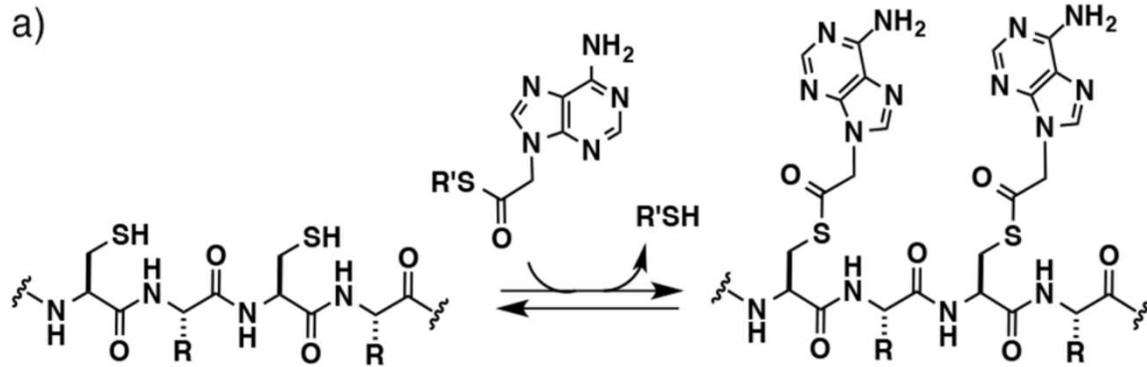
A polyamide responsive to selection pressure



Dynamic polymer responsive to template changes with high fidelity

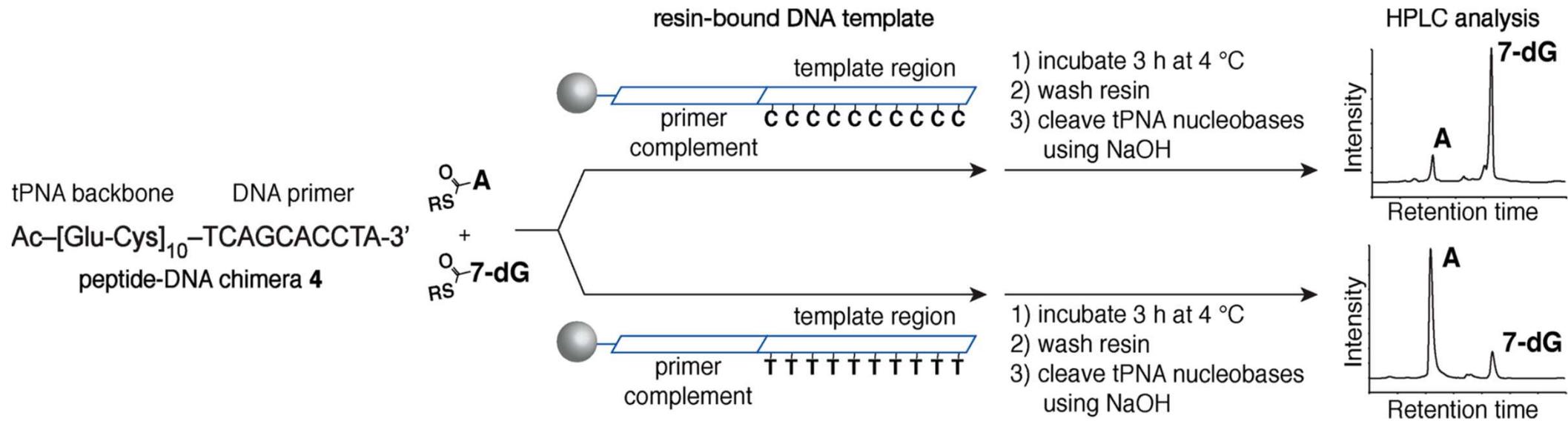
Ura Y, Beierle J, Leman L, Orgel LE, Ghadiri MR. *Science* **2009**, 325, 73-77.

Templated Self-Assembly of Dynamic Peptide Nucleic Acids



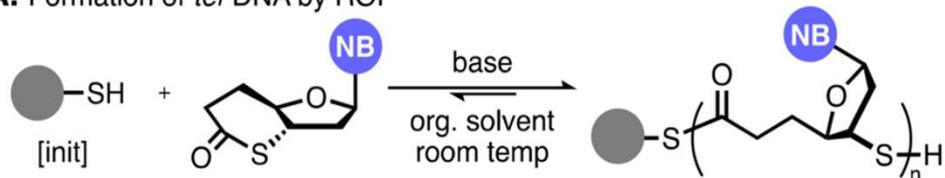
The DNA primer region affords a high level of control over the location and register of the tPNA backbone in relation to the template strand.

Templated Self-Assembly of Dynamic Peptide Nucleic Acids



Dynamic and Responsive DNA-like Polymers

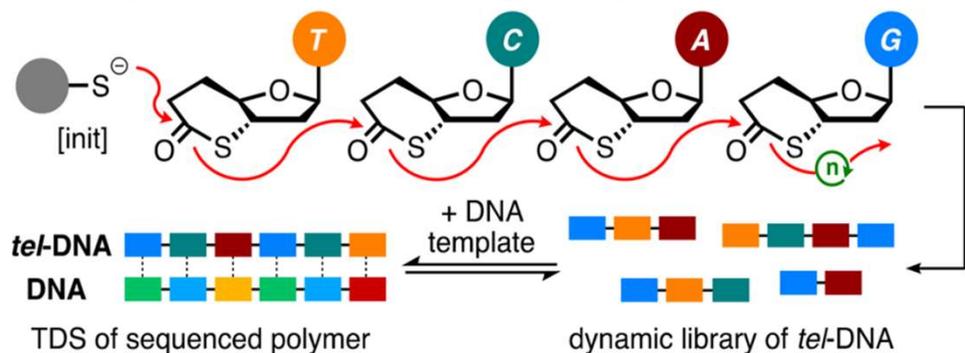
A. Formation of *tel*-DNA by ROP



B. Comparison of DNA with *tel*-DNA

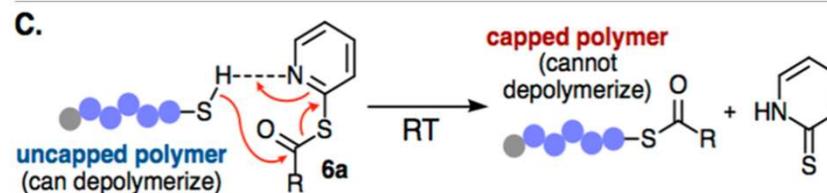
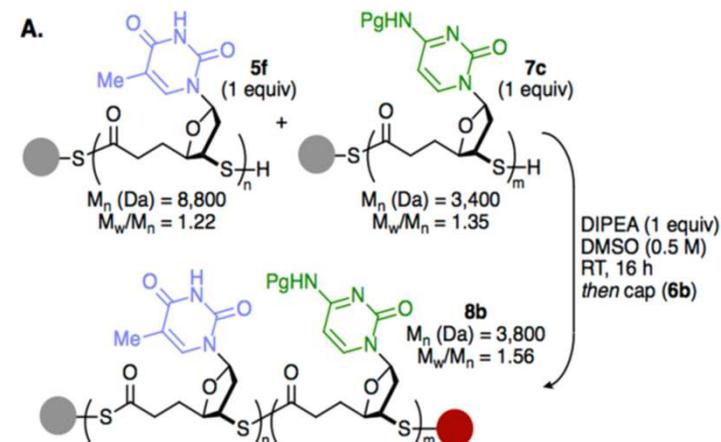
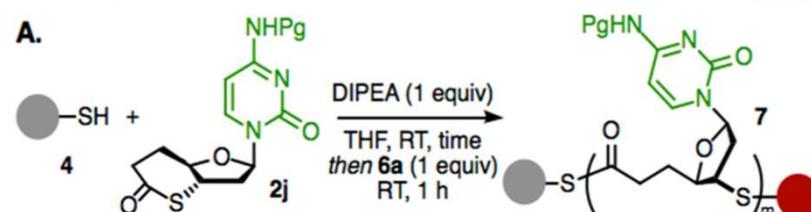
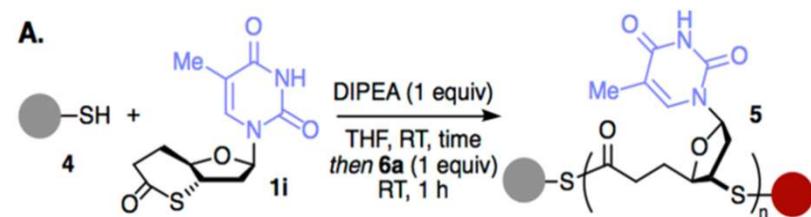


C. Concept towards template directed synthesis (TDS)

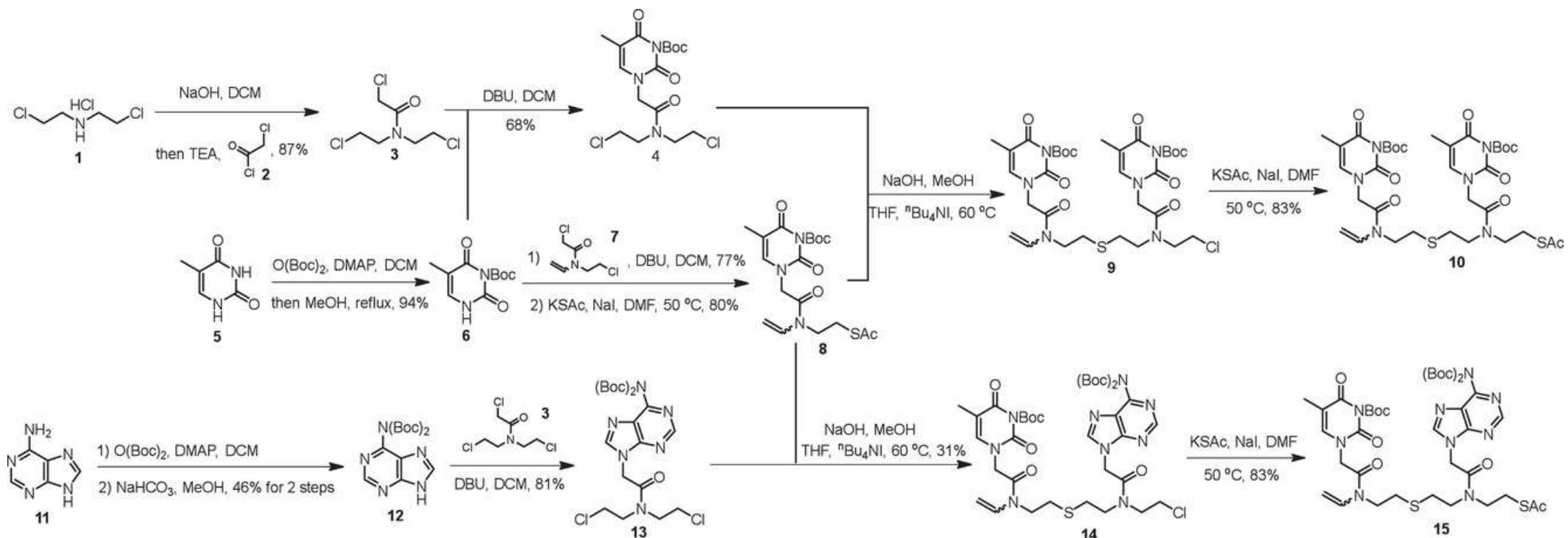


C: use of a thiolate initiator to form a dynamic library of *tel*-DNA oligomers in situ. Addition of a defined DNA template will equilibrate the dynamic mixture to a thermodynamically favorable complementary sequence defined polymer.

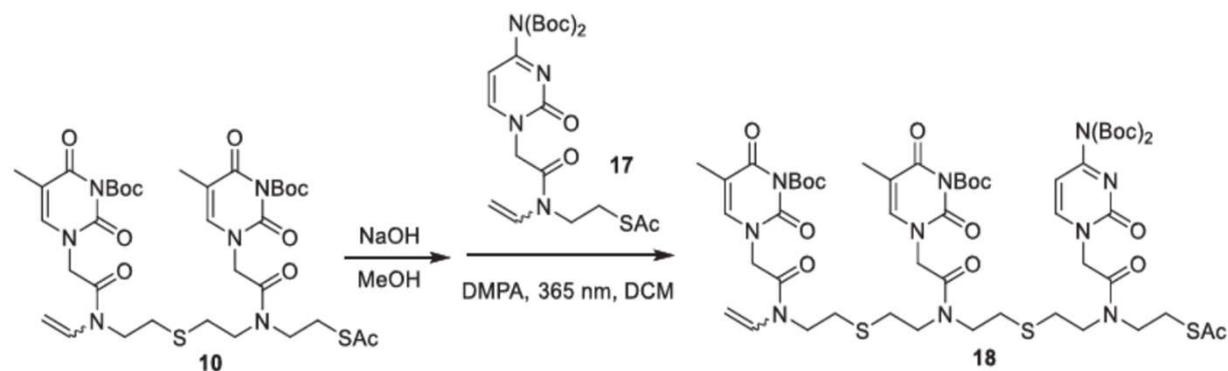
S. Mavila *et al.* *J. Am. Chem. Soc.* **2018**, *140*, 13594-13598



Sequence-Controlled Synthesis of Clickable Synthetic Oligonucleotides



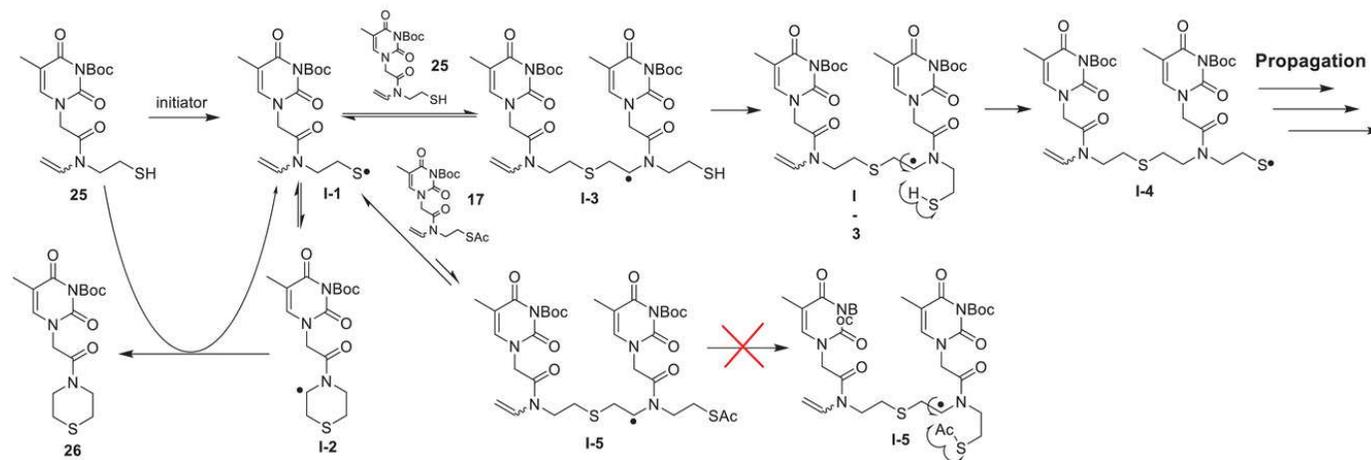
DCM = dichloromethane, TEA = triethylamine, DBU = 1,8-diazabicyclo(5,4,0)undec-7-ene, Boc = tert-butyloxycarbonyl, Ac = acetyl, DMF = dimethyl formaldehyde



X. Han et al. *Macromol. Rapid Commun.* **2020**, *41*, 2000327

Sequence-Controlled Synthesis of Clickable Synthetic Oligonucleotides

CNA-2G Monomer Propagation Mechanism:



CNA-2G Dimer Propagation Mechanism:

