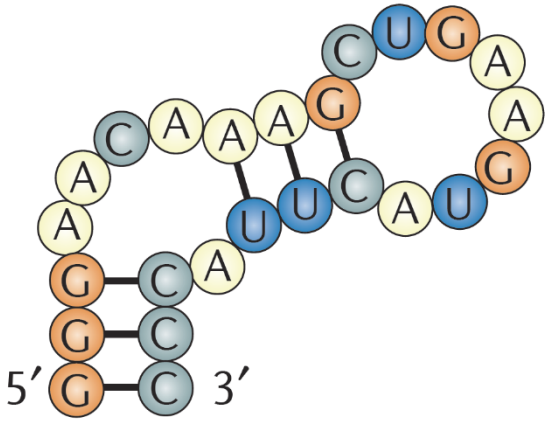
Aptamers as antibody alternatives: advances and applications

Journal Club – Lukas Frick

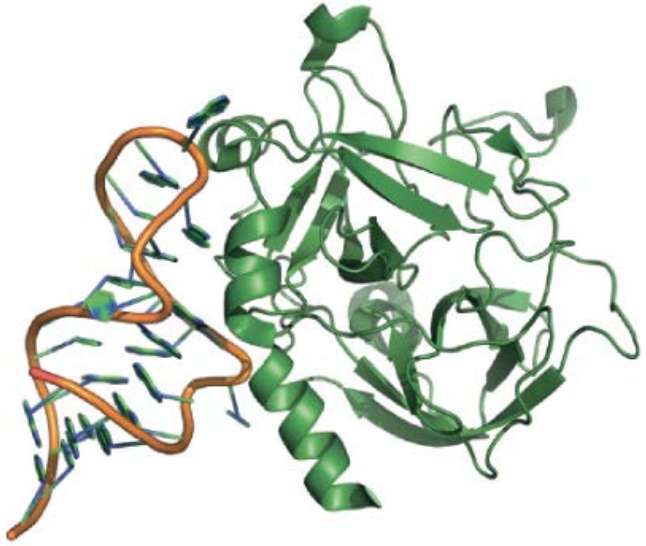
18 June 2019

What are aptamers?

Aptamers are **single-stranded oligonucleotides** (RNA or DNA) that fold into defined **3D structures** and recognize their target by their complementary **shape and charge**.



Secondary structure

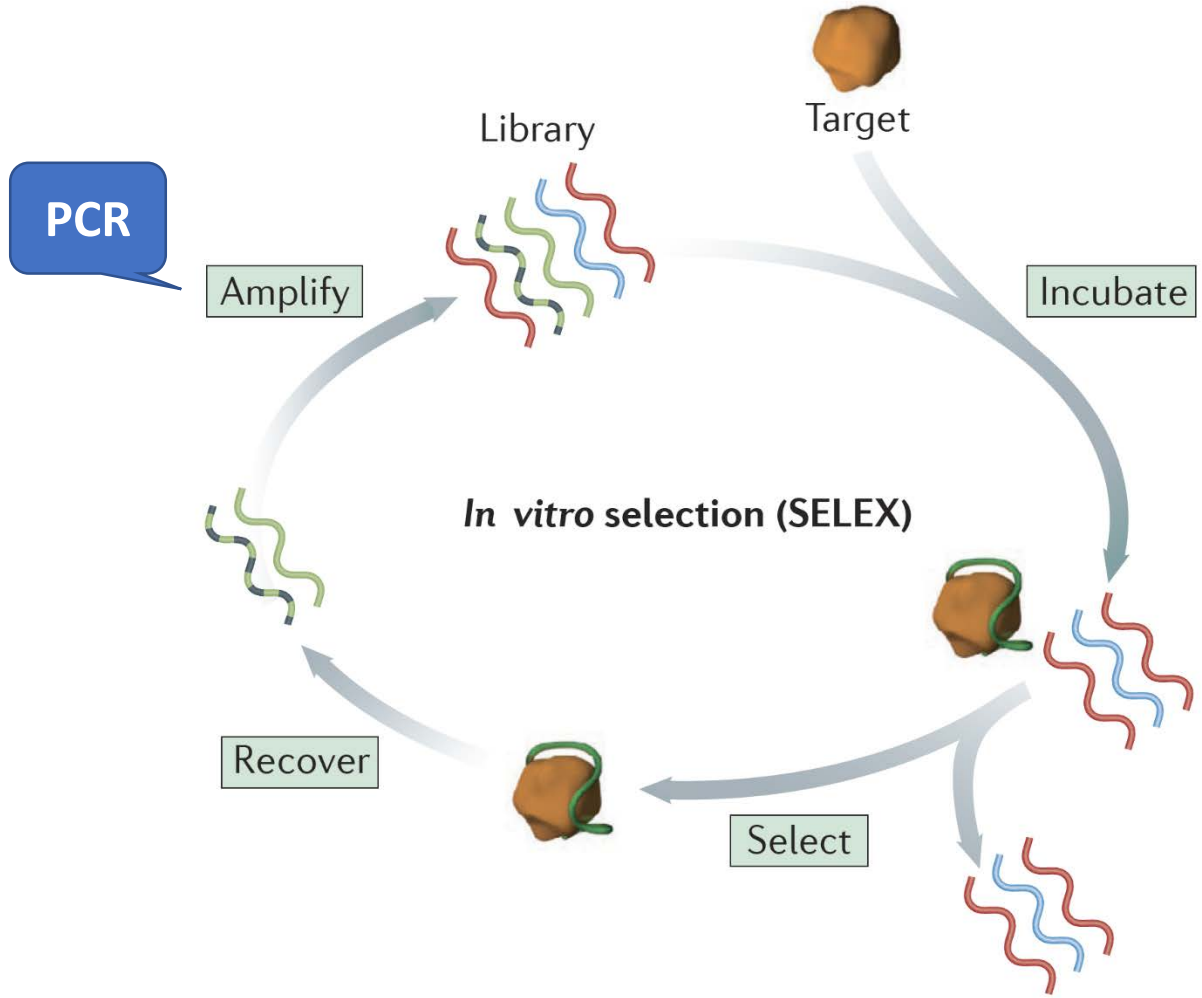


Human α-thrombin-aptamer complex

Aptamers are generated artificially using some form of **SELEX**

(systematic **e**volution of **l**igands by **ex**ponential enrichment)

- The starting point is a **huge library of random oligonucleotide species** (~10^{15}).
- Aptamers with the desired properties are **enriched** by multiple (~10) rounds of **selection, then amplification.**



Aptamers and SELEX were invented 30 years ago

***In vitro* selection of RNA molecules that bind specific ligands**

Andrew D. Ellington & Jack W. Szostak*

Department of Molecular Biology, Massachusetts General Hospital, Boston, Massachusetts 02114, USA

***Nature*, 1990**

Systematic Evolution of Ligands by Exponential Enrichment: RNA Ligands to Bacteriophage T4 DNA Polymerase

CRAIG TUERK AND LARRY GOLD

***Science*, 1990**

Since then, many small-protein antibody mimetics have been developed…

Antibody mimetic	Scaffold	Mass
Affibody molecules	Z domain of Protein A (Staph. aureus)	6 kDa
Affilins	Gamma-B crystallin	20 kDa
	Ubiquitin	10 kDa
Affimers	Cystatin	12–14 kDa
Affitins	Sac7d (from *Sulfolobus acidocaldarius*)	7 kDa
Alphabodies	Triple helix coiled coil	10 kDa
Anticalins	Lipocalins	20 kDa
Avimers	A domains of various membrane receptors	9–18 kDa
DARPins	Ankyrin repeat motif	10–19 kDa
Fynomers	SH3 domain of Fyn	7 kDa
Kunitz domain peptides	Kunitz domains of various protease inhibitors	6 kDa
Monobodies	10th type III domain of fibronectin	10 kDa
nanoCLAMPs	Carbohydrate Binding Module 32-2 (C. *perfringens*)	16 kDa

For comparison: **aptamers are 8 – 30 kDa**

In vitro selection of proteins requires a link to the encoding sequence using:

- **Phage display**
- **Ribosome display**

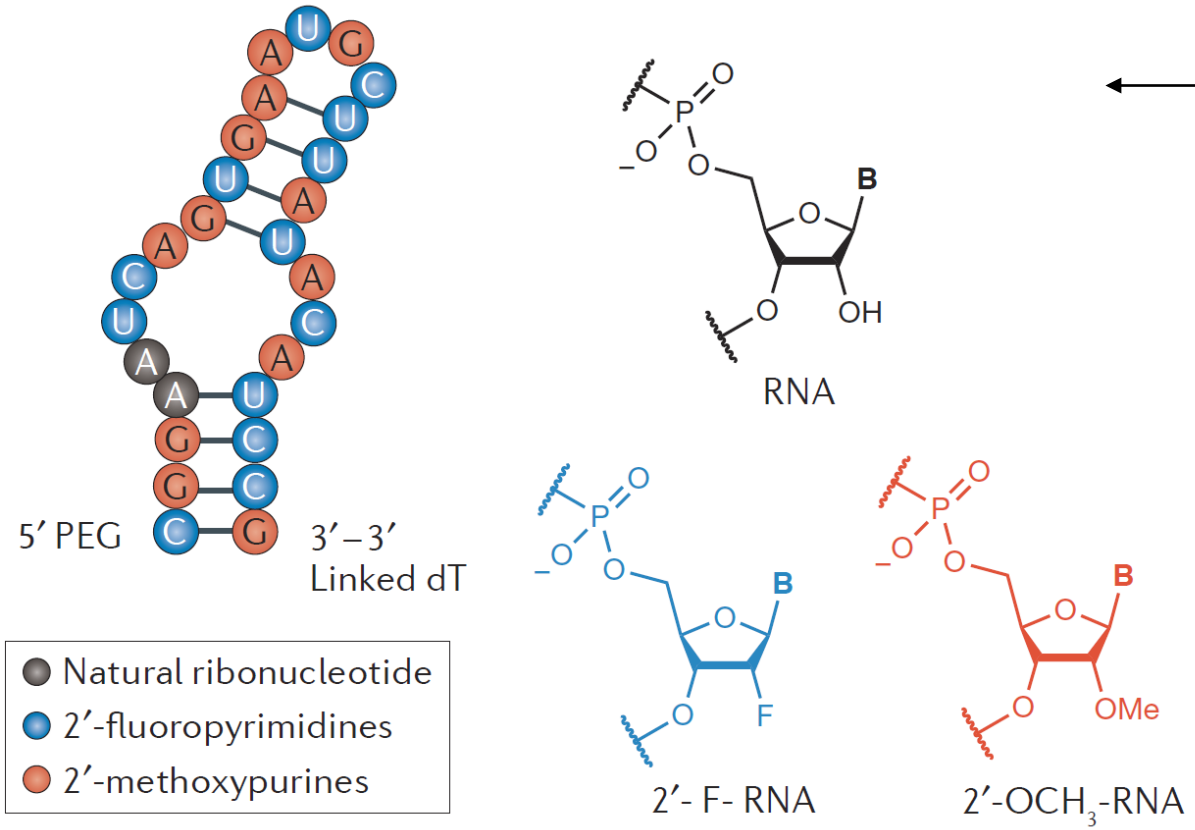
Advantages of aptamers

- Can be **chemically synthesized**
  - **Cheap**
  - **No batch-to-batch variability**
- Easy to chemically modify (fluorophores, biotin, etc.)
- Stable at room temperature
  - Refold after heat denaturation
- All technologies developed for DNA/RNA automatically apply to aptamers!
  - Next-gen sequencing, qPCR…

Disadvantages of aptamers

- **Limited diversity** of natural nucleic acids
  - For example, GFP is poorly aptagenic
- Nuclease sensitivity
  - ↓ stability in vivo

Pre- and post-SELEX modifications can increase aptamer stability



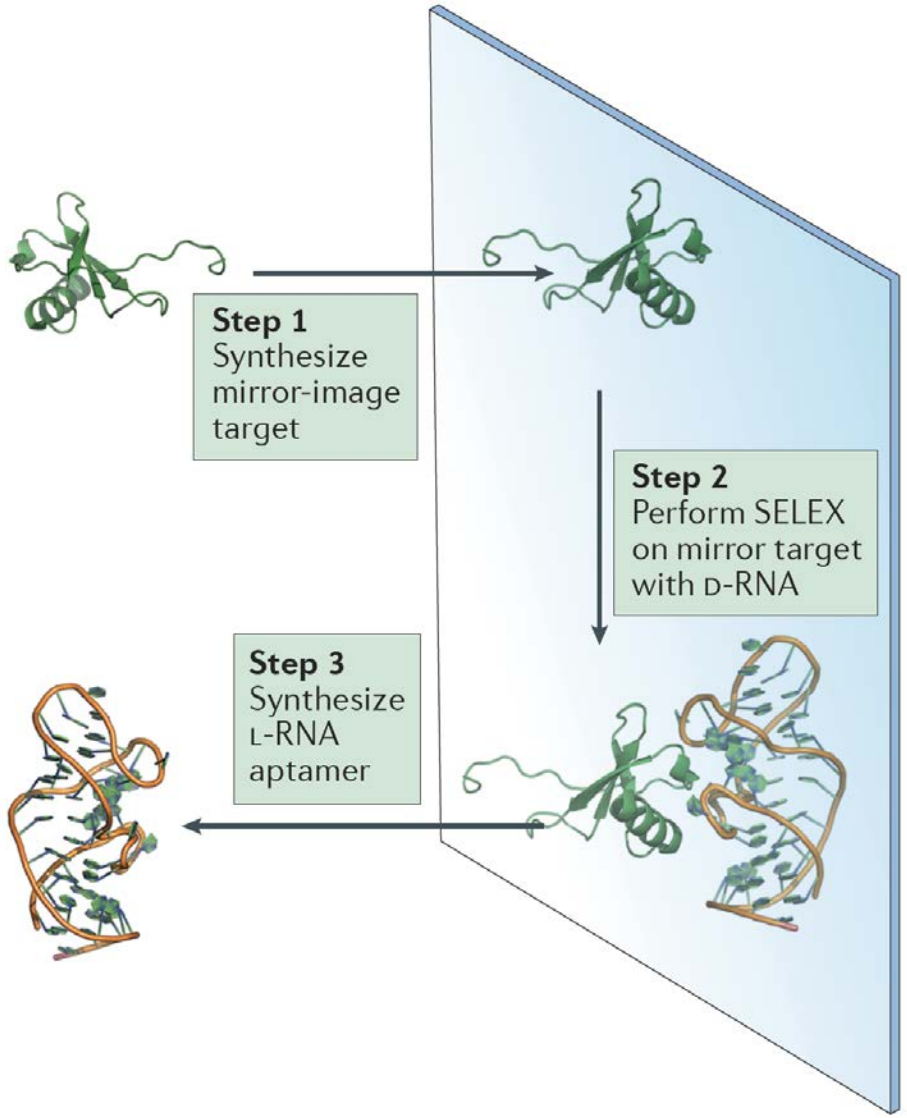
5’ polyethylene glycol (PEG)

3’ inverted nucleotide

Macugen (pegaptanib) is an anti-VEGF RNA aptamer approved for treating neovascular age-related macular degeneration (2004 in US, 2005 in Europe).

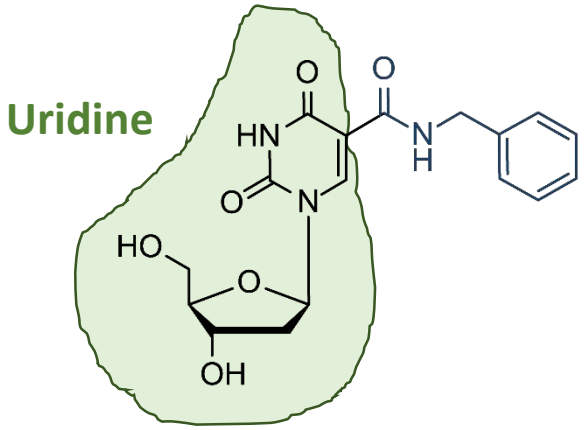
Ultimately, anti-VEGF antibodies (e.g. ranibizumab) proved more effective.

Spiegelmers made of L-enantiomer RNAs are resistant to nucleases

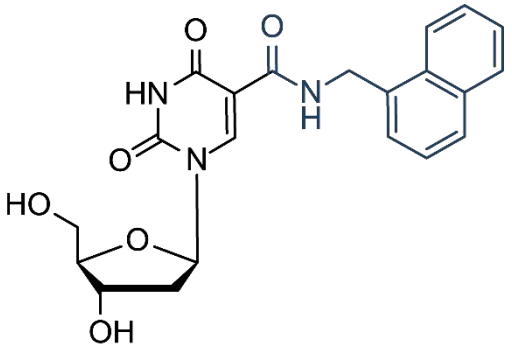


- Mirror-image proteins must be chemically synthesized from D-amino acids.
- SELEX is done with natural L-nucleic acids.
- In the future, mirror-image polymerases may allow SELEX with R-nucleic acids.
- Xenonucleic acids may offer another futuristic solution.

SOMAmers (Slow Off-rate Modified Aptamers) are chemically modified aptamers commercialized by SomaLogic

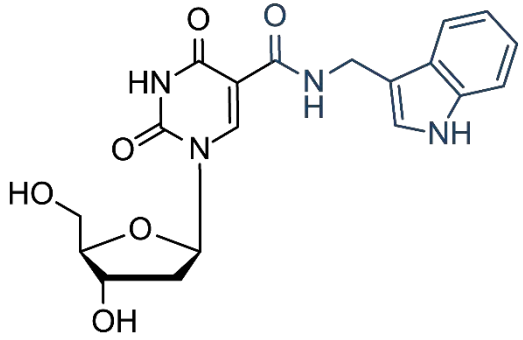


benzyl

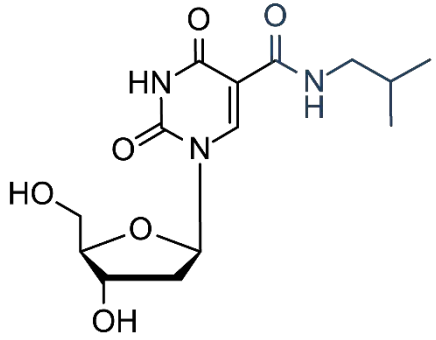


naphthyl

Hydrophobic side chains increase the chemical "possibility space"



tryptamino



isobutyl

nature chemical biology

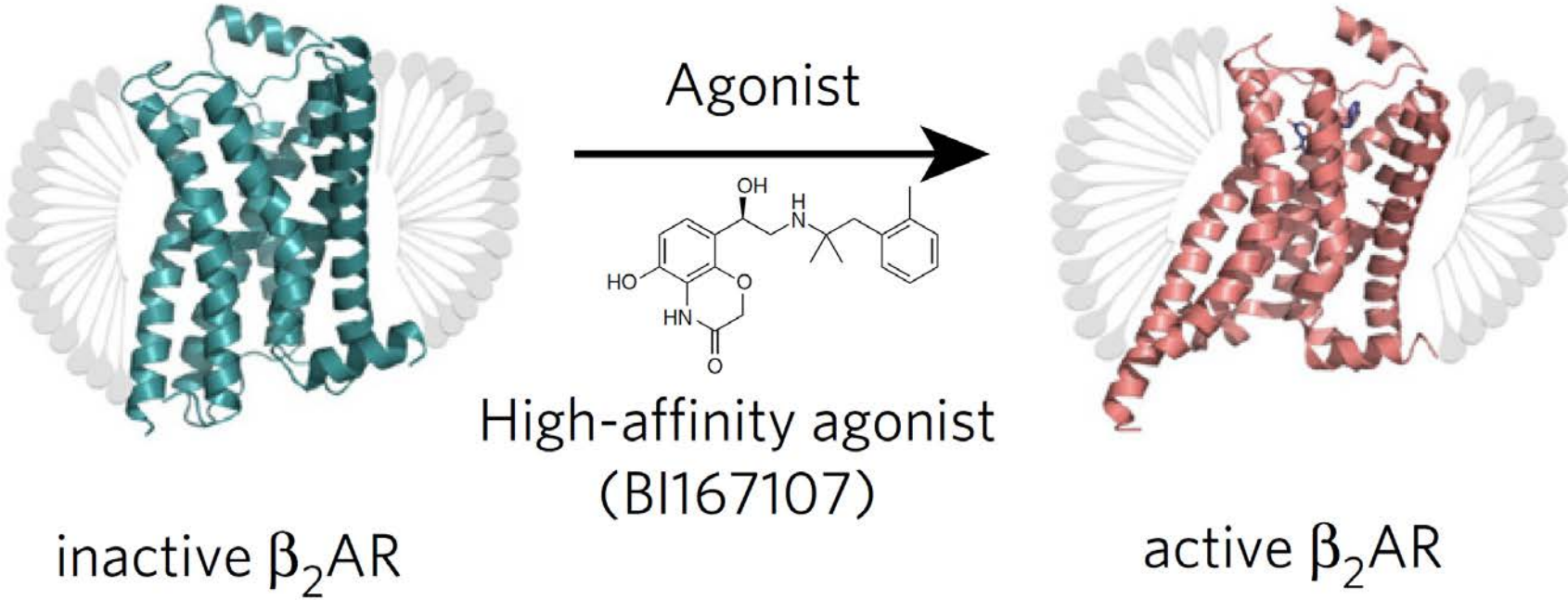
ARTICLE

PUBLISHED ONLINE: 11 JULY 2016 | DOI: 10.1038/NCHEMBIO.2126

Conformationally selective RNA aptamers allosterically modulate the β_2-adrenoceptor

Alem W Kahsai[1], James W Wisler[1], Jungmin Lee[1,2], Seungkirl Ahn[1], Thomas J Cahill III[1,3], S Moses Dennison[4], Dean P Staus[1], Alex R B Thomsen[1], Kara M Anasti[4], Biswaranjan Pani[1], Laura M Wingler[1], Hemant Desai[5], Kristin M Bompiani[6–8], Ryan T Strachan[9], Xiaoxia Qin[10], S Munir Alam[4], Bruce A Sullenger[6,7] & Robert J Lefkowitz[1,3,11]*

Aptamers were developed either against the ligand-bound/active or unbound/inactive form of β_2-adrenoceptor



Why develop aptamers against β_2-adrenoceptor?

- GPCRs have complex conformational states
  - Multiple active and inactive conformations
  - Specific conformations → biased signalling (G protein vs. β-arrestin)
  - Aptamer allosteric modulators may be **useful drugs**

- Stabilization of the active conformation permits crystallography

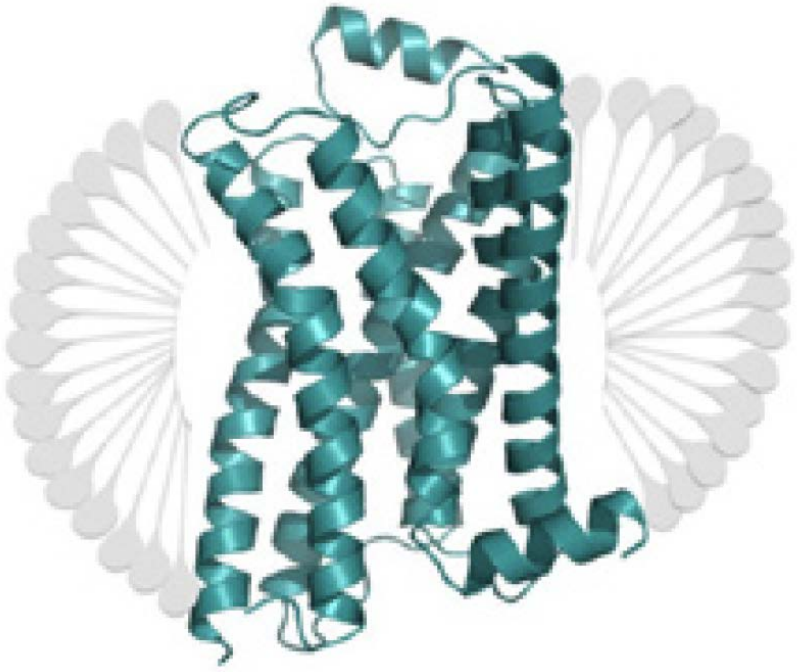
Structure of a nanobody–stabilized active state of the β_2 adrenoceptor

Søren G. F. Rasmussen[1,2]*, Hee-Jung Choi[1,3]*, Juan Jose Fung[1]*, Els Pardon[4,5], Paola Casarosa[6], Pil Seok Chae[7], Brian T. DeVree[8], Daniel M. Rosenbaum[1], Foon Sun Thian[1], Tong Sun Kobilka[1], Andreas Schnapp[6], Ingo Konetzki[6], Roger K. Sunahara[8], Samuel H. Gellman[7], Alexander Pautsch[6], Jan Steyaert[4,5], William I. Weis[1,3] & Brian K. Kobilka[1]

Nature, 2011

β_2AR was used as a well-characterized "model GPCR"

- Protein expression:
  - SF9 insect cells transfected with β_2AR baculovirus
  - N-terminal FLAG tag
- Protein purification:
  3-step affinity chromatography, with anti-FLAG antibodies and an **alprenerol** affinity purification step



inactive β_2AR

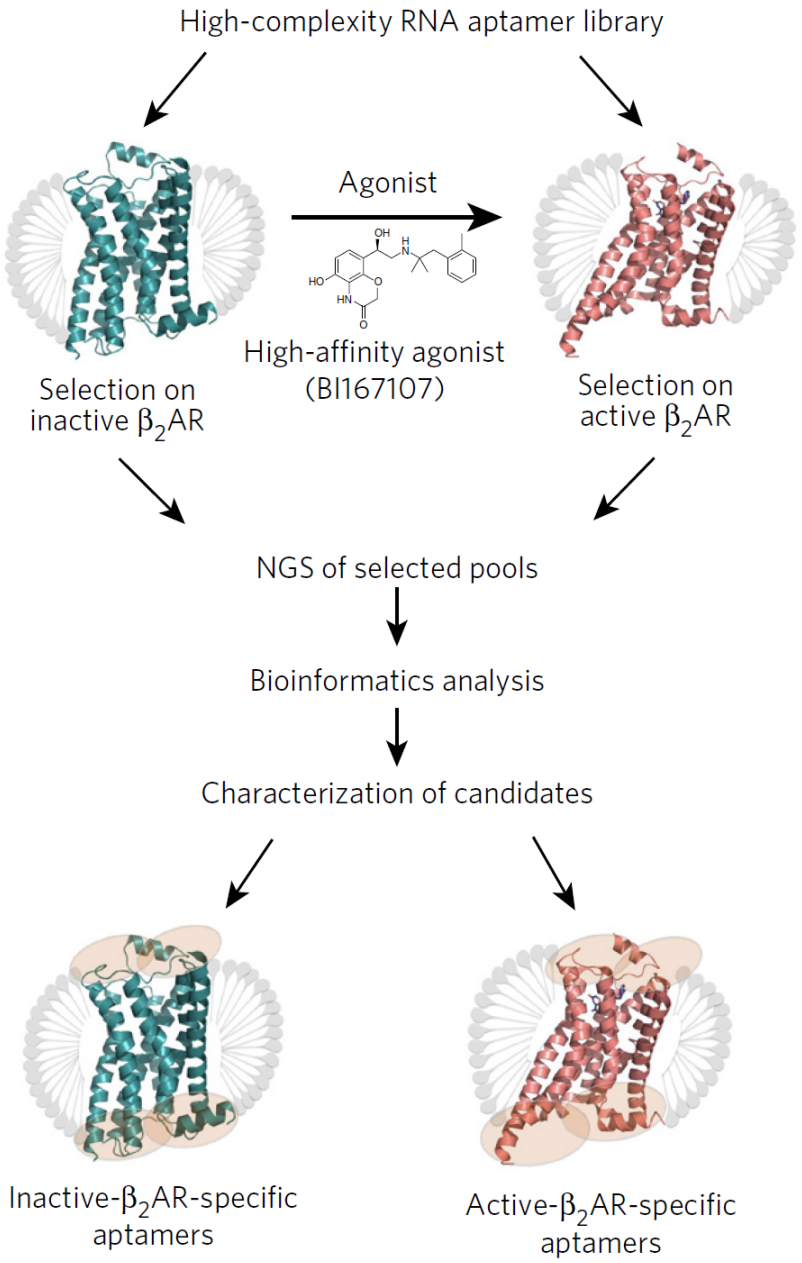
stabilized by MNG (maltose neopentyl glycol) detergent micelles

β_2AR ligand → only isolate functional receptors

Traditional SELEX was supplemented with next-generation sequencing

- 9 rounds of traditional SELEX were done (**twice**; against active and inactive β_2AR).

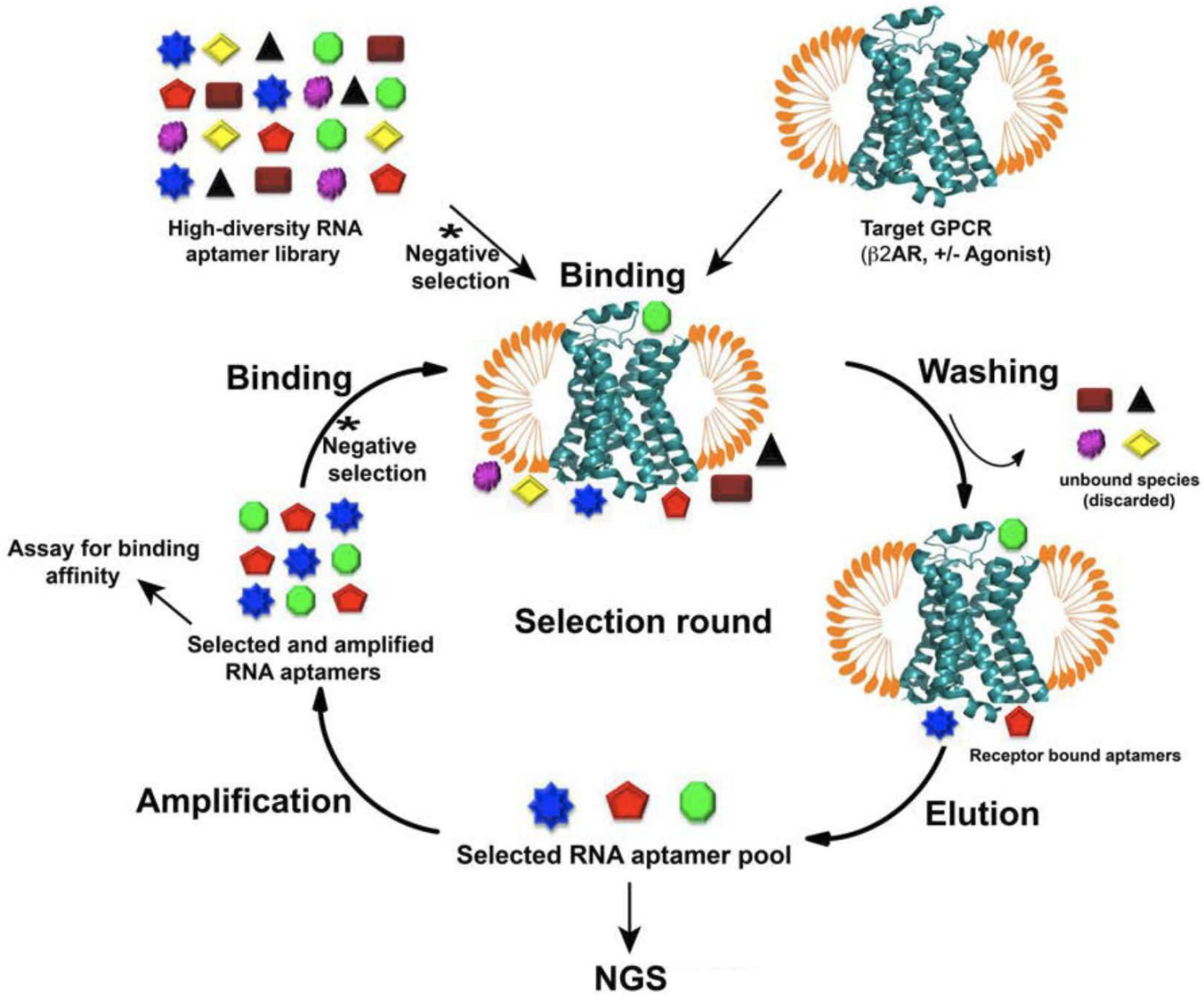
- In each round, aliquots were taken for:
  - Measurement of bulk K_d of the pool
  - Next-generation sequencing → individual species
    - → Not absolute copy number, but **enrichment from early to late** rounds!



The starting library consisted of ~10^{15} unique sequences.

Each had a 40-nucleotide random region, and flanking primer regions

- 15-base on 5’ side
- 25-base on 3’ side



The iterative SELEX process

1. A dsDNA library is **transcribed** *in vitro* to an RNA library overnight
   - Y639F mutant T7 RNA polymerase allows incorporation of 2’-F-pyrimidines

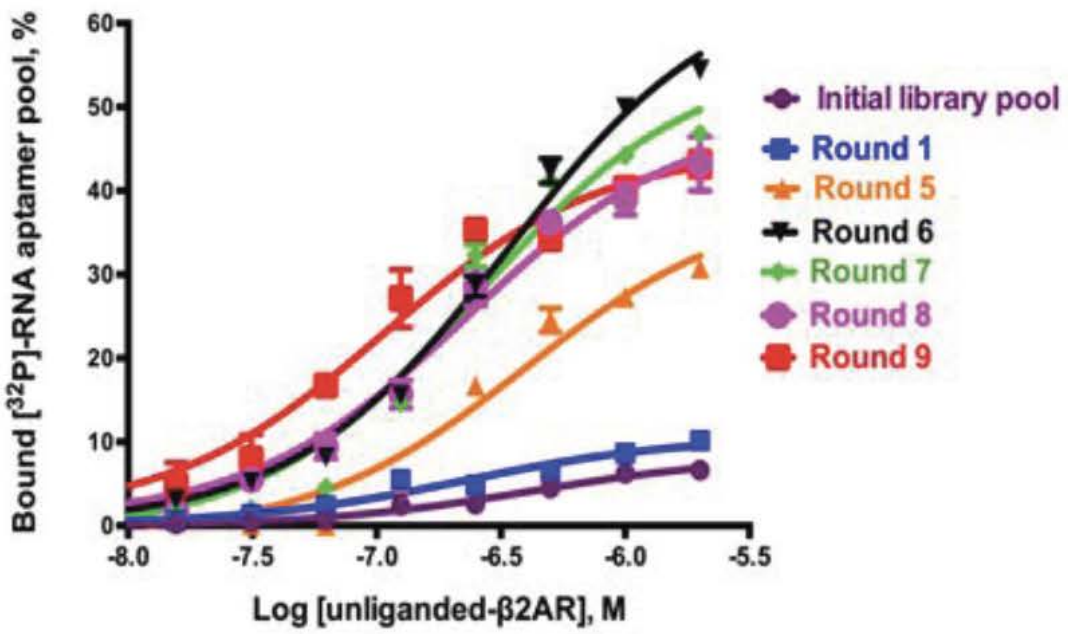
     → nuclease resistance
2. **Selection**
3. ¼ of the RNA pool is **reverse transcribed** to DNA
4. **PCR** amplification

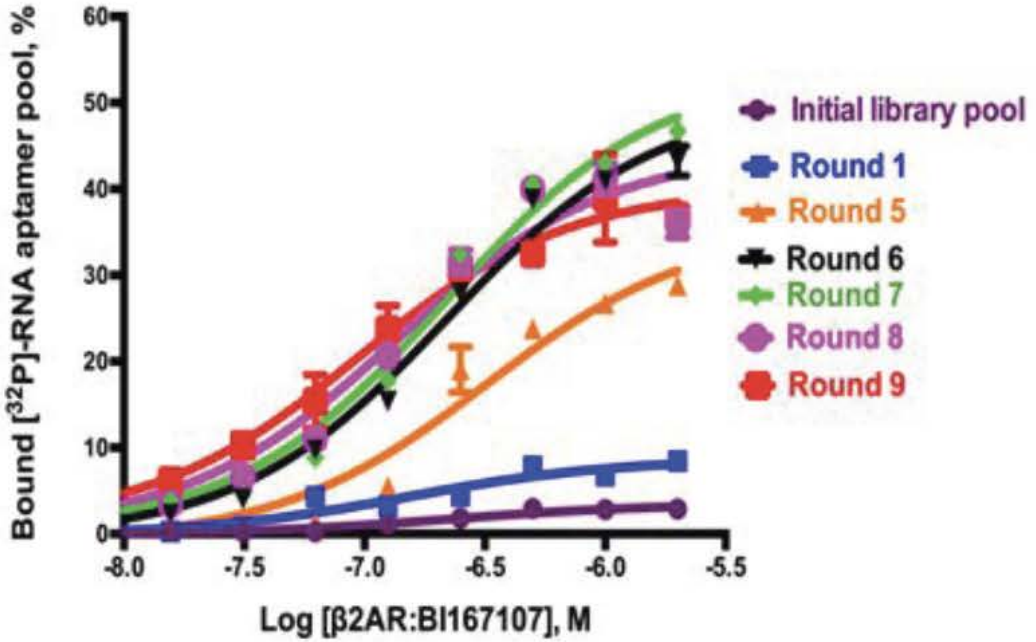
→ repeat

The selection process in detail

1. **Negative selection**: The RNA aptamer mix is incubated with the nitrocellulose matrix (without the target, in selection buffer, at 25°C).
   - Filter-binding aptamers are depleted
   - In the 5th round, negative selection was performed with a nontarget receptor (AT1aR bound to telmisartan).
2. **Positive selection**: aptamers are incubated with β_2AR (ligand-bound or unbound), then passed through nitrocellulose filters.
   - **Unbound aptamers are washed away**, aptamer-β_2AR complexes stick to the matrix.
3. Bound aptamers are extracted (incubated in phenol/chloroform/isoalyl alcohol, chloroform extracted, ethanol precipitated, and resuspended in Tris-EDTA buffer).

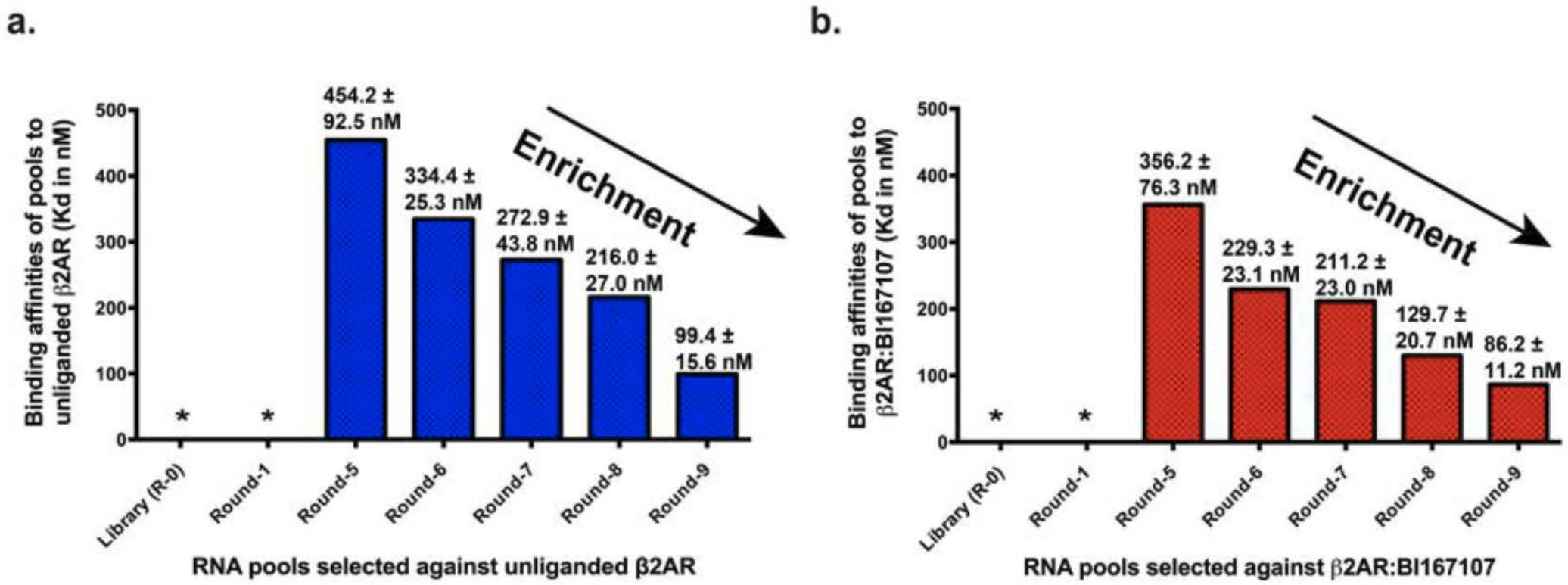
In later rounds, a larger fraction of the aptamer pool bound to the target





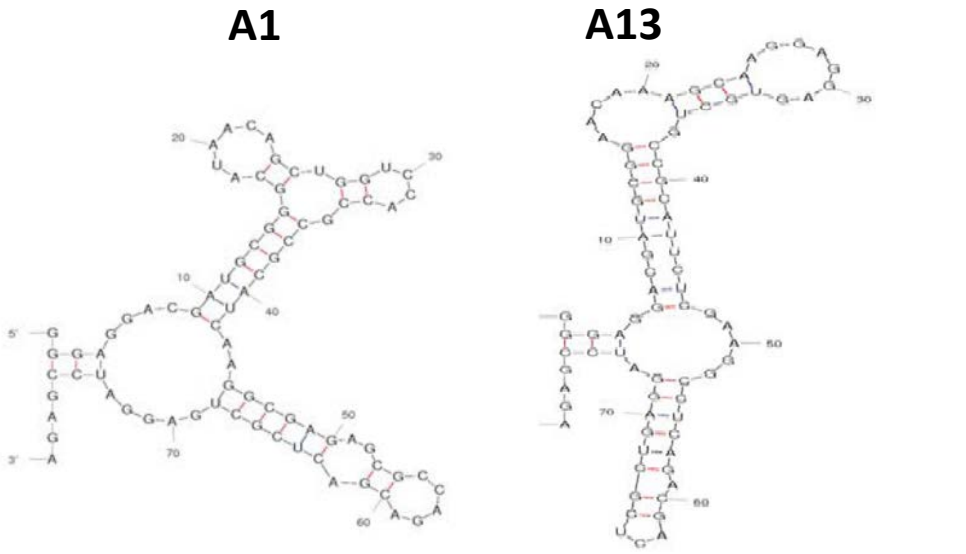
Nitrocellulose filter binding assay with 5’-^{32}P-radiolabelled aptamers

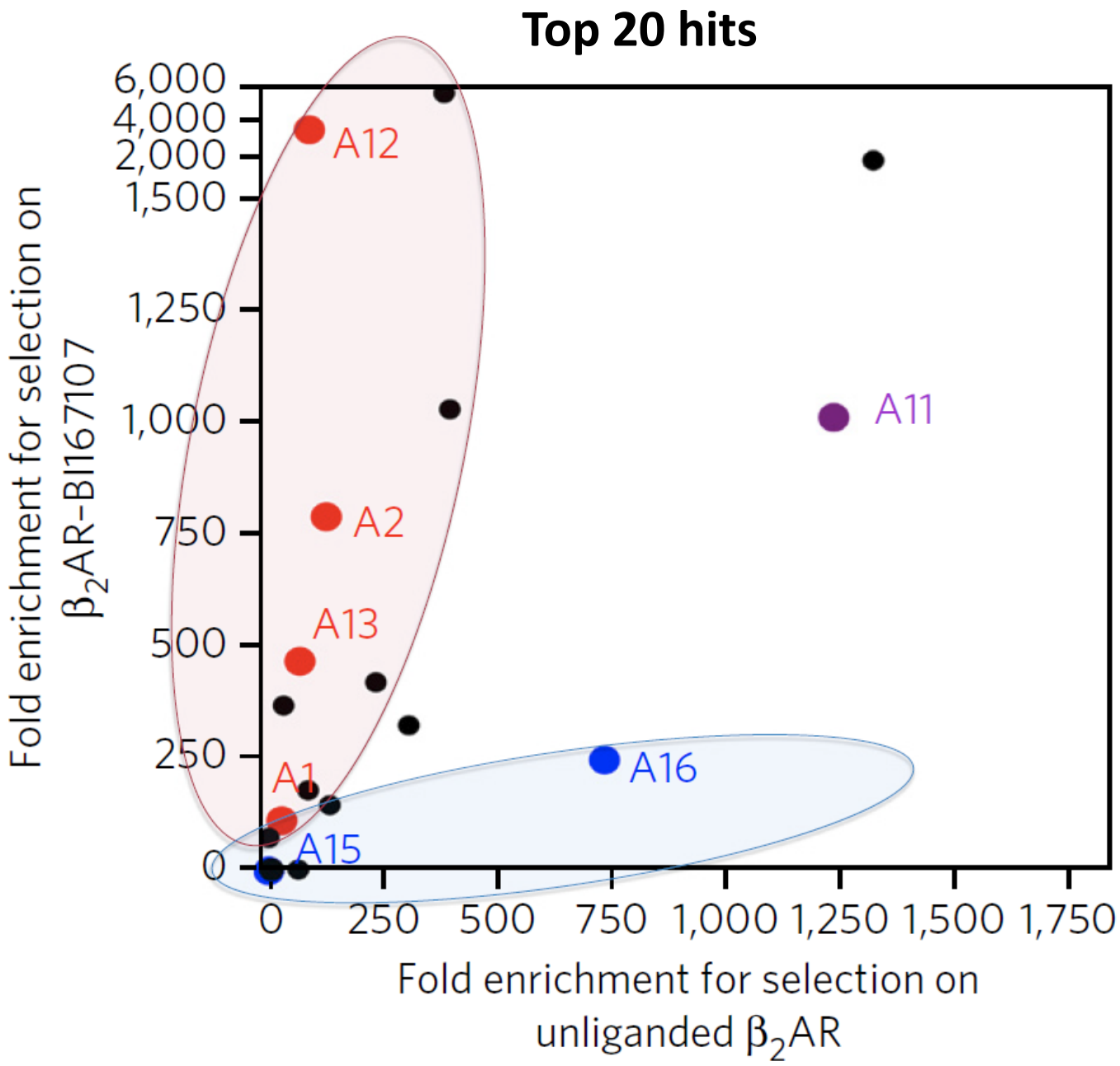
In later rounds, a larger fraction of the aptamer pool bound to the target



A list of top 20 aptamers was compiled

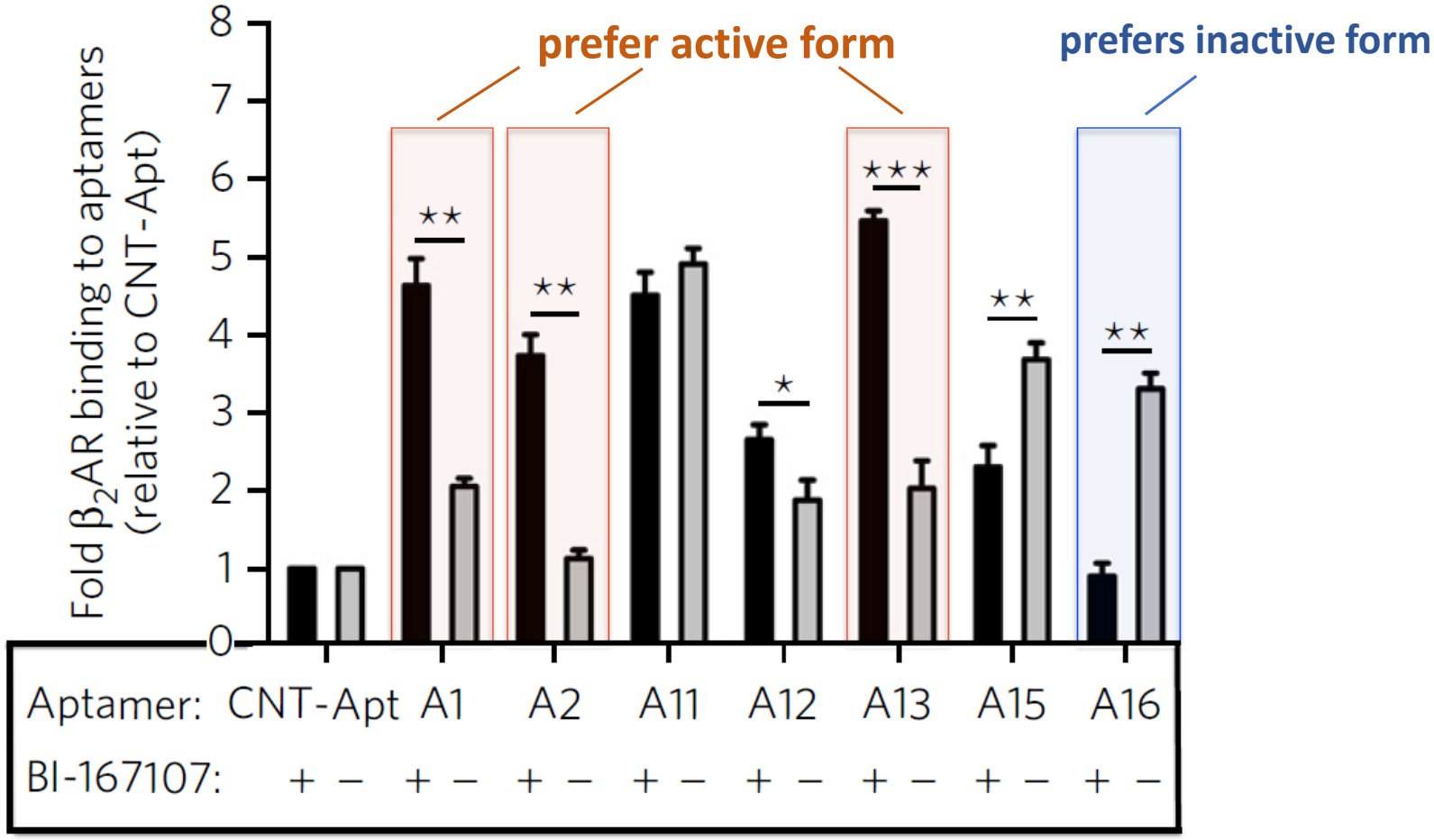
- Samples from multiple rounds were collected for multiplexed Illumina HiSeq 2000
  - PCR was done with primers containing a pool-specific barcode for multiplexing
- Criteria for picking the top species were:
  - **Fold enrichment** in later rounds (ratio of frequency in round 9 vs. round 4)
  - Rank-order copy number
  - Predicted structural stability (from *mfold* RNA folding algorithm)





Some aptamers had **high fold enrichment** (in rounds 9 vs. round 4) specifically **for bound or unbound** β_2AR.

A pulldown assay confirmed the predictions from bioinformatics



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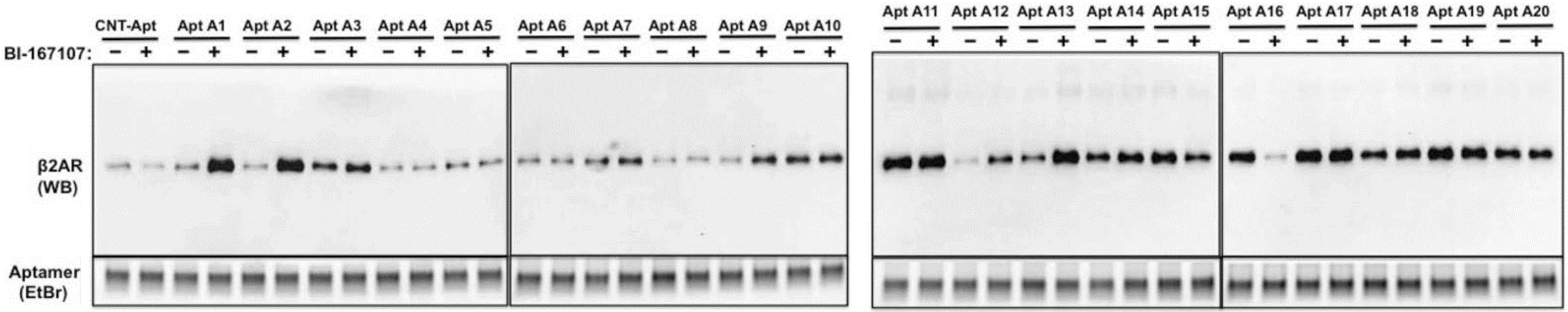
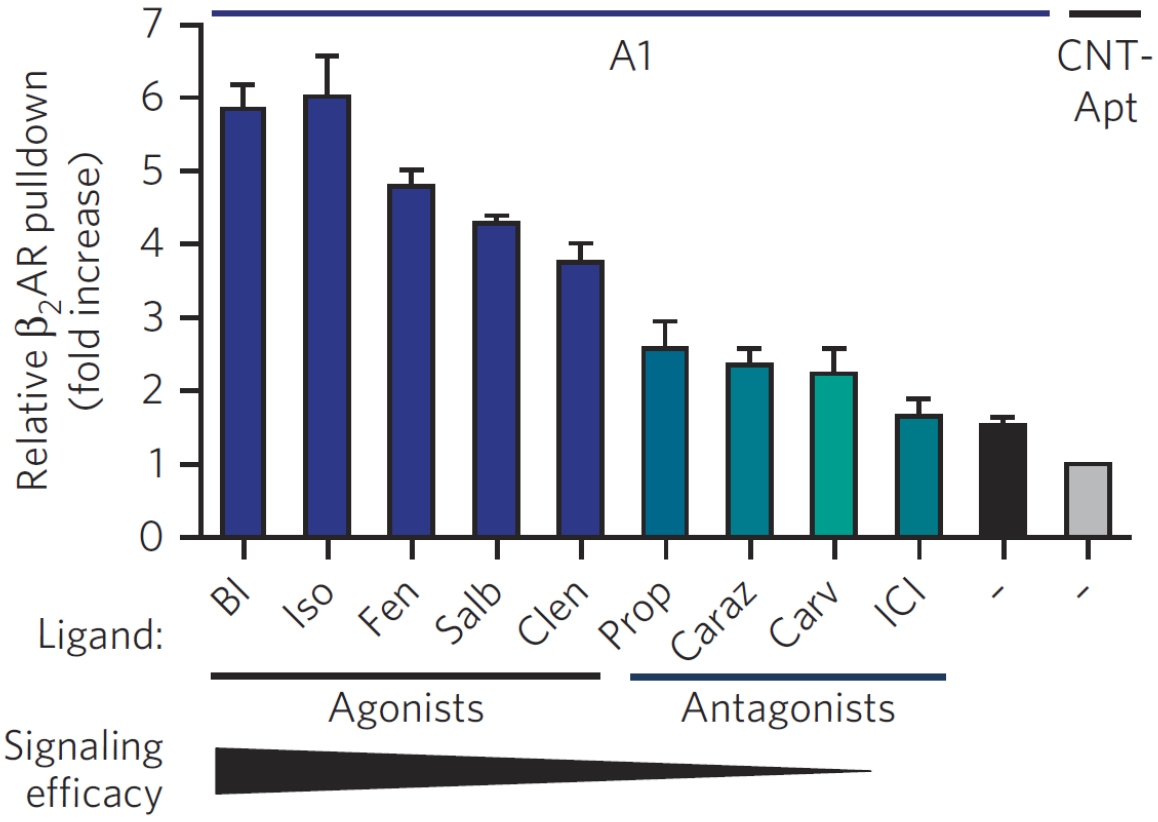
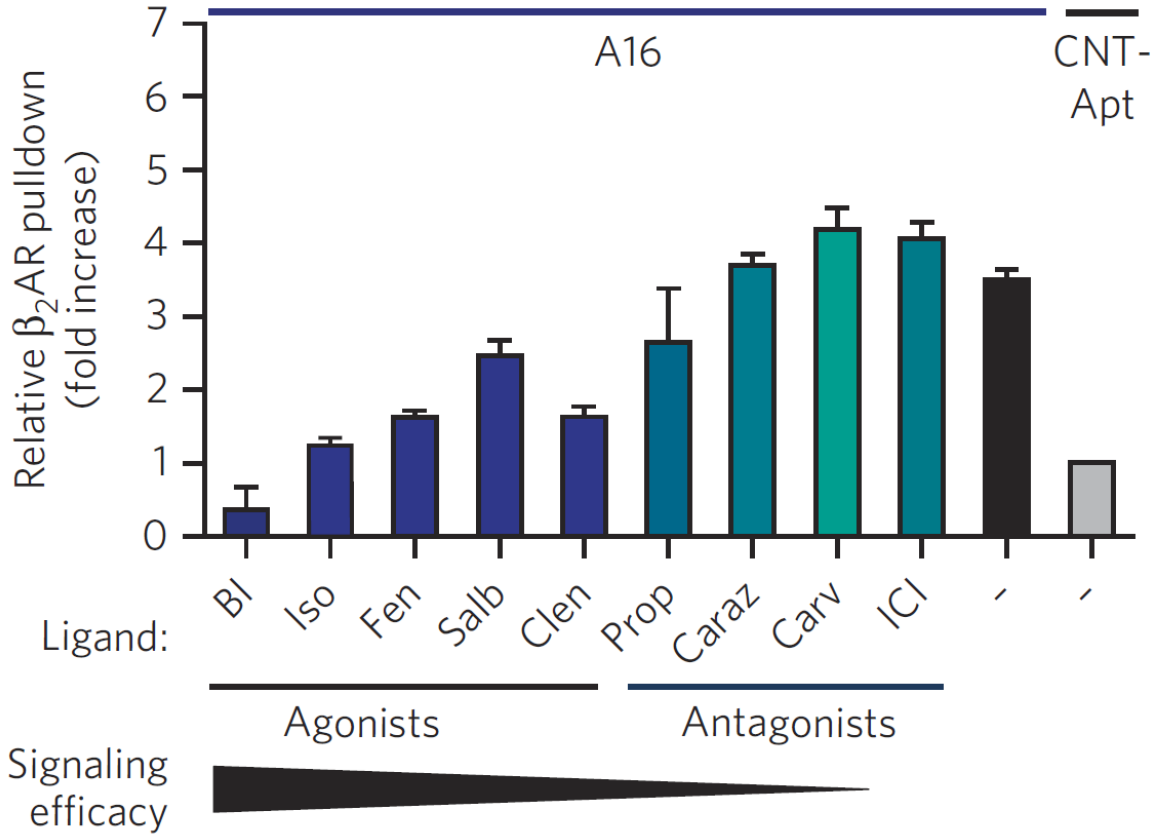


- Biotinylated aptamer species were immobilized onto NeutrAvidin beads, then incubated with β_2AR (± agonist) for 1 hour. Unbound β_2AR was washed away.
- Bound complexes were eluted. Western blot was done using anti-β_2AR antibody.

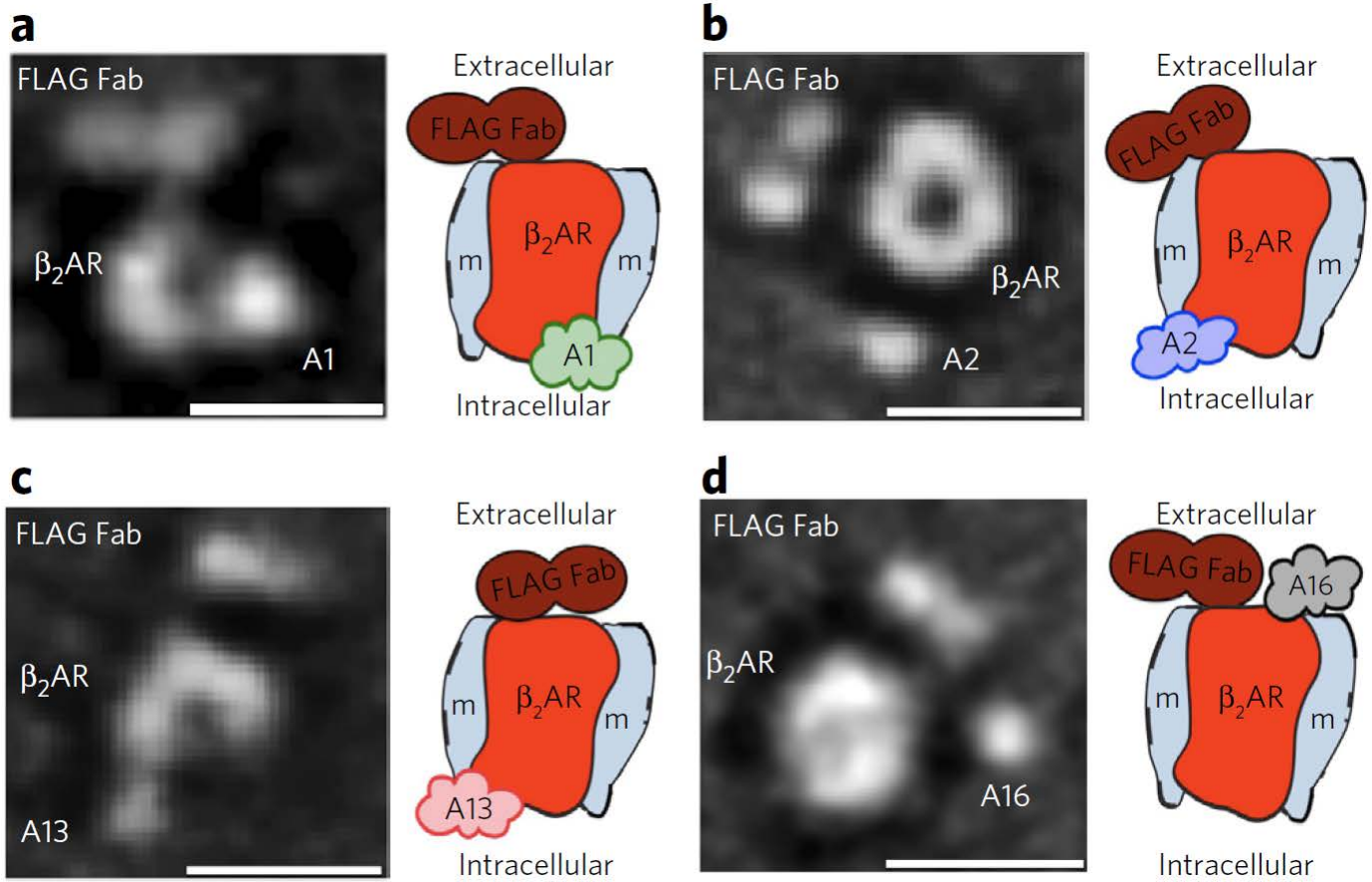
Incubated with A1 aptamer
(stabilizes **active** form)



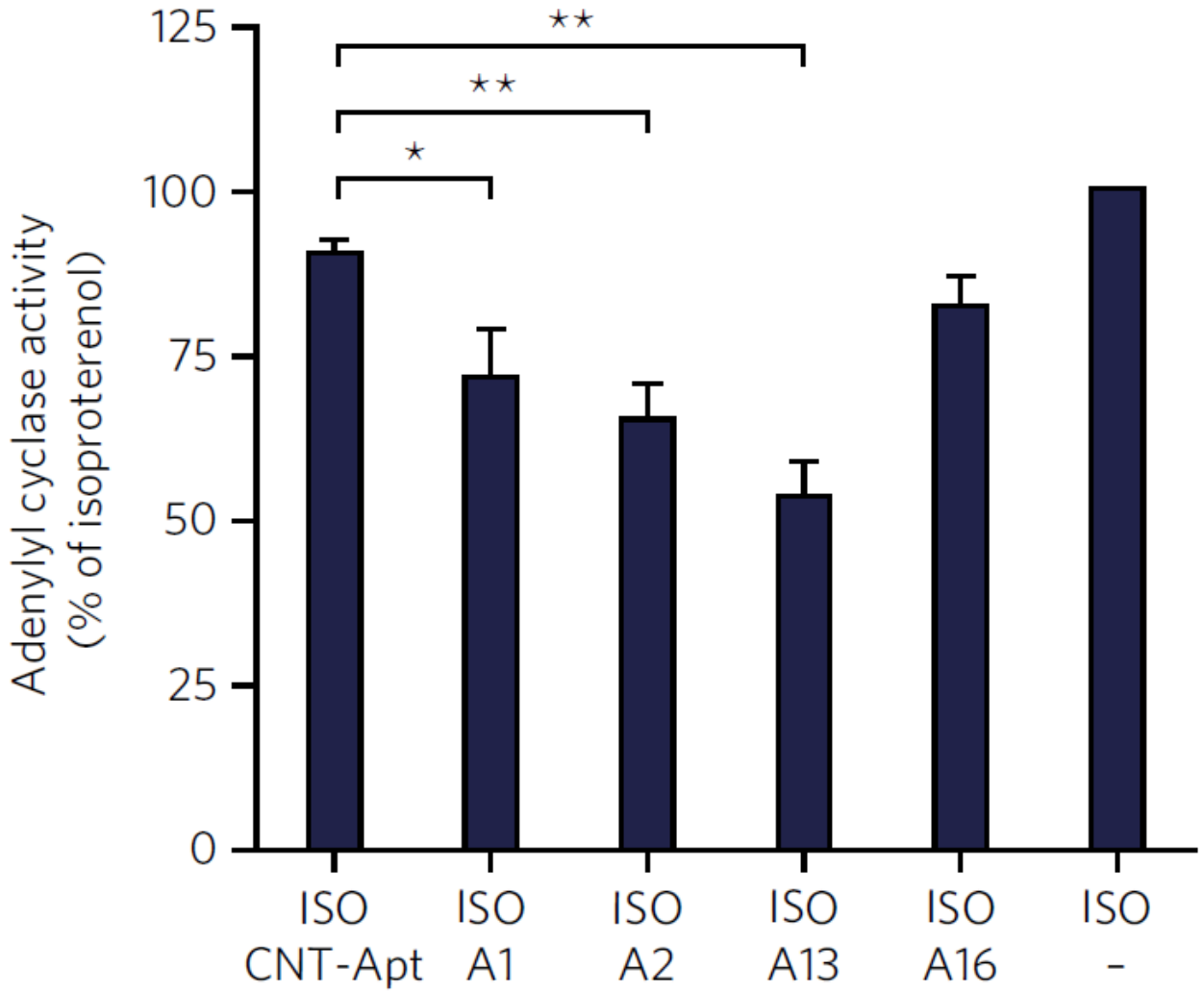
Incubated with A16 aptamer
(stabilizes **inactive** form)



Electron microscopy was used to visualize the binding locations of the aptamers (intra- versus extracellular)



cAMP assay



Paradoxically, the aptamers specific for active β_2AR actually decreased cAMP signalling.

Maybe their intracellular binding sites competed with the G protein?

Conclusions

nature chemical biology
ARTICLE
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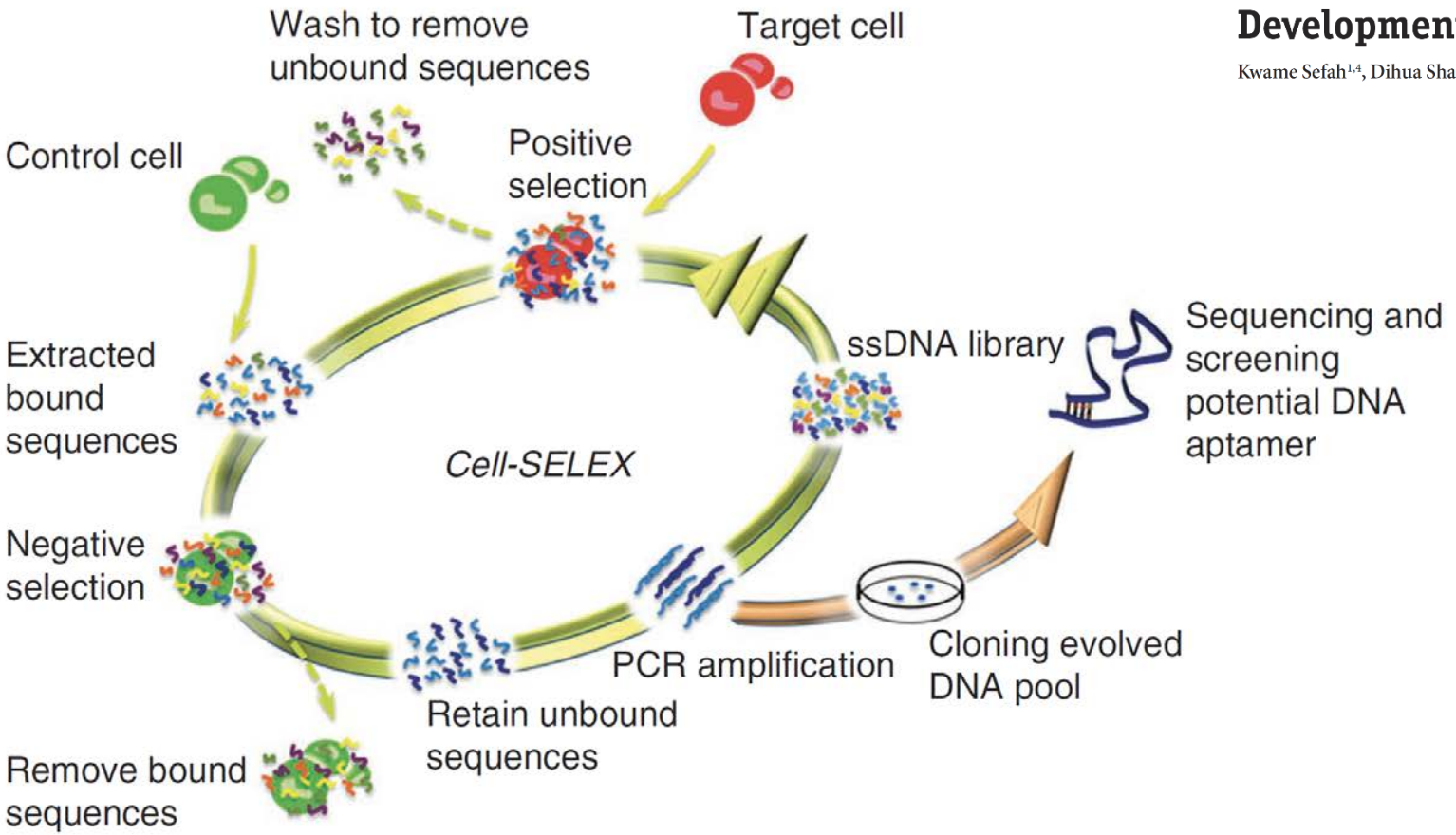
Conformationally selective RNA aptamers allosterically modulate the β_2-adrenoceptor

- It was possible to create aptamers that preferentially bound to the ligand-bound/active or inactive state of β_2AR.
  - This hold promise for aptamers as GPCR-targeting drugs.

- Next-generation sequencing was useful for identifying RNA species with the desired features.

Cell-SELEX isolates aptamers that bind to specific cells



Development of DNA aptamers using Cell-SELEX

Kwame Sefah[1,4], Dihua Shangguan[1,3], Xiangling Xiong[1], Meghan B O'Donoghue[1] & Weihong Tan[1,2,4]

Nature Protocols, 2010

Cell internalization SELEX can be used to develop aptamers that are taken up by specific cells!

PNAS

Tunable cytotoxic aptamer–drug conjugates for the treatment of prostate cancer

Bethany Powell Gray[a], Linsley Kelly[a,b], Douglas P. Ahrens[c], Ashley P. Barry[c], Christina Kratschmer[b], Matthew Levy[b,1], and Bruce A. Sullenger[a,2]

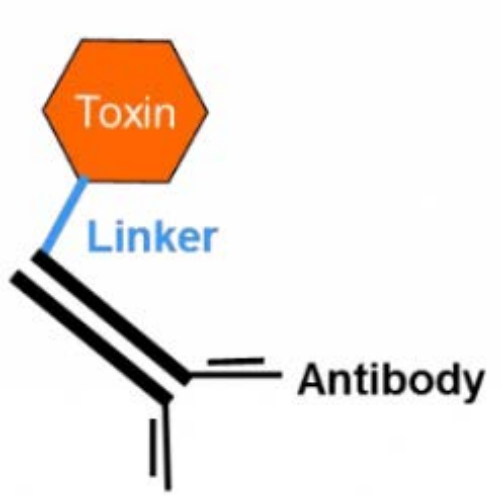
PNAS, 2018

Antibody-drug conjugates permit the use of highly toxic agents

- Antibody-drug conjugates target cancer cells specifically
  - An anti-PSMA-auristatin conjugate is in clinical trials for prostate cancer

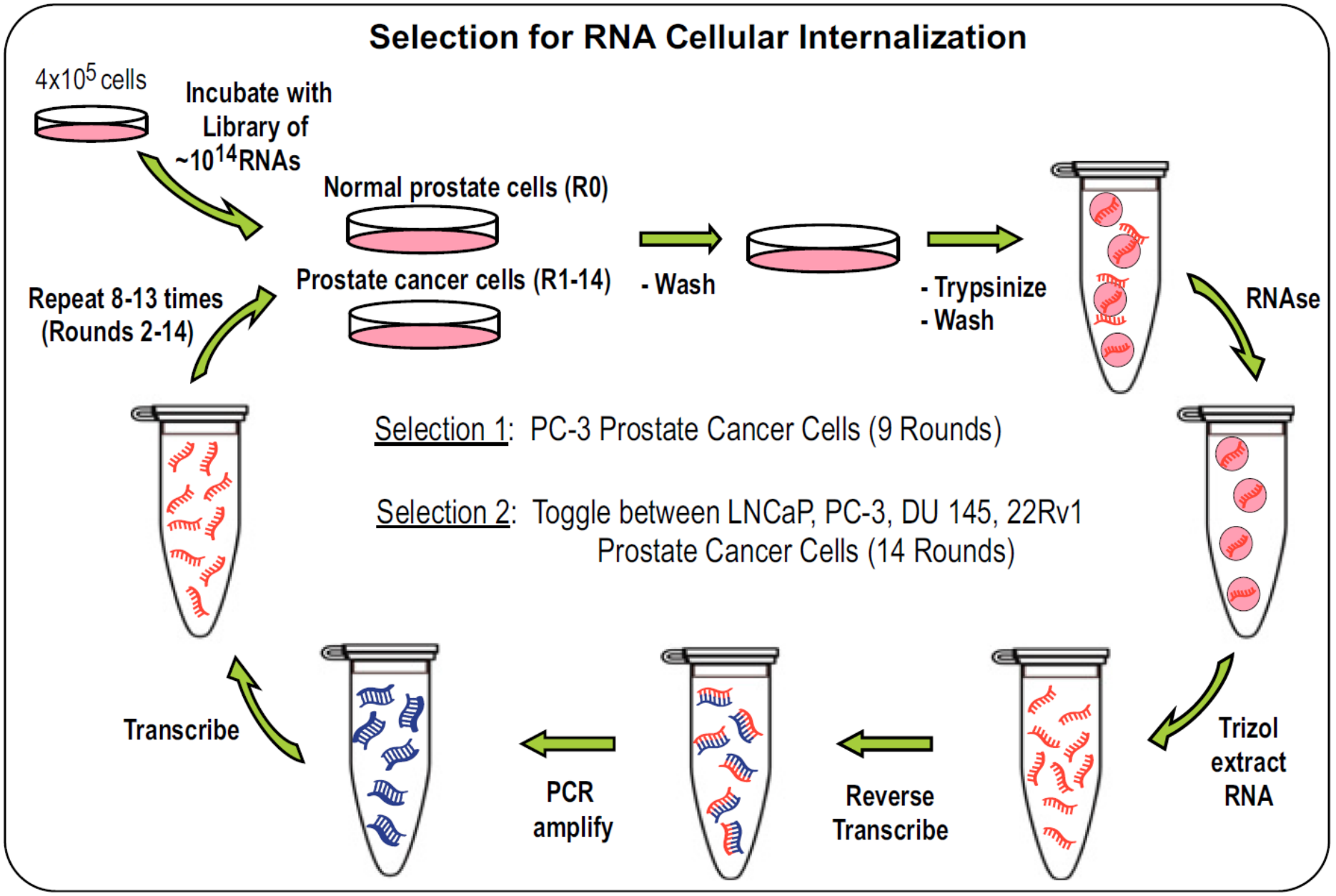
    PSMA = Prostate-specific membrane antigen

    auristatin = antimitotic agent, binds to tubulin



- Additional targets and better specificity would be desirable.
- Cell-SELEX can be used to design new **aptamer-drug conjugates**.

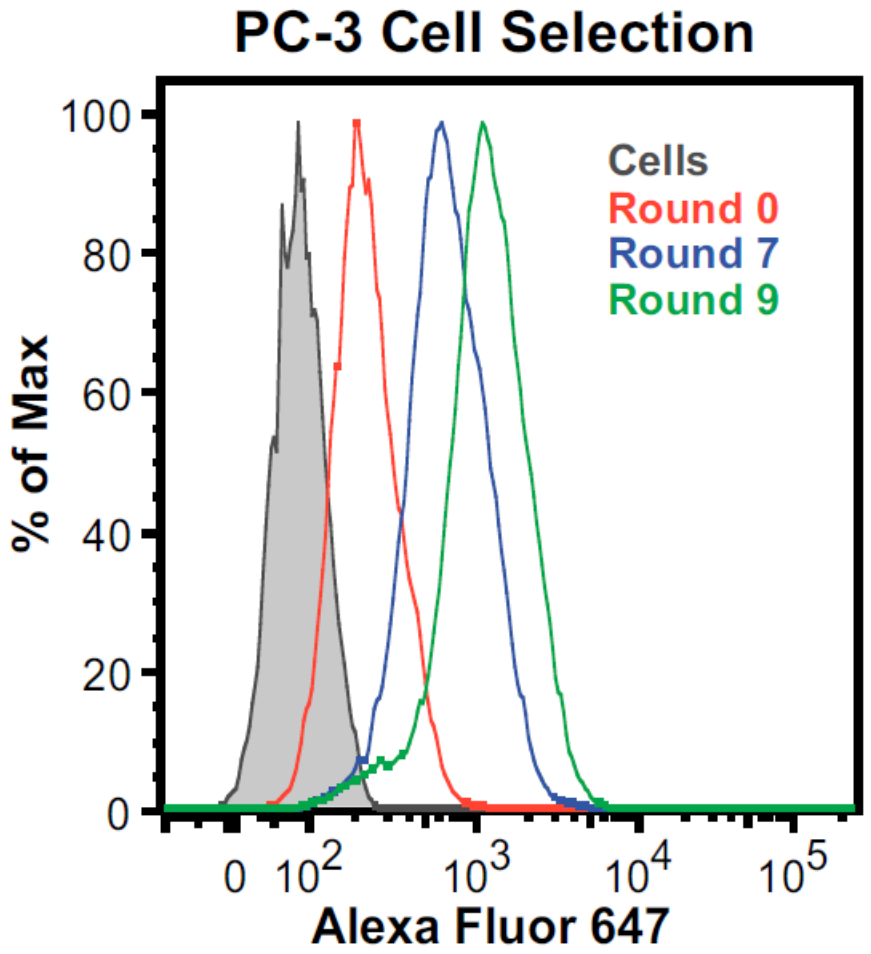
2’-F modified RNAs



Some cell lines were PSMA-negative (PC-3 & DU 145)

Flow cytometry reveals progressively better internalization of the RNA aptamer pool

RNA pools from different selection rounds were transcribed with a 3’ 22 nt tail that annealed to an Alexa Fluor 647-labeled reverse primer.



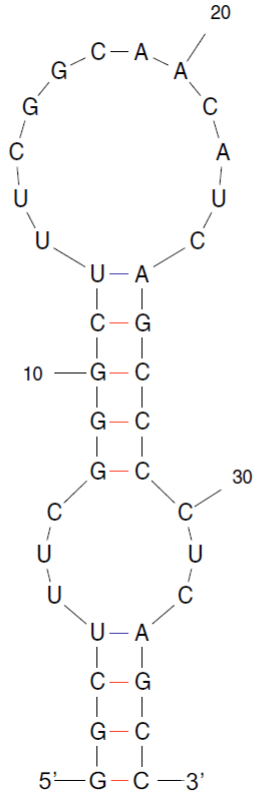
Of 12 unique RNAs that were evaluated, 3 were able to internalize.

The **E3 aptamer** showed the best internalization.

Selection	Selected Sequence ID	5' to 3' Nucleotide Sequence
PC-3 Round 9	E3	GGGAGGACGAUGCGGUACUUUCGGGCUUUCGGCAACAUCAGCCCCU CAGGACGCAAUUUCUCCUACUGGGAUAGGUGGAUUAU
	A3	GGGAGGACGAUGCGGACUUUUCGUUUCUAGACCUCUAGACCCAUCC CCGCCUUCCAUUUCUCCUACUGGGAUAGGUGGAUUAU
Toggle Round 14	E3	GGGAGGACGAUGCGGUACUUUCGGGCUUUCGGCAACAUCAGCCCCU CAGGACGCAAUUUCUCCUACUGGGAUAGGUGGAUUAU
	A3	GGGAGGACGAUGCGGACUUUUCGUUUCUAGACCUCUAGACCCAUCC CCGCCUUCCAUUUCUCCUACUGGGAUAGGUGGAUUAU
	D11	GGGAGGACGAUGCGGUCCCCGGAUUUCGGAUACGAUCCCUCAUCCC UUGACCGCAAUUUCUCCUACUGGGAUAGGUGGAUUAU

Two independent runs of SELEX both produced the E3 aptamer.

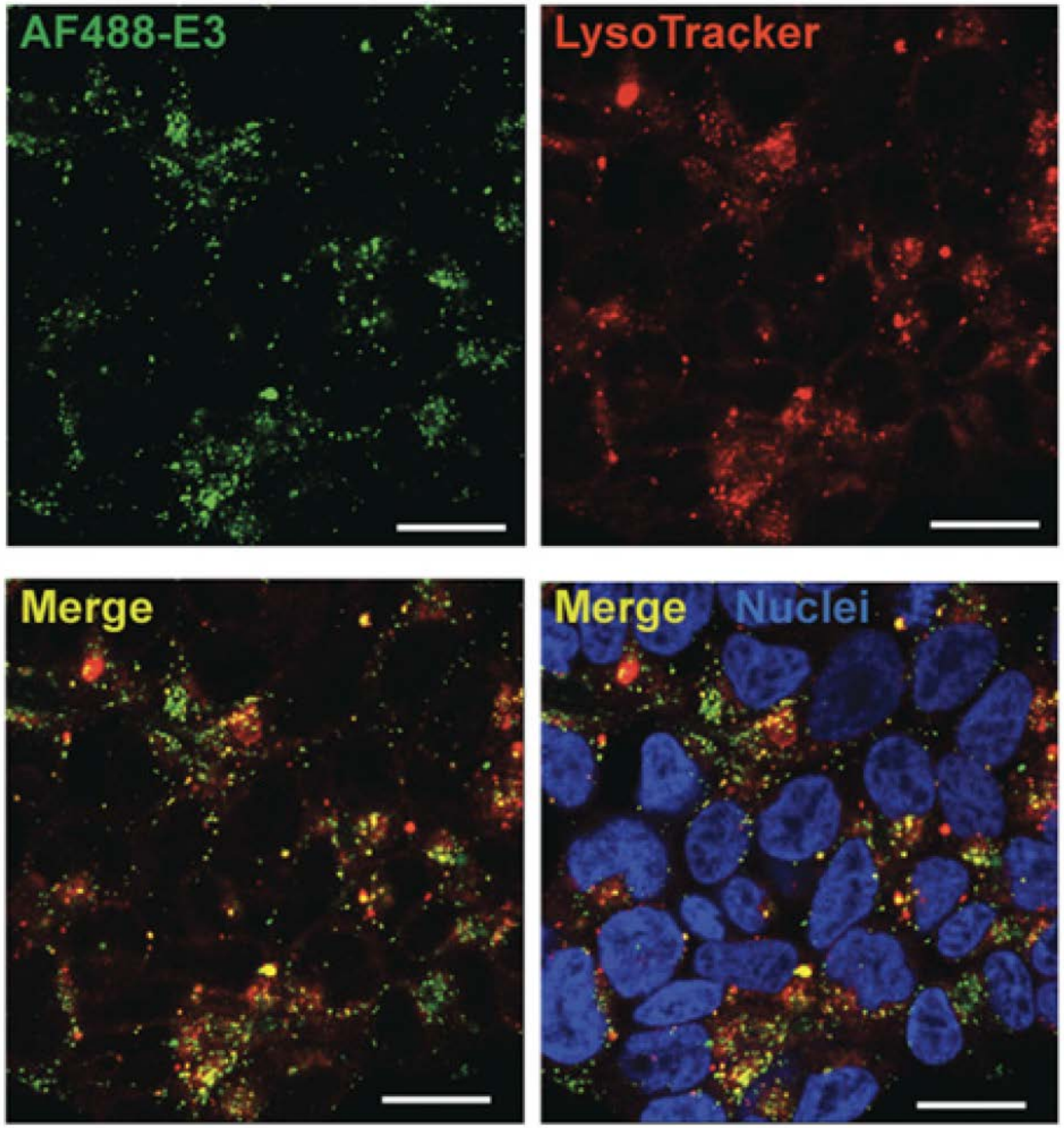
Truncated E3 Aptamer



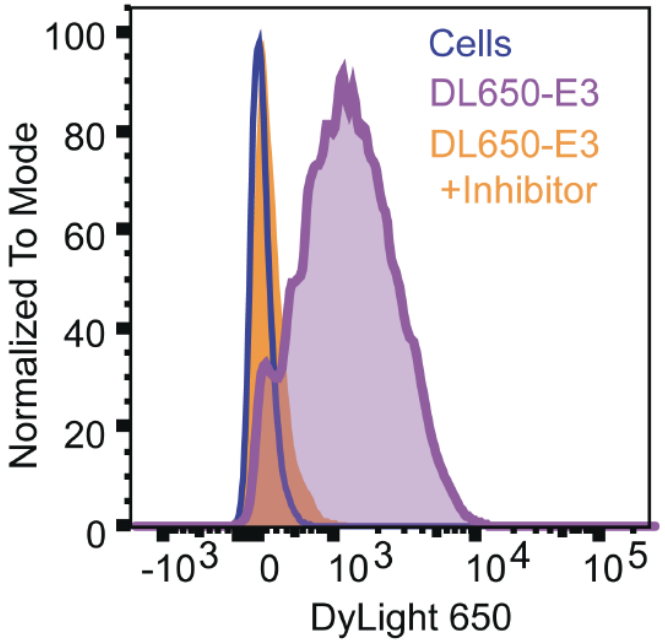
The minimal active sequence was isolated

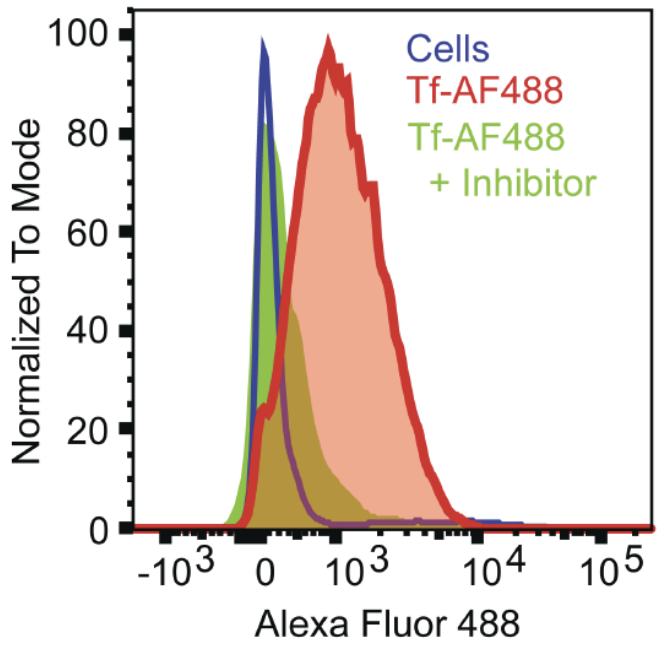
Internalized E3 seems to localize to the ribosomes

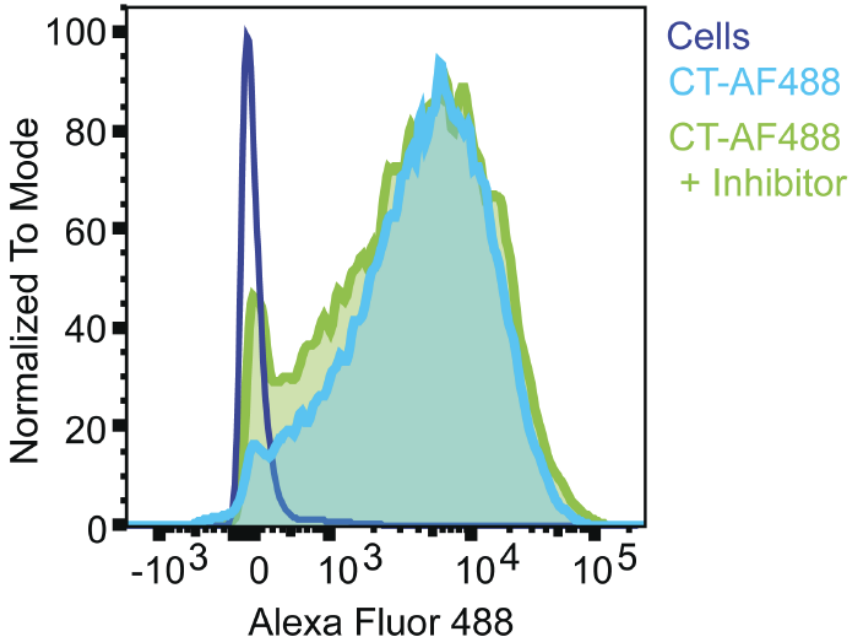
→ receptor-mediated endocytosis?



Internalization depends on clathrin-mediated endocytosis

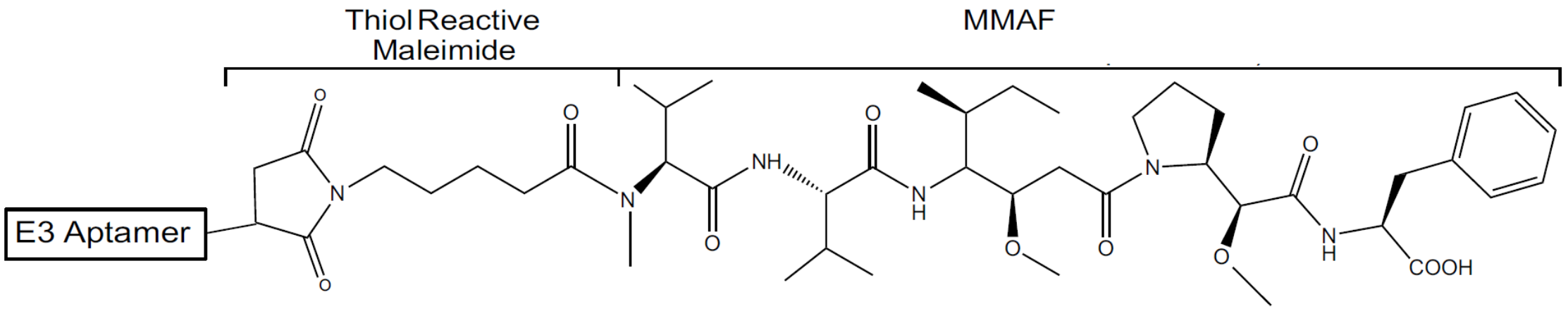






500 µM Dynasore (dynamin inhibitor)

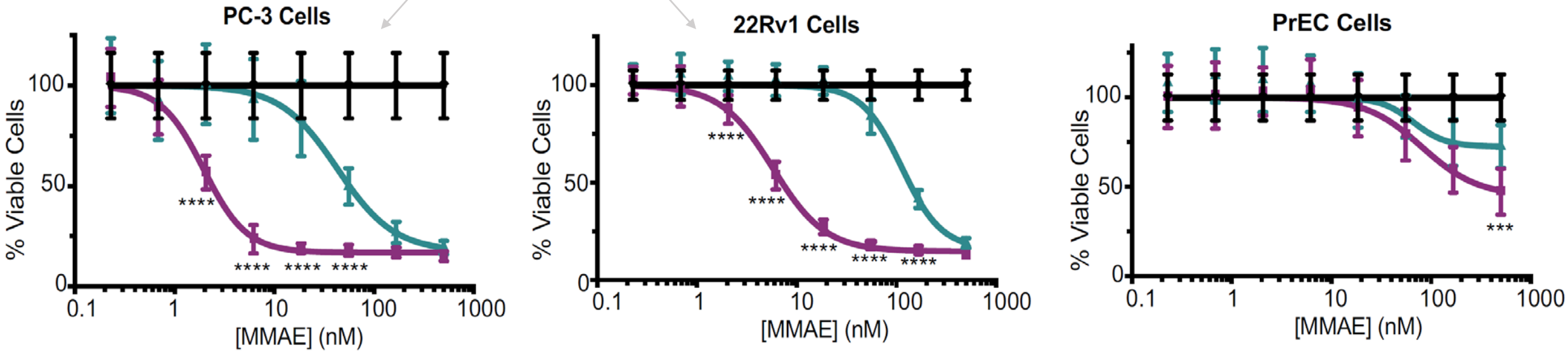
The E3 aptamer was covalently bound to monomethyl auristatin



The conjugate is toxic to prostate cancer cells, but spares normal prostate cells

prostate cancer cell line

normal prostate cell line

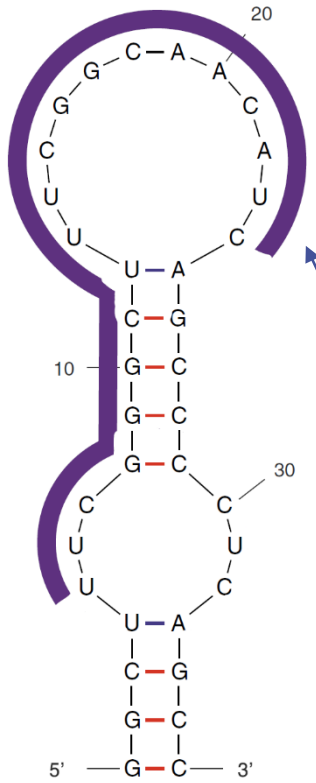


Cells Alone

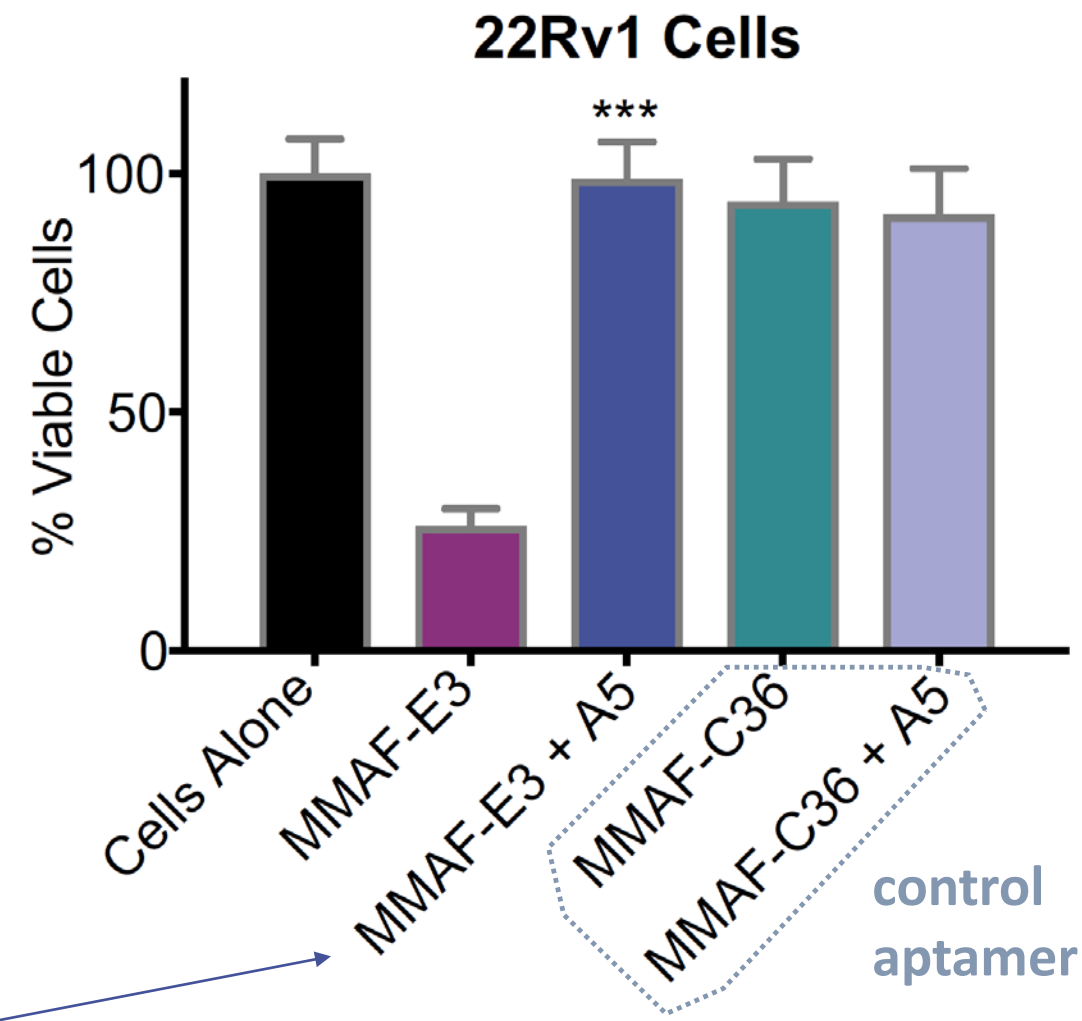
MMAE-E3

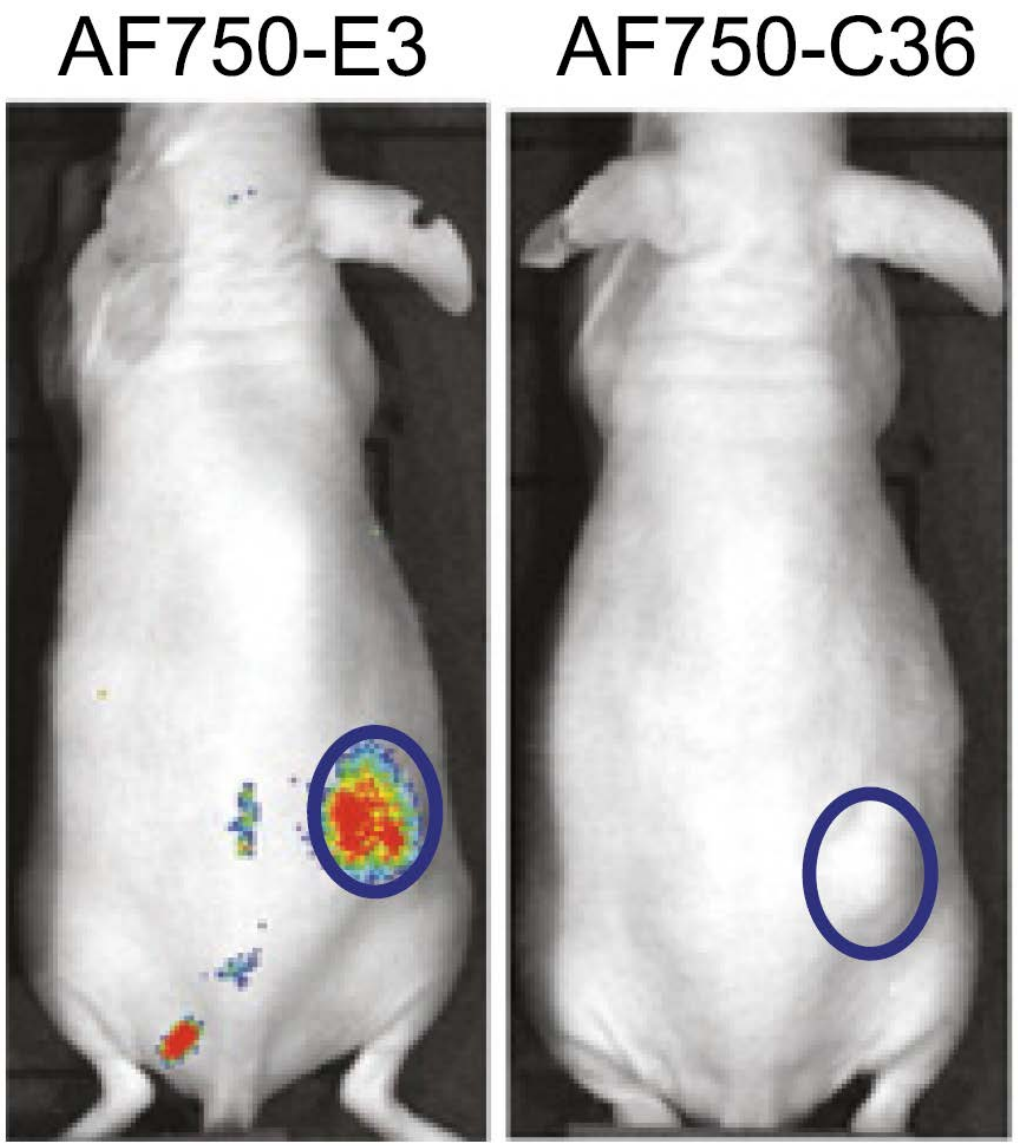
MMAE-C36 ⟶ **control aptamer**

A 20-nt RNA antidote was able to block toxicity of the conjugate *in vitro*.



antidote

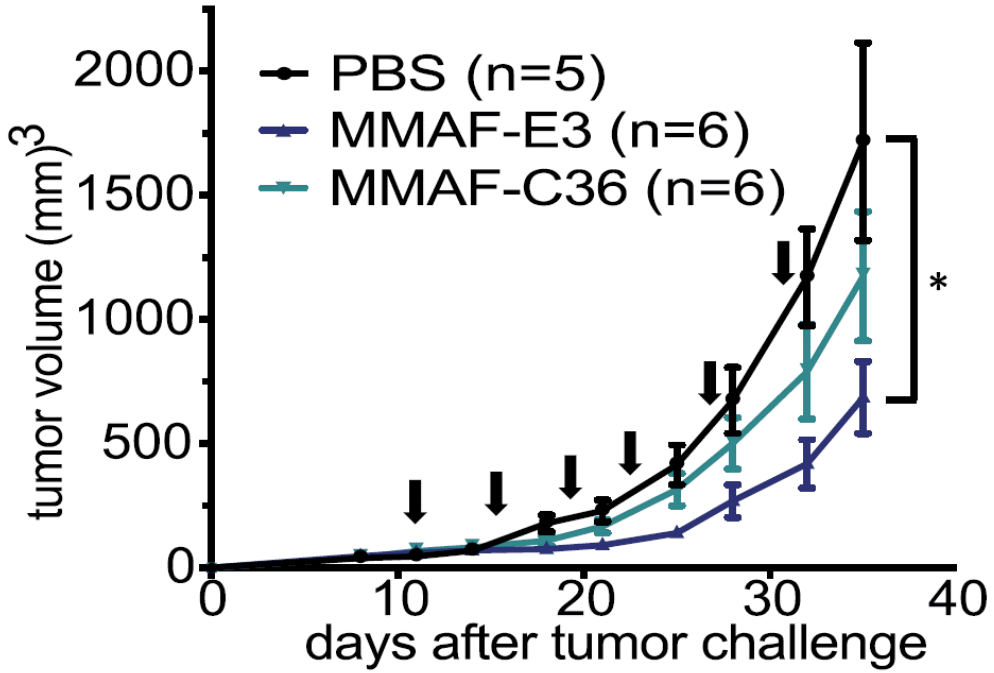




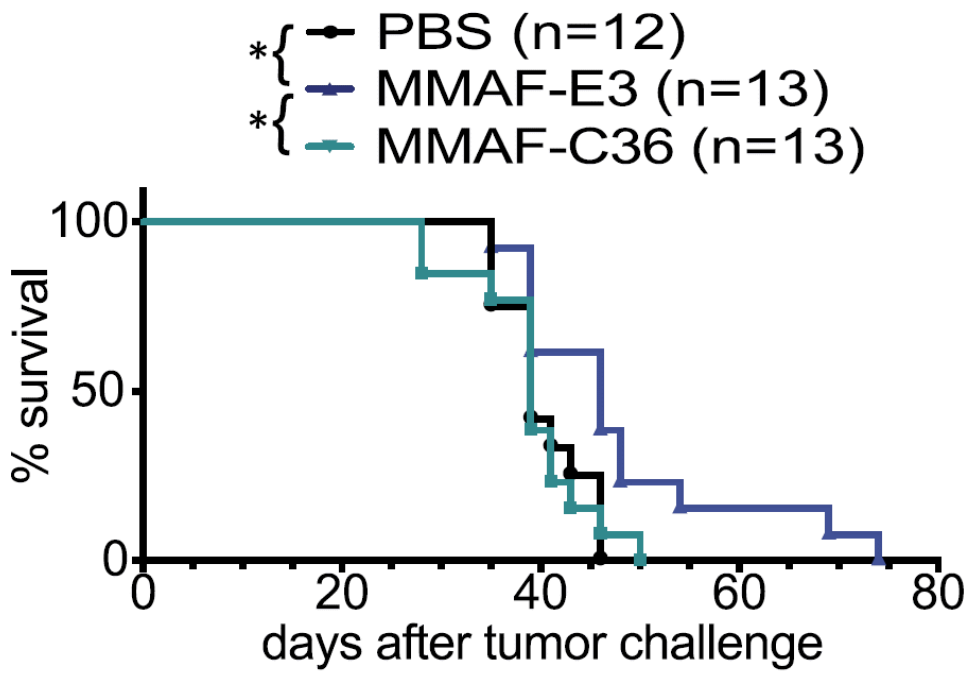
The E3 aptamer accumulates in prostate cancer xenografts in vivo.

Alexa-Fluor-750-labelled aptamer was visualized by near-infra-red epifluorescent imaging.

The conjugate showed **anti-tumor efficacy** *in vivo*



***p* = 0.03**



***p* = 0.02**

Conclusions

Tunable cytotoxic aptamer–drug conjugates for the treatment of prostate cancer

Bethany Powell Gray[a], Linsley Kelly[a,b], Douglas P. Ahrens[c], Ashley P. Barry[c], Christina Kratschmer[b], Matthew Levy[b,1], and Bruce A. Sullenger[a,2]

- A promising aptamer-drug conjugate was produced through cell-internalization SELEX.
  - Further optimisation of the half-life may increase efficacy in vivo.

- No attempt was made to find the target molecule or complex recognized by the aptamer.

ARTICLE

Genomic atlas of the human plasma proteome

Benjamin B. Sun[1,22], Joseph C. Maranville[2,20,22], James E. Peters[1,3,22], David Stacey[1], James R. Staley[1], James Blackshaw[1], Stephen Burgess[1,4], Tao Jiang[1], Ellie Paige[1,5], Praveen Surendran[1], Clare Oliver-Williams[1,6], Mihir A. Kamat[1], Bram P. Prins[1], Sheri K. Wilcox[7], Erik S. Zimmerman[7], An Chi[2], Narinder Bansal[1,8], Sarah L. Spain[9], Angela M. Wood[1], Nicholas W. Morrell[3,10], John R. Bradley[11], Nebojsa Janjic[7], David J. Roberts[12,13], Willem H. Ouwehand[3,14,15,16,17], John A. Todd[18], Nicole Soranzo[3,14,16,17], Karsten Suhre[19], Dirk S. Paul[1], Caroline S. Fox[2], Robert M. Plenge[2,20], John Danesh[1,3,16,17]*, Heiko Runz[2,21,23] & Adam S. Butterworth[1,17,23]*

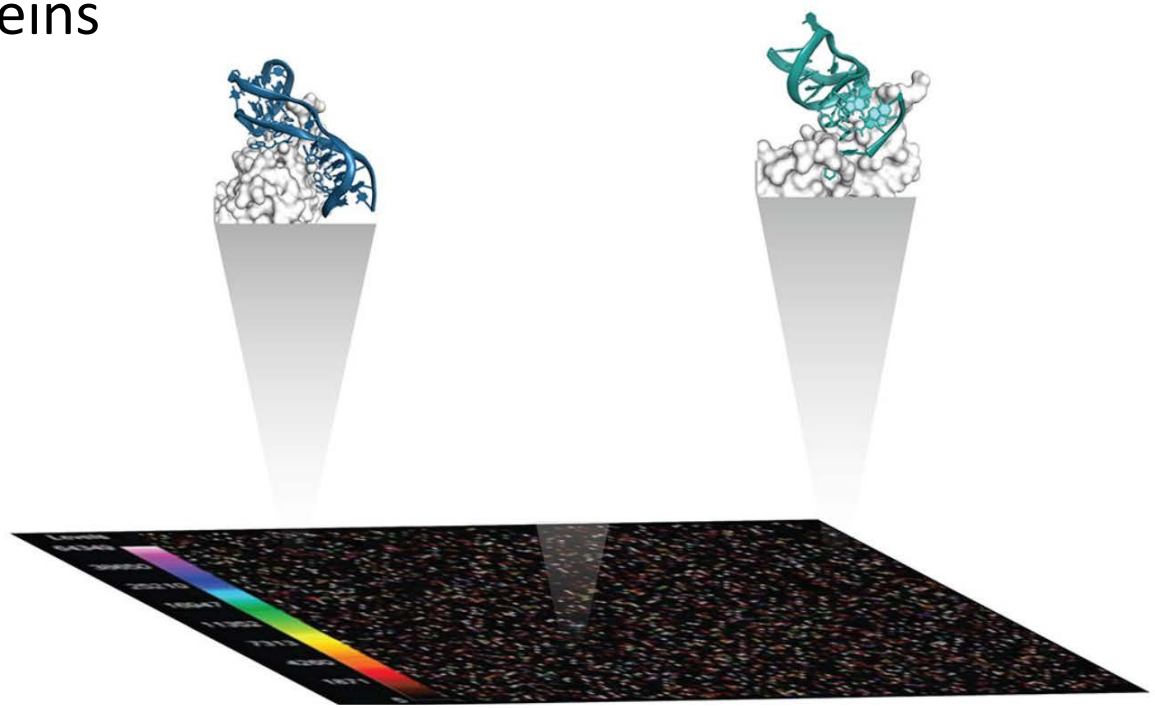
Nature, June 2018

Proteomics was done using SOMAscan®,

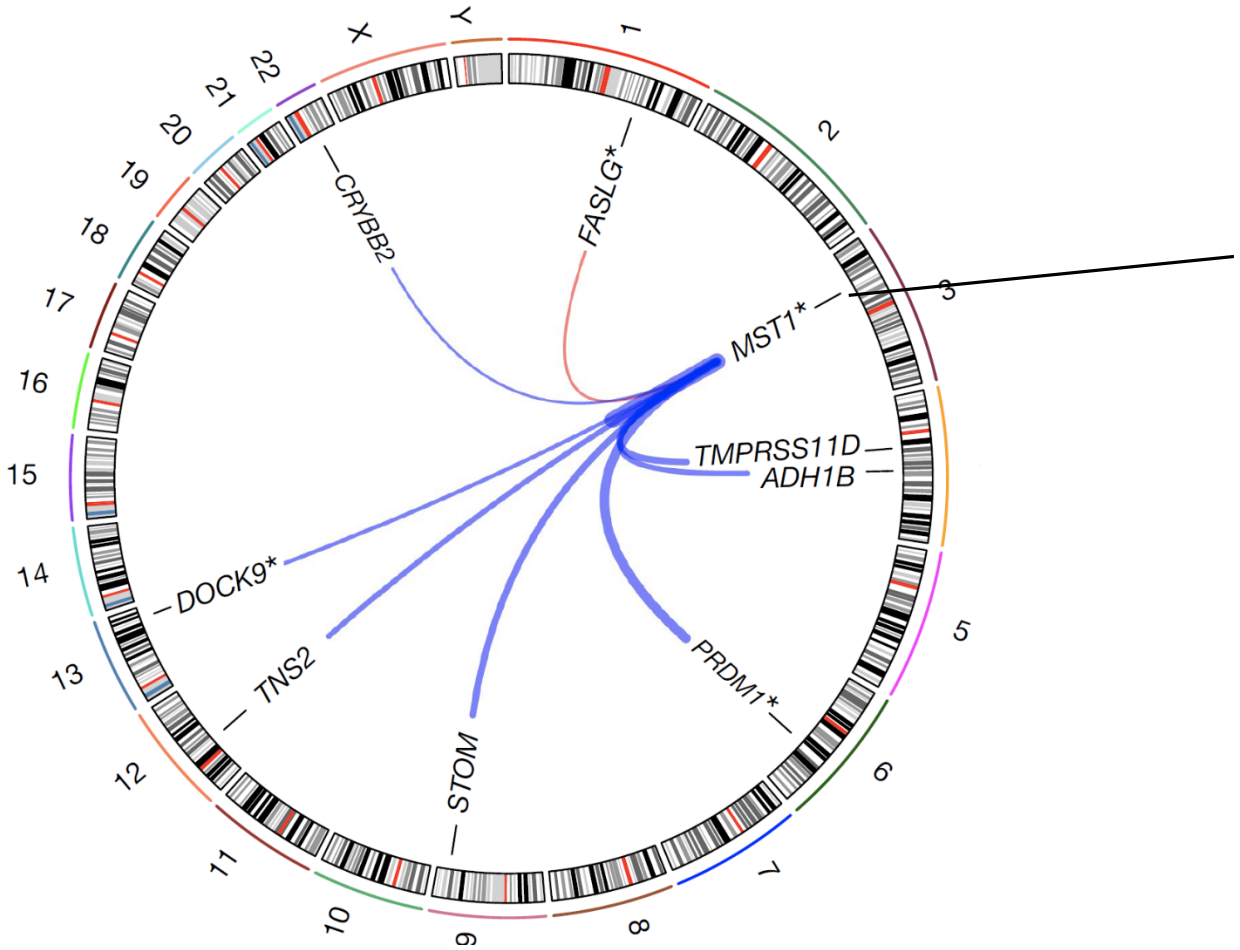
an assay based on chemically modified aptamers.

The SOMAscan assay contains aptamer species against thousands of protein targets

- In a multi-step process, stable aptamer-protein complexes are isolated, and unbound proteins and aptamers are washed away.
- The aptamer component of the complex is quantified using a fluorescence-based microarray chip.



Both SOMAscan (proteomics → 3622 plasma proteins) and a SNP microarray (genetics) was done for 3622 healthy blood donors



The rs3197999:A variant, located in the macrophage-stimulating 1 (*MST1*) gene, is known to be associated with inflammatory bowel disease.

This locus was associated with the plasma protein levels of 8 proteins (including MST1).

Thank you for your attention!