UniversitätsSpital Zürich

Successful replacement of animal models: production of antibodies

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May 3rd 2016

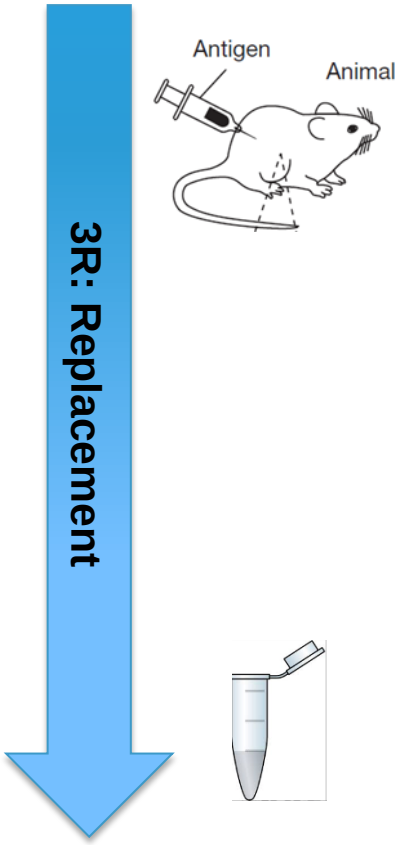
Outline

Historical overview concerning **methods for antibody production**

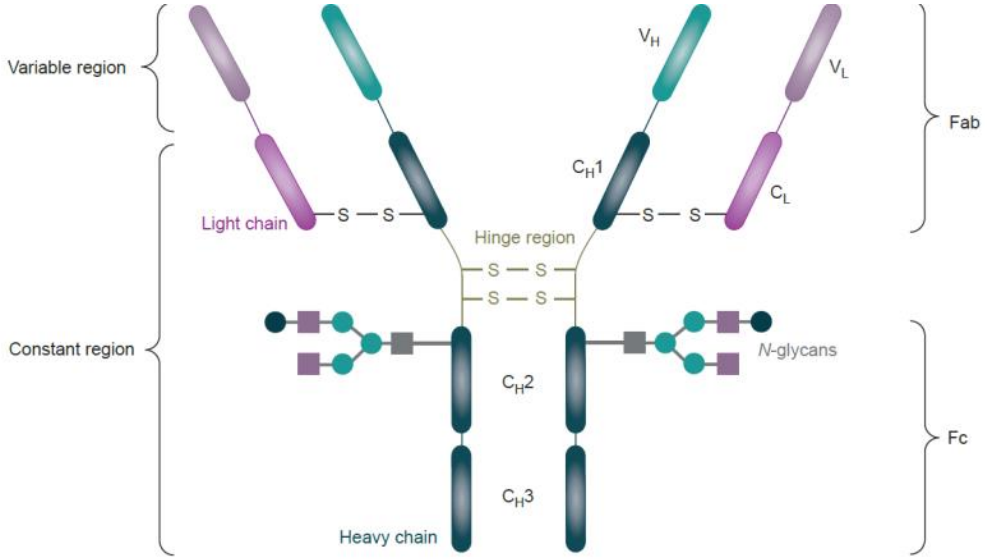
- Based on animal immunization:
  - Monoclonal antibody technology

- Recombinant binders:
  - Immunoglobulin derivatives: Fab/scFv libraries
  - Non immunoglobulin binders: DARPins

Derived from *in vitro* selection techniques



Immunoglobulin structure



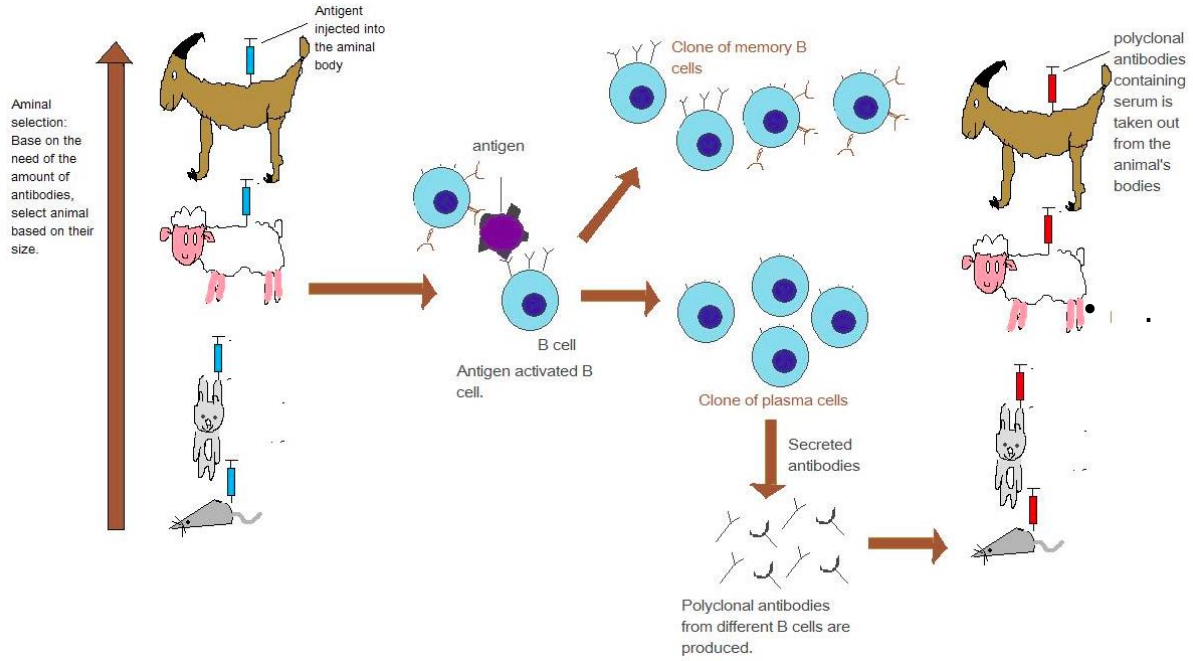
Naturally produced immunoglobulins or antibodies are macromolecular Y-shaped proteins of approximately 150 kDa.

In the animal, antibodies are produced primarily by plasma cells, a type of terminally differentiated B lymphocyte upon activation of the immune system.

They can be generated to selectively target a specific antigen binding partner and are universal weapons against pathogenic threats.

- **Diagnostic**
- **Research**
- **Therapy**

Polyclonal antibodies

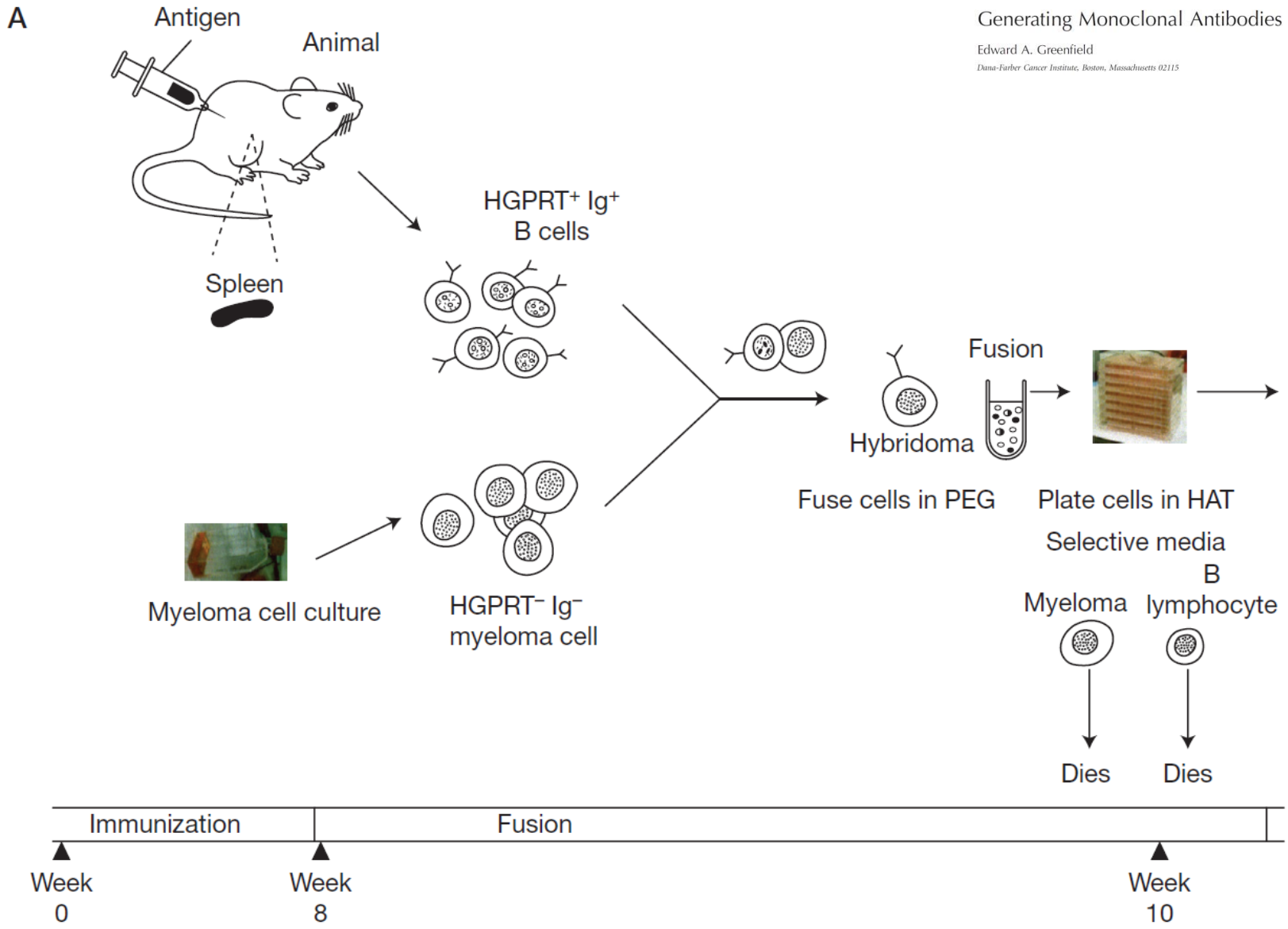


Serum contains mixed population of different types of antibodies (polyclonal) targeting the antigen in different epitopes.

- Prone to batch-to-batch variability.
- Produce large amounts of non-specific antibodies, which can create background signal in some applications.
- Multiple epitopes make it important to check immunogen sequence for cross-reactivity.
- Not useful for probing specific domains of antigen because antiserum will usually recognize many domains

As antibodies producing plasma cells from animals cannot be grown in tissue culture, they cannot be used as an in vitro source of antibodies.

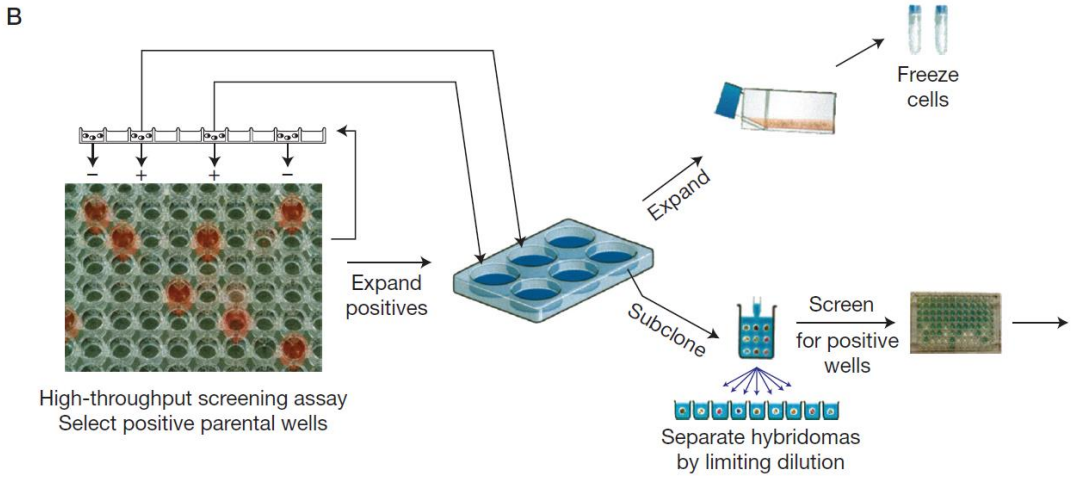
Mouse monoclonal technology:
From immunization to Hybridoma generation

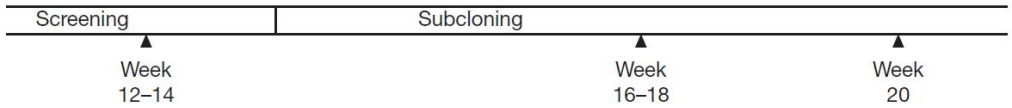


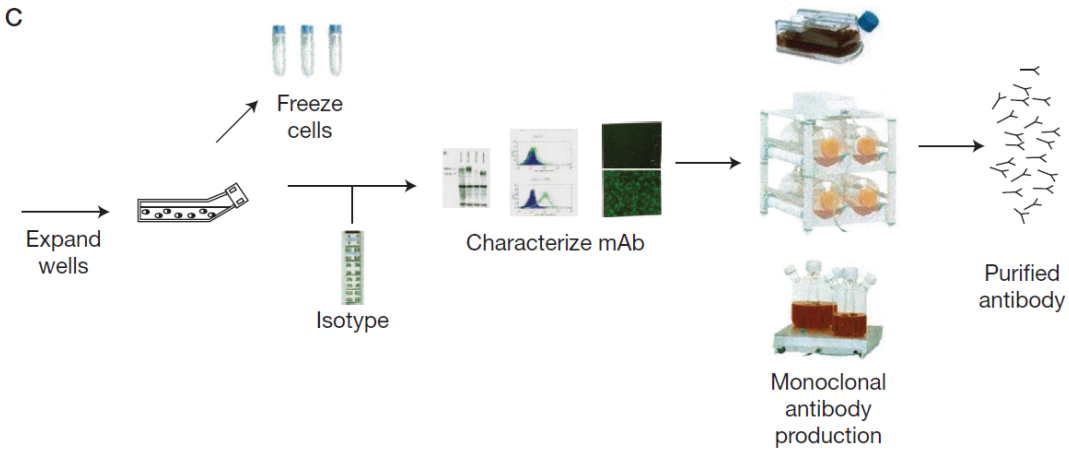
Generating Monoclonal Antibodies

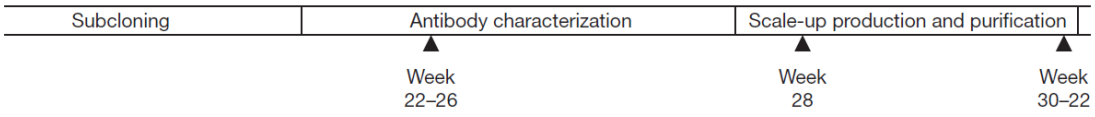
Edward A. Greenfield
Dana-Farber Cancer Institute, Boston, Massachusetts 02115

Mouse monoclonal technology:
From Hybridoma screening to antibody production









Generating Monoclonal Antibodies

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Dana-Farber Cancer Institute, Boston, Massachusetts 02115

Myeloma cell lines used as fusion partners

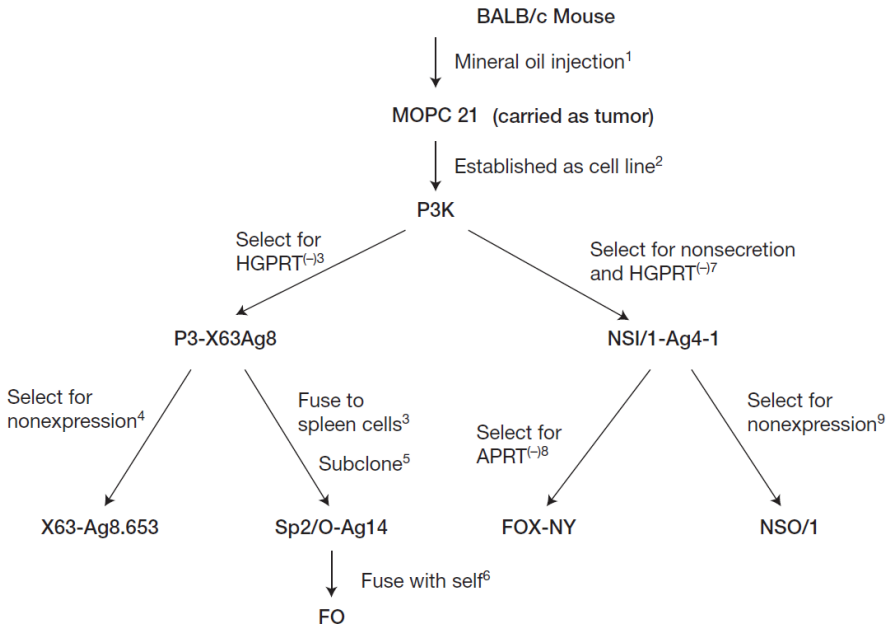


TABLE 2. Myeloma cell lines used as fusion parents

Cell line	Reference(s)	Derived from	Chains expressed	Secreting	Comments
Mouse lines					
P3-X63Ag8	Köhler and Milstein 1975	P3 K	γ1, κ	IgG1	Not recommended
X63Ag8.653	Kearney et al. 1979	P3-X63Ag8	None	No	Recommended
Sp2/0-Ag14	Köhler and Milstein 1976; Shulman et al. 1978	P3-X63Ag8× BALB/c	None	No	Recommended
FO	de St. Groth and Scheidegger 1980	Sp2/0-Ag14	None	No	Recommended
NSI/1-Ag4-1	Köhler et al. 1976	P3-X63Ag8	Kappa	No	Recommended
NSO/1	Galfre and Milstein 1981	NSI/1-Ag4-1	None	No	Recommended
FOX-NY	Taggart and Samloff 1983	NSI/1-Ag4-1	Kappa(?)	No	
Rat lines					
Y3-Ag1.2.3	Galfre et al. 1979	Y3	Kappa	No	Not recommended
YB2/0	Kilmartin et al. 1982	YB2/3HL	None	No	Recommended
IR983F	Bazin 1982	LOU/c rats	None	No	Recommended

Rabbit monoclonal technology (1): plasmacytoma cell lines

Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 9348–9352, September 1995
Immunology

Rabbit monoclonal antibodies: Generating a fusion partner to produce rabbit–rabbit hybridomas

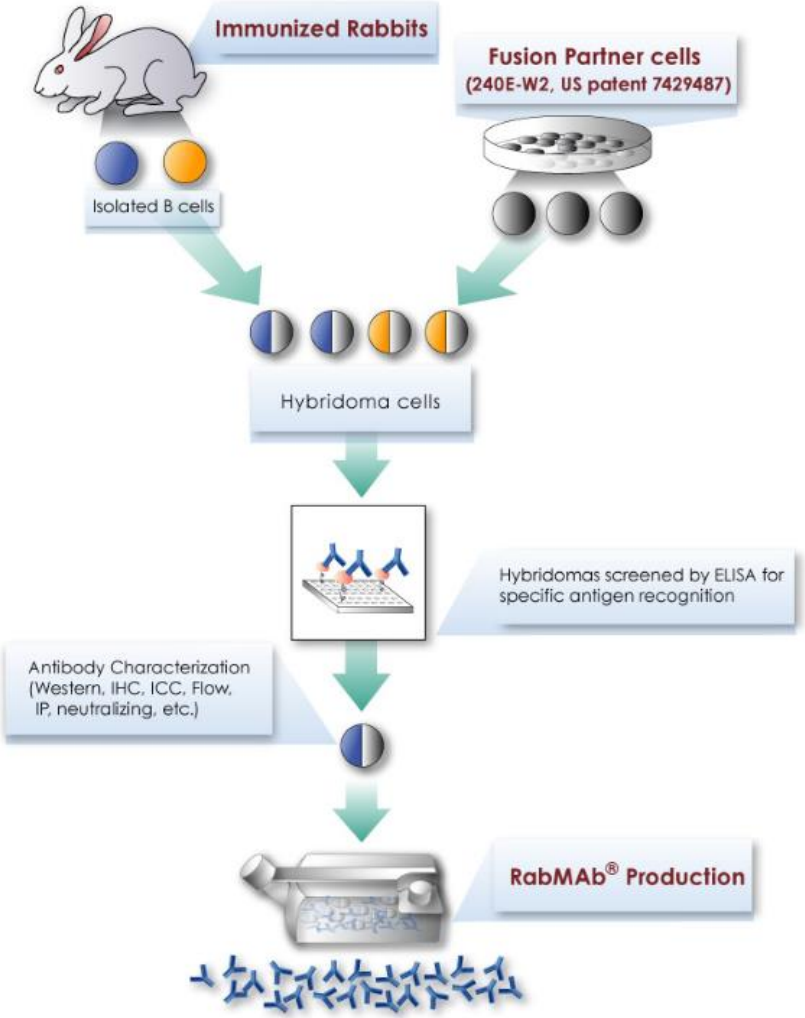
(*myc*/*abl*/transgenic rabbits/plasmacytoma/B cells)

HELGA SPIEKER-POLET, PERIANNAN SETHUPATHI, PI-CHEN YAM, AND KATHERINE L. KNIGHT*

- myc/abl double-transgenic rabbits developed plasmacytomas
- Establishement of **plasmacytoma cell lines 240E-1** able to develop into a usable fusion partner with spleen cells from hyperimmunized rabbits
- produce mAbs specific for mouse antigens and also for antigens or epitopes that are not immunogenic in mice
- **stability problems** with the fusion cell line 240E-1

Rabbit monoclonal technology (1): plasmacytoma cell lines

- After multiple rounds of subcloning and selection, **a new cell line 240E-W**, was identified which expressed better fusion efficiency and stability



(12) **United States Patent**
Pytela et al.

(10) **Patent No.: US 7,429,487 B2**
(45) **Date of Patent: Sep. 30, 2008**

(54) **FUSION PARTNER FOR PRODUCTION OF MONOCLONAL RABBIT ANTIBODIES**

(75) Inventors: **Robert Pytela**, San Francisco, CA (US); **Weimin Zhu**, San Francisco, CA (US); **Yaohuang Ke**, San Francisco, CA (US); **Qi Qian**, San Francisco, CA (US); **Harry C. Au**, San Francisco, CA (US)

(73) Assignee: **Epitomics, Inc.**, Burlingame, CA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **11/476,277**

(22) Filed: **Jun. 27, 2006**

(65) **Prior Publication Data**

US 2007/0015259 A1 Jan. 18, 2007

Related U.S. Application Data

(60) Provisional application No. 60/697,014, filed on Jul. 5, 2005.

(51) **Int. Cl.**
C12N 5/06 (2006.01)
C12N 5/16 (2006.01)

(52) **U.S. Cl.** **435/326**; 435/328

(58) **Field of Classification Search** ..... None
See application file for complete search history.

(56) **References Cited**

U.S. PATENT DOCUMENTS

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OTHER PUBLICATIONS

Rief et al., "Production and Characterization of a Rabbit Monoclonal Antibody Against Human CDC25C Phosphatase", Hybridoma (1998), 17(4), pp. 389-394.
Spieker-Polet et al., "Rabbit Monoclonal Antibodies: Generating A Fusion Partner to Produce Rabbit-Rabbit Hybridomas", Proc. Natl. Acad. Sci. USA. (1995), 26:92(20), pp. 9348-9352.
Liguori et al., "Recombinant Human Interleukin-6 Enhances the Immunoglobulin Secretion of a Rabbit-Rabbit Hybridoma", (2001), 20(3), pp. 189-198.

* cited by examiner

Primary Examiner—Phillip Gambel
Assistant Examiner—Sharon Wen
(74) *Attorney, Agent, or Firm*—James S. Keddie; Bozicevic, Field & Francis LLP

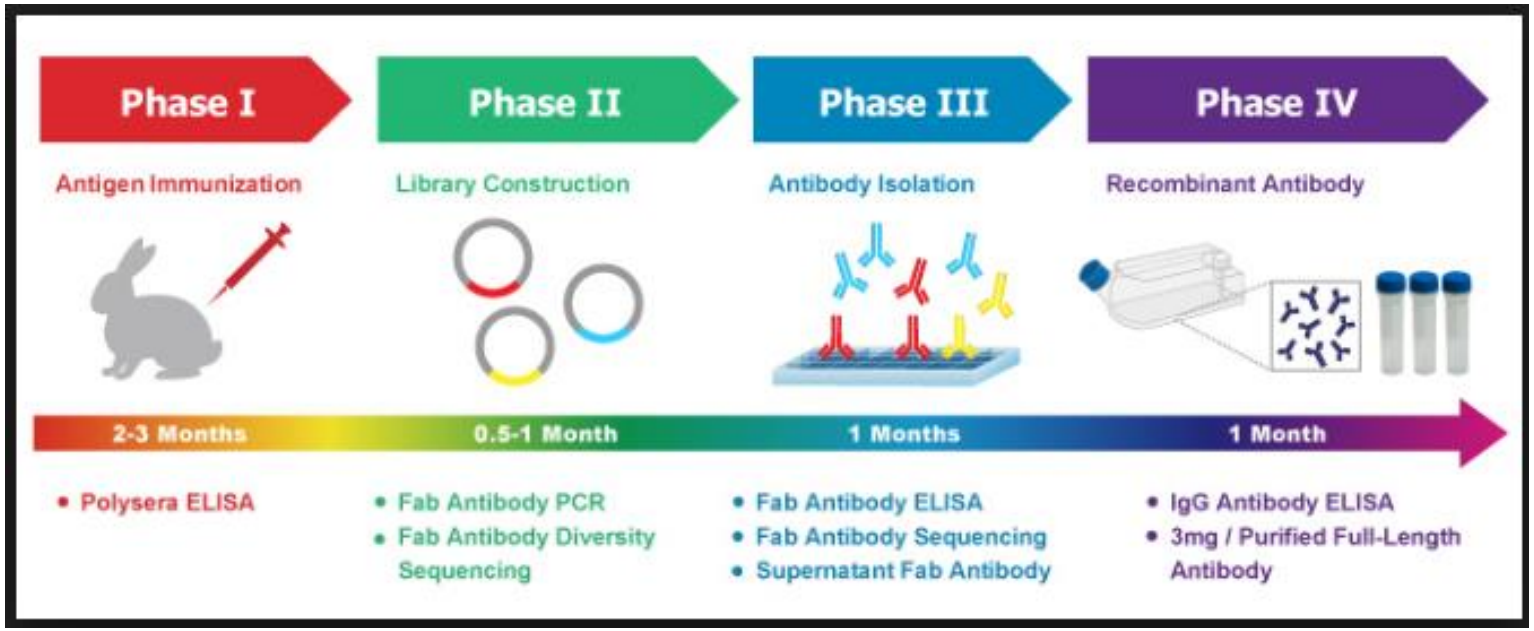
(57) **ABSTRACT**

The invention provides a rabbit-derived immortal B-lymphocyte capable of fusion with a rabbit splenocyte to produce a hybrid cell that produces an antibody. The immortal B-lymphocyte does not detectably express endogenous immunoglobulin heavy chain and may contain, in certain embodiments, an altered immunoglobulin heavy chain-encoding gene. A hybridoma resulting from fusion between the subject immortal B-lymphocyte and a rabbit antibody-producing cell is provided, as is a method of using that hybridoma to produce an antibody. The subject invention finds use in a variety of different diagnostic, therapeutic and research applications.

6 Claims, 5 Drawing Sheets

Rabbit monoclonal technology (2):

scFV or Fab library from immunized rabbit selection by in vitro display screening



Rabbit monoclonal technology (3):

Rabbit B cell from peripheral blood

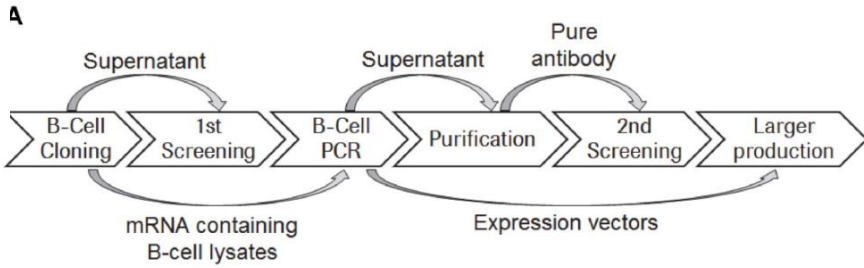
OPEN ACCESS Freely available online

PLOS ONE

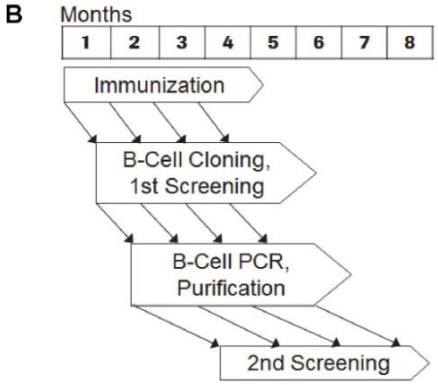
A Robust High Throughput Platform to Generate Functional Recombinant Monoclonal Antibodies Using Rabbit B Cells from Peripheral Blood

Stefan Seeber, Francesca Ros, Irmgard Thorey, Georg Tiefenthaler, Klaus Kaluza, Valeria Lifke, Jens André Alexander Fischer, Stefan Klostermann, Josef Endl, Erhard Kopetzki, Achal Pashine[¤a], Basile Siewe[¤b], Brigitte Kaluza, Josef Platzer, Sonja Offner*

Large Molecule Research, Pharma Research and Early Development, Roche Diagnostics GmbH, Penzberg, Germany

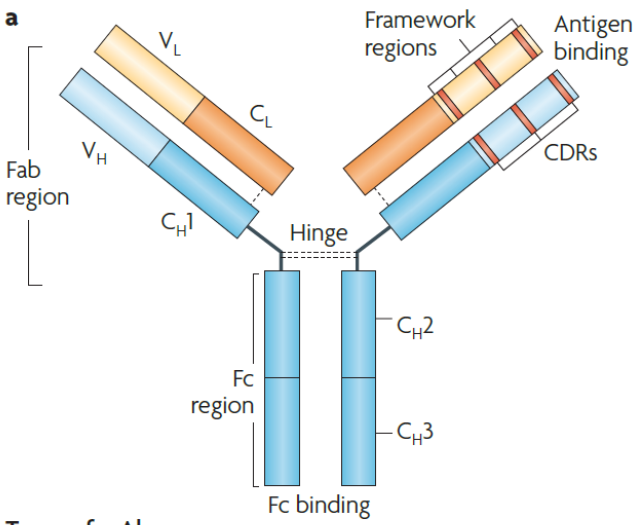


Peripheral B cells could be a preferred source for very good antibodies in terms of affinity maturation, since the emigration of the matured ASCs/plasma blasts from the spleen is an affinity driven process



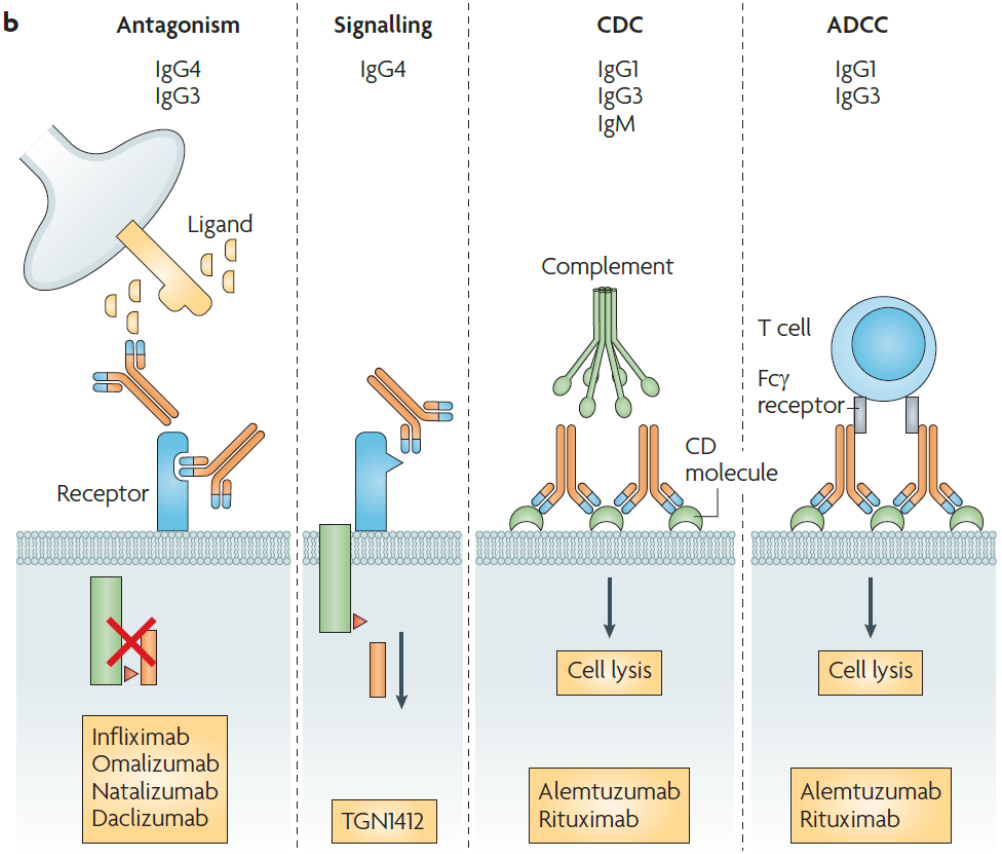
Method based on a combination of B-Cell Cloning and B-Cell PCR technology which is highly effective in isolating a large number of rabbit B-cell clones secreting sufficient monoclonal antigen specific IgG.

Going for therapy:
Humanization and and modulation of the Fc effector function



Types of mAbs

Murine	Entirely murine amino acids	'o' = mouse e.g. muromonab
Chimeric	Human constant (C) + murine variable (V) regions	'xi' = chimeric e.g. rituximab
Humanized	Murine complementarity determining regions (CDRs)	'zu' = humanized e.g. alemtuzumab
Human	Entirely human amino acids	'u' = human e.g. adalimumab



When developing therapeutic mAbs, the choice of IgG subclass is important, especially in oncology

Disadvantages of mammalian cell systems for mAb production

At present, he majority of mAbs approved for therapeutic applications are produced in:

- Chinese hamster ovary cells (12 out of 28),
- SP2/0 (7/28) and NS0 (5/28) mouse cell lines,
- hybridomas (2/28).

The remaining two are antigen-binding fragments (Fabs) that are produced periplasmically in Escherichia coli.

Advantages of mammalian cell expression systems:
endotoxin-free
high-level expression and stability,
Recently developed for the display of functional glycosylated immunoglobulin on the cell surface.

However, the selection of stable antibody-producing cell lines is very **time consuming** and results in **higher costs** relative to microbial expression systems that involve much faster growth rates and thus lower capital investment.

Procaryotic systems for mAb production?

nature COMMUNICATIONS

ARTICLE

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DOI: 10.1038/ncomms9072 OPEN

Efficient expression of full-length antibodies in the cytoplasm of engineered bacteria

Michael-Paul Robinson[1], Na Ke[2], Julie Lobstein[2,†], Cristen Peterson[1], Alana Szkodny[1], Thomas J. Mansell[1], Corinna Tuckey[2], Paul D. Riggs[2], Paul A. Colussi[2,†], Christopher J. Noren[2], Christopher H. Taron[2], Matthew P. DeLisa[1] & Mehmet Berkmen[2]

A major challenge facing the use of E. coli as an antibody expression platform is the production of mAbs with the correct disulfide bonds.

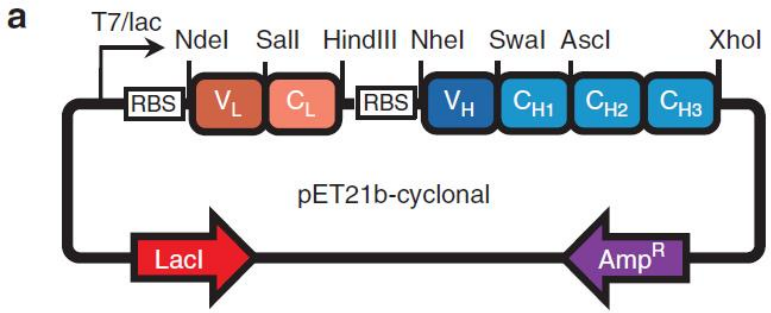
The formation of disulfide bonds in E. coli can be catalysed in either the naturally oxidative periplasmic compartment.

However, periplasmic expression limited by the smaller volume, the lack of ATP-dependent molecular chaperones and by inefficiently secretion of both the IgG HC and LC across the cytoplasmic membrane.

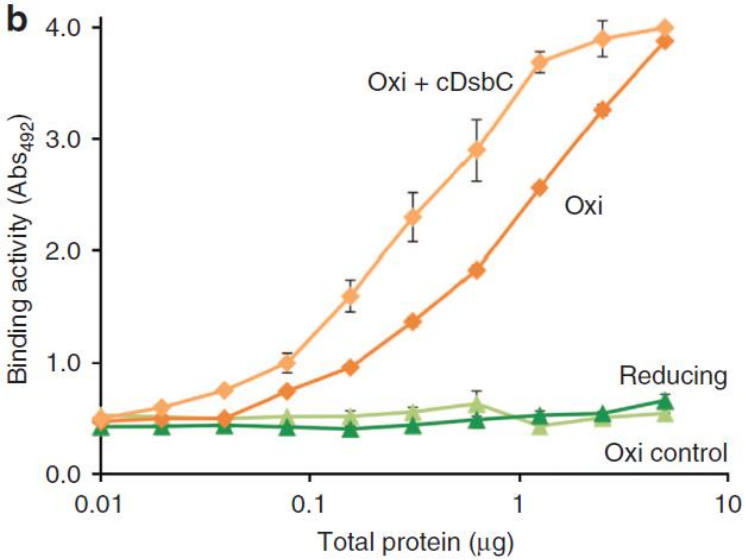
Biologically active IgGs can be obtained in **engineered oxidative cytoplasm** of the E. coli strain SHuffle.

Significantly higher titres of cytoplasmic IgGs named ‘cyclonals’ were obtained compared with periplasmic IgG expression.

Cytoplasmic expression of mouse anti-MBP cyclonals in SHuffle



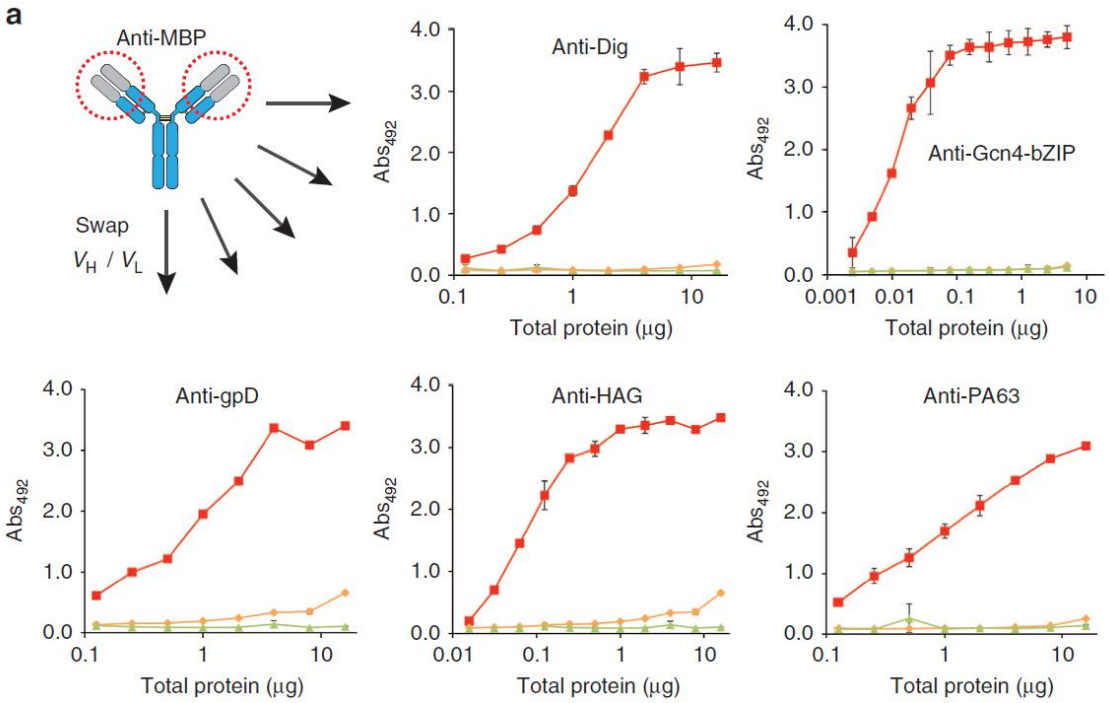
The genes encoding HC (VH–CH1–CH2–CH3) and LC (VL–CL) of anti-MBP were assembled into a synthetic, bicistronic operon under the control of a T7/lac promoter in plasmid pET21b.

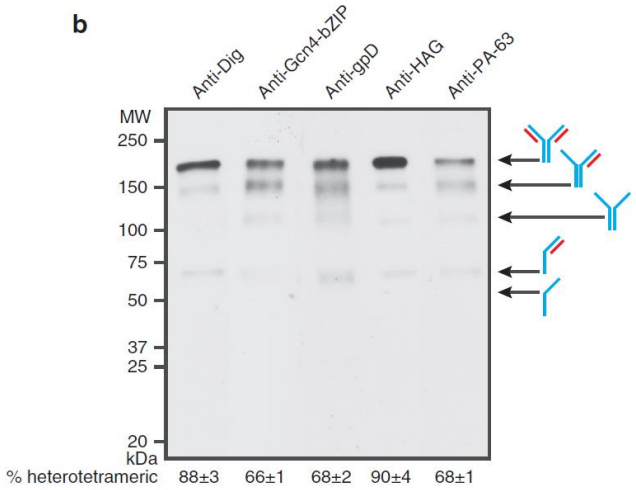


Test wild-type **(Reducing)** E. coli B strain vs the isogenic suppressor strain MB1731 **(Oxi),** whose cytoplasmic reductive pathways have been diminished, allowing the formation of disulfide bonds in the cytoplasm.

Cyclonal production is enhanced in **Shuffle strain (Oxi + cDsbC)** by expression of DsbC, an **oxidoreductase chaperone** capable of enhancing oxidative protein folding both in its native periplasmic compartment and when expressed cytoplasmically.

Redirecting cyclonals to new antigens with swapped variable regions





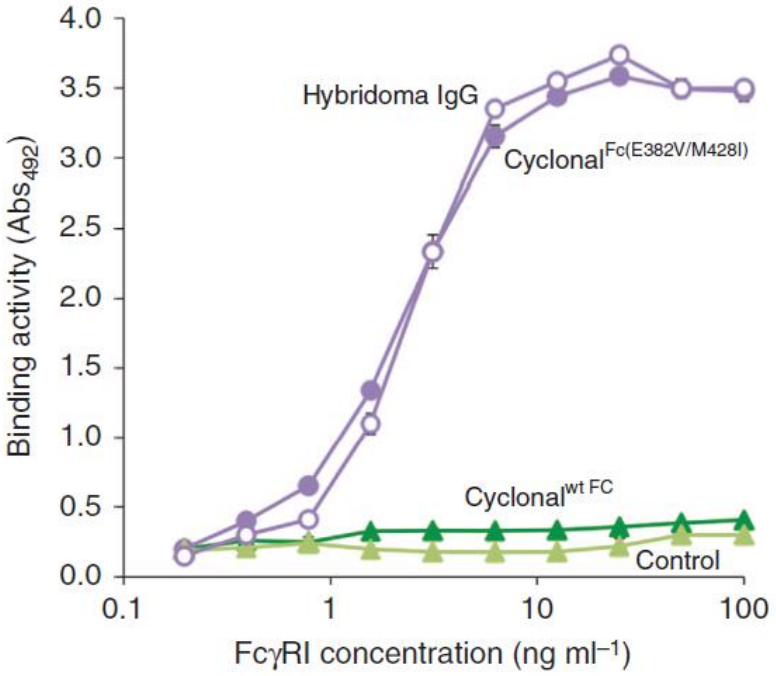
A panel of new cyclonals (less than a week).
This involved first replacing the VH and VL genes of the anti-MBP cyclonal with the same genes from a variety of existing antibodies and antibody fragments.

Purified 1–25 mg of highly active IgGs per litre of shake-flask culture using affinity chromatography on a protein-A colum.

Remodelling the Fc domain of cyclonals for binding to FcγRs.

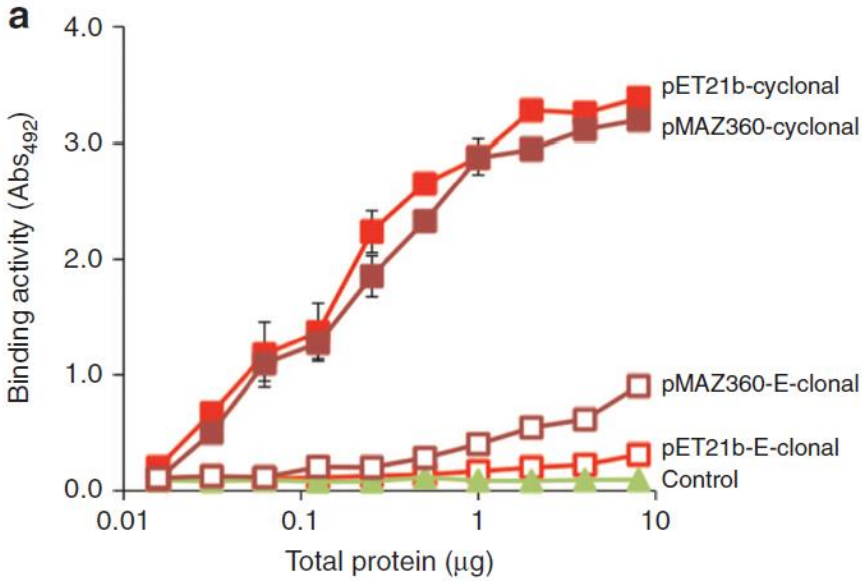
IgGs lacking glycosylation in their Fc domain, such as those produced in E. coli, are completely unable to bind to FcgRs, and do not induce FcγR-mediated effector functions.

Aglycosylated Fc with E382V/M428I mutation binds to FcγRI and is efficiently produced by cyclonals



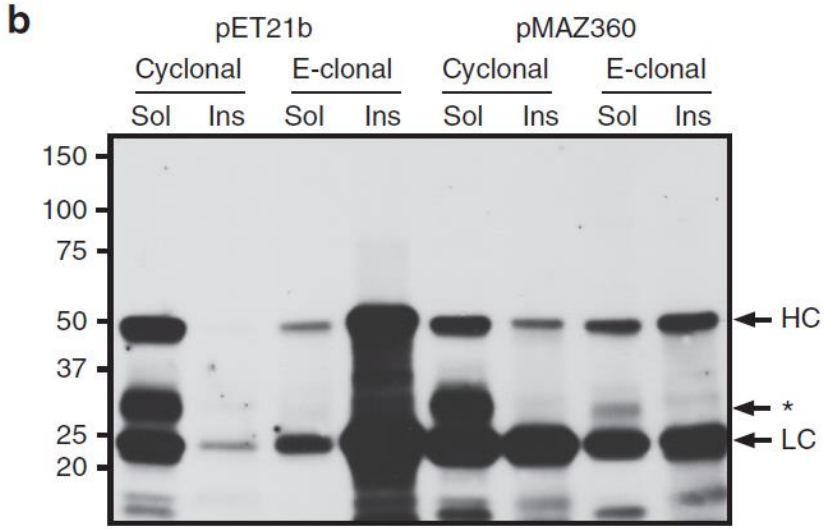
Antigen-binding activity of chimeric **anti-PA-63** cyclonal with either Wt or mutated Fc domain (cyclonal Fc(E382V/M428I)). Glycosylated IgGs with WT Fc were purified from hybridoma cultures and included as positive control

Comparison of cytoplasmic versus periplasmic IgG expression



Cytoplasm

Periplasm

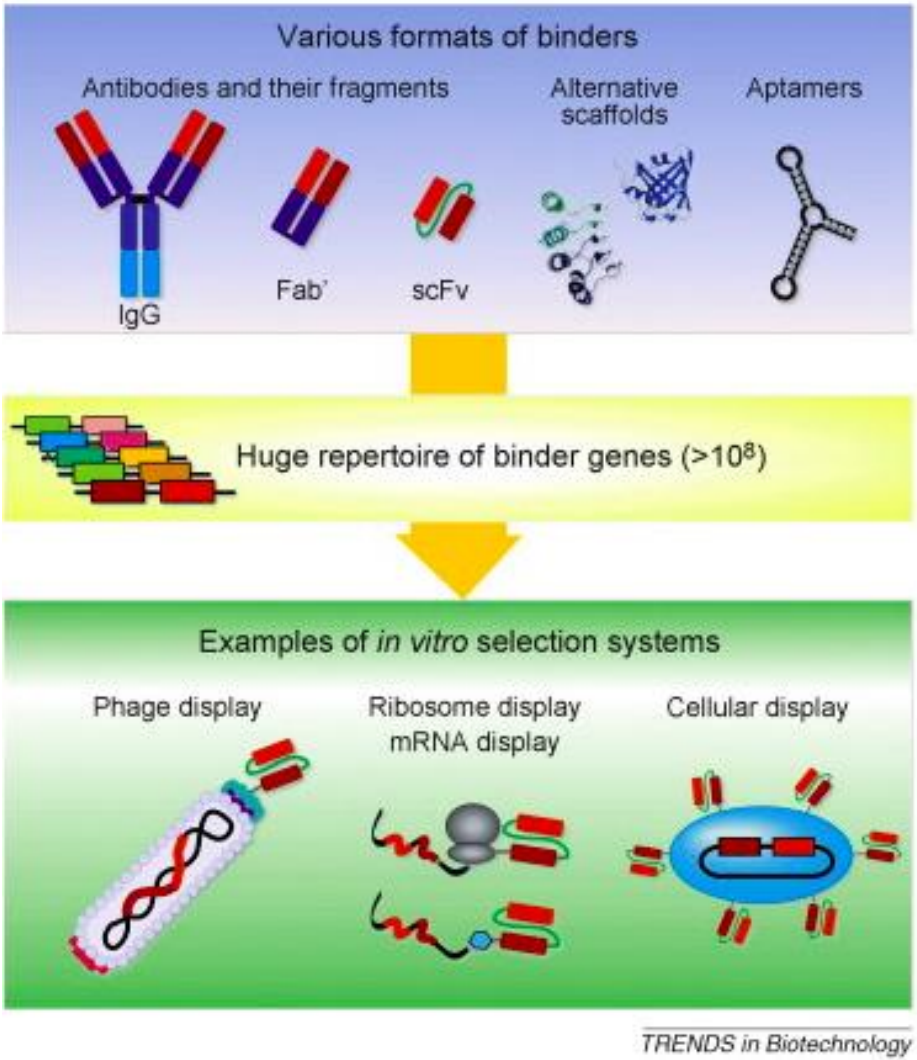


Comparison of the accumulation of the anti-MBP IgG in the mFab/hFc format following expression in the cytoplasm and periplasm

Shortcomings of monoclonal antibody technology

- Relies on animal immunization
- The antigen needs to be immunogenic but must be not lethal when injected
- The specificity and affinity of the selected antibody can be checked but not improved in hybridoma technology.

Generating recombinant antibodies and antibody mimetics



A large **library** of potentially interesting antibodies/mimetics is created, from which binders with desirable specificity and affinity can be selected against a specific antigen.

Prerequisite for ***in vitro* selection process**:
Link between the genotype (DNA sequence) and the phenotype (protein)

Construction of antibodies libraries

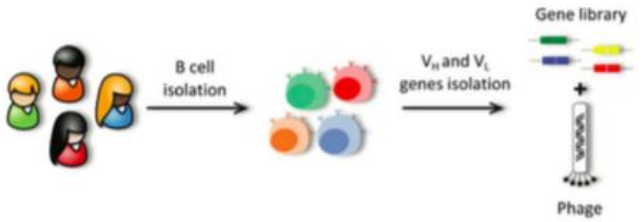
Antibody libraries

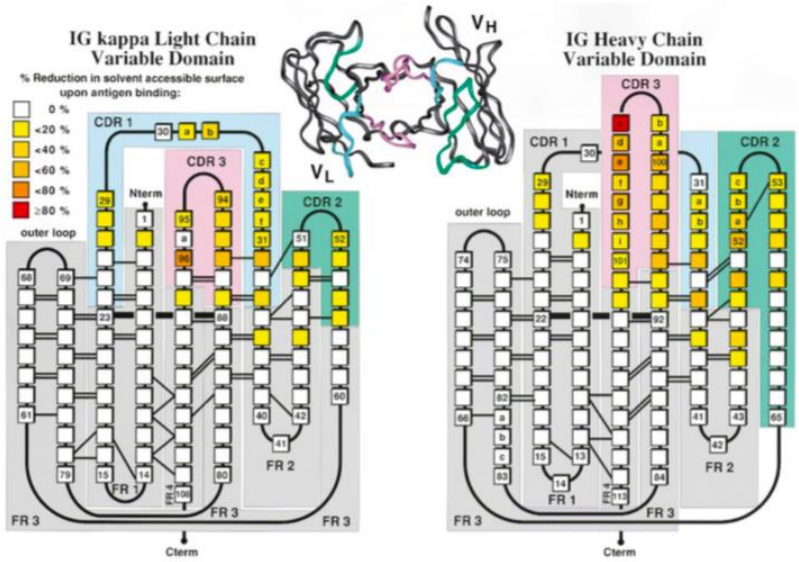
- **Natural**
  - **Naive**
  - **Immune**
- **Synthetic**

Modular Consensus Frameworks and randomized CDRs:
All the sequences are exactly known

The repertoire of antibody light and heavy chain mRNA from B cells is amplified via PCR using a set of specific primers covering all the V gene families.
The antibody fragments are generated by random combination of VL and VH chain genes.

Most commonly Fab and scFv can be cloned and displayed on phage/ribosome libraries.

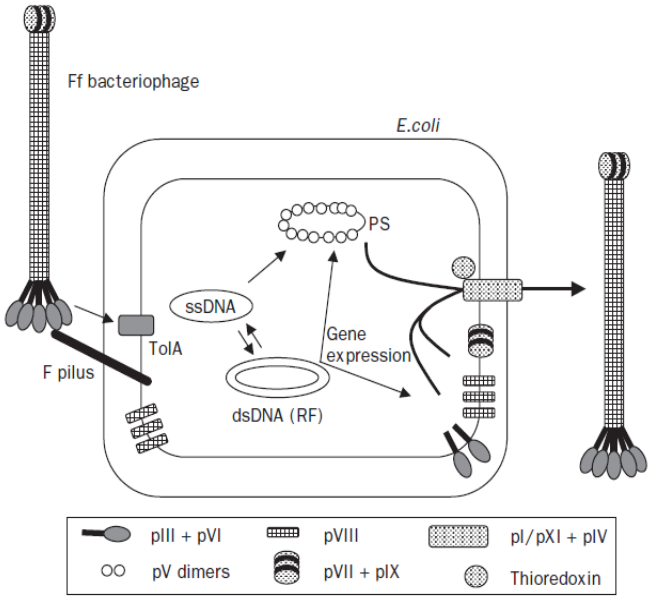


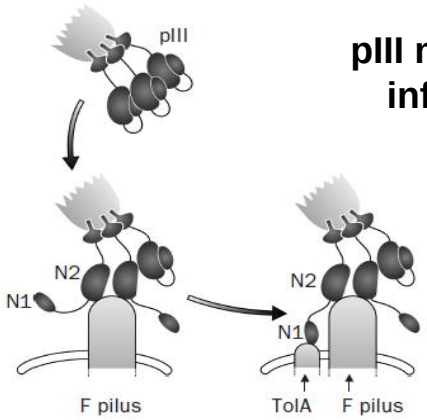


Knappik A. Et al., J. Mol. Biol. (2000) 296, 57-86

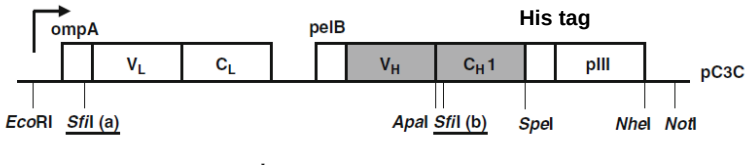
The **diversity of the library** is crucial for successful isolation of specific binders.

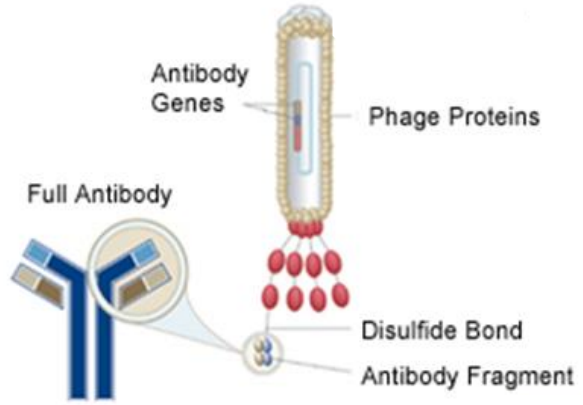
Phage display derived antibodies: The bacteriophage bio-technology





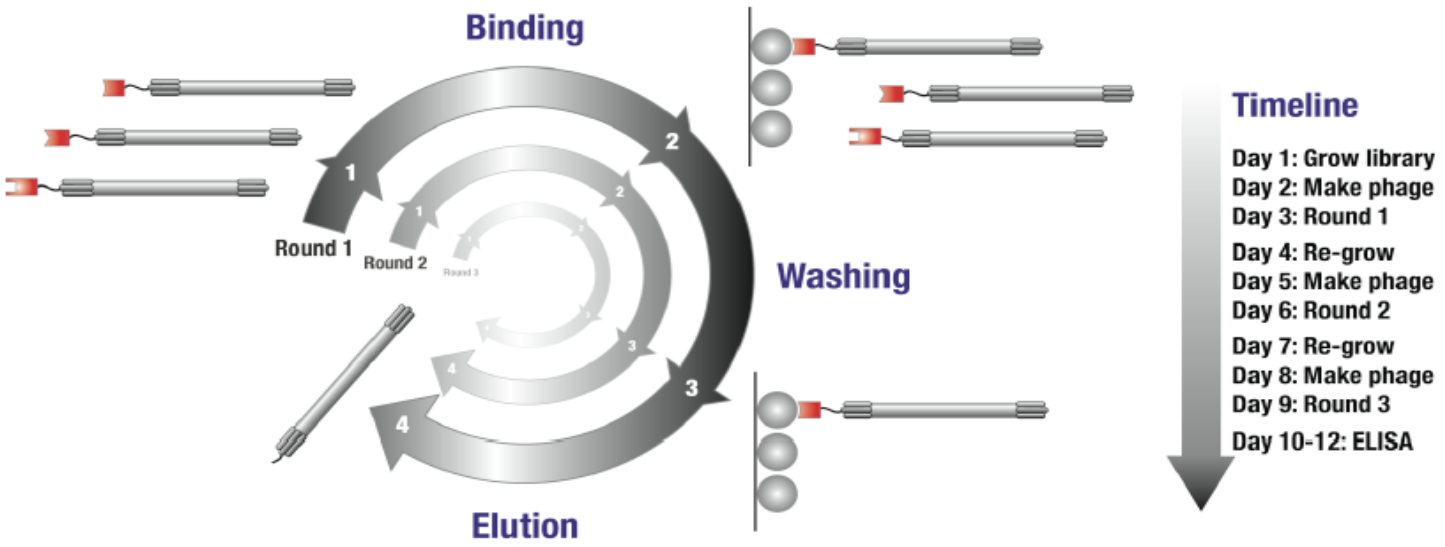
pIII mediated infection





Phage display format: **Fab fragment has its CH1 domain fused to the pIII coat protein** of the phage via an Amber codon. Using a bacteria strain that does not read the Amber codon as a Stop allows the fusion between the two proteins.

Phage selection: biopanning



PROTOCOL

The use of phage display to generate conformation-sensor recombinant antibodies

Aftabul Haque[1–3] & Nicholas K Tonks[1]

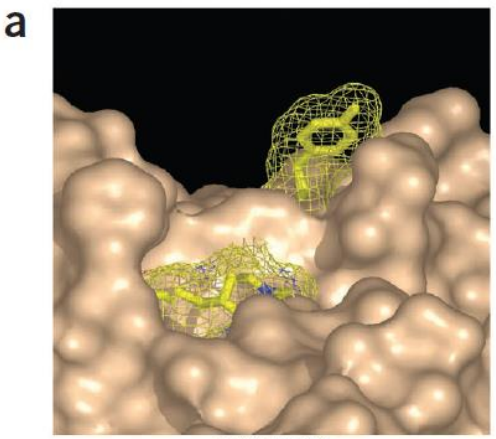
[1]Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, USA. [2]Molecular and Cellular Biology Graduate Program, Stony Brook University, Stony Brook, New York, USA. [3]Present address: Whitehead Institute for Biomedical Research, Cambridge, Massachusetts, USA. Correspondence should be addressed to N.K.T. (tonks@cshl.edu).

Published online 15 November 2012; doi:10.1038/nprot.2012.132

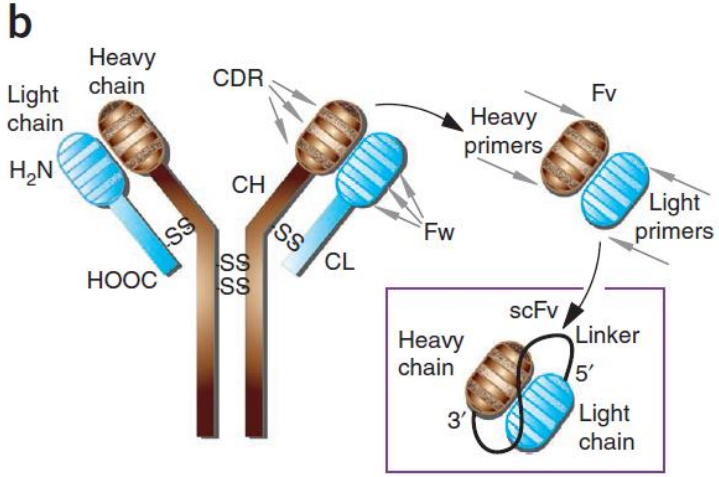
Generation of scFv for protein tyrosine phosphatase 1B (PTP1B)

PTP1B plays a pivotal role in insulin and leptin signaling.
PTP1B is oxidized by ROS in response to stimuli (insulin).
Elements of the catalytic cleft are exposed on the surface and the enzyme is inactive (the substrate binding is inhibited).
This reversible oxidation presents new unique binding surface.

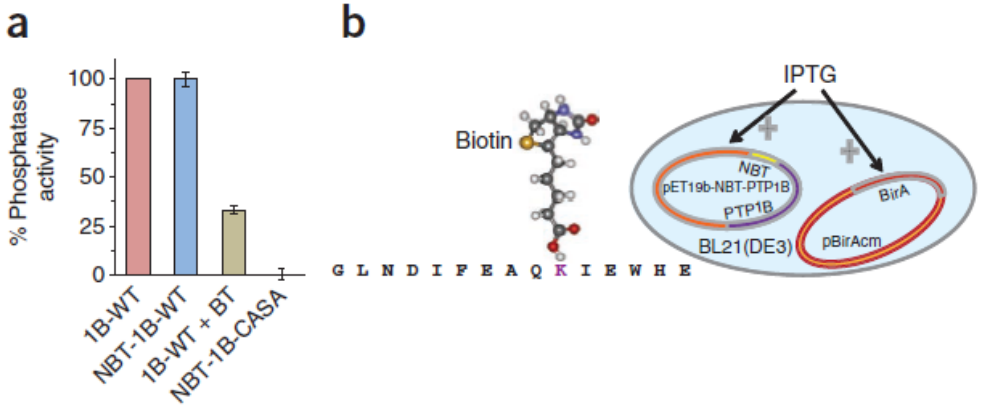
The goal was to generate scFV targeting oxidation-specific epitopes of PTP1B that could be expressed in cells as intrabodies to block PTP1B function.



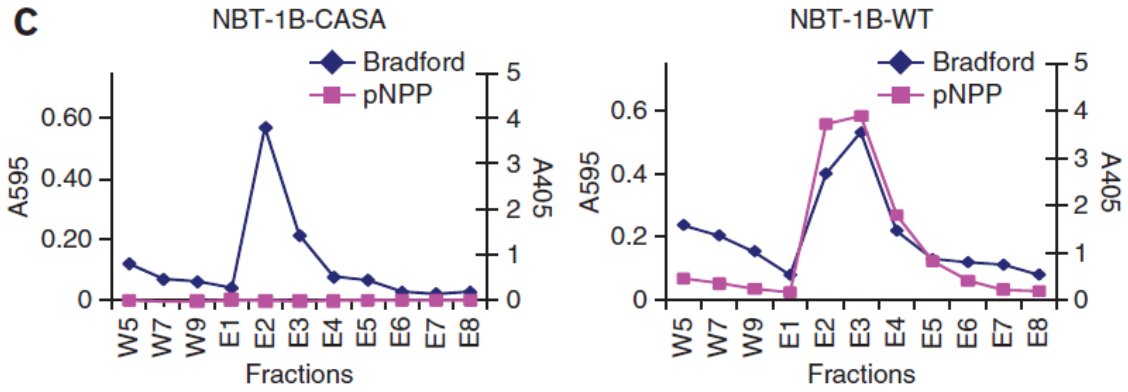
CASA mutation mimicking the conformation of oxidized PTP1B



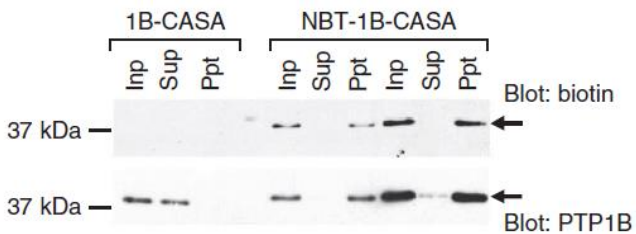
Site specific biotinylation of PTP1B WT and CASA mutant



PTP1B *in vivo* biotinylation

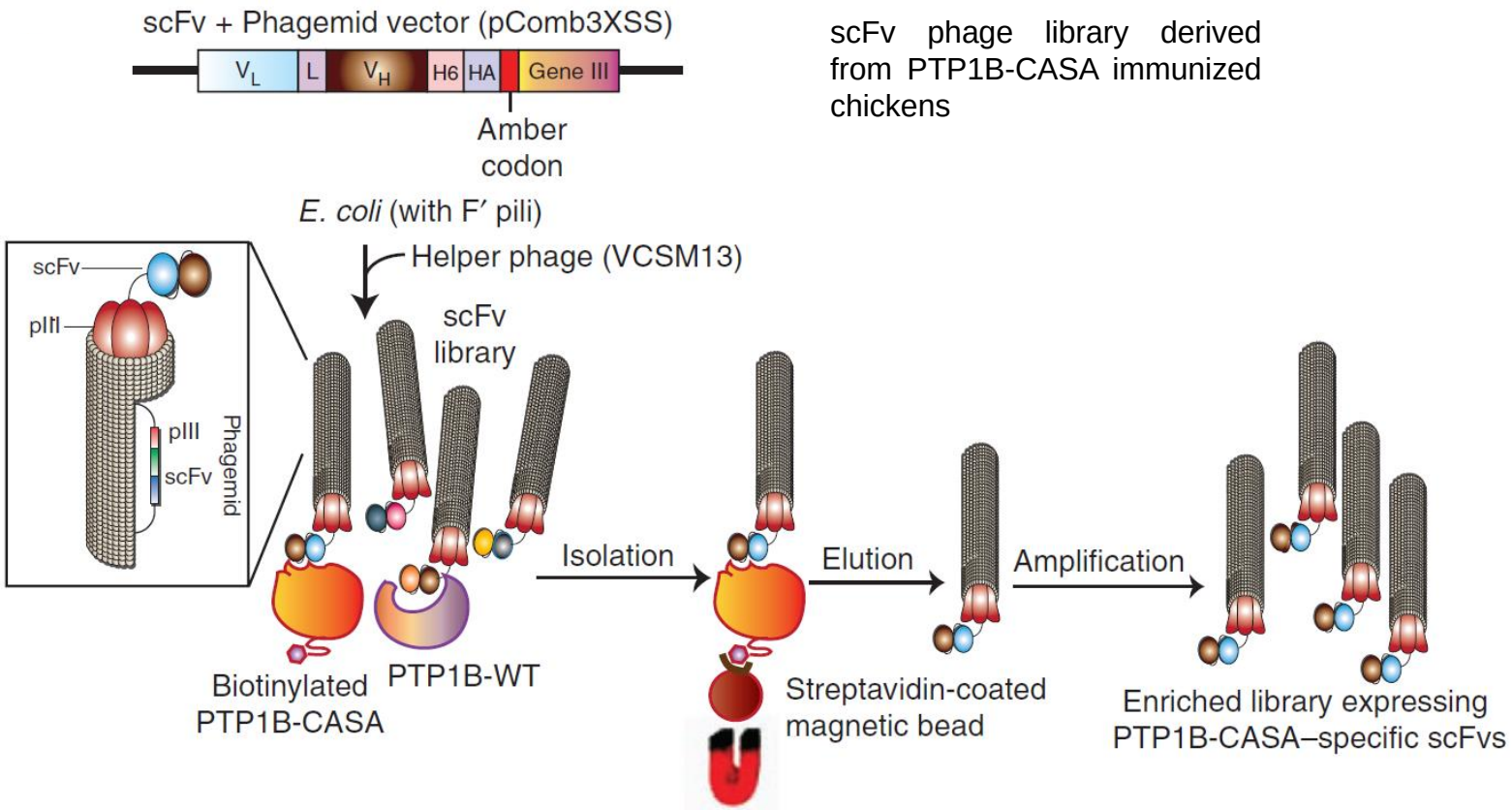


pNTT: Phosphatase activity



Pull-down of biotinylated by streptavidin beads

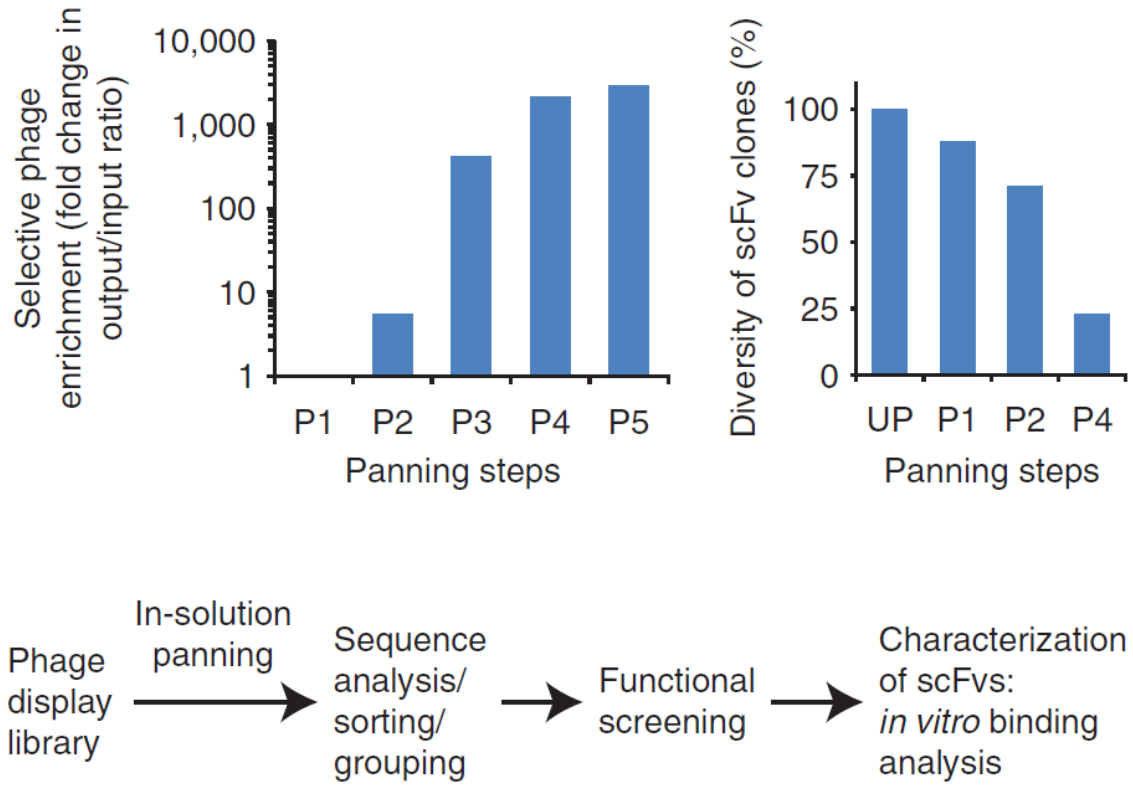
Schematic representation of the panning steps: In-solution panning to enrich PTP1B-OX-specific scFvs



scFv phage library derived from PTP1B-CASA immunized chickens

Solution-based in vitro selection strategy crucial to preserve specific interaction with a particular conformation of the antigen

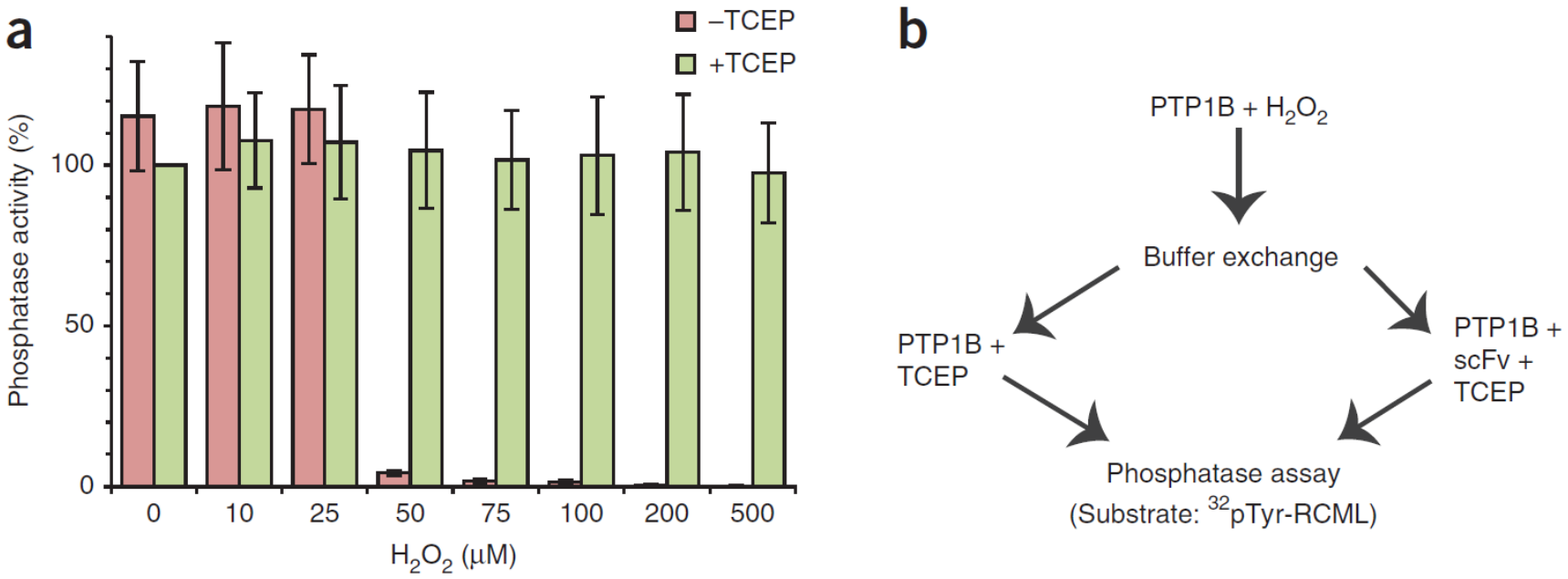
Phage output/input ratio and library diversity



Screening of scFvs specific to PTP1B-OX

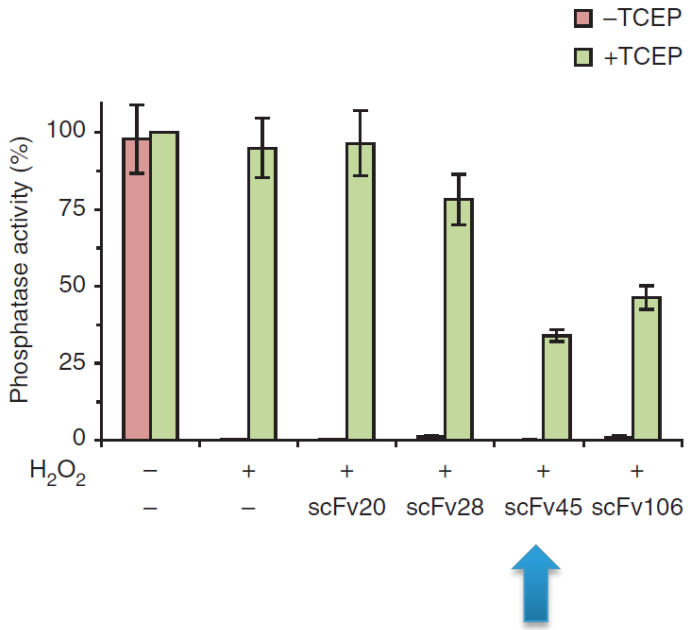
Standard screening such as ELISA are not sufficient to assess the function of selected scFV to inhibit PTP1B-OX reactivation.

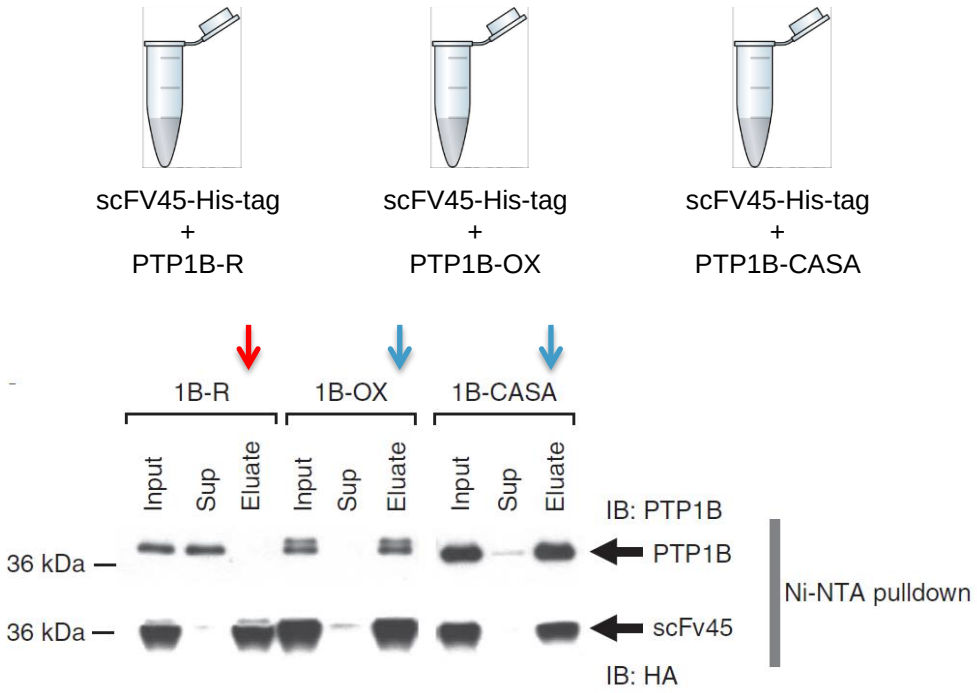
Screening based on phosphatase assay in solution



Conditions were established for reversible oxidation of PTP1B by H2O2 *in vitro*

Screening of scFvs specific to PTP1B-OX



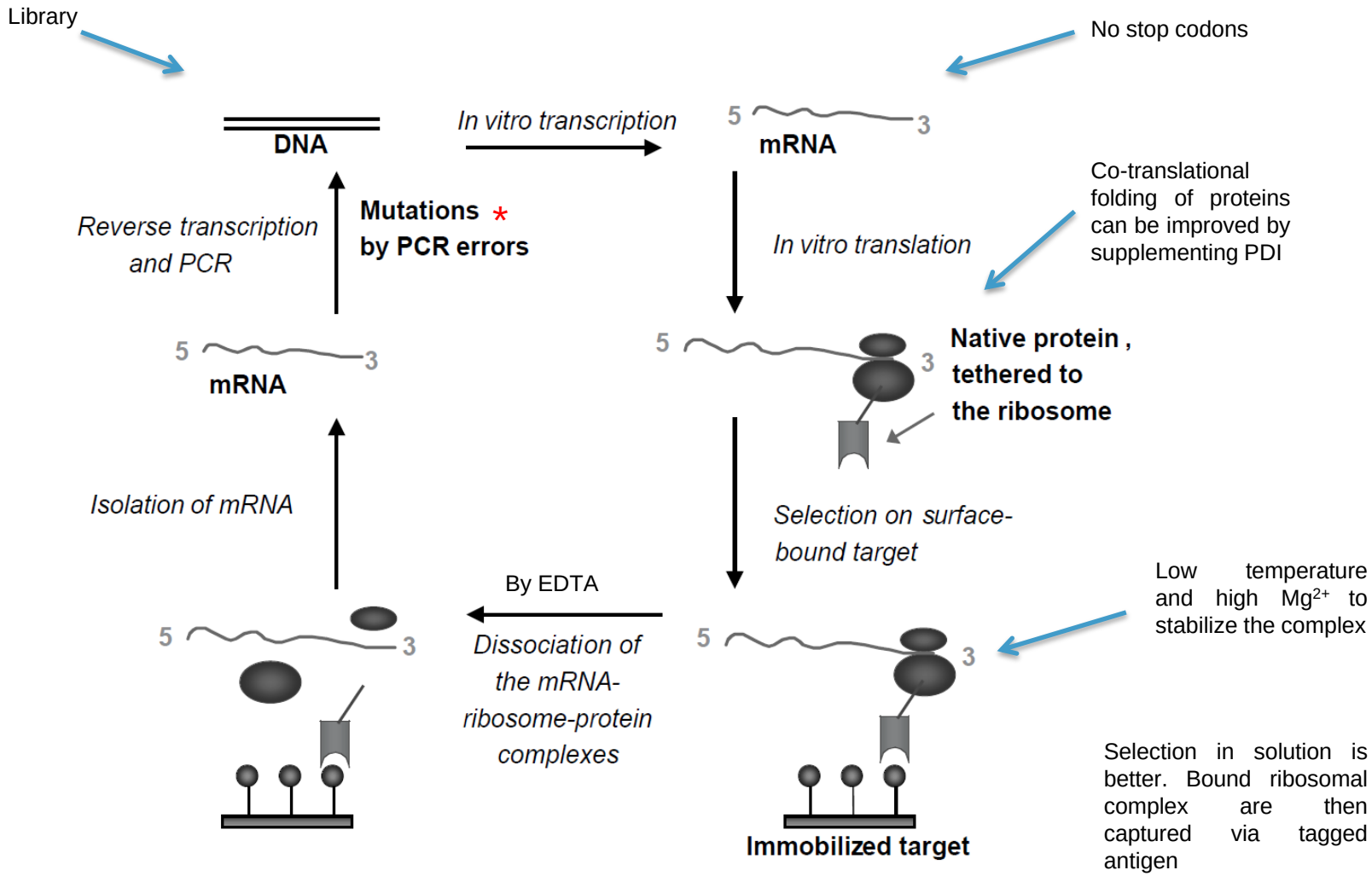


Specific binding of **scFv45** to oxidized PTP1B *in vitro*

Limitations of the phage display techonology

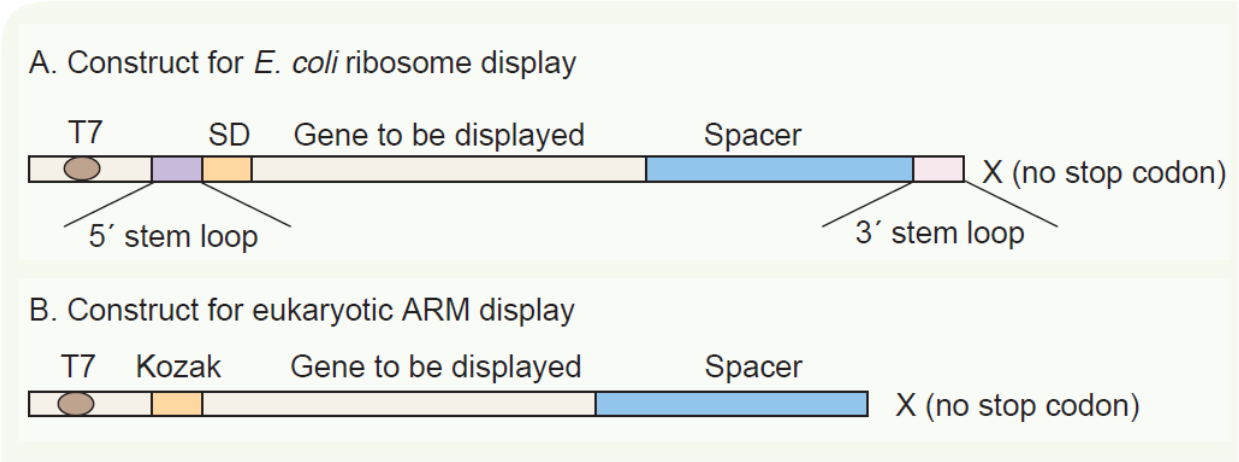
- Selection in the context of host environment cannot be avoided
- Loos of potential candidates due to growth disadvantage /toxicity
- Risk to lose the best binders (with picomolar affinity) if elution is not efficient
- Diversification step to evolve the antibody is possible but laborious:
  - mutator strains: it can create unwanted mutations in the plasmid and host genome
  - switch to diversification step in vitro (maturation libraries or mutagenesis by PCR) and subsequent new selection by phage display (laborious)
- Phage display is relatively demanding and time consuming technology

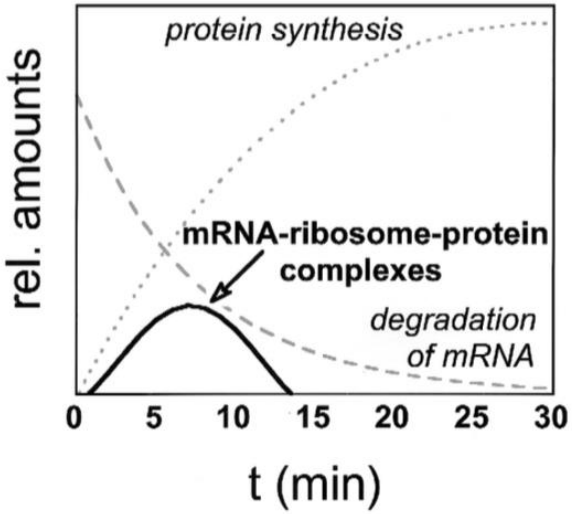
Principle of ribosome display:
Entirely in vitro cell free transcription, translation and selection system



Selection of very large **libraries** which do not need to be transformed into cells.

Characteristic of the constructs for ribosome display



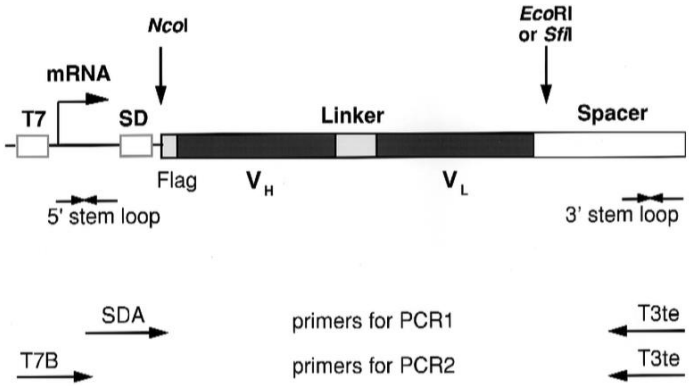


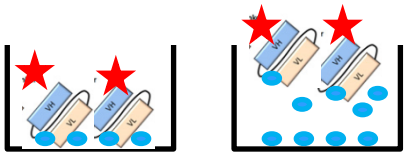
Picomolar affinity antibodies from a fully synthetic naive library selected and evolved by ribosome display

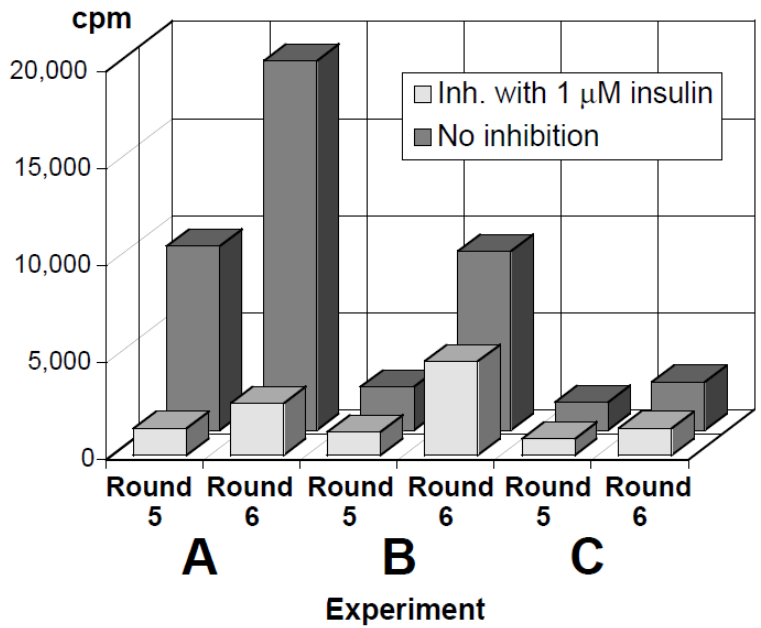
Jozef Hanes[1,2 †], Christiane Schaffitzel[1 †], Achim Knappik[3], and Andreas Plückthun[1*]

Ribosome display to **in vitro selection and evolution** of **scFvs** from a large **synthetic library** (Human Combinatorial Antibody Library; HuCAL) against bovine **insulin**

Selection of scFV binding to insulin from a synthetic library







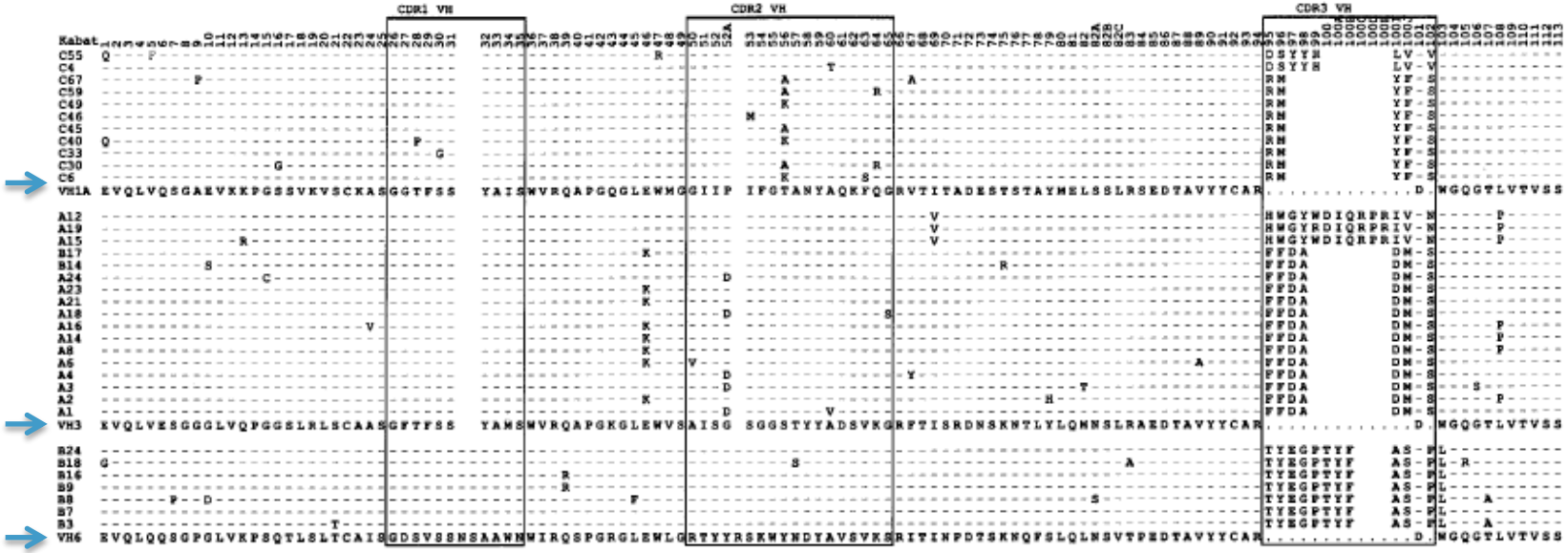
Radioimmunoassays of the pools after the fifth and sixth rounds of ribosome display.

Pooled RNAs from three independent ribosome display experiments A, B, and C were **translated in vitro in the presence of [35S]methionine**, and translation mixtures (labelled scFV) containing 0 and 1 µM bovine insulin (competition) were analyzed by RIA at room temperature.

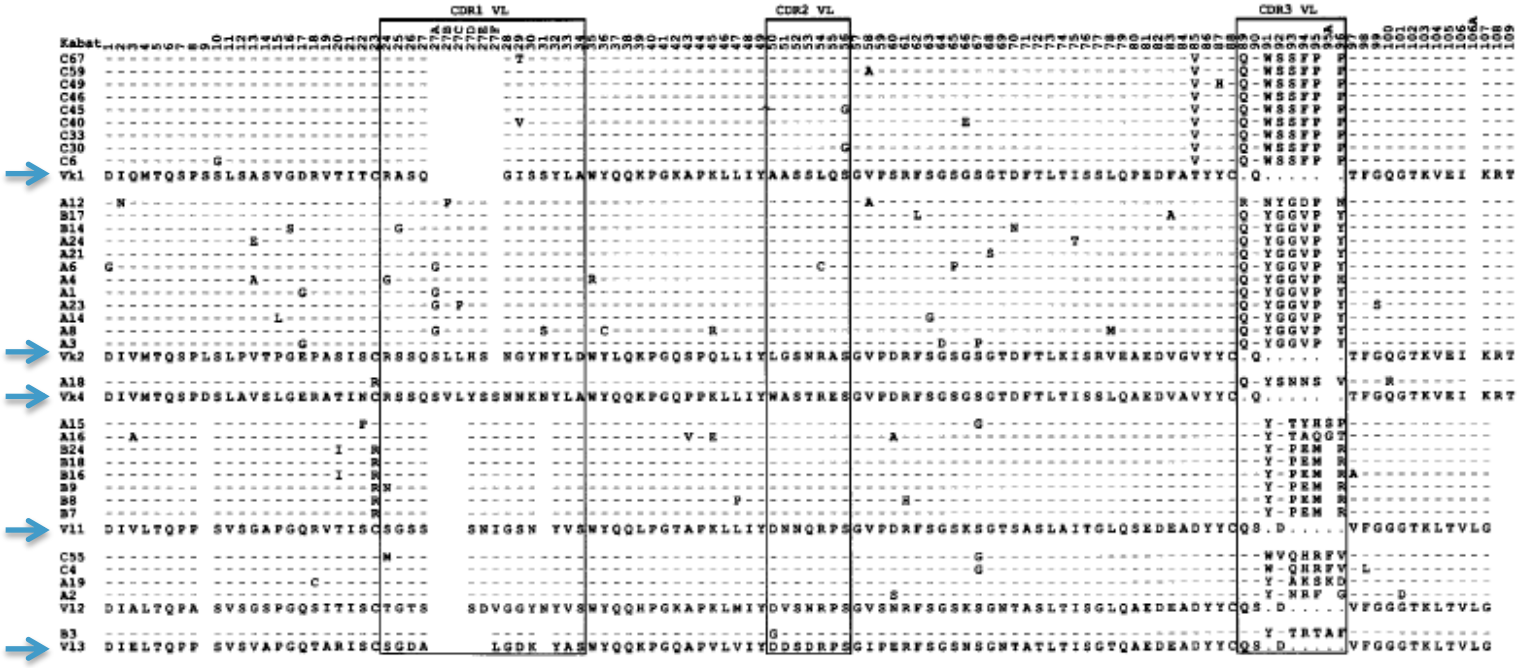
Alignment of the amino acid sequences of VH and VL of the scFvs binders:

All binders have mutation compared to the original HuCAL sequences

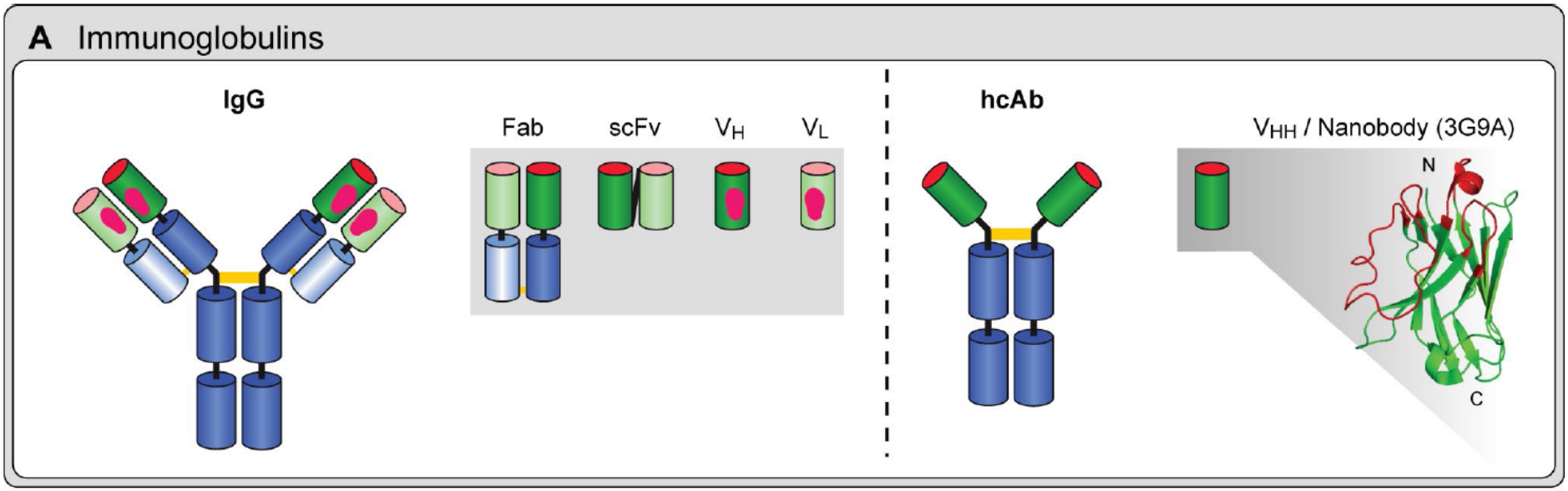
VH



VL



Immunoglobulins and their derivatives



The necessity of noncovalent interdomain interactions for functional domain assembly impairs the thermodynamic stability of Fabs and scFvs

Naturally evolved heavy chain antibodies (hcAbs) from camels: the functional antigen-binding unit of hcAbs consists of one single variable domain (VHH domain; nanobody).

VHHs have a size of ~13–14 kD and have evolved biochemical features:

the substitution of hydrophobic with hydrophilic residues in framework regions increases stability and solubility

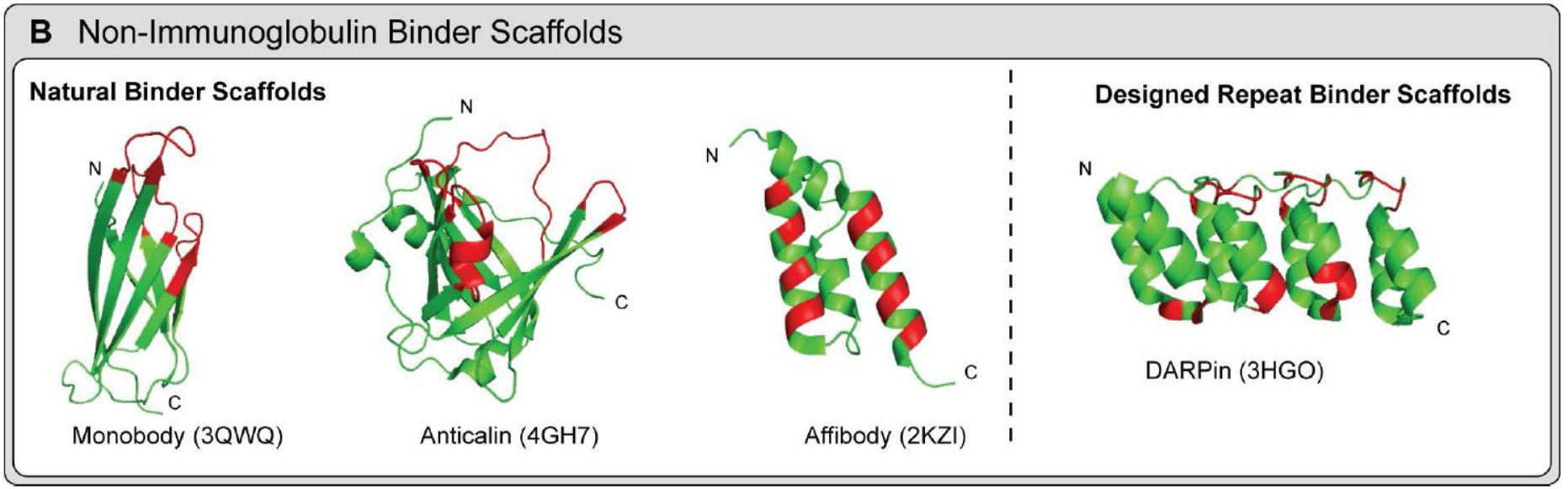
This allows robust, heterologous expression in bacterial hosts and functional expression in eukaryotic cells.

J. Cell Biol. Vol. 209 No. 5 633–644

Nanobodies and recombinant binders in cell biology

Jonas Helma,[1] M. Cristina Cardoso,[2] Serge Muyldermans,[3] and Heinrich Leonhardt[1]

Antibody mimetics: alternatives to animal sourced antibodies



Antibody drawbacks:

- Many antibodies have relatively low expression yields
- tendency to aggregate
- dependence on disulfide bonds for stability.

Antibody mimetics based on **alternative protein scaffold** would have none of these drawbacks while still exhibiting the same affinity and specificity

J. Cell Biol. Vol. 209 No. 5 633–644

Nanobodies and recombinant binders in cell biology

Jonas Helma,[1] M. Cristina Cardoso,[2] Serge Muyldermans,[3] and Heinrich Leonhardt[1]

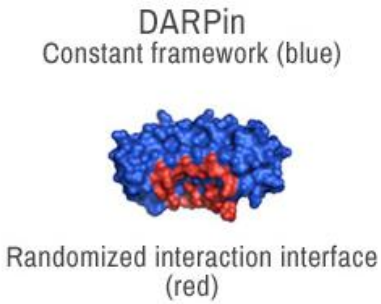
DARPins

nature biotechnology

High-affinity binders selected from designed ankyrin repeat protein libraries

H Kaspar Binz[1,2], Patrick Amstutz[1,2], Andreas Kohl[1,2], Michael T Stumpp[1], Christophe Briand[1], Patrik Forrer[1], Markus G Grütter[1] & Andreas Plückthun[1]

replicate the virtues of antibodies and address their shortcomings



Ankyrin repeat proteins

- Repeat proteins: consecutive homologous structural units (repeats), which stack to form an elongated protein domain with a continuous hydrophobic core
- They occur throughout all phyla and mediate protein-protein interactions in the nucleus or cytoplasm, or while anchored to the membrane or when secreted into the extracellular space
- AR are built from stacked, **33 amino acid repeats, each forming a β-turn that is followed by two antiparallel α-helices and a loop** reaching the β-turn of the next repeat.
- The β-turn and the first α-helix mediate the interactions with the target, and different numbers of adjacent repeats are involved in binding.
- The reported target binding affinities of natural AR proteins are in the low nanomolar range

Design of the DARPins library

Based on a **consensus strategy**:

Underlying assumption: residues important for maintaining the fold will be more conserved and thus show up prominently in an alignment.

A **consensus framework** was built **and surface residues were identified that might potentially interact with the target**—based on analogy of complexes of natural ankyrin repeat proteins with their targets.

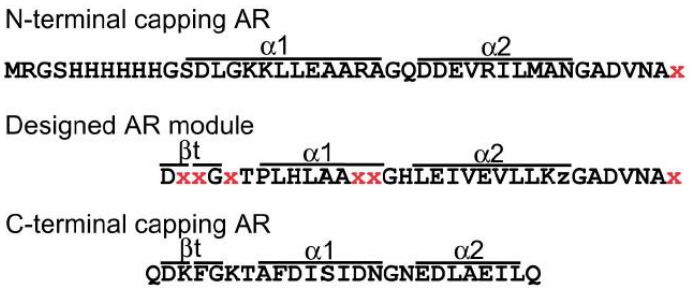
These residues were randomized, **avoiding the residues Cys** (to eliminate disulfide formation), **Pro, and Gly** (as some of the residues are located in a helix). This restriction was achieved by using trinucleotide building blocks during library generation.

(DARPin) library thus comprises fixed and variable positions
The fixed positions reflect structurally important framework positions, whereas the six variable positions per repeat module reflect non-conserved, surface-exposed residues that can be potentially engaged in interactions with the target.

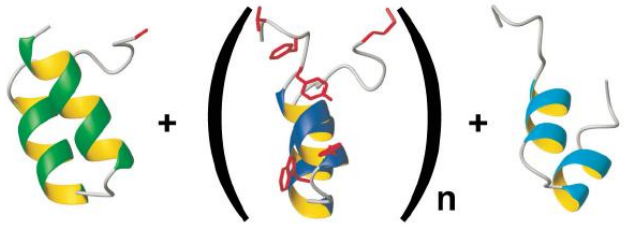
The theoretical diversities of the DARPin libraries are 5.2×10^{15} or 3.8×10^{23} for two-module or three-module binders, respectively.

Properties of the DARPins

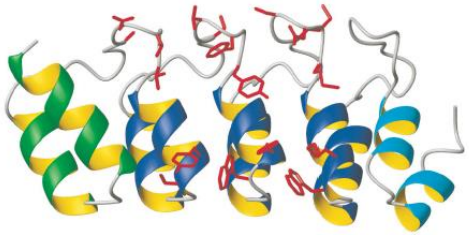
a



b

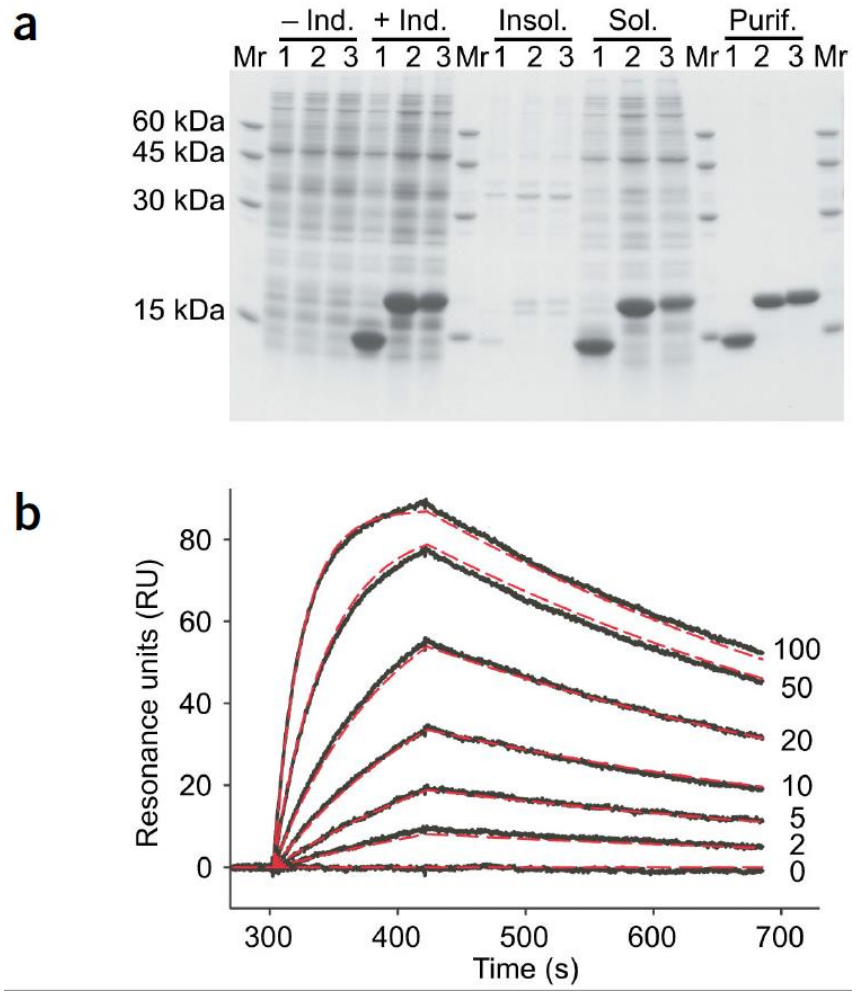


c



- DARPins proteins use both β-turns and a randomized surface and, because of their modular architecture, the interaction surface can be adapted by adding more repeat modules
- **Concave shape** of the binding site which binds **structural epitopes** on the target protein surface
- Next generation of **DARPins (Loop DARPins)** with extended epitope-binding properties: introduction of an elongated loop mimicking convex paratope
- With LoopDARPin library, binders with an affinity of 30 pM could be isolated with only a single round of **ribosome display** directly from the original library.
- N- and C-capping repeat flanking the binding modules are essential for DARPins to fold in *E. Coli.*
- High stability, no aggregation even at high concentration, expressed at very high yield in soluble form in the cytoplasm of *E. coli (*up to 200 mg per liter of shake-flask culture).
- Purified by IMAC

Expression, purification and SPR analysis of selected AR proteins

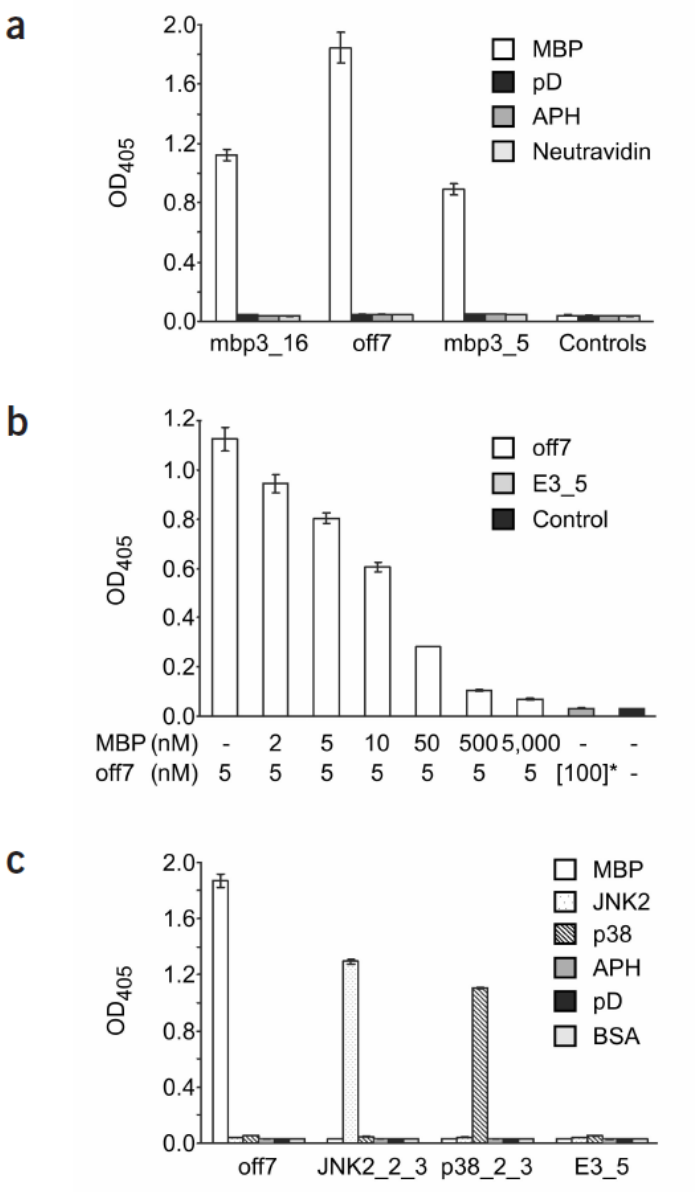


expressed in high amounts in soluble form and free of cysteines

Table 1 Kinetic binding data of selected clones determined by surface plasmon resonance

Target	Clone name (length)	k_{on} [$M^{-1}s^{-1}$]	k_{off} [s^{-1}]	K_D [M]
MBP	off7 (N3C)	$4.2 \cdot 10^{5}$	$1.9 \cdot 10^{-3}$	$4.4 \cdot 10^{-9}$
	mbp3_5 (N3C)	$2.0 \cdot 10^{5}$	$4.4 \cdot 10^{-3}$	$22 \cdot 10^{-9}$
	mbp3_16 (N2C)	$6.0 \cdot 10^{5}$	$1.0 \cdot 10^{-2}$	$17 \cdot 10^{-9}$
JNK2	JNK2_2_3 (N2C)	$9.7 \cdot 10^{5}$	$2.0 \cdot 10^{-3}$	$2.1 \cdot 10^{-9}$
p38	p38_2_3 (N2C)	$9.5 \cdot 10^{5}$	$3.5 \cdot 10^{-3}$	$3.7 \cdot 10^{-9}$

ELISAs with selected AR proteins



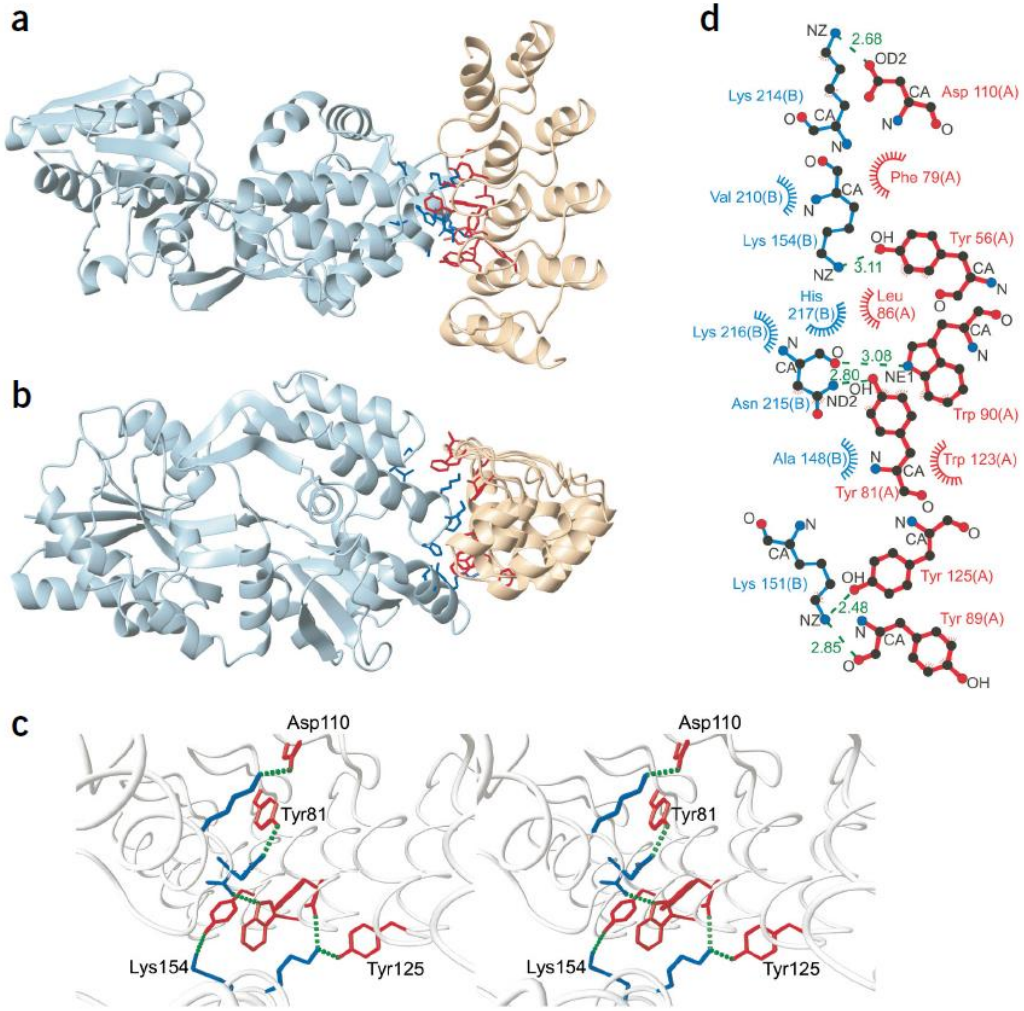
Specificity of MBP binders

Competition ELISA

Specificity comparison of
an MBP, a JNK2 and a p38 binder

Crystal structure of the designed AR protein off7 in complex with MBP

To validate our AR randomization scheme and to analyze the selected interaction at the atomic level



- An interaction interface that is comparable to that found in natural heterodimer and antibody-antigen complexes.
- AR protein binds its target with the randomized amino acids (hence validating our randomization scheme
- aromatic amino acids are involved in MBP binding, including four prominent tyrosines

Structural and functional analysis of phosphorylation-specific binders of the kinase ERK from designed ankyrin repeat protein libraries

Lutz Kummer[a], Petra Parizek[a], Peter Rube[b], Bastian Millgramm[b], Anke Prinz[b], Peer R. E. Mittl[a], Melanie Kaufholz[c], Bastian Zimmermann[c], Friedrich W. Herberg[b], and Andreas Plückthun[a,1]

[a]Department of Biochemistry, University of Zurich, 8057 Zurich, Switzerland; [b]Department of Biochemistry, University of Kassel, 34132 Kassel, Germany; and [c]Biaffin GmbH and Co. KG, 34132 Kassel, Germany

Ribosome display selected DARPins against either the unphosphorylated or the phosphorylated form of the MAPK ERK2 (ERK2 or pERK2).

Selections were carried out with **N2C and N3C libraries** consisting of two or three randomized ankyrin repeat modules between an N- terminal and a C-terminal capping repeat, respectively.

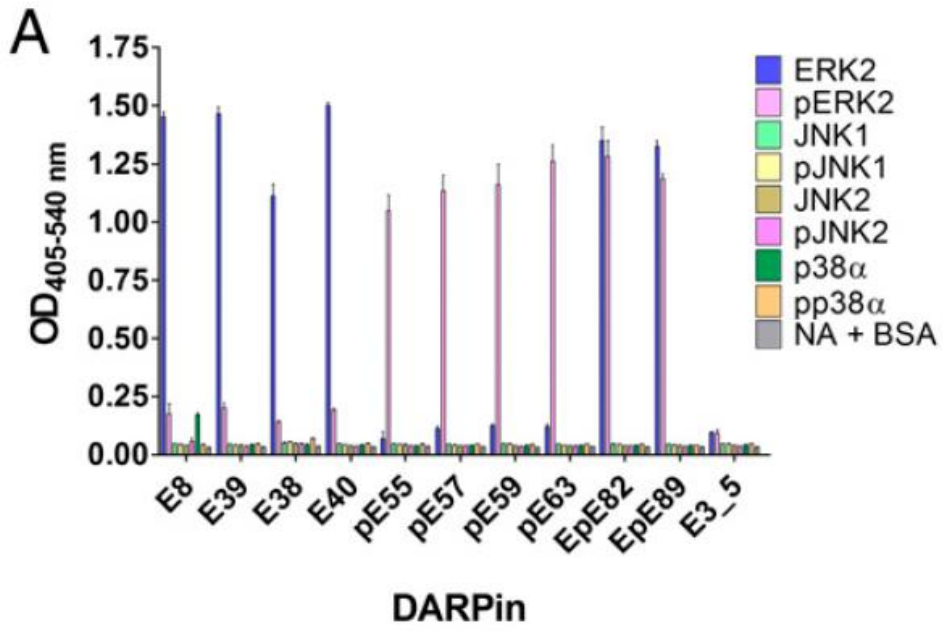
To be able to select for phosphorylation-specific DARPins, the **selection pressure** was increased by introducing a prepanning step, using the non desired ERK2 form.

Antigens: fusion proteins with an N-term avi tag for in vivo biotinylation followed by the respective MAPK and a C- terminal His tag for purification (**avi-MAPK-His6**).

Activated MAPKs required the coexpression of upstream kinases:
pLK1_ERK1+MEK1R4F and pLK1_ERK2+MEK1R4F in pAT222 vector.

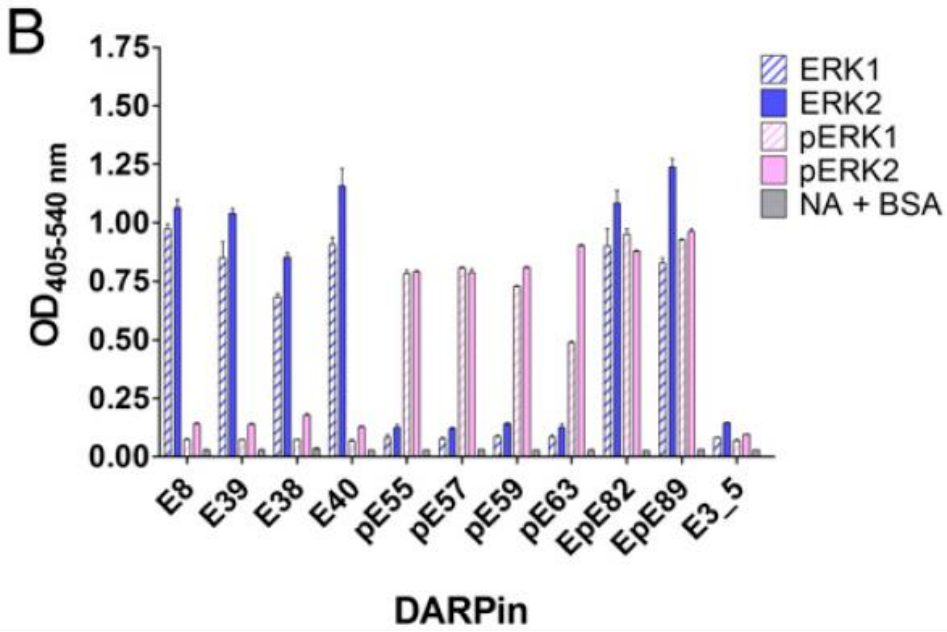
pBirAcm (Avidity) was used for **in vivo biotinylation** of MAPKs

Binding specificity analysis of selected DARPins by ELISA



Test MAPK family members with marked sequence homologies (sequence identity of >40% over the highly conserved catalytic core).

All tested DARPins were highly specific for their cognate antigens ERK2 and pERK2 and did not interact with the inactive or active form of any other MAPK

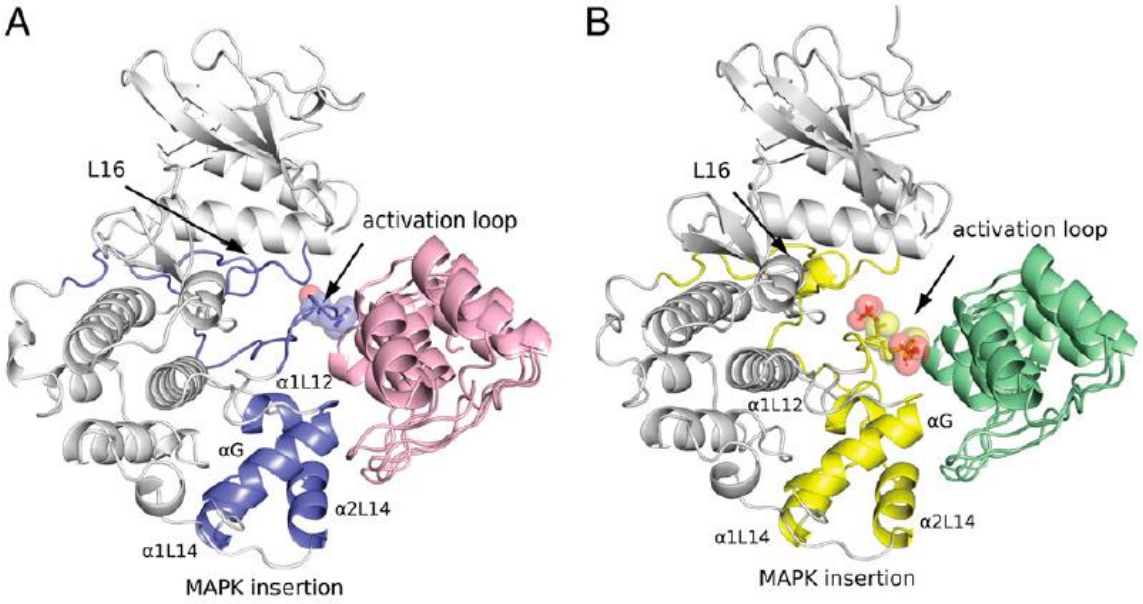


Binding of the selected DARPins to ERK2 was compared with that to ERK1, (85% sequence identity).

Tested DARPins did not discriminate between ERK1 and ERK2, but retained the phosphorylation status specificity observed for ERK2 and also for ERK1.

Crystal structures of phosphorylation status-specific DARPins E40 and pE59 in complex with ERK2 and phosphorylated ERK2

To Validate the selection strategy and explain the binding specificity on the atomic level



The binding region of DARPins includes the activation loop, the MAPK insertion, α-helix G, and partially α-helix 1L12.

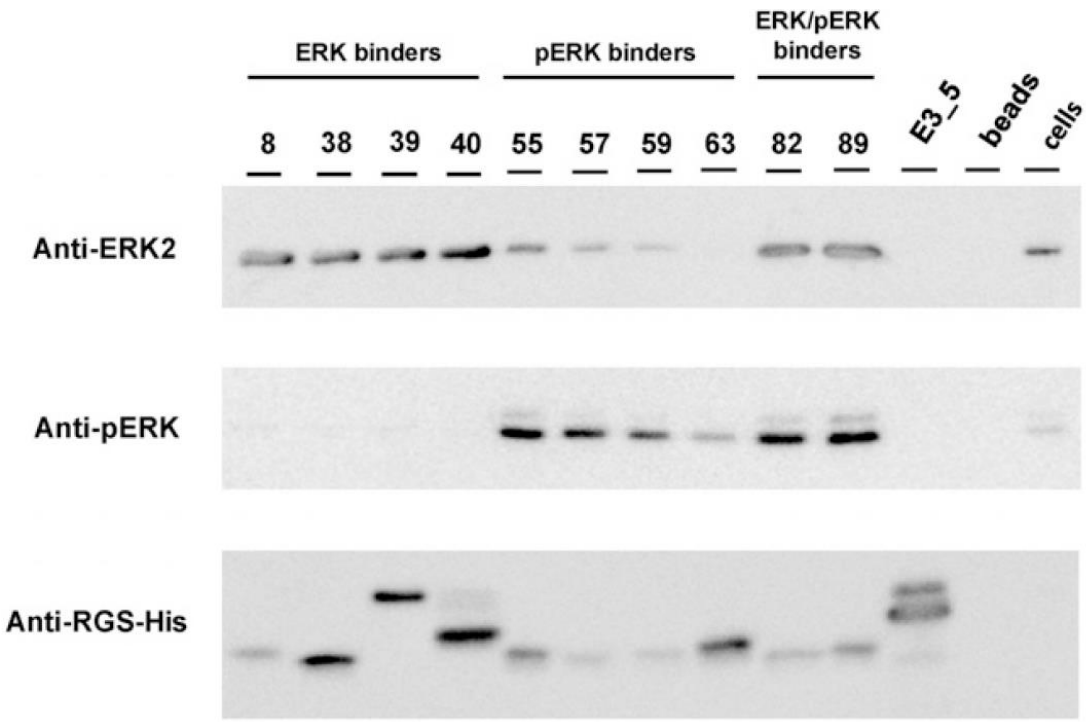
The interaction of pE59 with pERK2 relies on contact formation with identical regions and residues as identified for the E40/ERK2 complex.

Thus, **specificity of DARPins E40 and pE59 results from binding to discriminating structural elements, which have changed in spatial conformation**, but not from interaction with different amino acid residues on the target.

Affinity precipitation

Table 1. Affinity and selectivity of DARPins E40 and pE59

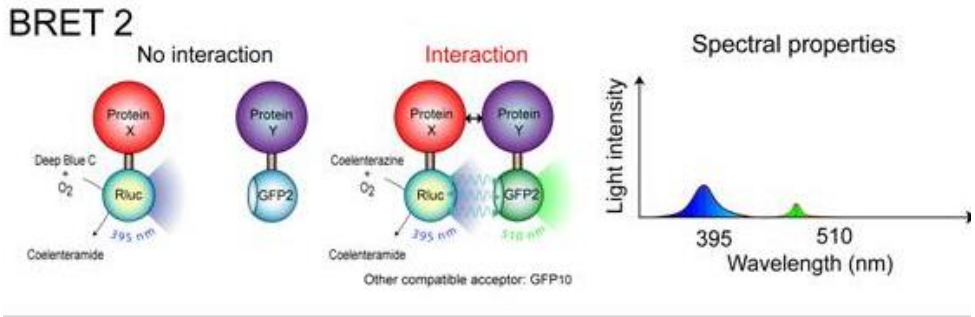
DARPin	K_D, M: ERK2	K_D, M: pERK2	Selectivity
E40	6.6×10^{-9}	1.2×10^{-6}	182
pE59	$>8.7 \times 10^{-6}$	117×10^{-9}	>74



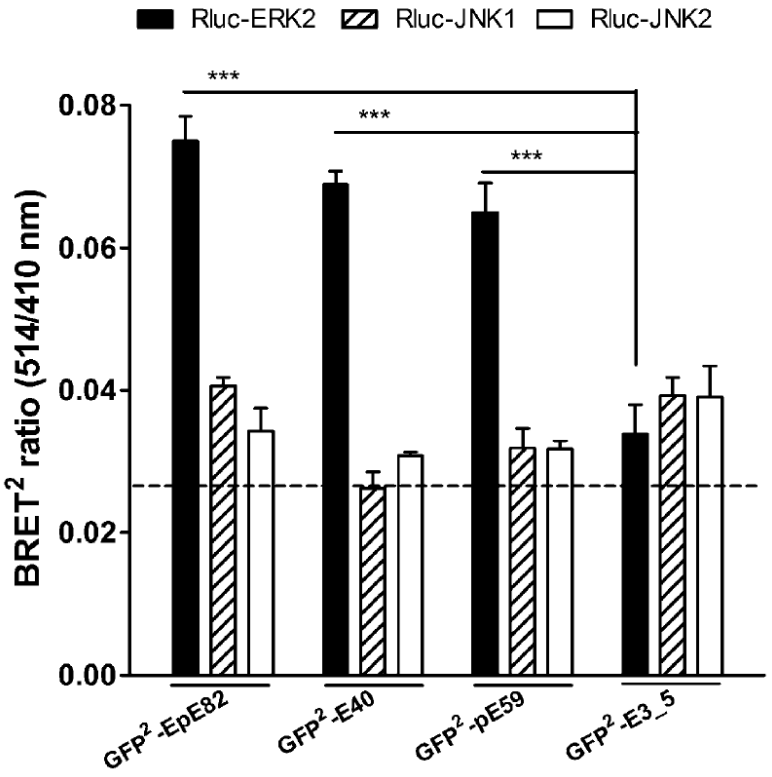
The differences in band intensities seen with ERK2 and pERK2 antibodies did not correlate with the amounts of captured DARPins, but may result from different binding affinities of the individual DARPins under the given experimental conditions

Cell lysates from HEK293T cells were incubated with His-tagged DARPins, which were subsequently captured by Ni-NTA beads. Affinity-precipitated ERK and pERK were detected on Western blots with ERK2- and pERK1/2-specific antibodies.

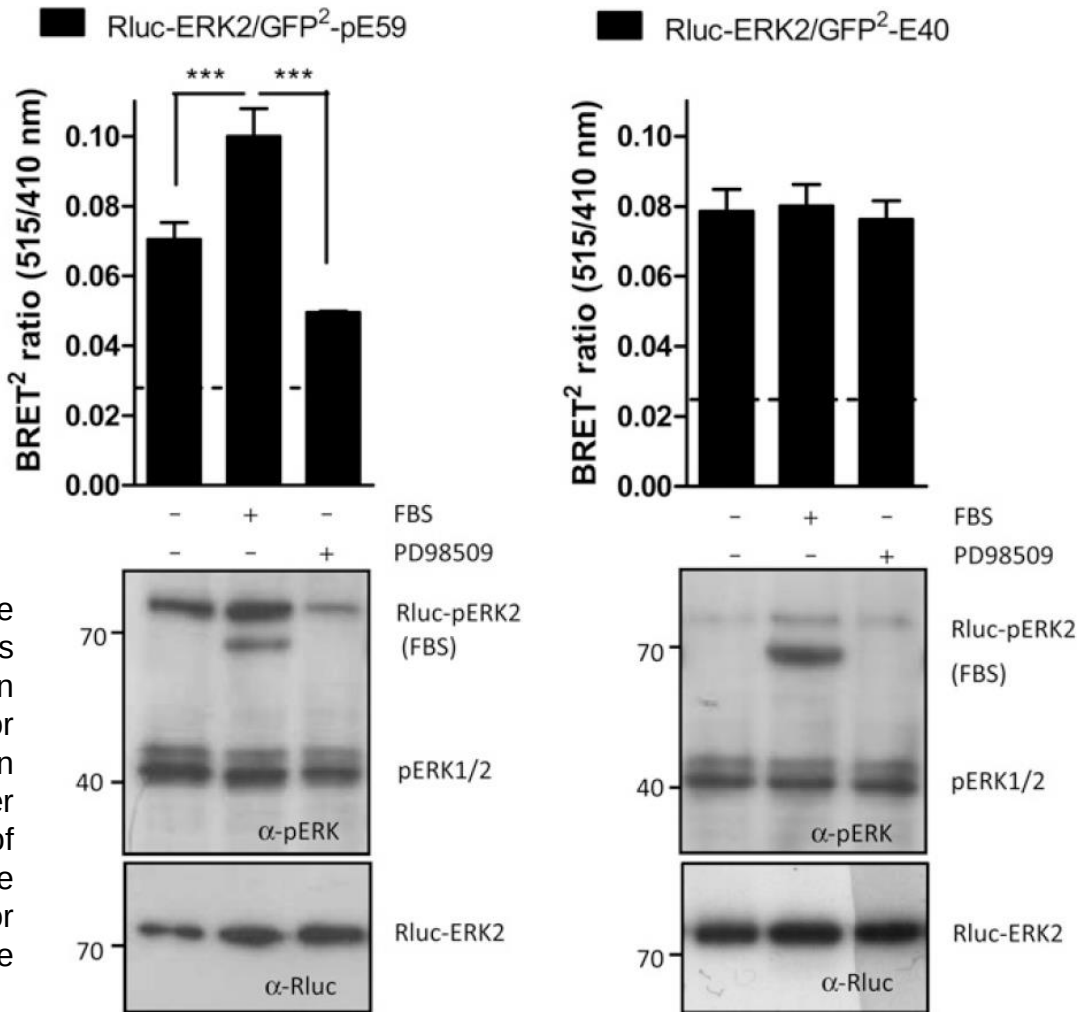
Specificity comparison of selected DARPins in living cells by Bioluminescence resonance energy transfer (BRET2) assays



DARPins fused to the N and C termini of either Renilla luciferase (Rluc) or a variant of green fluorescent protein (termed GFP2).



Functionality of selected DARPins in living cells



To determine whether the BRET2 DARPin reporters GFP2-E40 and -pE59 retain their specificity for ERK or pERK, ERK phosphorylation in COS-7 cells was either stimulated by addition of FBS or diminished by the ERK pathway inhibitor PD98509, which inhibits the upstream kinase MEK.

Both **sensors and intracellular inhibitors** can be obtained by selecting DARPins to the native targets.

DARPins are suited for **functional studies as intracellular protein-specific reagents (intrabodies),** because they do not require stabilizing disulfide bonds and because they can thus fold in the cytoplasm, where they neither aggregate nor are degraded.

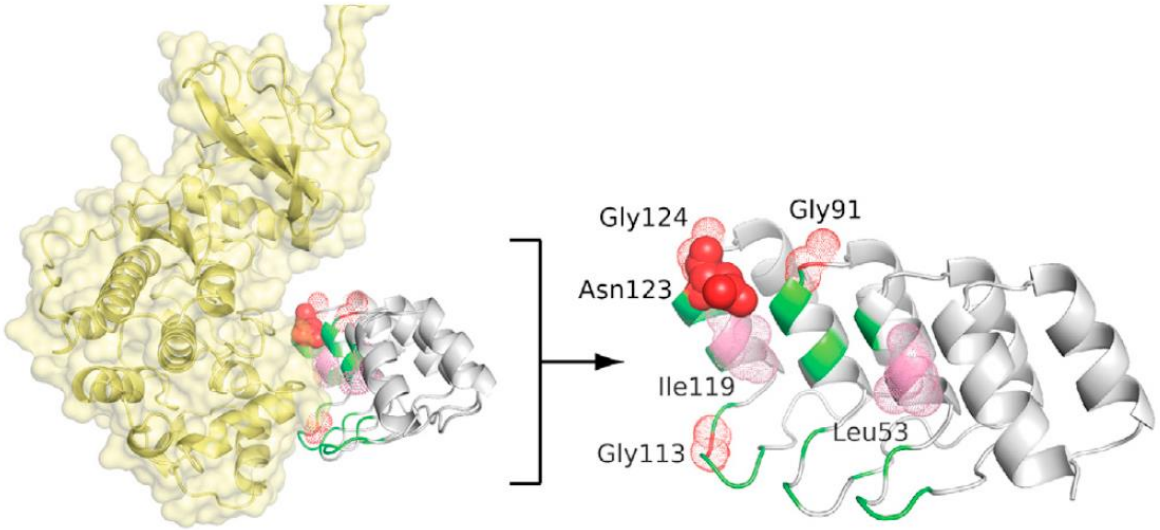
Chemistry & Biology
Resource

Knowledge-Based Design of a Biosensor to Quantify Localized ERK Activation in Living Cells

Lutz Kummer,[1] Chia-Wen Hsu,[2] Onur Dagliyan,[3] Christopher MacNevin,[2] Melanie Kaufholz,[4] Bastian Zimmermann,[4] Nikolay V. Dokholyan,[3] Klaus M. Hahn,[2] and Andreas Plückthun[1,*]

Biosensor based on the DARPin scaffold that responds specifically to active doubly phosphorylated ERK (pERK)

Sites for Dye Labeling in the Structure of DARPin pE59



Functional pE59 mutants were covalently derivatized with a diverse set of merocyanine dyes. pE59-C123m87 was selected and it is referred to as **pE59RFD biosensor**

DARPin binding to the target is detected by attachment of a bright **solvatochromatic fluorophore**, which has emissive properties that are dependent on the solvent environment.

When positioned appropriately in the binding protein, the exposure of the dye to a hydrophobic environment, which forms upon target binding within the new protein-protein interaction interface, causes a change in fluorescence intensity and/or lmax.

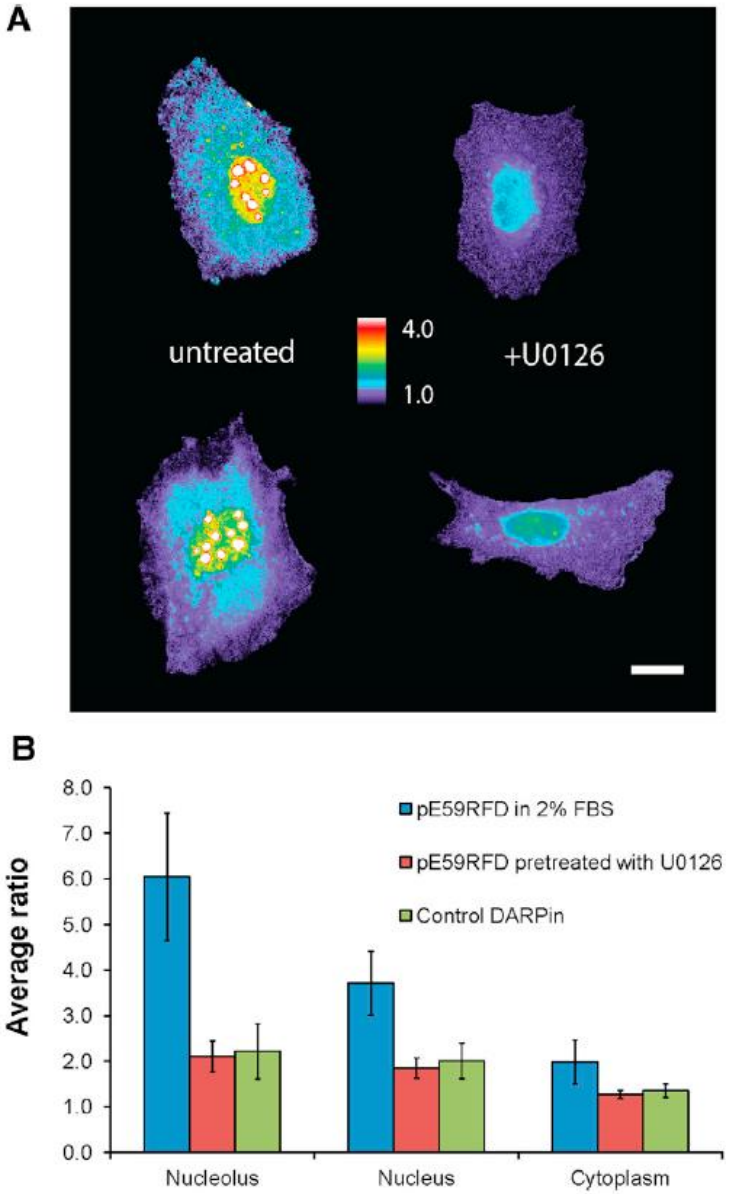
The dyes can be excited at long wavelengths (>580 nm) to avoid cell damage and diminish cellular autofluorescence.

Quantifying Activation of Endogenous ERK in Living Cells

The pE59RFD biosensor was tested in NIH 3T3 mouse embryo fibroblasts (MEFs) stably expressing YPet, a yellow fluorescent protein derivative for ratiometric imaging.

Only the altered conformation of the active kinase is detected, pointing to a concept applicable to many molecular species undergoing conformational changes in the cell.

Useful **chemical tools in studying subtle changes of protein dynamics in living cells**.

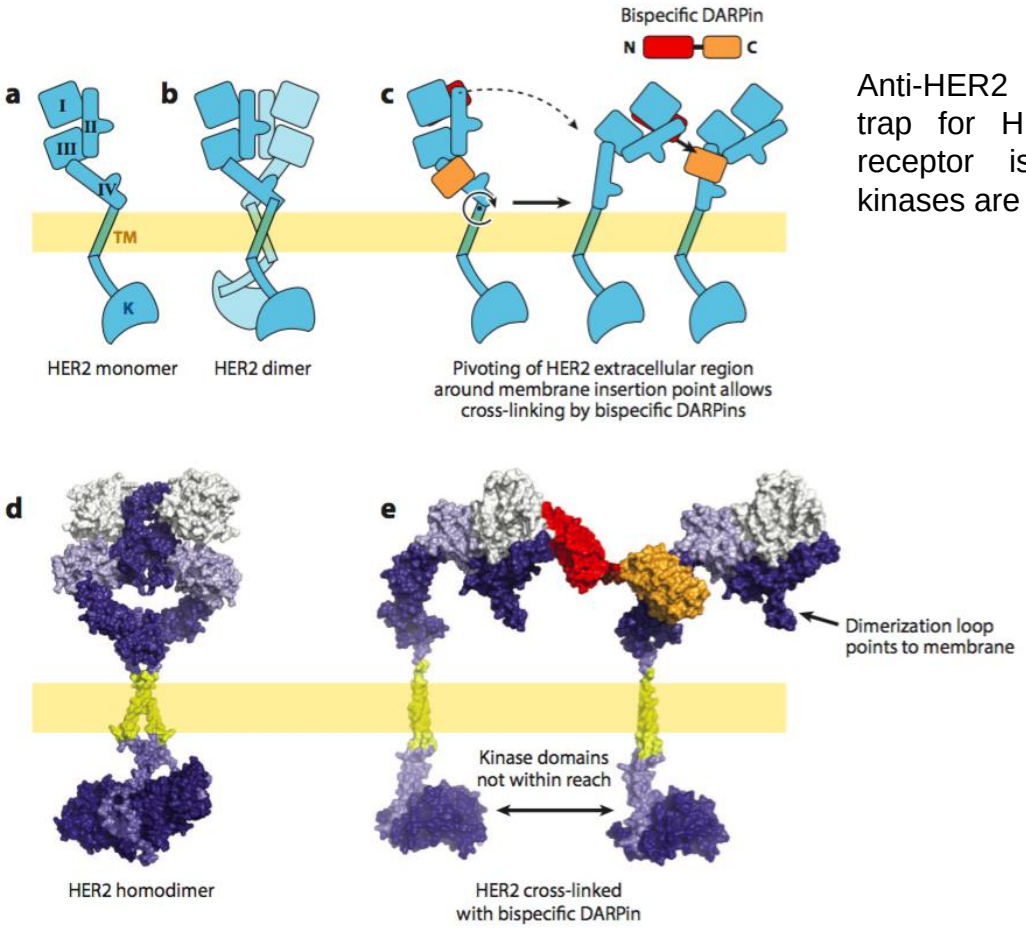


Kummer L. et al., Chemistry & Biology 20, 847–856, June 20, 2013

Bispecific DARPins to induce apoptosis in HER2-addicted tumor cells.

Human epidermal growth factor receptor-2 (HER2/ErbB2):

receptor tyrosine kinase without a known natural ligand, directly linked to the growth of malignancies from various tissues.



Anti-HER2 DARPins create a trap for HER2 in which the receptor is bent over and kinases are unable to interact.

All signaling from HER2 complexes is obstructed, leading to a pan-HER inhibition.

Jost C. et al., Structure 2013 Nov 5;21(11):1979-91

Advantages of Therapeutic DARPin

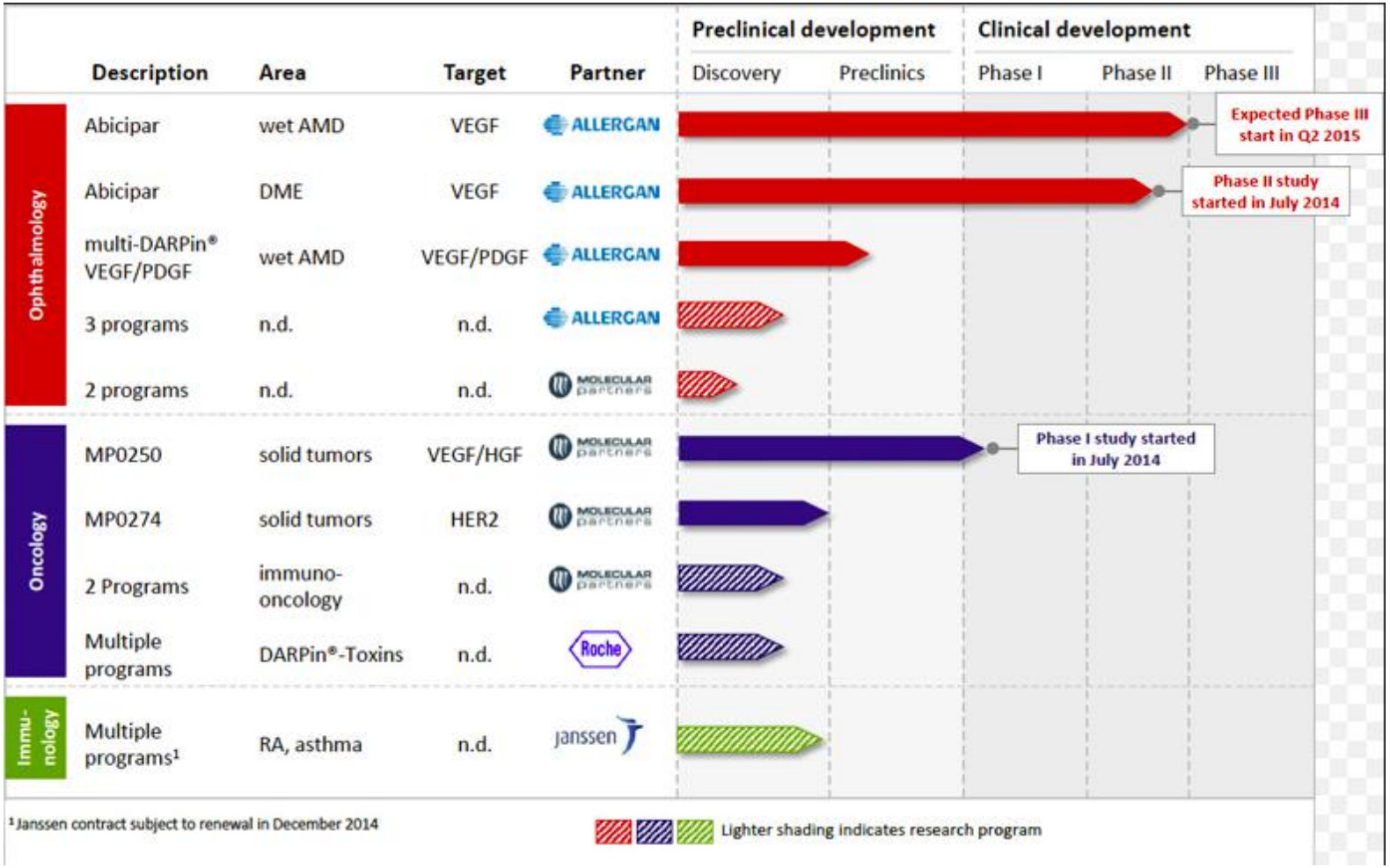
Monovalent DARPins

- High tissue penetration: easier reaching of targets outside the blood circulation.
- Absence of effector function: ideally suited for neutralization of soluble targets and undesirable side-effects emerging from binding membrane-associated variants of the target are minimized
- Adjustable pharmacokinetics (PK): Unmodified DARPins offer fast PK with predicted half-lives in the range of hours, which can be used to rapidly remove unwanted molecules from the blood stream. The half-life can be prolonged by fusion to PEG or serum protein binding molecules.
- Allosteric Inhibition as they bind their target proteins on a conformational epitope.
- New administration routes: routes in which very large amounts of drug is needed.

Conjugated DARPins

- **Deliver active moieties** to sites of disease tissue (in oncology DARPins are used to deliver toxins to tumors or in inflammation where DARPins inhibit cytokines in inflamed sites).
- **Multispecificity**: DARPins with different specificities can be fused allowing the combination of various functions in one molecule (to hijack a transcytosis receptor, binding a target in a disease tissue and recruit effector molecules in that tissue etc).

Therapeutic DARPins



Immunogenicity?

High stability and no aggregation tendency: prerequisites for **low immunogenicity**.

Possible **immunological tolerance:** the abundance of the anykrin protein itself in the erythrocytes suggests that ankyrin protein fragments are constantly in the circulation.

Thank you for your attention!