Review

Bernhard Valldorf, Steffen C. Hinz, Giulio Russo, Lukas Pekar, Laura Mohr, Janina Klemm, Achim Doerner, Simon Krah, Michael Hust and Stefan Zielonka*

Antibody display technologies: selecting the cream of the crop

https://doi.org/10.1515/hsz-2020-0377
Received November 25, 2020; accepted March 5, 2021; published online March 23, 2021

Abstract: Antibody display technologies enable the successful isolation of antigen-specific antibodies with therapeutic potential. The key feature that facilitates the selection of an antibody with prescribed properties is the coupling of the protein variant to its genetic information and is referred to as genotype phenotype coupling. There are several different platform technologies based on prokaryotic organisms as well as strategies employing higher eukaryotes. Among those, phage display is the most established system with more than a dozen of therapeutic antibodies approved for therapy that have been discovered or engineered using this approach. In recent years several other technologies gained a certain level of maturity, most strikingly mammalian display. In this review, we delineate the most important selection systems with respect to antibody generation with an emphasis on recent developments.

Keywords: antibody display; antibody engineering; antibody screening; antibody selection; genotype phenotype coupling.

Introduction

Monoclonal antibodies (mAbs) have proven to be remarkably versatile therapeutics for disease treatment including cancer and inflammatory disorders (Chan and Carter 2010; Scott et al. 2012; Yasunaga, 2020). This is exemplified by the fact that as of November 2019, around 80 mAbs have been granted marketing approval either in the US or the EU and 79 antibody-derived modalities were investigated in late stage clinical trials (Kaplon et al. 2020). Also, from a commercial perspective, antibody-based therapeutics are tremendously successful. In 2018, the global mAbs market was valued approximately 115 billion US dollars and is expected to grow to about 300 billion US dollars until 2025 (Lu et al. 2020). Moreover, the majority of top 10 drugs by global sales are antibody (Ab) therapeutics with Humira (Adalimumab) still being the most profitable entity (Urquhart 2020). As such, antibody-based therapeutics are one of the main drivers of revenues of the pharmaceutical market.

Antigen-specific mAbs can be obtained by immunization combined with hybridoma technology, which was pioneered in the 1970s by Köhler and Milstein and still remains state-of-the-art in Ab hit discovery (Köhler and Milstein 1975). Alternatively, Ab display systems such as phage display or yeast surface display enable the isolation of molecules with prescribed properties essentially resulting in several mAb therapeutics approved by healthcare authorities (Bradbury et al. 2011; Deng 2019; Frenzel et al. 2016; Lu et al. 2020). Herein, the key feature enabling the

Bernhard Valldorf, Steffen C. Hinz, Giulio Russo, and Lukas Pekar contributed equally to this work.

***Corresponding author: Stefan Zielonka**, Protein Engineering and Antibody Technologies, Merck KGaA, Frankfurter Strasse 250, D-64293 Darmstadt, Germany,
E-mail: Stefan.Zielonka@merckgroup.com. https://orcid.org/0000-0002-4649-2843
Bernhard Valldorf, Chemical and Pharmaceutical Development, Merck KGaA, Frankfurter Strasse 250, D-64293 Darmstadt, Germany
Steffen C. Hinz and Janina Klemm, Institute for Organic Chemistry and Biochemistry, Technische Universität Darmstadt, Alarich-Weiss-Strasse 4, D-64287 Darmstadt, Germany
Giulio Russo, Abcalis GmbH, Inhoffenstrasse 7, D-38124 Braunschweig, Germany; and Institut für Biochemie, Biotechnologie und Bioinformatik, Technische Universität Braunschweig, Spielmannstrasse 7, D-38106 Braunschweig, Germany. https://orcid.org/0000-0003-4099-6968
Lukas Pekar, Achim Doerner and Simon Krah, Protein Engineering and Antibody Technologies, Merck KGaA, Frankfurter Strasse 250, D-64293 Darmstadt, Germany. https://orcid.org/0000-0001-9259-0965 (L. Pekar)
Laura Mohr, Institute of Cell Biology and Neuroscience and Buchmann Institute for Molecular Life Sciences, University of Frankfurt, Max-von-Laue-Strasse 13, D-60438 Frankfurt am Main, Germany
Michael Hust, Institut für Biochemie, Biotechnologie und Bioinformatik, Technische Universität Braunschweig, Spielmannstrasse 7, D-38106 Braunschweig, Germany

isolation of a desired candidate relies on the linkage of the protein of interest (POI) (i.e. the phenotype) to its genetic information (i.e. the genotype). Hence, this principle of genotype phenotype coupling allows for 'barcoding' of up to several billion different protein variants of which binders can be selected via high-throughput identification in an iterative process. Several different display technologies are available today, comprising for instance ribosomal display, mammalian display, phage display or yeast surface display, with phage display being the most established one (Doerner et al. 2014). We aim at summarizing core principles of different platform technologies with an emphasis on recent developments and trends.

Antibody structure and formats utilized for display

Humans produce five different isotypes of immunoglobulins (Igs) referred to as IgG, IgM, IgA, IgD and IgE. Among those, IgG, which can be further divided into four different subclasses, is the most prevalent isotype in the serum (Schroeder and Cavacini 2010). Importantly, IgG is also the most relevant class in terms of therapeutic applications (Grilo and Mantalaris 2019). The Y-shaped IgG is a complex heterotetrameric protein composed of two identical heavy chains (HCs) as well as two identical light chains (LCs) with a molecular weight of approximately 150 kDa (Figure 1). Each light chain, either assigned to the κ- or λ-type, forms a heterodimer with a heavy chain and two of those resulting heterodimers, which are linked via disulfide bonds (LC-HC as well as HC-HC), represent the intact Ig. Within the molecule, each light chain is generally composed of a *N*-terminal variable domain (VL) as well as a constant domain (either CLκ or CLλ), whereas each of the heavy chains of an IgG1 consists of a *N*-terminal variable domain (VH) followed by three constant regions (CH1–CH3). From a functional perspective, this isotype has two identical fragments responsible for antigen binding (fragment antigen binding, Fab) and the fragment crystallizable (Fc part) that mediates effector functions as well as long serum half-life via binding to several distinct Fc receptors and complement proteins (Lu et al. 2018; van Erp et al. 2019).

Each Fab fragment is a heterodimer consisting of the VH-CH1 region of the heavy chain and the entire light chain (VL-CLκ or λ). Within the Fab region domain, VH and VL harbor the antigen binding site, i.e. the paratope. This paratope is composed of three hypervariable loops of the variable domain of the heavy chain and light chain, respectively. These hypervariable loops are referred to as complementarity determining regions (CDRs) and offer extraordinary variability with respect to loop length as well as amino acid composition (most strikingly in CDR-H3). Overall, those CDRs mediate highly specific antigen binding. Since Abs contain two identical Fab arms, antigen binding is bivalent and monospecific by nature. Simultaneous bivalent binding of two identical targets is enabled by the hinge region, which connects both Fab arms as wells as the Fc part and mediates sufficient flexibility (Thouvenin et al. 1997). Due to their complex nature, display of full-length IgGs is usually difficult to achieve using prokaryotic display systems or technologies engaging lower eukaryotes [of note, full IgGs can readily be displayed using mammalian systems (Dyson et al. 2020)]. Consequently, Ab fragments are typically utilized for those platform technologies. Those include Fab fragments (de Haard et al. 1999) as well as single chain variable fragments (scFvs) (Vaughan et al. 1996). ScFvs only consist of the VH and the VL domains connected by a flexible linker (Figure 1). In addition to this, camelids and sharks produce heavy chain only Abs, where antigen binding is mediated by a single variable domain (Jovčevska and Muyldermans 2019; Könning et al. 2017b; Zielonka et al. 2015). Single domain antibodies (sdAbs) display several beneficial attributes for instance multiple reformatting options when it comes to the construction of bi- and multispecific Abs (Bannas et al. 2017; Krah et al. 2016; Pekar et al. 2020; Ubah et al. 2018). sdAb repertoires can also be efficiently accessed employing genotype phenotype coupling (Roth et al. 2020; Sellmann et al. 2020; Uchański et al. 2019). Moreover, the display of bispecific entities as well as engineered Ab scaffolds has been described in literature (Fagète et al. 2017; Wozniak-Knopp et al. 2010).

Sources of antibody diversity

Different sources of diversity can be harnessed for the construction of Ab libraries. In principle, libraries can be divided into immune libraries and libraries of universal use. Immune libraries are typically constructed from tissues or blood samples of immunized animals (Clackson et al. 1991; Grzeschik et al. 2019; Rasetti-Escargueil et al. 2015; Rossant et al. 2014; Wang et al. 2016). With the advent of transgenic animals harboring the (partial) human Ig variable region gene repertoire (Brüggemann et al. 2015; Ching et al. 2018; Green et al. 1994; Lonberg 2005; Lonberg et al. 1994; Murphy et al. 2014; Osborn et al. 2013), fully human Abs can be obtained nowadays by the combination of immunization with an Ab display platform of choice

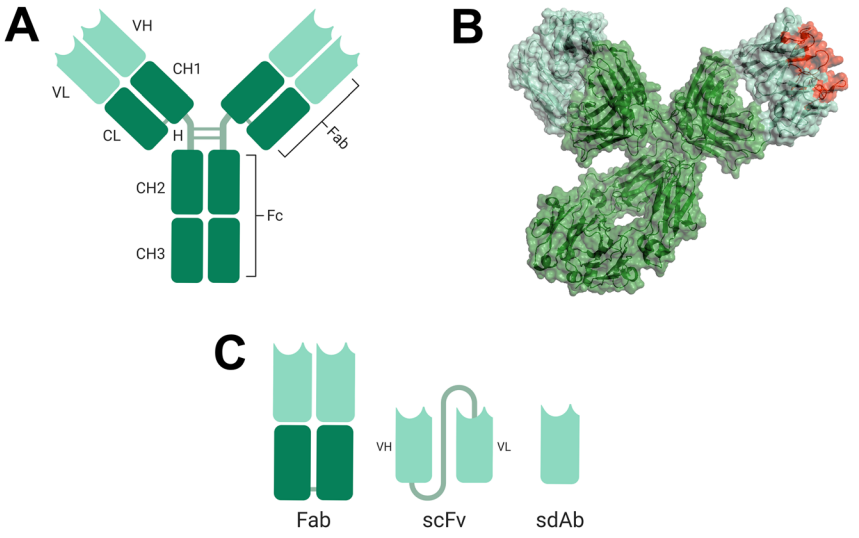

Figure 1: Antibody formats used in different display technologies.
(A) Schematic structure of an IgG antibody. VH, variable domain of the heavy chain; VL, variable region of the light chain; CL, constant domain of the light chain; CH1–CH3, constant regions of the heavy chain; H, hinge region; Fab, fragment antigen binding, Fc, fragment crystallizable.
(B) Model structure of an IgG based on pdb entry: 5dk3 (Scapin et al., 2015) generated using PyMOL v0.99. Variable domains colored in light green, constant regions in dark green and CDRs of one paratope are highlighted in red. (C) Schematic depiction of fragments used for display. ScFv, single chain fragment variable; sdAb, single domain antibody. Schemes depicted in (A) and (C) generated with biorender (www.biorender.com).

(Schröter et al. 2018). Moreover, those libraries can be generated based on humans that either have received vaccination or from patients who have suffered from a disease (Cai and Garen 1995; Coomber et al. 1999; Duan et al. 2005, 2009; Trott et al. 2014; Wenzel et al. 2020a, b). These kinds of libraries are heavily biased towards a given target since the Ab repertoire has already been pre-selected as well as affinity optimized *in vivo*. Hence, they typically contain high-affinity variants. However, their applicability is usually limited to single use only.

On the contrary, universal libraries can be considered as 'one-stop-shops' for the isolation of antigen-specific Abs against virtually every target. Accordingly, these types of libraries are of general use. Universal libraries can be sub-divided into naïve, synthetic and semi-synthetic approaches (Almagro et al. 2019). Naïve libraries make use of rearranged Ig V genes from non-immunized donors and, as consequence, typically access the IgM repertoire (de Haard et al. 1999; Kügler et al. 2015; Omar et al. 2018; Schofield et al. 2007). One of the hallmarks of such libraries relies on their combinatorial nature, i.e. during library construction heavy and light chains are randomly combined. While much efforts were made recently to keep the natural heavy and light chain pairing for immune libraries (Adler et al. 2018; Wang et al. 2018), this random i.e. unnatural pairing is generally considered to be advantageous in case of universal libraries (Lerner 2016). Many therapeutic antigens are self-molecules, but during B cell maturation autoreactive Abs expressed on the B cell surface (i.e. B cell receptors) are usually eliminated. Generally, it is considered that owing to the random combinatorial assembly of heavy and light chains, this immunological tolerance can be circumvented (at least partially), enabling the generation of Abs targeting self-antigens. Synthetic strategies are intended to maximize the functionality of libraries, e.g. by focusing on developable scaffolds or by precise control of loop length and amino acid composition using state-of-the-art methods of molecular biology (Almagro et al. 2019; Chen and Sidhu 2014; Fellouse et al. 2007; Knappik et al. 2000; Nilvebrant and Sidhu 2018; Prassler et al. 2011; Weber et al. 2014). Semi-synthetic approaches typically combine natural and synthetic Ab sequences or sequence parts (Frenzel et al. 2016). Regarding this, one extravagant example was described by Hoet and colleagues, who combined light chains from autoimmune patients with the human IGHV3-23 framework harboring autoimmune patient-derived CDR-H3 as well as synthetic CDR-H1 and CDR-H2 regions (Hoet et al. 2005). Beyond, libraries can also be constructed for a specific intended purpose, i.e. for humanization of candidates or for affinity maturation (Hu et al. 2015; Lai et al. 2018; Lou et al. 2010; Rader et al. 1998; Tiller et al. 2017).

Phage display

The most successfully applied platform technology in the context of Ab discovery is phage display (PD). As of today, 14 marketed therapeutic Abs were identified or engineered using this Nobel prize-awarded approach (Smith 2019) (Table 1), and a multitude of PD derived molecules are currently investigated in clinical trials (Frenzel et al. 2016).

Table 1: Antibodies granted marketing approval discovered or engineered using phage display or yeast surface display.

	Name	Trade name	Company	Display technology	Approach	Target	Approval	Indication(s)
1	Adalimumab	Humira	Abbott (Abbvie)	Phage display	Humanization by guided selections	TNF-α	2002 (USA), 2003 (EU)	Rheumatoide arthritis, psoriasis, Morbus Bechterew, Morbus Crohn
2	Ranibizumab	Lucentis	Novartis, Genentech/Roche	Phage display	Humanization, affinity maturation	VEGF-A	2006 (USA), 2007 (EU)	wet macular degeneration
3	Belimumab (LymphoStat-B)	Benlysta	CAT, human genome Sciences, GlaxoSmithKline (GSK)	Phage display	Initial selection	B-lymphocyte stimulator (BLyS, BAFF)	2011 (USA, EU)	Systemic lupus erythematosus (SLE)
4	Raxibacumab	ABThrax	Human genome Sciences/CAT, GSK	Phage display	Initial selection	*B. anthracis* PA	2012 (USA)	Anthrax
5	Ramucirumab	Cyramza	Imclone (Dyax)/ Lilly	Phage display	Initial selection	VEGFR2	2014 (USA, EU)	Stomach cancer, non-small-cell lung carcinoma (NSCLC)
6	Necitumumab	Portrazza	Eli Lilly	Phage display	Initial selection	EGFR	2015 (USA, EU)	NSLCC
7	Ixekizumab	Taltz	Eli Lilly	Phage display	Humanization	IL-17a	2016 (USA, EU)	Psoriasis
8	Atezolizumab	Tencentriq	Roche/Genentech	Phage display	Initial selection	PD-L1	2016 (USA), 2017 (EU)	Bladder cancer
9	Avelumab	Bavencio	Merck KGaA/EMD	Phage display	Initial selection	PD-L1	2017 (EU, USA)	Merkel-cell carcinoma
10	Guselkumab	Tremfya	Morphosys	Phage display	Initial selection	IL-23	2017 (EU, USA)	Psoriasis
11	Lanadelumab	Takhzyro	Dyax/Shire	Phage display	Initial selection	Plasma Kallikrein	2018 (USA, EU)	Hereditary angioedema
12	Caplacizumab	Cablivi	Ablynx	Phage display	Initial nanobody selection	von Willebrandt Faktor	2018 (USA), 2019 (EU)	Thrombotic thrombocytopenic purpura
13	Moxetumomab pasudotox	Lumoxiti	Medimmune/ AstraZeneca	Phage display	Murine scFv, affinity maturation	CD22	2018 (USA)	Hairy cell leukemia
14	Emapalumab	Gamifant	Novimmune	Phage display	Initial selection	INF-Gamma	2018 (USA)	Hemophagocytic lymphohistiocytosis (HLH)
15	Sintilimab	Tyvyt	Innovent Biologics/ Eli Lilly	Yeast surface display	Initial selection	PD-1	2018 (China)	Hodgkin lymphoma

PD has its origins in 1985, when George P. Smith was able to display and affinity purify short peptides (genetically) fused to the pIII minor coat protein of M13 filamentous phage (Smith 1985). With respect to Ab discovery, it was independently shown by three groups in the early 1990s that PD can be exploited for the *in vitro* selection of Ab fragments: the working groups around McCafferty and Chiswell in Cambridge (McCafferty et al. 1990), Barbas in La Jolla (Barbas et al. 1991) as well as Breitling and Dübel in Heidelberg (Breitlin et al. 1991). In general, the Ab candidate fused to pIII can be integrated into the phage genome (McCafferty et al. 1990). However, due to its enhanced flexibility, nowadays, the phagemid system is used much more frequently (Bass et al. 1990; Breitlin et al. 1991). Here, expression of the antibody::pIII fusion is uncoupled from phage replication and phage protein production. For single-stranded DNA replication and packaging into phage particles, a morphogenic signal is added. Assembly of functional phage particles is then facilitated by helper phage providing all crucial components for M13 phage packaging. This also allows for utilization of specialized helper phage, e.g. Hyperphage, enabling multicopy display and consequently avidity-based selections (Rondot et al. 2001; Soltes et al. 2007). Eventually, incorporation of

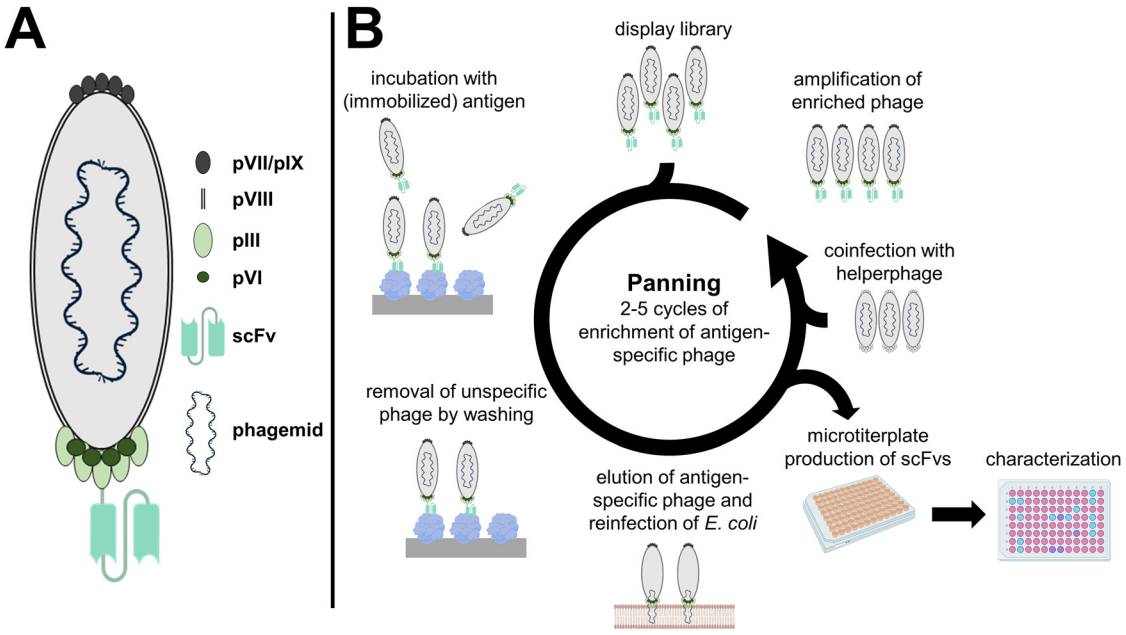

Figure 2: Schematic representation of scFv-displaying phage (A) and phage display procedure (B).
Main components of antibody-displaying phage are shown (A). pIII, phage protein III; pVI, phage protein VI; pVII, phage protein VII; pVIII, phage protein VIII; pIX, phage protein IX. (B) Overview of phage display panning. After library construction, antibody displaying phage are incubated with a particular antigen (here shown with surface immobilized antigen). Subsequently, unspecific phage are removed by washing followed by elution of antigen-specific phage *via* enzymatic treatment or pH-shift. Afterward, antigen-enriched phage are exploited for infection of *E. coli* for propagation. Co-infection with helper phage enables production and amplification of enriched phage. This procedure is commonly repeated for 2–5 cycles. Finally, after reinfection, scFvs can be produced in microtiter plates and utilized for characterization. Components created using biorender (www.biorender.com).

the antibody::pIII fusion into the phage coat results in display on the phage surface, while the corresponding genetic material resides within the phage particle (Figure 2). Albeit display of full-length Igs has been described (Mazor et al. 2010), usually Ab fragments such as scFvs (Vaughan et al. 1996), Fabs (Hoet et al. 2005) or single domain entities (Sellmann et al. 2020) are employed for PD, owing to a more robust and reliable processing by the bacterial expression host *Escherichia coli* (*E. coli*). In addition to this, fragment libraries can also be displayed on the surface of bacteriophage lambda (Garufi et al. 2005). Albeit being used less frequently, lambda display allows for N-terminal as well as C-terminal fusions of library candidates to phage proteins and might be especially beneficial when proteins cannot be secreted throughout the bacterial periplasm as phage fusion or when proteins are toxic for the host cell (Garufi et al. 2005; Gi Mikawa et al. 1996; Sternberg and Hoess 1995).

One of the main advantages of PD compared to most other platform technologies described in this review is the ease of library generation comprising huge diversities. In this respect, libraries with more than 10^{11} clones have been constructed (Table 2) (Schwimmer et al. 2013; Tiller et al. 2013). Hence, highly specific and high-affinity variants in the low nanomolar to sub-nanomolar range can be readily obtained not only from immune libraries (Miethe et al. 2014; Rasetti-Escargueil et al. 2015; Sellmann et al. 2020), but also from universal approaches, as elegantly reviewed by Almagro and co-workers (Almagro et al. 2019). Ab library generation can be conducted in several ways, for instance by sequential cloning of the light and heavy chain repertoire (Kügler et al. 2015), *via* splicing by overlap extension PCR (Davies et al. 1995; De Jaeger et al. 1997) or utilizing type IIs restriction enzymes for simultaneous introduction of heavy and light chain diversities (Nelson and Valadon 2017; Sellmann et al. 2020).

Once constructed, library candidates with desired characteristics such as affinity, selectivity or stability can be selected in a process referred to as panning (Figure 2), inspired by the process of gold mining. To this end, the library of Ab-displaying phage is incubated with the respective antigen. On the one hand, antigens can be immobilized to a solid surface e.g. to nitrocellulose (Hawlisch et al. 2001), magnetic beads (Moghaddam et al. 2003), column matrixes (Breitlin et al. 1991), polystyrene tubes (Hust et al. 2002) or microtiter plates (Barbas et al. 1991;

Table 2: Antibody gene library sizes per platform technology.

Display technology	Library size (unique clones)	References
Phage display	10^{11-12}	Schwimmer et al. 2013; Tiller et al. 2013
Bacterial display	10^{11}	Bessette et al. 2004
Yeast surface display	10^{9}	Benatuil et al. 2010; Rosowski et al. 2018
Mammalian display	10^{7-9}	Parthiban et al. 2019; Waldmeier et al. 2016
Ribosome display	10^{12-15}	Lipovsek and Plückthun 2004; Plückthun 2012

Russo et al. 2018) with high protein binding capacities. On the other hand, panning can alternatively be performed in solution using biotinylated antigens followed by 'pull-down' employing streptavidin-coated beads (Wenzel et al. 2020a, b). Moreover, selections can be carried out on primary cell lines or utilizing recombinant cells expressing the target of choice, clearly demonstrating the vast flexibility of PD. By applying stringent washing steps, the excess of target-unspecific phages can be removed and subsequently, antigen-bound phage are eluted [e.g. enzymatically (Kügler et al. 2015; Sellmann et al. 2020) or by pH-shift (Harrison et al. 1996; Smith 1985)] and used for re-infection and re-amplification of *E. coli*. Since phagemid-harboring *E. coli* cells only express the antibody::pIII fusion, helper phage infection is again needed for the production of functional phage particles. This iterative procedure is typically repeated for several rounds in order to highly enrich for antigen specific clones followed by soluble Ab fragment production and screening for specific binding either by ELISA (Russo et al. 2018), immunoblot (Hust et al. 2002) or flow cytometry (Ayriss et al. 2007). The ability to produce soluble Ab fragments simultaneously to the expression of the pIII fusion derives from the presence of an amber stop codon placed in frame between the Ab gene and the pIII gene in combination with the utilization of an amber-suppressor strain of *E. coli*. Afterward, the genetic information of positive hits is retrieved by sequencing. Moreover, sorting stringencies can be increased by implementing different depletion strategies (Droste et al. 2015; Even-Desrumeaux and Chames 2012).

Due to its high versatility, PD has been applied in manifold ways for Ab discovery and engineering. In this respect, this technology commonly is the method of choice for the *de novo* generation of candidates from universal library approaches and consequently a plethora of universal libraries is known today, as described elsewhere (Almagro et al. 2019; Hoet et al. 2005; Knappik et al. 2000; Kügler et al. 2015; Prassler et al. 2011; Schwimmer et al. 2013; Weber et al. 2014). Moreover, typical other applications of PD are Ab humanization (Baca et al. 1997; Nishibori et al. 2006; Rader et al. 1998) as well as affinity maturation of Abs. Affinity optimization of library candidates by phage display can be executed in many ways, for instance using different mutagenesis strategies (Gram et al. 1992; Tiller et al. 2017). Another prominent approach is referred to as light chain shuffling (Kang et al. 1991; Osbourn et al. 1996). Here, the heavy chain of a pre-selected binder (or heavy chains from a panel of binders) is paired with the light chain repertoire of a universal library (e.g. naïve) and re-selected under enhanced stringencies in order to obtain affinity-matured hits (Frenzel et al. 2017). A very elegant PD strategy relies in guided selection (Osbourn et al. 2005). Here, usually a rodent Ab is employed as template and each of its variable domains (e.g. the VH domain) is paired with the complementary human antibody variable domain repertoire (e.g. the human VL repertoire), respectively, and selected against the original target. Subsequently, both enriched human VH and VL repertoires are combined and further panned in order to isolate antigen-specific binders. Ultimately, this strategy leads to the identification of human Abs having similar properties as the parental non-human clone. Guided selection has been used in combination with PD for the generation of Adalimumab (Frenzel et al. 2016; Salfeld et al. 1998). However, it was shown that 'shuffling approaches' might result in a drift in the targeted epitope (Ohlin et al. 1996). Beyond, PD has proven to be versatile for a more extravagant engineering of 'next-generation' mAb entities such as common light chain bispecifics (Merchant et al. 1998), common heavy chain bispecifics (also known as κλ-bodies) (Fischer et al. 2015) or Abs comprising pH-dependent binding properties (Bonvin et al. 2015; Murtaugh et al. 2011).

Bacterial display

Bacteria have been utilized for surface display in order to combine fast growth rates as well as easy and cost-efficient handling with real-time multiparameter fluorescence analysis for cell sorting. Additionally, high library sizes have been reported for bacterial display, clearly representing a beneficial attribute (Table 2) (Bessette et al. 2004). Unlike PD or yeast surface display (described below), where mainly one specific methodology dominates the display field, many different systems of bacterial surface display have been developed in parallel of which some will be discussed in the following.

Francisco and colleagues, e.g., utilized the translocation properties of the major lipoprotein (lpp) of *E. coli* in combination with the outer membrane protein A (ompA) mediating the anchorage of the antibody on the outer membrane to achieve surface presentation (Francisco et al. 1993). Subsequently, this system was used to affinity mature an anti-digoxin scFv, which resulted in the isolation of a binder with sub-nanomolar affinity (Daugherty et al. 1998). Additional approaches employ alternative bacterial proteins either for display on the outer membrane, such as peptidoglycan associated lipoprotein (PAL) (Dhillon et al. 1999; Fuchs et al. 1991), β-domains of EhaA and intimin (Veiga et al. 2004), or for the display on the inner membrane.

In 2004, the anchored periplasmic expression technology (APEx) was published (Harvey et al. 2004). Here, in contrast to the lpp-ompA setup, the Ab fragment is either fused to lipoprotein NlpA (*N*-terminal setup) or to M13 phage gene 3 minor coat protein (g3p) (*C*-terminal setup) and displayed on the inner membrane. Another approach is the MAD-TRAP system, described by Karlsson and colleagues, which utilizes the twin-arginine translocation (tat) pathway to achieve inner membrane display of scFv molecules (Karlsson et al. 2012). Due to the location of the displayed Ab fragment, the outer membrane and cell wall need to be permeabilized or removed with EDTA and lysozyme in order to achieve accessibility for antigen and staining reagents.

The more sophisticated display of full-length Abs was achieved by Mazor and colleagues expanding the APEx system. To this end, the protein A-derived Ab-binding ZZ-domain was fused downstream of NlpA and displayed on the inner membrane of *E. coli* (Mazor et al. 2007). Additional expression of the light as well as the heavy chains and subsequent secretion into the periplasm allowed for capturing the full-length Igs via the ZZ-domain. In contrast to the display of immobilized Ab fragments, the periplasmic expression with cytometric screening (PECS) technology exploits periplasmic expression of Abs, which are retained by the outer membrane (Chen et al. 2001). Further, the antibody-antigen complex is detected by antigen-fluorophore conjugates which are limited to around 10 kDa owing to same permeability limitations retaining the Ab in the periplasm. More recently, Lombana and colleagues employed a lpp knock out *E coli* strain for non-immobilized full-length IgG display (Lombana et al. 2015). Due to the lpp gene deletion and supplementation with EDTA, the loss of outer membrane integrity allowed for antigens and immunostaining agents of up to 100 kDa to enter into the periplasm.

Besides surface display employing gram-negative bacteria, several approaches emerged relying on gram-positive bacteria such as *Staphylococcus xylosus*, *Staphylococcus aureus* or *Staphylococcus carnosus*. In these approaches, Ab fragments are either anchored by the cell surface attachment regions of protein A (XM domain) directly on the cell wall (Gunneriusson et al. 1996; Hu et al. 2018) or by endogenous sortase mediated attachment to peptidoglycan (Cavallari 2017). The capabilities of the gram-positive bacterial display method were demonstrated by e.g. Fleetwood and colleagues, who screened a VHH immune library against GFP and isolated several high affinity variants (Fleetwood et al. 2013). However, low transformation efficiencies for gram-positive bacteria still seems to limit their broad applicability in Ab discovery. Additionally, non-human-like post-translational modifications are drawbacks still existing for the different bacterial display platforms.

Yeast surface display

In addition to PD, another platform technology that successfully demonstrated to be suitable for the generation of therapeutic Abs is yeast surface display (YSD). YSD enabled the generation of the PD-1 blocking Ab molecule Sintilimab, which has gained marketing approval in China for the treatment of refractory classical Hodgkin's lymphoma (Table 1) (Hoy 2019; Wang et al. 2019; Zhang et al. 2018). This technology was pioneered in 1997 by Boder and Wittrup and relies on the genetic fusion of the library candidate to a microbial cell surface protein (Boder and Wittrup 1997). Albeit several different anchor proteins have been evaluated for displaying proteins on the surface of *Saccharomyces cerevisiae* (Kondo and Ueda 2004; Sato et al. 2002; Ueda and Tanaka 2000), the most commonly and successfully applied system is composed of the a-agglutinin complex subunits Aga1p and Aga2p (Figure 3). Here, the POI can be fused *N*-terminally or *C*-terminally to Aga2p, clearly enabling flexibility with respect to the optimal orientation that might be needed for a particular protein (Boder and Wittrup 1997; Wang et al. 2005). Typically, the resulting plasmid-encoded fusion protein is produced under the control of the galactose-inducible GAL1/10 promoter system, whereas Aga1p, which is also controlled by a galactose-inducible promoter, is integrated into the yeast genome. Upon expression, Aga2p forms two disulfide bonds with Aga1p (Pepper et al. 2008). Hence, covalent tethering of the POI is facilitated on the yeast surface, usually resulting in the display of 10^4–10^5 copies of the library candidate (Boder and Wittrup 1997).

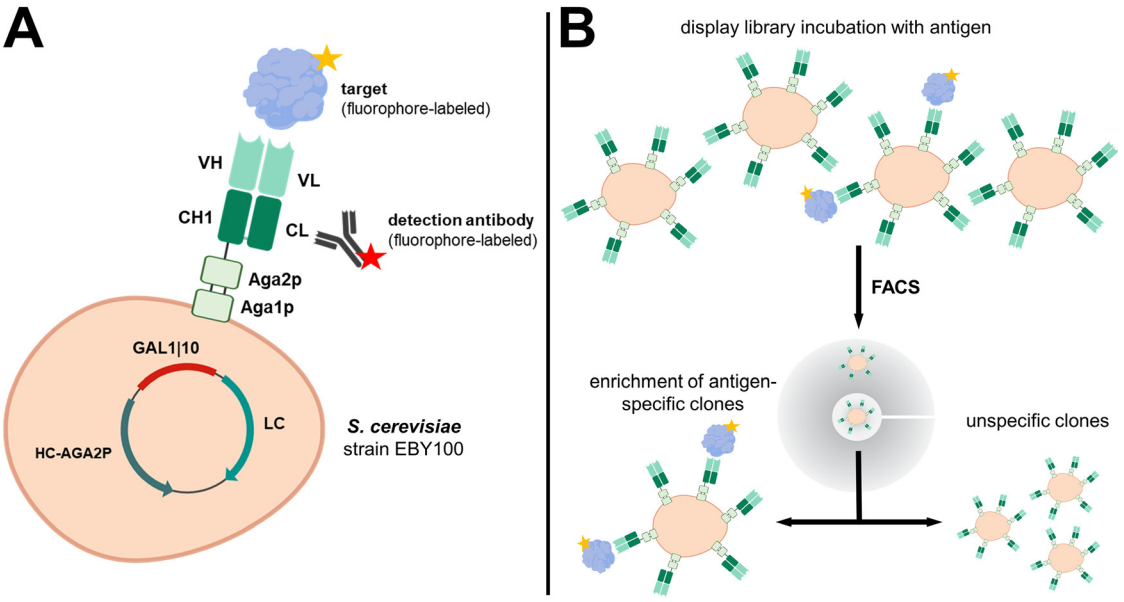

Figure 3: Overview of yeast surface display.
(A) Schematic illustration of yeast surface display based on *S. cerevisiae* strain EBY100. In this representation, expression of the heavy and the light chain for antibody Fab display is enabled by employing a one plasmid system under the control of galactose-inducible promoters. Surface display is achieved by genetically fusing the *C*-terminus of the CH1 domain to Aga2p, which upon secretion forms a covalent linkage with Aga1p. Of note, chromosomally integrated Aga1p is not shown. Utilization of fluorophore-labeled detection reagents (in this scheme directed against domain CL) as well as fluorescently labeled antigen allows FACS-based gating for functional surface expression simultaneous to antigen binding. (B) Process of library selection *via* FACS. After library generation, Fab-displaying yeast cells are incubated with the fluorophore-labeled antigen (labeling with detection reagents not shown). Hence, cells displaying an antigen-specific antibody fragment can be detected as fluorescence-positive event and sorted. Depending on the type of library, 2–5 rounds of selection can be carried out before the genetic information is obtained by sequencing. Scheme generated with biorender (www.biorender.com).

Although full-length Ig display has been achieved for YSD (Rakestraw et al. 2011; Rhiel et al. 2014; Rouha et al. 2015), Ab fragments, for instance scFvs and Fabs, are most commonly exploited in the context of Ab engineering *via* YSD (Boder and Wittrup 1997; Chao et al. 2006; Roth et al. 2019; Sivelle et al. 2018; van den Beucken et al. 2003; Weaver-Feldhaus et al. 2004). Moreover, sdAbs (Uchański et al. 2019; Zielonka et al. 2014) as well as scaffold proteins are frequently engineered using this platform approach (Könning and Kolmar 2018). YSD libraries can be constructed in manifold ways, e.g. by conventional cloning (Graff et al. 2004), in a homologous recombination-based process referred to as gap repair cloning (Swers 2004) or by Golden Gate Cloning involving type IIs restriction enzymes (Krah et al. 2018; Rosowski et al. 2018).

One of the main benefits of YSD is its compatibility with fluorescence activated cell sorting (FACS), enabling online and real-time analysis of library candidates and consequently precise control over selection on a quantitative basis (Doerner et al. 2014). In this regard, a two-dimensionally sorting strategy can be implemented using fluorescently labeled antigens as well as immunofluorescence staining to gate for clones with structural integrity (i.e. clones without frame shifts or stop codons). This can be accomplished by either employing epitope tags or by using fluorescently labeled detection reagents directed against constant components of the protein to be displayed, such as reagents targeting the constant region of the light chain in the case of Fab display (Figure 3) (Boder and Wittrup 1997; Schröter et al. 2018). Alternatively, reporter systems have been described which couple functional protein display to a fluorescence reporter read-out (Grzeschik et al. 2017; Lim et al. 2017) This allows for fine discrimination of clones based on binding affinities or kinetics. Moreover, due to the presence of more sophisticated quality control machineries for proper protein folding, it is believed that YSD enables the engineering of more complex molecules compared to technologies involving prokaryotes (Boder et al. 2012; Bowley et al. 2007; Doerner et al. 2014). After library sorting, enriched cell populations are expanded, followed by subsequent FACS-based selection rounds. Finally, the genetic information is obtained by sequencing. Additionally, enriched clones can directly be characterized in terms of binding functionality or affinity *via* flow cytometry without

the need for subcloning. In direct comparison to PD, the main bottleneck of YSD is confined library sizes (Table 2). While libraries in the range of 10^9 clones can be readily constructed for YSD (Benatuil et al. 2010; Blaise et al. 2004; Rosowski et al. 2018), libraries for PD are able to outperform this by several orders of magnitude (Almagro et al. 2019). Nevertheless, it was shown that YSD also allows for the isolation of antigen-specific Abs from universal libraries (Feldhaus et al. 2003; Puri et al. 2013; Zielonka et al. 2014). Moreover, the daily throughput of FACS is limited to the range of approximately 10^9 cells, which, however, can be increased for huge libraries by a pre-enrichment using magnetic-activated cell sorting.

One of the main applications of YSD in Ab engineering is affinity maturation (Boder and Wittrup 1997; Rappazzo et al. 2021; Tillotson et al. 2015b; van den Beucken et al. 2003). Similar to PD, this can be achieved utilizing different randomization strategies, such as CDR-targeted mutagenesis (Yang et al. 2018; Zielonka et al. 2014) or light chain shuffling (Blaise et al. 2004). In a recent work, we were able to demonstrate that also natural ligands for immune cell activating receptors can be affinity optimized by combining a semi-rational library approach with YSD (Pekar et al. 2021). Additionally, it has been shown that YSD allows for facile isolation of high-affinity candidates after animal immunization (Grzeschik et al. 2019; Roth et al. 2019; Schröter et al. 2018; Wang et al. 2016). Related to this, Johnson and colleagues demonstrated that the construction of natively paired Ab libraries (i.e. libraries retaining the original pairing of the heavy and the light chain from a distinct B cell) yield proteins with higher sensitivities and specificities after immunization than libraries with random paired chains (Adler et al. 2018). Consequently, sophisticated techniques have been established aiming at the maintenance of this natural heavy and light chain pairing (Adler et al. 2018; Wang et al. 2018). Additionally, YSD can be employed for mAb humanization (Elter et al. 2020). Like PD, YSD has proven to be versatile for the engineering of next-generation Abs such as pH-dependent mAbs (Bogen et al. 2019; Hinz et al. 2020; Könning et al. 2016; Schröter et al. 2015; Tillotson et al. 2015a), common chain entities (Krah et al. 2017; Rosowski et al. 2018), anti-idiotypic entities (Könning et al. 2017a) or antigen-binding Fc parts (Wozniak-Knopp et al. 2010). A very elegant approach for the isolation of mAbs exhibiting excellent developability properties originating from YSD libraries has been published by Krauland and colleagues (Xu et al. 2013). During Ab selection, the authors implemented a depletion step, i.e. a negative selection by employing a polyspecificity reagent, in order to enrich for clones with significantly reduced polyspecific binding. In another work, published by Lerner and colleagues, it was demonstrated that also complex antigens such as G protein-coupled receptors can be targeted by YSD in a FACS-based biopanning approach (Yang et al. 2019). In addition, yeast display can be utilized to scrutinize PD selection outputs in a high-throughput manner eventually improving selection by combining the high diversity of phage display libraries with specificity and affinity advantages provided by YSD (Ferrara et al. 2012).

Mammalian surface display

Similar to bacterial display, many different systems of mammalian surface display have been developed in parallel. With the advent of sophisticated tools of gene editing and genetic engineering, e.g. CRISPR/Cas9 or transposon technologies, tremendous progress has been made within recent years. This section aims at briefly giving an overview about some of the main techniques and recent trends.

Mammalian Ab display offers some distinct advantages compared to other selection techniques described in this review due to the use of cells not only suitable for selection but also for the production of the desired proteins. The mammalian expression and secretory apparatus is particularly favorable for the production of properly folded full-length Ig molecules with human-like glycosylation patterns and other post-translational modifications. The vast majority of marketed Ab therapeutics is produced in mammalian cells (Birch and Racher 2006; Wurm 2004). Hence, a mammalian platform technology for Ab selection enables processing of library candidates similar to the final production cell line. This facilitates a thorough selection with respect to developability besides the binding functionality by FACS (Dyson et al. 2020).

Mammalian cell surface display of Abs is typically enabled by the genetic fusion of the *C*-terminus of the heavy chain constant region to a transmembrane domain e.g. of the murine H-2Kk protein or the human platelet-derived growth factor (PDGF) receptor (Figure 4) (Bowers et al. 2014; Parthiban et al. 2019). While transient transfection has traditionally been used for library construction in mammalian cells (Higuchi et al. 1997), the field rapidly shifted to stable library generation due to a much more robust genotype phenotype coupling (Parthiban et al. 2019). Since transiently transfected cells generally produce the Ab only for a certain time period, the introduced plasmid DNA must typically be rescued after one round of selection, clearly impeding throughput and convenience in handling (Higuchi et al. 1997; Ho and Pastan 2009). This is in stark contrast to stable library generation, where, in

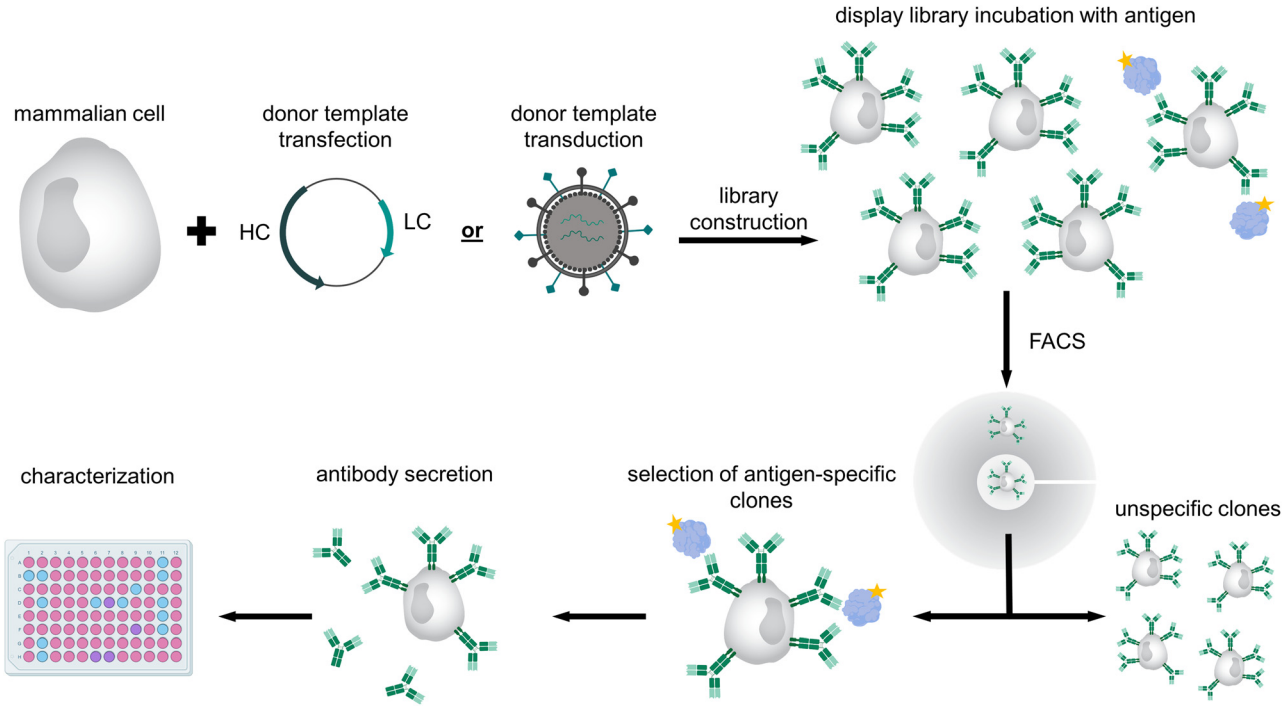

Figure 4: Schematic representation of mammalian surface display.
Antibody libraries can be generated by transfection of mammalian cells with plasmids encoding for antibody diversities. In this respect, the use of modern genetic engineering tools ensures mono-copy integrations into a predefined site and hence, monoclonal display. Moreover, libraries can also be constructed via transduction or transposase-mediated integration. Library sorting by FACS is similar to yeast surface display i.e. via incubation with fluorophore-labeled antigen and detection reagents (not shown). In several mammalian systems, owing to simultaneous display and secretion, library cells can be exploited directly for antibody production and characterization after several rounds of FACS-based enrichment. Partially generated with biorender (www.biorender.com).

theory, infinite rounds of selection can be carried out without plasmid loss (Beerli et al. 2008; Breous-Nystrom et al. 2014; Ho and Pastan 2009; Parthiban et al. 2019; Waldmeier et al. 2016).

In addition to surface display, simultaneous secretion can be achieved, for instance, by inclusion of LoxP sites flanking the sequence which encodes for the membrane anchoring protein domain, yielding cryptic splice donor sites. The alternatively spliced product lacks the transmembrane domain and is directly secreted into the medium, which enables further biophysical characterization of Abs expressed by selected cells (Bowers et al. 2014; Parthiban et al. 2019). As example, Waldmeier and colleagues used the supernatants from sorted clones for affinity determination but also checked their suitability as antibody-drug conjugates in a secondary ADC cell killing assay (Waldmeier et al. 2016). In a recent publication, Aebischer-Gumy and colleagues turned this concept upside-down resulting in a proportion of the secreted protein as displayed molecule on the cell surface by alternative splicing using the chicken cardiac troponin intron 4 between the coding sequence of the HC and the transmembrane domain (Aebischer-Gumy et al. 2020). Due to the small size (0.5 kb) of the chicken cardiac troponin intron 4, it can easily be used in plasmids for Ab display compared to the natural intron of the Ig gene (5 kb), which has been used with transposable vectors (Aebischer-Gumy et al. 2020; Waldmeier et al. 2016). The technology called SPLICELECT™ claims that the level of cell surface display can be correlated with expression titers and product quality attributes. In addition, the intron sequence can be modified in a nature either favoring display or mAb secretion, clearly enabling a certain degree of flexibility. Beyond, alternative strategies for simultaneous display and secretion have been described e.g. by a leaky translation termination signal between the HC of the Ab and a transmembrane domain (Lang et al. 2016), by non-sense codons (Bouquin et al. 2006) or by amber suppression (Chakrabarti et al. 2018).

One of the biggest challenges in mammalian display is the construction of large libraries comparable to those in bacteria or yeast. Depending on the strategy chosen for stable cell library generation, multiple or single copies of Ig genes can be integrated into the genome either randomly or

in a site-specifically manner. Standard transfection, viral transduction or transposon technologies for library generation can yield clones with multiple DNA integrations encoding for different Ab genes (Beerli et al. 2008; Breous-Nystrom et al. 2014; Waldmeier et al. 2016). Of note, polyclonal display negatively impacts genotype phenotype coupling, and consequently the isolation of the genetic information of a favored library candidate. Besides, due to the presence of different Ab chains, mispaired heavy and light chains are displayed and the enrichment of specific clones can be hampered substantially. To obviate this, the DNA utilized for transfection or viral particles can be titrated carefully, thereby enabling an average of one transfected cell displaying only one protein variant (Beerli et al. 2008; Breous-Nystrom et al. 2014; Waldmeier et al. 2016).

In general, irrespective of titration in order to obtain pseudo-monoclonal display, large libraries can be generated by random introduction of Ab genes into cells using viral transduction or transposon technology (Beerli et al. 2008; Breous-Nystrom et al. 2014; Waldmeier et al. 2016; Zhang et al. 2012). The Transpo-mAb display platform e.g. applies DNA transposition to generate mammalian cell libraries with a potential complexity of 10^9 by simple transfection (Table 2) (Waldmeier et al. 2016). Libraries of more than 10^9 clones have also been generated by B cell transduction with retroviral particles carrying light chain and heavy chain diversities, respectively, as described by Breous-Nystrom in 2014 (Breous-Nystrom et al. 2014). However, one of the main downsides of random Ab gene insertion is the variation in the expression level, which strongly depends on the transcriptional activity at the integration site.

In recent years, mammalian display made significant advances due to the application of progressive genetic engineering tools, i.e., CRISPR/Cas9 or TALE nucleases. Genetic engineering can be used to efficiently create libraries of up to 10^7 clones with strictly monoclonal display as well as gene insertion at a pre-defined integration site (Parthiban et al. 2019). In this respect, the group of McCafferty was able to create a functional chain-shuffled library of 1.8×10^6 clones using a population of VH genes pre-selected against PD-L1 combined with a repertoire of light chains. Novel high-affinity IgGs specific for PD-L1 could be selected from this mammalian Ab display library (Parthiban et al. 2019). In a different set-up Cas9-driven homology-directed repair (HDR) has been used to generate site-directed mutagenesis antibody libraries with about 10^5 variants (Mason et al. 2018). Herein, variations were introduced utilizing single-stranded oligonucleotides as the donor template at individual complementarity determining regions of a pre-integrated Ab gene in a modified hybridoma cell line, referred to as plug-and-(dis) play hybridomas (Mason et al. 2018; Pogson et al. 2016). The group was able to increase Cas9-mediated integration efficiency by more than 15-fold in the hybridoma cell line by improving an HDR plasmid donor setup that applies in situ linearization (Parola et al. 2019). Furthermore, this allowed for the efficient integration of complete VL and VH chain cassette libraries into the genome of the hybridoma cells. Interestingly, in another publication, the group combined mammalian library screening with machine learning. To this end, they trained a neural network by screening and deep-sequencing fractions of rational site-directed mutagenesis libraries based on the therapeutic entity Trastuzumab. By using this approach, antigen-binding could accurately be predicted based on Ab sequences from an *in silico* library of ~10^8 variants, which was confirmed by the expression and characterization of 30 mAbs (Mason et al. 2019). In future, this strategy could guide the rational design of mammalian Ab display libraries and help to overcome the remaining challenge of sampling the huge theoretical sequence space.

Another benefit of mammalian cell display relies on the possibility to directly implement somatic hypermutation (SHM) *in vitro*. Naturally, SHM is mediated by the enzyme activation-induced cytidine deaminase (AID) and is an essential process for optimizing Ab affinity (Di Noia and Neuberger 2007). In 2013, Bowers and colleagues have used SHM directed by AID to affinity mature and isolate antibodies (Bowers et al. 2014). In a first step, cells displaying Abs with a rather low antigen affinity were selected and their functionality was tested. In a second step, those variants were affinity optimized by *in vitro* SHM mediated by AID expression. This procedure enabled the evolution of functional high-affinity binders. In 2020, this technology has been further improved by Luo and colleagues through reengineering of the AID enzyme, optimizing the Ab sequence and the AID gene transfection procedure (Luo et al. 2020).

Besides selection, affinity maturation and production of antigen-specific Igs, mammalian display also allows to directly transduce reporter cells with an Ab library and, thereby, to directly screen for agonistic or antagonistic molecules as shown by Lerner and colleagues (Zhang et al. 2012). In this work, EpoR specific binders were enriched via PD and selected genes were switched afterward into lentiviruses for the transduction of reporter cells. These reporter cells were infected with one to four different lentiviruses resulting in an expression of the Abs inside the cell as well as a secretion of the molecules. A subsequent phenotypic screening enabled the selection of

heterodimeric bispecific agonistic molecules. The same group was able to confirm this concept in another publication by selecting an agonistic Ab specific for the granulocyte colony-stimulating factor receptor (G-CSFR) inducing human CD34+ stem cells to form neural progenitor cells (Xie et al. 2013).

Ribosome display

Besides cellular display technologies or approaches involving filamentous phage, cell-free *in vitro* methods were developed with ribosome display (RD) being the most established cell-free platform technology. Herein, compared to cellular display systems, the number of library members is not limited by the transformation efficiency of the host. This allows for the generation and screening of larger diversities in the range of 10^{12}–10^{15} variants (Table 2) (Lipovsek and Plückthun 2004; Plückthun 2012). RD mediates genotype phenotype coupling by fusing the library candidate to its corresponding mRNA, which is reverse-transcribed to DNA after selection, PCR-amplified and sequenced (Figure 5) (Yan and Xu 2006). Technically, the mRNA lacks the information of the terminal stop codon at the end of the sequence encoding for the POI. During translation, the ribosome is stalled at that position on the mRNA, because release-factors cannot enter the A-position of the ribosome, which further impedes the release of the respective polypeptide chain. This results in the formation of mRNA-ribosome-polypeptide complexes.

Similar to PD, selections can be carried out by using antigen coupled magnetic beads or antigen-coating on microtiter plates. The isolation of high affinity binders can be promoted by applying stringencies, such as a reduced antigen-concentration and an increased washing intensity during subsequent selection rounds. A limiting factor of RD is the accessibility of functional ribosomes per library reaction, which can reduce the library-size on the protein level (Kunamneni et al. 2020; Plückthun 2012).

Historically, Mattheakis and colleagues demonstrated the isolation of peptides binding a mAb with high affinity by using the display on polysomes in 1994 (Mattheakis et al. 1994). Hanes and Plückthun described RD in 1997 for the first time in the context of Ab selections and were able to 10^{8}-fold enrich a complete disulfide containing protein (i.e. an anti-hemagglutinin scFv) out of a mixture with another scFv, specific to β-lactam (Hanes and Pluckthun 1997). For *in vitro* translation, both groups utilized *E. coli* cell extracts. In the same year, generation of antibody-ribosome-mRNA (ARM) complexes using eukaryotic cell extracts was described. To this end, single chain VH/κ

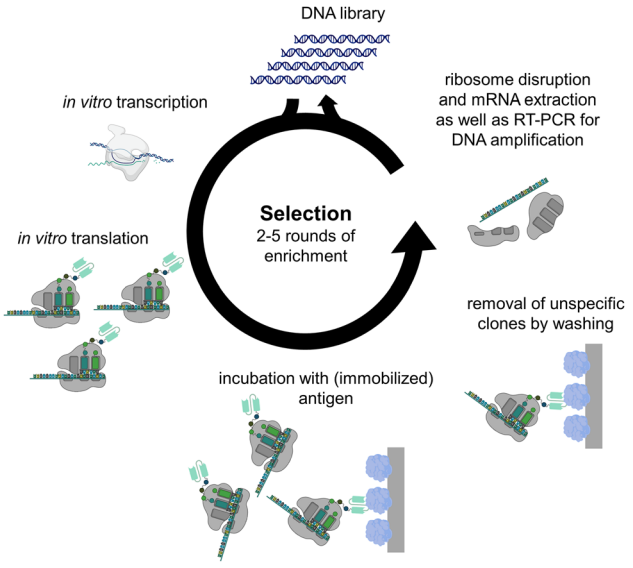

Figure 5: Principle of ribosome display.
The amplified DNA library consists of (5′ to 3′) a promotor, the ribosomal binding site, the gene of interest and no stop codon. DNA is transcribed *in vitro* to RNA followed by *in vitro* translation with cell extracts (prokaryotic or eukaryotic). At the end of translation, mRNA-ribosome complexes are formed that are incubated with immobilized antigen. After successive washing steps, non-binding complexes are removed, and *in situ* RT-PCR followed by PCR-amplification is applied to recover DNA of antigen binding variants. This DNA output can either be exploited for post selection characterization or another ribosome display cycle. Figure created with biorender (www.biorender.com).

fragments (i.e. a fusion of VH to a complete κ-light chain) specific to progesterone were selected by application of antigen-coupled magnetic beads enabling a 10^{4}–10^{5} enrichment in a single cycle (He 1997). In another approach, the yeast transcription factor GCN4 was utilized for the generation of a murine immune library, which was subsequently used for RD selection, enabling the isolation of a scFv binding the antigen with a double digit picomolar affinity (Hanes et al. 1998). This scFv exhibited a 65-fold higher affinity than its "presumed" progenitor with only one amino acid substitution in CDR-L1. Plückthun and colleagues hypothesized that the incorporation of this mutation occurred during the RD process itself and not during *in vivo* Ab maturation. Therefore, the use of RD for molecule selection may overcome tedious optimization steps, as features like the maturation of Ab affinity might be inherent in the RD process.

Today, RD has been extensively studied, especially for Ab affinity maturation (Dreier and Plückthun 2012; Jermutus 2001; Levin and Weiss 2006). Library candidates from immune and PD repertoires have been affinity optimized with improvements of more than 1000-fold (Groves

and Osbourn 2005). Moreover, RD has been applied to generate high-affinity Abs from a synthetic library (HuCAL) (Hanes et al. 2000). Unlike cellular display approaches RD allows the simple incorporation of additional diversity also throughout the screening process, by applying gene-shuffling or error-prone PCR amplification. In this regard, selected scFvs against insulin accumulated mutations introduced by Ab amplification with a low fidelity DNA-polymerase, resulting in 40-fold improved binding affinities compared to their progenitors, originally present in HuCAL (Hanes et al. 2000).

Besides RD, mRNA-display or cDNA-display represent complementary technologies, fusing mRNA to puromycin, which is an analog of a tyrosyl-tRNA and mimics tyrosine and adenosine with part of its structure. Essentially, the amide bond in the puromycin molecule is not hydrolyzable and resulting in a physical linkage between the mRNA and its protein at the end of translation (Josephson et al. 2014). As mRNA is unstable, a puromycin linker DNA is used as a primer for reverse transcription in cDNA-display, forming mRNA–cDNA hybrids (Yamaguchi et al. 2009). Finally, also CIS display enables the selection of library candidates (Odegrip et al. 2004). CIS display takes advantage of the cis activity of a DNA replication initiator protein referred to as RepA that exclusively binds to the DNA template from which it has been expressed. By genetically fusing the respective library candidate to the gene encoding RepA genotype phenotype coupling is preserved.

B cell selection

Albeit not being considered an engineered platform technology *per se*, the most natural kind of genotype phenotype coupling is provided by the B cell itself. Besides displaying an Ab variant on its surface in form of a B cell receptor, the B cell carries the corresponding genetic information inside the cell as mRNA. Hence, it is tempting to directly utilize B cells and their Ig repertoire (e.g. after immunization) for Ab discovery (Seeber et al. 2014). This also affords the benefit to preserve the natural heavy and light chain pairing of a particular B cell, which is advantageous since random reassembly during library generation may cause a loss of specificity and affinity (Adler et al. 2018) or even evoke self-reactive molecules (which is wanted for universal libraries as discussed above but may be considered as detrimental for immune approaches) (Moon et al. 2011). In contrast to hybridoma technology, selecting B cells directly obviates the need for cell fusion with tumor cell lines which is typically a tedious and inefficient procedure and might also result in polyclonal Ab variants (Bradbury et al. 2018). Moreover, with hybridoma one is kind of restricted to certain species, while exploiting B cells for selection enables mining of (in theory) every species imaginable.

In general, single B cell Ab selection is a consecutive multi-step process. At first (antigen-specific) single B cells must be detected within a population of different cells. After detection and isolation, the genetic information encoding for the Ab variable genes have to be obtained by molecular biology techniques, e.g. RT-PCR, and subcloned into adequate expression vectors. Finally, the identified variant can be produced and characterized in more detail. Single cell B cell isolation can either be performed in a random fashion, i.e. without selecting for the binding functionality, or in an antigen-selective manner which obviously is the more sophisticated and promising approach for hit discovery. To this end, several different techniques have been described (Gross et al. 2015) utilizing e.g. antigen-coated magnetic beads or fluorochrome-labeled antigens via multi-parameter FACS (Malkiel et al. 2016). In this regard, one of the most commonly applied methodologies for the isolation of antigen-specific B cells is fluorescence-activated cell sorting. Here, a multi-parameter labeling strategy can easily be employed. For instance, fluorescently labeled Abs against surface markers of B cells (such as CD19) can be utilized to gate for the specific B cell population within a tissue sample. Application of fluorochrome-labeled antigen further enables the isolation of antigen-positive B cells. In addition to this, the involvement of next generation sequencing (NGS) for high-throughput sequencing of Ig genes in single B cell approaches allows for gathering detailed information about the diversities and dynamics of B lymphocyte populations in individuals (Goldstein et al. 2019; Singh et al. 2019).

To this date, several human mAbs addressing different diseases have been developed based on single B cell selection, of which several are currently under clinical evaluation (Bailey et al. 2018; Cox et al. 2016; Fu et al. 2016; Morris et al. 2011; Yang et al. 2020).

Complementary to B cell selection and other *in vitro* display methodologies described herein, certain hit discovery campaigns demand screening of secreted molecules directly from mAb producing cells. Recent years have seen unparalleled development of microfluidic applications for Ab discovery, both in academic and pharmaceutical research (Shembekar et al. 2018; Fitzgerald and Leonard 2017; Seah et al. 2018). Microfluidics can support native chain paired library generation (Rajan et al. 2018; Tanno et al. 2020) as well as direct screening of Ig secreting cells such as plasma cells obtained by rodent immunization

(Gérard et al. 2020) or plasmablasts from human peripheral blood (Rogers et al. 2020) (Renn et al. 2020). Abs secreted from single cells are interrogated after compartmentalization in droplets or nanostructures, such as nanopens or nanowells, mostly by fluorescence-based methods allowing real-time and high-throughput sorting. Methodologies range from selection for binding on recombinant target or cells to internalization or functional screening utilizing reporter gene target cells, as exemplified by recent work from Gérard and co-workers (Gérard et al. 2020). While broad diversities of neutralizing Abs against infectious diseases such as HIV, Ebola or COVID-19 have been identified from convalescent individuals (Renn et al. 2020; Wang et al. 2018; Wippold et al. 2020), microfluidics can be valid for generating tool antibodies (Winters et al. 2019) as well as therapeutic molecules targeting cancer or immunological diseases (Asensio et al. 2019; Bounab et al. 2020). Several companies offer customizable microfluidic chips and Ab screening campaigns (Gérard et al. 2020; Shembekar et al. 2018; Wippold et al. 2020) and stand-alone devices are commercially available (Josephides et al. 2020; Winters et al. 2019), further driving development and diversification of microfluidic methodologies for therapeutic Ab discovery.

Conclusion

Display technologies enable the selection and engineering of Abs with therapeutic relevance. The fact that – to the best of our knowledge – 15 mAb-derived entities have been granted marketing approval either by US, European or Chinese healthcare authorities (Table 1), of which 10 molecules reached market access within the last five years (Frenzel et al. 2016), is clearly corroborating the major impact those platform technologies have, with respect to drug discovery. Among all therapeutics obtained by Ab display, PD is evidently the most successfully applied approach with 14 therapeutic molecules, either identified or engineered using this technology. Notwithstanding, meanwhile several mAb-derived entities obtained from other display techniques are under clinical investigation, culminating in the approval of Sintilimab in China (Deng 2019; Hoy 2019). Essentially, each display technology comes with its own benefits and downsides. For instance, YSD allows for a very precise control over selection due to the possibility of FACS-based real-time and on-line analysis of library candidates (Doerner et al. 2014). However, library sizes are appreciably restricted compared to PD or RD (Almagro et al. 2019; Benatuil et al. 2010; Blaise et al. 2004; Plückthun 2012). In recent years, mammalian display emerged as promising platform technology for Ab engineering. At least, this can be partially explained by the advent of sophisticated genetic engineering tools such as Nobel-prize awarded CRISPR/Cas9 or transposase-based systems allowing for stable generation of libraries with adequate sizes as well as mono-copy insertions at a predefined integration site (Ledford and Callaway 2020). In this respect, a plethora of different technologies have been described (Aebischer-Gumy et al. 2020; Parola et al. 2019; Parthiban et al. 2019; Pogson et al. 2016; Waldmeier et al. 2016). Ultimately, mammalian display aims at ensuring translatable features for a potential therapeutic candidate after reformatting (i.e. biophysical properties or expression yields), since the Ab production and processing apparatus of the library host is quite similar to the final production cell line. However, slow growth rates of mammalian cells can obviously be considered as one significant limitation compared to prokaryotic display systems or platform technologies based on lower eukaryotes.

Beyond display technologies, microfluidic systems were developed that have proven to support mAb discovery from Ig secreting cells, such as plasmablasts or plasma cells (Gérard et al. 2020; Renn et al. 2020). In addition to select exclusively for binding, those devices also enable the implementation of functional screenings. As such, microfluidics has the capability to enrich the toolbox for Ab hit discovery.

This also holds true for NGS which can be combined with literally every platform technology imaginable (Barreto et al. 2019; D'Angelo et al. 2014; Ferrara et al. 2020; Rouet et al. 2018; Yang et al. 2017). Additionally, NGS can be used as platform technology for Ab hit discovery on its own (Saggy et al. 2012; Valdés-Alemán et al. 2014).

Interestingly, also novel and rather unconventional techniques that are not within the scope of this work have been described for genotype phenotype coupling, such as peptide barcoding (Miyamoto et al. 2019). Eventually, it will be interesting to see how all these different approaches contribute to the global drug discovery engine within the near future.

Acknowledgments: This work is dedicated to Harald Kolmar on the occasion of his 60th birthday.

Author contributions: All the authors have accepted responsibility for the entire content of this submitted manuscript and approved submission.

Research funding: This work was partially funded by EU project ATAC (Horizon2020 number: 101003650).

Conflict of interest statement: G.S. is affiliated with Abcalis GmbH. M.H. is affiliated with Yumab GmbH. B.V.,

S.K., A.D. and S.Z. are employees of Merck KGaA. Besides, the authors declare that the work was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

References

Adler, A.S., Bedinger, D., Adams, M.S., Asensio, M.A., Edgar, R.C., Leong, R., Leong, J., Mizrahi, R.A., Spindler, M.J., Bandi, S.R., et al. (2018). A natively paired antibody library yields drug leads with higher sensitivity and specificity than a randomly paired antibody library. mAbs 10: 431–443.

Aebischer-Gumy, C., Moretti, P., Ollier, R., Ries Fecourt, C., Rousseau, F., and Bertschinger, M. (2020). SPLICELECT™: an adaptable cell surface display technology based on alternative splicing allowing the qualitative and quantitative prediction of secreted product at a single-cell level. mAbs 12: 1709333.

Almagro, J.C., Pedraza-Escalona, M., Arrieta, H.I., and Pérez-Tapia, S.M. (2019). Phage display libraries for antibody therapeutic discovery and development. Antibodies 8: 44.

Asensio, M.A., Lim, Y.W., Wayham, N., Stadtmiller, K., Edgar, R.C., Leong, J., Leong, R., Mizrahi, R.A., Adams, M.S., Simons, J.F., et al. (2019). Antibody repertoire analysis of mouse immunization protocols using microfluidics and molecular genomics. mAbs 11: 870–883.

Ayriss, J., Woods, T., Bradbury, A., and Pavlik, P. (2007). High-throughput screening of single-chain antibodies using multiplexed flow cytometry. J. Proteome Res. 6: 1072–1082.

Baca, M., Presta, L.G., O'Connor, S.J., and Wells, J.A. (1997). Antibody humanization using monovalent phage display. J. Biol. Chem. 272: 10678–10684.

Bailey, M.J., Duehr, J., Dulin, H., Broecker, F., Brown, J.A., Arumemi, F.O., Bermúdez González, M.C., Leyva-Grado, V.H., Evans, M.J., Simon, V., et al. (2018). Human antibodies targeting Zika virus NS1 provide protection against disease in a mouse model. Nat. Commun. 9, https://doi.org/10.1038/s41467-018-07008-0.

Bannas, P., Hambach, J., and Koch-Nolte, F. (2017). Nanobodies and nanobody-based human heavy chain antibodies as antitumor therapeutics. Front. Immunol. 8, https://doi.org/10.3389/fimmu.2017.01603.

Barbas, C.F., Kang, A.S., Lerner, R.A., and Benkovic, S.J. (1991). Assembly of combinatorial antibody libraries on phage surfaces: the gene III site. Proc. Natl. Acad. Sci. U.S.A. 88: 7978–7982.

Barreto, K., Maruthachalam, B.V., Hill, W., Hogan, D., Sutherland, A.R., Kusalik, A., Fonge, H., DeCoteau, J.F., and Geyer, C.R. (2019). Next-generation sequencing-guided identification and reconstruction of antibody CDR combinations from phage selection outputs. Nucleic Acids Res. 47: e50.

Bass, S., Greene, R., and Wells, J.A. (1990). Hormone phage: an enrichment method for variant proteins with altered binding properties. Protein Struct. Funct. Genet. 8: 309–314.

Beerli, R.R., Bauer, M., Buser, R.B., Gwerder, M., Muntwiler, S., Maurer, P., Saudan, P., and Bachmann, M.F. (2008). Isolation of human monoclonal antibodies by mammalian cell display. Proc. Natl. Acad. Sci. U.S.A. 105: 14336–14341.

Benatuil, L., Perez, J.M., Belk, J., and Hsieh, C.-M. (2010). An improved yeast transformation method for the generation of very large human antibody libraries. Protein Eng. Des. Sel. 23: 155–159.

Bessette, P.H., Rice, J.J., and Daugherty, P.S. (2004). Rapid isolation of high-affinity protein binding peptides using bacterial display. Protein Eng. Des. Sel. 17: 731–739.

Birch, J.R. and Racher, A.J. (2006). Antibody production. Adv. Drug Deliv. Rev. 58: 671–685.

Blaise, L., Wehnert, A., Steukers, M.P.G., van den Beucken, T., Hoogenboom, H.R., and Hufton, S.E. (2004). Construction and diversification of yeast cell surface displayed libraries by yeast mating: application to the affinity maturation of Fab antibody fragments. Gene 342: 211–218.

Boder, E.T., Raeeszadeh-Sarmazdeh, M., and Price, J.V. (2012). Engineering antibodies by yeast display. Arch. Biochem. Biophys. 526: 99–106.

Boder E.T. and Wittrup, K.D. (1997). Yeast surface display for screening combinatorial polypeptide libraries. Nat. Biotechnol. 15: 553–557.

Bogen, J.P., Hinz, S.C., Grzeschik, J., Ebenig, A., Krah, S., Zielonka, S., and Kolmar, H. (2019). Dual function pH responsive bispecific antibodies for tumor targeting and antigen depletion in plasma. Front. Immunol. 10, https://doi.org/10.3389/fimmu.2019.01892.

Bonvin, P., Venet, S., Fontaine, G., Ravn, U., Gueneau, F., Kosco-Vilbois, M., Proudfoot, A.E., and Fischer, N. (2015). *De novo* isolation of antibodies with pH-dependent binding properties. mAbs 7: 294–302.

Bounab, Y., Eyer, K., Dixneuf, S., Rybczynska, M., Chauvel, C., Mistretta, M., Tran, T., Aymerich, N., Chenon, G., Llitjos, J.F., et al. (2020). Dynamic single-cell phenotyping of immune cells using the microfluidic platform DropMap. Nat. Protoc.

Bouquin, T., Rasmussen, P.B., Bertilsson, P.-A., and Okkels, J.S. (2006). Regulated readthrough: a new method for the alternative tagging and targeting of recombinant proteins. J. Biotechnol. 125: 516–528.

Bowers, P.M., Horlick, R.A., Kehry, M.R., Neben, T.Y., Tomlinson, G.L., Altobell, L., Zhang, X., Macomber, J.L., Krapf, I.P., Wu, B.F., et al. (2014). Mammalian cell display for the discovery and optimization of antibody therapeutics. Methods 65: 44–56.

Bowley, D.R., Labrijn, A.F., Zwick, M.B., and Burton, D.R. (2007). Antigen selection from an HIV-1 immune antibody library displayed on yeast yields many novel antibodies compared to selection from the same library displayed on phage. Protein Eng. Des. Sel. 20: 81–90.

Bradbury, A.R.M., Sidhu, S., Dübel, S., and McCafferty, J. (2011). Beyond natural antibodies: the power of in vitro display technologies. Nat. Biotechnol. 29: 245–254.

Bradbury, A.R.M., Trinklein, N.D., Thie, H., Wilkinson, I.C., Tandon, A.K., Anderson, S., Bladen, C.L., Jones, B., Aldred, S.F., Bestagno, M., et al. (2018). When monoclonal antibodies are not monospecific: hybridomas frequently express additional functional variable regions. mAbs 10: 539–546.

Breitling, F., Dübel, S., Seehaus, T., Klewinghaus, I., and Little, M. (1991). A surface expression vector for antibody screening. Gene 104: 147–153.

Breous-Nystrom, E., Schultze, K., Meier, M., Flueck, L., Holzer, C., Boll, M., Seibert, V., Schuster, A., Blanusa, M., Schaefer, V., et al. (2014). Retrocyte Display® technology: generation and

screening of a high diversity cellular antibody library. Methods 65: 57–67.
Brüggemann, M., Osborn, M.J., Ma, B., Hayre, J., Avis, S., Lundstrom, B., and Buelow, R. (2015). Human antibody production in transgenic animals. Arch. Immunol. Ther. Exp. 63: 101–108.
Cai, X. and Garen, A. (1995). Anti-melanoma antibodies from melanoma patients immunized with genetically modified autologous tumor cells: selection of specific antibodies from single-chain Fv fusion phage libraries. Proc. Natl. Acad. Sci. U.S.A. 92: 6537–6541.
Cavallari, M. (2017). Rapid and direct VHH and target identification by staphylococcal Surface display libraries. Int. J. Mol. Sci. 18: 1507.
Chakrabarti, L., Zhuang, L., Roy, G., Bowen, M.A., Dall'Acqua, W.F., Hawley-Nelson, P., and Marelli, M. (2018). Amber Suppression coupled with inducible Surface display identifies cells with high recombinant protein productivity. Biotechnol. Bioeng., https://doi.org/10.1007/978-981-10-5759-5.
Chan, A.C. and Carter, P.J. (2010). Therapeutic antibodies for autoimmunity and inflammation. Nat. Rev. Immunol. 10: 301–316.
Chao, G., Lau, W.L., Hackel, B.J., Sazinsky, S.L., Lippow, S.M., and Wittrup, K.D. (2006). Isolating and engineering human antibodies using yeast surface display. Nat. Protoc. 1: 755–768.
Chen, G., Hayhurst, A., Thomas, J.G., Harvey, B.R., Iverson, B.L., and Georgiou, G. (2001). Isolation of high-affinity ligand-binding proteins by periplasmic expression with cytometric screening (PECS). Nat. Biotechnol. 19: 537–542.
Chen, G. and Sidhu, S.S. (2014). Design and generation of Synthetic antibody libraries for phage display. In: Ossipow, V. and Fischer, N. (Eds.), *Monoclonal antibodies*. Totowa, NJ: Humana Press, pp. 113–131.
Ching, K.H., Collarini, E.J., Abdiche, Y.N., Bedinger, D., Pedersen, D., Izquierdo, S., Harriman, R., Zhu, L., Etches, R.J., van de Lavoir, M.-C., et al. (2018). Chickens with humanized immunoglobulin genes generate antibodies with high affinity and broad epitope coverage to conserved targets. mAbs 10: 71–80.
Clackson, T., Hoogenboom, H.R., Griffiths, A.D., and Winter, G. (1991). Making antibody fragments using phage display libraries. Nature 352: 624–628.
Coomber, D.W., Hawkins, N.J., Clark, M.A., and Ward, R.L. (1999). Generation of anti-p53 Fab fragments from individuals with colorectal cancer using phage display. J. Immunol. 163: 2276–2283.
Cox, K.S., Tang, A., Chen, Z., Horton, M.S., Yan, H., Wang, X.-M., Dubey, S.A., DiStefano, D.J., Ettenger, A., Fong, R.H., et al. (2016). Rapid isolation of dengue-neutralizing antibodies from single cell-sorted human antigen-specific memory B-cell cultures. mAbs 8: 129–140.
D'Angelo, S., Kumar, S., Naranjo, L., Ferrara, F., Kiss, C., and Bradbury, A.R.M. (2014). From deep sequencing to actual clones. Protein Eng. Des. Sel. 27: 301–307.
Daugherty, P.S., Chen, G., Olsen, M.J., Iverson, B.L., and Georgiou, G. (1998). Antibody affinity maturation using bacterial surface display. Protein Eng. Des. Sel. 11: 825–832.
Davies, E.L., Smith, J.S., Birkett, C.R., Manser, J.M., Anderson-Dear, D.V., and Young, J.R. (1995). Selection of specific phage-display antibodies using libraries derived from chicken immunoglobulin genes. J. Immunol. Methods 186: 125–135.
de Haard, H.J., van Neer, N., Reurs, A., Hufton, S.E., Roovers, R.C., Henderikx, P., de Bruïne, A.P., Arends, J.W., and Hoogenboom, H.R. (1999). A large non-immunized human Fab fragment phage library that permits rapid isolation and kinetic analysis of high affinity antibodies. J. Biol. Chem. 274: 18218–18230.
De Jaeger, G., Buys, E., Eeckhout, D., Bruyns, A.-M., De Neve, M., De Wilde, C., Gerats, T., Van Montagu, M., Fischer, R., and Depicker, A. (1997). Use of phage display for isolation and characterization of single-chain variable fragments against dihydroflavonol 4-reductase from *Petunia hybrida*. FEBS (Fed. Eur. Biochem. Soc.) Lett. 403: 116–122.
Deng, M. (2019). The approval of sintilimab for classical Hodgkin's lymphoma: views and perspectives of anti-PD-1/PD-L1 antibodies in China. Antibody Ther. 2: 54–55.
Dhillon, J.K., Drew, P.D., and Porter, A.J. (1999). Bacterial surface display of an anti-pollutant antibody fragment. Lett. Appl. Microbiol. 28: 350–354.
Di Noia, J.M. and Neuberger, M.S. (2007). Molecular mechanisms of antibody Somatic hypermutation. Annu. Rev. Biochem. 76: 1–22.
Doerner, A., Rhiel, L., Zielonka, S., and Kolmar, H. (2014). Therapeutic antibody engineering by high efficiency cell screening. FEBS (Fed. Eur. Biochem. Soc.) Lett. 588: 278–287.
Dreier, B. and Plückthun, A. (2012). Rapid Selection of high-affinity binders using ribosome display. In: Douthwaite, J.A. and Jackson, R.H. (Eds.), *Ribosome display and related technologies*. New York, NY: Springer New York, pp. 261–286.
Droste, P., Frenzel, A., Steinwand, M., Pelat, T., Thullier, P., Hust, M., Lashuel, H., and Dübel, S. (2015). Structural differences of amyloid-β fibrils revealed by antibodies from phage display. BMC Biotechnol. 15, https://doi.org/10.1186/s12896-015-0146-8.
Duan, J., Yan, X., Guo, X., Cao, W., Han, W., Qi, C., Feng, J., Yang, D., Gao, G., and Jin, G. (2005). A human SARS-CoV neutralizing antibody against epitope on S2 protein. Biochem. Biophys. Res. Commun. 333: 186–193.
Duan, T., Ferguson, M., Yuan, L., Xu, F., and Li, G. (2009). Human monoclonal Fab antibodies against West Nile virus and its neutralizing activity analyzed *in vitro* and *in vivo*. J. Antivir. Antiretrovir. 1: 36–42.
Dyson, M.R., Masters, E., Pazeraitis, D., Perera, R.L., Syrjanen, J.L., Surade, S., Thorsteinson, N., Parthiban, K., Jones, P.C., Sattar, M., et al. (2020). Beyond affinity: selection of antibody variants with optimal biophysical properties and reduced immunogenicity from mammalian display libraries. mAbs 12: 1829335.
Elter, A., Bogen, J.P., Hinz, S.C., Fiebig, D., Palacios, A.M., Grzeschik, J., Hock, B., and Kolmar, H. (2020). Humanization of chicken-derived scFv using yeast surface display and NGS data mining. Biotechnol. J. 2000231.
Even-Desrumeaux, K. and Chames, P. (2012). Phage display and Selections on cells. In: Chames, P. (Ed.). *Antibody engineering*. Totowa, NJ: Humana Press, pp. 225–235.
Fagète, S., Botas-Perez, L., Rossito-Borlat, I., Adea, K., Gueneau, F., Ravn, U., Rousseau, F., Kosco-Vilbois, M., Fischer, N., and Hartley, O. (2017). Dual display: phage selection driven by co-engagement of two targets by two different antibody fragments. Protein Eng. Des. Sel. 30: 575–582.
Feldhaus, M.J., Siegel, R.W., Opresko, L.K., Coleman, J.R., Feldhaus, J.M.W., Yeung, Y.A., Cochran, J.R., Heinzelman, P., Colby, D., Swers, J., et al. (2003). Flow-cytometric isolation of human antibodies from a nonimmune Saccharomyces cerevisiae surface display library. Nat. Biotechnol. 21: 163–170.

Fellouse, F.A., Esaki, K., Birtalan, S., Raptis, D., Cancasci, V.J., Koide, A., Jhurani, P., Vasser, M., Wiesmann, C., Kossiakoff, A.A., et al. (2007). High-throughput generation of synthetic antibodies from highly functional minimalist phage-displayed libraries. J. Mol. Biol. 373: 924–940.

Ferrara, F., Naranjo, L.A., Kumar, S., Gaiotto, T., Mukundan, H., Swanson, B., and Bradbury, A.R.M. (2012). Using phage and yeast display to select hundreds of monoclonal antibodies: application to antigen 85, a tuberculosis biomarker. PloS One 7: e49535.

Ferrara, F., Teixeira, A.A., Naranjo, L., Erasmus, M.F., D'Angelo, S., and Bradbury, A.R.M. (2020). Exploiting next-generation sequencing in antibody selections – a simple PCR method to recover binders. mAbs 12: 1701792.

Fischer, N., Elson, G., Magistrelli, G., Dheilly, E., Fouque, N., Laurendon, A., Gueneau, F., Ravn, U., Depoisier, J.-F., Moine, V., et al. (2015). Exploiting light chains for the scalable generation and platform purification of native human bispecific IgG. Nat. Commun. 6, https://doi.org/10.1038/ncomms7113.

Fitzgerald, V. and Leonard, P. (2017). Single cell screening approaches for antibody discovery. Methods 116: 34–42.

Fleetwood, F., Devoogdt, N., Pellis, M., Wernery, U., Muyldermans, S., Ståhl, S., and Löfblom, J. (2013). Surface display of a single-domain antibody library on Gram-positive bacteria. Cell. Mol. Life Sci. 70: 1081–1093.

Francisco, J.A., Campbell, R., Iverson, B.L., and Georgiou, G. (1993). Production and fluorescence-activated cell sorting of *Escherichia coli* expressing a functional antibody fragment on the external surface. Proc. Natl. Acad. Sci. U.S.A. 90: 10444–10448.

Frenzel, A., Kügler, J., Helmsing, S., Meier, D., Schirrmann, T., Hust, M., and Dübel, S. (2017). Designing human antibodies by phage display. Transfus. Med. Hemother. 44: 312–318.

Frenzel, A., Schirrmann, T., and Hust, M. (2016). Phage display-derived human antibodies in clinical development and therapy. mAbs 8: 1177–1194.

Fu, Y., Zhang, Z., Sheehan, J., Avnir, Y., Ridenour, C., Sachnik, T., Sun, J., Hossain, M.J., Chen, L.-M., Zhu, Q., et al. (2016). A broadly neutralizing anti-influenza antibody reveals ongoing capacity of haemagglutinin-specific memory B cells to evolve. Nat. Commun. 7, https://doi.org/10.1038/ncomms12780.

Fuchs, P., Breitling, F., Dübel, S., Seehaus, T., and Little, M. (1991). Targeting recombinant antibodies to the surface of *Escherichia coli*: fusion to a peptidoglycan associated lipoprotein. Bio Technol. 9: 1369–1372.

Garufi, G., Minenkova, O., Passo, C.L., Pernice, I., and Felici, F. (2005). Display libraries on bacteriophage lambda capsid. In: *Biotechnology annual review*. New York: Elsevier, pp. 153–190.

Gérard, A., Woolfe, A., Mottet, G., Reichen, M., Castrillon, C., Menrath, V., Ellouze, S., Poitou, A., Doineau, R., Briseno-roa, L., et al. (2020). Screening and sequencing of antibodies using droplet microfluidics. Nat. Biotechnol. 38, https://doi.org/10.1038/s41587-020-0563-7.

Gi Mikawa, Y., Maruyama, I.N., and Brenner, S. (1996). Surface display of proteins on bacteriophage λ heads. J. Mol. Biol. 262: 21–30.

Goldstein, L.D., Chen, Y.-J.J., Wu, J., Chaudhuri, S., Hsiao, Y.-C., Schneider, K., Hoi, K.H., Lin, Z., Guerrero, S., Jaiswal, B.S., et al. (2019). Massively parallel single-cell B-cell receptor sequencing enables rapid discovery of diverse antigen-reactive antibodies. Commun. Biol. 2, https://doi.org/10.1038/s42003-019-0551-y.

Graff, C.P., Chester, K., Begent, R., and Wittrup, K.D. (2004). Directed evolution of an anti-carcinoembryonic antigen scFv with a 4-day monovalent dissociation half-time at 37°C. Protein Eng. Des. Sel. 17: 293–304.

Gram, H., Marconi, L.A., Barbas, C.F., Collet, T.A., Lerner, R.A., and Kang, A.S. (1992). In vitro selection and affinity maturation of antibodies from a naive combinatorial immunoglobulin library. Proc. Natl. Acad. Sci. U.S.A. 89: 3576–3580.

Green, L.L., Hardy, M.C., Maynard-Currie, C.E., Tsuda, H., Louie, D.M., Mendez, M.J., Abderrahim, H., Noguchi, M., Smith, D.H., Zeng, Y., et al. (1994). Antigen–specific human monoclonal antibodies from mice engineered with human Ig heavy and light chain YACs. Nat. Genet. 7: 13–21.

Grilo, A.L. and Mantalaris, A. (2019). The increasingly human and profitable monoclonal antibody market. Trends Biotechnol. 37: 9–16.

Gross, A., Schoendube, J., Zimmermann, S., Steeb, M., Zengerle, R., and Koltay, P. (2015). Technologies for single-cell isolation. Int. J. Mol. Sci. 16: 16897–16919.

Groves, M.A. and Osbourn, J.K. (2005). Applications of ribosome display to antibody drug discovery. Expet Opin. Biol. Ther. 5: 125–135.

Grzeschik, J., Hinz, S.C., Könning, D., Pirzer, T., Becker, S., Zielonka, S., and Kolmar, H. (2017). A simplified procedure for antibody engineering by yeast surface display: coupling display levels and target binding by ribosomal skipping. Biotechnol. J. 12: 1600454.

Grzeschik, J., Yanakieva, D., Roth, L., Krah, S., Hinz, S.C., Elter, A., Zollmann, T., Schwall, G., Zielonka, S., and Kolmar, H. (2019). Yeast surface display in combination with fluorescence-activated cell sorting enables the rapid isolation of antibody fragments derived from immunized chickens. Biotechnol. J. 14: 1800466.

Gunneriusson, E., Samuelson, P., Uhlen, M., Nygren, P.A., and Stähl, S. (1996). Surface display of a functional single-chain Fv antibody on staphylococci. J. Bacteriol. 178: 1341–1346.

Hanes, J., Jermutus, L., Weber-Bornhauser, S., Bosshard, H.R., and Pluckthun, A. (1998). Ribosome display efficiently selects and evolves high-affinity antibodies *in vitro* from immune libraries. Proc. Natl. Acad. Sci. U.S.A. 95: 14130–14135.

Hanes, J. and Pluckthun, A. (1997). In vitro selection and evolution of functional proteins by using ribosome display. Proc. Natl. Acad. Sci. U.S.A. 94: 4937–4942.

Hanes, J., Schaffitzel, C., Knappik, A., and Plückthun, A. (2000). Picomolar affinity antibodies from a fully synthetic naive library selected and evolved by ribosome display. Nat. Biotechnol. 18: 1287–1292.

Harrison, J.L., Williams, S.C., Winter, G., and Nissim, A. (1996). [5] Screening of phage antibody libraries. In: *Methods in enzymology*. New York: Elsevier, pp. 83–109.

Harvey, B.R., Georgiou, G., Hayhurst, A., Jeong, K.J., Iverson, B.L., and Rogers, G.K. (2004). Anchored periplasmic expression, a versatile technology for the isolation of high-affinity antibodies from *Escherichia coli*-expressed libraries. Proc. Natl. Acad. Sci. U.S.A. 101: 9193–9198.

Hawlisch, H., Müller, M., Frank, R., Bautsch, W., Klos, A., and Köhl, J. (2001). Site-specific anti-C3a receptor single-chain antibodies selected by differential panning on cellulose sheets. Anal. Biochem. 293: 142–145.

He, M. (1997). Antibody-ribosome-mRNA (ARM) complexes as efficient selection particles for in vitro display and evolution of antibody combining sites. Nucleic Acids Res. 25: 5132–5134.

Higuchi, K., Araki, T., Matsuzaki, O., Sato, A., Kanno, K., Kitaguchi, N., and Ito, H. (1997). Cell display library for gene cloning of variable regions of human antibodies to hepatitis B surface antigen. J. Immunol. Methods 202: 193–204.

Hinz, S.C., Elter, A., Rammo, O., Schwämmle, A., Ali, A., Zielonka, S., Herget, T., and Kolmar, H. (2020). A generic procedure for the isolation of pH- and magnesium-responsive chicken scFvs for downstream purification of human antibodies. Front. Bioeng. Biotechnol. 8, https://doi.org/10.3389/fbioe.2020.00688.

Ho, M. and Pastan, I. (2009). Mammalian cell display for antibody engineering. In: Dimitrov, A.S. (Ed.). *Therapeutic antibodies*. Totowa, NJ: Humana Press, pp. 337–352.

Hoet, R.M., Cohen, E.H., Kent, R.B., Rookey, K., Schoonbroodt, S., Hogan, S., Rem, L., Frans, N., Daukandt, M., Pieters, H., et al. (2005). Generation of high-affinity human antibodies by combining donor-derived and synthetic complementarity-determining-region diversity. Nat. Biotechnol. 23: 344–348.

Hoy, S.M. (2019). Sintilimab: first global approval. Drugs 79: 341–346.

Hu, D., Hu, S., Wan, W., Xu, M., Du, R., Zhao, W., Gao, X., Liu, J., Liu, H., and Hong, J. (2015). Effective optimization of antibody affinity by phage display integrated with high-throughput DNA synthesis and sequencing technologies. PloS One 10: e0129125.

Hu, F.J., Volk, A.-L., Persson, H., Säll, A., Borrebaeck, C., Uhlen, M., and Rockberg, J. (2018). Combination of phage and Gram-positive bacterial display of human antibody repertoires enables isolation of functional high affinity binders. N Biotechnol 2017: 80–88.

Hust, M., Maiss, E., Jacobsen, H.-J., and Reinard, T. (2002). The production of a genus-specific recombinant antibody (scFv) using a recombinant potyvirus protease. J. Virol. Methods 106: 225–233.

Jermutus, L. (2001). Tailoring in vitro evolution for protein affinity or stability. Proc. Natl. Acad. Sci. U.S.A. 98: 75–80.

Josephides, D., Davoli, S., Whitley, W., Ruis, R., Salter, R., Gokkaya, S., Vallet, M., Matthews, D., Benazzi, G., Shvets, E., et al. (2020). Cyto-mine: an integrated, picodroplet system for high-throughput single-cell analysis, sorting, dispensing, and monoclonality assurance. SLAS Technol 25: 177–189.

Josephson, K., Ricardo, A., and Szostak, J.W. (2014). mRNA display: from basic principles to macrocycle drug discovery. Drug Discov. Today 19: 388–399.

Jovčevska, I. and Muyldermans, S. (2019). The therapeutic potential of nanobodies. BioDrugs 34: 11–26.

Kang, A.S., Jones, T.M., and Burton, D.R. (1991). Antibody redesign by chain shuffling from random combinatorial immunoglobulin libraries. Proc. Natl. Acad. Sci. U.S.A. 88: 11120–11123.

Kaplon, H., Muralidharan, M., Schneider, Z., and Reichert, J.M. (2020). Antibodies to watch in 2020. mAbs 12: 1703531.

Karlsson, A.J., Lim, H.-K., Xu, H., Rocco, M.A., Bratkowski, M.A., Ke, A., and DeLisa, M.P. (2012). Engineering antibody fitness and function using membrane-anchored display of correctly folded proteins. J. Mol. Biol. 416: 94–107.

Knappik, A., Ge, L., Honegger, A., Pack, P., Fischer, M., Wellnhofer, G., Hoess, A., Wölle, J., Plückthun, A., and Virnekäs, B. (2000). Fully synthetic human combinatorial antibody libraries (HuCAL) based on modular consensus frameworks and CDRs randomized with trinucleotides. J. Mol. Biol. 296: 57–86.

Köhler, G. and Milstein, C. (1975). Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 256: 495–497.

Kondo, A. and Ueda, M. (2004). Yeast cell-surface display? applications of molecular display. Appl. Microbiol. Biotechnol. 64: 28–40.

Könning, D. and Kolmar, H. (2018). Beyond antibody engineering: directed evolution of alternative binding scaffolds and enzymes using yeast surface display. Microb. Cell Factories 17, https://doi.org/10.1186/s12934-018-0881-3.

Könning, D., Rhiel, L., Empting, M., Grzeschik, J., Sellmann, C., Schröter, C., Zielonka, S., Dickgießer, S., Pirzer, T., Yanakieva, D., et al. (2017a). Semi-synthetic vNAR libraries screened against therapeutic antibodies primarily deliver anti-idiotypic binders. Sci. Rep. 7, https://doi.org/10.1038/s41598-017-10513-9.

Könning, D., Zielonka, S., Grzeschik, J., Empting, M., Valldorf, B., Krah, S., Schröter, C., Sellmann, C., Hock, B., and Kolmar, H. (2017b). Camelid and shark single domain antibodies: structural features and therapeutic potential. Curr. Opin. Struct. Biol. 45: 10–16.

Könning, D., Zielonka, S., Sellmann, C., Schröter, C., Grzeschik, J., Becker, S., and Kolmar, H. (2016). Isolation of a pH-Sensitive IgNAR variable domain from a yeast-displayed, histidine-doped master library. Mar. Biotechnol. 18: 161–167.

Krah, S., Grzeschik, J., Rosowski, S., Gaa, R., Willenbuecher, I., Demir, D., Toleikis, L., Kolmar, H., Becker, S., and Zielonka, S. (2018). A streamlined approach for the construction of large yeast Surface display Fab antibody libraries. In: Nevoltris, D. and Chames, P. (Eds.), *Antibody engineering*. New York, NY: Springer New York, pp. 145–161.

Krah, S., Schröter, C., Eller, C., Rhiel, L., Rasche, N., Beck, J., Sellmann, C., Günther, R., Toleikis, L., Hock, B., et al. (2017). Generation of human bispecific common light chain antibodies by combining animal immunization and yeast display. Protein Eng. Des. Sel. 30: 291–301.

Krah, S., Schröter, C., Zielonka, S., Empting, M., Valldorf, B., and Kolmar, H. (2016). Single-domain antibodies for biomedical applications. Immunopharmacol. Immunotoxicol. 38: 21–28.

Kügler, J., Wilke, S., Meier, D., Tomszak, F., Frenzel, A., Schirrmann, T., Dübel, S., Garritsen, H., Hock, B., Toleikis, L., et al. (2015). Generation and analysis of the improved human HAL9/10 antibody phage display libraries. BMC Biotechnol. 15: 10.

Kunamneni, A., Ogaugwu, C., Bradfute, S., and Durvasula, R. (2020). Ribosome display technology: applications in disease diagnosis and control. Antibodies 9: 28.

Lai, J.Y., Loh, Q., Choong, Y.S., and Lim, T.S. (2018). Cassette hybridization for vector assembly application in antibody chain shuffling. Biotechniques 65: 269–274.

Lang, S., Drewello, D., Wichter, J., Nommay, A., Wilms, B., Knopf, H.-P., and Jostock, T. (2016). Surface display vectors for selective detection and isolation of high level antibody producing cells: surface display vectors for cell line development. Biotechnol. Bioeng. 113: 2386–2393.

Ledford, H. and Callaway, E. (2020). Pioneers of revolutionary CRISPR gene editing win chemistry Nobel. Nature 586: 346–347.

Lerner, R.A. (2016). Combinatorial antibody libraries: new advances, new immunological insights. Nat. Rev. Immunol. 16: 498–508.

Levin, A.M. and Weiss, G.A. (2006). Optimizing the affinity and specificity of proteins with molecular display. Mol. Biosyst. 2: 49–57.

Lim, S., Glasgow, J.E., Filsinger Interrante, M., Storm, E.M., and Cochran, J.R. (2017). Dual display of proteins on the yeast cell surface simplifies quantification of binding interactions and enzymatic bioconjugation reactions. Biotechnol. J. 12: 1600696.

Lipovsek, D. and Plückthun, A. (2004). In-vitro protein evolution by ribosome display and mRNA display. J. Immunol. Methods 290: 51–67.

Lombana, T.N., Dillon, M., Bevers, J., III, and Spiess, C. (2015). Optimizing antibody expression by using the naturally occurring framework diversity in a live bacterial antibody display system. Sci. Rep. 5: 17488.

Lonberg, N. (2005). Human antibodies from transgenic animals. Nat. Biotechnol. 23: 1117–1125.

Lonberg, N., Taylor, L.D., Harding, F.A., Trounstine, M., Higgins, K.M., Schramm, S.R., Kuo, C.-C., Mashayekh, R., Wymore, K., McCabe, J.G., et al. (1994). Antigen-specific human antibodies from mice comprising four distinct genetic modifications. Nature 368: 856–859.

Lou, J., Geren, I., Garcia-Rodriguez, C., Forsyth, C.M., Wen, W., Knopp, K., Brown, J., Smith, T., Smith, L.A., and Marks, J.D. (2010). Affinity maturation of human botulinum neurotoxin antibodies by light chain shuffling via yeast mating. Protein Eng. Des. Sel. 23: 311–319.

Lu, L.L., Suscovich, T.J., Fortune, S.M., and Alter, G. (2018). Beyond binding: antibody effector functions in infectious diseases. Nat. Rev. Immunol. 18: 46–61.

Lu, R.-M., Hwang, Y.-C., Liu, I.-J., Lee, C.-C., Tsai, H.-Z., Li, H.-J., and Wu, H.-C. (2020). Development of therapeutic antibodies for the treatment of diseases. J. Biomed. Sci. 27, https://doi.org/10.1186/s12929-019-0592-z.

Luo, R., Zhao, Y., Fan, Y., An, L., Jiang, T., Ma, S., and Hang, H. (2020). High efficiency CHO cell display-based antibody maturation. Sci. Rep. 10, https://doi.org/10.1038/s41598-020-65044-7.

Malkiel, S., Jeganathan, V., Wolfson, S., Manjarrez Orduño, N., Marasco, E., Aranow, C., Mackay, M., Gregersen, P.K., and Diamond, B. (2016). Checkpoints for autoreactive B cells in the peripheral blood of lupus patients assessed by flow cytometry: autoreactive B cell checkpoints assessed by flow cytometry. Arthritis & Rheumatology 68: 2210–2220.

Mason, D.M., Friedensohn, S., Weber, C.R., Jordi, C., Wagner, B., Meng, S., Gainza, P., Correia, B.E., and Reddy, S.T. (2019). *Deep learning enables therapeutic antibody optimization in mammalian cells by deciphering high-dimensional protein sequence space*, bioRxiv 617860.

Mason, D.M., Weber, C.R., Parola, C., Meng, S.M., Greiff, V., Kelton, W.J., and Reddy, S.T. (2018). High-throughput antibody engineering in mammalian cells by CRISPR/Cas9-mediated homology-directed mutagenesis. Nucleic Acids Res. 46: 7436–7449.

Mattheakis, L.C., Bhatt, R.R., and Dower, W.J. (1994). An *in vitro* polysome display system for identifying ligands from very large peptide libraries. Proc. Natl. Acad. Sci. U.S.A. 91: 9022–9026.

Mazor, Y., Van Blarcom, T., Carroll, S., and Georgiou, G. (2010). Selection of full-length IgGs by tandem display on filamentous phage particles and *Escherichia coli* fluorescence-activated cell sorting screening: selection of IgG by tandem phage panning-FACS. FEBS J. 277: 2291–2303.

Mazor, Y., Van Blarcom, T., Mabry, R., Iverson, B.L., and Georgiou, G. (2007). Isolation of engineered, full-length antibodies from libraries expressed in *Escherichia coli*. Nat. Biotechnol. 25: 563–565.

McCafferty, J., Griffiths, A.D., Winter, G., and Chiswell, D.J. (1990). Phage antibodies: filamentous phage displaying antibody variable domains. Nature 348: 552–554.

Merchant, A.M., Zhu, Z., Yuan, J.Q., Goddard, A., Adams, C.W., Presta, L.G., and Carter, P. (1998). An efficient route to human bispecific IgG. Nat. Biotechnol. 16: 677–681.

Miethe, S., Rasetti-Escargueil, C., Liu, Y., Chahboun, S., Pelat, T., Avril, A., Frenzel, A., Schirrmann, T., Thullier, P., Sesardic, D., and Hust, M. (2014). Development of neutralizing scFv-Fc against botulinum neurotoxin A light chain from a macaque immune library. mAbs 6: 446–459.

Miyamoto, K., Aoki, W., Ohtani, Y., Miura, N., Aburaya, S., Matsuzaki, Y., Kajiwara, K., Kitagawa, Y., and Ueda, M. (2019). Peptide barcoding for establishment of new types of genotype–phenotype linkages. PloS One 14: e0215993.

Moghaddam, A., Borgen, T., Stacy, J., Kausmally, L., Simonsen, B., Marvik, O.J., Brekke, O.H., and Braunagel, M. (2003). Identification of scFv antibody fragments that specifically recognise the heroin metabolite 6-monoacetylmorphine but not morphine. J. Immunol. Methods 280: 139–155.

Moon, S.A., Ki, M.K., Lee, S., Hong, M.-L., Kim, M., Kim, S., Chung, J., Rhee, S.G., and Shim, H. (2011). Antibodies against non-immunizing antigens derived from a large immune scFv library. Mol. Cell. 31: 509–513.

Morris, L., Chen, X., Alam, M., Tomaras, G., Zhang, R., Marshall, D.J., Chen, B., Parks, R., Foulger, A., Jaeger, F., et al. (2011). Isolation of a human anti-HIV gp41 membrane proximal region neutralizing antibody by antigen-Specific Single B cell Sorting. PLoS One 6: e23532.

Murphy, A.J., Macdonald, L.E., Stevens, S., Karow, M., Dore, A.T., Pobursky, K., Huang, T.T., Poueymirou, W.T., Esau, L., Meola, M., et al. (2014). Mice with megabase humanization of their immunoglobulin genes generate antibodies as efficiently as normal mice. Proc. Natl. Acad. Sci. U.S.A. 111: 5153–5158.

Murtaugh, M.L., Fanning, S.W., Sharma, T.M., Terry, A.M., and Horn, J.R. (2011). A combinatorial histidine scanning library approach to engineer highly pH-dependent protein switches: engineering pH-sensitive protein switches. Protein Sci. 20: 1619–1631.

Nelson, R.S. and Valadon, P. (2017). A universal phage display system for the seamless construction of Fab libraries. J. Immunol. Methods 450: 41–49.

Nilvebrant, J. and Sidhu, S.S. (2018). Construction of synthetic antibody phage-display libraries. In: Hust, M. and Lim, T.S. (Eds.), *Phage display*. New York, NY: Springer New York, pp. 45–60.

Nishibori, N., Horiuchi, H., Furusawa, S., and Matsuda, H. (2006). Humanization of chicken monoclonal antibody using phage-display system. Mol. Immunol. 43: 634–642.

Odegrip, R., Coomber, D., Eldridge, B., Hederer, R., Kuhlman, P.A., Ullman, C., FitzGerald, K., and McGregor, D. (2004). CIS display: *in vitro* selection of peptides from libraries of

protein-DNA complexes. Proc. Natl. Acad. Sci. U.S.A. 101: 2806–2810.

Ohlin, M., Owman, H., Mach, M., and Borrebaeck, C.A.K. (1996). Light chain shuffling of a high affinity antibody results in a drift in epitope recognition. Mol. Immunol. 33: 47–56.

Omar, N., Hamidon, N.H., Yunus, M.H., Noordin, R., Choong, Y.S., and Lim, T.S. (2018). Generation and selection of naïve Fab library for parasitic antigen: anti-*Bm* SXP antibodies for lymphatic filariasis. Biotechnol. Appl. Biochem. 65: 346–354.

Osborn, M.J., Ma, B., Avis, S., Binnie, A., Dilley, J., Yang, X., Lindquist, K., Ménoret, S., Iscache, A.-L., Ouisse, L.-H., et al. (2013). High-affinity IgG antibodies develop naturally in Ig-knockout rats carrying germline human IgH/Igκ/Igλ loci bearing the rat C_H region. J. Immunol. 190: 1481–1490.

Osbourn, J., Groves, M., and Vaughan, T. (2005). From rodent reagents to human therapeutics using antibody guided selection. Methods 36: 61–68.

Osbourn, J.K., Field, A., Wilton, J., Derbyshire, E., Earnshaw, J.C., Jones, P.T., Allen, D., and McCaffertya, J. (1996). Generation of a panel of related human scFv antibodies with high affinities for human CEA. Immunotechnology 2: 181–196.

Parola, C., Neumeier, D., Friedensohn, S., Csepregi, L., Di Tacchio, M., Mason, D.M., and Reddy, S.T. (2019). Antibody discovery and engineering by enhanced CRISPR-Cas9 integration of variable gene cassette libraries in mammalian cells. mAbs 11: 1367–1380.

Parthiban, K., Perera, R.L., Sattar, M., Huang, Y., Mayle, S., Masters, E., Griffiths, D., Surade, S., Leah, R., Dyson, M.R., et al. (2019). A comprehensive search of functional sequence space using large mammalian display libraries created by gene editing. mAbs 11: 884–898.

Pekar, L., Busch, M., Valldorf, B., Hinz, S.C., Toleikis, L., Krah, S., and Zielonka, S. (2020). Biophysical and biochemical characterization of a VHH-based IgG-like bi- and trispecific antibody platform. mAbs: 1812210, https://doi.org/10.1080/19420862.2020.1812210.

Pekar, L., Klausz, K., Busch, M., Valldorf, B., Kolmar, H., Wesch, D., Oberg, H.-H., Krohn, S., Boje, A.S., Gehlert, C.L., et al. (2021). Affinity maturation of B7-H6 translates into enhanced NK Cell–mediated tumor cell lysis and improved proinflammatory cytokine release of bispecific immunoligands via NKp30 engagement. J. Immunol. 206: 225–236.

Pepper, L.R., Cho, Y.K., Boder, E.T., and Shusta, E.V. (2008). A decade of yeast surface display technology: where are we now? Comb. Chem. High Throughput Screen. 11: 127–134.

Plückthun, A. (2012). Ribosome display: a perspective. In: Douthwaite, J.A. and Jackson, R.H. (Eds.), *Ribosome Display and related technologies*. New York, NY: Springer New York, pp. 3–28.

Pogson, M., Parola, C., Kelton, W.J., Heuberger, P., and Reddy, S.T. (2016). Immunogenomic engineering of a plug-and-(dis)play hybridoma platform. Nat. Commun. 7, https://doi.org/10.1038/ncomms12535.

Prassler, J., Thiel, S., Pracht, C., Polzer, A., Peters, S., Bauer, M., Nörenberg, S., Stark, Y., Kölln, J., Popp, A., et al. (2011). HuCAL PLATINUM, a synthetic Fab library optimized for sequence diversity and superior performance in mammalian expression systems. J. Mol. Biol. 413: 261–278.

Puri, V., Streaker, E., Prabakaran, P., Zhu, Z., and Dimitrov, D.S. (2013). Highly efficient selection of epitope specific antibody through competitive yeast display library sorting. mAbs 5: 533–539.

Rader, C., Cheresh, D.A., and Barbas, C.F. (1998). A phage display approach for rapid antibody humanization: designed combinatorial V gene libraries. Proc. Natl. Acad. Sci. U.S.A. 95: 8910–8915.

Rajan, S., Kierny, M.R., Mercer, A., Wu, J., Tovchigrechko, A., Wu, H., Dallacqua, W.F., Xiao, X., and Chowdhury, P.S. (2018). Recombinant human B cell repertoires enable screening for rare, specific, and natively paired antibodies. Communications Biology 1: 1–8.

Rakestraw, J.A., Aird, D., Aha, P.M., Baynes, B.M., and Lipovsek, D. (2011). Secretion-and-capture cell-surface display for selection of target-binding proteins. Protein Eng. Des. Sel. 24: 525–530.

Rappazzo, C.G., Tse, L.V., Kaku, C.I., Wrapp, D., Sakharkar, M., Huang, D., Deveau, L.M., Yockachonis, T.J., Herbert, A.S., Battles, M.B., et al. (2021). Broad and potent activity against SARS-like viruses by an engineered human monoclonal antibody. Science eabf4830.

Rasetti-Escargueil, C., Avril, A., Chahboun, S., Tierney, R., Bak, N., Miethe, S., Mazuet, C., Popoff, M.R., Thullier, P., Hust, M., et al. (2015). Development of human-like scFv-Fc antibodies neutralizing botulinum toxin serotype B. mAbs 7: 1161–1177.

Renn, A., Fu, Y., Hu, X., Hall, M.D., and Simeonov, A. (2020). Fruitful neutralizing antibody pipeline brings hope to defeat SARS-Cov-2. Trends Pharmacol. Sci.: 1–15, https://doi.org/10.1016/j.tips.2020.07.004.

Rhiel, L., Krah, S., Günther, R., Becker, S., Kolmar, H., and Hock, B. (2014). REAL-Select: full-length antibody display and library screening by surface capture on yeast cells. PLoS One 9: e114887.

Rogers, T.F., He, W., Limbo, O., Smith, C., Song, G., Woehl, J., Yang, L., Abbott, R.K., Callaghan, S., Garcia, E., et al. (2020). Animal model. Science 963: 956–963.

Rondot, S., Koch, J., Breitling, F., and Dübel, S. (2001). A helper phage to improve single-chain antibody presentation in phage display. Nat. Biotechnol. 19: 75–78.

Rosowski, S., Becker, S., Toleikis, L., Valldorf, B., Grzeschik, J., Demir, D., Willenbücher, I., Gaa, R., Kolmar, H., Zielonka, S., et al. (2018). A novel one-step approach for the construction of yeast surface display Fab antibody libraries. Microb. Cell Factories 17, https://doi.org/10.1186/s12934-017-0853-z.

Rossant, C.J., Carroll, D., Huang, L., Elvin, J., Neal, F., Walker, E., Benschop, J.J., Kim, E.E., Barry, S.T., and Vaughan, T.J. (2014). Phage display and hybridoma generation of antibodies to human CXCR2 yields antibodies with distinct mechanisms and epitopes. mAbs 6: 1425–1438.

Roth, L., Grzeschik, J., Hinz, S.C., Becker, S., Toleikis, L., Busch, M., Kolmar, H., Krah, S., and Zielonka, S. (2019). Facile generation of antibody heavy and light chain diversities for yeast surface display by Golden Gate Cloning. Biol. Chem. 400: 383–393.

Roth, L., Krah, S., Klemm, J., Günther, R., Toleikis, L., Busch, M., Becker, S., and Zielonka, S. (2020). Isolation of antigen-specific VHH single-domain antibodies by combining animal immunization with yeast surface display. Methods

Mol. Biol.: 173–189, https://doi.org/10.1007/978-1-4939-9853-1_10.

Rouet, R., Jackson, K.J.L., Langley, D.B., and Christ, D. (2018). Next-generation sequencing of antibody display repertoires. Front. Immunol. 9, https://doi.org/10.3389/fimmu.2018.00118.

Rouha, H., Badarau, A., Visram, Z.C., Battles, M.B., Prinz, B., Magyarics, Z., Nagy, G., Mirkina, I., Stulik, L., Zerbs, M., et al. (2015). Five birds, one stone: neutralization of α-hemolysin and 4 bi-component leukocidins of *Staphylococcus aureus* with a single human monoclonal antibody. mAbs 7: 243–254.

Russo, G., Meier, D., Helmsing, S., Wenzel, E., Oberle, F., Frenzel, A., and Hust, M. (2018). Parallelized antibody selection in microtiter plates. Methods Mol. Biol. 1701: 273–284.

Saggy, I., Wine, Y., Shefet-Carasso, L., Nahary, L., Georgiou, G., and Benhar, I. (2012). Antibody isolation from immunized animals: comparison of phage display and antibody discovery via V gene repertoire mining. Protein Eng. Des. Sel. 25: 539–549.

Salfeld, J., Kaymakçalan, Z., Tracey, D., Roberts, A., and Kamen, R. (1998). Generation of fully human anti-TNF antibody D2E7 [abstract]. Arthritis Rheum. 41(Suppl. 9): S57.

Sato, N., Matsumoto, T., Ueda, M., Tanaka, A., Fukuda, H., and Kondo, A. (2002). Long anchor using Flo1 protein enhances reactivity of cell surface-displayed glucoamylase to polymer substrates. Appl. Microbiol. Biotechnol. 60: 469–474.

Scapin, G., Yang, X., Prosise, W.W., McCoy, M., Reichert, P., Johnston, J.M., Kashi, R.S., and Strickland, C. (2015). Structure of full-length human anti-PD1 therapeutic IgG4 antibody pembrolizumab. Nat. Struct. Mol. Biol. 22: 953–958.

Schofield, D.J., Pope, A.R., Clementel, V., Buckell, J., Chapple, S.D., Clarke, K.F., Conquer, J.S., Crofts, A.M., Crowther, S.R., Dyson, M.R., et al. (2007). Application of phage display to high throughput antibody generation and characterization. Genome Biol. 8: R254.

Schroeder, H.W. and Cavacini, L. (2010). Structure and function of immunoglobulins. J. Allergy Clin. Immunol. 125: S41–S52.

Schröter, C., Beck, J., Krah, S., Zielonka, S., Doerner, A., Rhiel, L., Günther, R., Toleikis, L., Kolmar, H., Hock, B., et al. (2018). Selection of antibodies with tailored properties by application of high-throughput multiparameter fluorescence-activated cell sorting of yeast-displayed immune libraries. Mol. Biotechnol. 60: 727–735.

Schröter, C., Günther, R., Rhiel, L., Becker, S., Toleikis, L., Doerner, A., Becker, J., Schönemann, A., Nasu, D., Neuteboom, B., et al. (2015). A generic approach to engineer antibody pH-switches using combinatorial histidine scanning libraries and yeast display. mAbs 7: 138–151.

Schwimmer, L.J., Huang, B., Giang, H., Cotter, R.L., Chemla-Vogel, D.S., Dy, F.V., Tam, E.M., Zhang, F., Toy, P., Bohmann, D.J., et al. (2013). Discovery of diverse and functional antibodies from large human repertoire antibody libraries. J. Immunol. Methods 391: 60–71.

Scott, A.M., Wolchok, J.D., and Old, L.J. (2012). Antibody therapy of cancer. Nat. Rev. Cancer 12: 278–287.

Seah, Y.F.S., Hu, H., and Merten, C.A. (2018). Microfluidic single-cell technology in immunology and antibody screening. Mol. Aspect. Med. 59: 47–61.

Seeber, S., Ros, F., Thorey, I., Tiefenthaler, G., Kaluza, K., Lifke, V., Fischer, J.A.A., Klostermann, S., Endl, J., Kopetzki, E., et al. (2014). A robust high throughput platform to generate functional recombinant monoclonal antibodies using rabbit B cells from peripheral blood. PLoS One 9: e86184.

Sellmann, C., Pekar, L., Bauer, C., Ciesielski, E., Krah, S., Becker, S., Toleikis, L., Kügler, J., Frenzel, A., Valldorf, B., et al. (2020). A one-step process for the construction of phage display scFv and VHH libraries. Mol. Biotechnol.

Shembekar, N., Hu, H., Eustace, D., and Merten, C.A. (2018). Single-cell droplet microfluidic screening for antibodies specifically binding to target cells. Cell Rep. 22: 2206–2215.

Singh, M., Al-Eryani, G., Carswell, S., Ferguson, J.M., Blackburn, J., Barton, K., Roden, D., Luciani, F., Giang Phan, T., Junankar, S., et al. (2019). High-throughput targeted long-read single cell sequencing reveals the clonal and transcriptional landscape of lymphocytes. Nat. Commun. 10, https://doi.org/10.1038/s41467-019-11049-4.

Sivelle, C., Sierocki, R., Ferreira-Pinto, K., Simon, S., Maillere, B., and Nozach, H. (2018). Fab is the most efficient format to express functional antibodies by yeast surface display. mAbs 10: 720–729.

Smith, G. (1985). Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface. Science 228: 1315–1317.

Smith, G.P. (2019). Phage display: simple evolution in a Petri dish (Nobel Lecture). Angew. Chem. Int. Ed. 58: 14428–14437.

Soltes, G., Hust, M., Ng, K.K.Y., Bansal, A., Field, J., Stewart, D.I.H., Dübel, S., Cha, S., and Wiersma, E.J. (2007). On the influence of vector design on antibody phage display. J. Biotechnol. 127: 626–637.

Sternberg, N. and Hoess, R.H. (1995). Display of peptides and proteins on the surface of bacteriophage lambda. Proc. Natl. Acad. Sci. U.S.A. 92: 1609–1613.

Swers, J.S. (2004). Shuffled antibody libraries created by in vivo homologous recombination and yeast surface display. Nucleic Acids Res. 32: 36.

Tanno, H., McDaniel, J.R., Stevens, C.A., Voss, W.N., Li, J., Durrett, R., Lee, J., Gollihar, J., Tanno, Y., Delidakis, G., et al. (2020). A facile technology for the high-throughput sequencing of the paired VH:VL and TCRβ:TCRα repertoires. Science Advances 6, https://doi.org/10.1126/sciadv.aay9093.

Thouvenin, E., Laurent, S., Madelaine, M.-F., Rasschaert, D., Vautherot, J.-F., and Hewat, E.A. (1997). Bivalent binding of a neutralising antibody to a calicivirus involves the torsional flexibility of the antibody hinge. J. Mol. Biol. 270: 238–246.

Tiller, K.E., Chowdhury, R., Li, T., Ludwig, S.D., Sen, S., Maranas, C.D., and Tessier, P.M. (2017). Facile affinity maturation of antibody variable domains using natural diversity mutagenesis. Front. Immunol. 8, https://doi.org/10.3389/fimmu.2017.00986.

Tiller, T., Schuster, I., Deppe, D., Siegers, K., Strohner, R., Herrmann, T., Berenguer, M., Poujol, D., Stehle, J., Stark, Y., et al. (2013). A fully synthetic human Fab antibody library based on fixed VH/VL framework pairings with favorable biophysical properties. mAbs 5: 445–470.

Tillotson, B.J., Goulatis, L.I., Parenti, I., Duxbury, E., and Shusta, E.V. (2015a). Engineering an anti-transferrin receptor ScFv for pH-sensitive binding leads to increased intracellular accumulation. PLoS One 10: e0145820.

Tillotson, B.J., Lajoie, J.M., and Shusta, E.V. (2015b). Yeast display-based antibody affinity maturation using detergent-solubilized cell lysates. In: Liu, B. (Ed.). *Yeast surface display*. New York, NY: Springer New York, pp. 65–78.

Trott, M., Weiß, S., Antoni, S., Koch, J., von Briesen, H., Hust, M., and Dietrich, U. (2014). Functional characterization of two scFv-Fc antibodies from an HIV controller selected on soluble HIV-1 Env complexes: a neutralizing V3- and a trimer-specific gp41 antibody. PLoS One 9: e97478.

Ubah, O.C., Buschhaus, M.J., Ferguson, L., Kovaleva, M., Steven, J., Porter, A.J., and Barelle, C.J. (2018). Next-generation flexible formats of VNAR domains expand the drug platform's utility and developability. Biochem. Soc. Trans. 46: 1559–1565.

Uchański, T., Zögg, T., Yin, J., Yuan, D., Wohlkönig, A., Fischer, B., Rosenbaum, D.M., Kobilka, B.K., Pardon, E., and Steyaert, J. (2019). An improved yeast surface display platform for the screening of nanobody immune libraries. Sci. Rep. 9, https://doi.org/10.1038/s41598-018-37212-3.

Ueda, M. and Tanaka, A. (2000). Genetic immobilization of proteins on the yeast cell surface. Biotechnol. Adv. 18: 121–140.

Urquhart, L. (2020). Top companies and drugs by sales in 2019. Nat. Rev. Drug Discov. 19: 228.

Valdés-Alemán, J., Téllez-Sosa, J., Ovilla-Muñoz, M., Godoy-Lozano, E., Velázquez-Ramírez, D., Valdovinos-Torres, H., Gómez-Barreto, R.E., and Martinez-Barnetche, J. (2014). Hybridization-based antibody cDNA recovery for the production of recombinant antibodies identified by repertoire sequencing. mAbs 6: 493–501.

van den Beucken, T., Pieters, H., Steukers, M., van der Vaart, M., Ladner, R.C., Hoogenboom, H.R., and Hufton, S.E. (2003). Affinity maturation of Fab antibody fragments by fluorescent-activated cell sorting of yeast-displayed libraries. FEBS (Fed. Eur. Biochem. Soc.) Lett. 546: 288–294.

van Erp, E.A., Luytjes, W., Ferwerda, G., and van Kasteren, P.B. (2019). Fc-mediated antibody effector functions during respiratory syncytial virus infection and disease. Front. Immunol. 10, https://doi.org/10.3389/fimmu.2019.00548.

Vaughan, T.J., Williams, A.J., Pritchard, K., Osbourn, J.K., Pope, A.R., Earnshaw, J.C., McCafferty, J., Hodits, R.A., Wilton, J., and Johnson, K.S. (1996). Human antibodies with sub-nanomolar affinities isolated from a large non-immunized phage display library. Nat. Biotechnol. 14: 309–314.

Veiga, E., de Lorenzo, V., and Fernández, L.A. (2004). Structural tolerance of bacterial autotransporters for folded passenger protein domains. Mol. Microbiol. 52: 1069–1080.

Waldmeier, L., Hellmann, I., Gutknecht, C.K., Wolter, F.I., Cook, S.C., Reddy, S.T., Grawunder, U., and Beerli, R.R. (2016). Transpo-mAb display: transposition-mediated B cell display and functional screening of full-length IgG antibody libraries. mAbs 8: 726–740.

Wang, B., DeKosky, B.J., Timm, M.R., Lee, J., Normandin, E., Misasi, J., Kong, R., McDaniel, J.R., Delidakis, G., Leigh, K.E., et al. (2018). Functional interrogation and mining of natively paired human VH:VL antibody repertoires. Nat. Biotechnol. 36: 152–155.

Wang, B., Lee, C.-H., Johnson, E.L., Kluwe, C.A., Cunningham, J.C., Tanno, H., Crooks, R.M., Georgiou, G., and Ellington, A.D. (2016). Discovery of high affinity anti-ricin antibodies by B cell receptor sequencing and by yeast display of combinatorial V_H:V_L libraries from immunized animals. mAbs 8: 1035–1044.

Wang, J., Fei, K., Jing, H., Wu, Z., Wu, W., Zhou, S., Ni, H., Chen, B., Xiong, Y., Liu, Y., et al. (2019). Durable blockade of PD-1 signaling links preclinical efficacy of sintilimab to its clinical benefit. mAbs 11: 1443–1451.

Wang, Z., Mathias, A., Stavrou, S., and Neville, D.M. (2005). A new yeast display vector permitting free scFv amino termini can augment ligand binding affinities. Protein Eng. Des. Sel. 18: 337–343.

Weaver-Feldhaus, J.M., Lou, J., Coleman, J.R., Siegel, R.W., Marks, J.D., and Feldhaus, M.J. (2004). Yeast mating for combinatorial Fab library generation and surface display. FEBS (Fed. Eur. Biochem. Soc.) Lett. 564: 24–34.

Weber, M., Bujak, E., Putelli, A., Villa, A., Matasci, M., Gualandi, L., Hemmerle, T., Wulhfard, S., and Neri, D. (2014). A highly functional synthetic phage display library containing over 40 Billion human antibody clones. PloS One 9: e100000.

Wenzel, E.V., Bosnak, M., Tierney, R., Schubert, M., Brown, J., Dübel, S., Efstratiou, A., Sesardic, D., Stickings, P., and Hust, M. (2020a). Human antibodies neutralizing diphtheria toxin *in vitro* and *in vivo*. Sci. Rep. 10: 571.

Wenzel, E.V., Roth, K.D.R., Russo, G., Fühner, V., Helmsing, S., Frenzel, A., and Hust, M. (2020b). Antibody phage display: antibody selection in solution using biotinylated antigens. Methods Mol. Biol. 2070: 143–155.

Winters, A., McFadden, K., Bergen, J., Landas, J., Berry, K.A., Gonzalez, A., Salimi-Moosavi, H., Murawsky, C.M., Tagari, P., and King, C.T. (2019). Rapid single B cell antibody discovery using nanopens and structured light. mAbs 11: 1025–1035.

Wippold, J.A., Wang, H., Tingling, J., Leibowitz, J.L., de Figueiredo, P., and Han, A. (2020). PRESCIENT: platform for the rapid evaluation of antibody success using integrated microfluidics enabled technology. Lab Chip 20: 1628–1638.

Wozniak-Knopp, G., Bartl, S., Bauer, A., Mostageer, M., Woisetschläger, M., Antes, B., Ettl, K., Kainer, M., Weberhofer, G., Wiederkum, S., et al. (2010). Introducing antigen-binding sites in structural loops of immunoglobulin constant domains: Fc fragments with engineered HER2/neu-binding sites and antibody properties. Protein Eng. Des. Sel. 23: 289–297.

Wurm, F.M. (2004). Production of recombinant protein therapeutics in cultivated mammalian cells. Nat. Biotechnol. 22: 1393–1398.

Xie, J., Zhang, H., Yea, K., and Lerner, R.A. (2013). Autocrine signaling based selection of combinatorial antibodies that transdifferentiate human stem cells. Proc. Natl. Acad. Sci. U.S.A. 110: 8099–8104.

Xu, Y., Roach, W., Sun, T., Jain, T., Prinz, B., Yu, T.-Y., Torrey, J., Thomas, J., Bobrowicz, P., Vasquez, M., et al. (2013). Addressing polyspecificity of antibodies selected from an *in vitro* yeast presentation system: a FACS-based, high-throughput selection and analytical tool. Protein Eng. Des. Sel. 26: 663–670.

Yamaguchi, J., Naimuddin, M., Biyani, M., Sasaki, T., Machida, M., Kubo, T., Funatsu, T., Husimi, Y., and Nemoto, N. (2009). cDNA display: a novel screening method for functional disulfide-rich peptides by solid-phase synthesis and stabilization of mRNA-protein fusions. Nucleic Acids Res. 37: e108.

Yan, X. and Xu, Z. (2006). Ribosome-display technology: applications for directed evolution of functional proteins. Drug Discov. Today 11: 911–916.

Yang, L., Liu, W., Yu, X., Wu, M., Reichert, J.M., and Ho, M. (2020). COVID-19 antibody therapeutics tracker: a global online database of antibody therapeutics for the prevention and treatment of COVID-19. Antibody Therapeutics 3: 204–211.

Yang, W., Yoon, A., Lee, S., Kim, S., Han, J., and Chung, J. (2017). Next-generation sequencing enables the discovery of more diverse positive clones from a phage-displayed antibody library. Exp. Mol. Med. 49: e308.

Yang, Z., Du, M., Wang, W., Xin, X., Ma, P., Zhang, H., and Lerner, R.A. (2018). Affinity maturation of an TpoR targeting antibody in full-length IgG form for enhanced agonist activity. Protein Eng. Des. Sel. 31: 233–241.

Yang, Z., Wan, Y., Tao, P., Qiang, M., Dong, X., Lin, C.-W., Yang, G., Zheng, T., and Lerner, R.A. (2019). A cell–cell interaction format for selection of high-affinity antibodies to membrane proteins. Proc. Natl. Acad. Sci. U.S.A. 116: 14971–14978.

Yasunaga, M. (2020). Antibody therapeutics and immunoregulation in cancer and autoimmune disease. Semin. Cancer Biol. 64: 1–12.

Zhang, H., Wilson, I.A., and Lerner, R.A. (2012). Selection of antibodies that regulate phenotype from intracellular combinatorial antibody libraries. Proc. Natl. Acad. Sci. U.S.A. 109: 15728–15733.

Zhang, S., Zhang, M., Wu, W., Yuan, Z., Tsun, A., Wu, M., Chen, B., Li, J., Miao, X., Miao, X., et al. (2018). Preclinical characterization of Sintilimab, a fully human anti-PD-1 therapeutic monoclonal antibody for cancer. Antibody Therap. 1: 45–53.

Zielonka, S., Empting, M., Grzeschik, J., Könning, D., Barelle, C.J., and Kolmar, H. (2015). Structural insights and biomedical potential of IgNAR scaffolds from sharks. mAbs 7: 15–25.

Zielonka, S., Weber, N., Becker, S., Doerner, A., Christmann, A., Christmann, C., Uth, C., Fritz, J., Schäfer, E., Steinmann, B., et al. (2014). Shark Attack: high affinity binding proteins derived from shark vNAR domains by stepwise in vitro affinity maturation. J. Biotechnol. 191: 236–245.