REVIEW

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# Retroviral Display in Gene Therapy, Protein Engineering, and Vaccine Development

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**ABSTRACT** The display and analysis of proteins expressed on biological surfaces has become an attractive tool for the study of molecular interactions in enzymology, protein engineering, and high-throughput screening. Among the growing number of established display systems, retroviruses offer a unique and fully mammalian platform for the expression of correctly folded and post-translationally modified proteins in the context of cell plasma membrane-derived particles. This is of special interest for therapeutic applications such as gene therapy and vaccine development and also offers advantages for the engineering of mammalian proteins toward customized binding affinities and catalytic activities. This review critically summarizes the basic concepts and applications of retroviral display and analyses its benefits in comparison to other display techniques.

Proteins have evolved in nature to carry out a myriad of highly specialized tasks and can acquire improved or even novel functions by accumulating mutations that are favored and become stabilized under the appropriate selective pressures. Our knowledge of protein function at the molecular level is still limited and makes the rational design of proteins with tailored properties difficult. A highly successful means to engineer proteins with desired function in the laboratory is to adapt the principle of evolution, i.e., the generation of a large pool of mutant variants followed by selection or screening for the desired property. This concept of directed evolution has been widely implemented using protein display technologies such as phage display. In this approach, a protein-encoding DNA library (with up to $10^{12}$ variants) is generated and expressed as translational fusion to the coat proteins of phage particles (*1, 2*). Every particle packages the gene encoding the displayed protein variant, thereby physically linking phenotype and genotype, a prerequisite for all display platforms. Subsequently, the pool of particles is subjected to a selection pressure of choice (*e.g.*, binding affinity), followed by exclusion of inactive particles in addition to recovery and amplification of variants with the desired phenotype by infection of *E. coli* cells. Iterative rounds of selection allow for the enrichment and identification of library members with the desired property. Over the past two decades phage display not only revolutionized the development of recombinant antibody fragments for biotechnological and therapeutic applications but also enabled the identification of protease substrates and the engineering of proteins with catalytic activity (*3*). However, the prokaryotic expression system employed in phage display comes with certain limitations: (i) mammalian proteins are often prone to misfolding in bacterial systems and tend to

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form aggregates or to be insoluble; (ii) the lack of post-translational protein modifications, such as glycosylation, or inefficient disulfide bridge formation in bacteria might render displayed eukaryotic proteins inactive; (iii) bacterial and eukaryotic codon usage differs and might bias the selection. Nonetheless, the powerful application of phage display has inspired the development of a series of novel display platforms (*4*), such as *in vitro* ribosome (*5*) and mRNA (*6*) display, or cell surface display systems on bacteria (*7*), yeast (*8*), and mammalian cells (*9*).

Another class of display platforms that has been exploited in protein engineering and is unimpaired in protein expression/modification are eukaryotic viruses, namely, retroviruses (*10*), baculoviruses (*11*), and adeno-associated viruses (*12*). Among these, retroviruses were the first to be modified with foreign proteins expressed on the surface with the aim to extend and/or redirect their tropism to different cell types (*13*). Therefore, extensive research focused on the incorporation of foreign polypeptides into the viral membrane has facilitated the development of retroviral library display systems. Moreover, their replication to high titers in mammalian cell cultures and the ease of genomic manipulation, including a high capacity for transgene incorporation, make retroviruses ideal candidates to be used as display platform in protein engineering and for the study of protein–ligand interactions.

Rather unique features of retroviral display systems arise from the replication cycle of the virus. First, retroviral progeny bud at the host cell's plasma membrane, which ultimately constitutes the envelope of the viral particle. The composition of the viral membrane closely resembles the cell's plasma membrane (*14, 15*) and can therefore be considered as a metabolically inert minicell, which is an especially attractive surrounding to engineer and study mammalian membrane proteins (*16*). Second, after infection the retroviral genome gets stably integrated into the host cell's genome, meaning that genotype and phenotype of a transgene targeted for surface display is physically linked not only at the level of the retroviral particle but also at the level of the host cell that is used to produce the cognate virus. This dual linkage allows the development of exciting novel protein engineering applications that are difficult to achieve using other display platforms (*17*). Third, retroviral envelope proteins incorporated into the viral membrane provide highly exposed epitopes for the induction of protective immunity and, analogously the display of heterologous proteins on the viral surface can be exploited to engineer potent immunization vehicles.

Here we review recent applications of retroviral surface display with a focus on protein engineering toward the development of novel targeted viruses for gene therapy and the engineering of affinity ligands and enzymes. Additionally, we assess the use of antigen display on retroviruses as effective means to induce humoral and cellular immunity.

**Protein Display on Retroviruses, Retroviral Vectors, and Virus-Like Particles.** Retroviruses are enveloped viruses with two copies of a positive-sense, single-stranded RNA genome (*18*). The viral genome contains the genes *gag*, *pol*, and *env* encoding the core proteins (*gag*), the viral protease, integrase, reverse transcriptase (*pol*), and the envelope protein (*env*). Two identical long terminal repeats (LTR) harboring regulatory sequences such as promoters, enhancers, and polyadenylation signals flank the genome, and a single Ψ-site ensures selective packaging of the RNA genome into viral particles (Figure 1A). The retroviral life cycle is initiated by binding of the viral envelope protein Env to the cognate receptor on the target cell. Env is a trimeric type-1 transmembrane protein composed of heterodimers that are formed by the surface unit (SU) and the transmembrane protein (TM). Receptor binding induces conformational changes in SU and TM and ultimately leads to fusion of the viral and the host cell membranes and the release of the viral core into the cytoplasm. Here, the genomic RNA is reverse transcribed into double-stranded DNA, which gets integrated into the host genome upon transport into the nucleus. Full-length genomic viral RNA, transcribed by the cellular machinery, is packaged into viral particles during the assembly process, and progeny virions bud from the host cell plasma membrane containing incorporated Env proteins.

Retroviral vectors are used to stably transfer genomic information of choice to target cells but do not encode a functional genome and are therefore replication-incompetent. To establish retroviral vectors, the genetic information of interest is flanked by retroviral LTRs, equipped with a Ψ-site and cloned into a transfer plasmid. All retroviral genes required for particle formation (*gag*, *pol*, and *env*), are provided in *trans* either on separate plasmids or chromosomally encoded in a packaging cell line (Figure 1B). Transfection of the transfer plas-

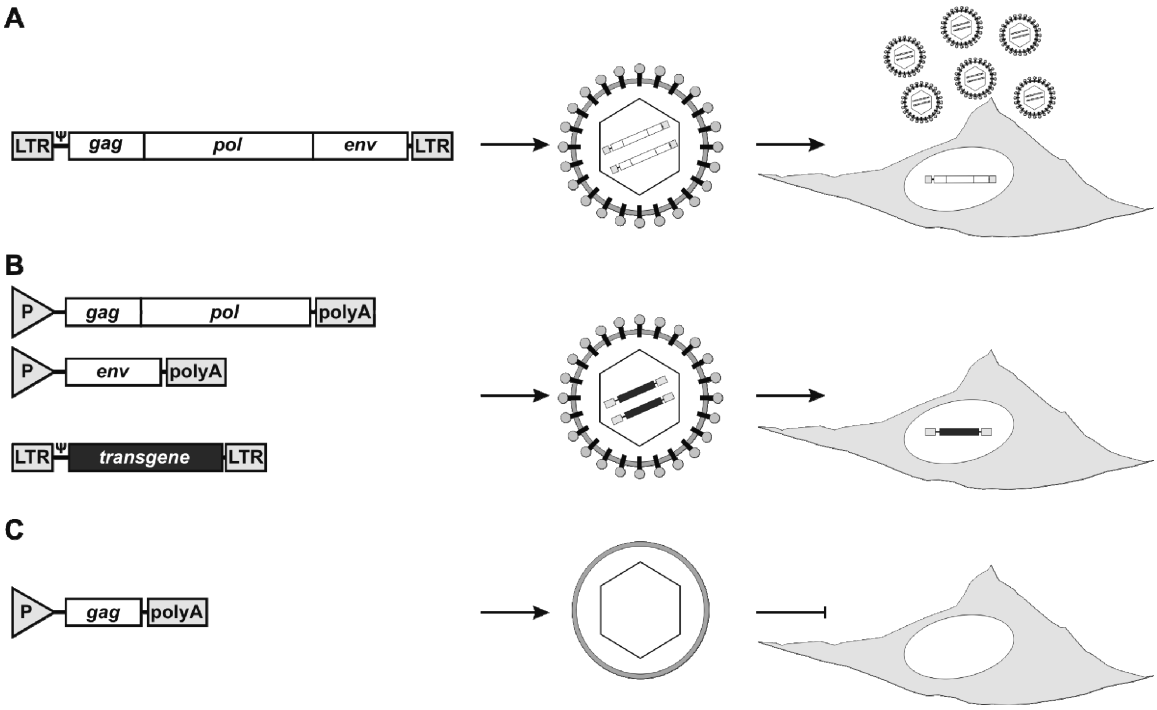


**Figure 1. Retrovirus, retroviral vector, and retrovirus-derived virus-like particle. (A) The genome of replication-competent C-type retroviruses encodes the genes *gag* (structural proteins), *pol* (protease, reverse transcriptase, integrase), and *env* (envelope protein) and is flanked by LTRs containing all regulatory elements. A Ψ-site ensures selective packaging of the genomic RNA into viral progeny. Transfection of DNA plasmids encoding full-length viral genomes (left) into producer cells results in the release of infectious replication-competent viruses carrying two copies of the viral RNA genome (middle). Following infection of target cells the viral genome is reverse transcribed into DNA and integrated into the host genome, and progeny virus are produced (right). (B) Retroviral vectors are produced by cotransfection of DNA plasmids providing the *gag*/*pol* and *env* genes under the control of constitutive promoters (P) and harboring polyadenylation and transcription termination signals (polyA) in *trans* along with a transfer plasmid encoding an LTR-flanked transgene of interest (left). Only the transfer vector RNA but no viral genes are packaged into particles by means of the Ψ-site (middle). Infection of target cells results in stable integration and LTR-driven expression of the transgene, but no viral progeny are produced (right). (C) Transfection of plasmids expressing the *gag* precursor (left) into producer cells results in the release of plasma membrane-derived VLPs lacking genomic information (middle) and being noninfectious (right).**

mid into a packaging cell or cotransfection along with plasmids providing *gag*/*pol*/*env* leads to formation of infectious particles that have packaged the transgene encoding RNA but lack the genomic information for viral replication.

Virus-like particles (VLPs) resemble mature virions in terms of structure and membrane composition but do not contain any viral RNA and are noninfectious. VLPs have been shown to efficiently bud from cells that express only the retroviral precursor protein encoded by the *gag* gene (*19*), even in the absence of a functional *env* gene (Figure 1C). Co-expression of *pol*, which encodes the retroviral protease, along with *gag* leads to proteolytic processing of the Gag precursor into the matrix (MA), capsid (CA), and nucleocapsid (NC) proteins and also supports the self-assembly and budding of VLPs (*20*).

Since retroviruses, retroviral vectors, and retroviral VLPs all have plasma membrane-derived envelopes, the display of foreign polypeptides on their surface is guided by the same principles. Although the rules for efficient membrane incorporation are not fully understood, the overexpression of proteins equipped with a signal sequence directing the polypeptide to the secretory pathway and a transmembrane domain is likely to result in particle display but needs to be tested and con-

firmed empirically. The mammalian expression vector pDisplay, for example, contains the murine Ig κ-signal sequence and the transmembrane domain derived from platelet derived growth factor receptor (PDGFR) and was designed to incorporate polypeptides of choice into the cell's plasma membrane (http://tools.invitrogen.com/content/sfs/manuals/pdisplay_man.pdf). Using this platform, the display of a 15 residue peptide with ~10,000 copies per particle, as well as the display of fully glycosylated polypeptides has been achieved on VLPs produced in HEK-293T cells (*21–23*). Alternatively, translational fusions of polypeptides inserted at various positions of SU of the retroviral Env have been successfully used to incorporate functional proteins into retroviral particles (reviewed in ref 24 and below). It should be noted that LTR-driven expression of retroviral wild-type *env* results in an estimated 14 to 75 Env-trimers per human immunodeficiency virus 1 (HIV-1) and murine leukemia virus (MLV) particle, respectively (*25, 26*), which can be tuned by the use of heterologous promoters in retroviral vector systems. Whether high or low copy numbers of the displayed protein are desirable obviously depends on the intended application, but needs to be taken into account for the design of appropriate strategies.

**KEYWORDS**

**Directed evolution:** A method that transfers the principles of natural evolution, *e.g.*, the establishment of a large mutant pool of closely related variants followed by selection for variants able to carry out a desired function to the test tube. In contrast to screening procedures a very large pool (library) of variants competes in the same reaction compartment to carry out the desired function with the highest efficiency, and poorly performing variants are eliminated from the pool. Because of the complex reaction conditions, the phenotype (*e.g.*, function) of each variant must be coupled to its genotype (*e.g.*, a replicable signature) to enable iterative rounds of selection/variant-amplification and ultimate identification of the desired variants.

**Mammalian expression system:** An *in vitro* expression system in mammalian cell cultures that mirrors human codon usage and reflects the full complexity of human protein expression with regard to post-translational protein modification.

**Post-translational modification:** The controlled chemical modification of a protein after translation may be of critical importance for its proper function. Prominent post-translational protein modifications in higher eukaryotes include but are not limited to the enzymatic addition of functional groups, such as glycosylation, and structural changes as introduced by disulfide bond formation.

**Protein engineering:** The development of proteins with improved or novel functions for biotechnological or therapeutic applications by means of rational design, functional screening, directed evolution, or combinations thereof.

**Retrovirus:** An enveloped virus with a diploid single-stranded positive-sense RNA genome replicating through a DNA intermediate that gets integrated into the host cell's chromosome. The envelope is derived from the host cell's plasma membrane and contains both cellular membrane proteins and the trimeric retroviral receptor.

**Screening:** A method that allows testing of a pool of different molecule variants for a desired function. In screening systems the desired function needs to translate into a detectable signal, and the cognate variant must have a unique signature for unambiguous identification. Screenings are often carried out in isolated reaction compartments to avoid interference between variants and to simplify signal detection.

**Applications in Gene Therapy.** Early examples of foreign proteins displayed on retroviral particles all had the aim to broaden or change the natural tropism of the virus to enable the infection of otherwise nonpermissive cell types or tissues. This idea is especially attractive in the field of virus-mediated gene therapy, which aims to introduce a transgene encoding a beneficial therapeutic function into target cells with the help of a viral vehicle in a safe and specific manner. Since the productive interaction between a retroviral Env protein and a cell surface receptor determines the tropism, the seemingly easiest way to alter the natural host range of the virus is to substitute its Env protein with a heterologous one from a foreign virus known to have a distinct host range, an approach termed pseudotyping. Successful pseudotypes include the substitution of HIV-1 Env by the vesicular stomatitis virus G protein (VSV-G), or amphotropic MLV Env on the surface of lentiviral vectors (*27*). These vectors combine the broad host range of VSV and amphotropic MLV with the ability of HIV-1 to even infect nondividing and terminally differentiated cells, a feature mediated by localization sequences for nuclear transport contained in components of the lentiviral preintegration complex (PIC) (*28*). Nonetheless, the options to engineer retroviral tropism by pseudotyping are limited because of the small number of characterized viral envelope proteins available and the loss of function associated with certain pseudotypes (*27, 29*).

Another approach to redirect retroviral tropism to new receptors is the genetic fusion of targeting domains, such as single chain antibody fragments (scFv) or growth factors (*13, 30*), to the viral envelope protein. However, while the expression of a targeting domain fusion to Env in general mediates binding specificity to the cell type expressing the cognate receptor, infectivity is often strongly reduced or abolished (*31*). Usually the complex postbinding events required for effective membrane fusion and infection are simply not compatible with the targeted receptor on the cell surface and/or cannot be carried out by the chimeric Env. Even if the chimeric Env protein supports infection *via* its natural receptor, tethering of the virus to an unnatural receptor expressed on the same cell might impair or abolish infection as the result of a sequestration mechanism and require co-expression of unmodified Env to restore infectivity (*32, 33*). The genetic fusion of the epidermal growth factor (EGF) close to the N-terminus of the envelope protein of MLV or spleen necrosis virus (SNV) leads

to specific binding of retroviral particles to cells expressing the EGF-receptor (EGF-R), such as tumor cells (*30, 34*). Interestingly, infectivity of the particles is strongly impaired on target cells co-expressing high levels of EGF-R along with the natural MLV receptor, whereas cells expressing low levels or no EGF-R along with the MLV receptor are infected with high efficacy. In this example, even though the chimeric Env supports infection *via* the natural receptor, Env gets functionally sequestered on EGF-R receptor and particles are most likely internalized and inactivated in lysosomal compartments. (*30*)

*Engineering of Protease-Activatable Env Proteins.* The aforementioned findings led to the concept of using polypeptides such as EGF as blocking domains on Env and to engineer protease-activatable retroviruses for tumor therapy. Tumor cells and stromal cells in the tumor microenvironment have a specific signature of protease expression (*35, 36*), which leads to the remodeling of the extracellular matrix, an event required for cell invasion, angiogenesis, and tumor growth. By fusing EGF to amphotropic Env *via* a linker peptide containing a matrix metalloproteinase (MMP) cleavage signal, retroviruses displaying these chimeric Env were shown to bind EGF-R expressing HT1080 human fibrosarcoma cell lines and regain infectivity upon proteolytic cleavage of the EGF domain by HT1080-associated MMPs (*37*). Similar results were obtained using a chimeric Env composed of a scFv targeting the c-Met receptor on glioma cells fused to amphotropic Env *via* a cleavable linker that provided the substrate for gliomo-associated urokinase (*38*). Propelled by the overall concept of protease-activatable retroviruses, the first retroviral display library has been generated with the aim of characterizing the cleavage sites of tumor-associated proteases (*10*). Herein, Buchholz *et al.* fused the EGF targeting/blocking domain to the N-terminus of ecotropic MLV Env *via* a randomized heptapeptide and passed the replication-competent retroviral library over human HT1080 cells expressing high levels of EGF-R and engineered to co-express the natural ecotropic MLV receptor Rec-1 (Figure 2). As mentioned above, even though EGF-R on HT1080 cells sequesters the chimeric viral envelope and infectivity *via* Rec-1, it can be restored upon proteolytic cleavage of the linker. Hence, variants encoding HT1080-specific protease substrates were thought to be favored to infect and replicate in this system. After three rounds of selection, sequencing of the viral genomes revealed an enrichment of arginine/lysine-rich linker peptides, which suggested the selection of variants displaying substrate sites for furin-like proteases and cleavage of the blocking domain already in intracellular compartments of the producer cells during transport of the chimeric Env to the cell surface. Even though substrate motifs for tumor-specific proteases could not be selected in this proof-of-concept study, this goal was achieved using later generations of retroviral display libraries. By randomizing a known motif for the tumor-associated MMP-2 at three positions and avoiding the incorporation of arginine residues, novel MMP-2 substrates were selected on HT1080 cells using an EGF-blocked amphotropic MLV library (*39*). Moreover, selected viruses spread more efficiently through HT1080 cell cultures than the viral clone displaying the parental MMP-2 substrate. Since the EGF blocking domain only prevents infection of cells with high EGF-R levels by sequestering the retroviral Env away from its natural receptor, MMP-2 activatable retroviruses selected in the latter study still efficiently infected cell types with low or no EGF-R expression and therefore lack the safety profile required for *in vivo* tumor therapy. To overcome this limitation, EGF was substituted by CD40-ligand (CD40L) as the blocking domain in a later selection for MMP-2 activatable retroviruses. N-Terminal fusions of CD40-ligand to MLV Env are blocked to interact with their natural receptor not only by sequestration to CD40 but also by steric hindrance and are therefore also noninfectious on CD40 negative cells (*40*). Selection of a CD40L blocked protease substrate library on HT1080 cells resulted in the identification of amphotropic MLV variants that are noninfectious on CD40 negative cells but gain infectivity on MMP-2 positive tumor cells after cleavage of the blocking domain (*41*). Additional biodistribution studies revealed that the engineered viruses had an up to 500-fold increased selectivity to infect MMP-2 positive tumor xenografts as compared to normal tissues (such as bone marrow, spleen, and liver) than the untargeted wild-type virus (*42*). In principle this selection scheme can be applied to every cell type of interest without prior knowledge of its protease profile and should result in the identification of retroviruses with a tropism restricted to tissues expressing the cognate proteases.

*Engineering of Env Proteins with Novel Specificities.* Another route to engineer retroviruses with a tailored tropism is to select retroviral Env display libraries for variants capable of infecting target cells of choice *via* novel cell surface receptors. In this regard, several stud-

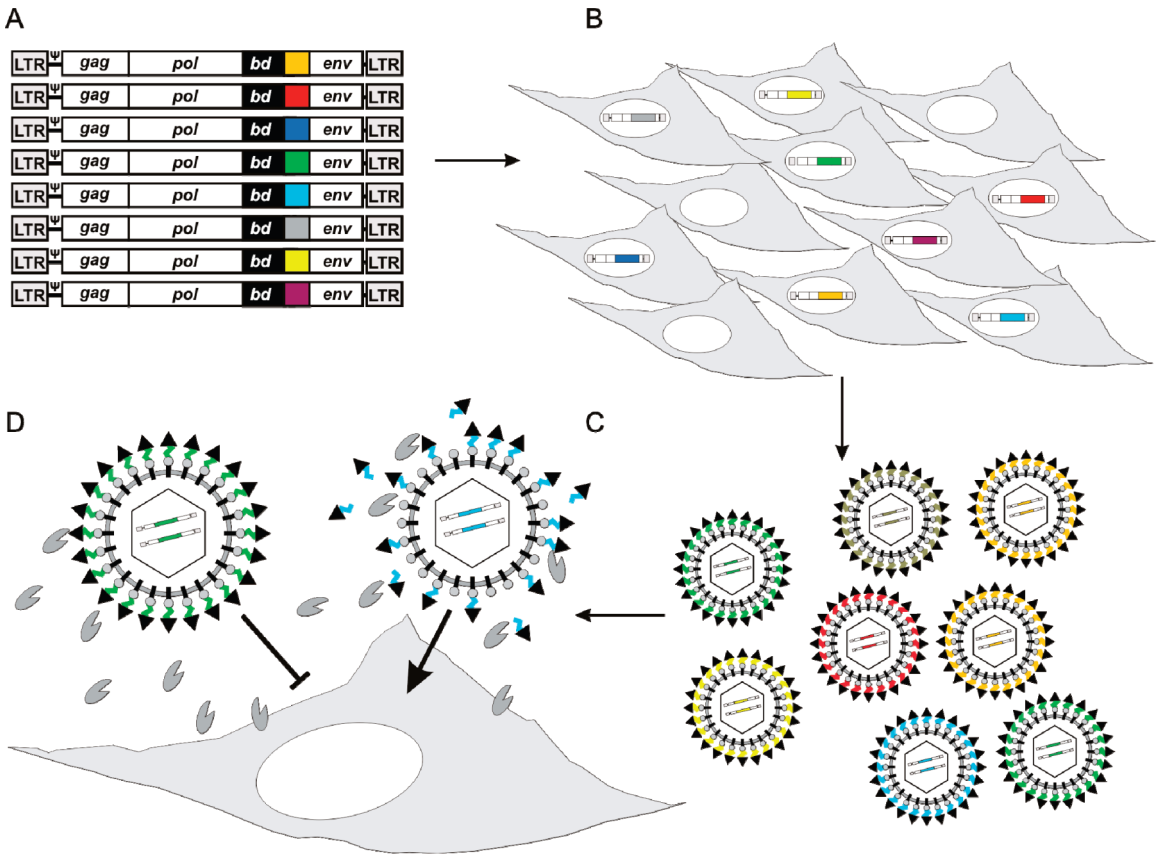


**Figure 2. Selection of protease-activatable retroviruses. (A) A plasmid library is generated encoding full-length retroviral genomes in which a blocking domain (*bd*) is fused to the retroviral envelope protein (*env*) by a randomized linker peptide (indicated by colored bars). (B) Transfection of the plasmid library into producer cells leads to expression of the viral genomes and (C) release of the viral library displaying the blocking domain (black triangles) on top of the envelope protein (gray lollipops) connected *via* the randomized linker peptide. (D) The viral library is then transferred to a protease-secreting (gray symbols) cell type of choice. Viruses displaying substrates for cell-associated proteases become infectious upon cleavage of the blocking domain and get amplified by replication, whereas non-substrate-displaying viruses remain noninfectious and fail to replicate. Iterative rounds of selection result in a strong enrichment of protease-activatable viruses and allow the cloning, sequencing, and characterization of the encoded protease substrates.**

ies successfully employed a vector-based feline leukemia virus (FeLV) Env library to select envelope variants mediating infection of target cells of interest. In contrast to other γ-retroviruses such as MLV, the receptor-binding domain (RBD) of subgroup A FeLV (FeLV-A) Env is suggested to be structurally simple, making it the ideal candidate to alter its tropism by introducing only limited changes (*43*). By selecting a FeLV Env vector library harboring 10 randomized residues in the variable region A (VRA) of its RBD on feline AH927 fibroblasts, Bupp *et al.* identified a Env variant with altered tropism as compared to parental FeLV-A Env (*43*). In this study, viruses displaying fully functional Env variants were recovered by infecting the AH927 selector cells with the retroviral vector library encoding a neomycin resistance cassette along with the modified *env* variant, followed by G418 selection. Even though experimental evidence indicated a switch from a subgroup A FeLV receptor to a subgroup C receptor utilized for infection, the isolated virus surprisingly revealed a more than 10,000-fold higher titer on D17 canine osteosarcoma cells as compared to the AH927 selector cells and negligible titers on five other tested canine cell lines. Selection of the same library on 143B ki-ras transformed human osteosarcoma

cells resulted in the isolation of Env variants that revealed high titers on human 143B and 293T cells but negligible infectivity on nine other human as well as on 3T3 murine or AH927 feline fibroblast cell lines (*44*). Receptor interference assays using the selected Env variant on both FeLV-A and FeLV-B preinfected 293T cells indirectly indicated the usage of a novel yet unidentified receptor for infection. In later studies, the Env display library was selected in parallel on different cell lines: selection on D17 canine osteosarcoma cells resulted in an Env variant using an FeLV-C receptor, whereas selection on feline AH927 fibroblasts identified an Env utilizing a novel receptor (*45*). This novel receptor was later identified by a cDNA library screen as the putative membrane protein SLC35F2 with unknown function, highlighting the possibility of defining and characterizing novel and functional Env/cell receptor pairs by selection of retroviral libraries (*46*). Interestingly, selection of the same library on feline AH927 cells identified an Env variant using a FeLV-C receptor in the initial study but uncovered another Env variant using the unrelated SLC35F2 protein as receptor in subsequent experiments (*43, 45, 46*). The different outcomes of the same selection schemes might be attributed to the size of the library used, since the theoretical diversity of $2 \times 10^{11}$ different variants in a peptide library with 10 randomized positions cannot be covered by retroviral surface display. However, the same group recently identified a novel Env/cell receptor pair by screening a related peptide library on human Caki-1 renal carcinoma cells, followed by a cDNA screen to characterize the targeted receptor (*47*), which underscores the success rate of the method. The receptor utilized by the Env variant selected in the latter study was identified as G-protein coupled receptor 172A (GPRi72A), a known receptor for the porcine endogenous retrovirus A (PERV-A).

An alternative to using random Env display libraries to engineer retroviruses with novel tropism is to recombine a subset of known *env* genes by DNA shuffling and select for infection of a cell type of interest which is nonpermissive for the parental Env variants. Soong *et al.* shuffled the *env* genes of six ecotropic MLVs by DNaseI fragmentation and PCR reassembly and selected the resulting display library for infection of Chinese Hamster Ovary (CHOK1) cells (*48*). The selection was carried out on co-cultures of CHOK1 and Lec8 cells, a CHOK1-derivative permissive for some ecotropic Envs due to defects in glycosylation pathways. Lec-8 cells were mixed with a large excess of CHOK1 cells in the selection to allow the initial propagation of virus variants with only low infectious activity on CHOK1 cells. After 5 passages on the co-cultures, retroviruses capable of infecting CHOK1 cell were selected from the shuffled display library, whereas a mixture of the parental variants passaged in parallel did not evolve infectious variants. Even though the cell receptor used for infection by the selected viruses was not further characterized, the study demonstrates that retroviral display libraries can be utilized to select for envelope proteins with novel cell targeting properties.

*Measles Virus-Derived Surface Glycoproteins.* Recent developments suggest that in the near future the targeting of retroviral vectors to cell types and tissues of choice will become feasible. An important step in this regard was the successful establishment of retroviral pseudotypes equipped with measles virus (MV)-derived surface glycoproteins. In MV the receptor binding and membrane fusion activities are separated into two distinct membrane-incorporated glycoproteins, the dimeric hemeagglutinin H and the trimeric fusion protein F, respectively. Upon attachment of the H protein to one of its natural receptors CD46 or SLAM on the cell surface, the fusion activity of the F protein is triggered and leads to the pH-independent fusion of the viral and cellular membranes. The identification of crucial amino acids for interaction of the H protein with CD46 and SLAM has allowed the engineering of mutant H proteins that are blinded for recognition of these natural receptors (*49*). Interestingly, binding of cell-targeting ligands, such as single-chain antibody fragments, displayed on the C-terminus of mutant H proteins to their cognate cell surface receptors is fully compatible to mediate F protein-triggered membrane fusion and MV infection (*50, 51*). While the natural MV H and F proteins are incorporated into retroviral particles with very poor efficacy, two research teams independently reported highly efficient pseudotyping of HIV-1 derived lentiviral vectors with MV H and F proteins that are truncated in their cytoplasmic tails and retain infectivity (*52, 53*). Lentiviral vectors pseudotyped with nonblinded MV H/F enabled efficient and stable transduction of quiescent T- and B-lymphocytes *via* the natural receptors CD46 and SLAM, a feature that has not been reported before using classical lentiviral vectors (*53, 54*). Using natural receptor-blinded lentiviral MV H/F vectors displaying a panel of different scFvs, Anliker *et al.* successfully trans-

duced human CD105$^+$ endothelial cells, human CD133$^+$ hematopoietic progenitors, and murine GluA-expressing neuron with high efficiency and in a cell-type specific manner (*55*). It is expected that this recent progress in the development of targeted lentiviral vectors will have fundamental influence in basic research and therapeutic gene therapy. Moreover, the *de novo* engineering of targeted lentiviral vectors by screening/selecting peptide or scFv libraries displayed on receptor-blinded H proteins on cell types of choice *in vitro* or even in living animals seems feasible.

**Protein Engineering.** In addition to therapeutic applications such as the development of novel gene therapy vectors, retroviral particles have also been used for classical protein engineering purposes. In particular, they have been exploited for the selection of protein variants with tailored binding affinities or catalytic activities. Even though many other platforms for these purposes exist, retroviral display offers conceptual advantages for some specialized applications.

*Screening for Binding Affinities.* Selections for tailored binding affinities are of special interest in regard to monoclonal antibodies, which constitute the fastest growing class of all prescribed drugs. According to the Food and Drug Administration (FDA; www.fda.gov), there were 22 approved antibodies in clinical use in the US in 2009, and the worldwide sales of these products exceeded $40 billion in the same year. As a first step toward exploiting retroviral display techniques for the screening of antibodies, short peptides have been used as a model system. Federspiel and co-workers randomized an octapeptide library ($2 \times 10^6$ different variants were generated) and fused it to the envelope protein of the avian leukosis virus (ALV). This viral species seemed perfectly suited since the ALV SU and TM envelope glycoproteins are covalently bound through a stable disulfide bond and therefore not subjected to shedding (the dissociation of the SU unit from TM (*56*)). Hence, displayed nonviral peptides and proteins remain on the particle surface even during ultracentrifugation as required for many purification or concentration protocols. Sequence analysis of the displayed octapeptide variants revealed no obvious bias, thus demonstrating potential advantages compared to the phage system, in which preferred codon usage and misfolding of eukaryotic polypeptides frequently results in sequence censorship (*57*). Using two different immobilized antibodies as targets, octapeptides with specific binding affinities were successfully selected (*58*).

In a similar approach, single-chain variable antibody fragments (scFvs) were selected from an MLV library. The antibodies were fused to the N-terminus of SU and the resulting viral particles were selected on laminin-coated plates. After three selection rounds particles displaying scFvs with high specificity for laminin were selected. In direct comparison to the phage system, the scFvs recovered from the MLV library showed strongly improved expression levels in human cells (up to 25 mg/L in the cell culture supernatant of transfected HEK293T cells) (*59*). Potentially even more important, retroviral libraries also facilitate the direct selection of antibodies expressed in human cells: Marasco and co-workers expressed bivalent scFvs on the transmembrane domain of HIV (gp41) and used the resulting retrovirus library for the stable transduction of HEK293T cells (*60*). The resulting antibody-displaying cells were subsequently incubated with the target antigen immobilized on magnetic beads, thus allowing for the specific enrichment of strong binders.

*Screening for Catalytic Activity.* In addition to selections for binding affinities, retroviral particles have also been used to screen proteins for catalytic activities. In particular, tissue plasminogen activator (tPA; a serine protease) was fused to the N-terminus of the transmembrane domain of PDGFR, which is easily incorporated into MLV particles (*22*). tPA requires glycosylation and disulfide bridging, for which reason a eukaryotic expression system seemed preferable. For selection, the tPA-displaying particles were encapsulated individually into aqueous droplets of a water-in-oil emulsion serving as independent miniaturized reaction vessels (Figure 3). By adding soluble substrates of a fluorescence assay for tPA activity, droplets hosting enzymatically active particles turned highly fluorescent. This allowed the quantitative screening and sorting of $1.8 \times 10^6$ samples (droplets) in just 1 h using microfluidic technology (*23*). Furthermore, the procedure enabled a roughly 1,300-fold enrichment of particles displaying active enzyme, when sorting a single time for catalytic activity under multiple turnover conditions.

Taken together, these examples demonstrate the successful use of retroviral display for classical protein engineering purposes, such as affinity selections or the screening for catalytic activity. However, it should be mentioned that in comparison to the phage system, the

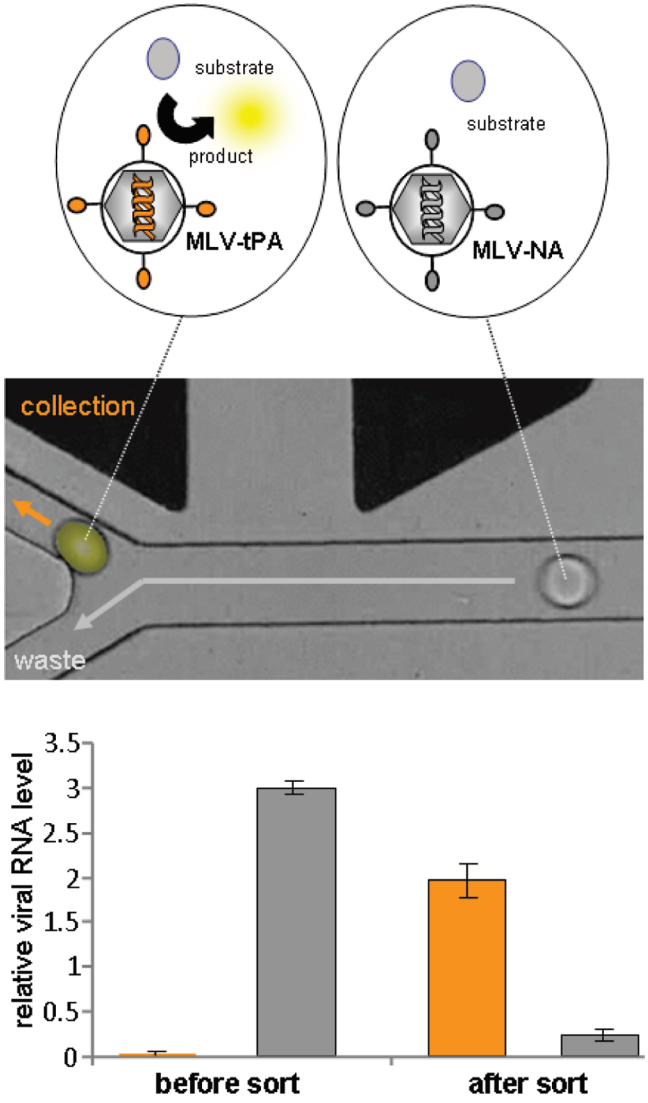


**Figure 3. Screening of retrovirally displayed enzymes for catalytic activity. MLV particles displaying tissue plasminogen activator (MLV-tPA) or an inactive control enzyme (MLV-NA, 1000-fold excess) were encapsulated into picoliter droplets of a water-in-oil emulsion in the presence of all components of a fluorescence assay for tPA activity (top). Subsequently highly fluorescent droplets (indicating strong enzymatic activity) were quantitatively sorted at a rate of 500 samples per second using a microfluidic device (center). The encapsulated viral particles were recovered and quantitatively analyzed by RT-PCR, revealing a more than 1300-fold enrichment of the enzymatically active MLV-tPA variant (bottom).**

actual diversity of the libraries is drastically decreased (typically ~$10^6$ for retroviruses and up to $10^{12}$ in the phage system). This is mainly due to limitations in the transfection efficiency of eukaryotic cultures (significantly lower cell numbers compared to bacterial cultures) and the fact that the applied plasmids are usually large (>6 kb), for which reason the cloning of highly diverse libraries becomes more difficult. Hence, retroviral display seems advantageous solely for applications in which a eukaryotic expression system is absolutely required (*e.g.*, if the protein of interest requires post-translational modifications).

**Development of New Vaccines.** Although vaccination is a well-established and highly cost-effective means to prevent infectious diseases, there is a continuous need for the development of novel candidate vaccines against emerging pathogens. Moreover, therapeutic vaccination against self-antigens to treat chronic diseases such as cancer and Alzheimer's disease has become a field of extensive research and is currently extended to other medical conditions such as arteriosclerosis, hypertension, and obesity upon identification of the respective self-antigens. VLPs are a promising novel class of candidate vaccines based on noninfectious particles that mimic functional virions derived from more than 30 eukaryotic viruses upon expression of their structural genes in the appropriate producer cell (*61*). A milestone in VLP-based immunization was the recent approval of two human papillomavirus (HPV)-derived VLP vaccines, Cervarix and Gardasil, by the FDA for the prophylactic vaccination against cervical cancer-causing HPV types 16 and 18 (*62*). Both vaccines are based on the HPV capsid protein L1, which is expressed in *Saccharomyces cervevisiae* followed by self-assembly into VLP in the case of Gardasil, or by baculovirus-mediated expression of VLPs in insect cells for Cervarix.

In contrast to conventional subunit or attenuated live virus vaccine, which often suffer from poor immunogenicity and the risk of reversion to a more virulent strain, respectively, VLP have proven to combine high levels of immunogenicity and safety. Antigens associated with VLP are presented to the immune system in an ordered, high-density form

that has been shown to elicit stronger humoral responses as the same antigen presented in a monomeric form (*63*). In addition, the particulate structure of VLPs supports uptake of these immunogens by professional antigen presenting cells (APCs), namely, dendritic cells (DC) and macrophages (MC) (*64*). Uptake and processing of the VLP-associated antigens by APCs not only stimulates $CD4^+$ T-helper cells by epitope presentation on major histocompatibility complex class II (MHC class II) but also activates $CD8^+$ cytotoxic T-cell (CTL) responses by presentation on MHC class I (*65*).

*Vaccination against Viral Antigens.* The potent stimulation of both an efficient humoral and a strong cell-mediated immune response as mediated by VLPs is widely believed to be a prerequisite for the development of vaccines against HIV (*66*). HIV-derived VLPs have been produced in a variety of systems, which include baculovirus-mediated or vaccinia virus-mediated expression in insect and monkey cells, respectively, but also expression in human embryonic kidney (293T) or monkey fibroblast (COS-7) cells following transient DNA plasmid transfection (*22, 67–69*). Most candidate HIV vaccines derived from retroviral VLPs consist of the Gag precursor, which is required for particle assembly, and the Env protein, which is the major target for neutralizing antibodies. Linking the SU subunit (gp120) of HIV-1 to the transmembrane domain of Epstein−Barr Virus (EBV) has been shown to increase the incorporation efficacy of the chimeric Env trimer into VLP about 10-fold as compared to wild-type HIV-1 Env upon baculovirus-mediated expression in insect cells (*67*). This formulation elicited neutralizing antibodies and a strong CD8+ T-cell response toward gp120 in the absence of additional adjuvants in rabbits and Balb/c mice, respectively (*70*). Another study compared the efficacy of humoral and cellular immune responses elicited by HIV-1 Env displaying VLP, soluble monomeric gp120, and soluble trimerized gp140 (gp140 consists of gp120 fused to the gp41 ectodomain *via* a mutated, uncleavable linker), all produced in COS-7 monkey cells. Env-displaying VLPs not only elicited a broader antibody response, recognizing more linear Env epitopes and resulting in overall higher titers of neutralizing antibodies as compared to soluble Env variants, but were also the only formulation which induced a robust Env-directed cellular immunity after intranasal immunization of BALB/c mice (*71*). The immunogenicity of retroviral VLPs can further be enhanced by the co-incorporation of immunostimulatory molecules as recently demonstrated for simian immunodeficiency virus (SIV)-derived VLPs (*72*). The co-incorporation of glycosylphosphatidylinositol (GPI)-anchored granulocyte-macrophage colony-stimulating factor (GM-CSF) or CD40L along with SIV Env on VLPs both lead to enhanced $CD4^+$ and $CD8^+$ T-cell responses in mice, whereas only GM-CSF co-incorporation additionally elicits a stronger humoral immune response. CD40L is primarily expressed on activated T cells and interacts with CD40 on the surface of APCs, thereby activating APCs to upregulate costimulatory molecules such as CD80 (B7.1) and CD86 (B7.2), as well as to secrete immunstimmulatory cytokines, which in turn facilitates the priming of naive $CD4^+$ T-helper and $CD8^+$ cytotoxic T-cells (*73*). GM-CSF acts as a growth factor and is known to lead to the expansion of myeloid-derived DC (*74*), which most likely accounts for the observed immunstimulatory effect *in vivo*. As with other subunit vaccines, the co-administration (instead of co-incorporation as described above) of adjuvants such as cholera toxin, GM-CSF, IL-12, and synthetic oligonucleotides containing unmethylated CpG motifs along with retroviral VLP has been shown to enhance the elicited humoral and cellular immune response (*75–78*). Moreover, it should be noted that VLPs *per se* are potent activators of innate immune responses and induce maturation of DCs as judged by the upregulation of surface expressed CD80, CD86 and the enhanced secretion of inflammatory cytokines (*79, 80*). This intrinsic adjuvant property of retroviral VLPs has been mainly investigated using VLPs expressed by baculovirus in insect cells and could be in part attributed to the impurities carried over from this heterologous expression system.

In addition to the extensive HIV-derived VLP research to develop candidate vaccines, preclinical studies have been carried out with MLV-derived VLPs pseudotyped with surface proteins of heterologous viruses. In this regard, VLPs displaying highly pathogenic avian influenza surface proteins hemagglutinin (HA), neuraminidase (NA) and the small ion channel forming M2 (M2) protein, as well as VLPs displaying VSV-G, have been shown to induce high titer neutralizing antibodies in mice (*81, 82*).

An alternative to *in vitro* engineered VLPs for vaccination was recently established by Bellier and co-workers. In this study, the authors displayed the MHC class I-restricted $GP^{33-41}$ epitope of the lymphocytic choriomeningitis virus glycoprotein as fusion to the 4070A envelope protein of amphotropic Mo-MLV on MLV-derived

VLP (*83*). Gene gun-mediated co-delivery of DNA plasmids encoding MLV *gag*/*pol* and *env-gp* (*33*–*41*), respectively, led to gene expression and VLP formation in mice *in vivo* and induced an epitope-specific cellular immune response. Furthermore, immunization with DNA plasmids encoding Mo-MLV *gag* and wild type ecotropic *env* fully protected 60% of mice from infection when challenged with the closely related myeloproliferative leukemia virus (MPLV). In contrast to classical DNA vaccines, which encode only selected immunogenic proteins, vaccination with VLP-encoding DNA combines the excellent immunogenicity of VLPs with the easy and cost-effective production of recombinant DNA.

*Vaccination against Self-Antigens.* VLP-based vaccinations are not restricted to viral immunogens and have been extended to induce immune responses against disease-associated self-antigens. A candidate prion protein (PrP) vaccine was developed by establishing MLV-derived VLPs displaying the native endogenous form $PrP^C$ (*22*). The infectious misfolded isoform $Prp^{Sc}$ propagates by converting normal $PrP^C$ into the infectious variant *in vivo*, thereby inducing amyloid formation in brain and neural tissue with ultimately fatal outcome (*84*). Although it is known that $Prp^{Sc}$ propagation can be inhibited by passive immunization with antibodies recognizing native $PrP^C$, the induction of $PrP^C$-specific antibodies *in vivo* largely failed. In contrast, vaccination of mice with $PrP^C$-displaying VLPs in the absence of adjuvant elicited a robust antibody response in PrP wild type mice for the first time and highlights the immunogenicity of retroviral VLP and their ability to overcome B-cell tolerance.

Using the same MLV-derived VLP display system for vaccination, a strong, long lasting antibody response was also induced against the β-amyloid peptide (Aβ), the main component of amyloid plaques associated with Alzheimer's disease (*21*). To avoid the induction of a cellular immune response against Aβ epitopes, which can result in adverse effects such as meningoencephalitis (*85*), only the 15 N-terminal residues were displayed on VLPs (Figure 4A). Single immunizations resulted in high Aβ-specific IgG titers and restimulatable B cell memory, while induction of autoreactive T cells and infiltrating lymphocytes was not observed. Moreover, a 40% reduction of brain Aβ plaque burden (Figure 4B and C) as well as of soluble Aβ was achieved in APP23 mice, which express about 10-fold higher levels of human Aβ as compared to endogenous murine

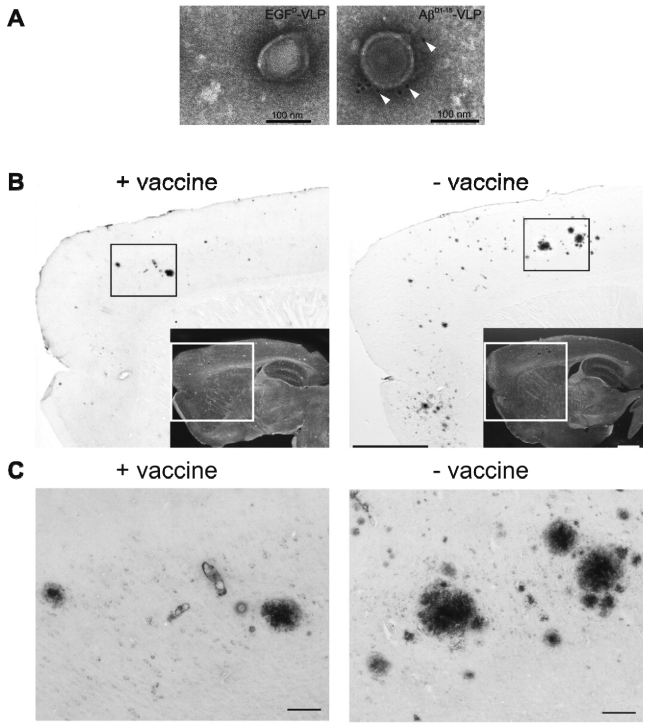


**Figure 4. Vaccination of APP23 mice with Aβ-displaying virus-like particles reduces plaque burden. (A) Immunoelectron microscopic analysis of EGF (left panel; negative control)- and Aβ (right panel)-displaying MLV-derived VLPs. Aβ (indicated by arrowheads) was labeled using anti-Aβ antibodies, and a gold particle-labeled secondary antibody. (B) Histological and biochemical analysis of vaccinated (left panel) and nonvaccinated (right panel) APP23 mice. Immunized mice received six i.v. injections of $10^{10}$ Aβ-VLP in monthly intervals. Two months after the last immunization mice were sacrificed, and sagittal brain sections were stained with anti-Aβ antibodies. The dark-field pictures are shown for orientation and highlight the sections shown in the bright-field by white squares (scale bar 1 mm). (C) Magnification of relevant sections of the bright-field pictures from B (indicated by black squares in B) to visualize Aβ plaque histology (scale bar 100 μm) (reproduced from ref 21).**

Aβ and develop Aβ deposits in the cerebral cortex at ~6 month of age (*86*). The ease with which retroviral immunogen-presenting VLPs can be engineered and purified, the high density of the displayed immunogenic epitopes, the efficient uptake into APC, and the resulting induction of both innate and adaptive immune responses makes retroviral VLP excellent candidates for the development of novel vaccines.

*Summary and Future Directions in Retroviral Protein Display.* Even though many display techniques have been developed for protein engineering purposes, only

a very few of these enable the expression in a fully mammalian or even human context. In case the protein of interest requires post-translational modifications such as glycosylation or disulfide bridging, retroviral display offers unique advantages since it not only allows the correct folding of structurally complex eukaryotic proteins but also their screening in the absence of the cellular machinery and metabolism. However, it must be noted that only a relatively small number of variants (typically about $1 \times 10^6$) can be screened using this technology, for which reason classical protein engineering applications (*e.g.*, affinity selections) are still preferentially carried out in other systems. In contrast, retroviral display has become an extremely efficient tool in research areas such as gene therapy or the development of vaccines. Here, the library size is far less relevant than the possibility of screening eukaryotic proteins for sophisticated biological functions such as targeted viral cell-entry or the evocation of an immune response. We envisage further applications in this area, potentially leading to novel therapeutic approaches in the clinic and further advances in fighting diseases such as cancer or HIV.

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