

REVIEW

Single domain antibodies for the knockdown of cytosolic and nuclear proteins

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Received 3 February 2017; Accepted 3 March 2017

DOI: 10.1002/pro.3154

Published online 8 March 2017 proteinscience.org

Abstract: Single domain antibodies (sdAbs) from camels or sharks comprise only the variable heavy chain domain. Human sdAbs comprise the variable domain of the heavy chain (VH) or light chain (VL) and can be selected from human antibodies. SdAbs are stable, nonaggregating molecules *in vitro* and *in vivo* compared to complete antibodies and scFv fragments. They are excellent novel inhibitors of cytosolic/nuclear proteins because they are correctly folded inside the cytosol in contrast to scFv fragments. SdAbs are unique because of their excellent specificity and possibility to target post-translational modifications such as phosphorylation sites, conformers or interaction regions of proteins that cannot be targeted with genetic knockout techniques and are impossible to knockdown with RNAi. The number of inhibiting cytosolic/nuclear sdAbs is increasing and usage of synthetic single pot single domain antibody libraries will boost the generation of these fascinating molecules without the need of immunization. The most frequently selected antigenic epitopes belong to viral and oncogenic proteins, followed by toxins, proteins of the nervous system as well as plant- and drosophila proteins. It is now possible to select functional sdAbs against virtually every cytosolic/nuclear protein and desired epitope. The development of new endosomal escape protein domains and cell-penetrating peptides for efficient transfection broaden the application of inhibiting sdAbs. Last but not least, the generation of relatively new cell-specific nanoparticles such as polymersomes and polyplexes carrying cytosolic/nuclear sdAb-DNA or –protein will pave the way to apply cytosolic/nuclear sdAbs for inhibition of viral infection and cancer in the clinic.

Keywords: intrabodies; single domain antibodies; scFv fragment; cytosolic/nuclear intrabodies; camelid VHHs; shark vNARs; human VH; human VL

Abbreviations: Nb, nanobody; NLS, nucleus leader sequence; sdAb, single domain antibody; vNAR, variable domain of the new antigen antigen receptor (IgNAR) from sharks.

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Introduction

New insights into the function of proteins can be obtained with nucleotide-based knockout and knockdown methods at the DNA or mRNA level respectively. Knockdown and knockout methods are essential to analyze the function of proteins.

Knockdown of proteins complement these methods and is essential if post-translational modifications,

ScFvs and sdAbs targeted to subcellular compartments

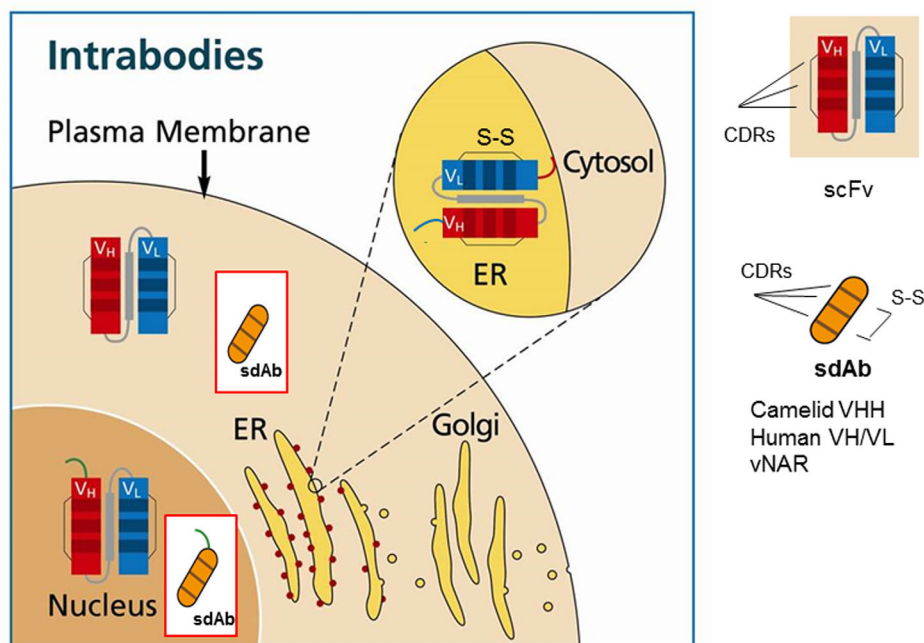


Figure 1. ScFvs and sdAbs targeted to subcellular compartments. Intrabodies can be targeted to the ER, mainly as scFv fragment, where they are retained by the KDEL sequence (red line). Binding of the specific antigen will lead to retention of the antigen-ER-intrabody complex inside the ER (not shown). Alternatively, scFvs or sdAbs can be targeted to the nucleus or cytosol via a nucleus leader peptide (green line) or without leader sequence, respectively. Complementarity-determining regions are shown by lines. sdAbs from shark have only CDR1 and CDR3.

conformations and interaction regions of proteins have to be studied. Regarding knockdown at the mRNA level this cannot be achieved with siRNA technology. Today, protein inhibitors are dominant negative mutants, small molecule inhibitors including peptides, allosteric modulators, short single-stranded DNA or RNA oligonucleotides (aptamers, intramers), neutralizing antibodies and intrabodies and nonimmunoglobulin binder scaffolds such as cysteine-knot proteins, adnectins, anticalins, affibodies, and DARPin^s.^{1,2}

Another class of promising knockdown molecules recently gaining importance are intracellular antibodies (intrabodies), i.e. antibodies that are expressed within the target cell that contains the corresponding antigen. Intrabodies are recombinant antibody fragments and the main advantages are their high specificity such that post-translational modifications, interaction regions, conformers, splice variants and isoforms can be targeted without off target effects. Long lasting functional inhibition of intracellular proteins can be achieved, which is not possible with classical neutralizing antibodies. Intrabodies can be specifically transported to the cytosol, nucleus or ER to bind their antigens at specific loci only (Fig. 1).

The development of cytosolic/nuclear intrabodies was hampered by the fact that intrabodies in the

scFv and Fab format cannot form disulfide bridges within the reducing environment of the cytosol and are therefore unstable.^{3,4}

Specific strategies were developed such as the yeast two-hybrid system⁵ to express functional cytosolic/nuclear scFv intrabodies and usage of these methods led to some remarkable functional cytosolic/nuclear scFv intrabodies.⁶ A breakthrough in antibody engineering was the discovery of stable expressed cytosolic/nuclear sdAbs derived from camel or shark antibodies comprising only the variable domain of the heavy chain. The variable domain of camelid heavy chain antibodies is called VHH, the variable domain of the new antigen receptor (IgNAR) from sharks vNAR. Camelid VHHs recognizing their antigens are called nanobodies.

Both single domain antibodies derived from camels or sharks constitute intact functional antigen-binding sites, the single domain VHH domain with 3 complementarity determining CDR regions⁷ and the vNAR domain with CDR1, CDR3 and HV2 and HV4.⁸⁻¹⁰ Camelid and shark single domain antibodies possess good solubility, high thermal stability and refolding capability.^{7,10,11} In addition, stable human VH and VL domains can be constructed from human antibodies after framework mutations and randomization of CDRs.¹²⁻¹⁸ Camelid nanobodies can be stably expressed inside the

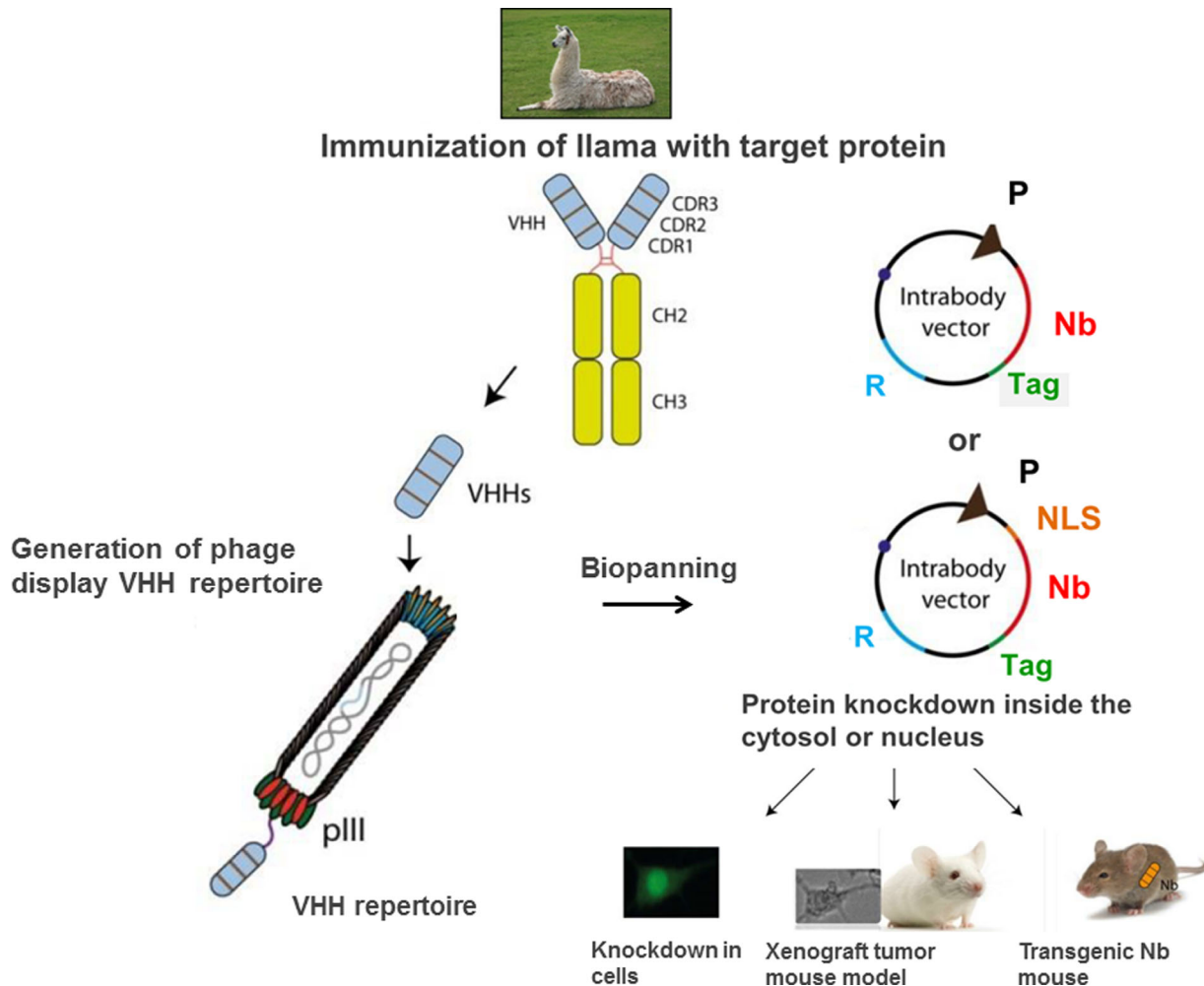


Figure 2. Construction and application of cytosolic/nuclear camelid nanobodies. Until now, most cytosolic camelid nanobodies have been constructed after immunization of camels. The VHH domains will be amplified from the cDNA isolated from the peripheral blood lymphocytes of immunized camels. Then a phage display repertoire is generated to display the VHH repertoire on the surface. Biopanning with the antigen will deliver specific recombinant nanobody phages from which the genes can be cloned into an appropriate targeting vector for expression in the cytosol or nucleus. The function of the intrabody can be tested in cell culture or applied in a xenograft tumor mouse model. Alternatively, a transgenic mouse expressing the nanobody can be generated. (P, promoter; R, resistant gene; Nb, nanobody; NLS, nucleus leader sequence).

cytoplasm even if the disulfide bridge is not formed.^{2,6} Folded nanobodies can find their antigen and are then stabilized by binding to the antigen.

As an analytical tool camelid antibodies have been used to illuminate *in vivo* trafficking of target molecules, to detect conformational states and post-translational modification of proteins. In addition, they have been employed as chaperons for crystallization in cases where unstable proteins are sensitive to degradation, unfolding or aggregation.^{19–23}

In general nanobodies are selected from naïve, immune, synthetic camelid VHH single domain antibody repertoires^{24–27} whereas shark sdAbs and human VH and VL sdAbs are mainly selected from synthetic antibody libraries.^{12–18,28–34}

Antibody fragments are displayed on filamentous phages or yeast. Selected sdAbs can then be converted to cytosolic/nuclear intrabodies with a

single cloning step (Figs. 2 and 3). In particular, universal synthetic nanobody libraries are very valuable because no immunization with the target antigen is needed. Low affinity binders can be affinity-matured by introducing CDR mutations, randomization of mutational hotspots or by error prone PCR.^{35–37}

The number of available inhibiting nanobodies against different cytosolic/nuclear proteins is increasing. So far most cytosolic/nuclear single domain intrabodies have been derived from camels, followed by some single domains VH and VL of human origin (Table I).

The targets comprise

- intracellular enzymes including kinases;^{53,54,58,59,62,76–78,81}
- oncogenic proteins^{56–60,62–65,68,69,81}

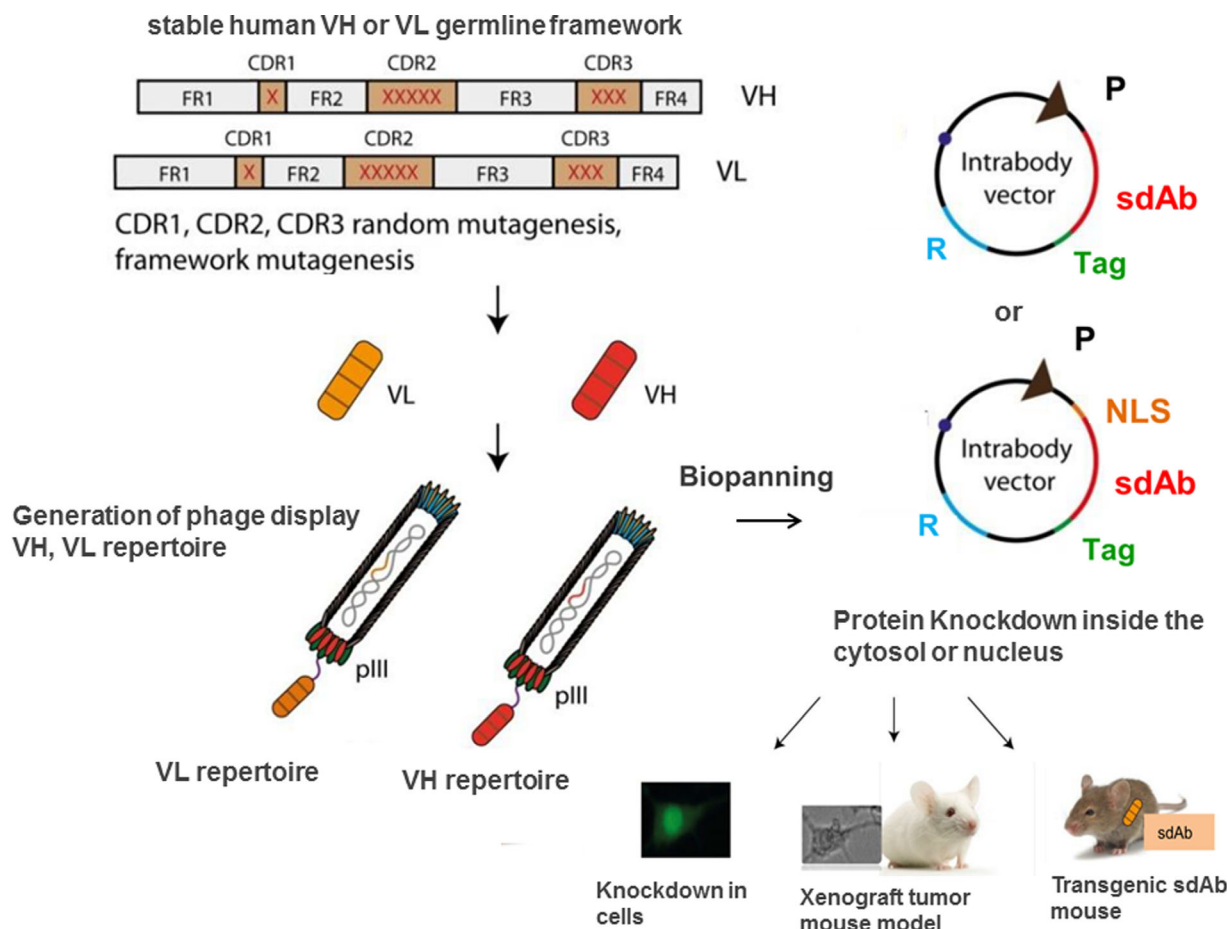


Figure 3. Construction and application of cytosolic/nuclear human VH and VL domains. Synthetic human VH/VL libraries are constructed from a stable human VH or VL germline framework or from a VH/VL scaffold of an existing antibody. After randomization of the CDRs (mainly CDR3) and possible introduction of framework mutations, human VH and VL are displayed on phages. Specific sdAbs are selected by biopanning and the sdAb-coding region is subsequently cloned into a targeting vector for expression inside the cytosol or nucleus. *In vivo* application follows. (P, promoter; R, resistant gene; NLS, nucleus leader sequence; sdAb, single domain antibody).

- toxins^{53–55}
- proteins of the nervous system^{70–73} and
- virus proteins^{38–52}
- proteins from plants and drosophila^{77–80}

Most cytosolic/nuclear sdAbs have been evaluated in living cells but *in vivo* application of single domain intrabodies in mice is well documented, too.^{57,60,81} In the future, generation of transgenic mice expressing cytosolic/nuclear sdAb is possible and very useful if a corresponding genetic knockout is lethal.⁸²

ER intrabodies versus cytosolic/nuclear intrabodies

ER-intrabody knockdown function is based on retention of molecules passing the secretory pathway via the KDEL sequence fused to the C-terminal end of the intrabody.^{6,83–85} In the past ER-intrabodies have been the most promising molecules because they are correctly folded in the ER environment.⁸⁶ Contrary

to ER intrabodies which have only to bind to the antigen inside the ER, cytosolic/nuclear intrabodies have to inactivate their targets by binding to the antigen and inducing a conformational change or interfering with the binding of the target protein to its specific binding partner. Intrabodies targeted to the ER (ER-intrabodies) are mostly expressed as scFv fragment, cytosolic/nuclear intrabodies as scFv or recently as much more stable single domain antibody.^{6,87,88} Due to the high stability of sdAbs in the cytosol/nucleus compared to scFv fragments and efficient reliable selection techniques based on phage and yeast display, the knockdown of cytosolic/nuclear proteins by sdAbs is very promising.

Cytosolic/nuclear intrabodies

To select stable correctly folded scFv fragments, specific *in vivo* selection in eukaryotes such as yeast or in prokaryotes such as *E.coli* has to be performed.⁶ Possible options are also expression of scFv-protein fusions and CDR grafting or introduction of

Table 1. Reports of Cytosolic/nuclear sdAb Mediated Knockdown

Target	Source	Characterization, cells and mice	Physiological knockdown readout	Ref.
Viral Proteins HIV Rev	Llama immunized with recombinant HIV Rev.	HeLa cells and human embryonal kidney 293T cells were cotransfected with Rev-GFP and intrabody-expression plasmid. Localization of REV-GFP and intrabody was determined by immunofluorescence. Cells stable expressing intrabody. Yeast cells co-transfected with intrabody and HA-Vpr- expression plasmids. Analyzation of the localization of Vpr by immunofluorescence.	Intrabody interferes with Rev multimerization and inhibits HIV-1 production inside cells. Suppression of the replication of different HIV subtypes.	Vercruyse et al. ³⁸ Boons et al. ³⁹
HIV Vpr	Llama immunized with synthetic Vpr peptide, selection of nanobodies by cytoplasmic yeast two-hybrid system.	Yeast cells co-transfected with intrabody and HA-Vpr- expression plasmids. Analyzation of the localization of Vpr by immunofluorescence.	Delocalization of Vpr from the nucleus to the cytoplasm.	Matz et al. ⁴⁰
HIV Vif	Rabbit immunized with HIV-1 Vif protein. Construction of camelized rabbit-derived VH single domain from anti-HIV-1 scFv.	Permissive Jurkat and non-permissive H9 cells transfected with recombinant HIV-1 virus encoding single domain intrabody. HIV-1 GAG p24 levels were determined by ELISA.	Inhibition of HIV-1 replication in non-permissive H9 cells and reduction of viral infectivity.	Aires da Silva et al. ⁴¹
HIV-1 Nef	Llama immunized with purified recombinant Nef protein.	HPB-ALL CD4 ⁺ T cells cotransfected with Nef-GFP and intrabody -expression plasmid. Cell surface expression of CD4 was determined by flow cytometry. In vivo: fetal liver cells from CD4 ⁺ /HIV ^{NEF} transgenic mice retroviral infected with nanobody were injected into lethally irradiated host animals. Analysis of CD4 ⁺ T-cell function. HPB-ALL T cells and RAW264.7	Intrabody inhibits Nef-induced down-regulation and endocytosis of CD4. Intrabody was able to rescue Nef-mediated thymic CD4 ⁺ T-cell maturation defect and peripheral CD4 ⁺ T-cell activation phenotypes of the transgenic mouse model.	Bouchet et al. ⁴²
HIV-1 Nef	Nanobody against HIV-1 Nef was modified by fusion of a Nef SH3 binding peptide.	Macrophages transiently transfected with Nef-GFP and modified intrabodies (Neffins).	Neffins inhibit Nef-induced MHC-I and CD4 downregulation in T cells and restores efficient phagocytosis. Down regulation of MHC I were more efficiently inhibited by Neffins compared to the parental intrabodies.	Bouchet et al. ⁴³
Influenza virus Nucleoprotein (NP)	Llama immunized with alcohol-fixed influenza virus PR8.	MDCK cells stable transfected with intrabody expression plasmid were infected with influenza virus and nuclear localization of viral ribonucleoprotein complex analyzed.	Inhibition of nuclear import of incoming viral ribonucleoprotein complex.	Ashour et al. ⁴⁴

Table I. Continued

Target	Source	Characterization, cells and mice	Physiological knockdown readout	Ref.
HCV NSB4	Usage of a naïve camelid VH/VHH phage display antibody repertoire. Biopanning was performed with recombinant NSB4.	Analysis of intrabody mediated inhibition of HCV replication using Huh7 cells electroporated with HCV RNA and transfected with VH/VHH fused with penetratin.	4 clones inhibited HCV replication.	Glab-Ampai et al. ⁴⁵
HCV NS3 helicase	Usage of a naïve camelid VH/VHH phage display antibody repertoire. Biopanning was performed with C-terminal NS3 helicase. VH/VHH gene sequences were fused to cell penetrating peptide penetratin.	Analysis of intrabody mediated inhibition of HCV replication performed as by Glab-Ampai et al. ⁴⁵	VH/VHH clones inhibited HCV replication by interfering with the virus helicase activity.	Phalaphol et al. ⁴⁶
HCV RNA dependent RNA polymerase NS5B	Usage of a naïve camelid VH/VHH phage display antibody repertoire. Biopanning was performed with recombinant NS5B. VH/VHH gene sequences were fused to cell penetrating peptide penetratin. Nanobodies were constructed from a mouse mAb.	Analysis of intrabody mediated inhibition of HCV replication performed as by K. Glab-ampai.	4 clones inhibited HCV replication.	Thueng-in et al. ⁴⁷
HCV Core antigen	Llama immunized with recombinant HBcAg.	Analysis of the HCV titer in HCV and recombinant intrabody adenovirus transfected CD81-defective Huh7-25 cells.	Intrabody expression reduced intracellular and extracellular infectious titres. Furthermore intrabody expression suppressed HCV-core induced NFκB promoter activity.	Suzuki et al. ⁴⁸
Porcine reproductive and respiratory syndrome virus (PRRSV) Nonstructural protein 9 (Nsp9)	Llama immunized with Nsp9.	Hepatocyte cell line HepG2 transfected with intrabody and HBcAg expression plasmid. Localization of HBcAg was determined by immunofluorescence.	Cotransfection with the intrabody directed to the nucleus showed a speckled HBcAg pattern demonstrating that nuclear nanobodies interferes with HBcAg trafficking.	Serruys et al. ⁴⁹
Porcine reproductive and respiratory syndrome virus Nsp4	Llama immunized with Nsp4.	Monkey kidney MARC-145 cells stable expressing nanobody were infected with PRRSV.	Nanobody protected cells from virus-induced cytopathic effect and fully block PRRSV replication.	Liu et al. ⁵⁰
Porcine endogenous retrovirus p15 Matrix Protein	Llama immunized with recombinant p15 Matrix protein.	MARC-145 cells stable expressing nanobody-EGFP fusionprotein infected with PRRSV.	Nanobodies inhibit PRRSV replication.	Liu et al. ⁵¹
		PK 15 cells for production of porcine endogenous retrovirus PERV-A/B transfected with nanobody.	PERV-A/B Virus production is blocked upon expression of intrabody.	Dekker et al. ⁵²

Table I. Continued

Target	Source	Characterization, cells and mice	Physiological knockdown readout	Ref.
Toxins				
SpvB toxin of <i>Salmonella typhimurium</i>	Llama immunized with SpvB Toxin of <i>Salmonella typhimurium</i> .	Raw 264.7 macrophages transiently transfected with intrabody were infected with <i>Salmonella typhimurium</i> . Vero cells transiently transfected with nanobody were infected with binary chimeric C2IN-C/SpvB fusionprotein. Cytopathic effects were analyzed by immunofluorescence.	Single domain intrabody protect cells from cytopathic effects of <i>Salmonella</i> infection.	Alzogaray et al. ⁵³
<i>Clostridium botulinum</i> neurotoxin protease	Llama immunized with recombinant <i>Clostridium botulinum</i> neurotoxin A and B proteases.	Neuronal cells, transfected with nanobody recognizing neurotoxin A protease was infected with botulinum neurotoxin A. Cleavage of SNAP25 was analyzed by Western Blot.	Cleavage of endogenous synaptic protein SNAP25 was inhibited.	Tremblay et al. ⁵⁴
Mycotoxin 15-Acetyldeoxynivalenol (15-AcDON)	Llama immunized with 15-AcDON.	<i>P. pastoris</i> transformed with nanobody and cultured with Mycotoxin 15-Acetyldeoxynivalenol. Analysis of the influence of Nb on cytotoxicity.	Intracellular expression of mycotoxin-specific VHH in <i>P. pastoris</i> causes significant resistance to 15-AcDON.	Doyle et al. ⁵⁵
Oncogenic Proteins				
C-MYC	Human VH libraries with randomized CDR3. Human VHs were selected using the yeast and mammalian two hybrid system.	Affinity maturation was achieved by additional stepwise randomization of CDR2 and CDR1.	Isolation of human single domain intrabodies recognizing the basic-helix-loop-helix and leucine zipper region of CMYC.	Zeng et al. ⁵⁶
LIM domain only 2 (LMO2)	Human single domain VH and VL library with randomized CDR3 regions generated from two human scFv genes recognizing the RAS oncogenic protein. Selection of appropriate single domain antibody using yeast two hybrid system.	In vivo: Neoplastic T cells from a Lck-Lmo2 transgenic mouse thymoma were transfected with retrovirus-VH and transplanted into nude mice. The amount of retrovirus comprising reporter GFP transfected T-cells was compared with retrovirus comprising reporter GFP and single domain intrabody transfected cells.	Anti-LMO2 VH inhibits T-cell neoplasia in a nude mouse. LMO2 is necessary for T-cell tumorigenesis.	Tanaka et al. ⁵⁷
Endothelial and epithelial kinase (Etk)	Human single domain VL library (Domantis) derived from a single human framework of a κ light chain variable region. Complete randomization at 13 residues of the CDR1 -CDR3 was performed.	NSR cells (mouse NIH3T3 cells overexpressing v-SRC) were transfected with single domain intrabodies and inhibition of the function of endogenous Etk assayed with [γ - ³³ P] ATP. Analyzing inhibition of cellular transformation by estimating colony formation of intrabody transfected NSR cells.	Significant inhibition of Etk function and cellular transformation by single domain intrabodies.	Paz et al. ⁵⁸

Table I. Continued

Target	Source	Characterization, cells and mice	Physiological knockdown readout	Ref.
Protein kinase C ϵ	Camels were immunized with the kinase C ϵ .	One inhibitory VHH domain with a mCherry tag was cotransfected with PKC ϵ -EGFP in HeLa cells and the translocation of the kinase to the cell membrane tested.	The inhibitory VHH inhibits PKC ϵ translocation from the cytosol to the cell membrane.	Summanen et al. ⁵⁹
F-actin capping protein CapG	Llama immunized with human recombinant CapG.	MDA-MB-231 breast tumor cells transfected with intrabodies. Analysis of the inhibition of migration, matrix invasion, multinucleation. In vivo: Breast tumor metastasis mouse model with immunodeficient NOD-SCID mice and MDA-MB-231 cells stable expressing single domain antibody GFP fusionprotein and luciferase reporter.	Significant inhibition of migration, matrix invasion, multinucleation. Significant inhibition of lungmetastasis in immunodeficient NOD-SCID mice.	Van Impe et al. ⁶⁰
RAS	Human single domain VH library was constructed from a consensus framework sequence of an anti-RAS scFv fragment. After randomization of the CDRs specific single domain VHs were selected using the yeast two hybrid system with mutated oncogenic RAS as antigen.	RAS transfected mouse NIH3T3 cells, RAS transfected human fibrosarcoma and RAS transfected human colorectal adenocarcinoma cells. Cells were lentiviral transfected with a selected nanobody. In vivo: Lung metastasis tumor mouse model. RAS transfected cells retroviral stable transfected with selected nanobody were injected subcutaneously into nude mice and tumor size was analyzed.	Nanobody reverts the RAS transformed phenotype of the cells. Furthermore it was shown that the nanobody prevents effector molecules binding to RAS. The variable domain binds to the regions where the effector ligands bind, demonstrated with the crystal structure of antibody-RAS. Intrabody inhibits tumorigenesis and metastasis of RAS transformed cells in mice.	Tanaka et al. ⁶¹
Caspase-3	VHH library derived from non-immunized llamas. Selection of nanobodies against recombinant purified Caspase-3.	SHSY-5Y cells transiently transfected with VHH antibodies. After induction of oxidative stress with H ₂ O ₂ the influence of the VHHs on apoptosis was analyzed microscopically. Activity assays with purified caspase-3 in the presence of purified VHHs.	Two VHHs were selected. One VHH inhibit apoptosis and activity of caspase-3, the other clone was an antagonist of apoptosis.	McGonigal et al. ⁶²
				Inoue et al. ⁶³

Table I. Continued

Target	Source	Characterization, cells and mice	Physiological knockdown readout	Ref.
Heterogenous nuclear ribonucleoprotein K (hnRNP-K)	Construction of a VHH library with randomized CDR 3 starting from a VHH recognizing hen egg white lysozyme. The aim was to use the library to identify targets involved in metastasis.	Establishment of a loss of cell migration screening procedure: (1) transfection of VHH library into metastatic human fibrosarcoma HT1080 cells, (2) separation of non-migrating cells from migrating cells, (3) isolation of antibody plasmids from non-migrating cells and trans-formation in <i>E.coli</i> .	After repeating the screening procedure three times one clone was selected that inhibits cell migration of HT 1080 cells. Further it was shown that the targeted protein is hnRNP-K. Nanobody lead to cytoplasmic accumulation of the protein.	
Bax	VHH library derived from non-immunized llamas. Panning was performed with recombinant Bax.	Isolation of mitochondria from SHSY-5Y cells and analyzing reactive oxygen species (ROS) and cytochrome c release in the presence of Bax and nanobodies. SHSY-5Y cells stable transfected with VHH-GFP fusionproteins. Induction of oxidative stress with H ₂ O ₂ and measurement of Caspase 3/7.	Bax activity was inhibited in isolated mitochondria. Caspase 3/7 activation was prevented by anti-Bax intrabodies. Anti-Bax VHHs can be applied for studying the role of Bax in oxidative-stress-induced apoptosis.	Gueorguieva et al. ⁶⁴
L-plastin	Llama immunized with recombinant L-plastin	Usage of human prostate cancer cell line PC-3 cells. Studying the effect of L-plastin on Actin binding and Actin bundling in the presence of VHHs. Analysis of cell migration and matrix invasion of PC-3 cells transiently transfected with nanobodies. THP-1 monocytic leukemia cells lentiviral stable transfected with anti-L-plastin nanobodies. Analysis of actin bundling and stability of the podosome. Jurcat T cells transfected with nanobodies and activated with anti-CD3/CD28 antibodies. Incubation of Jurcat T cells transfected with nanobody with Staphylococcus enterotoxin stimulated antigen presenting Raji cells.	VHHs inhibit binding of L-plastin to actin and actin bundling. Cell migration and matrigel invasion was inhibited by anti-L-plastin nanobodies. Nanobodies inhibit actin bundling leading to podosome instability. Nanobodies reduce proliferation and IL-2 secretion after primary T cell activation. Anti-L-plastin antibodies decrease T cell-Raji cell contact area. One nanobody inhibits phosphorylation of L-plastin and LFA-1.	Delanote et al. ⁶⁵ De Clercq et al. ⁶⁶ De Clercq et al. ⁶⁷

Table I. Continued

Target	Source	Characterization, cells and mice	Physiological knockdown readout	Ref.
Fascin and cortactin	Llama immunized with fascin or cortactin.	MDA-MB-231 breast cancer cells and PC-3 prostate cancer cells transiently transfected with nanobodies. Analysis of the influence of the anti-fascin nanobodies on actin-bundling and anti-cortactin nanobodies on WASP-interacting protein (WIP) recruitment toward the plasma membrane. Analysis of the floppin formation.	Fascin-actin bundling is affected by anti-fascin nanobodies and they have an effect on invadopodium array organization. WIP recruitment is inhibited by anti-cortactin antibodies and they inhibit invadopodium array organization as well as formation of invadopodium. The nanobodies prevent matrix degradation.	Van Audenhove et al. ⁶⁸
Gelsolin	Llamas immunized with human recombinant gelsolin.	MDA-MB-231 breast cancer cells stably lentiviral transfected with VHHs. Binding assays to analyze if VHHs inhibits binding of gelsolin to actin. Migration of PC-3 cells transfected with VHH was analyzed.	One nanobody inhibits interaction of gelsolin with actin and prevents migration of PC-3 cells.	Van den Abbeele et al. ⁶⁹
Proteins of the nervous system Huntington	ScFv fragments against exon 1 of huntingtin were isolated from a yeast displayed non-immune human scFv library. It was demonstrated that the paratope was only defined by VL. The function of affinity matured light chain was analyzed. The cysteine residues of yeast displayed VL (see D W Colby, P Garg above) were changed to valine and alanine and the variant affinity matured by Error Prone PCR.	Analyzing huntingtin aggregation in a cell-free <i>in vitro</i> assay in the presence of affinity matured VL or in HEK293 cells transiently transfected with affinity matured VL and huntingtin exon 1 (httex1) fragment with pathological-length of 67 polyglutamine residues-fused to GFP (httex1-Q ₆₇ - GFP). Human embryonic ST14A cells and human neuroblastoma SH-SY5Y cells co-transfected with disulfide bond-free VL and httex1Q ₆₇ -GFP were analyzed for huntingtin aggregation. Cytotoxicity assay with sdAb transfected ST14A and yeast cells.	Single domain VL inhibits huntingtin aggregation <i>in vitro</i> and in mammalian cells. Disulfide bond-free mutant of VL, affinity matured by Error Prone PCR, blocked huntingtin aggregation and inhibited toxicity.	Colby et al. ⁷⁰ Colby et al. ⁷¹
α -Synuclein				Lynch et al. ⁷²

Table I. Continued

Target	Source	Characterization, cells and mice	Physiological knockdown readout	Ref.
Other Targets β-catenin	Selection of anti-α Synuclein scFv fragments and VH scAbs from a yeast displayed non-immune human scFv library. VH single domain antibody derived from a yeast-displayed non-immune human scFv library was not soluble. The PEST sequence was fused to the scAb to favour solubility and intracellular degradation of the formed antigen-scAb complex. Llama immunized with recombinant β-catenin.	Transfection of a truncated version of WTsyn-EGFP into stable sdAb expressing ST14A cells. Inhibition of aggregation of WTsyn-EGFP was tested. (WTsyn = wildtype Synuclein). In human embryonic ST14A cells co-transfected with sdAb-Pest and α-synuclein-GFP, degradation of α-Synuclein-GFP was analyzed.	Aggregation of α-Synuclein was inhibited. Fusion of the PEST sequence to the nanobody significantly increased steady-state soluble intrabody levels and α-synuclein-GFP is significantly degraded in presence of nanobody-PEST.	Joshi et al. ⁷³
Nuclear poly(A)-binding protein 1 (PABPN1)	Llamas immunized with recombinant PABPN1.	Wnt induced luciferase bioassay was performed with HEK293 cell transfected with anti-β-catenin VHHs. HeLa cells were co-transfected with nuclear targeted VHH-NLS and aggregation prone mutant PABPN1 comprising 17 alanines. Aggregation of mutant PABPN1 in the presence of nanobody was analyzed by immunofluorescence.	4 nanobodies inhibited β-catenin activity. Nanobody inhibits aggregation of mutant PABPN1 and cleared existing aggregates.	Newnham et al. ⁷⁴ Verheesen et al. ⁷⁵
2,5 dihydroxy pyridine dioxigenase (NicX)	Llamas immunized with NicX.	Bacterial strain <i>P.putida</i> transformed with VHH fused to a thioredoxin domain, which function as chaperon. Bacterial growth and NicX activity was measured.	Nanobodies inhibited bacterial growth and enzyme activity of NicX.	Jimenez et al. ⁷⁶
β ₂ – adrenergic Receptor	Llamas immunized with a truncated form of β ₂ – adrenergic receptor.	HEK293 cells transiently transfected with nanobodies were tested for G protein activation and β-arrestin recruitment.	Approximately half of the intrabodies showed inhibitory effects on G protein activation and β-arrestin recruitment.	Staus et al. ²¹

Table I. Continued

Target	Source	Characterization, cells and mice	Physiological knockdown readout	Ref.
Plants Potato starch branching enzyme A (SBE A)	A naive single-domain llama antibody library with randomized CDRs was screened with recombinant SBE A.	VHHs were cloned into plant transformation vectors, introduced into <i>Agrobacterium tumefaciens</i> and transgenic potato lines generated. SBE A activity was measured.	VHHs inhibited significantly enzyme activity.	Jobling et al. ⁷⁷
Nla protease of potato virus Y	Llama immunized with Nla protease.	The expression plasmid was transferred into potato plants via <i>Agrobacterium tumefaciens</i> mediated transformation. Transgenic plants were inoculated with potato virus Y. Effect of virusinfection in the presence of nanobody was tested.	Expression of nanobody leads to partial protection against the virus.	Bouaziz et al. ⁷⁸
<i>Drosophila</i> Myosin regulatory light chain (Sqh)-GFP and transcriptionfactor apterous (ap)-GFP from <i>Drosophila</i>	Llama immunized with GFP. To selected nanobody was fused the F-box domain contained in the N-terminal part of Slmb (F Box Protein) from <i>Drosophila</i> . Fusion protein NSlmb-VHHGFP leads to recruitment of E2 which fuse Ubiquitin to the nanobody target. The target is degraded inside the proteasome.	Transgenic <i>Drosophila</i> knock in lines carrying the NSlmb-VHHGFP gene and the Sqh-GFP-gene or ap-GFP-gene were generated and the phenotype analyzed.	Degradation of Sqh and apterous protein fused to GFP mediated through ubiquitination led to significant phenotype of transgenic intrabody flies. This method is universal and the GFP tag can be used to monitor the protein knockout in living cells.	Caussinus et al. ⁷⁹
<i>Drosophila</i> Decapentaplegic-(Dpp) GFP	Into the framework of an anti- β -lactamase VHH were transplanted the CDRs of an anti-GFP VHH	An anti-eGFP VHH - CD8 transmembrane domain - cytoplasmic mCherry fluorescent tag-fusion protein (morphotrap) was expressed in a transgenic mouse expressing DPP-GFP to trap GFP-Dpp on the cell surface. The wing disc development was analyzed.	When Dpp is immobilized on the cell surface, wing disc patterning is lost and lateral cells still divide at normal rates.	Harmansa et al. ⁸⁰

synthetic CDRs in stable frameworks.⁶ The most frequently used method to select functional scFv's is the Antibody Capture Technology (IACT) based on the yeast 2-hybrid system.⁵ The next paragraph summarizes the targets against which scFv fragments have been selected by IACT and describes some new scFv's inhibiting the degradation of p53 and restoring the activity of mutated p53, respectively.

ScFv format

Many different scFv's have been isolated by IACT, for example against Tau, amyloid- β peptide, α -synuclein, proNGF, gephyrin, Syk, EGFR, BCR-ABL, caspase 3, aggrecanase-2 and NSP5 from rotavirus.⁶

Recently, a scFv fragment against the E6 oncoprotein of HPV selected by IACT was targeted to the nucleus where it inhibits the ability of HPV16 E6 (16E6) to mediate p53 degradation and causes cell death of HPV16-positive cells.⁸⁹ Additionally, the nucleus-targeted intrabody inhibited tumor activity of HPV16-positive tumor cells in two pre-clinical mouse models. Interestingly, two bispecific scFv fragments have been constructed that (1) comprises a scFv that targets the nucleus and a scFv fragment that restored the function of mutated p53 and (2) comprises the intracellular nuclear transport scFv fragment and a scFv fragment that binds Mdm2 and prevents destruction of p53 by Mdm2.⁹⁰ All three scFv fragments were constructed from corresponding hybridoma clones.

Single domain format

In contrast to the time-consuming strategies for selection of functional cytosolic/nuclear scFv's, nanobodies can be directly selected from immune, naive or synthetic libraries displayed on phage or yeast which significantly shortens the isolation of binders. Single domain antibodies are composed of only one V region (variable domain of the heavy or light chain) and in numerous examples it was shown that sdAbs can be stably expressed inside the cytoplasm or nucleus (Table I). In the next paragraph, current methods for construction and selection of camelid, shark and human sdAbs including new developments will be summarized.

Generation of cytosolic/nuclear single domain intrabodies

Single domains antibodies can be generated from camelid heavy-chain IgGs (VHHs), from cartilaginous fish IgNARs (V_{NAR}^S) or from conventional human IgGs (VH and VL). The antibody repertoires can be derived after immunization of camels or sharks, build up from naive natural antibody sequences or synthetically constructed based on stable scaffolds.^{10,24,56–58,61,63,81} However, the process of

immunization of sharks is demanding in comparison to immunization of mammals. Therefore, shark nanobodies have mostly been selected from synthetic repertoires. Human single domain VH and VL domains were selected from synthetic human VH or VL antibody repertoires.

Strategies to Construct Camelid VHH Repertoires

Construction of immune camelid VHH libraries

Camelid VHHs repertoires for the selection of single domain antibodies are mostly generated after immunization of camels. PCR amplification of VHH can be performed with primers binding to the N-terminus and CH2 region of camel heavy chain IgG cDNA, followed by nested PCR amplifying the VHH coding region. The product is cloned into a phagemid vector and a phage antibody repertoire established⁹¹ (Fig. 2).

Examples of cytosolic/nuclear nanobodies from immune camelid VHH libraries

So far, most nanobodies and cytosolic/nuclear nanobodies have been selected after immunization of llamas with the target protein. Nanobodies inhibited the function of HIV Rev,^{38,39} HIV Vpr,⁴⁰ HIV-1 Nef,^{42,43} influenza virus nucleoprotein,⁴⁴ hepatitis B Core antigen (HBcAg),⁴⁹ nonstructural proteins 9 and 4 of porcine reproductive and respiratory syndrome virus (PRRSV),^{50,51} p15 matrix protein of porcine endogenous retrovirus,⁵² SpvB toxin of *Salmonella typhimurium*,⁵³ neurotoxic protease of *Clostridium botulinum*,⁵⁴ mycotoxin 15-Acetyldeoxynivalenol (15-AcDON),⁵⁵ protein kinase C,⁵⁹ F-actin capping protein CapG,⁶⁰ L-plastin,⁶⁵ fascin and cortactin,⁶⁸ gelsolin,⁶⁹ β -catenin,⁷⁴ 2.5 dihydroxy pyridine dioxygenase (NicX),⁷⁶ β_2 -adrenergic receptor,²¹ Nla protease of potato virus Y⁷⁸ and GFP for depletion of target GFP-fusions in eukaryotes.⁷⁹

Naive and synthetic single domains libraries have the advantage that no immunization of animals is needed. A disadvantage is that the selected sdAbs are not affinity matured. Nevertheless, if these libraries contain a high number of clones with high diversity, sdAbs against all possible targets and epitops should be identifiable. Selected sdAbs can then easily be converted into cytosolic/nuclear antibody fragments by one cloning step (Fig. 3). In the next paragraphs, the principles of the generation of naive and synthetic libraries together with some new developments are shown.

Construction of naive camelid VHH libraries

Naive llama VHH libraries are single pot or universal libraries generated from blood samples from several animals for the selection of a large number of different specificities.⁹² Naive libraries with different

sizes have been constructed ranging from 10^7 to 5×10^9 clones.^{25,93} Recently, ribosome display with nanobodies has been performed with an expression cassette that can easily be used for *in vitro* synthesis of ribosome-VHH complexes in different expression systems.⁹⁴

Examples of cytosolic/nuclear naïve VHHs

Some interesting naïve cytosolic/nuclear nanobodies have been generated particularly against non-structural membrane protein NSB4 of HCV,⁴⁵ Bax,⁶⁴ NS3 helicase of HCV,⁴⁶ RNA dependent RNA polymerase NS5B,⁴⁷ nuclear poly(A)-binding protein 1 (PABPN1)⁷⁵ and Caspase-3.⁶²

Construction of synthetic VHH libraries

Synthetic camelid VHH libraries have been constructed based on natural VHH frameworks. Antigen binding diversity has been introduced by randomization of the CDRs, mainly CDR3.⁹⁵ Interestingly, a semi-synthetic library has been constructed from 3 unimmunized llamas. In this case, the CDRs and frameworks were diversified by error-prone PCR and then these sequences were spliced together through the common framework overlaps by PCR.^{96,97}

Examples of synthetic cytosolic/nuclear nanobodies

A specific nanobody that leads to cytoplasmic accumulation of heterogenous nuclear ribonucleoprotein K (hnRNP-K) has been selected.⁶³ Furthermore, a nanobody that significantly inhibits the enzyme activity of potato starch branching enzyme has been generated.⁷⁷ Recently, a very stable variant of an anti-GFP nanobody was constructed by transplantation of the CDRs from an anti-GFP VHH to a stable framework of an anti- β lactamase VHH.⁹⁸ This antibody was part of the morphotrap that was used to analyze the function of *Drosophila* Decapentaplegic (Dpp) on wing disc development.⁸⁰

Non camelid sdAb libraries

Construction of synthetic human VH/VL libraries

Synthetic human VH/VL libraries are constructed from a stable human VH/VL germline framework or from a VH/VL scaffold of a human monoclonal antibody. Randomization of the CDRs, especially of CDR3, leads to synthetic human VH libraries with high diversity.^{6,12-16,99} VL single domain antibody libraries have been constructed similarly¹⁶⁻¹⁸ (Fig. 3). Recently a synthetic library was constructed comprising the Ig1 domain of human neural cell adhesion molecule 1 (NCAM) in which randomized CDR1 and CDR3 from a shark clone were transplanted.¹⁰⁰ Chimeric antibodies were selected that block the signaling activity of chemokine receptor CXCR4.

Examples of synthetic human VH/VL cytosolic/nuclear sdAbs. Anti-myc human VH single domain antibodies have been selected from a synthetic human VH library based on a VH consensus framework of an anti-RAS scFv. Stepwise randomization of CDR3, CDR2 and CDR1 followed by yeast two hybrid system selection led to specific anti-myc clones.^{56,61} Similarly, an anti-RAS human VH and anti-LIM domain only 2 (LMO2) VH domain was selected.^{57,81} The anti-RAS VH inhibited RAS-dependent tumorigenesis in a mouse tumor model and the anti-LMO2 antibody fragment inhibits T-cell neoplasia in a nude mouse. An anti-epithelial kinase (Etk) human VL was selected from a synthetic library based on a human κ domain framework with randomized CDR3s.⁵⁸

Furthermore, camelization of human VHs can be performed by introduction of point mutations in the VH framework of an human antibody, mainly framework 2 to render the overall structure more hydrophilic.⁸⁸ Such camelized human VH frameworks have been used to generate CDR3 randomized single domain antibody repertoires displayed on phages for selection of new binders with improved solubility and refolding efficiency.^{101,102} Interestingly a rabbit-derived anti-HIV Vif VH single-domain intrabody was active in its camelized format.⁴¹

Construction of synthetic IgNAR variable domain (vNAR) libraries

In general, vNAR libraries have been selected from semi-synthetic libraries based on naturally occurring vNAR sequences and randomized CDR 3 loops.²⁸⁻³² A shark anti-hen egg-white lysozyme IgNAR VH clone was used as the scaffold for the generation of a semi-synthetic library with randomized CDR 3 loops.³³ Interestingly, a neutralizing VNAR single-domain antibody against BAFF that blocked the binding of BAFF to all three of its receptors was selected recently. In this case, the shark antibody repertoire was based on Type 2 nurse shark VNAR frameworks with a number of framework mutations and partial randomization of CDR3.³⁴ At present, no shark cytosolic/nuclear sdAb have been reported in the literature.

Selection of cytosolic/nuclear sdAbs

For selection of antigen-specific sdAbs, the sdAb repertoires are displayed on phages or on the cell surface of yeast cells. Then, by incubation of the recombinant phage antibody repertoire with immobilized antigen (biopanning), specific binders will be selected and cloned into a cytosolic or nuclear targeting vector (Figs. 2 and 3). The cytosolic or nuclear intrabodies will be evaluated in target cells, in xenograft tumor mouse models or transgenic sdAb mice.

Recently, a promising screening method to identify functional sdAbs has been developed.¹⁰³ Alpacas were immunized with a mixture of inactivated influenza A virus (IAV) and vesicular stomatitis virus (VSV). Generated VHH cDNAs were subsequently directly cloned into a retroviral vector to produce lentivirus particles. Then, A549 cells were infected with recombinant viruses and after 48 h treated with a lethal dosis of IAV and VSV. Surviving colonies were further characterized and the sequences of the VHHs were determined. After this screen, 19 VHHs that specifically block infection of cells with IAV or VSV were isolated. This screening approach should be applicable to identify inhibitors of any pathogen or biological pathway.

Most sdAbs have been selected by phage display without additional usage of the bacterial-, yeast- or mammalian-two hybrid system. This approach is usually reliable and can be used for selection of cytosolic/nuclear scFv fragments as well as sdAbs because cytosolic/nuclear selection will favor well-folded scFv fragments or sdAbs rather than the high affinity ones. However, it is still unclear if the affinity of antibodies is the only prerequisite for efficient intrabody engineering. Usage of bacterial-, yeast- or mammalian-two hybrid systems enables direct in vivo selection of functional cytosolic/nuclear intrabodies without the need of purified antigen.^{104–106} Another two-hybrid assay that allows studying the interaction of two proteins in living cells directly has been developed.¹⁰⁷ Here, a fluorescently labeled prey protein and the corresponding bait protein fused to fluorescently labeled lacI are coexpressed in mammalian cells that contain chromosomally integrated lac operator. Interaction of both molecules results in co-localization of two different fluorescent signals at the lac operator, which can be observed as a spot within the nucleus. The next paragraph provides a list of the selection and description of all known cytosolic/nuclear sdAbs derived from camels and human antibodies. Cytosolic sdAbs are described which have been successfully applied in xenograft tumor models and in addition a nanobody-GFP fusion protein is highlighted which is universally applicable for monitoring protein knockout in living cells.

Examples of cytosolic/nuclear single domain antibodies

Cytosolic/nuclear nanobodies that interfere with the function of approximately 40 cytosolic/nuclear targets have been generated successfully (Table I: reports of cytosolic/nuclear sdAb mediated knockdown). Compared to this number of cytosolic/nuclear targets ER-intrabodies against approximately 35 transitory proteins have been reported. Most sdAb-targets belong to viral or oncogenic proteins, followed by toxins and proteins of the nervous system.

Cytosolic intrabodies have also successfully been expressed in plants and in *Drosophila*. Single domain intrabodies partially protected potato plants against potato virus Y.⁷⁸ A universal method has been developed to monitor protein knockout in living cells.⁷⁹ To achieve this anti-GFP VHHs were fused to an F-box domain of *Drosophila* Slimb for degradation of targets fused to GFP via the ubiquitin depletion pathway. The technique was applied for the degradation of a transcription factor and a protein involved in morphogenesis of transgenic *Drosophila melanogaster* flies expressing GFP-target protein and anti-GFP VHH. In addition an anti-eGFP – CD8 transmembrane domain – cytoplasmic mCherry-fusion protein (morphotrap) was developed to study wing disc development in transgenic flies expressing eGFP-morphogenetic protein Decapentaplegic (Dpp) and the morphotrap. The morphotrap immobilized eGFP-Dpp on the cell surface, leading to inhibition of wing disc patterning.⁸⁰ Even gene silencing using sequence-selective and nucleic-acid hydrolyzing nanobodies is now possible.¹⁰⁸

Most sdAbs have been tested in cell culture and only few have been investigated in mice as an animal model. SdAbs against LIM domain only 2 (LMO2), F-Actin capping protein CapG and RAS were tested in appropriate xenograft tumor mouse models by transplanting tumor cells expressing the corresponding intrabody in nude mice or NOD-SCID mice.^{57,60,81} In addition an anti-HIV-1 Nef intrabody was able to rescue Nef-mediated thymic CD4⁺ T-cell maturation defect and peripheral CD4⁺ T-cell activation phenotypes in CD4⁺/HIV^{NEF} transgenic mice.⁴² Interestingly, fusion of a loop of a SH3 domain to one selected nanobody improved some inhibiting HIV-1 Nef functions.⁴³ Furthermore a cysteine-free human VL has been selected⁷¹ with better activity as the original antibody fragment. Interestingly changing the cysteine residues to valine and alanine led to lower affinity as the original antibody domain with the cysteins. An improved cysteine-free variant could be selected after random mutagenesis using error-prone PCR.

Several nanobodies against viral proteins, toxins, oncogenic proteins and proteins of the nervous system have therapeutic potential (table: reports of cytosolic/nuclear sdAb mediated knockdown). In the next paragraph new developments in protein delivery of scFv fragments/sdAbs and model proteins into the cytosol/nucleus of target cells will be shown.

Protein transfection with cytosolic/nuclear sdAbs

Alternatively to viral transfection or plasmid transfection, intrabodies can be delivered as proteins into the cytosol (profection). Delivery of intrabodies in the scFv or nanobody format into the cytosol of a target cell has the advantage that the intrabodies

retain their disulfide bridges for correct folding. Further, this method might be a safe alternative to viral gene transfer for clinical approaches.

The delivery of antibodies to the cytosol using different protection reagents has been studied in detail and was compared to electroporation, which was found to be the most effective reliable technique.¹⁰⁹

Alternatively, fusion of cell penetrating peptides or targeting proteins (ligands or antibodies) together with translocation domains or usage of cell penetrating peptides fused to endosome escape domains has been investigated.^{109,110} Fusion of peptides and proteins to reach the cytoplasm is often not very efficient because of inefficient endosomal escape.¹¹⁰ However, a cell penetrating anti-DNA antibody localizes to the nucleus through a nucleoside salvage pathway and is used as a delivery vehicle and combined with specific targeting scFvs.⁹⁰

Some highlights referring to cell-penetrating peptides should be mentioned. Highly efficient delivery was achieved using a cell-penetrating peptide-adaptor system built of TAT-calmodulin fusion and a fusion consisting of calmodulin binding site and cargo.¹¹¹ TAT-calmodulin non-covalently binds, delivers and releases cargo into the cytoplasm. Another system uses cell-penetrating peptides binding to heparan sulfate glycosaminoglycan fused to a protein transduction domain. Cell types that are generally difficult to transduce, such as mouse embryonic stem cells (mESCs), human ESCs and human induced pluripotent stem cells (hiPSCs), have been efficiently transfected.¹¹² Furthermore, camelid nanobodies against HCV proteins have been fused to cell penetrating penetrin and were successfully applied in HVC infected cells.^{45-47,113}

It was shown that the osteoblast transcription factor Runx2 can be targeted to the nucleus of mesenchymal cells by the peptide dentin phosphophoryn, bypassing the lysosomal degradation pathway.¹¹⁴ Recently, an efficient serum-stable and low-toxic molecular transporter comprising one rigid planar core and four flexible arms with one guanidinium group on each arm was constructed to efficiently deliver small cargoes and large active proteins into cytosol.¹¹⁵ A very interesting approach combined the TAT cell penetrating peptide to an endosomal escape sequence and GFP β 11 peptide. Endosomal escape can be measured by binding of GFP β 11 to non-fluorescent GFP β 1-10, inducing chemical formation of the GFP fluorescent chromophore. The assay enables screening of the next generation of endosomal escape domains.¹¹⁶

In addition, highly efficient cyclic cell-penetrating peptides have been designed. These cyclic peptides are characterized by efficient release from the endosome.¹¹⁷ Last but not least, the solvent exposed residues of the framework region of nanobodies have been mutated to positively charged arginines or

lysines (cationic resurfacing), leading to cell penetration.¹¹⁸ This approach has the potential to build a general applicable scaffold to transport nanobodies into the cell.

A very promising additional approach is the usage of the type VI secretion pathway of pathogenic bacteria to transport sdAbs into human cells.¹¹⁹ The injection of the sdAbs does not require bacterial invasion or the transfer of genetic material.

The approach to deliver drugs or toxins specifically to tumors mediated by nanoparticles decorated with nanobodies has been demonstrated in many *in vitro* examples and is now beginning to be tested *in vivo*.⁸⁸ Polymersomes and polyplexes are relatively new artificial vesicles that can be modified simply by modifying the polymer-building blocks.^{120,121} It might be very attractive to encapsulate recombinant cytosolic sdAb or sdAb expression plasmids into nanoparticles decorated with an specific anti-receptor nanobody (for example EGFR¹²² to deliver cytosolic anti-cancer single domain antibodies to tumor cells. Furthermore, nanoparticles/nanotubes coated with transferrin or Angiopep-2, a ligand for the low-density lipoprotein receptor-related protein-1 (LRP1), demonstrated translocation across the blood-brain barrier.^{123,124} The delivery of cytosolic/nuclear single domain intrabodies into the brain (for example recognizing huntington protein or α -Synuclein)^{71,72} might be possible in the future.

Conclusion

Single domain antibodies from camels, shark and humans are very useful to efficiently and specifically knock down cytosolic and nuclear proteins *in vivo*.

The option to specifically target interaction regions, conformers, splice variants, isoforms and even posttranslational modifications such as phosphorylation sites^{21,22,34,58,125-127} makes intrabodies very unique and valuable. RNAi, on the other hand, has the disadvantage of demonstrating off-target effects^{128,129} even if allele specific inhibition of Huntington protein and inactivation of oncogenic mutations of p53 by RNAi have been reported.^{130,131}

The availability of synthetic universal, single-pot camelid, shark and human single domain antibody libraries displayed on phages or yeast cells enables selection of a huge number of different single domain intrabodies theoretically directed against any antigen and desired epitope.^{12-18,28-31,33,34,95,96,132} By using antibody affinity maturation strategies, binders with high affinity can be isolated from these nonaffinity-maturated universal libraries by introducing CDR mutations, randomization of mutational hotspots or error prone PCR.³⁵⁻³⁷ Usage of a recently published method based on selection of specific inhibitory dAbs by recombinant lentiviruses to omit phage display selection will be attractive in the future.¹⁰³

Strategies to apply cytosolic/nuclear single domain intrabodies in the clinic may become possible with cell penetrating peptides,^{45–47,113} new optimized endosomal escape domains,¹¹⁶ sdAb expression plasmids or recombinant sdAbs embedded into nanoparticles such as polymersomes and polyplexes.⁸⁸ For cell- and tissue-specific transductional targeting, nanoparticles can be decorated with anti-receptor sdAbs. Application of mRNA coding for cytosolic/nuclear single domain intrabodies might also be possible in the future.^{133,134}

In summary, cytosolic/nuclear sdAbs of camelid, shark and human origin can be applied to clarify the function of uncharacterized proteins such as virus proteins and host cell factors, oncogenic proteins and cofactors, proteins of the nervous system, intracellular enzymes involved in signaling, transcription factors and proteins involved in differentiation and development.

Acknowledgments

Figures 1 and 2 were prepared by Oliver Backhaus. I thank Peter P Müller for reading the manuscript. Special thanks to S. Muyldermans and Wulf Blankenfeldt for critical reading the manuscript and giving very helpful comments.

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