Chapter 7

# ER-targeted Intrabodies Mediating Specific *In Vivo* Knockdown of Transitory Proteins in Comparison to RNAi

Oliver Backhaus and Thomas Böldicke

Additional information is available at the end of the chapter

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**Abstract**

In animals and mammalian cells, protein function can be analyzed by nucleotide sequence-based methods such as gene knockout, targeted gene disruption, CRISPR/Cas, TALEN, zinc finger nucleases, or the RNAi technique. Alternatively, protein knockdown approaches are available based on direct interference of the target protein with the inhibitor.

Among protein knockdown techniques, the endoplasmic reticulum (ER) intrabodies are potent molecules for protein knockdown *in vitro* and *in vivo*. These molecules are increasingly used for protein knockdown in living cells and transgenic mice. ER intrabody knockdown technique is based on the retention of membrane proteins and secretory proteins inside the ER, mediated by recombinant antibody fragments. In contrast to nucleotide sequence-based methods, the intrabody-mediated knockdown acts only on the posttranslational level.

In this review, the ER intrabody technology has been compared with the RNAi technique on the molecular level. The generation of intrabodies and RNAi has also been discussed. Specificity and off-target effects (OTE) of these molecules as well as the therapeutic potential of ER intrabodies and RNAi have been compared.

**Keywords:** Knockdown techniques, intracellular antibodies, ER intrabodies, RNA interference, off-target effects

## 1. Introduction

For the study of protein function in animals and mammalian cells, DNA-based methods such as gene knockout, targeted gene disruption, CRISPR/Cas, TALEN, zinc finger nucleases [1],

as well as the RNAi technique [2] were proven and reliable tools. Besides the RNAi technique, approaches with miRNA are also very attractive [3]. Silencing of target mRNA can be achieved using siRNA, miRNA, or shRNA (Box 1).

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**Box 1**

***siRNA***

Small interfering RNA (siRNA) are small pieces of double-stranded (ds) RNA, usually about 21 nt long, with 2-nt-long 3′ overhangs at each end. They can be applied for the interference with the protein translation by binding to the messenger RNA (mRNA), whereby promoting the degradation or destabilization of the mRNA.

***shRNA***

shRNAs form hairpin structures, which consist of a stem region of paired antisense and sense strands, connected by unpaired nucleotides building a loop. They are converted into siRNAs by the same RNAi machinery that processes miRNAs.

***miRNA***

MicroRNAs are small RNA molecules, encoded in the genome of plants and animals. These highly conserved, ~21-mer RNAs regulate the expression of genes by binding to the 3' untranslated regions (3'-UTR) of specific mRNAs.

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Protein knockdown is possible with small molecule inhibitors including peptides, neutralizing and intracellular antibodies, and allosteric modulators [4–8]. In addition, aptamers and intramers, in general short single-stranded DNA or RNA oligonucleotides are also potent molecules for specific inhibition of small molecules, peptides, proteins, or even whole living cells [9].

Currently, RNAi is the most often used gene-silencing technique in functional genomics [2]. In this article, we described an emerging protein knockdown technology using intracellular antibodies (intrabodies) targeted to the ER and compared the advantages and disadvantages of this promising technique with the RNAi technology. We tried to make scientists, who are interested in protein research or have very specific protein-related questions, familiar with the ER intrabody technology [10]. The molecular mechanisms of both methods are different. RNAi-mediated knockdown is based on the interference of siRNA with mRNA (Figure 1), whereas the protein knockdown by ER intrabodies is exerted upon binding of a recombinant antibody fragment to its specific antigen inside the ER [10] (Figure 2).

Intrabodies are recombinant antibody fragments targeted to a cell expressing the specific antigen. Intracellular binding of the intrabody to the antigen results in inhibition of antigen function. Moreover, intrabodies can specifically be targeted to subcellular compartments such

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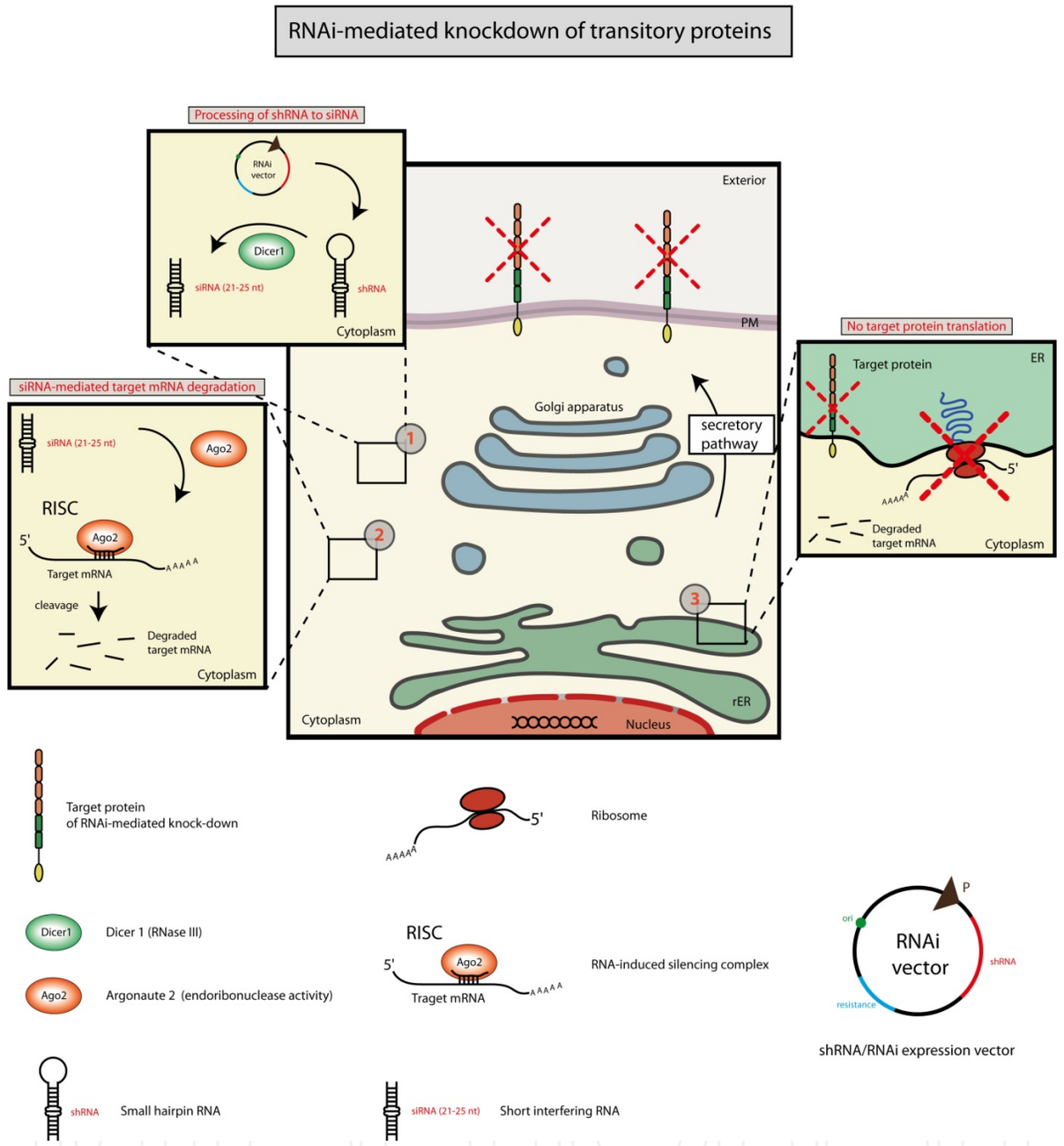


**Figure 1. Principle of the knockdown of transitory proteins using the RNA interference technique.** For knockdown of the mRNA of transitory proteins, transfection with a specific shRNA-expressing plasmid is sufficient. Although by using the RNA interference technology all kinds of proteins could be targeted, only knockdown of transitory proteins is illustrated. (1) Specific shRNA is transcribed and processed by the RNase III Dicer-1 enzyme in mammalian cells in order to form the mature siRNA. (2) The Argonaute 2 protein (Ago2) is loaded with the siRNA and forms together with additional proteins the RNA-induced silencing complex (RISC), which is a multiprotein complex consisting of effector (Argonaute proteins), accessory proteins, and si/miRNA. During the loading of the Argonaute protein, one strand of the siRNA duplex is discarded. Next, the RISC complex associates with its target mRNA via complementary base pairing of the siRNA and the target mRNA. In many cases, the recognition site comprises the 3′ untranslated regions (UTR) of the mRNA. Finally, target binding leads to mRNA degradation or translational inhibition [11]. mRNA degradation is mediated through the endonuclease activity of the Argonaute proteins. (3) As a result of mRNA knockdown, the target protein is not expressed on the cell surface [11].

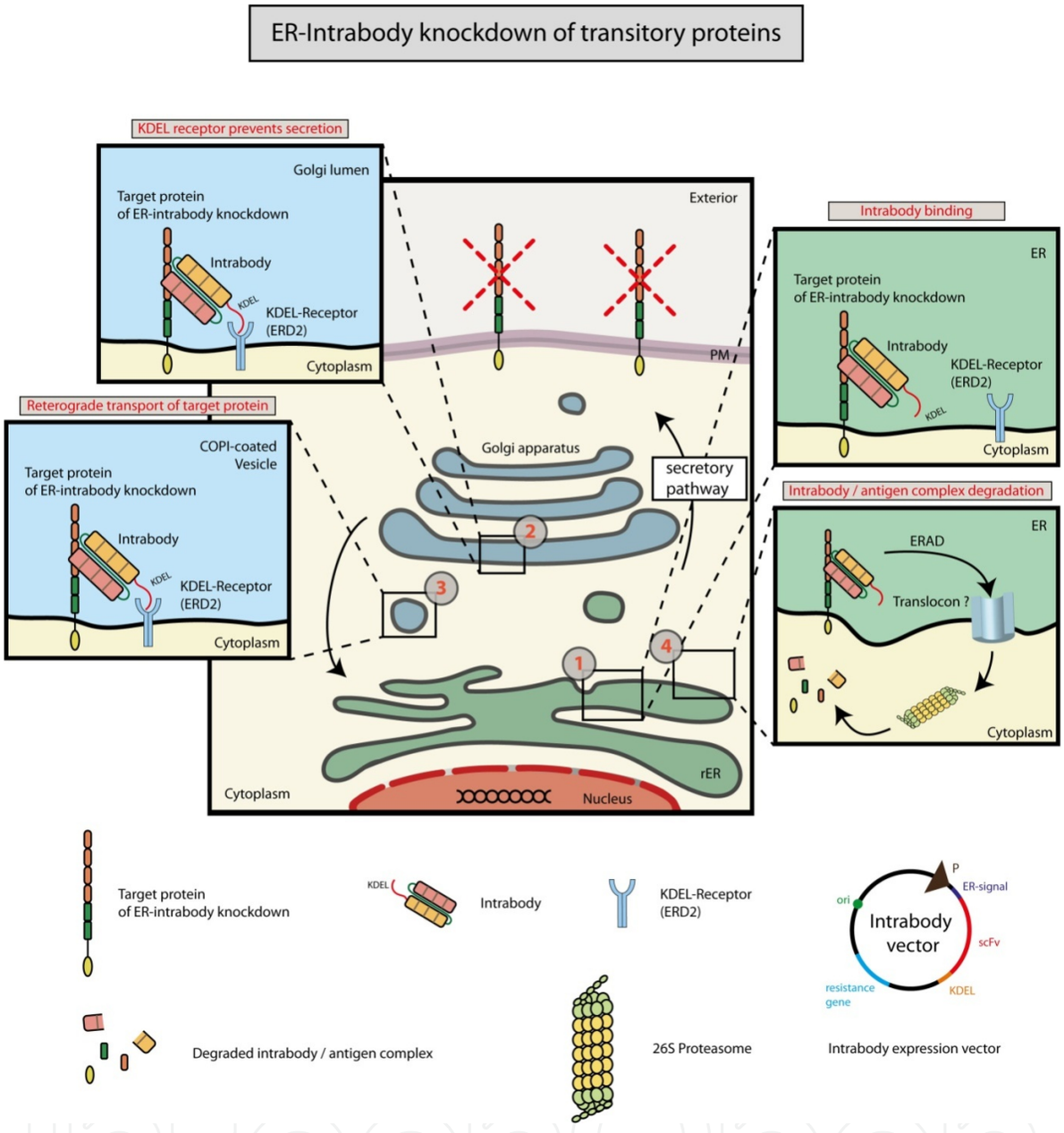


**Figure 2. Principle of the specific knockdown of transitory proteins with endoplasmic reticulum (ER)-retained intrabodies.** In wild-type cells, transitory proteins are transported through the ER and can be further processed (e.g., glycosylated) in the Golgi apparatus. These proteins could reside in the secretory cell compartments, secreted through the plasma membrane (PM), or become integrated in the PM as a membrane protein. For functional inhibition of these proteins, transfection with an ER intrabody expressing plasmid is sufficient. The intrabody construct consists of an N-terminal secretion sequence for the translocation in the ER (leader sequence) and the C-terminal retention signal (KDEL). (1) The intrabody inside of the ER binds to the target protein. This complex of antibody and target protein is further processed and transported through the secretory pathway. (2) In the cis-cisterna of the Golgi stack, the hERD2 receptor binds to the KDEL sequence and (3) initiates the retrograde transport back to the ER compartment. This continuous binding of the intrabody and retrograde transport prevents the target protein to reach its localization where it normally acts. (4) The accumulated intrabody–antigen complex in the ER might be transported into the cytoplasm, where it is marked for degradation by the 26S proteasome [12, 13]. Böldicke and Burgdorf have shown that an anti-toll-like receptor 2 (TLR2) ER intrabody is degraded by the proteasome (unpublished data).

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as the nucleus, cytoplasm, mitochondria, or ER [10] (Box 2). Currently, the most used and promising intrabodies are the ER intrabodies, because of the correct folding in the oxidative environment of the ER [14]. This contrasts with cytosolic intrabodies, in which disulfide bridges are not formed in the reducing environment of the cytoplasm [15, 16].

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**Box 2**

***Intrabodies*** are intracellularly expressed recombinant antibody fragments, which specifically inhibit the function of target proteins produced in the same cell [10].

***ER Intrabodies*** retain their corresponding antigen inside the ER by inhibiting the translocation of the antigen to the cell compartment where it normally acts.

***Cytosolic Intrabodies*** are expressed in the cytoplasm. They inactivate their targets or interfere with the binding of the target protein to its corresponding binding partner.

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The effect of ER intrabodies is based on retention of proteins passing the secretory pathway. Secretory proteins, membrane proteins, and even Golgi or endosomal-located proteins can be targeted [17–19], which cannot be reached by classical antibodies, due to the extracellular presence. Successful functional knockdown was achieved for oncogenic receptors, viral proteins for preventing virus assembly, cellular virus receptors to block virus entry, and receptors of the immune system as well as of the nervous system [20–24].

The format of expressed intrabodies is, in general, the single-chain variable fragment (scFv) or less common the antigen-binding fragment (Fab) [25]. The only prerequisite of ER intrabodies is the efficient binding to the antigen, and the method to select and generate an ER intrabody is greatly simplified by phage display. On the contrary, functional cytoplasmic intrabodies have to inactivate the antigen or have to interfere with the binding of the target protein to its corresponding binding partner [10].

The starting material for construction of an ER intrabody is an scFv or Fab, which can be obtained by amplification of the variable domains from a hybridoma clone [26], or scFv fragments can be selected from phage or yeast display [27, 28].

Early attempts using the intrabody approach failed frequently due to the lack of reliable techniques for the identification of the correct functional antibody sequence from a hybridoma clone. The genes of the variable domains for construction of recombinant antibody fragments can be amplified from hybridoma clones using mixtures of consensus primers [29]. This approach was used in the beginning. As hybridoma cells could secrete several different antibodies, it was sometimes difficult to isolate the correct functional sequences of the variable domains. Presently, with reliable protein sequencing techniques, next generation of DNA sequencing and optimized consensus primer sequences, the functional antibody DNA can much better be identified. Furthermore, optimized strategies for amplification of the correct functional antibody sequence are available [30–32].

In the case of using *in vitro* display systems, like phage or yeast cell surface display, the selected scFv fragment has only to be cloned into the ER-targeting vector. For preliminary characterization of the intrabody function, co-transfection of the intrabody expression plasmid with the corresponding antigen expression plasmid into HEK 293 cells is sufficient and followed by co-immunoprecipitation and immunofluorescence analysis [33].

In contrast to the ER intrabody technology, the advantage of the RNAi is that it can be applied for almost every mRNA and also non-coding RNAs. Here, we further compared the RNAi with the intrabody technology, regarding specificity, off-target effects, and therapeutic approaches.

## 2. Intracellular intrabodies versus RNA interference

### 2.1. Generation of ER intrabodies

The prerequisite for generating intrabodies is the availability of a hybridoma antibody clone or scFv/Fab fragments selected from *in vitro display* systems [10]. Starting from a hybridoma clone, the variable domains of the heavy and light chain are amplified by PCR from the cDNA. This can be achieved by (1) PCR amplification using consensus primer [29, 34–36], (2) rapid amplification of cDNA ends (RACE) [30], (3) PCR amplification using adaptor-ligated cDNA [31], or (4) inverse PCR with constant region heavy chain and light chain primer, amplifying the corresponding antibody sequence from circularized double-stranded cDNA [32] (Figure 3 A).

In most cases, using consensus primers is a fast and efficient approach for amplification of the correct functional antibody sequence from a hybridoma clone. However, less-common non-consensus antibody sequences cannot be amplified and primer mismatching could be a problem. Approaches (no. 2–4 shown in Figure 3) for amplifying the variable antibody domains are more time-consuming; however, the correct functional antibody gene sequence can be obtained. The variable domains are compiled by assembly PCR, linking both variable domains together by a short flexible linker sequence, for example $(Gly_4Ser)_3$, resulting in the scFv fragment. Next, the scFv fragment will be cloned into the ER targeting vector, providing the ER signal sequence, an myc tag for detection of intrabody, and the KDEL retention sequence localized at the C-terminus of the intrabody gene [26].

Following the *in vitro* display pipeline, an scFv fragment or Fab fragment selected by phage or yeast cell surface display can directly be cloned into the ER targeting vector. Most recombinant antibody fragments in the scFv or Fab format are selected by phage display or also the frequently used yeast cell surface display [27, 28, 41]. Other *in vitro* display systems are bacterial, mammalian cell surface display, or ribosome display [42–44]. Cytoplasmic intrabodies are generated from hybridoma clones or scFv/Fab fragments from *in vitro* display libraries in a similar way and cloned into an appropriate cytosolic targeting vector [10]. The main difference in comparison to ER intrabodies is that cytosolic intrabodies have to demonstrate neutralizing activity, and furthermore stable folding antibody fragments have to be selected [10].

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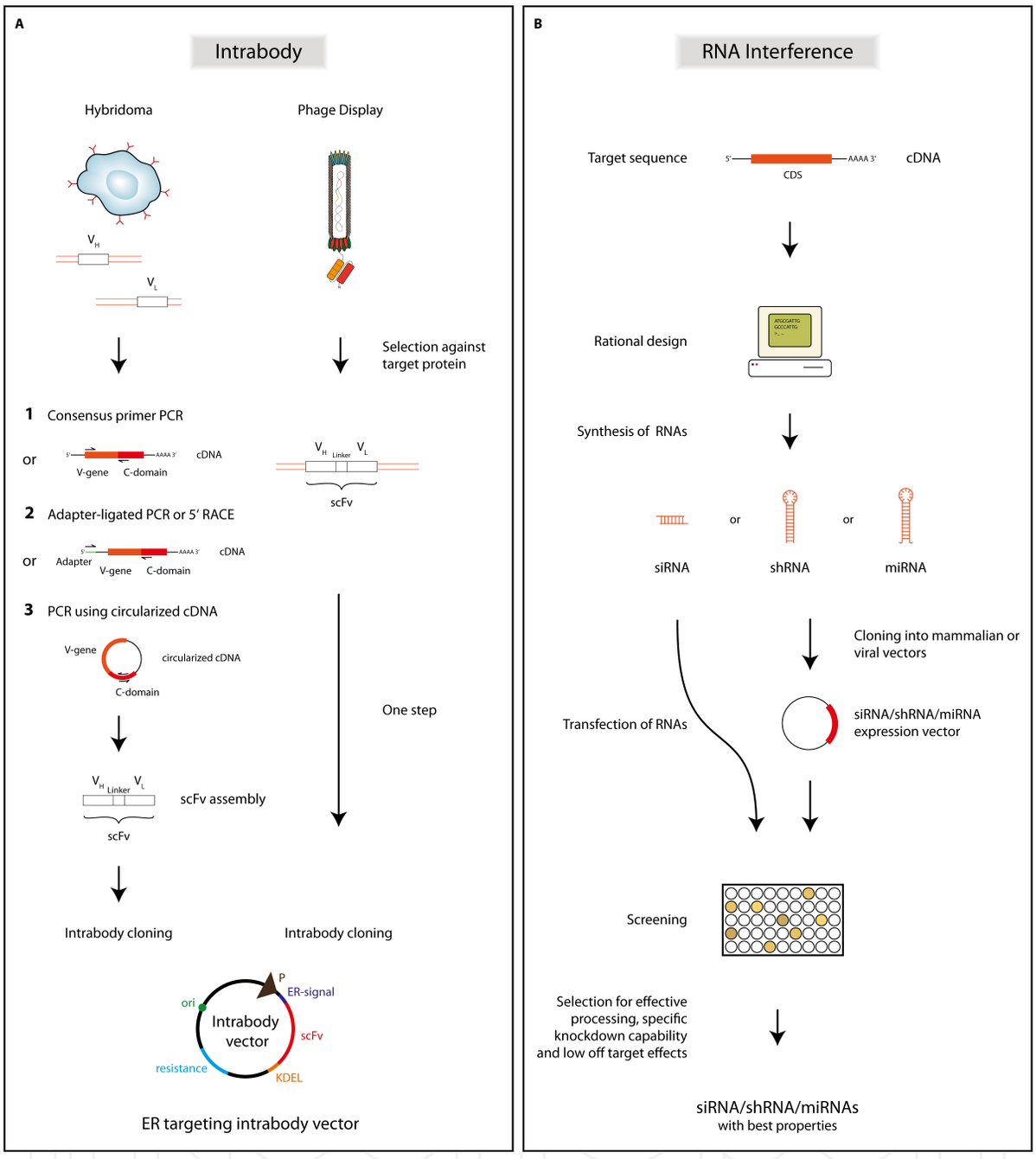


**Figure 3. Generation of intrabody and RNA interference knockdown constructs.** (**A**) Generation of intrabody knockdown vectors. The scFv fragment could be either cloned from hybridoma cell lines or selected from huge human naive phage display libraries. The antibody variable domain of the light chain (VL) and heavy chain (VH) is amplified from cDNA using consensus primer mixtures (1), 5′ adapter-ligated PCR or rapid amplification of cDNA ends (RACE) (2) or with constant domain-specific primer from circularized cDNA (3). The antibody VL and VH genes are assembled as scFv by fusing both domains with a flexible $(Gly_4Ser)_3$-linker sequence and cloned into the ER targeting intrabody vector. The scFvs are cloned between an upstream secretion signal and a downstream retention sequence (KDEL). Using the phage display system, selected scFvs can directly be cloned into the intrabody vector in one cloning step. Shown is an ER-targeting vector. (**B**) Generation of siRNA/shRNA/miRNA knockdown vectors. Rational *in silico* design of siRNA, shRNA, or miRNA mimics using software algorithms like those mentioned in Ref. [37] or in Ref. [38], a recent publication, deduced from the target cDNA. The algorithms are designed to select appropriated sequences by means of empiric criteria. Main criteria are an siRNA length of 19–21 nucleotides (nt) in conjunction with 2 nt overhangs at

their 3′ ends, as well as thermodynamic properties of target mRNA hybridization. Rational design can be expanded by testing *in silico* the potential off-target effects of the designed sequences by using genome-wide enrichment of seed sequence matches (GESS) [39] or Haystack [40]. Designed sequences are chemically synthesized and cloned into appropriate mammalian or viral knockdown expression vectors. Alternatively, siRNA can be used for direct cell transfection. The siRNA/shRNA/miRNA sequences originated from rational design are screened for effective processing, specific knockdown capabilities, and potential off-target effects. Corresponding clones are selected, and for most applications 3–4 different targeting sequences were chosen and theses libraries are used for the RNA interference knockdown. ER: endoplasmic reticulum, CDS: coding DNA sequence, p:promoter.

### 2.2. Generation of siRNA, miRNA, and shRNA

In order to generate siRNAs for a specific target, only the mRNA information about the target sequence is needed [45] (Figure 3 B). siRNA-mediated mRNA knockdown can be performed in several ways. In general, cells can directly be transfected with siRNA, using transfection reagents like lipofectamine. Cells can also be transfected using siRNA/shRNA/miRNA-expressing plasmids or viral vectors. Long-lasting gene silencing can be achieved with shRNAs expressed from stably transfected plasmids or from integrated retro- or lentiviral vectors [46]. Several approaches exist for RNA interference-mediated knockdown, the principle workflow of *in silico* design, screening, and selection of siRNA/shRNA/miRNA, with best knockdown properties shown in Figure 3 B. Currently, software algorithms mentioned in Ref. [37] or [38] can help to find the appropriate knockdown sequences of 19–21 nt length siRNA by analysis of the optimal thermodynamic properties of mRNA hybridization. Potential off-target effects can be reduced by *in silico* optimization with GESS [39] or Haystack [40]. Resulting siRNA/shRNA/miRNA sequences are tested for effective processing, specific knockdown capability, and low off-target effects.

### 2.3. Stability

Intrabodies are stably expressed inside the ER [14], whereas most cytosolic intrabodies are not correctly folded [15, 16]. On the other hand, siRNA can be cleaved by nucleases, present in the blood serum and cellular cytoplasm.

### 2.4. Specificity and Off-Target Effects (OTE)

For the knockdown of distinct target proteins, the specificity of the process is crucial. Otherwise, the resulting phenotypes of the induced knockdown experiment might be superimposed with off-target effects. The specificity of the RNAi and the ER intrabody knockdown technique is the main difference between them.

Intrabodies, which are also known as intracellular antibodies, are generated from monoclonal antibodies (mAbs) and phage or yeast antibody repertoires. Intrabodies are very specific to their targets due to antibody–antigen interactions.

The high specificity of ER intrabodies has been demonstrated for the specific knockdown of members of the TLRs. The knockdown of toll-like receptor 2 (TLR2) and TLR9, which functions as a part of the innate immunity and recognizes pathogen-associated molecular patterns (PAMP), did not influence the expression of other TLRs. The developed anti-TLR2 intrabody

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did not inhibit TLR3-, TLR4-, and TLR9-driven signal transduction [33] and the anti-TLR9 intrabody did not inhibit TLR3-, TLR4-, TLR7-, and TLR8-driven signaling, respectively [18].

Stress response induction in the endoplasmic reticulum (ER), due to the accumulation of retained and partially unfolded target proteins upon intrabody–antigen complex formation, was analyzed by measuring the unfolded protein response (UPR) for an overexpressed anti-p75NTR ER intrabody and could not be proven [24]. No off-target effects of expressed intrabodies are known yet, particularly any activation of the immune system.

On the other hand, unspecific silencing is a major problem using RNAi-mediated gene silencing, due to the expression of short-interfering RNA sequences, such as miRNA, siRNA, shRNA, or dsRNA [47]. The short seed region of these silencing RNAs recognizes and hybridizes with 2–8 nt to the target mRNA. Even with specific alignment software, it is practically impossible to exclude any possible transcript, which aligns with the target seed sequence, because statistically the chance is high to have the same sequence or secondary structure in other non-target mRNA transcripts too. However, at least software algorithms such as GESS [39] and Haystack [40] are able to predict potential off-targeted genes. By computer-aided optimization of the miRNA, siRNA, or shRNA, the OTEs can be reduced to a minimum.

siRNA can bind to TLR3, TLR7, and TLR8, resulting in secretion of type I interferon and pro-inflammatory cytokines [48–50]. Aberrant expression of up to more than 1000 genes has also been described [51].

Fortunately, some progress has been made in the repression of the RNAi-induced immune response. When siRNA is *in vitro* transcribed by the T7 polymerase, a 5′-triphosphate group is added. The 5′ triphosphate is recognized by the innate immunity, and it activates the type I interferon response. This can be prevented by chemical synthesis of siRNA, which misses the 5′-triphosphate group. Furthermore, the siRNA molecules can be modified by adding 2′-O-methyl groups, in order to reduce the recognition by toll-like receptors (TLRs) [52]. Interestingly, this modification additionally hampers degradation of the siRNA by RNases, leading to an increase in serum half-life [53]. Finally, strong destabilizing unlocked nucleic acids (UNAs), which were altered to have an acyclic ribose, also reduce the recognition by TLRs [54].

The specific suppression of one allele in heterozygous genes is of concern in dominantly inherited genetic disorders. Huntington's disease (HD) is caused by a dominant mutation of the huntingtin protein (Htt) and an excellent target for the examination of allele-specific knockdown of the mutated Htt, with high therapeutic potential. Huntington's disease is based on a long stretch of CAG triplets on one disease-caused allele [55]. Most of the patients are heterozygous for the *htt* gene mutation and 48% of the American and European HD patients are heterozygous at a single nucleotide polymorphism (SNP) site, making this genetic disease a *bono fide* target for specific protein knockdown. Approaches to inhibit the appearance of Huntington's disease is silencing of wild type and mutant Htt or silencing of only the disease-causing allele.

Although it was found in HD mice that co-silencing of wild type and mutant Htt provides therapeutic benefit, nothing is known of such a long-term suppression of huntingtin [56]. Thus,

the effect and safety over decades have yet to be proven in clinical trials. Therefore, there is still a need for high allele-specific inhibition of the mutant Htt protein, which is toxic due to an expanded polyglutamine (polyQ) motif (CAG motif). Targeting of the CAG motif is not selective for the mutant allele and affected both alleles. Genotyping of the Huntington's disease patients resulted in three single nucleotide polymorphisms (SNP) in huntingtin [57]. Therefore, an alternative strategy when using RNAi is targeting a single nucleotide mutation localized in the disease-caused sequence [58]. Furthermore, targeting of the mutant huntingtin SNPs or the expanded CAG motif by designed artificial miRNAs was recently demonstrated *in vitro*, using an allele-specific reporter system and *in vivo* in a transgenic mouse model [59].

For the RNAi technique, it is possible to discriminate between very similar targets with a specific reduction on the RNA and protein level [58, 60, 61]. However, there are still some concerns and limitations. The RNAi-mediated allele-specific knockdown may result in a broad off-targeting and therefore has to be further evaluated in appropriate preclinical model systems [62]. Using both target strategies, CAG motif and prevalent mutant SNPs, in the case of huntingtin, the wild-type allele is also affected by the knockdown, and the knockdown ratio between the wild type and mutant allele remains unsatisfactory. Furthermore, the shift to *in vivo* delivery systems can have a substantial impact on the specificity, as was demonstrated in the mouse model [59]. Next, a limited expression of the miRNA vectors is important to avoid saturation of the miRNA processing machinery, as the selectivity seems to be reduced when miRNAs are highly expressed *in vivo* [59].

Different alleles can also be targeted and discriminated using specific intracellular antibodies (intrabodies) and represent a valuable alternative to RNA interference. Intrabodies targeting, for example, huntingtin have to recognize an epitope common in most disease-associated huntingtin SNP forms, which also has to be different in the translated amino acid between the mutant and wild-type allele. Alternatively, they could target the expanded polyglutamine (polyQ) motif associated with misfolding and aggregation [63]. Furthermore, cytoplasmic intrabodies have been developed, which efficiently inhibited aggregation of mutant HD [64]. Interestingly, a disulfide bond-free single-domain intracellular antibody with high affinity was developed after affinity maturation [65] from a specific anti-HD scFv fragment, demonstrating the power of antibody engineering.

For the allele-specific knockdown, the intrabody technology utilizes the high specificity of monoclonal antibodies, with no or low concerns about off-target effects and activation of the immune system. In the case of huntingtin, no RNAi approach was able to discriminate effectively between the wild-type and mutant expanded polyglutamine stretch [59]. Here, intracellular antibodies could, in principle, recognize different conformational epitopes formed by polyglutamine and might be able to discriminate between the length of the polyQ motifs [63]. However, in the case of the cytoplasmic huntingtin protein, it is more difficult to generate and select cyto-intrabodies, due to the reducing environment of the cytoplasm. In general, the allele-specific knockdown strategy should be also applied with ER intrabodies.

The kind of mismatches introduced into siRNAs or artificial miRNAs, in order to increase allele specificity for preference of the mutant allele, can differ. Purine-to-purine mismatches, for example, are more effective than purine-to-pyrimidine mismatches. This limitation can be

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overcome by introduction of a second mismatch, preferentially into the seed or cleavage region of the siRNA/miRNA [59]. Using a set of SNP sites, common in disease-associated alleles, might enable reaching many patients [57], but it is hard to access the whole population. For those genotypes that could not be cured by using mutant SNP-targeting siRNA, intrabody-mediated protein knockdown, recognizing a prevalent mutant epitope could be superior. Whereby, in the case of SNPs due to the posttranslational targeting, the intrabody technology demonstrates one of its weaknesses. Discrimination between mutant and wild-type SNP could only be achieved when the mutant SNP induces a change of the encoded amino acid. In addition, mutant SNPs in introns and untranslated regions (UTR) cannot be addressed, as it is in the case of HD.

| **Features** | **Intrabodies** | **siRNA, shRNA, miRNA** |
|---|---|---|
| **Requirements** | Monoclonal antibody or scFv/Fab selected by phage or yeast cell surface display | Sequence of the mRNA |
| **Very high specificity to the antigen** | + | Off-target effects |
| **Stability** | Stable in the ER | Susceptibility to nucleases |
| **Inhibition of post-translational modifications** | + | - |
| **Inhibition of splice variants** | + | + |
| **Inhibition of several protein isoforms with one intrabody or siRNA** | + | + |
| **Targeting of specific protein domains** | + | + |
| **High-throughput screening** | - | + |
| **In vivo knockdown** | + | + |

**Table 1.** Intrabodies versus siRNA

## 2.5. High-throughput screening

Oligonucleotide and cDNA microarrays can be applied for simultaneous quantitative monitoring of gene expression of thousands of genes [66]. A combination of cDNA microarrays and

RNA interference was used to validate upregulated genes, playing an important role in cancer development [67]. In this case, a pre-screening with cDNA microarrays is performed followed by silencing of selected upregulated mRNAs using RNAi. This might also be possible with intrabodies.

Although high-throughput RNAi screening is very useful in order to validate new genes involved in cancer pathogenesis or infection processes [68, 69], such high-throughput screening is not possible with intrabodies.

### 2.6. Therapeutic potential of siRNA and ER intrabodies

The therapeutic potential of siRNA and ER intrabodies has been shown in different mouse models [70–73]. It has been shown that siRNA protected mice from fulminant hepatitis [74], viral infection [75], sepsis [76], tumor growth [77], and macular degeneration [78]. In these mouse models, synthetic siRNA was delivered systemically, peritoneally, or subretinally.

Furthermore, in an Alzheimer's and spinocerebellar ataxia disease-related mouse model, RNAi suppresses the expression of amyloid-β peptide or ataxia, respectively [79, 80]. In these mouse models, target-specific RNAi was virally delivered using adeno-associated virus or Herpes simplex virus. Interestingly, the knockdown of angiopoietin-2 mRNA in a mouse model with pancreatic carcinoma and xenotransplantation suppresses metastasis and downregulates metalloproteinase-2 [81].

Many ER intrabodies have shown therapeutic potential against relevant targets in cancer, infection, and brain diseases, for example, ErbB-2, EGFR, VEGFR-2, Tie-2, VEGFR-2 × Tie-2, metalloproteinases MMP-2, MMP-9, E7 oncoprotein of human papillomavirus, CCR5, TLR2, TLR9, and amyloid-β protein [18, 33, 82–90]. Nevertheless, only four of these antigens have been applied in xenograft tumor mouse models so far, using an anti-Tie intrabody [85], a bispecific VEGFR-2 × Tie-2 intrabody [86], an anti-amyloid-β protein intrabody in an Alzheimer's disease mouse model [90], and an anti-E7 oncoprotein intrabody in a mouse infection model with human papillomavirus [89]. Intrabody delivery was performed via adenovirus, adeno-associated virus, and retrovirus, respectively.

#### *2.6.1. Transgenic mice*

Transgenic RNAi mouse against p120-Ras GTPase-activating protein [91] and cytokine-activated IκB kinase 1 (IKK1) has been established [92]. Furthermore, RNAi transgenic mice and non-germline genetically engineered RNAi cancer mouse models were established [93]. In contrast to constitutive RNAi transgenic mice, generation of conditional RNAi in mice is also possible [94].

Recently, two transgenic ER intrabody mice have been generated against VCAM and gelsolin [71, 72]. In addition, a transgenic mouse expressing an anti-EVH1 intrabody has been published [73]. However, the inhibitory results obtained with these mice have been criticized because the intrabody was directed to the secretory pathway, but confusingly recognized a cytosolic protein [95]. Interestingly, the transgenic VCAM intrabody mouse was viable in

contrast to the lethal knockout mice generated by targeted homologous recombination [96]. The intrabody mice were deficient in VCAM-1 cell surface expression.

#### *2.6.2. Clinical approaches*

Different clinical approaches have been performed with siRNA. RNAi-based clinical trials are ongoing (phase I–III) [62, 97]. For example, a Bevasiranib RNAi targeting VEGF has been applied to heal macular degeneration [98] and RNAi targeting the RSV nucleocapsid SPC3649 has shown significant anti-viral activity [99].

In comparison to the RNAi, only one example of an ER intrabody targeting erbB-2 has been applied in a clinical phase I study [82]. As demonstrated, none of the patients treated in this study exhibited a dramatic clinical benefit.

Both methods share the limitations of viral and non-viral delivery methods. Using integrating vectors, insertional mutagenesis is still the main problem [100]. Concerning non-viral delivery methods, lipid-based and peptide polymer-based delivery systems have been applied [101]. However, for some diseases like HD, the non-neurotropic feature of many delivery systems and the lack of passing the blood–brain barrier (BBB) remain problematic.

Cell- and tissue-specific targeting is also always a concern; however, transductional and transcriptional targeting is promising [102]. Tissue-specific carrier for siRNA includes aptamers, antibodies, peptides, proteins, and oligonucleotide agonists [101]. Referring to ER intrabodies, the use of mRNA in clinical approaches is promising [103].

### 2.7. Other features

Intrabodies are able to inhibit posttranslational modifications, such as phosphorylation sites [104, 105]. This is not possible using RNAi. Besides the high specificity of intrabodies, this is an important advantage of intrabodies over RNAi.

Recently, single-stranded siRNA was used to suppress the spliced variants of proteins [106]. This might also be possible with specific intrabodies (Table 1). In addition, targeting of specific protein domains and isomers of a protein might also be feasible. For example, miRNA suppresses specifically an oncogenic isoform [107]. Intriguingly, the suppression of different protein isoforms with only one intrabody or one siRNA, recognizing a common epitope within all isoforms, might be possible, for example, the knockdown of all interferon alpha isoforms (13 different subtypes in human).

### 2.8. miRNA

It is known that miRNA influences tumorigenesis [3], and therefore miRNA and combined miRNA/siRNA pharmacological approaches are attractive [108]. miRNA has been applied in cancer mouse models as for lymphoid malignancies [109]. Furthermore, important studies using miRNA has been performed for diagnosis, prognosis, and prediction of cancer [108]. One of the most developed microRNA-based candidates is MRX34, a miR-34 mimetic that restores the function of miR-34 in cancer cells [110], which is applied in an ongoing multicenter

phase I clinical trial. The repression of expression of several potential miR-34 target oncogenes was demonstrated [111]. Finally, miRNAs can be used to reprogram somatic cells into pluripotent stem cells [112]. However, siRNA and miRNA share the same silencing machinery and microRNA causes also off-target effects [113].

## 3. Conclusions and perspectives

siRNA and ER intrabody technology are both efficient knockdown techniques. siRNA is acting on the mRNA level, whereas ER intrabodies are acting on the protein level. The strength of the RNAi technology results from the possibility that nearly all mRNAs of a cell can be targeted. Currently, the knockdown of proteins mediated by intrabodies is most promising with ER intrabodies, because they are correctly folded inside the ER and can be generated more easily than in the past. Because of the availability of many new scFv fragments, generated by research consortia, one cloning step is sufficient to convert selected scFv fragments into ER intrabodies.

Stable cytosolic intrabodies have to be selected with considerable effort. Two approaches are successful and reliable: the intracellular antibody capture technology, based on an antigen-dependent two-hybrid system [114] and single-domain antibodies [115], which are stably folded in a reducing environment for inhibition of cytoplasmic proteins. Single-domain antibodies comprise only one V region, the variable domain of the heavy or light chain. Most successfully applied are camelid single-domain antibodies ($V_HHs$) [115–118]. Alternatively, human VL and VH domains are also potent molecules and their successful construction is ongoing [119, 120].

The number of ER intrabodies will increase due to the fact that international research consortia as the "Affinomics" initiative [121] in the European Union and similar initiatives in the United States have already generated several thousands of recombinant antibodies, including the V-region genes, which can be used to build up a new repertoire of intrabodies. Using this pipeline, the duration for development of intrabodies is similar to that of siRNA/shRNA/miRNAs. In the future, scFvs against very valuable disease-related targets have to be provided.

The main advantage of intrabodies is their specificity, no off-target effects, and posttranslational modification inhibition. The specificity of an intrabody can be estimated by immunoassays such as ELISA, flow cytometry, and immunoprecipitation. On the contrary, the specificity and off-target effects of RNAi are often more difficult to predict.

Conferring to *in vivo* application, RNAi has been currently applied predominantly in phase 1 and 2 studies [62, 97]. In the future, the success of clinical approaches using RNAi and ER intrabodies is dependent on the development of safe viral vectors and the development of non-viral vectors possessing high transfection efficiency [122].

Two attractive applications of RNAi, hardly to perform with ER intrabodies, are genome-wide screening [68, 69] and reprogramming of somatic cells into pluripotent stem cells [112].

Thus, the ER intrabody approach has demonstrated its huge potential for *in vitro* and *in vivo* analysis of protein function [10]. The ER intrabody technique can complement the RNAi

technique in cases where siRNA, shRNA, and miRNA molecules demonstrate unwanted unspecificity and off-target effects.

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## Author details

Oliver Backhaus and Thomas Böldicke*

*Address all correspondence to: thomas.boeldicke@helmholtz-hzi.de

Helmholtz Centre for Infection Research, Department of Structural and Functional Protein Research, Braunschweig, Germany

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