Target validation through protein-domain knockout – applications of intracellularly stable single-chain antibodies

Peter Lichtlen, Adrian Auf der Maur and Alcide Barberis

The human genome project has delivered a large number of genes and respective proteins that await to be validated as potential drug targets. Such a complexity has made target validation the main bottleneck in today's drug development process. In addition, the majority of these new potential targets are proteins that function intracellularly. Approaches such as gene knockout, antisense RNA or RNA interference (RNAi) are currently used to validate candidate drug targets by analysing the effects of their deletion. Intrabodies (single-chain antibodies expressed within the cell) present an attractive alternative for directly modulating protein function *in vivo*. In particular, intrabodies can be used to target specific domains of a protein and perform so-called 'protein-domain knockouts', thus allowing the dissection of the varied functions of multi-domain proteins.

Peter Lichtlen*
Adrian Auf der Maur
Alcide Barberis
ESBATech AG
Wagistrasse 21
CH-8952 Zurich-Schlieren
Switzerland
Tel.: +41 1 733 49 00
Fax: +41 1 733 49 90
*e-mail: lichtlen@esbatech.com

▼ Sequencing of the human genome has provided the pharmaceutical industry with thousands of new potential drug targets, most of which operate intracellularly [1,2]. This fact opens new perspectives for therapy of human diseases but also demands new approaches in target validation. Different experimental tools are currently used for validation of these emerging intracellular drug targets. The classical way to investigate the function and thereby determine the physiological and pathological relevance of gene products is to interfere with the expression of the gene.

Gene knockout in mice by performing homologous recombination in mouse embryonic stem cells (ES cells) is a commonly applied approach for target validation, acting at the genomic level. The strength of this technology is its power for investigation of the function of a gene in the whole mammalian organism. Nevertheless, the achievement of a gene knockout is time-consuming, laborious and not applicable in human cells. In addition, compensatory effects or, conversely, complex phenotypes caused by the lack of varied protein functions frequently impair the dissection of specific functions of a gene product (for a review see Ref. [3]). Hence, from the phenotype of a knockout mouse, one often cannot extrapolate the effects of a putative drug action, as 'smart' modern drugs usually influence specific functions of protein domains (e.g. by binding to the ATP-binding cassette of a kinase domain, as exemplified by Gleevec) rather than eliminate all interactions of the target protein with the proteome.

Antisense and RNA interference (RNAi) approaches intend to perform mRNA knockdowns. In principle, the idea behind an antisense approach is very simple: Base pairing of the antisense RNA (or DNA) with its corresponding mRNA is thought to block translation of the mRNA either by preventing the access of the protein translation machinery or by marking the RNA for degradation by RNase H [4,5]. An advantage of this technology is the possibility of rapid and cost-effective generation of specific antisense oligonucleotides. Nevertheless, antisense technology has to overcome several obstacles such as low stability, toxicity, identification of accessible mRNA sequences, specificity problems and the generation of immune responses (for a comprehensive review see Refs [5,6]). Today, some but not all of these problems have been solved by chemical modifications of the antisense RNA backbones [5]. Yet,

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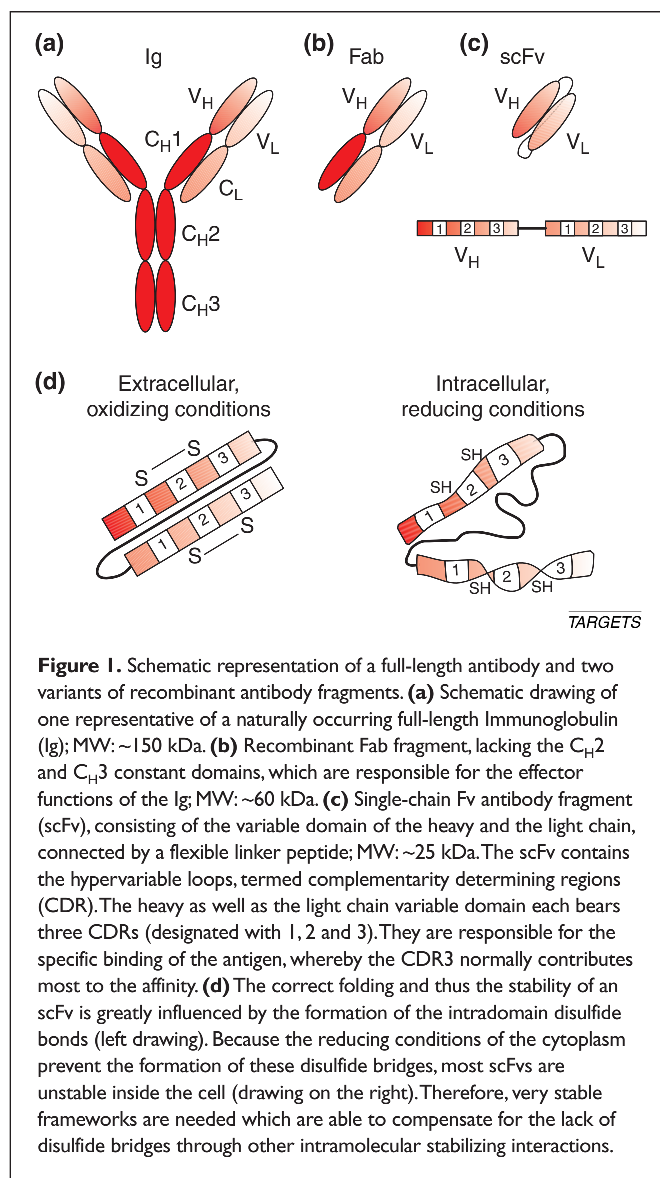

Figure 1. Schematic representation of a full-length antibody and two variants of recombinant antibody fragments. **(a)** Schematic drawing of one representative of a naturally occurring full-length Immunoglobulin (Ig); MW: ~150 kDa. **(b)** Recombinant Fab fragment, lacking the C_H2 and C_H3 constant domains, which are responsible for the effector functions of the Ig; MW: ~60 kDa. **(c)** Single-chain Fv antibody fragment (scFv), consisting of the variable domain of the heavy and the light chain, connected by a flexible linker peptide; MW: ~25 kDa. The scFv contains the hypervariable loops, termed complementarity determining regions (CDR). The heavy as well as the light chain variable domain each bears three CDRs (designated with 1, 2 and 3). They are responsible for the specific binding of the antigen, whereby the CDR3 normally contributes most to the affinity. **(d)** The correct folding and thus the stability of an scFv is greatly influenced by the formation of the intradomain disulfide bonds (left drawing). Because the reducing conditions of the cytoplasm prevent the formation of these disulfide bridges, most scFvs are unstable inside the cell (drawing on the right). Therefore, very stable frameworks are needed which are able to compensate for the lack of disulfide bridges through other intramolecular stabilizing interactions.

even if antisense molecules are constantly improving, their action also eliminates all functions of a target gene product at once, thus making it difficult to mimic the effects of a small molecule that presumably will act at a specific domain of the protein. In other words, antisense RNA approaches might not clarify whether the target qualifies for specific interference by agonistic or antagonistic compounds.

Gene silencing by RNAi is a very powerful tool to study gene function in invertebrates [7,8]. RNAi is a process whereby double-stranded RNA induces the homology-dependent degradation of the cognate mRNA and appears to act by a different, more complex mechanism than antisense RNA [9]. Until recently, there was one major drawback: RNAi did not work in mammalian cells and, when applied in non-embryonic mammalian cells, triggered profound sequence-unspecific physiological reactions leading to activation of the interferon pathway [10]. However, a recent report on the efficacy of short RNA molecules (so-called siRNAs, that are essentially too short to induce the interferon response) in mammalian cells has dramatically changed the situation [11]. In summary, target validation based on siRNA holds great promises for the future, although it also holds its limitations regarding the mimicking of putative effects of highly specific drugs.

The concept of validating drug targets at the protein level harbours conceptual advantages. First of all, by acting at the protein level one attends to the target directly. Moreover, by specifically blocking the function of protein subdomains, the action of highly specific drugs, which presumably are associated with fewer side effects, can be mimicked (for a review see Ref. [12]). An impressive example of this concept has been provided by Rao and colleagues [13]. In this study it was shown that selective blocking of the NFAT-binding site of calcineurin, the molecular target of the immunosuppressive drugs cyclosporine A and FK506, potently inhibits the expression of NFAT-dependent cytokines in T cells. At the same time, other cytokines whose expression is dependent on non-NFAT-mediated calcineurin activity are not affected. Cyclosporine A and FK506 inhibit the activation of all genes dependent on calcineurin activity, independently of whether they're mediated by NFAT or not. Their action leads to effective immunosuppression (through inhibition of NFAT activation) but also to severe side effects, such as progressive loss of renal function, neurotoxicity or increased risk of malignancy [14]. As these side effects are assigned to the inhibition of non-NFAT-mediated actions of calcineurin, Rao and colleagues have given a nice example on how targeting protein-subdomains can be useful for development of therapeutic agents that are less toxic than current drugs.

The advantage of dealing with a protein target directly and the potential to functionally neutralize specific protein subdomains has made the use of single-chain antibody fragments (scFvs) for intracellular applications increasingly important in the field of functional genomics [15]. Such so-called intrabodies have been generally derived from specific monoclonal antibodies or from scFvs (Fig. 1) that were initially selected by *in vitro* techniques, such as phage or ribosome display, and afterwards tested for their biological activity within eukaryotic cells [16–18]. Both procedures do not ensure that the isolated antibodies do also bind their cognate antigen in the cytoplasm of a cell, because antibodies are not created to function in the cell cytoplasm. In fact, the cytoplasm is a non-physiological environment for the antibodies. The reducing conditions within the cytoplasm prevent the formation of disulfide bonds. Thus, the cytoplasmic expression of scFvs is generally confronted with difficulties concerning the stability, solubility and aggregation tendency of the intrabody because the

intra-chain disulfide bridges are necessary for the stability and proper folding of the scFv molecule (Fig. 1d). For this reason most scFvs have been found to be inactive inside eukaryotic cells [19,20]. So far, no reliable rules are available to make predictions about antibody structures that can tolerate the reducing environment of the cytoplasm, and notwithstanding remain functional even in the absence of disulfide bonds. Moreover, individual biochemical analysis of scFvs is a long and tedious procedure. A technology enabling the use of antibodies under reducing conditions is thus required to benefit from the main advantage of this approach: Intrabodies can be used to perform highly specific functional protein-domain knockdowns, thus mimicking drug action *in vivo*.

One approach to negotiate the problem of proper intracellular folding is to express selected single-chain antibodies in the bacterial periplasm, wherein disulfide bonds are formed. After protein purification, such scFvs are chemically coupled to laser-sensitive chromophores and used for protein transduction of tissue culture cells. Upon irradiation, chemically reactive species can be generated that modify amino acid residues, a process that can lead to inactivation of particular protein functions [21]. The need for protein purification, the limited efficiency of chemical coupling and the restriction of the technology to a certain amount of transduced scFvs within a certain time frame sets the limits of this approach.

How does IMMUNA work?

ESBATech (Zurich-Schlieren, Switzerland) approaches the problem of practicability of intrabodies using its IMMUNA platform technology, which selects for intracellular stable, high-affinity single-chain antibodies *in vivo*. IMMUNA provides the construction of randomized CDR (complementary-determining regions) libraries on scFv frameworks that have been selected for high stability and solubility in an intracellular environment.

One part of IMMUNA, named ‘Quality Control’, allows the selection of such intrinsically stable and soluble intrabody frameworks in yeast independently of their antigen-binding properties. To this end, we have constructed fusion proteins composed of scFv sequences fused to a constant marker that provides a selectable activity in yeast by controlling expression of defined reporter genes [22] (Fig. 2a–b). The activation of the reporter genes, which stimulate yeast colony formation on a plate, or growth in liquid culture, is directly proportional to the stability and solubility of the scFv moiety of the fusion protein (Fig. 2c). Therefore the degree of reporter gene activation is dependent on the presence of a stable and soluble single-chain Fv antibody fragment inside the cell. It is worth noting that the assessment of solubility and stability of a recombinant antibody fragment by means of cellular expression is not necessarily restricted to transcriptional activity. These properties might also be validated with alternative types of ‘constant markers’ that provide certain selectable activity which is dependent on the stability and solubility of the intrabody.

A further implementation of the ‘Quality Control’ comprises the isolation of ‘super stable frameworks’. These serve as scaffolds or acceptor backbones to construct CDR libraries by randomization of one or more hypervariable loops (CDRs). Such random CDR libraries are subsequently screened in a different selection system in yeast for identifying novel antigen-binding single-chain antibodies. This selection assay allows the isolation of functional intrabodies based on their ability to bind the cognate antigen *in vivo*. In this assay, the scFv is linked to a transcriptional activation domain, whereas the target antigen is linked to a DNA-binding domain (Fig. 3a). A specific interaction between the scFv fusion protein and the antigen leads to expression of selectable reporter genes. The expression levels of the reporter genes can be conveniently monitored, allowing an efficient screen by yeast cell-growth selection (Fig. 3b).

Applications of IMMUNA

Intrabodies can interfere with protein functions in several ways, affording them great potential for use in target validation. Intrabodies directed against specific domains can, for example, block protein–protein interactions. In collaboration with the laboratory of Andreas Plückthun, we have shown that intracellular stable single-chain antibodies targeted against the dimerization domain of the yeast transcriptional activator Gcn4 significantly reduced its activation function [16]. Gcn4 belongs to the family of proteins with a leucine zipper dimerization domain that is required for efficient and sequence-specific DNA binding [23]. Two point mutations in the leucine zipper motif that prevented dimer formation *in vitro* [24] also abolished the transcription activation function of Gcn4 *in vivo* [16]. Our results show that intracellular expression of single-chain antibodies directed against the Gcn4 leucine zipper, which has been shown to interfere with dimerization of this protein *in vitro* [25], could reduce activation of a Gcn4-dependent gene to various extents down to ~15% (Fig. 4) [16]. The different inhibitory effects of these various intrabodies directed against the Gcn4 dimerization domain correlated with their stability and solubility [16], thus indicating the importance of these parameters for designing or selecting effective intrabodies.

Domain-specific intrabodies have also been shown to interfere with protein–DNA interactions. An intracellular scFv derived from a monoclonal antibody, which was characterized for its ability to specifically prevent DNA

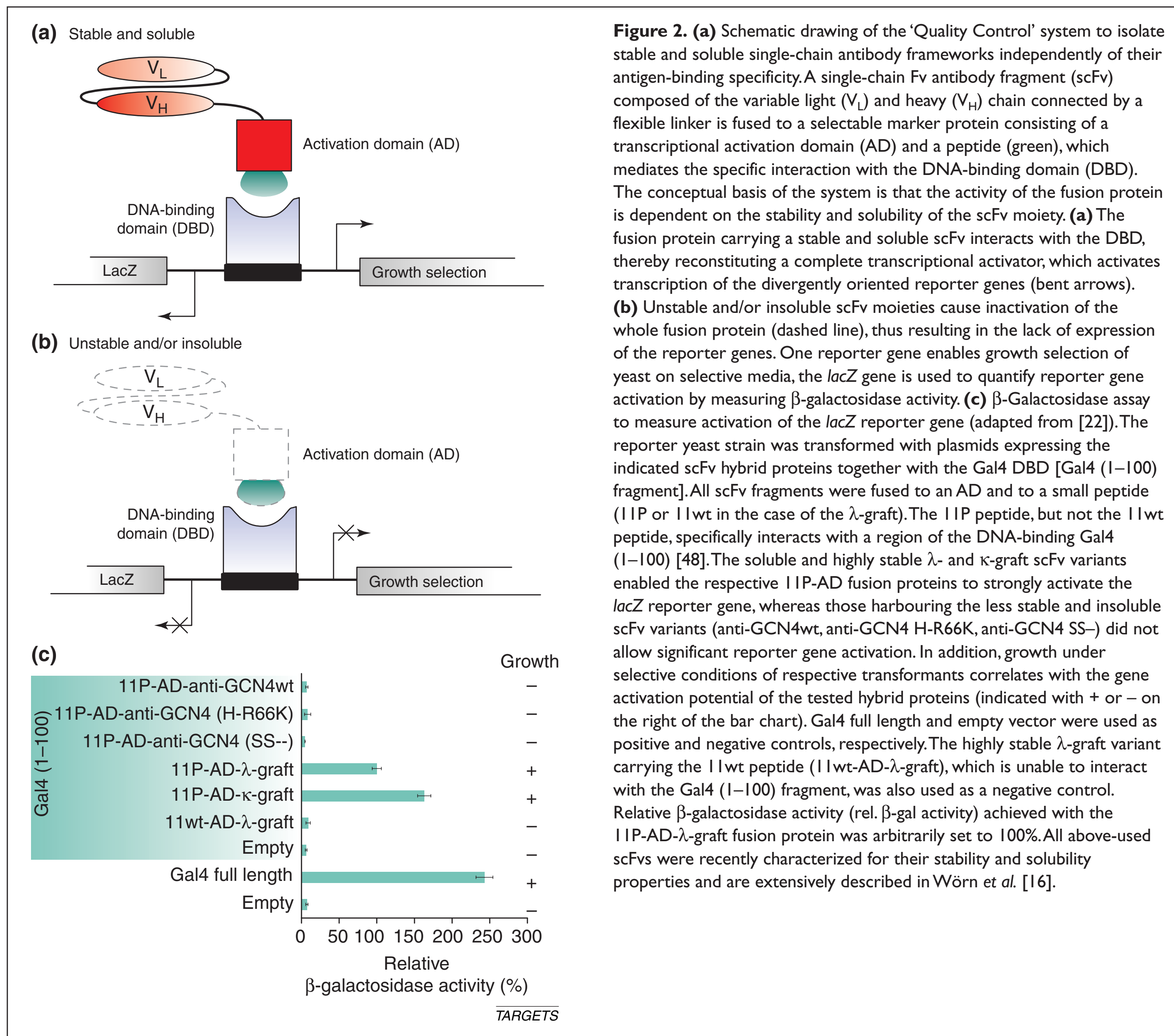

Figure 2. (a) Schematic drawing of the 'Quality Control' system to isolate stable and soluble single-chain antibody frameworks independently of their antigen-binding specificity. A single-chain Fv antibody fragment (scFv) composed of the variable light (V_L) and heavy (V_H) chain connected by a flexible linker is fused to a selectable marker protein consisting of a transcriptional activation domain (AD) and a peptide (green), which mediates the specific interaction with the DNA-binding domain (DBD). The conceptual basis of the system is that the activity of the fusion protein is dependent on the stability and solubility of the scFv moiety. **(a)** The fusion protein carrying a stable and soluble scFv interacts with the DBD, thereby reconstituting a complete transcriptional activator, which activates transcription of the divergently oriented reporter genes (bent arrows). **(b)** Unstable and/or insoluble scFv moieties cause inactivation of the whole fusion protein (dashed line), thus resulting in the lack of expression of the reporter genes. One reporter gene enables growth selection of yeast on selective media, the *lacZ* gene is used to quantify reporter gene activation by measuring β-galactosidase activity. **(c)** β-Galactosidase assay to measure activation of the *lacZ* reporter gene (adapted from [22]). The reporter yeast strain was transformed with plasmids expressing the indicated scFv hybrid proteins together with the Gal4 DBD [Gal4 (1–100) fragment]. All scFv fragments were fused to an AD and to a small peptide (11P or 11wt in the case of the λ-graft). The 11P peptide, but not the 11wt peptide, specifically interacts with a region of the DNA-binding Gal4 (1–100) [48]. The soluble and highly stable λ- and κ-graft scFv variants enabled the respective 11P-AD fusion proteins to strongly activate the *lacZ* reporter gene, whereas those harbouring the less stable and insoluble scFv variants (anti-GCN4wt, anti-GCN4 H-R66K, anti-GCN4 SS–) did not allow significant reporter gene activation. In addition, growth under selective conditions of respective transformants correlates with the gene activation potential of the tested hybrid proteins (indicated with + or – on the right of the bar chart). Gal4 full length and empty vector were used as positive and negative controls, respectively. The highly stable λ-graft variant carrying the 11wt peptide (11wt-AD-λ-graft), which is unable to interact with the Gal4 (1–100) fragment, was also used as a negative control. Relative β-galactosidase activity (rel. β-gal activity) achieved with the 11P-AD-λ-graft fusion protein was arbitrarily set to 100%. All above-used scFvs were recently characterized for their stability and solubility properties and are extensively described in Wörn *et al.* [16].

binding by the human transcription factor ATF-1 *in vitro*, inhibited ATF-1-activated transcription *in vivo* as well as ATF-1-dependent tumorigenicity and metastatic potential of transfected melanoma cells in mice [26].

In addition to blocking protein activities, and in contrast to gene or RNA knockout techniques, intrabodies directed against specific domains can also have agonistic effects, thus stimulating or even restoring target protein function. For example, two intrabodies able to associate with p53 and to restore DNA binding activity of some p53 mutants *in vitro* were shown to recover the transcriptional activity of these p53 mutants in transfected tumour cells [27].

Several studies have demonstrated the ability of intrabodies to interfere with cellular processes by relocating specific proteins so as to take them away from their natural site of action. For example, redirecting the cytoplasmic Tau protein to the nucleus neutralized its function [28]. Diverting Ras from its natural location by Ras-specific intrabodies also caused neutralization of its function [29]. It is worth noting that this particular antibody was shown to have no effect on Ras in an *in vitro* system and, therefore, its neutralizing effect *in vivo* was solely because of its ability to relocate the target to a 'silencing' site. A further example for this mode of action of intrabodies is provided by the results of Zhu *et al.*, who showed that relocation of Caspase 7 by nuclear-targeted specific intrabodies significantly inhibited staurosporine-induced apoptosis [30].

In a further application of intrabodies, the high degree of specificity of antibody–antigen interactions has been exploited to selectively induce apoptosis of only those cells

that bore the specific antigen. Tse and Rabbits have indeed shown that when intracellular antibodies fused to Caspase 3, the so-called executioner in the apoptosis pathway, specifically bind a dimerizing target antigen, the caspase moieties are self-activated and induce apoptosis [31].

Intrabodies have been shown to affect intracellular protein functions in a variety of biological systems. To date, most of the data regarding the use of intrabodies to analyse protein function, as well as to test their potential therapeutic application, have been obtained from experiments performed with cultured mammalian cells [32,33]. However, the first application of intrabodies in functional genomics was established in yeast [34].

In addition to their broad use in single-cell biological systems, intrabodies have also been shown to function in multicellular organisms. For example, constitutive expression in transgenic plants of an intrabody directed against a plant virus caused reduction of viral infection incidence and significantly delayed the development of symptoms [35]. Intrabodies have also been successfully applied in the fruit fly *Drosophila melanogaster* to functionally neutralize the transcription factor Poxn, leading to the reproduction of the poxn⁻ phenotype [36].

Strengths and weaknesses of IMMUNA

IMMUNAs' 'Quality Control' system opens new possibilities to obtain single-chain antibodies suitable for intracellular applications. It facilitates a high-throughput screen of a large number of diverse scFv sequences in a rapid and efficient manner to isolate a set of 'super-stable' frameworks that can cope with intracellular (reducing) conditions. In the IMMUNA technology, the super-stable frameworks isolated by the 'Quality Control' are then used as backbones to construct intrabody libraries by randomization of the hypervariable loops. These libraries are then screened *in vivo* to identify antigen-specific binders. IMMUNA also allows to improve the intracellular performance of an existing binder through an 'intrabody evolution process' by random diversification of the primary sequence and subsequent growth selection in yeast. It is important to note that the system is not restricted to yeast cells only. It has been successfully applied to mammalian cells as well [22]. It might well be that an intrabody that is stable in yeast is not tolerated in certain types of mammalian cells. Because this system is an antigen-independent approach, such unpredictable limitations can quickly be investigated by testing the antibody in all cell types in which it finally has to work. The combination of all parts of the IMMUNA technology described above constitutes a powerful tool to obtain single-chain antibody fragments, which retain their activity even in the reducing environment of the cytoplasm. In addition, the selection systems take the particular requirements for the functionality of an intrabody (namely stability and solubility in addition to the affinity for the epitope) into account. Therefore, it allows a straightforward selection for intrabodies directly under the relevant *in vivo* conditions, and properties relevant for subsequent intracellular application can be optimised this way.

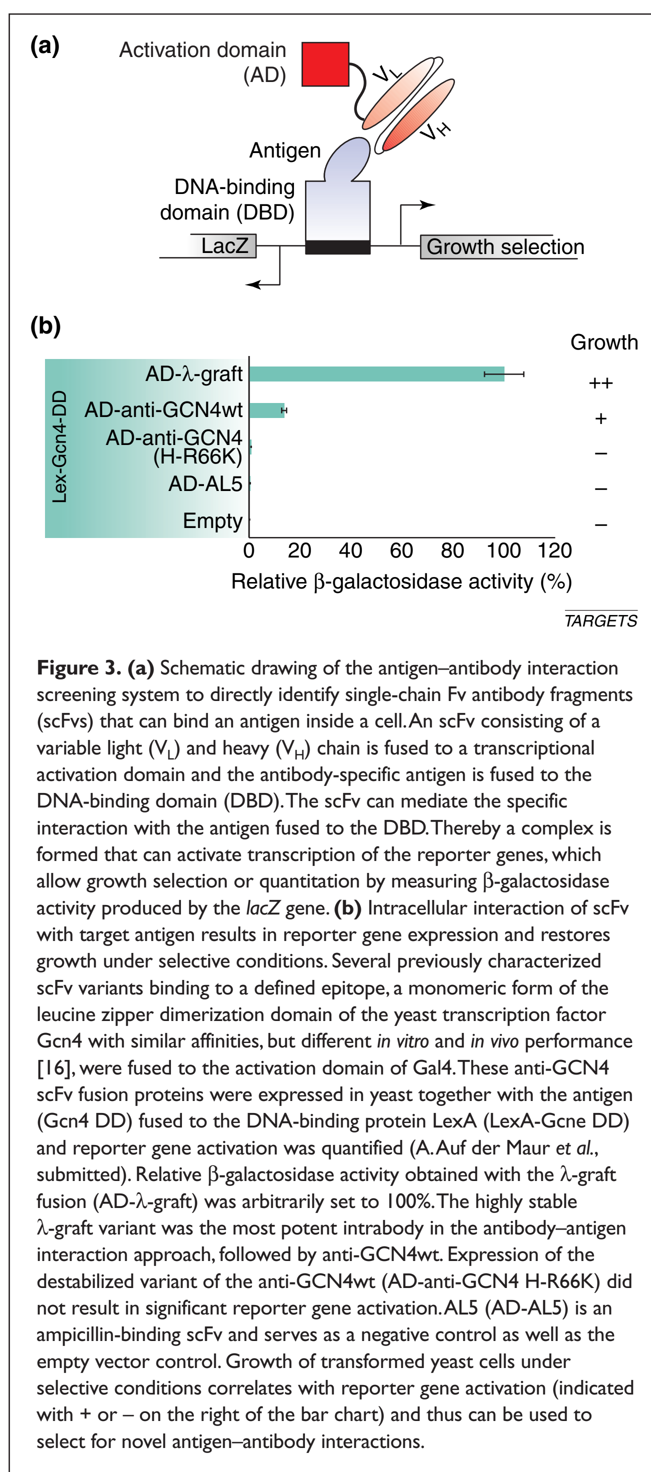

Figure 3. (a) Schematic drawing of the antigen–antibody interaction screening system to directly identify single-chain Fv antibody fragments (scFvs) that can bind an antigen inside a cell. An scFv consisting of a variable light (V_L) and heavy (V_H) chain is fused to a transcriptional activation domain and the antibody-specific antigen is fused to the DNA-binding domain (DBD). The scFv can mediate the specific interaction with the antigen fused to the DBD. Thereby a complex is formed that can activate transcription of the reporter genes, which allow growth selection or quantitation by measuring β-galactosidase activity produced by the *lacZ* gene. **(b)** Intracellular interaction of scFv with target antigen results in reporter gene expression and restores growth under selective conditions. Several previously characterized scFv variants binding to a defined epitope, a monomeric form of the leucine zipper dimerization domain of the yeast transcription factor Gcn4 with similar affinities, but different *in vitro* and *in vivo* performance [16], were fused to the activation domain of Gal4. These anti-GCN4 scFv fusion proteins were expressed in yeast together with the antigen (Gcn4 DD) fused to the DNA-binding protein LexA (LexA-Gcne DD) and reporter gene activation was quantified (A. Auf der Maur *et al.*, submitted). Relative β-galactosidase activity obtained with the λ-graft fusion (AD-λ-graft) was arbitrarily set to 100%. The highly stable λ-graft variant was the most potent intrabody in the antibody–antigen interaction approach, followed by anti-GCN4wt. Expression of the destabilized variant of the anti-GCN4wt (AD-anti-GCN4 H-R66K) did not result in significant reporter gene activation. AL5 (AD-AL5) is an ampicillin-binding scFv and serves as a negative control as well as the empty vector control. Growth of transformed yeast cells under selective conditions correlates with reporter gene activation (indicated with + or – on the right of the bar chart) and thus can be used to select for novel antigen–antibody interactions.

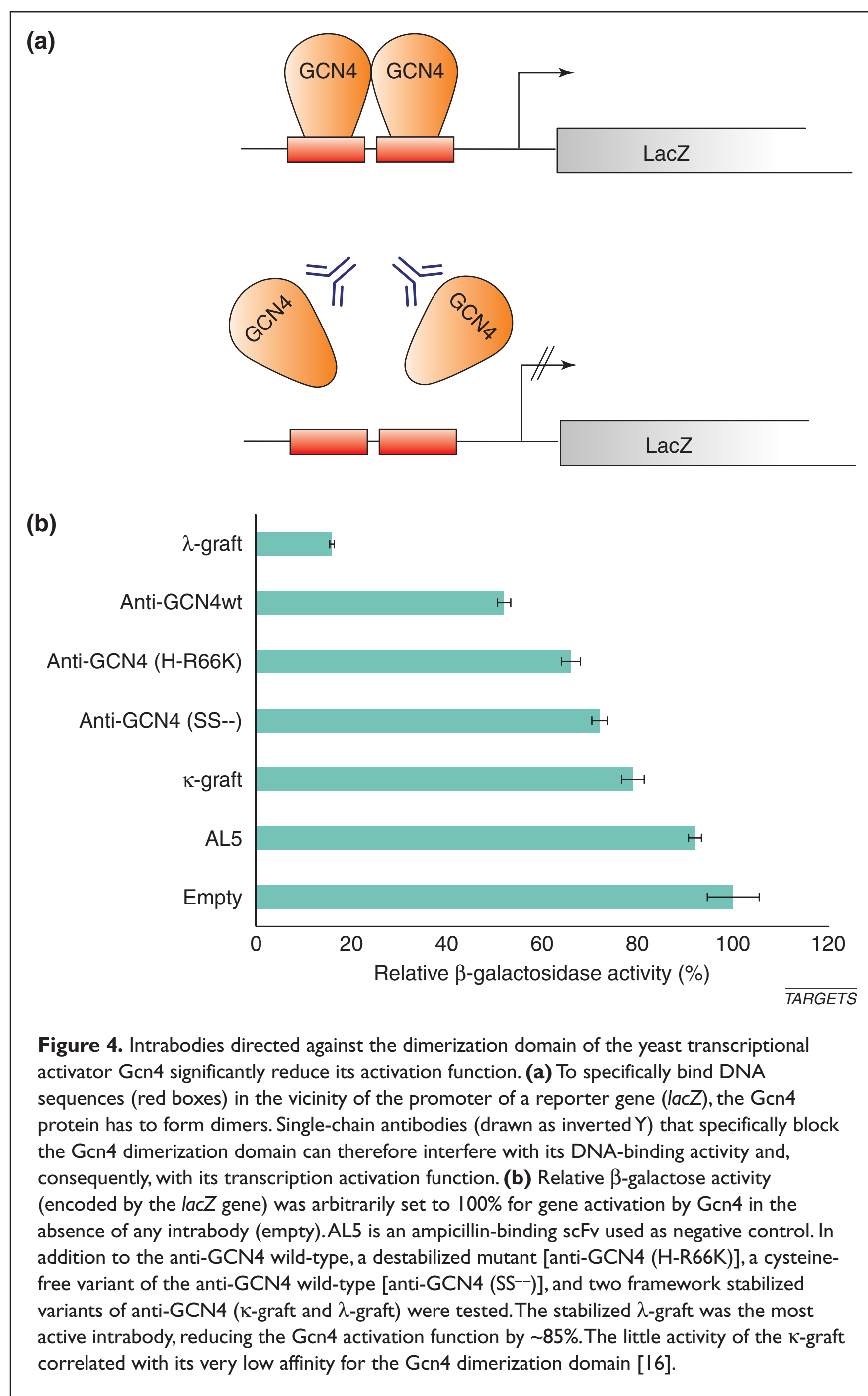

Figure 4. Intrabodies directed against the dimerization domain of the yeast transcriptional activator Gcn4 significantly reduce its activation function. **(a)** To specifically bind DNA sequences (red boxes) in the vicinity of the promoter of a reporter gene (*lacZ*), the Gcn4 protein has to form dimers. Single-chain antibodies (drawn as inverted Y) that specifically block the Gcn4 dimerization domain can therefore interfere with its DNA-binding activity and, consequently, with its transcription activation function. **(b)** Relative β-galactose activity (encoded by the *lacZ* gene) was arbitrarily set to 100% for gene activation by Gcn4 in the absence of any intrabody (empty). AL5 is an ampicillin-binding scFv used as negative control. In addition to the anti-GCN4 wild-type, a destabilized mutant [anti-GCN4 (H-R66K)], a cysteine-free variant of the anti-GCN4 wild-type [anti-GCN4 (SS−−)], and two framework stabilized variants of anti-GCN4 (κ-graft and λ-graft) were tested. The stabilized λ-graft was the most active intrabody, reducing the Gcn4 activation function by ~85%. The little activity of the κ-graft correlated with its very low affinity for the Gcn4 dimerization domain [16].

A major bottleneck for the screening of large scFv libraries in yeast is the comparatively low transformation efficiency that can be achieved in this organism. By contrast, *in vitro* screening techniques such as ribosome display [37] or mRNA display [38] can explore libraries to ~10^{13}–10^{14}. This low transformation efficiency might be disadvantageous for screening highly complex CDR libraries. However, given that a typical intrabody library is based on a framework that is optimised for the reducing environment of the cytoplasm and the antigen–antibody interaction is selected *in vivo*, it is conceivable that weak binders are identified from less complex libraries. One straightforward approach is to randomize just a few but crucial amino acids, starting, for example, with the CDR3 of the variable heavy chain, which is the most important antigen-interacting domain. Thereby, the library acquires a complexity, which can still be completely screened in yeast. Because the chance to immediately isolate a high-affinity binder is rather low, it is reasonable to follow a stepwise approach. Thus, in a first screening round low-affinity binders are isolated and in a second round randomization of another CDR is performed and the derived library is again selected *in vivo* under increased stringency.

One additional problem of IMMUNA is the difficulty to directly correlate antigen-binding with a potential biological effect of the intrabody. Thus, for each identified antibody that binds a given target, a functional test has to be established to point out and characterize the biological effect that the interaction could cause. The average probability of finding an effective antibody among several binders is not known. It is conceivable that such probability can vary according to the structure and function of the target antigen.

Who are considered to be major competitors in the field?

As target validation is a major bottleneck in today's drug discovery process, the number of competitors in the field is steadily growing. Nevertheless, the technologies discussed above constitute the basis of approaches used by the vast majority of competitor companies. The following section is intended to give an overview on products and applications of selected companies considered to be core competitors to ESBATech's IMMUNA platform technology in the target validation field, but does not claim completeness (see also Table 1).

Lexicon Genetics (Princeton, NJ, USA) and Deltagen (Redwood City, CA, USA) are two world-leading companies using gene knockout technology to discover the functions

of genes and move them into drug discovery programmes. Lexicon has generated a library of 200,000 frozen mouse embryonic stem cell clones, estimated to contain knockout clones for ~50% of all genes in the mouse genome. A focus is set to knockouts of genes that encode for 'druggable' target proteins [such as G-protein coupled receptors (GCPRs), enzymes, kinases or secreted proteins] and knockout mice are routinely screened for a variety of parameters relevant for human disease using advanced medical technologies. Lexicon has several collaborations regarding target validation with big pharma companies, but also follows its in-house drug development programme with successfully validated targets ([39], http://www.lexgen.com).

Table 1. Major competitors for ESBATech's IMMUNA technology (Selection)

Company	Technology
Lexicon Genetics	Gene knockout in mice
Deltagen	Gene knockout in mice
Atugen	Antisense RNA
Hybridon	Antisense RNA
ISIS	Antisense RNA
Cenix BioSciences	RNAi in *C. elegans* and *Drosophila*
Nucleonics	RNAi in zebrafish and mice
Xerion	Chromophore-coupled scFvs in human cells

Abbreviations: RNAi, RNA interference; scFvs, single-chain Fv antibody fragments

Deltagen is following a similar approach by performing up to several hundred gene knockouts in mice per year. Again, a focus is set on druggable targets and knockout mice are carefully monitored for physiological and behavioural phenotypes. All phenotype information is consolidated in a database that is accessible for subscribers. Major pharma companies, such as GlaxoSmithKline, Merck or Pfizer, have obtained access to this database ([40], http://www.deltagen.com).

Germany-based Atugen (Berlin, Germany) is validating therapeutic targets using antisense technology *in vitro* and *in vivo*. By the use of small, synthetically stabilized DNA/RNA molecules, specific, transient mRNA knockdown is performed. The antisense molecules are designed using specific computer algorithms to yield at least 70% knockdown of mRNA. Atugens's antisense molecules have been shown to be non-toxic in several cellular assays and animal models. In a rat corneal model, vascular endothelial growth factor (VEGF)-induced angiogenesis could be reduced *in vivo* by targeting expression of vascular endothelial growth factor (VEGFR) (http://www.atugen.com). In another example, the technology was applied to inhibit nF-κB activation by down-regulation of IKKγ protein levels [41].

Hybridon (Cambridge, MA, USA) is another company with a proven track record in designing antisense molecules. Recently, antisense oligonucleotides designed by Hybridon have been shown to mediate significant inhibition of MDM2 in MCF-7 breast cancer cells, leading to upregulation of the anti-oncogenic proteins p53 and p21 [42]. In another application, activity of cAMP-dependent protein kinase could be inhibited to such an extent that growth inhibition, apoptosis and cell morphology changes were induced in prostate cancer cells [43].

ISIS (Carlsbad, CA, USA) is a leading company in target validation with an extraordinarily strong IP-position on the antisense technology [6]. Besides development of antisense molecules for target validation, the company also has a focus on the use of antisense oligonucleotides as therapeutic tools. ISIS has several collaborations with big pharma companies for target validation (programme partners include Eli Lilly & Company, Celera Genomics, Abbott Laboratories, Johnson and Johnson and Aventis), giving ISIS a strong position in the field. Recently, antisense oligonucleotides designed by the company were successfully used to perform mRNA knockdowns of survivin, an inhibitor of apoptosis [44] and of IL-2, a cytokine involved in allograft rejection [45].

Cenix (Dresden, Germany) is one of several young companies exploiting RNAi technology. The company is focusing on experimental systems in which RNAi has shown to work efficiently, such as *C. elegans* and *Drosophila*. The company is shifting its activity towards application of RNAi in cultured human cells and mice in the light of the new perspectives for applications of RNAi [11] (http://www.cenix-bioscience.com).

Nucleonics Inc. (Malvern, PA, USA) has its core competence in RNAi and siRNA technology for target validation. The company has successfully applied its technological know-how to silence transgenic green fluorescent protein (GFP) as well as endogenous genes in zebrafish embryos. In addition, Nucleonics recently presented data showing efficient silencing of prostate-specific antigen (PSA) expression in human cells and silencing of IL-12 in adult mice, without provoking the interferon response [46].

Xerion Pharmaceuticals (Martinsreid, Germany) is using the concept of single-chain antibodies for target validation to exploit its proprietary CALI (Chromophore assisted laser inactivation) approach (for details see first section). CALI was successfully used to underline the power of single-chain antibodies for protein-domain targeting, as it was shown that CALI was capable of inactivating individual subunits of a multimeric receptor complex in living cells [21]. An interesting recent application of the CALI technology was its use for investigation of the role of the microtubule-associated protein tau in axon growth [47]. Most importantly, in this setting the technology was able to

clarify controversial results previously obtained with antisense RNA on one hand and genetic knockout experiments on the other hand, thus further supporting the advantages of using single-chain antibodies for functional genomics.

Partnerships to date

To date (April 2002), ESBATech uses its proprietary IMMUNA platform technology for internal target validation projects only. Strategic alliances with pharma partners are initiated.

References

1 Lander, E.S. *et al.* (2001) Initial sequencing and analysis of the human genome. International Human Genome Sequencing Consortium. *Nature* 409, 860–921
2 Venter, J.C. *et al.* (2001) The sequence of the human genome. *Science* 291, 1304–1351
3 Ihle, J.N. (2000) The challenges of translating knockout phenotypes into gene function. *Cell* 102, 131–134
4 Chiang, M.Y. *et al.* (1991) Antisense oligonucleotides inhibit intercellular adhesion molecule 1 expression by two distinct mechanisms. *J. Biol. Chem.* 266, 18162–18171
5 Estibeiro, P. and Godfray, J. (2001) Antisense as a neuroscience tool and therapeutic agent. *Trends Neurosci.* 24(Suppl. 11), S56–S62
6 Dove, A. (2002) Antisense and sensibility. *Nat. Biotechnol.* 20, 121–124
7 Fire, A. *et al.* (1998) Potent and specific genetic interference by double-stranded RNA in Caenhorabditis elegans. *Nature* 391, 806–811
8 Kennerdell, J.R. and Carthew, R.W. (1998) Use of dsRNA-mediated genetic interference to demonstrate that frizzled and frizzled-2 act in the wingless pathway. *Cell* 95, 1017–1026
9 Zamore, P.D. (2001) RNA interference: listening to the sound of silence. *Nat. Struct. Biol.* 8, 746–750
10 Stark, G.R. *et al.* (1998) How cells respond to interferons. *Annu. Rev. Biochem.* 67, 227–264
11 Elbashir, S.M. *et al.* (2001) Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* 411, 494–498
12 Kamb, A. and Caponigro, G. (2001) Peptide inhibitors expressed *in vivo. Curr. Opin. Chem. Biol.* 5, 74–77
13 Aramburu, J. *et al.* (1999) Affinity-driven peptide selection of an NFAT inhibitor more selective than cyclosporin A. *Science* 285, 2129–2133
14 Ruhlmann, A. and Nordheim, A. (1997) Effects of the immunosuppressive drugs CsA and FK506 on intracellular signalling and gene regulation. *Immunobiology* 198, 192–206
15 Cattaneo, A. and Biocca, S. (1997) *Intracellular Antibodies: Development and Applications*, Springer
16 Wörn, A. *et al.* (2000) Correlation between *in vitro* stability and *in vivo* performance of anti-GCN4 intrabodies as cytoplasmic inhibitors. *J. Biol. Chem.* 275, 2795–2803
17 Visentin, M. *et al.* (1999) Selection of antibodies for intracellular function using a two-hybrid *in vivo* system. *Proc. Natl. Acad. Sci. U. S. A.* 96, 11723–11728
18 Pörtner-Taliana, A. *et al.* (2000) *In vivo* selection of single-chain antibodies using a yeast two-hybrid system. *J. Immunol. Methods* 238, 161–172
19 Martineau, P. *et al.* (1998) Expression of an antibody fragment at high levels in the bacterial cytoplasm. *J. Mol. Biol.* 280, 117–127
20 Biocca, S. *et al.* (1995) Redox state of single chain Fv fragments targeted to the endoplasmic reticulum, cytoplasm and mitochondria. *Biol. Technol.* 13, 1110–1115
21 Liao, J.C. *et al.* (1995) Chromophore-assisted laser inactivation of subunits of the T-cell receptor in living cells is spatially restricted. *Photochem. Photobiol.* 62, 923–929
22 Auf der Maur, A. *et al.* (2001) Antigen-independent selection of stable intracellular single-chain antibodies. *FEBS Lett.* 508, 407–412
23 Hope, I.A. and Struhl, K. (1987) GCN4, a eukaryotic transcriptional activator protein, binds as a dimer to target DNA. *EMBO J.* 6, 2781–2784
24 Leder, L. *et al.* (1995) Spectroscopic, calorimetric, and kinetic demonstration of conformational adaptation in peptide-antibody recognition. *Biochemistry* 34, 16509–16518
25 Berger, C. *et al.* (1999) Antigen recognition by conformational selection. *FEBS Lett.* 450, 149–153
26 Jean, D. *et al.* (2000) Inhibition of tumor growth and metastasis of human melanoma by intracellular anti-ATF-1 single chain Fv fragment. *Oncogene* 19, 2721–2730
27 Caron de Fromentel, C. *et al.* (1999) Restoration of transcriptional activity of p53 mutants in human tumour cells by intracellular expression of anti-p53 single chain Fv fragments. *Oncogene* 18, 551–557
28 Visentin, M. *et al.* (2002) The intracellular antibody capture technology (ICAT): towards a consensus sequence for intracellular antibodies. *J. Mol. Biol.* 317, 73–83
29 Lener, M. *et al.* (2000) Diverting a protein from its cellular location by intracellular antibodies. The case of p21Ras. *Eur. J. Biochem.* 267, 1196–1205
30 Zhu, Q. *et al.* (1999) Extended half-life and elevated steady-state level of a single-chain Fv intrabody are critical for specific intracellular retargeting of its antigen, caspase-7. *J. Immunol. Methods* 231, 207–222
31 Tse, E. and Rabbits, T.H. (2000) Intracellular antibody-caspase-mediated cell killing: an approach for application in cancer therapy. *Proc. Natl. Acad. Sci. U. S. A.* 97, 12266–12271
32 Marasco, W.A. (1997) Intrabodies: turning the humoral immune system outside in for intracellular immunization. *Gene Ther.* 4, 11–15
33 Cattaneo, A. and Biocca, S. (1999) The selection of intracellular antibodies. *Trends Biotechnol.* 17, 115–121
34 Carlson, J.R. (1988) A new means of inducibly inactivating a cellular protein. *Mol. Cell. Biol.* 8, 2638–2646
35 Tavladoraki, P. *et al.* (1993) Transgenic plants expressing a functional single-chain Fv antibody are specifically protected from virus attack. *Nature* 366, 469–472
36 Hassanzadeh, G.G. *et al.* (1998) Isolation and characterization of single-chain Fv genes encoding antibodies specific for Drosophila poxn protein. *FEBS Lett.* 437, 75–80
37 Hanes, J. and Plückthun, A. (1997) *In vitro* selection and evolution of functional proteins by using ribosome display. *Proc. Natl. Acad. Sci. U. S. A.* 94, 4937–4942
38 Wilson, D.S. *et al.* (2001) The use of mRNA display to select high-affinity protein-binding peptides. *Proc. Natl. Acad. Sci. U. S. A.* 98, 3750–3755
39 Zambrowicz, B.P., Lexicon Genetics (2002) Transgenic knockouts. Drug Discovery Europe, IBC Lifesciences Conference, 16–18 April, Stuttgart, Germany
40 Klein, R., Deltagen (2002) Combining *in vivo* mammalian gene function with genome-wide gene expression analysis to identify the best new targets for drug discovery. Drug Discovery Europe, IBC Lifesciences Conference, 16–18 April, Stuttgart, Germany
41 Krappmann, D. *et al.* (2000) The IκB kinase (IKK) complex is tripartite and contains IKKγ but not IKAP as a regular component. *J. Biol. Chem.* 275, 29779–29787
42 Wang, H. *et al.* (2001) Antisense anti-MDM2 oligonucleotides as novel therapeutic approach to human breast cancer: *in vitro* and *in vivo* activities and mechanisms. *Clin. Cancer Res.* 7, 3613–3624
43 Cho, Y.S. *et al.* (2002) Protein kinase A RIalpha antisense inhibition of PC3M prostate cancer cell growth: Bcl-2 hyperphosphorylation, bax up-regulation, and bad-hyposphosphorylation. *Clin. Cancer Res.* 8, 607–614
44 Chen, J. *et al.* (2000) Down-regulation of survivin by antisense oligonucleotides increases apoptosis, inhibits cytokinesis and anchorage-independent growth. *Neoplasia* 2, 235–241
45 Tian, L. *et al.* (2001) Selective inhibition of IL-2 mRNA blocks allograft rejection by limiting T cell clonal expansion. *Transplant. Proc.* 33, 330
46 Giordano, T., Nucleonics Inc. (2002) Cambridge Healthtech's Fifth Annual Genomic Partnering Conference, 23–24 February, Santa Clara, USA
47 Liu, C.W. *et al.* (1999) Tau is required for neurite outgrowth and growth cone motility of chick sensory neurons. *Cell Motil. Cytoskeleton* 43, 232–242
48 Barberis, A. *et al.* (1995) Contact with a component of the polymerase II holoenzyme suffices for gene activation. *Cell* 81, 359–368