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## DEVELOPMENT OF ANTIBODY FRAGMENTS FOR IMMUNOTHERAPY OF PRION DISEASES

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### SHORT TITLE: ScFv IN PRION DISEASE THERAPY

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## SYNOPSIS

Prions are infectious proteins responsible for a group of fatal neurodegenerative diseases called transmissible spongiform encephalopathies (TSEs), or prion diseases. In mammals, prions reproduce themselves by recruiting the normal cellular protein (PrP<sup>C</sup>) and inducing its conversion to the disease-causing isoform denominated PrP<sup>Sc</sup>. Recently, anti-prion antibodies have been shown to permanently cure prion-infected cells. However, the inability of full-length antibodies and proteins to cross the blood-brain barrier (BBB) hampers their use in the therapy of TSEs *in vivo*. Alternatively, brain delivery of prion-specific scFv by adeno-associated virus (AAV) transfer delays the onset of the disease in infected mice, although protection is not complete.

We investigated the anti-prion effects of a recombinant anti-PrP (D18) single-chain variable fragment (scFv) by direct addition to scrapie infected cell cultures or by infection with both lentivirus and adeno-associated virus (AAV) transducing vectors. We show that recombinant anti-PrP scFv is able to reduce proteinase K-resistant PrP content in infected cells. In addition, we demonstrate that lentiviruses are more efficient than AAV in gene transferring of the anti-PrP scFv gene and in reducing PrP<sup>Sc</sup> content in infected neuronal cell lines. Finally, we have used a bioinformatic approach to construct a structural model of D18scFv-PrP<sup>C</sup> complex. Interestingly, according to the docking results, Arg<sub>PrP</sub>151 (Arg151 from prion protein) is the key residue for the interactions with D18scFv, anchoring the PrP<sup>C</sup> to the cavity of the antibody.

Taken together, these results indicate that combined passive and active immunotherapy targeting PrP might be promising strategies for therapeutic intervention in prion diseases.

**KEYWORDS:** Adeno-associated Virus, Immunotherapy, Lentivirus, PrP<sup>C</sup>, PrP<sup>Sc</sup>, ScFv.

**ABBREVIATIONS FOOTNOTE:** AAV: adeno-associated virus; BBB: blood-brain barrier; BSE: bovine spongiform encephalopathy; CJD: Creutzfeldt-Jakob disease; CNS: central nervous system; Fab: antigen-binding fragment; IPTG: isopropyl-beta-thio-galactoside; PK: proteinase K; PrP<sup>C</sup>: cellular prion protein; PrP<sup>Sc</sup>: scrapie prion protein; scFv: single-chain variable fragment; TSE: transmissible spongiform encephalopathy

## INTRODUCTION

Transmissible spongiform encephalopathies (TSEs) or prion diseases are fatal neurodegenerative disorders that affect humans and animals. The mechanisms involved in their pathogenesis are not completely understood, but they are associated with the accumulation in the brain of a misfolded form, PrP<sup>Sc</sup>, of the endogenously expressed cellular prion protein (PrP<sup>C</sup>) [1]. Industrial cannibalism has been responsible for one of the most infamous TSE epidemics, bovine spongiform encephalopathy (BSE), paralleled with an increasing number of human cases with a variant form of Creutzfeldt-Jakob Disease (vCJD) from BSE-contaminated beef products [2].

Direct interaction between the pathogenic PrP<sup>Sc</sup> template and the endogenous PrP<sup>C</sup> substrate and the following conformational conversion of PrP<sup>C</sup> into PrP<sup>Sc</sup> are proposed to drive the formation of nascent infectious prions [3]. Various lines of evidence indicate that the process of conversion is the key element in pathogenesis. Knockout mice (*Prnp*<sup>0/0</sup>) are resistant to prion diseases and do not propagate infectivity [4], as well as brain tissue homogenate from *Prnp*<sup>0/0</sup> cows are resistant to prion propagation in vitro [5]. Moreover, *Prnp*<sup>0/0</sup> brain tissue surrounding prion-infected *Prnp*<sup>+/+</sup> neurografts does not develop neuropathological changes typical of prion diseases [6]. Over the past several years, many anti-prion compounds have been identified in *in vitro* models of prion replication, such as polysulphated polyanionic compounds, polyamine, tetrapyrroles, polyene antibiotics, peptides, tetracyclic and tricyclic compounds. These prion antagonists can be targeted towards the selective binding of PrP<sup>C</sup> or PrP<sup>Sc</sup> and/or to the process of conversion (reviewed in [7-9]). However, most of these molecules were found to be toxic or ineffective in animal models of prion diseases (reviewed in [7-9]).

Interestingly, by taking advantage of the 'prion-resistant' polymorphisms Q171R and E219K that naturally exist in sheep and humans, respectively, a therapeutic approach based on the use of lentiviral vectors carrying mouse *Prnp* mutated gene has recently been evaluated [10]. The expression of these PrP variants, acting as dominant negative, inhibits prion replication in permanently prion-infected cells and in animals [10]. In addition, suppression of PrP expression by lentiviral vector-mediated RNAi prolongs survival of scrapie infected mice [11].

Alternatively, anti-prion protein (PrP) antibodies have been shown to have an anti-prion effect in cellular and animal models [12-15]. Early indications of the potential of antibody therapy for prion disease came from *in vitro* studies, showing reduction in infectivity of prions after incubation with an anti-PrP antibody [16]. Immunization with PrP peptides reduced PrP<sup>Sc</sup> in a peripheral model of mouse scrapie [17]. Different publications report that antibodies directed against the middle portion of PrP (residues 91-110 and 132-156) can cure scrapie infected cells in culture and reduce proteinase K (PK)-resistant PrP in spleens of infected mice (reviewed in [12, 13, 18]). In cell systems, anti-prion antibodies were able to purge the cells entirely of PrP<sup>Sc</sup> [12] and their potency correlated with their ability to recognize the total population of PrP<sup>C</sup> molecules on the cell surface. Despite these encouraging results, development of effective immunotherapy presents several problems both in active and passive approaches. An important obstacle in the development of efficacious regimens for active immunization is host tolerance to endogenous PrP<sup>C</sup>, which limited the therapeutic efficacy of this immunization approach [15, 19]. In comparison, passive immunization suffers from the intrinsic problem of poor antibody diffusion from vessels into tissues, especially in the nervous tissue: administration of monoclonal antibodies has been shown to prevent the pathogenesis only when applied

simultaneously, or shortly after, peripheral prion infection [18]. Moreover, production of large amounts of monoclonal antibodies for therapy is technically challenging and expensive.

In 2001, Peretz and co-workers [12] analyzed the ability of some recombinant antibody antigen-binding fragments (Fabs) to inhibit prion propagation in a cultured mouse neuroblastoma cell line infected with PrP<sup>Sc</sup> (ScN2a). The most effective Fab, D18, was found to abolish prion replication and to clear pre-existing PrP<sup>Sc</sup>, eliminating 50% of PrP<sup>Sc</sup> from the cells within about 24 hours. The activity of D18 was attributed to its ability to specifically recognize the total population of PrP<sup>C</sup> molecules on the cell surface. In PrP<sup>C</sup>, the D18 epitope spans residues 132-156 and incorporates helix A. This sequence lies within the region of the protein thought to bind PrP<sup>Sc</sup>, an essential step for prion propagation: therefore, it can be argued that D18 operates mechanistically by directly blocking or modifying interaction of PrP<sup>C</sup> with PrP<sup>Sc</sup> [12]. Compared to full-length antibodies, Fabs are smaller molecules and their monovalent nature may be beneficial, since intracerebral injection of some anti-PrP immunoglobulin G (IgG) antibodies seemed to provoke neuronal apoptosis [20]. However, their effectiveness in prion disease therapy *in vivo* has not yet been evaluated. The major problem in using anti-prion molecules *in vivo* is that peptide and protein therapeutics are generally excluded from transport to the brain, owing to the negligible permeability of these drugs to the brain capillary endothelial wall, which makes up the blood-brain barrier (BBB) *in vivo*. Fab fragments did not penetrate through the BBB, unless they are hydrophobized by adding stearyl chloride [21] or cationized [22]. One promising solution to this problem is offered by the use of single chain variable fragments or scFv. ScFv are monovalent mini-antibodies, constituted by a single fusion polypeptide comprising the variable region of the light and heavy chain (V<sub>L</sub>, V<sub>H</sub>); they maintain antigen specificity and can be engineered for intracellular expression or secretion. So far, several groups have investigated the use of scFv in prion-infected cell culture systems. One group showed the paracrine inhibition of prion replication by RD-4 cells expressing and secreting scFv(6H4) on co-cultured ScN2a [23]. Another interesting study showed the retention of PrP<sup>C</sup> in the endoplasmic reticulum (ER) of HEK 293 and PC12 cells after the expression of a scFv from antibody 8H4 and 8F9, containing the ER retention sequence KDEL [24]. Starting from these results a subsequent set of *in vivo* analyses was carried out showing that mice intracerebrally injected with KDEL-8H4-NGF-differentiated PC12 cells infected with scrapie did not develop scrapie clinical signs or show any brain damage [25]. Moreover, also scFvs directed against the 37/67 kDa laminin receptor (LRP/LR) have been recently used as therapeutic approach in prion diseases. Delivery of these scFv both by passive immunotransfer [26] or by adeno-associated virus (AAV)-mediated gene transfer [27] resulted in significant reduction of the peripheral PrP<sup>Sc</sup> propagation. Therefore, these data support the use of scFv as a therapeutic approach.

Additional support to the potential therapeutic value of intracerebral antibody production has recently been given by two studies showing that brain delivery of either prion-specific scFv by AAV transfer [28] or of a soluble prion antagonist (PrP-Fc<sub>2</sub>) by lentivirus transfer [29] delay the onset of the disease in infected mice, although protection in these studies was not complete. Because infectious prion replication occurs peripherally within the lymphoreticular system organs, the incomplete protection given by gene transferring of anti-prion molecules in the brain may be consequent to the fact that the peripheral pool of PrP<sup>Sc</sup> continues to replicate and then migrate to the CNS by neuroinvasion [28].

In order to develop a system that could have therapeutic and not only prophylactic

utility, in this study we combined active and passive immunotherapeutic approaches. First, we have produced a monovalent version of the anti-prion D18Fab, D18scFv, and we have tested it in prion-infected cells. The big improvement of our strategy compared to the previous gene transferring is that our molecule might have effects both peripherically and into the brain. We show here that this recombinant anti-prion scFv is able to reduce proteinase K (PK)-resistant PrP accumulation in different infected cell models of prion diseases. Furthermore, in order to provide a localized supply of D18scFv (that could obviate the needs of repetitive injection into the brain), we evaluated and compared the *in vitro* efficiency of two virus-mediated gene transfer systems. We produced two viral vectors, one based on recombinant AAV serotype 2 (AAV2) and the other on HIV-1, carrying the D18scFv gene and showed that the latter is more efficient in reducing prion levels in cultured infected cells *in vitro*. These results provide additional support to use small antibody fragments for active and passive immunotherapy in prion diseases and encourage the *in vivo* evaluation of an anti-prion scFv-based treatment for the therapy of prion disorders.



## EXPERIMENTAL

### Vector production and protein expression and purification

The D18scFv sequence was assembled by fusion PCR of the  $V_L$  and  $V_H$  of the D18Fab by inserting a 20-amino acid (AA) linker. The resulting sequence was cloned into a pET22b(+) vector (Novagen) digested by *Bam*HI and *Not*I in order to maintain the pelB leader in the N-terminal and the 6-his tag in the C-terminal part. X11-blue (Stratagene) and BL21(DE3) *E.coli* (Stratagene) were transformed both to amplify and to express the D18scFv, respectively.

For protein expression, transformed BL21(DE3) were scooped into LB broth (Ampicillin 100  $\mu$ g/ml) and grown overnight at 37°C. The overnight culture was diluted 1:50 and grown at 37°C up to  $OD_{600} = 0.6$ , before induction with 0.5 mM isopropyl-beta-thio-galactoside (IPTG) for 3 hours at 37°C or overnight at 30°C.

Bacteria aliquots (1 ml) were centrifuged and analysed for total protein expression by SDS-PAGE on 12% acrylamide gels and either Coomassie staining and Western blot by using 1:50 HisProbe-HRP (PIERCE Biotechnology, Inc. Rockford, IL)

To test the recovery of D18scFv from periplasm, bacteria were submitted to osmotic shock with 5 mM  $MgSO_4$  at 4°C in order to allow the release of periplasmic proteins in the aqueous phase. For cytosolic fraction preparation, bacteria pellet was resuspended in BugBuster Master Mix (Novagen) by pipetting and vortexing and then incubated on a shaking platform for 10-20 min at room temperature, pelleted, resuspended in BugBuster Master Mix and shaken again twice. Aliquots of pellets and supernatants were analysed by SDS-PAGE followed by either Coomassie staining and Western blot, in order to analyse insoluble and soluble cytosolic fractions. The last pellet was resuspended three times in 1:10 diluted BugBuster Master Mix (2.5 ml/gr of the original weight) and centrifuged at 4°C twice at 5.000xg and finally at 16.000xg. Since protein was found insoluble, it was purified by IMAC in denaturing conditions and then refolded by fast dilution. Briefly, pellet was resuspended in 10 ml (for a 1-L culture) of denaturing buffer A (6M Gdn-HCl, 0,1 M sodium phosphate buffer, 0,01 M Tris-HCl pH 8) and incubated with of 1X Ni-NTA His-bound resin (Novagen) (1 ml/4 ml of denatured protein) by gently shaking 3-4 hours at room temperature. After incubation, protein-bound resin was washed twice with buffer B (8M Urea, 0,1 M sodium phosphate buffer, 0,01 M Tris-HCl pH 6,3) and then the D18scFv was eluted with 4 x 1 ml buffer C (8M Urea, 0,1 M sodium phosphate buffer, 0,01 M Tris-HCl pH 5.9), followed by 4 x 1 ml buffer D (8M Urea, 0,1 M sodium phosphate buffer, 0,01 M Tris-HCl pH 4.5). Aliquots of sample were taken at each step for analysis by SDS-PAGE followed by either Coomassie staining and Western blot by using 1:50 HisProbe-HRP. Fractions containing the D18scFv were pulled and diluted about 35 times in 25 mM Tris-HCl pH 8.0, 5 mM EDTA for fast refolding. The purified protein was dialysed against 20 mM Tris-HCl at different pH (pH 6,5; 7,5 and 8.5) at 4°C overnight and then quantified and concentrated. Solubility of the refolded protein was tested by 1 hour of cold ultracentrifugation at 100,000xg in Beckman Optima MAX ultracentrifuge (MLA-130 rotor) (Beckman Coulter Inc., Palo Alto, CA) before/after concentration.

### Activity assays

**ELISA:** D18scFv was tested for binding to recombinant PrP-Fc.

Plates were coated with 20 $\mu$ g/ml of goat  $\alpha$ -human Fc in 0,1 M  $NaHCO_3$ , pH 8.6 overnight at room temperature, washed three times with TBST (20 mM TRIS, 137 mM NaCl, 0,05% Tween 20, pH 7.5) and then incubated with PBS 0,25% BSA, 0,05% Tween 20 for 1 hour at room temperature. PrP-Fc supernatant (containing 1-10  $\mu$ g/ml of protein) [30] was pre-incubated with several amounts of D18scFv (0,05 up

to 10 µg/well) for 15 min at room temperature and this solution was then incubated with the goat  $\alpha$ -human Fc present in the wells for 2 hours at room temperature. After washing with TBST and incubation with 1:50 HisProbe-HRP for 1 hour at room temperature, plates were developed with TMB solution (Pierce) for 15 min and 2 M H<sub>2</sub>SO<sub>4</sub> and optical density was measured at 450 nm. Several concentrations of D18Fab were tested as controls.

**Western blot competition assay:** Aliquots of 5 µl 10% brain homogenate from FVB mice were loaded on 12,5% acrylamide gels and subjected to SDS-PAGE and Western blot. Membranes were saturated with 5% milk/TBST and incubated for 1 hour with several concentration of D18scFv (0,05 up to 2,5 µg/ml) together with D18Fab by utilising Fab/scFv ratio of 1:1; 1:10; 1:50 and 1:100 and then with a goat a-human IgG-F(ab')<sub>2</sub>-HRP.

#### **Adeno-associated Virus 2 production**

The D18scFv fragment, including the C-terminal 6-his tag and the N-terminal secretion signal, was cloned into the AAV backbone of the plasmid pAAV-MCS (Stratagene) to obtain pAAV- D18scFv.

Infectious AAV2 vector particles were generated by the AAV Vector Unit (AVU) at ICGEB Trieste (<http://www.icgeb.org/RESEARCH/TS/COREFACILITIES/AVU.htm>) in HEK293 cells, using dual plasmid cotransfection procedure with pDG as packaging helper plasmid (kindly provided by J. A. Kleinschmidt, DKFZ, Germany), as previously described [31]. AAV-D18scFv vector stocks were purified by cesium chloride centrifugation and titration of viral particles was performed by real-time PCR quantification of the number of viral genomes, as described previously [31]; the viral preparations used in this work had titers between 1x10<sup>11</sup> and 1x10<sup>12</sup> viral genome particles (vgp) per ml.

#### **Lentivirus production**

D18scFv cDNA was cloned into the lentiviral vector pRRL.sin.cPPT.hCMV.GFP.Wpre in place of GFP by digestion with *Age*I and *Sa*II. Signal peptide from PrP<sup>C</sup> sequence was inserted at the N-terminal (*Bcl*II/*Age*I). Vectors stocks were generated by cotransfection of 293T with pCMV-{Delta}R8.91,13 pMD.G,13 and pRRL.sin.cPPT.hCMV.D18scFv vectors. 293T-conditioned media were filtered and ultracentrifuged to concentrate the vector. Particle content was measured by HIV-1 p24 antigen immunocapture. HIV-1 p24 concentration was about 70 ng/ml (corresponding to 100,000 infectious units per ng of p24).

#### **Cell cultures**

ScN2a cells and mouse gonadotropin-releasing hormone (GnRH) neuronal cells (ScGT1-1) were maintained in DMEM, 10% fetal bovine serum (FBS) in a humidified 37°C incubator with 5% CO<sub>2</sub>.

#### **Studies of D18scFv inhibition**

Several concentrations (0,1 up to 5 µg/ml) of D18scFv were added to ScGT1-1 cells and incubated for 1 week. The cells were fed three times per week with replacement media containing the appropriate amount of scFv. Cells were collected by washing three times with calcium- and magnesium-free PBS and resuspended in 500 µl lysis buffer (10 mM Tris, pH 7.5, containing 150 mM NaCl, 0.5% sodium deoxycholate, 0.5% nonident P-40). Cell nuclei were removed from the lysate by centrifugation at 2,000xg for 2 min, and the protein concentration of the supernatant measured by Bradford assay (Pierce) and subjected to PK-resistance assay.

#### **Studies of D18scFv-virus inhibition**

Different titers of lentivirus (10x10<sup>4</sup> up to 10x10<sup>6</sup> TU) and rAAV2 (10x10<sup>10</sup> up to 10x10<sup>12</sup> TU) carrying D18scFv gene were used to infect both ScGT1-1 and ScN2a



cells. For AAV2 infection, cells were pre-treated with 1 mM idrossiurea for about 16 hours. One week post-infection, cells were collected, resuspended in 500  $\mu$ l lysis buffer and subjected to PK-resistance assay as described below.

#### **Proteinase K (PK) resistance assay**

ScN2a and ScGT1 cell lysates (500  $\mu$ g) were treated with 20  $\mu$ g/ml of PK (total protein:enzyme ratio=50:1) for 30 min at 37°C. Proteolytic digestion was terminated by the addition of phenylmethyl sulphonyl fluoride (PMSF) to a final concentration of 2 mM. PK-resistant material was pelleted by centrifugation at 18,000xg for 1 hour and then resuspended in reducing SDS sample buffer, boiled for 5 min, cleared by centrifugation and resolved by SDS-PAGE (12%). Samples were transferred on nitrocellulose membrane and blocked with 5% milk in TBST. Prion protein was detected using SAF61 antibody. Blots were developed with enhanced chemiluminescence (ECL) reagent (Amersham) and exposed to ECL Hypermax film (Amersham).

#### **Bioinformatic models**

The sequence of the D18scFv [32] was compared to the sequences of known 3D structures collected in Protein Data Bank [33] in order to find templates for homology modeling. The anti-prion protein P scFv fragment (PDB code: 2HH0) [34] was identified and further used as the template for the modeling. 3D models were built using MODELLER 6v2 [35]. Amino acids of a signal sequence and poly-glycine parts of the sequences were neglected in the modeling. The structure of the D18scFv model was then used to dock the prion protein (PrP; PDB code: 1HJM) [36] by means of the HADDOCK 2.0 program [37, 38]. Residues determined to be important for protein-protein recognition between PrP and D18scFv were reported in the literature [32]. For the purpose of docking, they were filtered according to the solvent accessibility with the NACCESS program. All His residues were deprotonated. Cluster analysis were then performed with programs available in HADDOCK 2.0 [37, 38].

## RESULTS AND DISCUSSION

It has been recently shown that anti-prion scFvs could be delivered to the central nervous system (CNS) of mice by AAV and are able to delay the onset of the disease when used in prophylaxis but not when used after prion-exposure [28]. Contribution to the incomplete protection could derive from down-regulation of the scFv expression by PrP<sup>Sc</sup> neuroinvasion. In addition, because infectious prions replicate peripherally within the lymphoreticular system organs, the incomplete protection may be consequent to the fact that the peripheral pool of PrP<sup>Sc</sup> continues to replicate and migrate to the CNS by neuroinvasion. These observations make inefficient anti-prion treatments based exclusively on active immunotherapy. Since it has been shown that small proteins can be rapidly delivered and spread in the brain after i.c. injection or after nasal administration [39, 40], we have evaluated, in cell culture, an alternative approach to treat TSEs based on passive immunotherapy.

To this aim, we have produced a smaller version of the D18Fab, the D18scFv in which the variable regions of the light and heavy chains of Fab were fused together by PCR, by inserting a 20-AA linker and adding a 6-histidine tag in the C-terminal part (Figure 1). The sequence corresponding to the scFv (Figure 1A) was cloned in a bacterial expression system (Figure 1B) and its expression was induced by using 0.5 mM IPTG at 30 or 37°C. A higher expression of the molecule was found at 37°C, as shown in Figure 2A. To test the recovery of D18scFv from periplasm or cytosol, bacteria were submitted to osmotic shock and treated with 5 mM MgSO<sub>4</sub> at 4°C in order to allow the release of periplasmic proteins in the aqueous phase recovered after a following step of centrifugation (Figure 2B). For cytosolic fraction preparation, bacteria were resuspended in lysis buffer and after shaking, centrifugation was performed to separate a soluble from an insoluble protein fraction (Figure 2B). Equivalent aliquots of different fractions were analysed by Coomassie staining and Western blot (Figure 2B). Since the larger amount of D18scFv was recovered in the insoluble cytosolic fraction, it was purified by IMAC in denaturing conditions. In all recovered fractions, D18scFv was shown to be highly homogeneous and in considerable amounts (Figure 2C) and therefore refolded by fast-dilution. In order to test for functional refolding after fast dilution at different pH (6.5; 7.5 and 8.5) D18scFv was analysed for its solubility by ultracentrifugation at 100,000xg (Figure 2D). We found that D18scFv is more soluble at pH 8.5, suggesting that, at this pH, it has lower tendency to precipitate and aggregate and therefore its conformation is more stable.

In order to directly test for interaction and anti-prion activity, D18scFv was evaluated for its binding to PrP. Specifically, we tested the ability of this molecule to bind recombinant PrP-Fc [30] in an ELISA assay and no significant differences was found for the different pH refolding conditions (Figure 3A, right graph). Promisingly, D18scFv was found to maintain the same affinity for PrP of the original D18Fab used as control (Figure 3A, left graph). We also tested the ability of the scFv to bind PrP<sup>C</sup> present in brain homogenate from FVB mice. We set up a competition assay between the D18scFv and the D18Fab, which could be detected in western blot by an anti-human (Fab')<sub>2</sub>-HRP (Figure 3B). The reduction of PrP signal by utilising a Fab/scFv ratio of 1:50 and 1:100 clearly showed the ability of the scFv to specifically interact with PrP and to compete with the Fab for its binding. Moreover, we did not find any difference among the scFv refolded at the different pH (data not shown).

Due to the interest on how D18scFv interacts with the PrP<sup>C</sup>, we have used bioinformatic approaches to construct a structural model of D18scFv-PrP<sup>C</sup> complex. This kind of approach is commonly used as a first attempt to identify 3D structures of

the proteins and protein-protein interactions in the absence of detailed experimental data. Firstly, the 3D structure of D18scFv was modeled based on anti-prion protein P scFv fragment (PDB code: 2HH0) [34]. This fragment was crystallized in a complex with a small peptide. Due to the presence of a small peptide, the complementarity determining regions 3 (CDR3) are farther apart than in non-complexed antibody structures. Therefore, this structure was taken as a template for the model of the D18scFv since it allows an easy approach of the PrP<sup>C</sup> to the cavity of the antibody in the docking procedure (Figure 4A). Then a D18scFv-PrP<sup>C</sup> complex was built by docking of PrP<sup>C</sup> (PDB code: 1HJM) [36] to the model of D18scFv. According to the docking results, Arg<sub>PrP</sub>151 (Arg151 from PrP) is the key residue for the interactions with D18scFv. It anchors the PrP<sup>C</sup> to the cavity of the antibody forming H-bonds with Tyr<sub>VH</sub>32 (Tyr32 in heavy variable chain), Asn<sub>VH</sub>33, Asp<sub>VH</sub>35 and Tyr<sub>VL</sub>210 (Tyr210 in light variable chain), along with van der Waals interactions with Trp<sub>VL</sub>205 (Figure 4B). A series of other H-bonds and hydrophobic interactions contribute further to the D18scFv-PrP<sup>C</sup> complex stability.

Due to the lack of available mutational data it is difficult to evaluate accurately our models. However, the modeled structure of D18scFv-PrP<sup>C</sup> complex might provide an insight to the relevant interactions between the two proteins and give new suggestions for mutational studies.

The next step was to test D18scFv for its effect on scrapie replication in scrapie infected cell lines (Figure 5). After incubation of ScGT1 cells with different concentrations (0.1; 0.5; 1 and 5 µg/ml) of scFv for 1 week, scFv was found to reduce PK-resistant PrP levels in a concentration-dependent manner, similarly to what was found for the Fab in ScN2a cells [12]. In particular, we found 20%±4, 50%±7, 80%±3 and 95%±1 PK-resistant PrP reduction by using respectively 0.1, 0.5, 1 and 5 µg/ml D18scFv. We have used R2Fab (10 µg/ml) that was shown to be ineffective on reducing PK-resistant PrP in ScN2a cells [12] and D18Fab (1 and 5 µg/ml) was used as positive control. Interestingly, the same concentration of Fab and scFv (compare lanes 5 µg/ml, respectively) is required to achieve the same extend of reduction in PrP<sup>Sc</sup> level. However, since the scFv is approximately half molecular weight compared with the Fab, D18scFv is 2-fold less effective than Fab with respect to the reduction of the PrP<sup>Sc</sup> level in ScGT1 cells. After 1 week treatment, PK-resistant PrP was reduced to undetectable levels but was not completely eliminated from the cells because it reappeared after 1 additional week of growth in the absence of the scFv (data not shown). However, after 2 or 3 weeks of scFv treatment PK-resistant PrP remained undetectable for up to 3 weeks of culture without adding any scFv (data not shown), as it has been shown for the original Fab [12]. Also in this case we found no difference among the scFv refolded at the different pH (Figure 5). These data indicate that the small monovalent antibody fragment might be a very useful tool in therapy because it is able to interfere with prion metabolism (either reducing conversion or promoting degradation) in infected cells. The big improvement of our strategy compared to the previous gene transferring-based approaches [28] is that our molecule might have effects both peripherically and into the brain, both of them sites of prion replication.

In vivo the putative anti-prion effects of the D18scFv expressed as recombinant protein might be enhanced by combining passive and active immunotherapeutic approaches.

Recently, different gene transfer approaches in anti-prion prophylaxis of infected animals have been successfully used by using either lentivirus carrying a soluble prion antagonist [29] or AAV transducing either anti-LPR/LR [27] or anti-prion protein

specific scFvs [28]. Therefore, in order to have a continuous supply of our recombinant scFv to be eventually used in immunotherapy in animals and to test the effectiveness of these two viral systems, we cloned the gene encoding for D18scFv (Figure 1A) either in a defective lentiviral vector (pTTLsin.PPT.hPGK.GFPpre) (Figure 6A, left panel) or in an AAV vector (pAAV-MCS) (Figure 6A, right panel). The lentivirus vector was transfected in association with a three-plasmid expression system in 293T cells in order to produce a functional lentivirus. A lentivirus containing a GFP reporter gene was used to test the efficiency of viral transduction. At 48 hours post-transduction, ScGT1 cells were analysed by immunofluorescence and we found that the  $35\% \pm 5$  of ScGT1 cells were GFP positive (data not shown). Lentivirus carrying the D18scFv was titrated and tested for its effectiveness in reducing PK-resistant PrP content in infected cell cultures. Titers of  $10 \times 10^4$  up to  $10 \times 10^6$  TU were used to transduce both ScGT1 and ScN2a cells. One week post-transduction D18scFv was expressed (Figure 6B) and the levels of PK-resistant PrP were reduced of  $80\% \pm 7$  and  $90\% \pm 3$  respectively in ScGT1 and ScN2a cells by using the highest viral titer tested ( $10 \times 10^6$  TU) (Figure 6C). Interestingly, the effect on PK-resistant PrP content after lentivirus transduction was more pronounced in ScN2a cells, where even a lower titer of  $10 \times 10^4$  TU was effective (Figure 6C). Alternatively, we also produced a recombinant (rec) AAV2 (see Experimental) expressing D18scFv and subsequently packaged into virus stocks for CNS delivery (see Experimental). Titers of  $10 \times 10^{10}$  up to  $10 \times 10^{12}$  TU were used to transduce both ScGT1 and ScN2a cells and transgene expression (Figure 6B) and PK-resistant PrP levels (Figure 6C) were checked one week post-transduction. Interestingly, only the highest titer tested ( $10 \times 10^{12}$  TU) was able to reduce PK-resistant PrP level of  $20\% \pm 5$  in ScN2a cells (Figure 6C). Same result is achieved after longer period of viral transduction (up to 3 weeks) (data not shown). Also in this case we used an AAV2-containing GFP to evaluate the efficiency of viral transduction. At 48 hours post-transduction,  $15\% \pm 8$  of ScGT1 cells were GFP positive (data not shown). These data indicate that, at least in cell culture models, lentivirus transduces D18scFv in a functional form able to prevent or interfere with PrP<sup>Sc</sup> conversion and seem to represent a better delivery system compared to AAV. In comparison, due to the well-known high tropism of rAAVs for brain neurons *in vivo*, recently supported by its efficacy in different clinical trials for neurodegenerative disorders (see, among others: [41]), AAV-scFv remains a valuable potential tool for the *in vivo* treatment of prion disease. How these viruses act in reducing PK-resistant PrP levels has to be still elucidated. One possibility could be that anti-PrP scFvs block conversion reaction directly. Alternatively, PK-resistant PrP reduction could be an indirect effect of down-regulation of PrP<sup>C</sup> expression. Nevertheless, the eventual regulation of PrP<sup>C</sup> expression is specifically due to the scFv, because viruses expressing other constructs (GFP-PrP) do not reduce PK-resistant PrP content in scGT1 cells (data not shown). In conclusion, we validated here the use of D18scFv as potential anti-prion immunotherapeutic both as purified protein and after viral transduction in cultured neuronal cells. The availability of our lentiviral and AAV2 vectors expressing the same therapeutic molecule will now allow us to directly compare the efficacy of the two viral vectors in sick animals *in vivo*.

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## FIGURE LEGENDS

**Fig.1: Schema of the D18scFv in pET-22b(+).** **A)** Sequence of the D18scFv. The different regions of the sequence are indicated with different colors: the *pelB* leader is light gray, the  $V_H$  and  $V_L$  sequences are red, the 20-AA linker is grey and the 6-histidine tag is yellow. **B)** Cloning of the D18scFv in pET-22b(+). The D18 scFv was obtained by PCR ligation of the variable regions of the light [42] and heavy ( $V_H$ ) chains of FabD18, by inserting a 20-AA linker. The sequence corresponding to the scFv (A) was inserted between BamHI and NotI restriction enzyme sites present in the multiple cloning site of pET-22b(+) expression vector by keeping the *pelB* leader and the 6-histidine tag already present in the vector.

**Fig.2: Expression and purification of the D18scFv.** **A)** Expression of the D18scFv in *E. coli*. BL21 (DE3) Rosetta-gami *E. coli* strain was transformed and induced by using 0.5 mM IPTG (+) at 30 or 37°C. Note that a higher expression of the molecule was found at 37°C. **B)** Cytoplasmic extraction of the D18scFv. D18scFv was recovered from periplasmic (P) or both soluble (S) and insoluble (I) cytosolic preparations. Equivalent aliquots of the different obtained fractions were analysed by Coomassie staining (top panel) and Western blot by using an anti-6his probe (bottom panel). Note that the larger amount of D18scFv was recovered in the insoluble cytosolic fraction. **C)** Purification of insoluble D18scFv. Insoluble D18scFv was purified by IMAC after denaturation by using 6 M Guanidium-HCl. After elution, 8 different fractions were collected and analysed by Coomassie staining (top panel) and Western blot by using an anti-6his probe (bottom panel). Note that the scFv shows a high degree of homogeneity and is in consistent amount in all the analysed fractions. **B:** IPTG-induced bacteria. **D)** Solubility of the D18scFv after ultracentrifugation. Purified D18scFv refolded at different pHs (6,5; 7,5 and 8,5) was ultracentrifuged at 100,000xg (+) and analysed by Coomassie staining. Note that D18scFv is more soluble at pH 8,5. Arrows indicate D18scFv.

**Fig.3: Analysis of activity of the D18scFv.** **A)** D18scFv binding to recombinant PrP-Fc. ELISA assays were performed to test the binding to recombinant PrP-Fc of D18scFv (left graph) in comparison to FabD18 (right graph). Note that no significant difference was found for the different pH refolding conditions and that D18scFv maintains the same affinity for PrP of the original D18Fab. **B)** D18scFv binding to PrP<sup>C</sup> present in mouse brain homogenate. Western blot competition assay between the D18scFv and the D18Fab was set up as described in Experimental. Different concentrations of D18scFv were tested in competing D18Fab detection of PrP<sup>C</sup>. Note that the reduction of PrP signal by utilising a Fab/scFv ratio of 1:50 and 1:100 clearly showed the ability of the scFv to specifically interact with PrP and to compete with the Fab for its binding.

**Fig. 4: Model of interaction between D18scFv and PrP.** **A)** Complex between D18scFv (silver) and PrP (blue). Epitope residues of PrP (yellow) interact with the six CDR loops (red – CDR3, green – CDR1,2) of the antibody. **B)** Arg151 anchors the PrP to the cavity of the antibody through the H-bond and van der Waals interactions with Tyr32, Asn33, Asp35, Trp205 and Tyr210.

**Fig. 5: Effect of D18scFv in curing scrapie infected GT1 cells.** ScGT1 cells were incubated for 1 week with different concentrations (0.1; 0.5; 1 and 5 µg/ml) of scFv refolded at different pH (pH 6.5; 7.5 and 8.5). As controls, R2 (10µg/ml) and D18Fab (1 and 5 µg/ml) were used. PrP<sup>Sc</sup> present in cell lysates was digested with Proteinase K (PK +) as described in Experimental and detected by western blot by using SAF61

antibody. Note that scFv reduces PK-resistant PrP level of  $20\% \pm 4$ ,  $50\% \pm 7$ ,  $80\% \pm 3$  and  $95\% \pm 1$  by using respectively 0.1, 0.5, 1 and 5  $\mu\text{g/ml}$  D18scFv. Blots of PK-resistant assays were quantified by ImageJ ( $n=3$ ) and Student T-test was used to calculate significance of data ( $p<0.05$ ). No difference was found among the scFvs refolded at the different pH.

**Fig. 6: Production and cellular test of lentivirus and AAV2 containing the D18scFv.** **A)** Cloning of the D18scFv in pTTLsin.PPT.hPGK.GFPpre and in pAAV-MCS. For lentiviral vector production (left panel), the gene encoding for D18scFv (Figure 1A) was cloned in defective lentiviral vector (pTTLsin.PPT.hPGK.GFPpre) between AgeI and SalI restriction enzyme sites. The signal peptide of the PrP sequence was cloned upstream between BclI and AgeI. For AAV vector production (right panel), the gene encoding for D18scFv was cloned in pAAV-MCS between HindIII and BglII restriction enzyme sites. For secretion, the signal peptide of the immunoglobulin (Ig) sequence was added between HindIII and ApaLI. **B)** Expression of D18scFv after transduction of ScGT1 and ScN2a cells.  $10 \times 10^4$  TU of lentivirus and  $10 \times 10^{10}$  TU of rAAV2 were used to transduce both ScGT1 and ScN2a cells. One week post-infection, D18scFv expression was tested by western blot by using an anti-6his tag antibody. **C)** Effect of the lentivirus and rAAV2 carrying the D18scFv in curing scrapie infected neuronal cell lines. Titers of  $10 \times 10^4$ - $10 \times 10^6$  TU of the lentivirus (left panels) and  $10 \times 10^{10}$ - $10 \times 10^{12}$  TU of rAAV2 (right panels) carrying the D18scFv were used to infect both ScGT1 and ScN2a cells. One week post-infection, PrP<sup>Sc</sup> present in cell lysates was digested with proteinase K (PK +) as described in Experimental and detected by western blot by using SAF61 antibody. Blots of PK-resistant assays were quantified by ImageJ ( $n=3$ ) and Student T-test was used to calculate significance of data ( $p<0.05$ ). By using increasing titer of lentivirus, levels of PK-resistant PrP were reduced of  $2\% \pm 7$ ,  $75\% \pm 3$  and  $80\% \pm 7$  in ScGT1 and  $76\% \pm 2$ ,  $80\% \pm 7$  and  $90\% \pm 3$  in ScN2a cells. By using increasing titer of AAV2, levels of PK-resistant PrP were reduced of  $2\% \pm 3$ ,  $5\% \pm 3$  and  $5\% \pm 4$  in ScGT1 and  $4\% \pm 2$ ,  $4\% \pm 7$  and  $20\% \pm 5$  in ScN2a cells. Note that levels of PK-resistant PrP were significantly reduced in both cell systems by using the highest lentiviral titer tested ( $10 \times 10^6$  TU).

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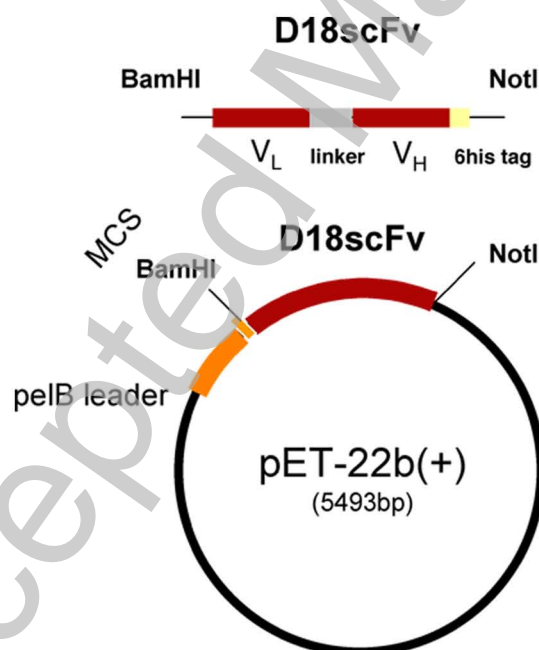


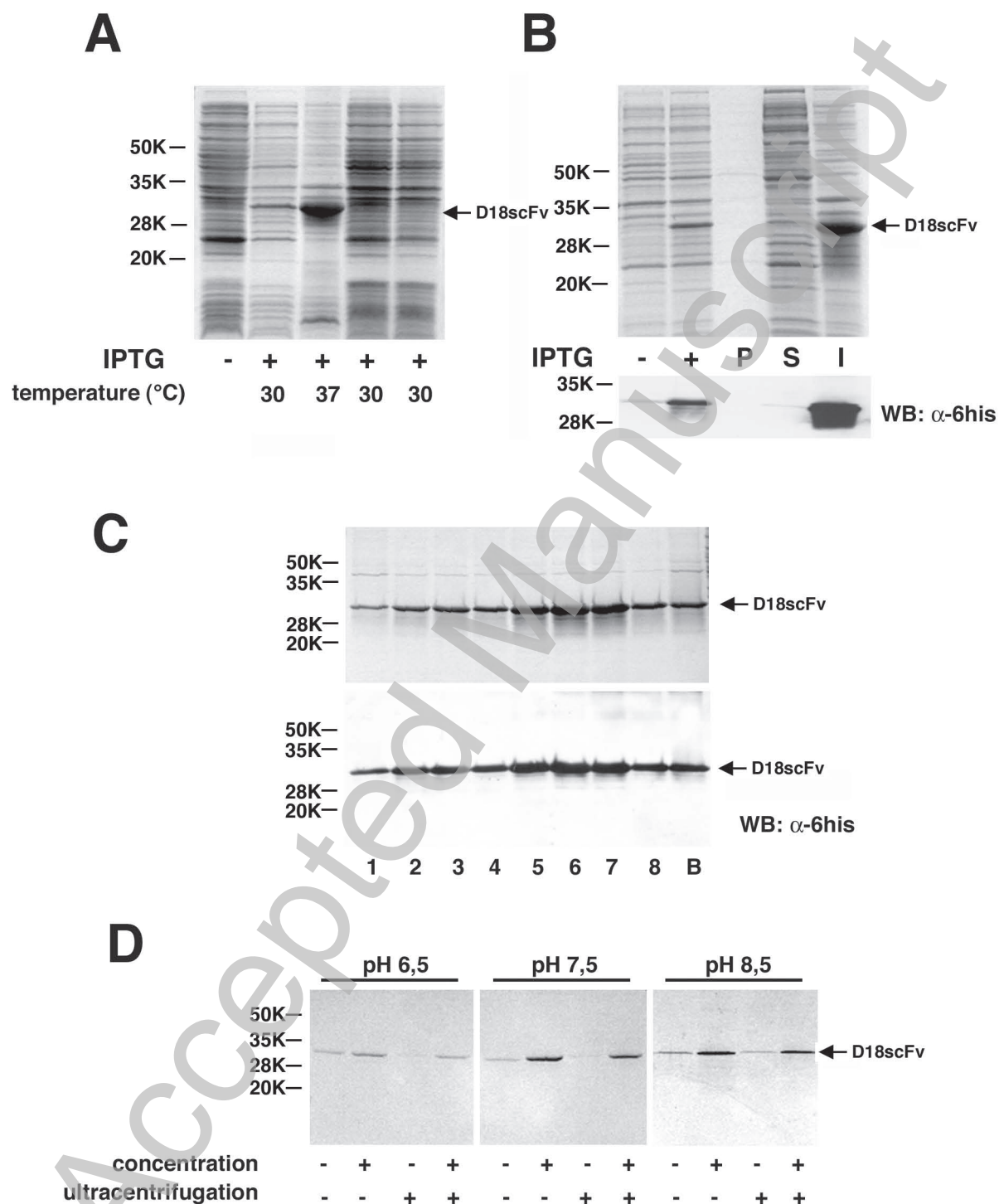
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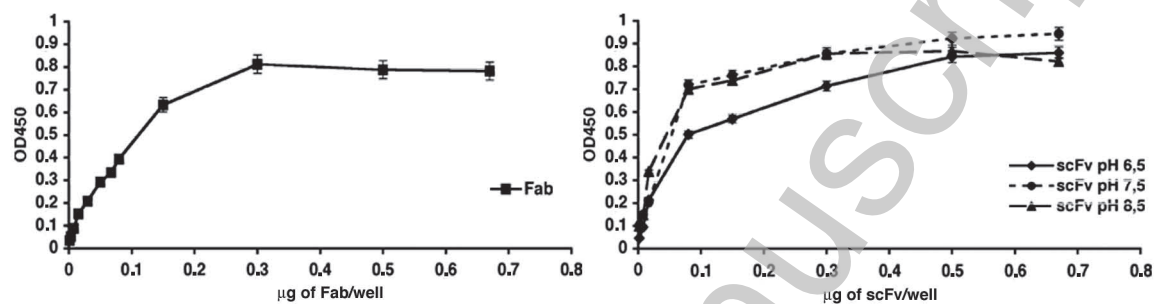
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**B****Figure 1**

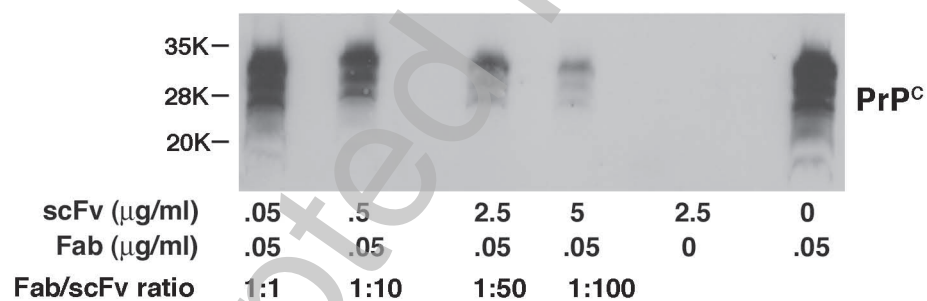


**Figure 2**

**A**

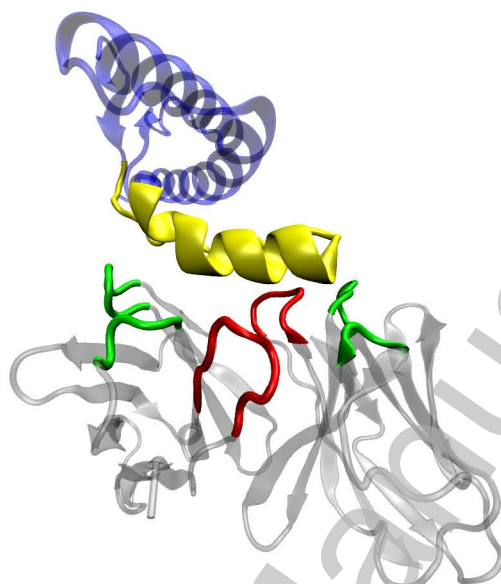


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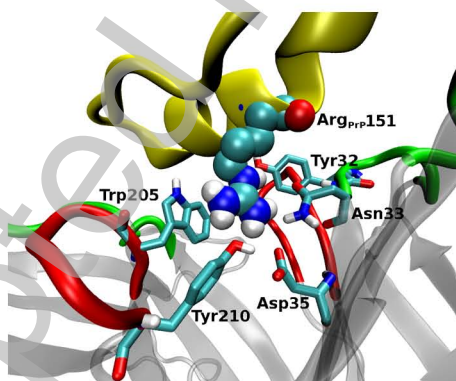


**Figure 3**

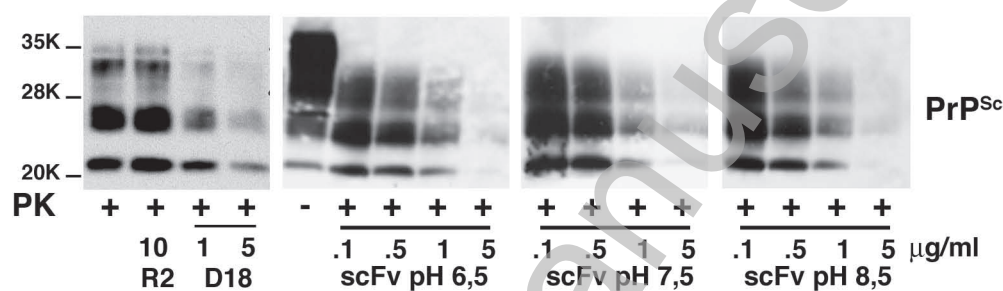
**A**



**B**



**Figure 4**



**Figure 5**



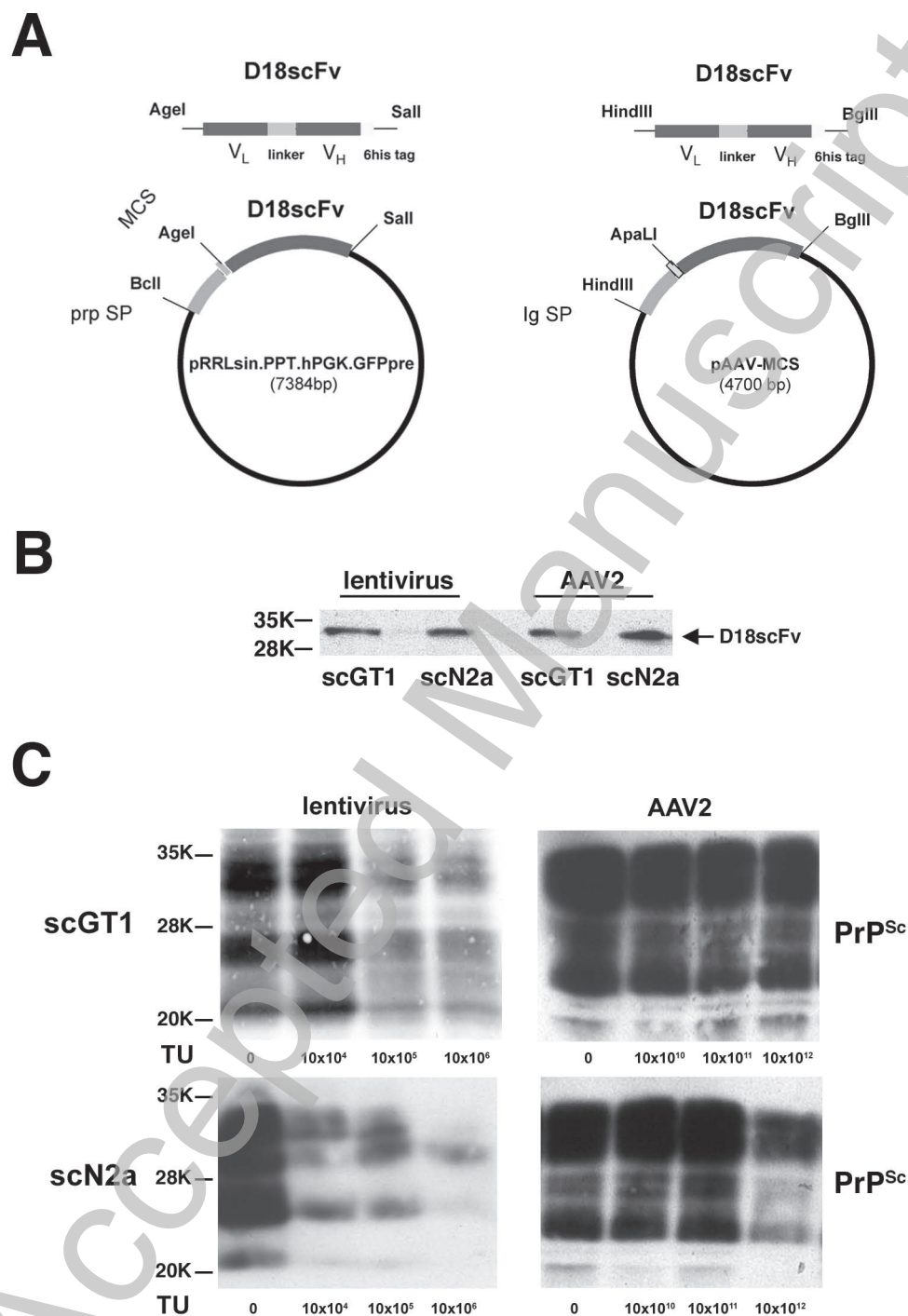


Figure 6