Using artificial binding proteins to inhibit protein-protein interactions

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Introduction

Antibodies are the best-studied group of biological binding molecules to date. They are important in a wide variety of biological and medical applications, but as molecular biology reagents they are limited by their significant size, poor stability, production costs and batch-to-batch variation. To overcome these issues a number of alternative binding reagents (protein, RNA and DNA aptamers) have been developed. These can bind to epitopes on target proteins and so have potential as molecular biology tools, therapeutic agents and as diagnostic tools for detection and imaging of proteins in patient samples. The BioScreening Technology Group at Leeds was established to exploit a novel artificial binding protein (ABP) library. Our ABP is called an adhiron and is based on a constant small 91 amino acid scaffold protein that constrains two randomised nine amino acid loop regions for molecular recognition (Figure 1A). The scaffold protein is extremely stable with a T_m of 101^{o}C and is the most stable ABP scaffold to date (Figure 1B), and maintains the beta structure following loop insertion (Figure 1C). We have developed a large naïve phage display library (>3×10^{10}) of adhirons that is of very high quality (86 % full length clones). The loop regions in the library contain an even distribution of each of the 19 amino acids excluding cysteines (Figure 1D).

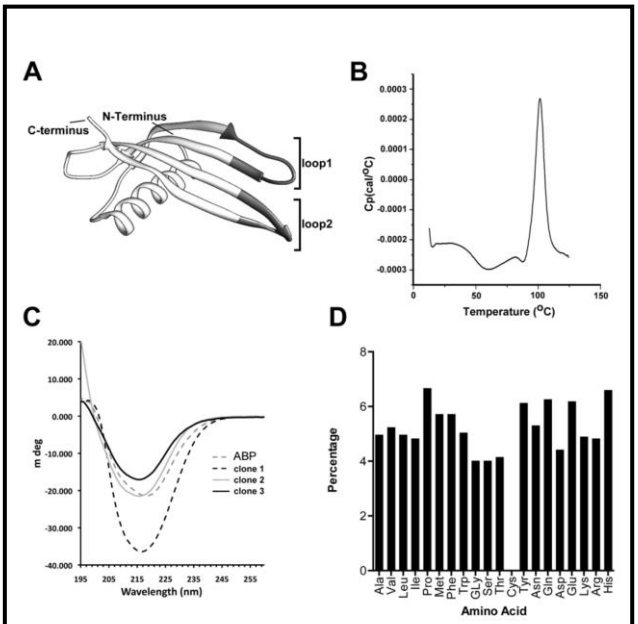

Figure 1: (a) The ABP is shown in white, the loop regions are shown in grey. **(b)** differential scanning calorimetry to measure melting temperature of scaffold protein. **(c)** circular dichroism examining structures of the ABP and three random clones. **(d)** amino acid composition of the loop regions showing an even distribution with little bias.

Developing specific reagents for inhibiting human SUMO2 interaction with RNF4

Small Ubiquitin-like Modifier (SUMO) regulates post-translational modifications involved in many aspects of cell function, including stress response, cell proliferation and apoptosis. To date the lack of suitable isoform specific reagents has limited understanding of the specific function of the isoforms. Recently, hSUMO1 specific binding reagents were identified but the same group failed to identify hSUMO2 specific binders. For the first time we have developed ABP reagents which differentiate between hSUMO1 and hSUMO2 isoforms (Figure 2A). To confirm this specificity we have developed assays that test ABPs ability to inhibit SUMO interactions in an isoform-specific manner. SUMO-targeted ubiquitin ligases (STUbL) are a unique class of E3 ubiquitin ligase enzymes that only recognise substrates modified with polymeric chains of hSUMO2. RNF4 is the only known cellular STUbL and contains two domains – one with SUMO Interacting Motifs (SIM) for binding SUMO and a RING domain responsible for its ubiquitination activity. *In vitro* recombinant RNF4 ubiquitinates polymers of hSUMO2 (poly-hSUMO2$_{2-8}$). ABPs specific for GFP (irrelevant control) and for hSUMO1 were unable to inhibit RNF4s ability to ubiquitinate poly-hSUMO2$_{2-8}$, whereas ABPs specific for hSUMO2 robustly inhibited this activity at less than 1 μM (Figure 2B).

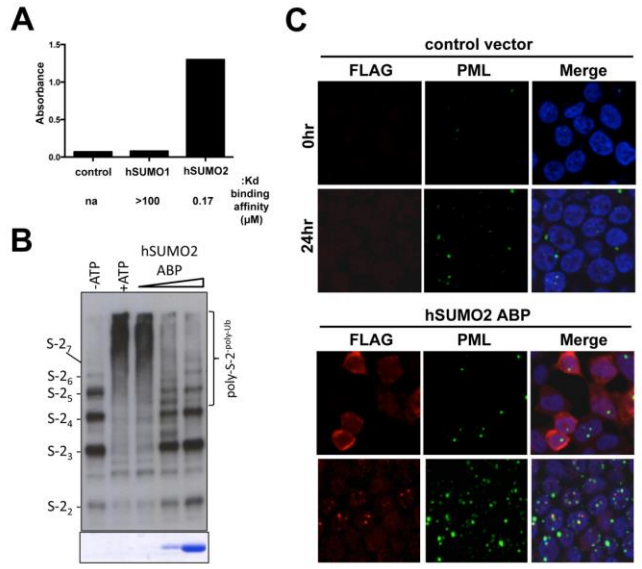

Figure 2: ABP targeting hSUMO2. **(a)** an ABP raised against hSUMO2 was tested to determine specificity for hSUMO1 and hSUMO2 by ELISA. The hSUMO2 ABP specifically bound to hSUMO2, which was reflected in the binding affinity. **(b)** blot showing the ability of the hSUMO2 ABP to inhibit RNF4's SUMO-targeted ubiquitin ligase activity. **(c)** a control vector or hSUMO2 binder was expressed in cells (red – anti-FLAG antibody) and cultured with arsenic to induce nuclear bodies, PML (green). RNF4 promotes degradation of PML. Blocking the interaction between hSUMO2 and RNF4 inhibits PML degradation.

To assess the hSUMO ABPs in a more physiological context, we expressed them in mammalian cell lines. A major role of cellular RNF4 is to maintain the levels of nuclear domain 10 (ND10) components such as promyelocytic leukaemia protein (PML). PML is SUMO-modified with both hSUMO-1 and hSUMO-2, but its modification with polymeric hSUMO-2 is critical for recruiting RNF4 in a SIM-dependent manner, leading to its ubiquitination and subsequent degradation by the 26S proteasome. Brief treatment of cells with arsenic trioxide (As_2O_3) induces the expression and SUMO modification of PML and when arsenic is removed PML levels return to normal in an RNF4-dependent manner. In cells expressing control or hSUMO1-ABPs, PML levels recover after removal of arsenic. By contrast in treated cell expressing hSUMO2 ABPs the levels of PML fail to return to normal (Figure 2C) demonstrating the specificity of the hSUMO2 ABPs.

Publications

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Tomlinson, D., Baxter, E., Loadman, P., Hull, M. & Knowles M. (2012) FGFR1-induced epithelial to mesenchymal transition through MAPK/PLCγ/COX-2-mediated mechanisms. *PloS One.* **7:**e38972.

Tomlinson, D., Knowles, M. & Speirs, V. (2012) Mechanisms of FGFR3 actions in endocrine resistant breast cancer. *Int J Cancer*. **130:**2857-66.

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