**ABSTRACT**

Antibodies are routinely used to study the activity of transcription factors, using various *in vitro* and *in vivo* approaches such as electrophoretic mobility shift assay, enzyme-linked immunosorbent assay, genome-wide method analysis coupled with next generation sequencing, or mass spectrometry. More recently, a new application for antibodies has emerged as crystallisation scaffolds for difficult to crystallise proteins, such as transcription factors. Only in a few rare cases, antibodies have been used to modulate the activity of transcription factors, and there is a real gap in our knowledge on how to efficiently design antibodies to interfere with transcription. The molecular function of transcription factors is underpinned by complex networks of protein-protein interaction and in theory, setting aside intra-cellular delivery challenges, developing antibody-based approaches to modulate transcription factor activity appears a viable option. Here, we demonstrate that antibodies or an antibody single-chain variable region fragments are powerful molecular tools to unravel complex protein-DNA and protein-protein binding mechanisms. In this study, we focus on the molecular mode of action of the transcription factor SOX18, a key modulator of endothelial cell fate during development, as well as an attractive target in certain pathophysiological conditions such as solid cancer metastasis. The engineered antibody we designed inhibits SOX18 transcriptional activity, by interfering specifically with an 8-amino-acid motif in the C-terminal region directly adjacent to α-Helix 3 of SOX18 HMG domain, thereby disrupting protein-protein interaction. This new approach establishes a framework to guide the study of transcription factors interactomes using antibodies as molecular handles.

**Introduction**

Forward and reverse genetics approaches to identify new pathways critical for developmental and pathological processes have reached a high level of routine and standardization due to advances in next-generation sequencing and gene editing methods. As a research field, developmental biology has contributed the most to the identification of key molecular switch transcription factors (TFs), in the major SOX, FOX and HOX classes. In contrast, study of the modes of action of TFs is still in its infancy, involving complex, empirical, multipronged cross disciplinary approaches, in need of refinement before they can gain a similar momentum. Twenty years ago, TFs were defined in a simple canonical manner, as proteins that bind to genomic DNA transactivating target genes expression. This definition was enough to allow Venter and collaborators to estimate the total number of transcription factors to 1500, later refined to a little less than 2000 in the sequenced human genome. In the restrictive context of this canonical definition (DNA binding and target gene transactivation), however, this number of TFs seems small to account for life’s complexity. While the definition is still used to differentiate TFs from other nuclear proteins, new insights have been gathered on their features and functionalities, i.e., TFs do not work alone, but instead they are embedded into complex protein-protein interaction networks involving other transcription factors and nuclear proteins (chromatin remodellers, cofactors). Transcription factors are packed with modular protein-protein interfaces and display intrinsically disordered structural characteristics allowing...
flexibility and interchangeability in elicited protein-protein
interactions.2-10

The study of this complex system is extremely challeng-
ing and any new investigational tool added to the current
toolbox is a worthy improvement.11,12 Many developmental
TFs have their expression resumed in adults under patho-
logical conditions,9,13-15 while not required to maintain cell
phenotypes under normal conditions. One such class of
developmental factors, the SOX (SRY-related HMG-box) TFs,
have emerged as pivotal aetiological components in
cancer-related conditions.16-21 Structural biology studies22-24
and attempts at modulating their activity24-28 have provided
useful insights in their mode of action, paving the way for
the conceptual development of new therapeutics applica-
tions. Study of TFs using antibodies is one attractive option
because of the intrinsic selectivity and affinity of antibodies,
but also for their ability to help elucidate the structure, traf-
ficking and molecular functions of TFs in one single con-
certed approach.19,20 Antibodies are already fundamental
tools heavily used in the study of TFs’ DNA binding activ-
ity, via techniques such as electrophoretic mobility shift
assay (EMSA), ELISA-based DNA-binding assays and
ChIP-seq.31 In a few rare instances, as early as 1994, mono-
clonal antibodies (mAbs) were even used to interfere with
transcription factors activity (DNA binding), to elucidate
their mode of action.32 More recently, some studies have
focused on “the use of antibody fragments as crystalization
chaperones to aid the structural determination of otherwise
‘uncrystallizable’ or ‘undruggable’ target proteins”, such as
TFs.30 Others have focussed on developing antibodies
against TFs for the academic community, using an auto-
mated phage display approach.29 In this study, we used a
phage display library screening to develop an antibody that
was used to explore the molecular mode of action and the
functional protein-protein interaction network of the TF
SOX18, which has been at the center of recent pharma-
modulation attempts with small molecules.27,28

Results

To select antibodies specific to the SOX18 protein, we screened
a human phage display library of antibody scFv using a 109-
amino-acid (aa) peptide of mouse SOX18 HMG domain
(Fig. 1A), centered on a-Helix 3 and its direct surroundings.
Several binders were isolated, and, after sequencing, two unique
binders were identified, F5 and B11 (Suppl. to Fig. 1, panels A
and B). These two binders were expressed as scFvs and also
reformatted into complete human IgG1 antibodies (Suppl.
to Fig. 1, panel C). The binder F5, selected for its higher affinity
than B11 (Suppl. to Fig. 1, panel D), showed no cross reactivity
with the human SOX2 protein, since a counter-screen against
this related SOX transcription factor was performed in parallel
(Fig. 1C). ScFv and full-length antibodies selectively recognized
mouse 109aa SOX18 HMG fragment and full-length human
and mouse SOX18 (Fig. 1B-C, and Suppl. to Fig. 1, panel D).
These results were cross-validated using a surface plasmon
resonance (SPR)-based approach using histidine-tagged mouse
full-length SOX18 immobilized on a nitrilotriacetic acid (NTA)
sensor chip (Fig. 1D). Taken together these data confirm that

SOX18 is a small (44 kDa) yet complex protein, with only
one structurally well-defined functional DNA binding domain,
the HMG-box. When expressed as a separate fragment, the
HMG-box is easy to crystallize and conserves its native ternary
structure and functions. This enabled detailed biochemical
analyses and in-silico crystal molecular dynamics simulations
on SOX18 HMG-box.24,28 Conversely, other SOX18 functional
domains, such as C-terminal transactivation domain(s) were
arbitrarily suggested based on phylogenetic studies, and the
presence of consensus sequences or local enrichments in spe-
cific amino acids.31 The boundaries of these functional domains
are therefore hypothetical, at best ill-defined.

Therefore, to gain a better understanding of the possible
mode of action of the putative blocking antibody, we first
assessed the roles and level of conservation of the three a-hel-
ciral structural components of SOX18 DNA-binding domain
(HMG-box, Figure 1A), using in silico analysis based on variant
databases and conservation across evolution. As shown in the
box plot of Figure 2A, comparing frequency of cancerous
somatic mutation (COSMIC variants) to exome variants (EV
database), somatic mutations of human SOX HMG domains
are strongly linked to cancer, and human HMG domains dis-
play very little “tolerance” for exome variations. Both evidences
point towards the HMG domain’s functional importance
(Fig. 2A, and Suppl. to Fig. 2A). Based on 10 nanoseconds
molecular dynamic simulations performed on HMG models of
20 human SOX, in the presence or absence of DNA, amino
acids displaying significant differences in carbon alpha root-
mean-square deviation (RMSD) of atomic position (A) were
observed in a-Helix 1 and 2 and accounted for two-thirds of all
COSMIC variants identified in human SOX HMG domains
(Fig. 2B, Suppl. to Fig. 2A, and Suppl. to Fig. 2B). While
a-Helix 1 and 2 seem mostly involved in DNA binding and
HMG structural configuration, a-Helix 3’s functional impor-
tance could not clearly be linked to either (Fig. 2B-C, and
Suppl. to Fig. 2C). Further, an open reading frame (ORF)-based
natural selection analysis revealed that a-Helix 3 and its direct
surroundings were by far the most conserved region in verte-
brate SOX18 HMG domains (Fig. 2D, and Suppl. to Fig. 2D,
panels A and B), yet did not interact with DNA (Fig. 2B). This
last observation suggests that a-Helix 3 is likely involved in
protein-protein interactions. In summary, comparison of
human SOX variants and computational analysis of phyloge-
etic, functional and structural features of SOX18 protein sug-
gest that protein-protein interaction might be driven by a
region conserved in the a-Helix 3 or in its vicinity. This in silico
approach enabled us to predict that the a-Helix 3 region of the
SOX18 protein is likely to contain the F5 binding epitope.

To establish experimentally the mechanism by which the
antibody likely interferes with SOX18 protein activity, we first
used a fluorescence polarisation (FP)-based assay able to mea-
sure SOX18 DNA binding activity to a specific oligonucleotide
harbouring a SOX binding site previously reported in the Prox1
gene.34 FP-based binding competition assay demonstrated that
neither antibody nor scFv competed with the binding of HMG
domain to its Prox1 DNA consensus element, instead forming
Figure 1. F5 anti-sox18 MAb selectively recognizes full-length human and mouse SOX18 and mouse 109-aa SOX18 HMG-Box peptide but not SOX2. A. Alignment of 109-aa mouse Sox18 fragment (109mSOX18-HMG) corresponding to residues 69 to 177 of full-length protein with its human counterpart corresponding to residues 75 to 183 of full-length protein. A 93% ClustalW similarity score was measured between the two fragments. B. Lysate of HEK cells overexpressing human SOX18 and a peptide representative of the mouse SOX18 HMG-Box were run on PAGE, transferred to PVDF membrane and probed with F5 mAb and HRP-anti-human kappa secondary antibody. The F5 mAb binds to the hSOX18 protein and the mouse SOX18 HMG-Box peptides (monomer and dimer) as indicated by the arrows. Negative controls with HRP anti-human kappa secondary antibody alone have also been performed. C. Western blot analysis of F5 mAb reactivity to human SOX18 and SOX2 transiently expressed in HEK cell lysates. Samples were resolved on 4–12% NuPAGE Bis-TRIS Gel (ThermoFisher) and subsequently transferred onto PVDF membrane and probed with the F5 mAb followed by detection with the anti-kappa light chain HRP. The F5 mAb binds to the hSOX18 protein as indicated by the arrowhead, but does not react with human SOX2. D. Insert – Dot-blot ELISA of full-length F5 mAb on lysate of naïve Sf9 insect cells or expressing full-length mouse Sox18. Main panel/ Estimation of antibody binding affinity to full-length mouse Sox18 by Surface Plasmon Resonance. Binding affinity of the F5 mAb was estimated by single cycle kinetics on a Biacore T200 (GE, US). 100 nM murine Sox18 engineered with a 6xHis tag was immobilised onto one channel of a nitrilotriacetic acid (NTA) sensor chip activated with 0.5 mM NiSO₄. The sensorgram of binding for five antibody concentrations increasing from 0.1 nM to 11 nM was corrected against two blank runs before curve fitting using a 1:1 surface binding model. Association constant (ka) and dissociation constant (kd) were calculated with a standard error of approximately 1% and then used to determine the affinity constant (K_D). Goodness of fit as measured with Chi2 (mean square of the residual profile) and uniqueness value (U-value; uniqueness of the calculated rate constants and Rmax) indicate reliable data (Chi2 = 0.105, U-value = 2).
a “SOX18:DNA:mAb” ternary complex (Fig. 3A). The difference in plateau height (Fig. 3A) is due to the fact that the mP index value increases with the molecular weight of the final assembly. In one condition, it reached ~215 kDa for SOX18:DNA:mAb (40 kDa SOX18 + 25 kDa DNA + 150 kDa IgG), but only 90 kDa for SOX18:DNA:scFv (40 kDa SOX18 + 25 kDa DNA + 25 kDa scFv). Here, we took advantage of reformatting the scFv into IgG to show that a specific increase of the mP index value correlates with a change in molecular weight. Further, these concentration-dependent data fitted very well to a Hill slope monovalent (1:1) model (R² > 0.9), returning dissociation constants in the low- to mid-nanoMolar range (Suppl. to Fig. 3, panel B). This finding suggests that while SOX18-HMG domain binds to the DNA via α-Helix 1 and 2, it may remain capable of eliciting protein-protein interaction in a DNA-bound state via its α-Helix 3 region, as predicted by our in-silico analysis.

To further investigate the effect of the F5 scFv antibody on SOX18 protein partner recruitment, we next took advantage of an ALPHAScreen-based technology. This approach is founded on the analysis of pairwise interaction in a cell-free protein expression system using a human proteome library. Protein-protein interaction (PPI) measurement of full-length SOX18 with itself and various protein binding partners showed that the scFv antibody was able to selectively disrupt SOX18 homodimerization, but none of SOX18 heterodimerizations with known protein partners such as MEF2C, XRCC5, RBPJ or SOX1727,28 (Fig. 3B). The homodimerization of SOX18, a well-characterized dimer in the SOX family, was not affected either, suggesting different interaction modalities for SOX9 and SOX18 homodimers (Fig. 3B). The scFv antibody failed, however, to recognize a 31-aa peptide corresponding to just the α-Helix 3 of the HMG box (Suppl. to Fig. 3, panel C). This could be due to the weak helicity of α-Helix 3 peptide when removed from the remainder of the HMG structure, but even in that case, some “weak” recognition would still be expected (Suppl. to Fig. 3, panel C). Altogether, these results suggest that the antibody and scFv, which were generated from a 109-aa
As previously described.38,39 The assay for disruption of protein-protein interaction (IC50) was conducted by expressing the protein pairs in

Figure 3. F5 scFv and mAb binding does not compete with DNA and selectively disrupts SOX18 homodimerization. A. Fluorescence polarization-based measurement of F5 mAb concentration-dependent binding with 109-aa Sox18 fragment used for phage library affinity screening, denotes the formation of a ternary “DNA-

Figure 4. scFv F5 mAb expressed in situ in fibroblastic cells inhibits Sox18-mediated luciferase expression. Luciferase reporter assay performed in fibroblast cells (COS-7) transiently transfected with SOX18 and a vector containing 1889 bp of the proximal Vcam1 promoter construct fused to the firefly luciferase reporter gene. Cells were transfected for 7 hours with aforementioned vectors along with an empty pcDNA 3.1 vector or the same vector containing the ORF of C-terminal myc-tagged scFv F5 mAb. Following an 18 hour-recovery and expression period, Sox18-mediated luciferase activity was measured as depicted in bar graph. Data are corresponding to three independent experiments with 6 internal replicates, error bars are SD of the mean.

peptide of mouse SOX18 HMG domain, most likely interact with the C-terminal region directly adjacent to SOX18 HMG domain α-Helix 3 (Suppl. to Fig. 3, panel A).24

To pinpoint the exact binding location of the scFv, we next performed an epitope mapping approach using the mouse SOX18 109-aa fragment used for the initial biopanning of the human scFv phage library. We used various truncated versions of the same 6HIS-MBP-tagged 109-aa construct, concentrating on α-Helix 3 and directly adjacent C-terminal region (Suppl. to Fig. 3, panel D). This approach confirmed that the antibody and scFv antibody fragment interact with an 8-aa motif, directly adjacent to SOX18 HMG domain (Fig. 3C, Suppl. to Fig. 3, panels E-G).

The fact that F5 antibody disrupts SOX18 homodimer but not SOX18 heterodimer formation suggests that this 8-aa motif is likely to be involved in protein self-recruitment. To gain a better understanding of the difference between homo- and hetero-dimer formation, a SOX18:RBPJ complex was investigated by in silico docking using ClusPro (Fig. 3D). This analysis takes advantage of the SOX18/DNA:Notch-RBPJ transcription complex generated from SOX18/DNA crystal,24 and Notch-RBPJ transcription complex crystal.23 In this model, the epitope sequence binding to the scFv is located outside the SOX18: RBPJ interface, exposed to the solvent. This spatial configuration might enable the SOX18/RBPJ complex to recruit the antibody without interfering with its own heterodimer interface, mainly involving α-Helix 3. This in silico analysis further defines a hot spot interface that could be targeted to disrupt a specific PPI, essential to mediate the activity of this transcriptional complex (Fig. 3B-D).

We previously demonstrated using small molecule inhibitors, that modulation of SOX18 protein-protein interactions disrupts its transcriptional activity.27,28 Here, we tested whether the scFv antibody co-expressed with full-length SOX18 and a
Discussion

Here, we report the discovery and characterization of a new biologic that proves to be useful for deciphering the molecular mode of action of a transcriptional regulator, the SOX18 transcription factor. The novel human antibody recognizes a highly conserved 8-aa motif directly positioned on the C-terminal extremity of the HMG-Box of the SOX18 protein. This antibody displays selective disruption of SOX18 self-assembly and inhibits SOX18-mediated transcriptional activation in cells. As expected, the reformating into a complete human IgG1 antibody (Suppl. to Fig. 1, panel C) substantially improved affinity as measured by the dissociation constant (Fig. 1D, Suppl. to Fig. 3, panel B). However, the overall effect of reformating on SOX18-blocking efficacy remains to be evaluated in vitro and in vivo. If the F5 mAb affinity level can be preserved during affinity maturation, its efficacy in blocking SOX18 homodimerization could be further improved by decreasing its concentration-independent off rate, currently estimated at $10^{-3}$ s$^{-1}$ (Fig. 1D). Despite dissociation constants in the low to mid nanoMolar range, denoting strong affinity from full-length antibody or scFv for their epitope, neither were able to competitively displace the HMG-box from its DNA binding site (Fig. 3A, and Suppl. to Fig. 3, panel B). These data combined with our results obtained on protein partner recruitment using ALPHA screen assay (Fig. 3B) strongly suggest that the mode of action of this antibody is via disruption of SOX18’s protein-protein interaction. The possibility that the epitope was located on $\alpha$-Helix 1 or 2 was rapidly dismissed, as these two helices are largely involved in protein-DNA interaction (Suppl. to Fig. 3, panel A, red crosses indicate regions involved in protein-DNA binding), which would not fit with observed non-competitive binding. One potential position for the F5 epitope was $\alpha$-Helix 3, involved only in a limited manner in protein-DNA interaction (Suppl. to Fig. 3, panel A), and already described in literature as engaged in protein-protein interactions. However, a $\alpha$-Helix 3 peptide was not recognized by the scFv F5 antibody (Suppl. to Fig. 3, panel C). This pointed towards an epitope located in the N-terminal region outside the SOX18 HMG-box or in the C-terminal region adjacent to the HMG-box, near $\alpha$-Helix 3. On the 109-aa peptide, the N-terminal region adjacent to the HMG domain consists of only 9 amino acids, compared to the 28-aa C-terminal region. In addition, SOX9 homodimer is not disrupted by the scFv F5 antibody (Fig. 3B), and a SOX9 dimerization domain has been identified on the N-terminal side of SOX9 HMG. Taken together, this evidence prompted us to prioritize epitope mapping on the C-terminal region of SOX18-HMG.

Across all SOX proteins, the HMG-box shares at least 46% of sequence homology. Just outside of this domain, however, SOX homology diverges due to decreased selection pressure. In this context, the antibody did indeed recognize both human and mouse SOX18 (Fig. 1B-D), which share 93% homology in this region (Calculated ClustalW similarity score, Fig. 1A), while it did not recognize divergent human SOX2. Interestingly, codon sequence conservation analysis showed that the 8-aa epitope coincides with a highly conserved region (Fig. 2D, black arrow), suggesting an important role in the binding of some SOX18 protein partner.

The protein SOX18 is a key molecular switch driving angio- and lymphangio-genesis during development, and a molecular target in various pathophysiological conditions. We hereby demonstrated that it can be pharmacologically modulated using an antibody that inhibits its transcriptional activity by disrupting its ability to recruit other proteins (in this case homodimerization) required to activate gene trans-activation. TF protein-protein interactions do not occur exclusively between transcription factors and cofactors in the nucleus. In 2010, Malki et al. identified nucleocytoplasmic shuttling canonical sequences in SOX protein sequences, especially in and around the HMG domain. This study identified an Importin-β binding motif, which precisely overlaps the 8 aa epitope reported in this study. It is therefore possible that blockade of SOX18 activity occurs either via interference with protein nuclear trafficking or co-factor recruitment. Further evidence of the sub-cellular localization of the scFv antibody will help to pinpoint the exact mode of action. The control of SOX proteins access to their target genes is potentially a powerful approach to modulate specific genetic programs. Further, the disruption of specific transcriptional complex is an alternative methodology that would allow subtle targeting of gene subsets. Both avenues open new attractive molecular strategies to study TF mechanisms.

Material and methods

In silico analysis of SOX18 natural selection and human SOX variants

Codon selection analysis of SOX18

For each codon, estimates of the number of inferred synonymous (s) and nonsynonymous (n) substitutions are presented along with the number of sites that are estimated to be synonymous (S) and non-synonymous (N). These estimates are produced using the joint Maximum Likelihood reconstructions of ancestral states under a Muse-Gaut model of codon substitution and Tamura-Nei model of nucleotide substitution. Maximum Likelihood computations of dN and dS were conducted using HyPhy software package. The analysis involved 102 nucleotide sequences with a total of 384 positions in the final dataset using MEGAS.
**Analysis of genetic variants in 20 human SOX proteins**

Amino acid variant saturation was compared in the HMG-box domain of 20 human SOX proteins between COSMIC database and EVS database (Exome Variant Server, NHLBI GO Exome Sequencing Project (ESP), http://evs.gs.washington.edu/EVS/), relative to the amino acid percentage of the HMG-box for SOX proteins. A significant difference of \( P < 0.05 \) using repeated measures ANOVA followed by post-hoc Wilcoxon signed rank test or Bonferroni corrected pairwise \( t \)-test was determined for the COSMIC dataset to EVS and for the COSMIC dataset to the percentage of HMG box in total protein. A table of the sequence alignment of the human SOX proteins across groups A-H, and the variant details for COSMIC and EVS can be found in material supplemental to Fig. 2A.

**Molecular dynamic simulation across 20 human SOX HMG models**

Molecular dynamic simulations were performed for 10 nanoseconds (ns) on all 20 human SOX HMG protein models with or without DNA. Difference in movement (root-mean squared fluctuation, RMSF) was analysed for each amino acid in presence or absence of DNA across all 20 models. Amino acids displaying significant difference between simulations with DNA and without DNA were identified (\( t \)-test, \( P < 0.01 \)).

**SOX18-RBPJ in silico docking**

The protein-protein docking between Notch1 transcription complex and SOX18 was performed with ClusPro online server version (cluspro.bu.edu), using pdb: 3V79 and pdb: 4Y60 for the structures of Notch1 transcription complex and SOX18-HMG, respectively. DNA molecules were removed before docking, as ClusPro is unable to process them, and restored after docking. Docking solutions clashing with DNA molecules were rejected. The model was further refined by rejecting docking solutions clashing with ScFv epitope location considering its inability to inhibit SOX18-RBPJ interaction. The resulting top docking pose was used as starting conformation in a 50 ns MD simulation to optimize the docking pose, and validate the stability of the new multi protein complex.

**SOX18 fragments preparation**

**SOX18 peptide fragment preparation for phage library panning**

The 109-aa mouse SOX18 HMG fragment used for phage library panning was BP cloned from cDNA templates (IMAGE cDNA clone IDs, Sox18: 3967084) into a pDONRTM221 pENTRY vector, sequenced and recombined into a pETG20A or a pDEST-HisMBP gateway vector for expression with an N-terminal His-MBP fusion partner. The His-MBP-Sox18-109 and His-MBP-Sox18-79 gene constructs were transformed into BL21 DE3 Star chemically competent cells (Invitrogen, ThermoFisher Scientific) using standard heat shock protocol and plated on LB-Agar with 100 \( \mu \)g/ml Ampicillin. Colonies were inoculated into 3 ml LB medium with 100 \( \mu \)g/ml ampicillin and grown for 2–4 hrs until \( OD_{600} \) of 0.4–0.6 was obtained. Fusion protein expression was induced by the addition of IPTG to the cultures to a final concentration of 1 mM and cultures were induced at 20°C for 20hrs. IPTG-induced cultures for His-MBP-Sox18-79 and His-MBP-Sox18-109 were mixed with PAGE loading dye with reducing agent, heated at 95°C for 5 mins and samples run on 4–12% NuPAGE Bis-Tris PAGE (Invitrogen, ThermoFisher Scientific). Proteins were transferred to PVDF membranes, the membranes washed with 0.05% Tween-20 in 1x phosphate-buffered saline (PBST) and blocked in 2% (w/v) skim milk in PBST for 30 mins at room temperature (RT). The membranes were probed for 60 mins at RT with the anti-Sox18 MAb F5 (50 \( \mu \)g/ml in block solution) to map its Sox18 binding site. The membranes were then washed 3xPBST/5 mins and probed for 60 mins at RT with horseradish peroxidase (HRP)-labelled anti-human kappa antibody (The Binding Site, Ltd, Birmingham, UK, Cat# AP015) diluted 1/1000 in blocking solution. The proteins were probed on separate PVDF membranes with HRP anti-His (Miltenyi Biotech AG, Germany, Cat# 130-092-785) diluted 1/2000 in block solution to confirm fusion protein expression. The membranes were washed 3xPBST/5 mins and the proteins detected by chemiluminescence with ECL substrate (Invitrogen, ThermoFisher Scientific). The binding of F5 MAb to human SOX18 and human SOX2 expressed in HEK lysates (Origene) was also determined by immunoblot with HEK lysate alone as a control.

**SOX18-109 truncations and deletion for F5 mAb epitope mapping**

Site Directed Mutagenesis PCR (SDM-PCR) was used to generate variants of Sox18-109 to determine the epitope recognized by the anti-sox18 MAb F5. For initial epitope mapping studies, SDM-PCR with specific oligonucleotides was used to introduce stop codons at specific sites in the gene encoding Sox18-109 to create C-terminal truncation variants. This included Sox18 truncated at residues 20, 56, 63 and 72. A variant with a deletion of residues 80–87 “YRPRRKQ” was also generated for the epitope mapping of F5 mAb. PCR products representing the variants of His-MBP-Sox18-109 were gel-purified, treated with Dpn1 (New England Biolabs, Inc.) for 3 hrs at 37°C and was transformed into BL21 DE3 Star chemically competent cells (Invitrogen, ThermoFisher Scientific). The fusion protein expression and immunoblots were performed similarly to His-MBP-Sox18-109 and His-MBP-Sox18-79 as already outlined.

**Phage library panning**

A human naive scFv antibody library in phagemid vector pHEN1, with a reported diversity of \( 6.7 \times 10^{9} \), was kindly provided by Prof. James Marks (University of California, San Francisco, USA). Biopanning was conducted against the mSOX18 109-aa fragment for three rounds based on the standard method. In brief, immunotubes (Nunc Maxisorp,
ThermoFisher Scientific) were coated overnight with 1 mL of truncated mSox18 in PBS at 10 μg/mL, followed by a series of washes with PBS. The tubes were subsequently blocked with 2% skim milk PBS for 1 hour at room temperature. In parallel, 10^13 phage particles from the naive scFv antibody library were blocked in 2% skim milk PBS for one hour and subsequently transferred to the immunotubes and incubated for a further 1 hour. Unbound phage was removed by washing the tubes three times with 0.1% PBS-T and three washes with PBS alone. Bound phage was eluted with 200 mM glycine pH 2.5 and neutralized with the addition of 1 M Tris-HCl pH 7.4. The eluate was then used to infect log phase XL1-blue cells grown in 2YT medium supplemented with 3 ug/mL tetracycline for 30 minutes. The cells were centrifuged, resuspended in 2x YT media and spread onto 2YT agar plates supplemented with 100 μg/mL ampicillin and 2% glucose (2YT-AmpGlu). Plates were incubated at 30°C overnight. Cells were detached from the plate with 2YT AmpGlu media and 20% glycerol and stored at −80°C.

Phage particles were rescued from the E. coli glycerol stocks and grown to log phase at 37°C in 2YT-AmpGlu followed by an addition of 10^11 M13KO7 helper phage particles and incubated for 30 minutes at 30°C. The cells were centrifuged, resuspended in 2YT supplemented with 100 μg/mL ampicillin and 30 μg/mL kanamycin (2YT-AmpKana) and incubated overnight at 30°C. Phage particles were recovered from the culture supernatant by two rounds of precipitation with 20% PEG, pH 5.6, followed by a series of washes with 0.1% PBST and three washes with PBS alone. Bound phage was eluted with 200 mM glycine pH 2.5 and neutralized with the addition of 1 M Tris-HCl pH 7.4. The eluate was then used to infect log phase XL1-blue cells grown in 2YT medium supplemented with 3 ug/mL tetracycline for 30 minutes. The cells were centrifuged, resuspended in 2x YT media and spread onto 2YT agar plates supplemented with 100 μg/mL ampicillin and 2% glucose (2YT-AmpGlu). Plates were incubated at 30°C overnight. Cells were detached from the plate with 2YT AmpGlu media and 20% glycerol and stored at −80°C.

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Analyses of individual clones were done by randomly selecting individual phage infected E. coli colonies and rescuing the phage as previously described, with the omission of the precipitation step. The supernatants containing the secreted phage particles were then assessed for reactivity to the mSO18 109 aa fragment through standard ELISA as described above. The scFv DNA sequence was then determined from the positive clones by Sanger sequencing at the Australian Genome Research Facility, using pHEN1-specific primers flanking the scFv sequence.53

Production of scFvs
DNA from two clones (B11 and F5) selected from the Sheets library panning was recovered by (miniprep) and used as templates in PCR with primers to incorporate a C-terminal cysteine residue, a 6xHis tag and HindIII and XbaI sites. PCR products were subsequently cloned into pCDNA3.1 (+) (Life Technologies, Inc., CA, USA). ScFvs were produced in CHO-S by incubating the plasmid DNA with PEI Max in OptiPro media. The DNA-PEI complex was subsequently added to the CHO-S cells at 3.0 × 10^6 cell/mL and incubated for 4 hours at 37°C. Efficient Feed A and Feed B were added at a final concentration of 7.5%, and anti-clumping agent at 0.4% followed by a further incubation at 32°C for 10 days. Cells were removed by centrifugation and His-tagged scFv protein was purified from the culture supernatant by His Trap FF affinity chromatography, eluting the product with 350 mmol L^-1 imidazole in phosphate buffer, pH 7.4. This was followed by a buffer exchange into PBS pH 7.4, using a GE Hi Prep 26/10 desalting column. The final product was filtered through 0.22 μm membrane and stored at −20°C.

Reformatting of IgG molecules
The two selected clones (B11 and F5) were reformatted to complete human IgG1 as previously described.55 Briefly the heavy and light chain variable fragments were individually amplified from the phagemid DNA, using primers specific to the variable regions and containing 15 bp overhangs at the 5’ ends to allow ligation-independent cloning into heavy and light chain Refo- mAB mammalian expression vectors (Acythe Biotech, Brisbane Australia) using the “In Fusion” system™ (Clontech, Inc, CA, USA). The heavy and light chain plasmids for each antibody were co-transfected, at 1:1 ratio, into CHO-S cells using PEI- Max as described above. Cells were removed by centrifugation and IgG was purified from the culture supernatant by protein A HiTrap affinity chromatography, eluting with 0.1 M glycine pH 3.0 followed by buffer exchange into PBS pH 7.4, using a GE Hi Prep 26/10 desalting column and the final product was filtered through 0.22 μm membrane and stored at −20°C.

Homogeneous assays
Surface plasmon resonance
A single cycle kinetic and affinity study of full-length mAb F5 dissociation from a murine His-StrepII-TEV-SOX18 (N-term) immobilized onto a nitroloacetic acid (NTA) sensor chip was performed using a Biacore T200 instrument (Biacore Life Sciences, GE Healthcare, Uppsala, Sweden). NTA sensor chip was activated with 0.5 mM NiSO₄ and immobilization was performed with a 100 nM Sox18 solution. The sensorgram of association and dissociation of antibody F5 consisted in a series of five analyte concentrations ranging from 0.1 to 11 nM. Data were corrected against two blank runs before curve fitting using a 1:1 surface binding model. Association constant (kₐ) and dissociation constant (k₈) were calculated using BIAevaluation software (Biacore Life Sciences, GE Healthcare, Uppsala, Sweden), with a standard error of 1% to determine the affinity constant (K₅).

Fluorescence polarization-based DNA binding assay
Fluorescence polarization (FP) was used as a homogenous in vitro assay to assess protein / DNA interaction using light polarization as a readout.56 We used an E coli recombinantly expressed 109-aa peptide, corresponding to the HM domain of mouse SOX18 and a 40bp-long double-stranded oligonucleotides harboring SOX responsive elements and labeled with 5’ fluorescein amidite (FAM) label (GeneWorks). The FP assay was run in 384-well black plates (BD Biosciences, USA) in a 25–30 μL final volume using 1FP assay buffer (30 mM HEPES pH 7.5, 100 mM KCl, 40 mM NaCl, 10 mM NH₄OAc, 10 mM guanidinium, 2 mM MgCl₂, 0.5 mM EDTA, 0.01% NP-
40). All data are displayed in milliP (mP) fluorescence polarization index (mP index).

Briefly, 100 nM mSOX18-HMG was preincubated in 384-well plates for 10–15 minutes with full-length antibody or corresponding scFv at concentrations ranging from 10 to 1000 nM. Labeled DNA probe was then added at 5 nM, and the mix briskly agitated for 5 minutes at room temperature protected from ambient light. 384-well plates were sealed (TopSeal™-A, PerkinElmer, USA) and following a further 30-minute incubation at room temperature, fluorescence polarization was read on a Tecan M1000 Pro (λexc = 480 nm, λem = 535 nm). All experiments were performed in internal as well as independent triplicate. Data analysis and plotting were performed with Prism 6 for Mac OS X (version 6.0d, GraphPad Software, Inc., CA, USA) using a basic one site specific binding model;

\[
mP = B_{max} \times \frac{[\text{antibody} \ \text{conc}]}{(Kd + [\text{antibody} \ \text{conc}])}.
\]

**ALPHAScreen based protein-protein interaction assay**

**Plasmid preparation and cell-free expression**

Transcription factor proteins were genetically encoded with enhanced green fluorescent protein (GFP), mCherry and cMyc (myc) tags, and cloned into cell-free expression Gateway destination vectors: N-terminal GFP tagged (pCellFree_G03), N-terminal Cherry-cMyc (pCellFree_G07) and C-terminal Cherry-cMyc tagged (pCellFree_G08). Human RBPJ (BC020780) and RBPJ Recombining binding protein suppressor of hairless (RBPJ) (BC020780) and MEF2C (BC026341) ORFs were sourced from the Human ORFeome collection, version 1.1 and 5.1, and the Human Orfeome collaboration OCAA collection (Open Biosystems), as previously described and cloned at the ARVEC facility, UQ Diamantina Institute. The entry clones pDONOR223 or pENTR201 vectors were exchanged with the ccdB gene in the expression plasmid by LR recombination (Life Technologies, Australia). The full-length human SOX18 gene was synthesized and the transfer to vectors was realized using Gateway PCR cloning. Translation competent *Leishmania tarentolae* extract (LTE) was prepared as previously described. Protein pairs were co-expressed by adding 30 nM of GFP template plasmid and 60 nM of Cherry template plasmid to LTE and incubating for 3 hours at 27 °C.

**ALPHAScreen**

ALPHAScreen was performed as previously described, using the cMyc detection kit and Proxiplate-384 Plus plates (PerkinElmer). The LTE lysate co-expressing the proteins of interest was diluted in buffer A (25 mM HEPES, 50 mM NaCl). For the assay, 12.5 μL (0.4 μg) of anti-cMyc coated Acceptor Beads in buffer B (25 mM HEPES, 50 mM NaCl, 0.001% NP40, 0.001% casein) were aliquoted into each well. This was followed by the addition of 2 μL of diluted sample, at different concentration, and 2 μL of biotin-labelled GFP-Nanotrap in buffer A. The plates were incubated for 45 min at room temperature, before adding 2 μL (0.4 μg) of streptavidin-coated Donor Beads diluted in buffer A, and incubation in the dark for 45 min at room temperature. The ALPHAScreen signal was measured on an Envision Plate Reader (PerkinElmer), using manufacturer’s recommended settings (λexc = 680/30 nm for 0.18 s, λem = 570/100 nm after 37 ms). The resulting bell-shaped curve is an indication of a positive interaction, while a flat line reflects a lack of interaction between the proteins. The measurement of each protein pair was repeated in triplicate.

The Binding Index was calculated as: \( BI = \frac{I - I_{neg}}{I_{ref} - I_{neg}} \times 100. \) 

\( I \) is the highest signal level (top of the hook effect curve) and \( I_{neg} \) is the lowest (background) signal level. The signals were normalized to the \( I_{ref} \) signal obtained for the strongest interaction. The assay for disruption of protein-protein interaction (IC50) was conducted by expressing the protein pairs in LTE and incubating with a dilution range of tested scFv antibody (0.01 to 7.5 μM) in buffer B for 1h. Percentage of interaction was calculated as: \( \frac{I_{ref}}{I_{max}} \times 100. \) Data from 3 independent experiments were fitted in GraphPad Prism version 6.0 using 3-parameter non-linear regression.

**Cell-based assay**

Monkey kidney fibroblast-like cells (COS-7) were cultured at 37 °C, 5% CO2 in DMEM (Life technologies, 11995) with fetal bovine serum (FBS), sodium pyruvate, L-glutamine, penicillin, streptomycin, non-essential amino acids and HEPES. Cells were grown in 96-well plates to 80% confluence, and transfected with mouse plasmids pGL2 Vcam-1 promoter construct (VC1889), pSG5 Sox18, and either an empty pcDNA 3.1 vector or containing the ORF of scFv F5. Transfection was performed using X-tremeGENE 9 DNA transfection reagent (Roche, 06356787001). After 7 h transfection, cells could recover in 0.5% FBS medium for another 18 h, before lysis and luciferase assay (Perkin Elmer, 6016711).

**Abbreviations**

| Abbreviation | Description |
|--------------|-------------|
| aa           | amino-acid  |
| FOX          | Forkhead box|
| HOX          | Homeo-box   |
| RBPJ         | Recombining binding protein suppressor of hairless |
| scFv         | single-chain variable fragment |
| SOX          | Sry-related HMG-box |
| TFs          | Transcription Factors |

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