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# 1 **Structural characterization of stem cell factors Oct4, Sox2, Nanog and** 2 **Esrrb disordered domains, and a method to identify their phospho-** 3 **dependent binding partners.**

4

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18

19 **Keywords:** Pluripotency Transcription Factors, Intrinsically Disordered Proteins, Post-  
20 Translational Modifications, kinases, NMR, Proteomics, Quantitative Mass-Spectrometry

21

## 22 **Abstract**

23 The combined expression of a handful of pluripotency transcription factors (PluriTFs) in  
24 somatic cells can generate induced pluripotent stem cells (iPSCs). Here, we report the  
25 structural characterization of disordered regions contained in four important PluriTFs, namely  
26 Oct4, Sox2, Nanog and Esrrb. Moreover, many post-translational modifications (PTMs) have  
27 been detected on PluriTFs, whose roles are not yet characterized. To help in their study, we  
28 also present a method i) to produce well-characterized phosphorylation states of PluriTFs,  
29 using NMR analysis, and ii) to use them for pull-downs in stem cell extracts analyzed by  
30 quantitative proteomics to identify of Sox2 binders.

31

## 32 **1. Introduction**

33 The possibility of reprogramming somatic cells to an induced pluripotency state was revealed  
34 in the 2000s, carrying great expectations in the fields of Biology and Medicine [1–3]. Induced  
35 pluripotent stem cells (iPSCs) and embryonic stem cells (ESCs) are characterized by the  
36 active state of a pluripotency network, whose core comprises the pluripotency transcription

1 factors (PluriTFs) Oct4, Sox2, Nanog and Esrrb (OSNE). These bind to enhancer sequences,  
2 and thus activate or repress, or even “bookmark” during mitosis, a wealth of genes related to  
3 pluripotency or cell differentiation [4–9]. Consistently, their misregulation correlates with  
4 cancer malignancy and stemness [10–14].

5 Comprehensive structural descriptions of OSNE are missing to our knowledge. The folded  
6 DNA-binding domains (DBDs) of OSNE have been structurally characterized, in complex  
7 with their DNA target sequences [15–19], together with the ligand-binding domain (LBD) of  
8 Esrrb [20]. Recent studies depicted even the splendid structures of Oct4 and Sox2 DBDs  
9 binding to nucleosomes, hence deciphering their “pioneer factor” abilities [21–29]. Another  
10 structure of Sox2 bound to the importin Imp $\alpha$ 3 has also been published, showing how its two  
11 Nuclear Localization Sequences (NLSs) flanking the DBD are involved in Sox2 nuclear  
12 import [30]. The other segments of OSNE have been predicted to be intrinsically disordered  
13 regions of proteins (IDRs) [31], i.e. they should have no stable tertiary fold when isolated  
14 [32–36].

15 These IDRs appear to have important roles in binding partners involved in epigenetic  
16 reprogramming, chromatin reorganization and in recruiting transcription or repression  
17 machineries [5,37–41]. These functions are poorly understood, and, to the best of our  
18 knowledge, no experimental characterization of the structural behavior of these regions in N-  
19 and C-terminal of DBDs has been released yet. Recent studies have shown that C-terminal  
20 regions of Oct4 and Sox2 are important for their reprogramming capacities [42,43], notably  
21 by contributing to the engagement in molecular phase-separated condensates with the  
22 Mediator complex [44]. More generally, the activating or repressive activities of IDRs of  
23 transcription factors (TFs) have been scarcely studied at the structural level: these segments  
24 are thought to contain hydrophobic patches flanked by acidic amino acids, which favors  
25 DNA-binding specificity, phase separation and low-specificity interactions, notably with the

1 Mediator subunit Med15 [44–53]; more specific interactions have been described in some  
2 cases [45,54,55].  
3 Post-translational modifications (PTMs) add a layer of complexity, by being often responsible  
4 of the regulation of IDRs' interactions [32,33,56,57], and notably of TFs activity [58–61].  
5 Post-translational modifications (PTMs) are classical carriers of cell signaling by regulating  
6 the stability and the interactions of proteins. An increasing number of PTMs have been  
7 described on OSNE IDRs in the recent years [5,37,38,62–72] [73–77], notably  
8 phosphorylation by Cyclin-dependent kinases (CDKs) [66,78–87] or by Mitogen-activated  
9 protein kinases (MAPKs) [83,88,89], or their complementary Ser/Thr O-GlcNAcylation by  
10 OGT [65,90–96]. In order to prompt future studies on this topic, we thought to establish and  
11 test an experimental strategy i) for producing well-characterized samples of post-  
12 translationally modified IDRs of PluriTFs and ii) to use these as baits in pull-down assays for  
13 identifying PTMs' related binding partners.  
14 Hence, we characterized some of the phosphorylation reactions of Esrrb and Sox2 by p38 $\alpha/\beta$ ,  
15 Erk2 and Cdk1/2. Then, we showed that biotinylated chimera of Sox2 and Esrrb coupled to an  
16 AviTag peptide could be attached to streptavidin-coated beads. Finally, we loaded truncated  
17 segments of the C-terminal IDR of Sox2 (phosphorylated or not) on these beads, and exposed  
18 them to extracts of mouse Embryonic Stem Cells (mESCs) in pull-down assays, which we  
19 analyzed using quantitative mass spectrometry-based proteomics. Among the quantified  
20 (phospho)-Sox2 binders, we verified the phospho-dependent interaction between the proline  
21 cis-trans isomerase Pin1 and Sox2 using NMR spectroscopy.

22

## 23 **2. Material and methods**

### 24 *2.1. Production of recombinant fragments of Oct4, Sox2, Nanog and Esrrb*

1 We used human protein sequences, unless specified. Codon-optimized (for expressing in  
2 *Escherichia coli*) genes coding for human Oct4(aa1-145) and Oct4(aa286-380) were  
3 synthesized in the context of larger genes coding for Tev-Oct4(aa1-145)-Tev-GB1 and Tev-  
4 Oct4(aa286-380)-Tev-GB1 by Genscript and cloned into pET-41a(+) vector between SacII  
5 and HindIII restriction sites, hence permitting the expression of GST-His6-Tev1-Oct4(aa1-  
6 145)-Tev2-GB1 and GST-His6-Tev1-Oct4(aa286-380)-Tev2-GB1; Tev1 and Tev2 are the  
7 heptapeptide ENLYFQG cleavage site of the TEV protease, Tev2 is separated by GAGGAGG  
8 from GB1 (T2Q variant of the immunoglobulin binding domain B1 of the protein G from  
9 group G *Streptococcus* [97,98]). The C-terminal GB1 tag was added to avoid any C-terminal  
10 proteolysis of the IDR of interest during the expression and the first purification steps; we did  
11 not test constructs without this supplementary folded domain, whose necessity for the stability  
12 of the IDR is thus not proven.

13 The same rationale (cDNA synthesis, cloning, vectors, chimera constructs) was used for  
14 producing Nanog(aa154-305), Nanog(aa154-215), Nanog(aa154-272), Nanog(aa154-305\_  
15 C185A-C227A-C243A-C251A), Nanog(aa154-272\_C185A-C227A-C243A-C251A), and a  
16 very similar rationale (chimera constructs missing the C-terminal Tev2-GB1) for Sox2(aa1-  
17 42), Sox2(aa115-317\_C265A), Sox2(aa115-187), Sox2(aa115-236), Sox2(aa115-  
18 282\_C265A), Esrrb(aa1-102\_C12A-C72A-C91A), Esrrb(aa1-102\_C12A-C91A), Nanog(aa1-  
19 85) (this latter was cloned in the MfeI/HindIII restriction sites from pET-41a(+)).

20 The recombinant production and the purification of the protein constructs followed the  
21 procedures described previously [99], using the soluble fraction of bacterial lysates, except for  
22 the constructs containing the Sox2 C-terminal fragments. These latter constructs were  
23 recovered from the insoluble fractions of the lysates, and resolubilized in 8 M urea; these  
24 were submitted to a His-Trap purification in urea, and the last size-exclusion chromatography  
25 (SEC) had to be carried out in 2 M urea, which avoided clogging of the column and permitted

1 to obtain regular elution peak widths (these were otherwise extremely broad, up to 100 mL for  
2 the longest Sox2(aa115-317\_C265A) construct). The samples were concentrated and stored at  
3 -20 °C, and thawed just before the NMR experiments. The Sox2 samples containing 2 M urea  
4 were submitted to 2-3 cycles of concentration/dilution in Hepes at 20 mM, NaCl at 75 mM to  
5 generate samples in urea at 0.25 or 0.125 M.

6 All purification steps were carefully carried out at 4 °C; protein eluates from every  
7 purification step were immediately supplemented with protease inhibitors (EDTA-free  
8 cOmplete, Roche) (together with DTT at 10 mM for cysteine-containing protein constructs),  
9 before being submitted to a concentration preparing the next purification step.

10 Chimera constructs of Sox2 and Esrrb IDR fragments containing a 15-mer peptide AviTag  
11 GLNDIFEAQKIEWHE were produced using procedures similar to those described earlier for  
12 OSNE constructs. The construct Sox2(aa234-317)-AviTag-His6 was soluble, and did not  
13 require to be purified in urea.

14 More details about the production of OSNE peptides are given in the Supplementary Material.

## 16 *2.2. Production of the biotin ligase BirA and specific biotinylation of the AviTag-peptide* 17 *chimera*

18 The biotin ligase BirA was produced using recombinant production in *E. coli* BL21(DE3)Star  
19 transformed with a pET21-a(+) plasmid containing a gene coding for BirA cloned at EcoRI  
20 and HindIII restriction sites. pET21a-BirA was a gift from Alice Ting (Addgene plasmid #  
21 20857)[100]. The expression was carried out over night at 20 °C in a Luria-Bertani culture  
22 medium. The construct was containing a His6 tag in C-terminal and was purified using a two-  
23 step purification procedure including a His-trap followed by a SEC. Details about the  
24 production of BirA are given in the Supplementary Material.

1 The biotinylation was executed using a rationale inspired from a published protocol [101], at  
2 room temperature during 90 minutes, in samples containing the AviTag-chimera of interest at  
3 100  $\mu$ M and BirA at 0.7  $\mu$ M in a buffer containing ATP at 2 mM, biotin at 600  $\mu$ M, MgCl<sub>2</sub> at  
4 5 mM, DTT at 1 mM, HEPES at 50 mM, NaCl at 150 mM, protease inhibitors (EDTA-free  
5 cOmplete, Roche), at pH 7.0. To remove some eventual proteolyzed peptides and BirA, the  
6 biotinylated constructs were purified using a SEC in a column (Superdex 16/60 75 pg, Cytiva)  
7 preequilibrated with a buffer containing phosphate at 20 mM, NaCl at 150 mM at pH=7.4  
8 (buffer called thereafter Phosphate Buffer Saline, PBS). The eluted fractions of interest were  
9 concentrated and stored at -20 °C.

10

### 11 *2.3. Assignment of NMR signals from OSNE fragments, and structural propensities*

12 The assignment strategy was the same than in previous reports from our laboratory [99]. The  
13 <sup>15</sup>N relaxation data were recorded and analyzed according to the methods described in  
14 previous reports [102]. Details are given in the Supplementary Material.

15 Disorder prediction were calculated using the ODINPred website ([https://st-](https://st-protein.chem.au.dk/odinpred)  
16 [protein.chem.au.dk/odinpred](https://st-protein.chem.au.dk/odinpred)) [103]. Experimental secondary structure propensities of  
17 unmodified OSNE peptides were obtained using the neighbor-corrected structural propensity  
18 calculator ncSCP [104,105] (<http://www.protein-nmr.org/>, [https://st-](https://st-protein02.chem.au.dk/ncSPC/)  
19 [protein02.chem.au.dk/ncSPC/](https://st-protein02.chem.au.dk/ncSPC/)) from the experimentally determined, DSS referenced C $\alpha$  and  
20 C $\beta$  chemical shifts as input, with a correction for Gly-Pro motifs (-0.77 ppm instead of -2.0  
21 ppm) [106]. Some signals were too weak in 3D-spectra from Sox2(aa115-317\_C265A)  
22 recorded at 950 MHz, and their chemical shifts were not defined. In these cases, chemical  
23 shifts from 3D-spectra of Sox2(115-236) or His6-AviTag-Sox2(234-317\_C265A) were used  
24 to complete the lists of chemical shifts used to calculate the chemical shift propensities shown  
25 in Figure 2.

1

2        2.4. NMR monitoring of phosphorylation reactions and production of phosphorylated  
3            peptides

4 We performed the phosphorylation kinetics presented in Figure 4a using commercial  
5 recombinant kinases GST-p38 $\beta$  at 10  $\mu$ g/mL (Sigma-Aldrich, ref. B4437), GST-Erk2 at 20  
6  $\mu$ g/mL (Sigma-Aldrich, ref. E1283), GST-Cdk1/CyclinA2 at 20  $\mu$ g/mL (Sigma-Aldrich, stock  
7 ref. C0244) and GST-Cdk2/CyclinA2 at 20  $\mu$ g/mL (Sigma-Aldrich, ref. C0495). Then, we  
8 used kinases produced in house in *E. coli*, using plasmids containing optimized genes coding  
9 for p38 $\alpha$ (aa1-360, full-length) and Erk2(aa8-360); these were produced, activated and  
10 purified in house as described previously [99]; in-house p38 $\alpha$  was used at 40  $\mu$ g/mL for the  
11 experiments shown in Figure 4.

12 Phosphorylation reactions were carried out using <sup>15</sup>N-labeled IDRs at 50  $\mu$ M, in Hepes 20  
13 mM, NaCl 50 mM, DTT or TCEP at 4 mM, ATP 1.5 mM, MgCl<sub>2</sub> at 5 mM, protease  
14 inhibitors (Roche), 7.5% D<sub>2</sub>O, pH6.8 at 25°C in 100  $\mu$ L using 3 mm diameter Shigemi tubes.  
15 We monitored the phosphorylation kinetics by recording time series of <sup>1</sup>H-<sup>15</sup>N SOFAST-  
16 HMQC spectra at 600 or 700 MHz, and quantifying the NMR signal intensities of the  
17 disappearing unphospho- and the appearing phospho-residues. We applied the methods that  
18 we described progressively in earlier publications [107–110]. More details are given in the  
19 Supplementary Material.

20

21        2.5. Pull-down assays

22 The extracts from mouse Embryonic Stem Cells (mESCs) were obtained from mESCs  
23 cultured in the conditions previously described [111]. Homogeneous extracts were obtained  
24 using DNA shearing by sonication in presence of benzonase, as described by Gingras and  
25 colleagues [112].

1 The pull-down assays were executed using 25  $\mu$ L of streptavidin-coated magnetic beads  
2 (Magbeads streptavidine, Genscript) loaded with 1 nmol of the biotinylated bait-peptides of  
3 interest. These were incubated during one hour at room temperature with mESCs extracts,  
4 washed in PBS and eluted using a 2x Laemmli buffer. Details are given in the Supplementary  
5 Material.

6

#### 7 *2.6. Mass spectrometry-based proteomics analysis of pull-down assays*

8 The pull-down samples were treated on-beads by trypsin/LysC (Promega). The resulting  
9 peptides were loaded and separated on a C18 columns for online liquid chromatography  
10 performed with an RSLCnano system (Ultimate 3000, Thermo Scientific) coupled to an  
11 Orbitrap Fusion Tribrid mass spectrometer (Thermo Scientific). Maximum allowed mass  
12 deviation was set to 10 ppm for monoisotopic precursor ions and 0.6 Da for MS/MS peaks.  
13 The resulting files were further processed using myProMS v3.9.3 ([https://github.com/bioinfo-](https://github.com/bioinfo-pf-curie/myproms)  
14 [pf-curie/myproms](https://github.com/bioinfo-pf-curie/myproms); Pouillet et al., 2007 [2]). False-discovery rate (FDR) was calculated using  
15 Percolator [3] and was set to 1% at the peptide level for the whole study. Label-free  
16 quantification was performed using peptide extracted ion chromatograms (XICs), computed  
17 with MassChroQ [4] v.2.2.1. The complete details are given in the Supplementary Material.

18

#### 19 *2.7. Recombinant production of Pin1 and NMR analysis of its interaction with Sox2 or* 20 *phospho-Sox2*

21 The plasmid containing the gene coding for the Pin1-WW domain was a kind gift from  
22 Isabelle Landrieu. The production was executed according to the previously published  
23 protocol [113]. The NMR analysis of binding with phosphoSox2(aa115-240) were performed  
24 at 283K and pH 7.0 with the GST-Pin1-WW construct and  $^{15}$ N-labeled Sox2(aa115-240)  
25 mixed in stoichiometric proportions, either at 50 or 10  $\mu$ M for non-phospho and

1 phosphoSox2, respectively. The details on the NMR acquisition, processing and analysis are  
2 given in the Supplementary Information.

3

### 4 **3. Results**

#### 5 *3.1. Structural characterization of the N- and C-terminal regions of Oct4*

6 We produced and purified protein constructs containing the fragments of human Oct4(aa1-  
7 145) and Oct4(aa286-360), which were both predicted to be mostly disordered (Supp. Mat  
8 3.2.3.). The 2D  $^1\text{H}$ - $^{15}\text{N}$  HSQC NMR spectra showed crosspeaks in the spectra region where  
9 random coil peptides resonances are usually found (Figure 1). We assigned the backbone  
10 NMR signals of  $^1\text{H}_\text{N}$ ,  $^{15}\text{N}$ ,  $^{13}\text{C}\alpha$ ,  $^{13}\text{C}\beta$ ,  $^{13}\text{CO}$  for both segments, which permitted to calculate  
11 experimentally derived secondary structure propensities from the  $^{13}\text{C}\alpha$  and  $^{13}\text{C}\beta$  chemical  
12 shifts. We did not find any sign of a stable secondary structure, the highest  $\alpha$ -helical  
13 propensities reaching about 25 % in short stretches of about 5 consecutive amino acids.  
14 Hence, we verified experimentally that these N- and C-terminal fragments of human Oct4 are  
15 IDRs.

16 We can notice that Oct4 IDRs contain a high density of Prolines, which are not directly  
17 detectable in the present  $^1\text{H}_\text{N}$ -detected experiments, even though most of the  $^{13}\text{C}\alpha$ ,  $^{13}\text{C}\beta$  and  
18 resonances were characterized via HNCAB and HNCO experiments. We have shown  
19 previously that the  $^{13}\text{C}$ -detected experiments  $^{13}\text{C}\alpha$  $^{13}\text{CO}$  permitted to observe all these Pro  
20 residues in Oct4(aa1-145) [99], whose chemical shifts were those of random coil peptides.

21

#### 22 *3.2. Structural characterization of the N- and C-terminal regions of Sox2*

23 We produced and purified peptide fragments of human Sox2, namely Sox2(aa1-42),  
24 Sox2(aa115-187), Sox2(aa115-236), Sox2(aa115-282), Sox2(aa234-317\_C265A), and  
25 Sox2(aa115-317\_C265A). We also produced and purified chimera peptides His6-AviTag-

1 Sox2(aa115-240) and His6-AviTag-Sox2(aa234-317\_C265A). These were all predicted to be  
2 disordered (Supp. Mat 3.3.3.).  
3 We had solubility issues with all of them but Sox2(aa1-42), Sox2(aa234-317\_C265A) and  
4 His6-AviTag-Sox2(aa234-317\_C265A). We had to recover these troublesome peptides from  
5 the insoluble fraction of the bacterial extract, after overexpression at 37 °C. We had even to  
6 carry out our final size-exclusion chromatography (SEC) purification step in a buffer  
7 containing urea at 2 M (at 4 °C) for Sox2(aa115-236), Sox2(aa115-282), His6-AviTag-  
8 Sox2(aa115-240), and Sox2(aa115-317-C265A). The assignments of these latter constructs  
9 were achieved in 0.25-0.5 M urea, after executing 2 to 3 concentration-dilution steps.  
10 Aggregates were forming during the acquisition, which made the assignment rather painful.  
11 This behavior correlated with liquid-liquid phase separation (LLPS) propensities, which we  
12 observed a few months before such a behavior was reported by Young and collaborators [44].  
13 The assignment of Sox2(aa115-317-C265A) was possible only at 950 MHz with the help of  
14 the previously assigned smaller fragments Sox2(aa115-236) and His6-AviTag-Sox2(aa234-  
15 317\_C265A). Some stretches of amino acids were particularly difficult to observe in 3D  
16 spectra, e.g. the region aa160-185, because of an apparent fast T2 relaxation. We may  
17 investigate these phenomena in later reports.  
18 We observed crosspeaks in the 2D <sup>1</sup>H-<sup>15</sup>N HSQC NMR spectra that were all resonating in the  
19 spectral region of random coil peptides resonances (Figure 2). The spectra of the short  
20 fragments of the C-terminal region of Sox2 were exactly overlapping with those of  
21 Sox2(aa115-317\_C265A) (Supp. Fig. 1). This shows that all these fragments have very  
22 similar local conformational behaviors, a phenomenon regularly observed with IDRs. We  
23 assigned the backbone NMR signals of <sup>1</sup>H<sub>N</sub>, <sup>15</sup>N, <sup>13</sup>C<sub>α</sub>, <sup>13</sup>C<sub>β</sub>, <sup>13</sup>CO for Sox2(aa1-42),  
24 Sox2(aa115-236), His6-AviTag-Sox2(aa234-317\_C265A) and partially for Sox2(aa115-  
25 317\_C265A). We aggregated the lists of chemical shifts of the C-terminal fragments, and

1 used them to calculate the experimental secondary structure propensities from the  $^{13}\text{C}\alpha$  and  
2  $^{13}\text{C}\beta$  chemical shifts. This confirmed the absence of any stable secondary structure elements  
3 in Sox2 N- and C-terminal region. The C-terminal region is poorly soluble below 0.25 M  
4 urea; this should not affect a stable fold, so we can affirm that these regions of Sox2 are  
5 experimentally proven IDRs.

6

### 7 *3.3. Structural characterization of the N-terminal region of Nanog*

8 We produced and purified the N-terminal peptide fragment of human Nanog(aa1-85). All our  
9 attempts to purify C-terminal regions of Nanog failed, even after alanine-mutation of  
10 cysteines in Nanog(aa154-305), Nanog(aa154-272) and Nanog(aa154-215). We managed to  
11 resolubilize our construct GST-His6-Tev-Nanog(aa154-305) from the insoluble fraction of the  
12 bacterial extract, to partially purify it and cleave it using the TEV protease. However, the  
13 resulting Nanog(aa154-305) peptide was barely soluble in a detergent (NP-40 at 2% v/v), and  
14 not in high salt buffers, or not even in presence of urea at 4 M. The 10 Tryptophane residues  
15 are probably playing a role in this behavior, in the context of a primary structure containing  
16 not enough hydrophobic amino acids favoring stable folds.

17 The crosspeaks of Nanog(aa1-85) in the 2D  $^1\text{H}$ - $^{15}\text{N}$  HSQC NMR spectrum were all in the  
18 spectral region of random coil peptides resonances (Figure 3). The assignment of the  
19 backbone NMR signals of  $^1\text{H}_\text{N}$ ,  $^{15}\text{N}$ ,  $^{13}\text{C}\alpha$ ,  $^{13}\text{C}\beta$ ,  $^{13}\text{CO}$  permitted to calculate experimental  
20 secondary structure propensities, which were low through the whole peptide. This Nanog N-  
21 terminal is thus confirmed to be an IDR.

22

### 23 *3.4. Structural characterization of the N-terminal region of Esrrb*

24 We produced and purified the N-terminal fragment of human Esrrb(aa1-102), which was  
25 predicted to be disordered (Supp Mat. 3.5.3.), in the alanine-mutated versions Esrrb(aa1-

1 102\_C12A-C72A-C92A), Esrrb(aa1-102\_C12A-C92A). This was a strategic choice to  
2 attenuate the formation of disulfide bonds; the wild-type N-terminal fragments might however  
3 be workable too. Mutating cysteines permitted to work in more comfortable conditions and to  
4 maintain our construct monomeric for longer periods of time in the next phosphorylation and  
5 biotinylation experiments. Cysteines are indeed highly solvent-accessible in IDRs and they  
6 are consequently difficult to keep in their thiol, non-disulfide forms, even in presence of fresh  
7 DTT or TCEP at neutral pH. We also produced chimera constructs Esrrb(aa1-102\_C12A-  
8 C72A-C92A)-AviTag-His6 and Esrrb(aa1-102\_C12A-C72A)-AviTag-His6.  
9 All the 2D  $^1\text{H}$ - $^{15}\text{N}$  HSQC NMR spectra revealed crosspeaks in the spectral region of random  
10 coil peptides (Figure 3). These spectra are overlapping to a large extent, confirming the weak  
11 influence of the mutations of cysteines: the mutation Cys72Ala has almost no consequences  
12 on the chemical shifts, below 0.05 ppm even for the neighboring amino acids (Supp. Fig  
13 2a,b); the mutation Cys91Ala has more impact, with chemical shifts perturbations of about  
14 0.1 ppm for the next 5 amino acids (Supp. Fig. 2c,d), which is at least partially due to the fact  
15 that the Ala substitution favors an increase of local  $\alpha$ -helicity (about 25 %, see Supp. Fig.  
16 2e,f). This N-terminal fragment of human Esrrb is thus an IDR, according to the calculated  
17 secondary structure propensities (Figure 3f, Supp. Fig. 2e,f).

18

### 19 *3.5. Phosphorylation of Esrrb and Sox2 by p38 $\alpha$ , Erk2, Cdk1/2 as monitored by NMR* 20 *spectroscopy*

21 We reported recently the site-specific phosphorylation kinetics of Oct4 by p38 $\alpha$  using  $^{13}\text{C}$ -  
22 direct NMR detection [99]. Here, we used more standard  $^1\text{H}$ -detected/ $^{15}\text{N}$ -filtered experiments  
23 to rapidly characterize the site preferences of MAPKs and CDKs on Esrrb and Sox2, which  
24 we thought to use as baits for performing phospho-dependent pull-down assays (see below).

1 To start with, we used commercial aliquots of MAPKs, namely p38 $\beta$  and Erk2, and CDKs,  
2 namely Cdk1/CyclinA2 and Cdk2/CyclinA2, on Esrrb(aa1-102). We observed the progressive  
3 phosphorylation of its three Ser-Pro motifs, i.e. at Ser22, Ser34 and Ser58, in agreement with  
4 the consensus motifs of these kinases [114,115]. Ser22 is the preferred target in all cases,  
5 while Ser34 is the least processed by CDKs, if at all: the commercial CDKs are poorly active  
6 in our hands, which we have verified with a number of other targets for years in the  
7 laboratory; this makes it difficult to distinguish between sites that are only mildly disfavored  
8 or those that are more stringently ignored by CDKs in NMR monitored assays.  
9 The limited activities and high costs of commercial kinases motivated us to develop in-house  
10 capacities in kinase production. p38 $\alpha$  was the most accessible to produce among the MAPKs  
11 and CDKs; we produced it and activated it using recombinant MKK6. We tested our home-  
12 made p38 $\alpha$  on His6-AviTag-Sox2(aa115-240) and His6-AviTag-Sox2(aa234-317). It  
13 phosphorylated all the Ser/Thr-Pro motifs of these two peptides, and also T306 in a PGT<sub>306</sub>AI  
14 context, which shows a favorable Proline in position -2 [116], and a less common S212 in a  
15 MTS<sub>212</sub>SQ context.  
16 Hence, we were able to generate AviTag-IDR chimera in well-defined phosphorylated states.  
17 To produce phosphorylated <sup>14</sup>N-AviTag-IDR dedicated to pull-down assays, we executed the  
18 same protocol, and monitored in parallel “identical”, but <sup>15</sup>N-labeled samples by NMR.  
19 Hence, we could not determine the exact phosphorylation status of the <sup>14</sup>N-AviTag-IDR for the next  
20 experiments.

21

### 22 *3.6. Structural characterization of AviTag-peptide chimera and biotinylated versions* 23 *thereof*

24 We aimed at identifying new partners of OSNE using pull-down assays. We thought to use  
25 chimera containing GST at the N-terminus, which appeared as a convenient approach: vectors

1 integrating a GST-coding DNA sequence for overexpression in *E. coli* are available and of  
2 common use; glutathione-coated beads are also accessible and permit efficient and specific  
3 binding of GST-containing chimera peptides. However, we were unsatisfied by the  
4 performances of the method: GST binding to glutathione-coated beads is slow at low  
5 temperature (necessary to avoid IDR proteolysis); moreover, it appeared that GST-IDRs  
6 chimera are hampered by the IDR “molecular-cloud” and are even weaker and slower to bind  
7 the beads. Our attempts to bind GST-IDRs to the beads were thus resulting in poor yields,  
8 which were not very reproducible. In the context of our aims, i.e. to establish a method  
9 allowing semi-quantitative detection of IDR binding partners, this unsatisfying lack of  
10 reproducibility was only promising supplementary variable parameters.

11 Thus, we decided to switch to another strategy: the use of the specifically biotinylated 15-mer  
12 peptide tag called AviTag [101]. This is efficiently and specifically biotinylated by the biotin  
13 ligase BirA (Figure 5d), which permits the high-affinity binding to streptavidin-coated beads.  
14 We designed AviTag-IDR chimera, with the AviTag in N-terminal position for Sox2-IDRs  
15 constructs, and in C-terminal for Essrb-IDR constructs. We characterized the AviTag and its  
16 impact on the IDRs of interest using NMR: the AviTag is unfolded and it does not affect the  
17 Sox2 and Essrb fragments, according to the observed negligible chemical shift perturbations  
18 (Fig 5) – the GST-Tag provoked also only very weak chemical shift changes on Essrb(aa1-  
19 102) (Fig S3). The biotinylated AviTag peptide appears to be slightly less mobile than the  
20 common IDPs on the ps-ns timescale, according to the heteronuclear  $^1\text{H}^{15}\text{N}$ -nOes (Figure 5b).  
21 We observed that the biotinylation of the AviTag provokes weak, but distinguishable  
22 chemical shift perturbations for the close neighbors of the biotinylated lysine, but had no  
23 effect on the peptides of interest (Figure 5c). It generated also the appearance of a HN-ester  
24 NMR signal, similar to that of acetylated lysine [107,117]. Hence, we could quantify and  
25 monitor the reaction advancement using NMR, and determine the incubation time that was

1 necessary and sufficient to obtain a complete biotinylation of our chimera AviTag-IDRs (see  
2 2.2.). This was one among many optimization steps permitting the production of sufficient  
3 quantities of intact IDRs for the pull-down assays.

4 Next, we tested the binding of the biotinylated AviTag-IDRs on streptavidin-coated beads.

5 This produced very satisfying results, i.e. stoichiometric, specific binding in one hour with no  
6 leakage (Fig. S4). This approach was thus selected for the pull-down assays.

7

### 8 *3.7. Identification of Sox2 binding partners in extracts from mouse Embryonic Stem Cells*

9 We prepared the four peptides AviTag-Sox2(aa115-240) and AviTag-Sox2(aa234-317) in  
10 their non-phosphorylated and phosphorylated versions, using p38 $\alpha$  to execute the  
11 phosphorylation reactions (Figure 6a). These peptides were also biotinylated, and later bound  
12 to streptavidin-coated beads, which we used as baits for pull-down assays in extracts from  
13 mESCs (Figure 6b). Importantly, size-exclusion chromatographies were carried out between  
14 every step to discard proteolyzed peptides, the enzymes (kinases of BirA) and their  
15 contaminants as much as possible. We performed the pull-down assays with the four samples  
16 in parallel with the same cell extract, in duplicate, and then analyzed the bound fractions using  
17 quantitative LC-MS/MS analysis (see Supp. Mat 1.6. for the full description). Hence, we  
18 could identify and evaluate the relative quantities of proteins retained by the four AviTag-  
19 peptides (Figure 6). On paper, this presents the important advantage of removing false-  
20 positive binders, which can interact unspecifically with the streptavidin-coated beads.  
21 We present here results that should be interpreted carefully: we produced only duplicates for  
22 every condition, using one single cell extract. To deliver trustful information, the common  
23 standards in the field recommend 3 to 5 replicates, and the use of independent samples. We  
24 can still comment the results, which we consider to be interesting preliminary data. We  
25 quantified proteins with at least 2 distinct peptides across the two replicates (Figure 6C).

1 Among the relative quantifications with an adjusted p-value  $< 0.05$ , we observed a two-fold  
2 change or more ( $FC > 2$ ) of a number of transcription factors in the proteins pulled out by  
3 AviTag-Sox2(aa115-240), among which the PluriTFs Oct4 and Klf5 are significantly  
4 enriched ( $> 4$  peptides, p-value  $< 0.02$ ). Most of these TFs appear to bind slightly less to the  
5 phosphorylated pSox2(aa115-240). However, pSox2(aa115-240) kept apparently a capacity to  
6 bind Oct4 and Klf5 to some extent, in comparison to pSox2(aa234-317). Also, we found out  
7 that pSox2(aa115-240) was pulling out the three isoforms of CK1 ( $> 3$  peptides, p-values  $< 10^{-5}$ )  
8 and the proline isomerase Pin1 ( $> 2$  peptides, p-values  $< 2 \cdot 10^{-4}$ ), while Sox2(aa115-240) and  
9 pSox2(aa234-317) did not or much less.

10

### 11 3.8. NMR characterization of the interaction between Pin1 and phospho-Sox2

12 We decided to test the interaction between pSox2(aa115-240) and Pin1. We recorded  $^1H$ - $^{15}N$   
13 NMR spectra of  $^{15}N$ -labeled Sox2(aa115-240) or pSox2(aa115-240) alone or in presence of  
14 the Pin1-WW domain (natural abundance peptide, i.e. 0.6%  $^{15}N$ , 99.4%  $^{14}N$ , hence “NMR  
15 invisible” in  $^{15}N$ -filtered experiments). We observed localized losses in signal intensities for  
16 the residues neighboring the three pSox2(aa115-240) phosphosites when mixed with Pin1  
17 (Figure 7b); in contrast, no significant differences showed up in the spectra obtained with  
18 non-phosphorylated Sox2(aa115-240) in absence or presence of Pin1-WW (Figure 7a).  
19 Hence, these signal losses are the typical signs of a position-specific interaction between an  
20 IDR and a folded protein in the intermediate or slow NMR time-scale, i.e.  $\mu s$ -s timescale,  
21 which corresponds to submicromolar affinities for this type of molecules. This shows that our  
22 pull-down assays were capable of isolating and identifying binding partners of an IDR of  
23 Sox2 in this range of affinities.

24

## 25 4. Discussion

1 The structural biochemistry analysis reported here can be applied to the broad field of  
2 transcription factors (TFs). These are essential actors of cell signaling: they are key elements  
3 for inducing or maintaining pluripotency or differentiation, for cell proliferation or cell-cycle  
4 arrest, by activating or repressing gene transcription [61,118]. About 90% of the ~1,600 TFs  
5 contain large disordered segments (> 30 consecutive amino acids), which is particularly true  
6 for PluriTFs [31,119], and this correlation between TFs and IDRs exist in all kingdoms of life  
7 [45,120]. IDRs of TFs recruit transcription co-factors or the transcription machinery, which is  
8 still not very well characterized in detail [44–55].

9 Indeed, the fine understanding of TFs interactions via their IDRs appears to be hampered by  
10 the nature of these interactions: weak affinities, multivalency, possible redundancy between  
11 TFs and coacervation; post-translational modifications (PTMs), which can switch on or off  
12 IDRs' interactions, are a supplementary source of confusion when searching for binding  
13 partners. Among the difficulties, we should also mention the basic biochemistry issues: IDRs  
14 are difficult produce and manipulate *in vitro*, because they are prone to degrade or aggregate.  
15 Here, we have tried to demonstrate the feasibility and the interest of some biochemical and  
16 spectroscopic approaches to better characterize IDRs of TFs, their phosphorylation and the  
17 associated binding partners.

18 Like other TFs, pluripotency TFs Oct4, Sox2, Nanog and Esrrb are post-translationally  
19 modified (see the introduction), notably by CDKs and MAPKs [66,78–89]. These two classes  
20 of kinases are fundamental actors in all aspects of eukaryotic cellular life, and understanding  
21 their activity and regulation in pluripotency or differentiation is of high significance.

22 Interesting questions are still pending: what is the phosphorylation status of OSNEs' IDRs in  
23 pluripotent cells, what is the impact on their interaction networks, and how does it affect  
24 pluripotency or differentiation? The inhibition of MAPK Erk signaling is necessary to  
25 maintain pluripotency in the standard culture conditions of ESCs and iPSCs [66,82–84], while

1 these cells show a high CDK activity [8,86,87]; these two kinases family have the same core  
2 consensus sites, i.e. Ser/Thr-Pro motifs, which are abundant in OSNEs' IDRs and whose  
3 phosphorylation has apparently consequences for initiating differentiation [73,78,80,83,85].  
4 We have shown that we could produce well-defined phosphorylation status of these peptides,  
5 using recombinant kinases and NMR analysis, which makes it possible to study their  
6 interactions *in vitro*. We have also demonstrated our capacity to use these peptides as baits in  
7 pull-down assays for detecting new binding partners.  
8 Using this approach, we have identified an interaction between phospho-Sox2(aa115-240)-  
9 pS212-pS220-pT232 and the prolyl cis-trans isomerase Pin1. Interactions between Pin1 and  
10 Oct4 or Nanog have been published earlier [81,121]. This does not represent a major surprise,  
11 because Pin1 recognize the consensus motif pSer/pThr-Pro, which is very degenerate (about  
12 one third of all the detected sites in phosphoproteomics) [122,123]; dozens of Pin1 binding  
13 partners have been reported, which leaves suspicions about the functionality of all these, and  
14 about the capacity of the intracellular pool of Pin1 molecules to engage in so many  
15 interactions. We also detected an affinity between phospho-Sox2(aa115-240)-pS212-pS220-  
16 pT232 and three CK1 isoforms. This capacity of CK1 kinases to recognize prephosphorylated  
17 in position -3 of a potential Ser/Thr is well-known (with a hydrophobic residue in +1), and a  
18 recent structural characterization of CK1 $\delta$  has shown that it was even capable of keeping  
19 some affinity for a phosphorylated product after the phosphate transfer [124].  
20 Hence, we feel that these results are at the same time satisfying, but do reveal degenerate  
21 interactions, whose biological significance is questionable. This corresponds to one of the  
22 major drawbacks in the field of IDRs' studies: they generate interactions of weak affinities,  
23 which can be easily released during the washes of our pull-down assays. In this regard, the  
24 « proximity labeling » approaches (BioID, APEX and their derivatives) appeared recently to be  
25 quite adapted to transient interactions: these methods, developed in the last ten years, use

1 chimera constructs containing enzymes that transfer chemical groups to their intracellular  
2 neighbors, which can be later identified by mass-spectrometry [125–128]. IDRs are very  
3 flexible, solvent-exposed and establish a lot of poorly specific transient interactions. This was  
4 raising concerns about the possible production of many false-positive if one used proximity  
5 labeling methods to detect IDRs' binding partners. This has been partially confirmed by a  
6 recent study, but this bias appears to be limited [129]. Interactomes of 109 TFs have actually  
7 been described using Bio-ID and affinity-purification MS, showing the complementarity  
8 between proximity-labeling and the pull-down approach proposed here [130]. Yeast double-  
9 hybrid, which can detect ~20  $\mu$ M affinity interactions, and novel phage display approaches  
10 will also help in this task [131–134].

11 Another difficulty in studying IDRs of TFs is their propensity to coacervate [44–53]. Here, we  
12 have tried to use Sox2 as a bait in pull-down assays, a protein that has been later recognized to  
13 favor liquid-liquid phase separation [44,135]. We met this difficulty during the production  
14 steps, which forced us to purify most of the Sox2 constructs in urea at 2 M. We could  
15 straightforwardly observe liquid-liquid phase separation of Sox2(aa115-317) at 4  $\mu$ M using  
16 DIC microscopy in presence of Ficoll-70 (data not shown), but also progressive aggregation  
17 and low solubility thresholds while working with our purified samples. These are clear  
18 limiting factors for sample production and NMR characterization, which will hamper a  
19 number of other studies on IDRs of TFs. This might also affect the results of pull-down  
20 assays: we noticed an enrichment in TFs in the samples obtained from pull-downs using  
21 Sox2(aa115-240) as a bait, which has a much higher coacervation propensity than  
22 Sox2(aa234-317). Is it possible to generate local surface liquid-liquid phase separation on the  
23 surface of streptavidin-coated beads? This might be at the same time a blessing and a curse for  
24 future studies, by helping the formation of biologically significant assemblies, or by favoring  
25 unspecific, non-native macromolecules interactions.

1 A final bottleneck in the studies of these IDRs is the capacity to produce post-translationally  
2 modified samples. The commercial enzymes are not very well adapted to our NMR studies,  
3 because of the required quantities. Here, we have used in-house production of the kinase  
4 p38 $\alpha$ . Since we carried out the present work, we have developed our capacities in producing  
5 activated Erk2, Cdk2/CyclinA1 and Cdk1/CyclinB1. These will be part of our future studies.  
6 TFs are indeed quite adapted to NMR investigations: they are 300 to 500 residues long and  
7 contain large IDRs (~100 amino acids) separating small folded domains (also ~100 amino  
8 acids) [31,45]. Their structural characterization would permit to understand a number of cell  
9 signaling mechanisms at the atomic scale, and possibly to identify new therapeutic targets,  
10 even though this class of proteins is notoriously difficult to inhibit [60,136,137].

11

## 12 **5. Conclusion**

13 We have applied NMR techniques to carry out a primary analysis of the pluripotency  
14 transcription factors Oct4, Sox2, Nanog and Esrrb, in particular of their intrinsically  
15 disordered regions. We have shown experimentally that they did not adopt a stable fold when  
16 isolated, and that we were able to conduct a residue-specific analysis. This relies on the  
17 delicate production and purification of these peptides, which are prone to proteolysis and  
18 aggregation; producing them in a well-defined post-translational modification status was an  
19 even arduous challenge. We have demonstrated the feasibility of these tasks using  
20 recombinant kinases and NMR analysis. We have also evaluated the usefulness of such  
21 protein constructs as baits in pull-down assays to identify new binding partners of IDRs.  
22 These characterizations and the associated methods provide firm basis for future  
23 investigations on transcription factors.

24

## 25 **Declaration of Competing Interest**

1 The authors declare that they have no competing interest.

2

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20

21

### 22 **Supplementary material**

23 Supporting information for this article is available online at ...

24

25

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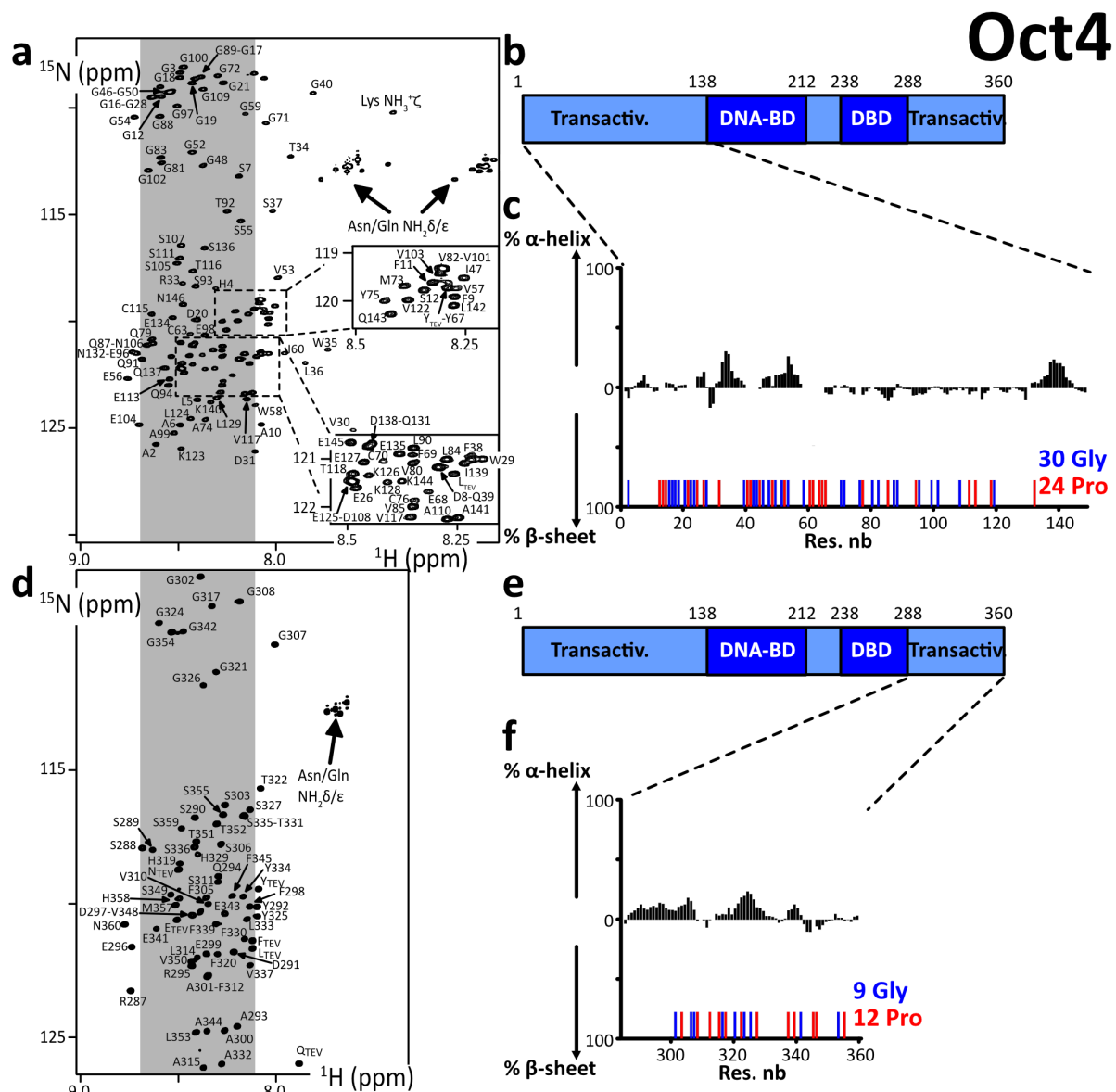
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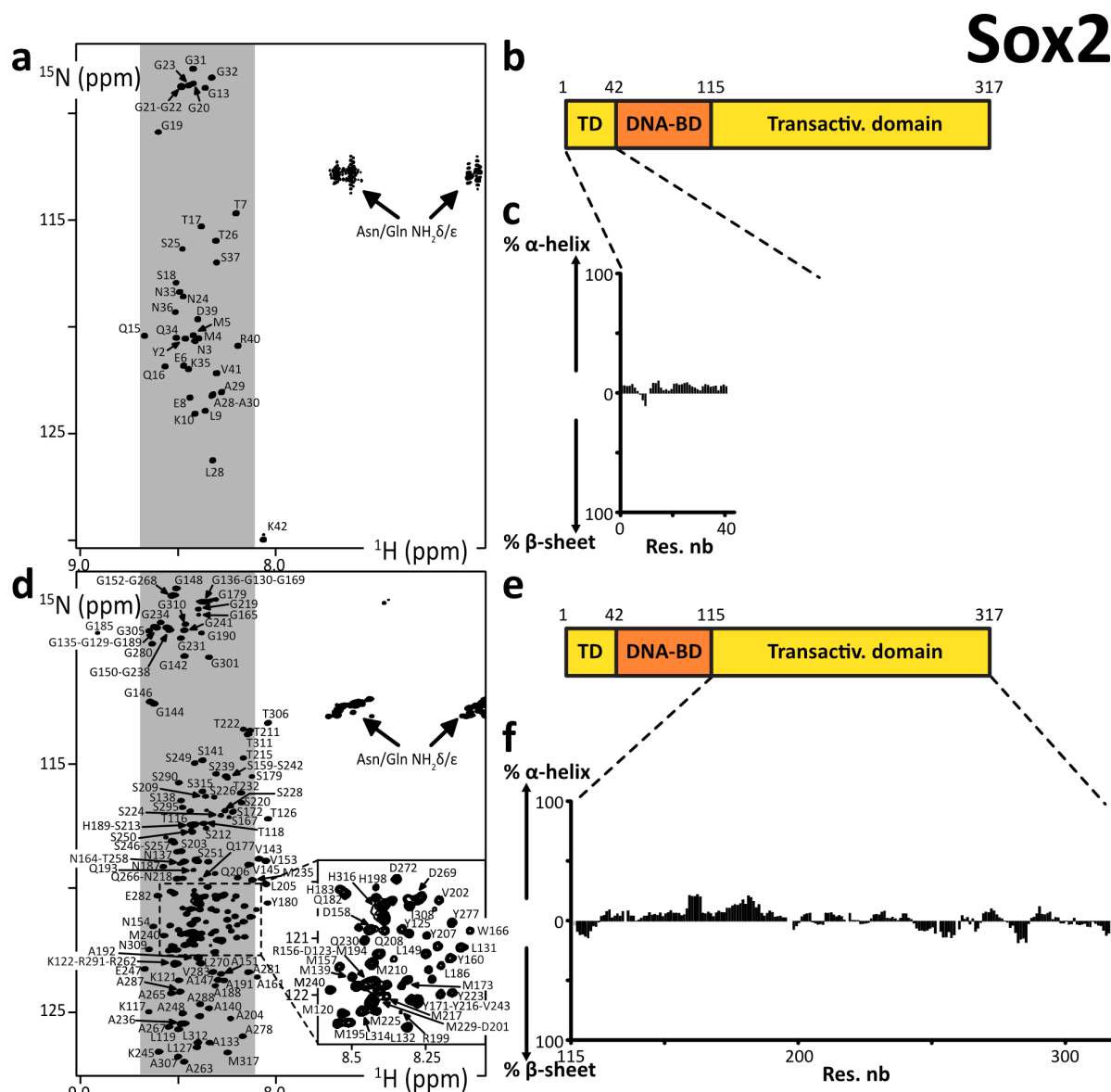
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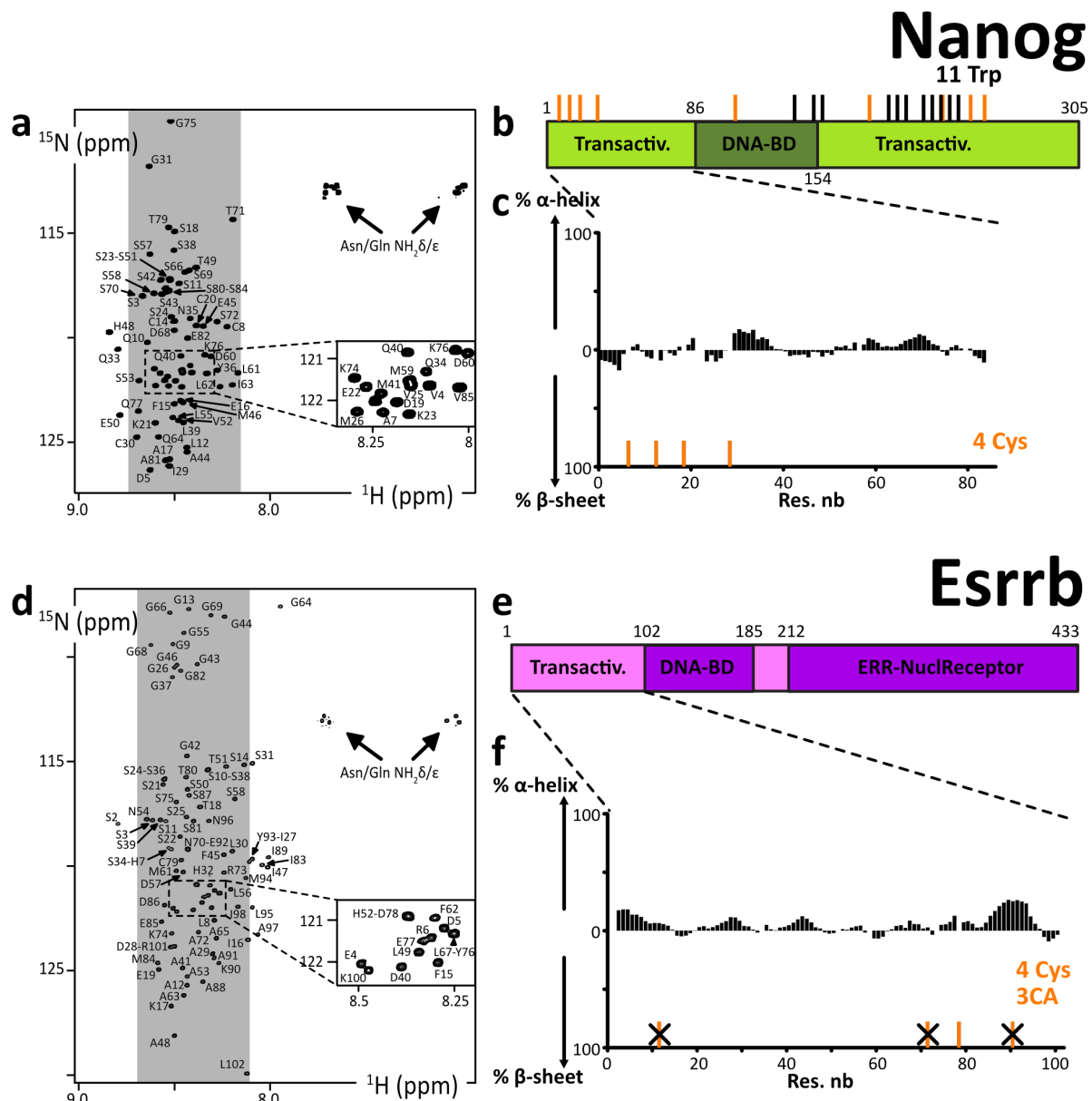
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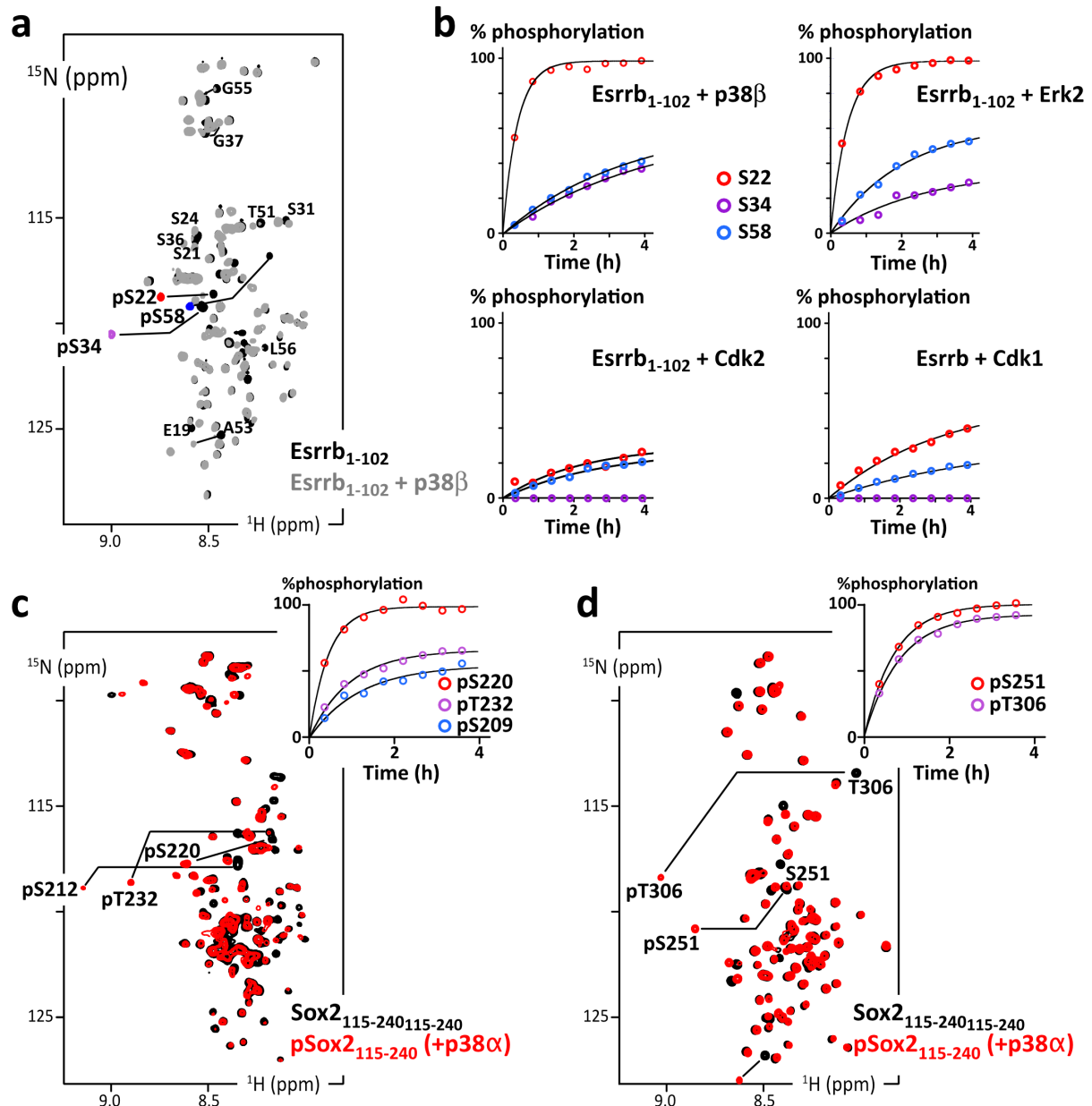
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2 **Figure 1** : **a. d.** 2D  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectra of the N- and C-terminal IDRs of human Oct4, the labels  
3 indicating the assignments; the grey areas show the spectral regions where random coil amino acids  
4 resonate usually; **b. c.** Primary structures of Oct4; dark and light colors indicate the folded and  
5 disordered domains, respectively; blue and red sticks indicate the positions of Glycines and Prolines,  
6 respectively; **c. e.** Secondary structure propensities calculated from the experimental chemical shifts  
7 of the peptide backbone  $C\alpha$  and  $C\beta$ , using the ncSPC algorithm [104,105].



1  
 2 **Figure 2:** *a. d.* 2D  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectra of the N- and C-terminal IDRs of human Sox2, the labels  
 3 indicating the assignments; the grey areas show the spectral regions where random coil amino acids  
 4 resonate usually; *b. c.* Primary structures of human Sox2; dark and light colors indicate the folded and  
 5 disordered domains, respectively; *c. e.* Secondary structure propensities calculated from the  
 6 experimental chemical shifts of the peptide backbone  $C\alpha$  and  $C\beta$ , using the ncSPC algorithm  
 7 [104,105].



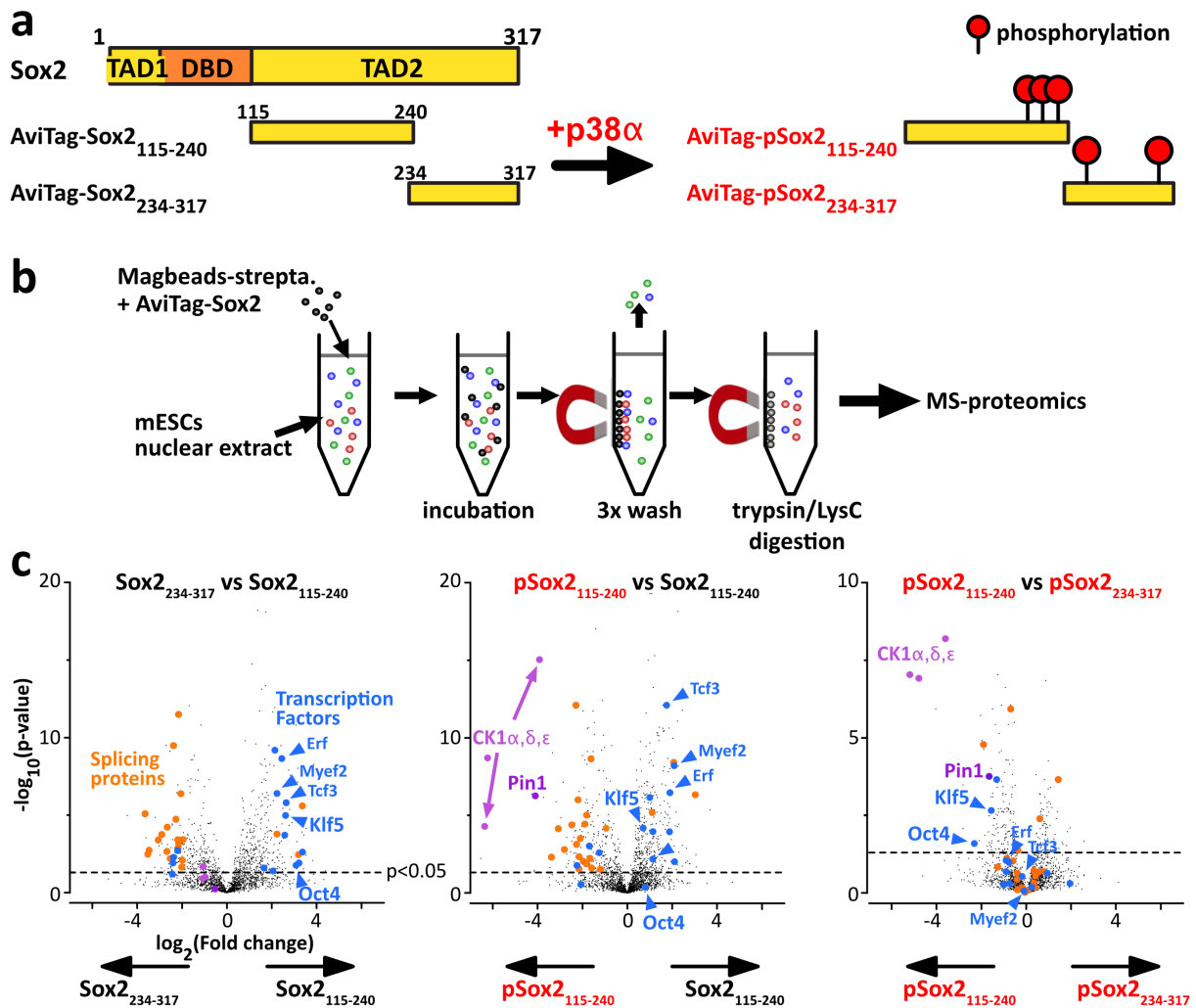
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2 **Figure 3:** **a. d.** 2D  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectra of the N-terminal IDRs of human Nanog and Esrrb, the labels  
3 indicating the assignments; the grey areas show the spectral regions where random coil amino acids  
4 resonate usually; **b. c.** Primary structures of human Nanog and Esrrb; dark and light colors indicate  
5 the folded and disordered domains, respectively; orange and black sticks indicate the positions of  
6 cysteines and tryptophanes, respectively; **c. e.** Secondary structure propensities calculated from the  
7 experimental chemical shifts of the peptide backbone  $C\alpha$  and  $C\beta$ , using the ncSPC algorithm  
8 [104,105].



1  
2 **Figure 4:** a. Overlay of 2D  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectra of Esrrb(aa1-102\_C12A-C72A-C91A) before (black)  
3 and after (grey and phosphosites colored in red/purple/blue) phosphorylation by p38 $\beta$ ; b. Residue  
4 specific time courses of the phosphorylation of Esrrb(aa1-102) executed by commercial kinases p38 $\beta$ ,  
5 Erk2, Cdk2 or Cdk1, as measured in time series of 2D  $^1\text{H}$ - $^{15}\text{N}$  SOFAST-HMQC spectra recorded during  
6 the reaction; c. Overlay of 2D  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectra of AviTag-Sox2(aa115-240) before (black) and  
7 after (red) phosphorylation by p38 $\alpha$ ; the inset at the top-right shows the residue specific  
8 phosphorylation build-up curves, as measured in time series of 2D  $^1\text{H}$ - $^{15}\text{N}$  SOFAST-HMQC spectra; d.  
9 Same as c. with AviTag-Sox2(aa234-317\_C265A).

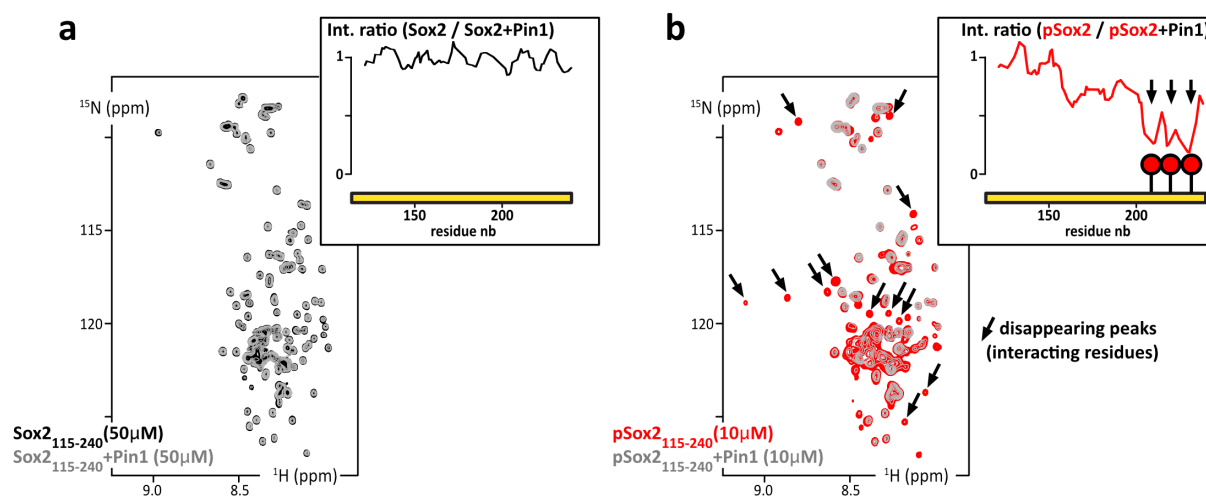
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1  
2 **Figure 6: Differential interactomics of Sox2 constructs upon p38 $\alpha$  phosphorylation.** *a.* We have  
3 produced unmodified/phospho-Sox2 truncations carrying N-ter biotinylation on the AviTag, and later  
4 attached on streptavidin-magnetic beads. *b.* We have generated mESCs nuclear extracts for pull-  
5 down assays using our Sox2 constructs as baits. *c.* The volcano plots of the log<sub>2</sub> ratios, showing a  
6 quantitative analysis of the proteins present at the end of the pull-down assays; the dashed line  
7 indicates the threshold of p-value < 0.05; we highlighted interesting partners in: blue: transcrip-  
8 tion factors associating with Sox2(aa115-240); magenta: phospho-dependent partners of Sox2(aa115-  
9 240) and or Sox2(aa234-317). Pull-downs have been performed in duplicates, using 15 million cells  
10 per sample (extract protein conc.: 5 mg/mL), and 1 nmol of bait protein. Experimental conditions may  
11 be improved (higher number of replicates, cells, washing conditions, ...).

12  
13



1  
2 **Figure 7: a.** Overlay of 2D  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectra of  $^{15}\text{N}$ -Sox2(aa115-240) alone at 50  $\mu\text{M}$  (black) or  
3 mixed with Pin1 in isotopic natural abundance and in stoichiometric amounts (grey) ; inset up-right :  
4 residue specific NMR signal intensity ratios as measured in the two HSQC spectra. **b.** Overlay of 2D  $^1\text{H}$ -  
5  $^{15}\text{N}$  HSQC spectra of  $^{15}\text{N}$ -phosphoSox2(aa115-240) alone at 10  $\mu\text{M}$  (red) or mixed with the Pin1-WW  
6 domain in stoichiometric amounts (grey); insets, up-right : residue specific NMR signal intensity ratios  
7 as measured in the two HSQC spectra (in absence/presence of Pin1-WW domain).  
8