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A cell-free system toward deciphering the post-translational modification barcodes of Oct4 in different cellular contexts

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ABSTRACT

The octamer-binding transcription factor 4 (Oct4) is essential for maintaining the self-renewal and pluripotency of embryonic stem cells (ESCs). Post-translational modifications (PTMs) of Oct4 critically control its structure, function and intracellular localization. However, determination of Oct4 PTM profiles has largely been restricted by the quantity and purity of the Oct4 protein samples required for mass spectrometric analyses. In this study, by incubating the *Escherichia coli*-derived His-tagged Oct4 proteins with the whole cell lysates of a variety of human cells followed by retrieving the reacted Oct4 proteins with the Ni–NTA beads, we developed a labor- and cost-effective *in vitro* PTM method that allowed for mass spectrometric determination of the phosphorylation profiles of Oct4 proteins exposed to various cell-free systems. A number of Oct4 phosphorylation sites that were commonly present in all the cell-free systems or specifically present in a particular cellular context were identified, indicating that Oct4 is controlled by both common and distinct PTM regulatory pathways. Our work provided a proof-of-concept that such a cell-free system-based *in vitro* PTM approach can be applied to systematically map out the physiologically-relevant PTM sites in Oct4 proteins, which opened up an avenue to fully decipher the Oct4 PTM barcodes in various cellular contexts.

1. Introduction

The octamer-binding transcription factor 4 (Oct4, also known as Oct3/4) is a homeodomain transcription factor of the POU family that is encoded by the *POU5F1* gene. It plays an essential role in the self-renewal of inner-cell-mass cells or epiblast stem cells *in vivo* as well as embryonic stem cells (ESCs) *in vitro*, and is indispensable for generating the induced pluripotent stem cells (iPSCs) [1]. Although Oct4 is undetectable in most differentiated tissues and cells [1], growing evidence documented its low-level expression in certain tissue stem cells [2] and stem-like cancer cells [3]. However, the exact roles and the regulation of Oct4 in those cells remain elusive.

In contrast to the transcriptional regulation of Oct4 expression that has been extensively studied [1,4,5], far less is known about its post-translational regulation that emerges to be a crucial regulatory mechanism for many transcription factors. Post-translational modifications (PTMs) refer to chemical modifications of a protein by covalently and reversibly adding a functional group or a protein to its specific amino acid residue(s) [6]. Thus far, over 200 types of PTMs have been identified and the most common ones include phosphorylation, ubiquitination, sumoylation, glycosylation, acetylation, and methylation, which play essential roles in regulating the structure, activity and localization of the proteins and their interaction with other cellular components [6]. The reversible PTMs are well placed to sense, relay and integrate a variety of extracellular and intracellular signals. The studies on histones have exemplified how combinations of PTMs can function in concert to allow for the storage and transduction of highly specific signals to control epigenetics-based gene transcription. Such combinatorial histone PTMs serve much like a bar code, providing great potential for signal diversity [7]. The PTM bar code concept has also been used to correlate specific biological behaviors of the tumor suppressor p53 with various stimuli [8]. However, because the sample preparation for mass spectrometric measurements of the combinatorial PTM codes on a specific protein is technically demanding, only a very limited number of successful cases have been reported so far.

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Although a variety of PTMs including phosphorylation, ubiquitination, sumoylation, glycosylation have been identified at multiple residues in Oct4 present in ESCs and embryonal carcinoma cells (ECCs) [9], in most cases only sporadic and individual sites were reported. For instance, Lin et al. reported that the phosphorylation of Oct4-T235 by Akt orchestrates the regulation of its stability, subcellular localization, and transcriptional activities, which collectively promotes the survival and tumorigenicity of ECCs [10]. Spelat et al. found that the phosphorylation of Oct4-S111 by ERK1/2 enhances Oct4 degradation and distribution in cytoplasm [11]. The most in-depth PTM identification study carried out for Oct4 so far was from Brumbaugh et al. where 14 phosphorylation sites were collectively identified in multiple human ESC samples by liquid chromatography mass spectrometry [12]. However, neither a time course-dependent profiling of the Oct4 PTMs in response to certain stimuli in a given cell type, nor a systematic profiling and comparing of the Oct4 PTMs in different cellular contexts has been reported. The main hurdle is likely to be the difficulty in obtaining endogenous or over-expressed Oct4 proteins with sufficient quantity and purity for mass spectrometric analyses.

In the present study, by incubating a large quantity of highly purified *Escherichia coli*-derived recombinant Oct4 proteins with the whole lysates of a variety of human cells, we developed a labor- and cost-effective *in vitro* PTM method for mass spectrometric determination of the Oct4 PTM profiles in different cellular contexts. A number of Oct4 phosphorylation sites that were commonly present in all the cellular contexts or specifically present in a particular cellular context were identified, indicating that Oct4 in different cellular environment is controlled by both common and distinct PTM regulatory pathways. Our work indicated that such a cell-free system-based *in vitro* PTM approach has the potential to fully decipher various PTM barcodes of Oct4 in different cellular contexts.

2. Materials and methods

2.1. Antibodies and reagents

Polyclonal anti-His antibody (A00174) was from GenScript (Piscataway, NJ, USA). Peroxidase-conjugated anti-rabbit secondary antibody (31460) was from Thermo Fisher Scientific (Waltham, MA, USA). Polyclonal anti-phospho-Oct4 (Oct4-pT235) was custom-made by GenScript (order ID: 134164-1). Adenosine triphosphate (ATP, AB0020) was from Sangon Biotech Shanghai Co., Ltd. (Shanghai, China). Most chemicals were from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Cell culture and transfection

NCCIT, U87, 293T and H1 cells were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA) and cultured as described previously [10]. For transfection, 293T cells were grown on 10 cm plates to a 30–50% confluence, and transfected with 5–10 μg of DNA per plate using GenEscort II reagent (Wisegen, Nanjing, China) with a ratio of 1:3 (w/v).

2.3. Plasmids

The human Oct4 construct with the 6 × His tag (N terminal) was subcloned into the EcoRI and SacI sites of the pET28a vector using the following primers:

Forward primer, 5′-ATGCGAATTCATGGCGGGACACCTGGCTTCGG-3′
Reverse primer, 5′-ATGCGTCGACTCAGTTTGAATGCATGGGAG-3′

The Flag-Oct4-6 × His portion was subcloned into the EcoRI and XbaI sites of the pLKO.1-TRC-shOct4-Flag-Oct4 plasmid [10] using the following primers:

Forward primer, 5′-GGAATTCATGGCGGGACACCTGGCTT-3′
Reverse primer, 5′-CACTACTACTACTACTACCGACGACGGCGACGGCGCGCCGTGGTTCAGTTTGAATGCATGGGAGAGC-3′

2.4. Immunoblotting and immunoprecipitation

Immunoblotting and immunoprecipitation was conducted as described previously [10].

2.5. Recombinant protein expression and purification

The His-tagged human Oct4 construct was transformed into Rosseta *E. coli* strain and the purification procedures were as described previously [10]. After sonication, the *E. coli* lysate was centrifuged at 12,000*g* for 10 min at 4 °C and the supernatants were mixed with 1 ml of a 50% (w/v) slurry of Ni–NTA beads (QIAGEN, Germantown, MD, USA), and incubated at 4 °C on a rotary shaker for 2 h. The mixture was then centrifuged at 800*g* for 2 min and the supernatant was discarded. The beads were then washed with lysis buffer containing 50 mM imidazole. The proteins binding on Ni–NTA beads were used for subsequent *in vitro* PTM assay.

2.6. Cell-free system-based in vitro PTM of recombinant Oct4 proteins

An aliquot (5 μg) of purified recombinant His-Oct4 protein binding to the Ni–NTA beads (100 μl) was incubated with 1 mg of the whole lysate derived from NCCIT, H1 or U87 cells at 30 °C for 1 h in 1 ml PMA buffer (50 mM Tris–HCl, pH 7.4, 50 mM KCl, 5 mM $MgCl_2$, 0.5% NP-40, 5 mM ATP, 100 μM S-adenosyl methionine, 100 μM acetyl-CoA, 1% EDTA-free protease inhibitor cocktail and phosphatase inhibitor cocktail). The His-Oct4 conjugated beads were sedimented by centrifugation at 800*g* for 2 min, washed three times with ice-cold PMA washing buffer (50 mM Tris–HCl, pH 7.4, 50 mM KCl, 5 mM $MgCl_2$, 0.5% NP-40), eluted and denatured in SDS–PAGE sample loading buffer by heating at 100 °C for 5 min. Pooled samples (10–20 μg) were loaded and separated by SDS–PAGE and stained with Coomassie brilliant blue R250. After destaining, the Oct4 bands (with a molecular weight of 45 kDa) were excised and analyzed by mass spectrometry.

2.7. Mass spectrometric identification of PTMs in Oct4 proteins

Oct4 samples were subjected to overnight digestion with trypsin or chymotrypsin as described by Liu et al. [13]. The peptides were extracted with acetonitrile containing 0.1% formic acid and vacuum dried. Proteolytic peptides were reconstituted with mobile phase A (2% acetonitrile containing 0.1% formic acid) and then separated on an on-line C18 column (75 μm inner diameter, 360 μm outer diameter, 10 cm, 3 μm C18). Mass spectrometry analysis was carried out on an LTQ-Orbitrap Velos mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) operated in data dependent scan mode. Survey scan (m/z 375–1300) was performed at a resolution of 60,000 followed by MS2 scans to fragment the 50 most abundant precursors with collision induced dissociation. The activation time was set at 30 ms, the isolation width was 1.5 amu, the normalized activation energy was 35%, and the activation q was 0.25. Mass spectrometry raw file was searched by Proteome Discovery version 1.3 using MASCOT search engine with percolator against the human ref-sequence protein database (updated on 07-04-2013). A filter of 90% peptide confidence was applied according to the Peptide-Prophet and Protein-Prophet parsimony algorithms.

Fragment assignment of each modified peptide was subject to manual inspection and validation using the original tandem mass spectra acquired in profile mode using Xcalibur software.

3. Results

3.1. Bacterially-expressed Oct4 proteins can be phosphorylated in vitro in a human cell-free system

Our general experimental strategy is summarized in Fig. 1A. To test if bacterially-expressed recombinant Oct4 proteins have the potential to be phosphorylated by a human cell-free system, we incubated the *E. coli*-derived 6 × His-tagged Oct4 proteins with the NCCIT whole cell lysates for varying periods, pulled down the reacted His-Oct4 proteins, subjected them to SDS–PAGE, and determined the levels of a site-specific phosphor Oct4 (Oct4-pT235) using immunoblotting (Fig. 1B). The Oct4-pT235 band was absent in His-Oct4 samples without NCCIT lysate incubation, but appeared at 10 min and became saturated at 60 min after incubation, suggesting that the bacterially-expressed Oct4 proteins can be phosphorylated by enzymes present in NCCIT cell lysates. In a parallel experiment in which Flag-Oct4-His was over-expressed in 1×10^7 of 293T cells and affinity purified using the Ni–NTA beads, only a small quantity (~1 μg) of Flag-Oct4-His proteins were obtained which was not sufficient for mass spectrometric analysis of the PTM profiles (Fig. 1C), highlighting the technical difficulties of the routine approach and the advantages of the *in vitro* PTM approach we developed in this study.

3.2. Comparing phosphorylation profiles of Oct4 in different cellular contexts

Next, we determined by mass spectrometry the phosphorylation profiles of Oct4 incubated with the whole cell lysates of H1, NCCIT, or U87 representing human ESCs, ECCs or differentiated cancer cells, respectively. In the absence of *in vitro* PTM, *E. coli*-derived His-Oct4 proteins only contained one phosphor site (S303) indicative of a low level of basal phosphorylation in bacterial context. In sharp contrast, 15, 16 or 11 phosphor sites were identified when the His-Oct4 proteins were exposed to the H1, NCCIT, or U87 cellular context, respectively. 6 phosphorylation sites were present in all cellular contexts, 7 sites were shared between H1 and NCCIT, 2 sites shared between H1 and U87, and 2 sites shared between NCCIT and U87 (Table 1, Fig. 2A). Intriguingly, T331 was a unique phosphorylation site in NCCIT cells, and phosphor T322 was only identified in U87 cells (Table 1).

Collectively, we identified 19 phosphorylation sites spanning the whole Oct4 sequence (Figs. 2B and 3A), of which 7 have been reported previously and 12 are novel (including 8 serine sites and 4 threonine sites), comparing to 17 phosphorylation sites listed at the PhosphoSitePlus website (Table 1 and Fig. 2B). We identified a total of 15 phosphorylation sites in Oct4 from the context of human ESCs (H1 cells), of which 5 overlapped with the 14 phosphor sites identified in H1 cells by Brumbaugh et al. [12] (Table 1 and Fig. 2C).

3.3. Characterization of Oct4 phosphorylation sites

We further compared all the experimentally identified Oct4 phosphorylation sites thus far (both by others and us) with those bioinformatically predicted by the NetPhos 2.0 Server. Among the total of 21 predicted sites, 15 were confirmed by the experiments. Remarkably, 13 out of the 14 predicted phosphor-serine sites, and 2 out of the 3 predicted phosphor-threonine sites were validated experimentally, while none of the 4 predicted phosphor-tyrosine sites were identified in the real experiments. The other 8 phosphor-threonine sites were not predicted but experimentally identified (Supplementary Table 1 and Fig. 2D).

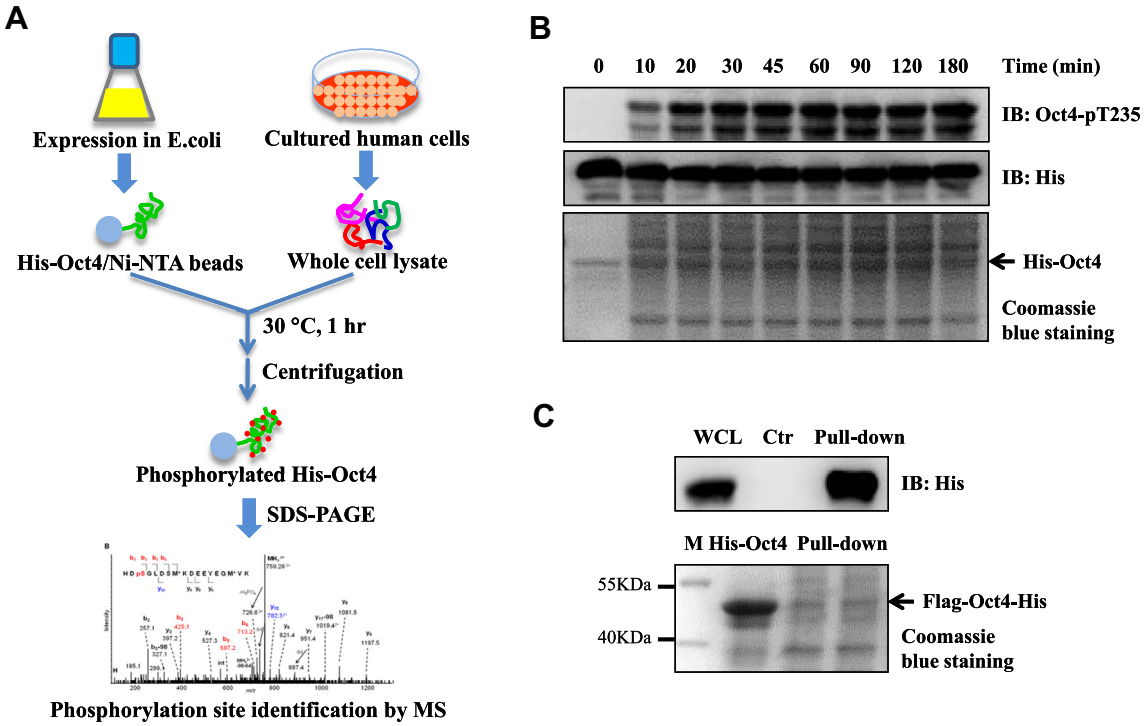

Fig. 1. Cell-free system-based *in vitro* PTM approach. (A) Schematic representation of the general strategy. (B) 1 μg of purified *E. coli*-derived His-Oct4 protein binding to the Ni–NTA beads (20 μl) was incubated with 0.2 mg of NCCIT whole cell lysate in 1 ml PMA buffer at 30 °C for varying periods. Samples were subjected to SDS–PAGE, immunoblotted with the indicated antibodies or stained with the Coomassie brilliant blue R250. (C) The Flag-Oct4-His construct was transfected into 1×10^7 of 293T cells (with non-transfected cells being the negative control, Ctr) and the over-expressed Flag-Oct4-His proteins were affinity purified using the Ni–NTA beads. Samples were subjected to SDS–PAGE, immunoblotted with anti-His or stained with the Coomassie brilliant blue R250.

Table 1
Comparison of Oct4 phosphorylation sites identified in this study and from other sources.

Type	Site	Phospho-SitePlus[a]	H1 lysate[b]	NCCIT lysate[b]	U87 lysate[b]	293T expression	Brumbaugh et al. study [12]
Ser	S12	S12	S12	S12	–	–	–
	S55	–	S55	S55	–	–	–
	S93	S93	–	–	–	–	S93
	S105	S105	–	–	–	–	S105
	S107	S107	–	–	–	–	S107
	S111	S111	–	–	–	–	S111
	S136	–	–	S136	S136	–	–
	S180	S180	–	–	–	–	–
	S193	–	S193	S193	S193	–	–
	S236	S236	S236	S236	–	S236	S236
	S288	S288	–	–	–	–	S288
	S289	S289	S289	S289	–	–	S289
	S290	–	S290	S290	S290	–	–
	S303	–	**S303**	**S303**	**S303**	–	–
	S306	–	S306		S306	–	–
	S327	–	S327	S327	S327	–	–
	S349	–	S349	S349		–	–
	S355	S355	S355	S355	S355	–	S355
	S359	S359	–	S359	S359	–	S359
Thr	T92	T92	–	–	–	–	T92
	T116	T116	–	–	–	–	T116
	T118	T118	–	–	–	–	T118
	T163	T163	–	–	–	–	–
	T225	–	T225	T225	T225	–	–
	T235	T235	T235	T235	–	–	T235
	T322	–	–	–	T322	–	–
	T331	–	–	T331	–	–	–
	T351	–	T351	T351	–	–	–
	T352	T352	T352	–	T352	–	T352

293T expression – data from this study where Oct4 was ectopically over-expressed in 293T cells and affinity purified for mass spectrometric analysis.
Phosphor S303 (highlighted in bold) was detected in *E. coli*-expressed His-Oct4 proteins without *in vitro* PTM.
[a] Data from Cell Signaling Technology Company database "PhosphoSitePlus".
[b] Data from this study where *E. coli*-expressed His-Oct4 proteins were incubated with whole cell lysates of H1, NCCIT or U87 cells in an *in vitro* PTM assay followed by mass spectrometric analysis.

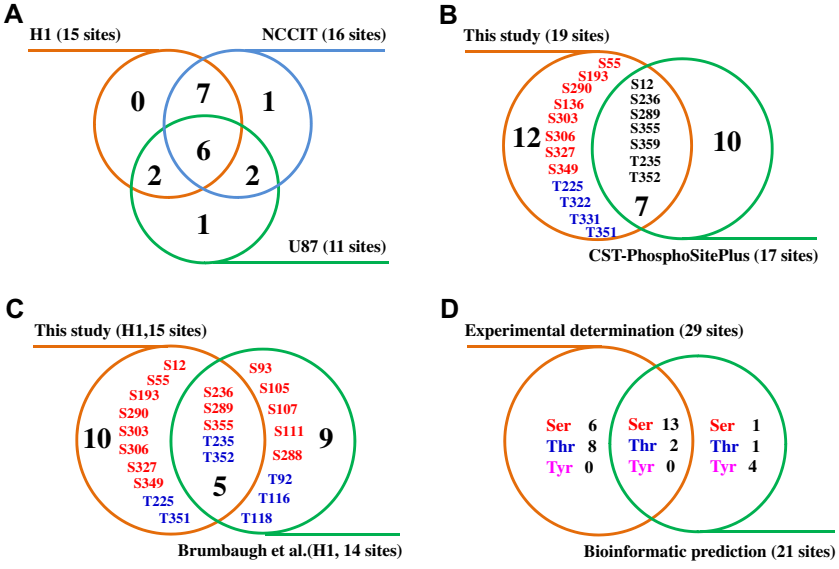

Fig. 2. Overview and various comparisons of the Oct4 phosphorylation sites. (A) A comparison of Oct4 phosphorylation sites in different cellular contexts. The overlapping phosphor sites indicate common regulatory mechanisms while the non-overlapping sites indicate unique regulatory mechanisms. (B) A comparison between the phosphor sites identified in this study with those reported in the CST-PhosphoSitePlus. (C) A comparison between the phosphor sites in H1 cells identified in this study with those reported by Brumbaugh et al. (D) A comparison of the proportions of the three phosphorylated residues (Ser: serine, Thr: threonine, Tyr: tyrosine) between experimental determinations and bioinformatic predictions.

Since Oct4 POU domains bind to target DNA sequences [14], the phosphorylation sites at these domains may play an important role in regulating the interaction between Oct4 and its target genes. We identified 1 phosphor-serine (S193) at the POU_S domain, 1 phosphor-threonine (T235) and 1 phosphor-serine (S236) at the POU_H domain (Fig. 3A). Hydropathy plot showed that the whole region of the human Oct4 POU domains spanning AA138 to AA289 (corresponding to mouse Oct4 AA131 to AA282) was overall hydrophobic but T235 and S236 were located at a highly hydrophilic area right next to the nuclear localization signal (NLS) sequence (Fig. 3B) and

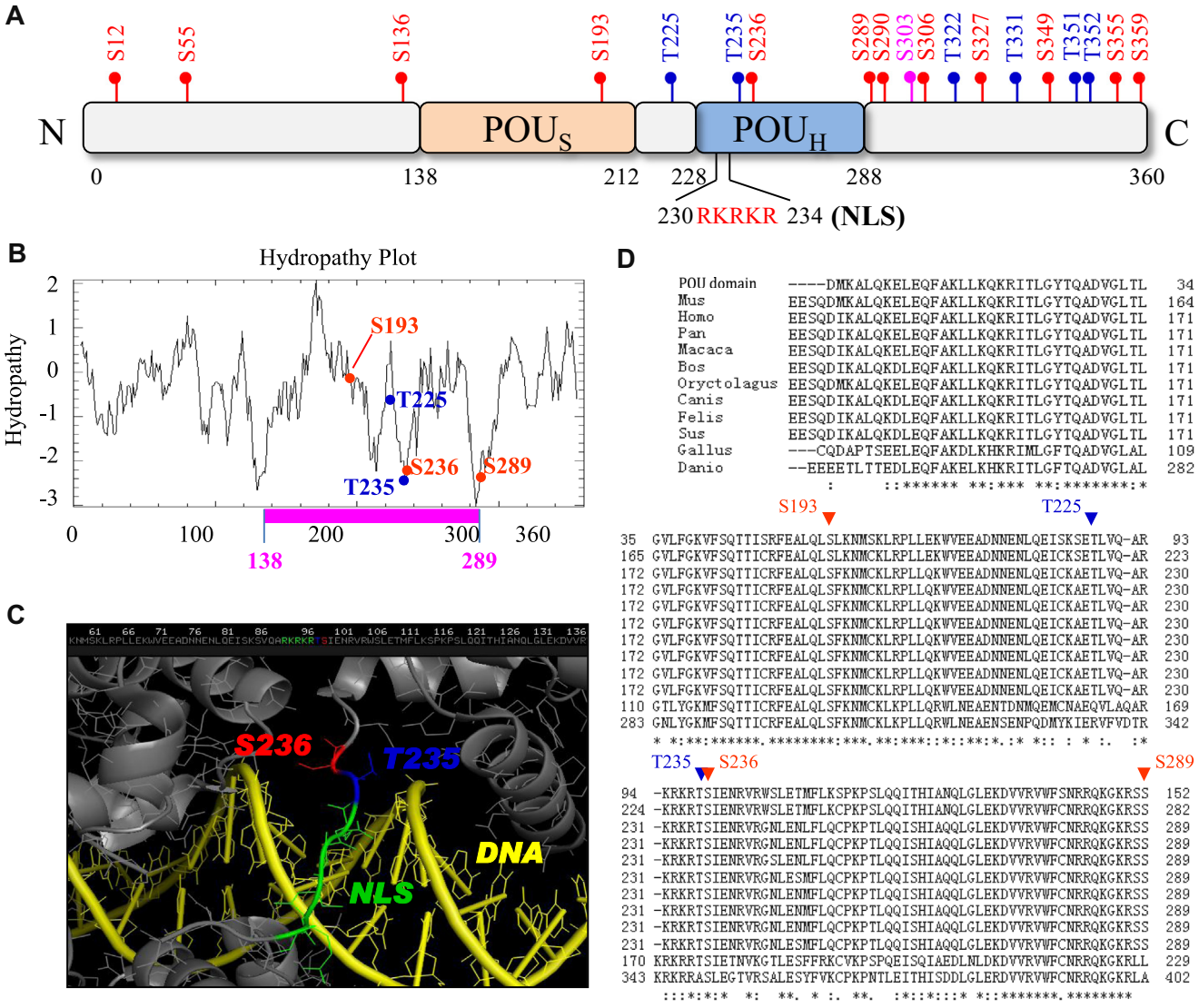

Fig. 3. Characterization of Oct4 phosphorylation sites. (A) All Oct4 phosphorylation sites collectively identified from three cellular contexts in this study. Phosphor-S303 (highlighted in pink) is the only phosphor site also identified in recombinant Oct4 proteins without *in vitro* PTM, indicating a low level of basal phosphorylation in bacterially-expressed Oct4. (B) Hydropathy plot for all the residues of Oct4. The phosphorylated sites at the POU domains are marked in red or blue. (C) Structural modeling showing close proximity of Oct4-T235 and -S236 to one of the target DNA strands. (D) Amino acid sequence alignment showing high conservativity of the phosphorylated sites at the POU domains.

in close proximity to the DNA strand (Fig. 3C), and therefore phosphorylation of these two residues is likely to have profound effect on the Oct4-DNA interaction. Amino acid sequence alignment further showed that all the phosphor-sites at the POU domains were highly conserved across multiple species, implicating important functional roles (Fig. 3D).

3.4. Regulatory network of Oct4 phosphorylation

To explore the regulatory mechanisms for Oct4 phosphorylation, we searched for kinases and phosphatases from Oct4 binding partners reported in the literature and predicted by the NetPhosK 1.0 Server, and attempted to build connections between those phosphorylation enzymes and all individual phosphor sites in Oct4. As shown in Fig. 4A, only a few connections have been established so far. For instance, Oct4-T235 was reported to be phosphorylated by Akt/PKB [10,15], Oct4-S236 (equivalent to mouse Oct4-S229) was phosphorylated by PKA [16], and a total of 5 sites (S111, S236, S289, S355 and T118) were phosphorylated by Erk1/2 or p38/MAPK [11,12] (Fig. 4A). Thus, Erk1/2 or p38/MAPK can phosphorylate multiple sites, either Ser or Thr, and the same Oct4-S236 site could be phosphorylated by either Erk1/2 or PKA, suggestive of cross-talk at multiple sites via different signaling pathways.

From the Oct4 binding partners reported in multiple studies [17–19], we found kinases and phosphatases regulating a variety of important cellular processes, such as cell cycle progression [20], cytoskeleton regulation, signaling pathways mediated by PKA [16], PI3K/Akt [10,15], MAPK8/JNK, p53, MAPK/ERK [12] and estrogen-related receptors (Fig. 4B). This indicated that Oct4 can be regulated by many signaling pathways to make the cell fate decisions in response to various extracellular and intracellular cues.

4. Discussion

In this study, we developed a cell-free system-based *in vitro* PTM approach to accommodate the identification of phosphorylation sites in Oct4 exposed to various cellular contexts. The main

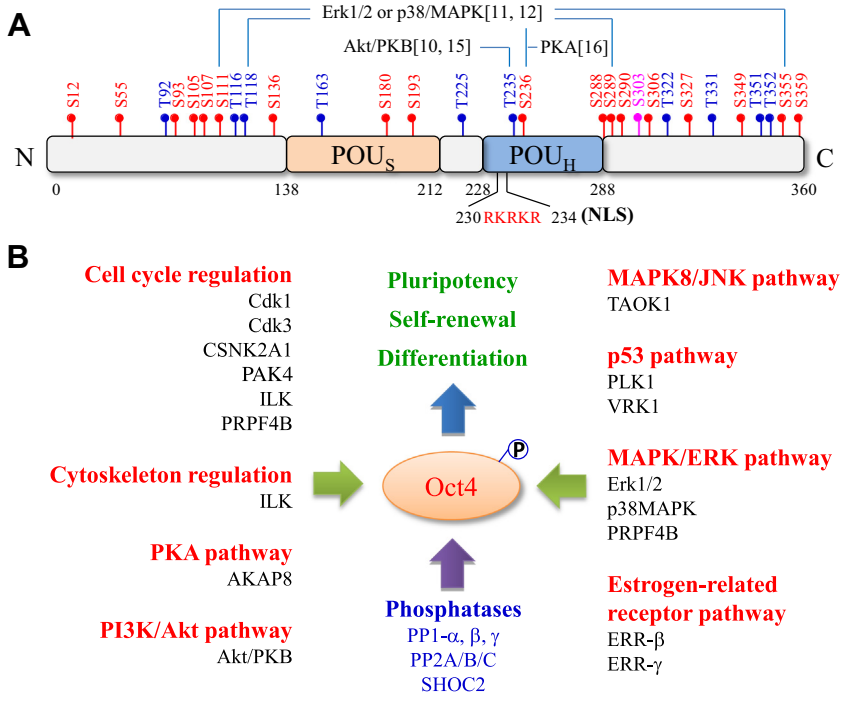

Fig. 4. Regulatory network of Oct4 phosphorylation. (A) The protein kinases and their corresponding phosphor sites in Oct4 that have been reported in the literature. (B) Signaling pathways and binding partners that could potentially regulate Oct4 phosphorylation profiles.

advantages of our system are as follows: First, by using bacterially-expressed Oct4 protein, it offers sufficient quantity and quality of the target protein for mass spectrometric analysis of the PTM profiles. It overcomes the main limitations for obtaining enough target proteins: a high affinity antibody to immunoprecipitate (IP) sufficient endogenous target proteins, or a highly efficient gene delivery system to allow ectopic expression of target proteins in certain hard-to-transfect cells. Second, it is highly cost-effective, saving considerable costs for cell cultures, transfection reagents, antibodies, and the beads for IP or pull-down. For example, compared to the Brumbaugh et al. study where approximately 1×10^8 H1 cells were used for each mass spectrometric measurement, only 1 mg of the H1 whole cell lysates (equivalent to 5×10^6 H1 cells) were required for each measurement in our experimental system. Third, it offers high flexibility of choosing the cell types and cell statuses for investigation, and can examine hard-to-transfect primary cells, or even hard-to-obtain clinical tissue samples. Fourth, it is a tractable system with relatively high efficiency and reproducibility. Given all these advantages, our system would allow for a time-dependent profiling of the PTMs in response to certain stimuli in a given cell type, or a systematic profiling and comparing of PTMs in different cellular contexts.

Inevitably, there are a number of uncertainties and limitations with our system. For example, in intact cells, protein PTMs occur in spatially-segregated subcellular compartments and also in a sequential manner as a result of signaling cascades. However, in the whole cell lysate-based cell-free system, the boundaries among the subcellular compartments are removed, and the target proteins are exposed to an environment that is perhaps not available *in vivo*. In addition, in order to manifest the phosphor sites, phosphatase inhibitors are usually added into the cell lysates or extracts, which probably alters the balance between a kinase and its corresponding phosphatase in an intact cellular environment. These raise the possibility that some PTM sites identified with our system may be artificially represented. It remains to be seen if such artificial conditions may account for the non-overlapping phosphor sites identified in our current study and by Brumbaugh et al. [12]. A possible and partial solution might be to incubate the bacterially-expressed target proteins with a particular subcellular fraction (e.g., nuclear extraction for Oct4) in the absence of any phosphatase inhibitors. Furthermore, the basal PTMs occurred at the bacterially-expressed target proteins need to be taken into account. Although our current results showed that the basal phosphorylation level of *E. coli*-derived Oct4 proteins is minimal, other PTMs may not be the same case, and therefore appropriate negative controls would be important for proper interpretation of the mass spectrometric results.

Cellular contexts can critically affect the PTM profiles and hence the behaviors and functions of the target proteins. For instance, phosphorylation of Oct4-T235 by Akt in NCCIT cells stabilized the Oct4 protein and promoted self-renewal and pluripotency [10], while the identical phosphorylation in HEK293A cells promoted the degradation of Oct4 [15]. A more thorough PTM profiling may offer important clues to this discrepancy. Our current study identified a number of phosphor sites commonly or differentially regulated in different cellular contexts that could form specific PTM barcodes. Thus, the 6 phosphor sites commonly present in all three cellular contexts may correspond to fundamental regulatory pathways such as MAPK/ERK, PI3K/Akt and cell cycle pathways. The 7 phosphor sites shared between H1 and NCCIT cells may constitute a self-renewal and pluripotency PTM barcode. Likewise, the 2 sites shared by NCCIT and U87 cells (S136 and S359) might be part of the carcinogenesis PTM barcode. Furthermore, Oct4-T331 was a unique phosphor site in NCCIT cells that may serve as a biomarker for testicular germ cell tumors [21], and Oct4-T322 that was uniquely phosphorylated in U87 cellular context might be associated with the formation of glioblastoma stem-like cancer cells [22]. Future work is required to examine the above assumptions. Although we only focused on phosphorylation of Oct4 in this study, the experimental system developed here may be adapted to other PTMs and other proteins if individualized optimization and appropriate controls can be applied. Such system is well suited to deciphering the PTM barcodes or the PTM atlas of most target proteins exposed to various cellular contexts.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.12.043.

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