Electronic Supporting Information

Proteomic analysis of cisplatin- and oxaliplatin-induced phosphorylation in proteins bound to Pt-DNA adducts

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1. Supplementary figures and tables

Table S1. IC_{50} values (μM) of cisplatin and oxaliplatin for different cell lines at 24 h.

Compound	cisplatin	oxaliplatin
HCT116	16.4 ± 1.8	6.2 ± 0.5
HepG2	7.5 ± 0.4	12.8 ± 0.3
PANC-1	37.1 ± 1.7	31.5 ± 3.0
L02	21.9 ± 0.9	17.3 ± 1.4

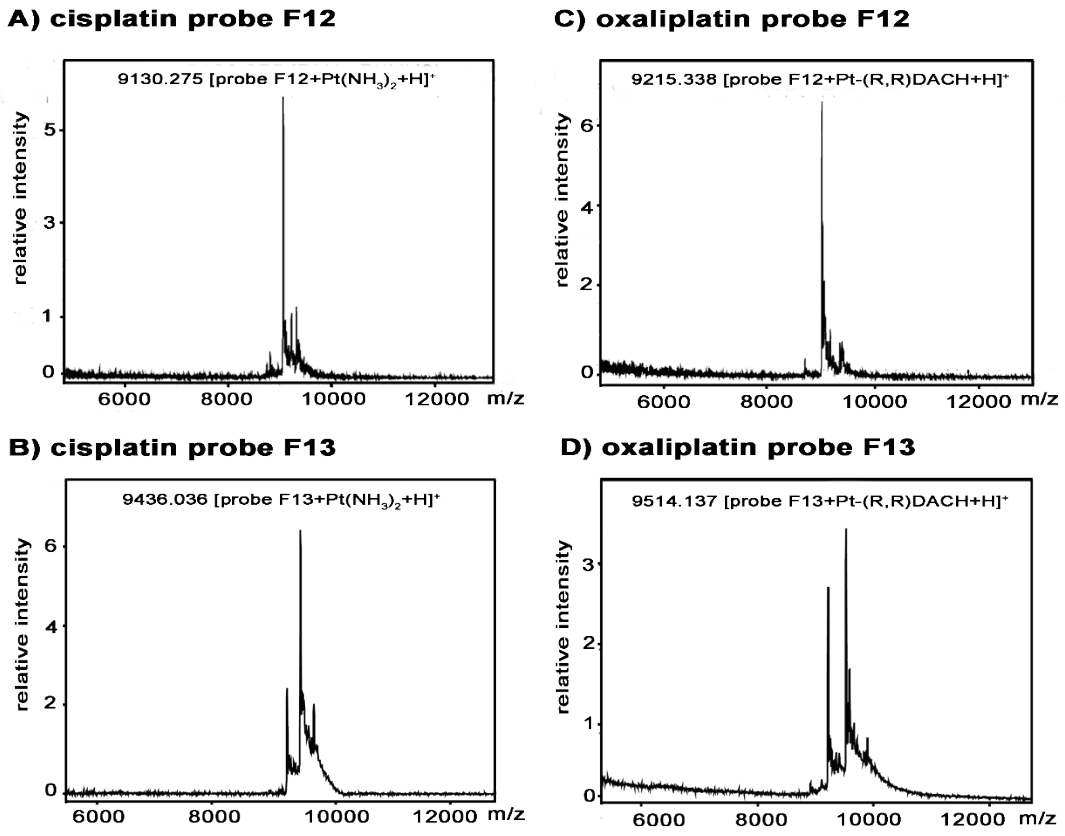

Figure S1. MALDI-TOF mass spectra of Pt-modified DNA strands containing 1,2- and 1,3-crosslinked adducts with cisplatin and oxaliplatin. The calculated mass of A-D is 9131.918, 9436.118, 9212.048, 9516.248, respectively.

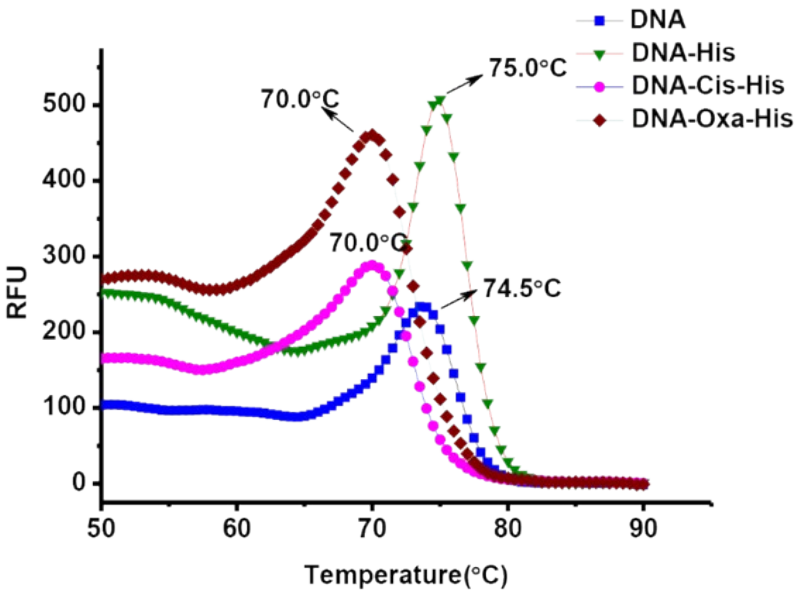

Figure S2. Melting curves of double-stranded DNA (DNA), dsDNA with poly(histidine) modification (DNA-His), poly (His)-modified dsDNA with cisplatin and oxaliplatin adducts (DNA-Cis-His and DNA-Oxa-His). Platination lowered the melting temperatures by 5°C.

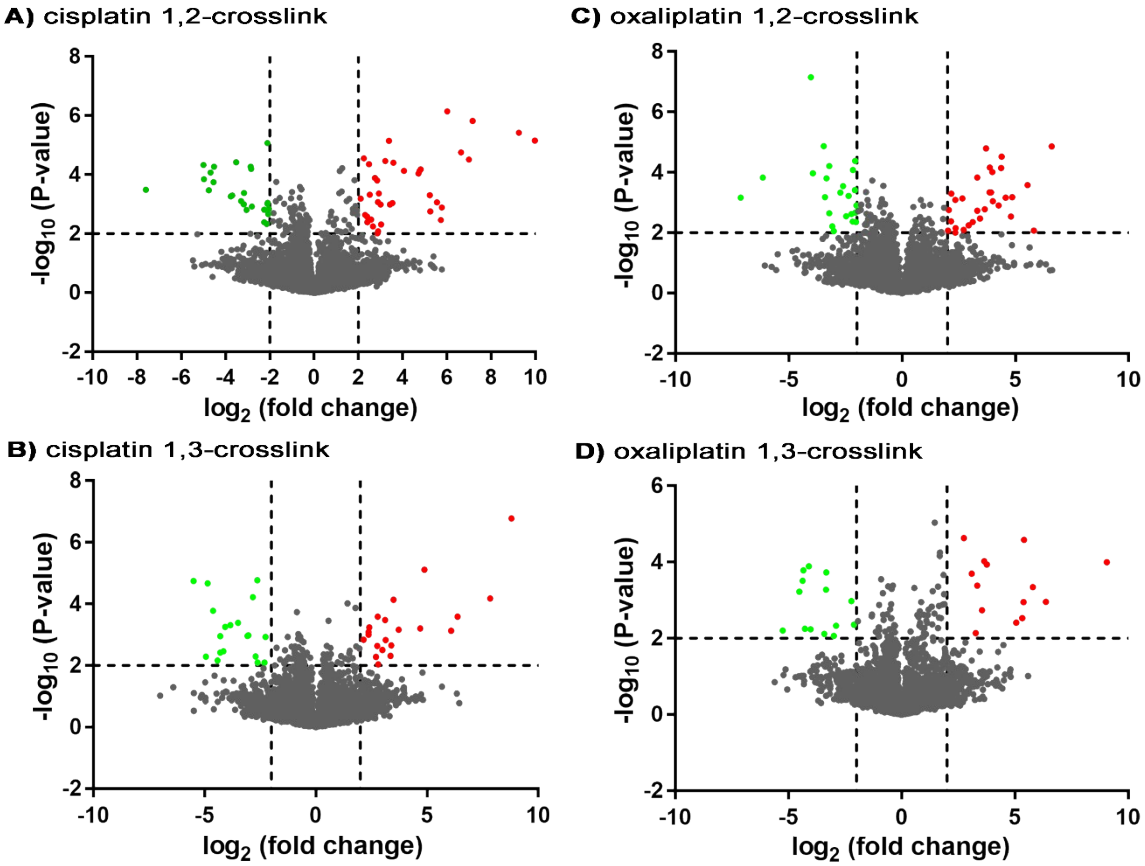

Figure S3. Volcano Plot of phosphorylated peptides identified by four Pt-DNA probes. Only peptides that meet the following two criteria were chosen for further analysis: i) change in abundance is at least 4-fold; ii) P value is less than 0.01. Green and red spots represent down- and up-regulated phosphorylated sites, and red spots represent up-regulated phosphorylated sites, respectively.

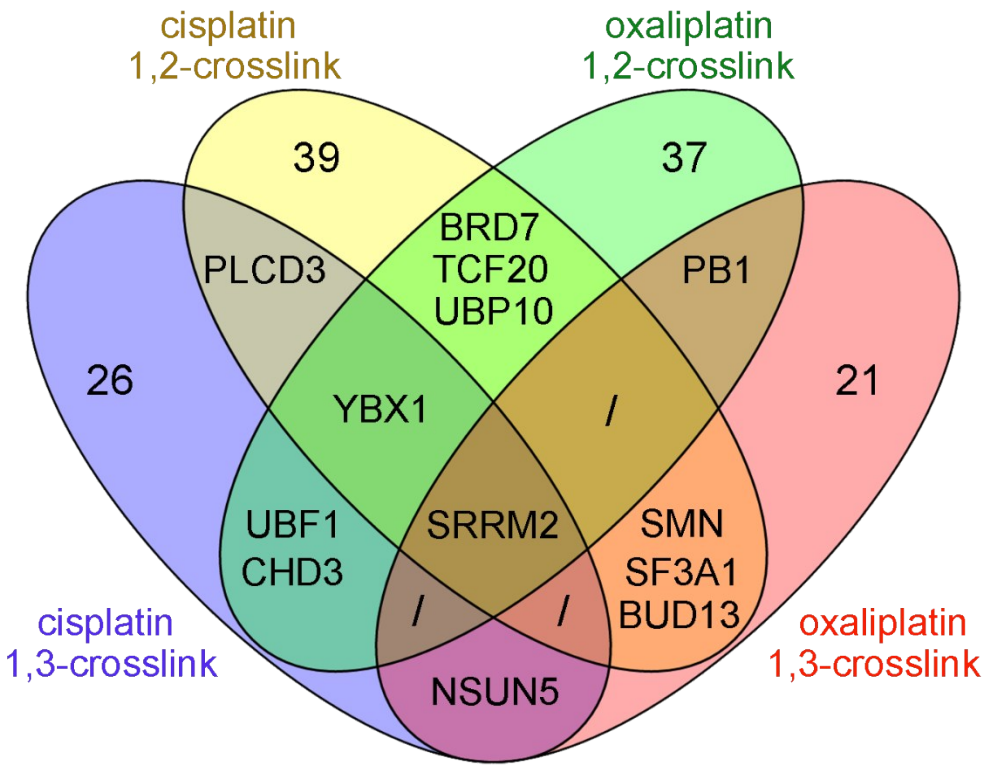

Figure S4. Venn diagram showing the distribution of the phosphorylated proteins identified by the four Pt-DNA probes. Proteins that are identified by at least two probes are listed.

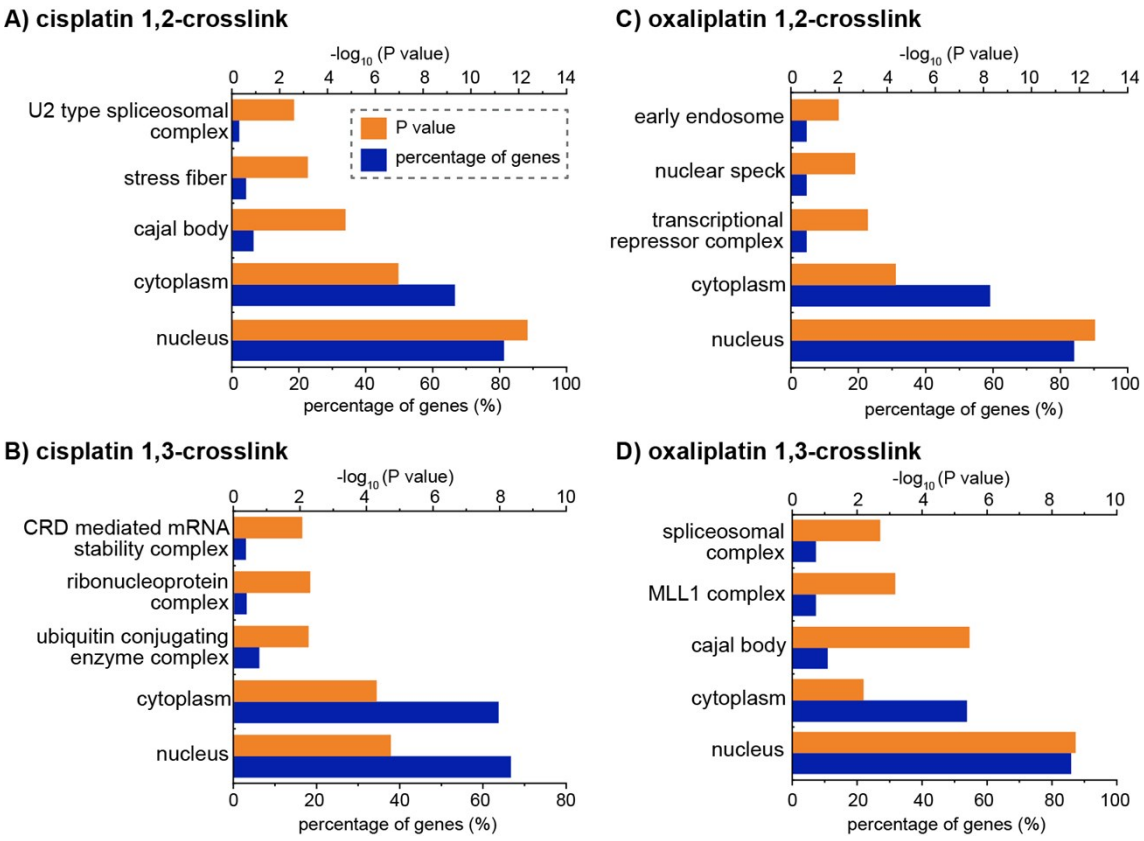

Figure S5. Gene ontology analysis of cellular component on differentially phosphorylated proteins identified by four Pt-DNA probes. Due to the dual distribution in both nucleus and cytoplasm of certain proteins, the fraction of cytoplasmic localization appeared higher and the sum of percentage from all categories may exceed 100%.

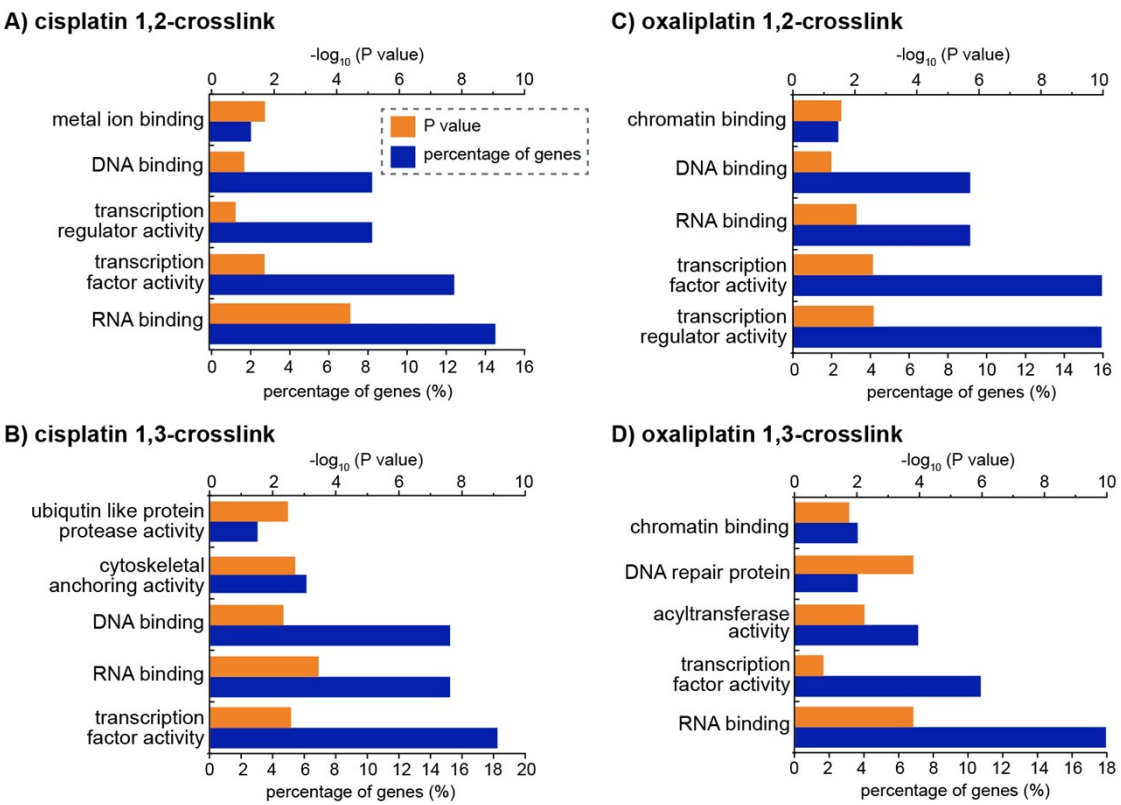

Figure S6. Gene ontology analysis of molecular function on differentially phosphorylated proteins identified by four Pt-DNA probes. As expected, majority of the identified proteins was involved in DNA- and RNA-binding and transcription factor/regulator activities.

2. Experimental section

Materials

DNA oligonucleotides were purchased from Shanghai Generay Biotech. Cisplatin and oxaliplatin were purchased from Shandong Boyuan Pharmaceutical. GMBS (N-γ–maleimidobutyryloxysuccinimide ester) was purchased from Speed Chemical. The poly(histidine) peptide was purchased from Genscript. The Ni-NTA agarose beads were purchased from GE Healthcare. The ZipTip C18 micro-column was purchased from Millipore. All cell lines including human colon carcinoma cells HCT116, liver cancer HepG2, pancreatic cancer PANC-1 and normal liver L02 were purchased from American Type Culture Collection. Cell culture reagents were purchased from Nanjing Sunshine Biotechnology and KeyGen Biotech. All the other solvents were HPLC-grade and were purchased from J&K Scientific.

DNA platination

To prepare the diaqua cisplatin and oxaliplatin derivative, 1.97 equiv. of $AgNO_3$ was mixed with cisplatin and [$PtCl_2$(1(R),2(R)-DACH)] in water in the dark, respectively. After shaking at 37°C for 4 h, the mixture was filtered using 0.22 μm filter membrane to remove AgCl. The filtrate was incubated in the dark for another 12 h and filtered again. The activated platinum solution was mixed with oligonucleotides S1 or S2 in 10 mM $NaClO_4$ at 37°C in the dark for overnight. The final concentration of platinum and DNA in the mixture was 100 μM and 50 μM, respectively. The mixture was concentrated to 50 μL using 3 kDa ultrafiltration tube. The platinated DNA were purified by 20% 8 M urea PAGE. Platinated DNA band was excised from gel and incubated in 20 mM Tris (pH 8.0) in the dark at 37°C for 8 h. The mixture was concentrated to 50 μL using 3 kDa ultrafiltration tube. All the platinum-DNA adducts were fully characterized by MALDI-TOF.

S1: 5’-TCTCTTCCTCCTCTGGTCTCCTCTCTCTCTCT-3’

S2: 5’-TCTCTTCCTCCTCTGTGTCTCCTCTCTCTCT-3’

DNA-peptide conjugation

10 OD oligonucleotides with 5’-amino modification were mixed with GMBS dissolved in acetonitrile. The final concentration of GMBS and DNA was 280 μM and 560 μM respectively. After incubation at 37°C for 50 min, the mixture was diluted by 20 mM NH_4Ac to 2 mL to remove free GMBS. The mixture was concentrated to approximately 50 μL. Next, 0.5 equiv. of peptide was incubated with the fraction in PBS buffer in the dark at 37°C for 10 min. This step was repeated for another 4 times. Finally, 20 mM NH_4Ac was added to

remove free peptide. The DNA-peptide conjugate was purified on a Histrap HP affinity column.

Probe annealing

DNA-peptide conjugate was mixed with 1 equiv. platinated DNA in an annealing buffer including 20 mM Tris (pH 8.0), 5 mM $MgCl_2$, 100 mM NaCl, and 50 mM KCl. Double-stranded DNA annealing was conducted by first heating the mixture to 95°C and then cooling slowly to 4°C (approximately 2 h).

MALDI-TOF MS

The purified probe was characterized by MALDI-TOF. After desalted with ZipTip pipette tip C18, 1 μL probe was mixed with 1 μL HPA matrix at Anchorchip. The target plate was dried and measured with a Bruker Autoflex II MALDI-TOF MS, using a 337 nm N_2 laser source, linear positive ion mode, and a delay time of 320 ns.

Thermal stability

The probes were divided into 10 mL aliquots and mixed with 1:1000 diluted SYBR green I dye solution, ensuring that the final concentration of all ingredients were 10 μM. The experiments were performed on CFX96 Touch Real-Time PCR Detection System, with the temperature increasing from 25°C to 95℃ at step of 0.1°C per minute. The fluorescent signal was recorded with the increasing temperature. The derivatives of the curve gave melting temperature of dsDNA probe.

Cell culture and protein extraction

HCT116 cells were cultured in McCoy's 5A media while HepG2, PANC-1 and L02 cells were cultured in DMEM media. All cell cultures were supplemented with 10% FBS and maintained at 37°C in a 5% CO_2 atmosphere. For proteomic study, HCT116 cells were treated with 10 μM platinum drugs for 24 h. For immunoblot analysis, HCT116 cells were treated with two concentrations (5 and 10 μM) of platinum drugs for 24 h; HepG2, PANC-1 and L02 cells were treated with 10, 35 and 18 μM platinum drugs for 24 h. After removing the medium, the cells treated with platinum complex were harvested by scraper with 3 mL Hypotonic Buffer (20 mM Tris-HCl pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$) followed by resting on the ice for 15 min and adding 150 μL 10% NP-40. A 10-sec vigorous vortex was performed to break cytomembrane. The nuclei were obtained by centrifugation at 3,000 rpm for 10 min. Next, a homogenizer was used to break nuclear membrane with 15 strokes every

10 min on ice. After centrifugation at 12,000 rpm at 4°C for 10 min, supernatants were transferred to clean tubes and used as the nucleoprotein for the next step.

Affinity isolation of nucleoproteins

The dsDNA probe (platinated or not) was incubated with 60 μL Ni-NTA sepharose beads at 4°C for 30 min in binding buffer (20 mM Tris pH 7.4, 100 mM NaCl). This incubation allowed the probes to bind to the beads. Freshly prepared nucleoprotein extracts (with 75 mM imidazole, pH 7.4) were added into sepharose beads. The mixture was gently rotated at 4°C overnight, followed by centrifugation at 3,000 rpm for 1 min to remove the supernatant. The beads were then washed three times with binding buffer containing 75 mM imidazole. After removing the supernatant, the beads were incubated with 100 μL SDS loading buffer at 95°C for 3 min. Supernatant was collected as much as possible.

Phosphorylated peptide sample preparation

Nucleoproteins isolated by dsDNA probes were digested by the Filter-Aided Sample Preparation (FASP) method. For phosphorylated peptide enrichment, peptides obtained from FASP was incubated with TiO_2 beads. Briefly, the enzymatically digested samples were first treated with 0.1% formic acid (FA) and then treated with TiO_2 micro-treating tips. The tip was first rinsed with 100% acetonitrile and equilibrated with 50% acetonitrile containing 0.1% FA. Sample was adsorbed onto the packing by pipetting up and down for 5 times using a pipette tip, followed by 50% acetonitrile containing 0.1 M KCl and 0.1 % FA immersion. Finally, the eluted peptides were reacted with 0.5% ammonia in water to neutralize the FA.

LC-MS/MS Analysis

The enriched phosphorylated peptides were analyzed by mass spectrometry after liquid phase separation. An online liquid chromatography-tandem mass spectrometry (LC-MS/MS) setup consisting of an EasynanoLC system and an Orbitrap Fusion Tribrid mass spectrometer (Thermo Scientific, Germany) equipped with a nano-electrospray ion source was used for all LC-MS/MS experiments. The composition of two mobile phase solutions used for the chromatographic separations was 2% ACN/0.1% FA for buffer A and 98% ACN/0.1% FA for B in water. The flow rate is 300 nL/min and the gradients as follows:

Time(min)	0	8	58	70	71	78	80
B%	5	8	22	32	90	90	5

The source was operated at 2 kV. For full MS survey scan, AGC target was 3e6. MS and MS/MS scans were acquired in m/z range of 300-1400 m/z with resolution of 70,000. The 50 most intense peaks with charge state 2 and above were selected for sequencing and

fragmented in the ion trap by HCD with normalized collision energy of 27 %. Exclude isotope item was on and dynamic exclusion time was set as 18 s. The secondary collision voltage is automatically adjusted according to the charge condition of the parent ion and the m/z ratio.

Database search and analysis

Raw MS files were performed against uniprot database using Maxquant software (version 1.5.2.8). The fixed modification was C(carbamidomethyl) and the variable modification was M(oxidation), protein N-term(acetyl) and S/T/Y(phosphorylation). The initial maximum precursor and fragment mass deviations were 6 ppm and 0.5 Da. The false discovery level in PSM and protein was 1%. Match between runs was used and minimum score for modified peptides was 40.

Western Blotting

The nuclear protein extracts were resolved on 12% SDS-PAGE and transferred to PVDF membranes in Towbin buffer containing 0.033% SDS. The PVDF membranes were blocked in blocking buffer (5% BSA; 0.1% Tween-20; TBS) at ambient temperature for 1 h. The primary antibodies in appropriate dilutions were incubated with the membranes at 4°C overnight. The membranes were washed with TBST (0.1% Tween-20; TBS) and incubated with peroxidase-conjugated secondary antibody in washing buffer for 1 h. After washing three times with TBST, the blots were visualized by enhanced chemiluminescence. The membranes were first probed with phosphorylation-specific antibodies including YBX1-S102phos and UBF1-S484phos. After visualization, the membranes were washed in stripping buffer (Tris pH 6.7, 0.07 M SDS, 0.1 M mercaptoethanol) at 50°C for 0.5 h, blocked again and incubated with anti-human YBX1 or anti-human UBF1 antibody. Total YBX1 or UBF1 proteins were detected after secondary antibody incubation and chemiluminescence imaging.