Cisplatin reprogramming of protein phosphorylation

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ABSTRACT

Cisplatin is a DNA-targeting chemotherapeutic. We have utilized a forward chemical genetics strategy to map cisplatin-damaged genes (CDGs) in A549 human lung cancer cells. Importantly, we found that cisplatin targets 77% of protein kinase genes and 96% of protein phosphatase genes in the human genome, suggesting that cisplatin can reprogram protein phosphorylation genome-wide. Further, we profiled the differentially expressed proteins (DEPs) and differentially phosphorylated proteins (DPPs) in NCCIT human testicular cancer cells subjected to cisplatin treatment. We demonstrated that ca.75% of downregulated proteins are encoded by CDGs, which caused the changes in the expression of a series of protein kinases and protein phosphates, leading to significant changes in the phosphorylation level of >600 proteins in NCCIT cells, and evidencing the proteome-wide reprograming of protein phosphorylation by cisplatin. More importantly, the reprogrammed protein phosphorylation activated the G2/M DNA damage checkpoint regulation and ATM signalling pathways, causing cell cycle arrest at G2/M phase and allowing the cell apoptosis/death via cisplatin inhibition of protein synthesis.

INTRODUCTION

Cisplatin, *cis*-[$PtCl_2(NH_3)_2$], is a first-line chemotherapeutic drug, in particular by systemic administration in combination therapy for solid tumours[1,2]. After hydrolysis in cells, cisplatin binds strongly to a variety of biological molecules, e.g., proteins, thiol-containing small molecules, and nucleic acids[3,4]. The major target is believed to be nuclear DNA (nDNA)[3,5], although the formation of nDNA adduct is not the sole determinant of cisplatin-induced cytotoxicity[6] and has been linked to side effects and toxicity[7]. The formation of 1,2-intrastrand -GG-/-AG- (90%) adducts, 1,3-intrastrand -GNG- adducts and interstrand crosslinks unwinds and bends DNA helices, inhibiting DNA replication and transcription, and in turn inducing cell apoptosis and death[3].

The human high mobility group box 1 (HMGB1) protein is a non-specific DNA binding protein, and well recognized for its specific binding to cisplatin-crosslinked double–stranded DNA in both cell-free media[8,9] and in cells[9-11]. HMGB1 functions as a protector of cisplatin-induced DNA damage from nucleotide excision repair (NER)[5], and may serve as a negative transcription cofactor to block the binding of transcription factors such as Smad3 to DNA, as revealed by single cell imaging[11]. Box a domain of HMGB1 has been demonstrated to have a higher affinity than box b for 1,2-cisplatin-crosslinked DNA[9,12]. Based on such a high affinity of HMGB1a domain for cisplatin-bound DNA, Shu et al. developed a "cisplatin-seq" method to map cisplatin lesion sites with a single base resolution throughout the whole genome[12]. They revealed that cisplatin was more likely to attack mitochondrial DNA (mDNA) than nDNA, and that the cisplatin damage was enriched within promotors and regions harbouring transcription termination sites[12].

Despite technical challenges arising from the extremely low incidence of cisplatin damage on DNA on a genome-wide scale[13], and impacts of cisplatin damage on the PCR-based sequencing, e.g., stalling PCR process and making base mismatch, there have been a number of innovative strategies that provide insights into the cisplatin damage sites at the genome-wide level[14]. Apart from the cisplatin-seq approach described above, Hu et al. developed a set of methods, termed "damage-seq" and "eXcision Repair-seq" (XR-seq), to map, at single-nucleotide resolution, the location and repair of cisplatin lesions in genomic DNA adducts[15], respectively. Their mapping data demonstrated that cisplatin damage was uniform and dominated by the underlying sequence, but the repair of cisplatin lesions was heterogeneous throughout the whole genome and affected by multiple factors such as transcription and chromatin states. Therefore, they thought that the overall effect of cisplatin damage in the genome is not primarily decided by cisplatin damage but by the effectiveness of DNA damage repair[15]. However, similar to the cisplatin-seq approach, the damage-seq method was not able to assign cisplatin lesions to specific gene loci. To address this issue, the same group integrated their "damage-seq", "XR-seq" and RNA-seq approaches to study comprehensively the genome-wide profiles of cisplatin lesions in mouse organs[16]. They revealed that the expression of 12 genes was commonly downregulated due to cisplatin damage across mouse kidney, liver, lung, and spleen[16]. However, the combined use of transcriptomics strategies did not produce a full map of cisplatin lesions throughout the whole genome due to the tissue specificity of transcriptomics.

The aim of the research reported here was to map the exact genomic distribution of cisplatin-damaged gene loci by using forward chemical genetics strategy[17,18]. The biotin-streptavidin system is often applied to enrich and isolate target DNA and proteins at ultra trace levels in complicated biological samples[17-21]. However, to avoid the effect of biotin modification on the chemical properties and pharmacological functions of the drug cisplatin itself, we combined HMGB1 box a (HMGB1a)-based affinity microprobes and high throughput sequencing to screen gene lesion loci induced by cisplatin on a whole-genome scale (Fig. 1), and to correlate the gene lesions with the action of the drug. Significantly, we showed that cisplatin attacks 400 out of 518 human putative protein kinase genes (77%)[22] and 131 out of 136 protein phosphatases (96%) in human genome[23], suggesting that cisplatin may reprogram protein phosphorylation of cells to exert its pharmacological function. Our further global quantitative proteomics and phosphoproteomics analysis of NCCIT human testicular cancer cells subjected to cisplatin treatment evidenced that it was cisplatin reduced

reprogramming of protein phosphorylation that caused cell cycle arrest at G2/M phase, and led to cell apoptosis and death due to cisplatin-induced inhibition of protein synthesis. These findings provide new insights into the mechanisms of action of this front-line anticancer drug and new targets for drug design.

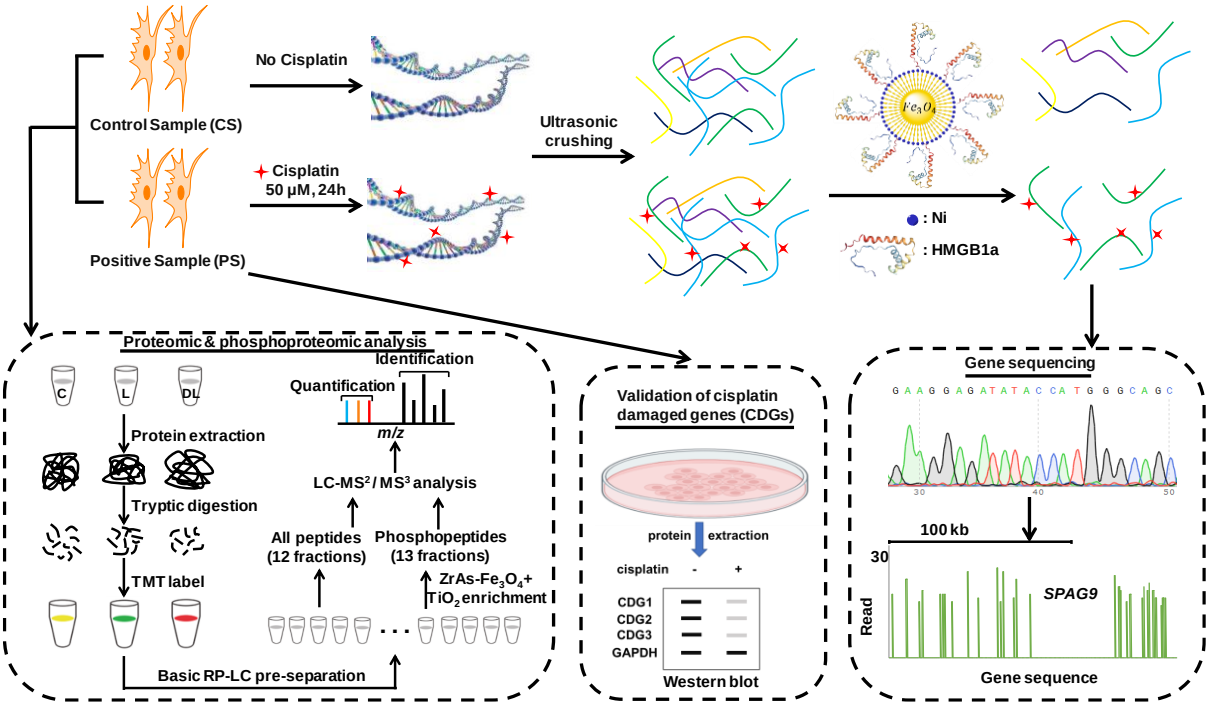

Fig. 1. Diagrammatic illustration of the workflow for capture and mapping of gene loci attacked by cisplatin. The procedure includes cell culture, extraction and fragmentation of genomic DNA, capture, sequencing and alignment of cisplatin-damaged DNA fragments, identification of cisplatin-damaged genes (CDGs), validation of CDGs by Western Blot assays, and MS-based quantitative proteomics and phosphoproteomics analysis.

RESULTS

Enrichment and sequencing of cisplatin-damaged DNA

We functionalized Ni-based magnetic microbeads with HMGB1a to assemble affinity microprobes for capturing and isolating 1,2-cisplatin-crosslinked DNA[3,9] from genomic DNA fragments derived from human A549 non-small cell lung cancer cells treated with 50 μM cisplatin for high throughput Illumina® sequencing (Fig. 1). Details of the expression and purification of HMGB1a, construction of affinity microprobes, cell harvesting, and DNA extraction and fragmentation are provided in Methods and Details.

Since HMGB1 binds to cisplatin-crosslinked DNA with high affinity, but can also bind to bent native DNA[3], we extracted and fragmented genomic DNA from A549 cells with (positive samples) and without (control samples) exposure to cisplatin for DNA sequencing. ICP-MS analysis for Pt showed that the platination level of genomic DNA extracted from cisplatin-treated cells reached 320 Pt atoms per 10^6 base pairs (Fig. 2A), consistent with our previous report[13]. Furthermore, gel-electrophoresis data demonstrated that the size of the DNA fragments gained from both the positive sample (PS) and the control sample (CS) ranged from 250 to 1000 bp, but the number of larger DNA fragments from positive samples was less than those from control samples (Fig. 2B). This is likely ascribed to platination, which could render genomic DNA more susceptible to sonication and more prone to aggregation[24]. Given that HMGB1a binds to both non-platinated and platinated DNA, it is not surprising that the same amount of the microprobes captured similar amounts of DNA fragments from positive and control samples (Extended Data Table 1). However, the subsequent sequencing revealed that there were significant differences between the DNA fragments enriched from the positive and control samples.

Next, we constructed DNA libraries based on the captured DNA fragments. Considering that cisplatin damage on DNA may stall DNA replication and/or cause base mismatch[14,25]. In this work, we tried to remove Pt bound to DNA bases by using excess of GSH (glutathione) because Pt(II) has a high affinity for S-donor ligands[26]. However, we did not observe significant difference between the amplification rates of GSH-treated and intact platinated DNA (data not shown). Moreover, previous reports also demonstrated that the replication of DNA can still reach a similar level for cisplatin-damaged DNA compared to intact DNA by extension of the replication time[25]. Therefore, we extended the PCR expansion time of both positive and negative samples to 30 min. The results showed that the DNA sequences in DNA libraries of the positive samples and control samples have similar sizes (Fig. 2C). This means that possible Pt-modification-induced bias for PCR amplification of DNA was circumvented (details given in Methods and Details), which minimized the risk of false negative results from gene mapping. Indeed, the total read counts and total base counts of the positive samples did not show a pronounced difference from those of the control samples. However, because cisplatin prefers to crosslink -GG- sites on DNA, the GC base-pair counts of the positive samples accounted for a slightly higher ratio to the total base counts than those of the control samples (Fig. 2D; Extended Data Table 1). Consequently, the mapping rates of total reads in proper base pairs of positive samples were slightly lower than those of control samples (Fig. 2E; Extended Data Table 1). Nevertheless, there are no differences in the

total mapping rates (≈99%) and unique mapping rates (>91%) of genomic DNA fragments from both positive and control samples (Fig. 2E; Extended Data Table 1).

To identify cisplatin-damaged genes (CDGs), we compared the total reads of all peaks mapped to a gene in the positive sample with those of all peaks mapped to the same gene in the corresponding control sample. If the ratio, which refers to the fold-enrichment (FE_P) of a peak mapped to a gene, was ≥ 1.5, the peak was counted to the gene. The fold-enrichment (FE_G) of a gene was the sum of the FE_P of all peaks mapped to the gene (more details given in Methods and Details). Notably, to eliminating the bias arising from the different total reads of all peaks mapped in the positive and corresponding negative samples, we used the normalized value of the read of a peak to the total read of all in the sample (Extended data Table 1). Following this method, we mapped 246,424 and 362,594 peaks with $FE_P \geq 1.5$ from replicates 1 and 2, respectively. As shown in Fig. 2F, the number of peaks mapped to promoter region, which is located at 3k bp upstream to a gene, accounts for the highest percentage (32.48%) of all peaks mapped genome-wide. Considering the length of each region in genome, the coverage of peaks mapped to the promotor region of all CDGs accounts for 82.5% of the total length of promotor region 1 (≤ 1 kb), 35.0% of promotor region 2 (1-2 kb), 27.8% of promotor region 3 (2-3 kb), respectively (Fig. 2G). These coverages are much higher than those of the peaks mapped to other regions, e.g. exon and intron regions. These results evidence that cisplatin damage was enriched at the promotor region, in particular, at the region close to the encoding region, in line with previous reports[12]. Since HMGB1a binds to double-stranded DNA over 5 base-pairs[3], we calculated the occurrence frequency of pentanucleotide motifs in all peaks containing platination sites, and found that the top 5 most-frequently platinated pentanucleotide motifs are CTGGG, CCAGG, GCTGG, GGAGG and CAGGC (Figure 2H).

When we calculated the FE of a CDG, we discarded the peaks mapped to the distal intergenic region of a gene. Following this method, we mapped totally 16216 CDGs with $FE_G \geq 1.5$ which commonly identified in the two replicates (Fig. 2I; Extended Data Table 2). Moreover, the fold-enrichment of CDGs mapped in two replicates was linearly correlated, with R = 0.804, as shown in Fig. 2J, evidencing good reproducibility of our sequencing. The four CDGs with the highest FE_G mapped in this way are shown in Extended Data Fig. 1. Remarkably, *PTPRN2,* the CDG with an FE_G of 33.3 (Extended Data Table 2), contains 82 (A/T)GGGG repeats instead of the five pentanucleotide motifs described above. Nevertheless, both the five most-frequently platinated pentanucleotide motifs and the pentanucleotide repeats in *PTPRN2* contain at least a GG site, consistent with the well-established finding that cisplatin prefers to bind to GG sites of DNA[3,5].

The 16216 CDGs are unevenly distributed over all 23 pairs of chromosomes (Fig. 2K), showing a moderate dependence on the average G-density of the chromosomes, i.e., the higher the average G-density of a chromosome, the more the genes in it were attacked by cisplatin (Fig. 2L), again consistent with the known preference of cisplatin for crosslinking -GG- sites. It is notable that due to the large difference in the length of genes, the fold-enrichment (FE_G) of a CDG calculated based on the fold-enrichment of all peaks to the gene does not correspond simply to the base coverage of all identified peaks on the gene (Extended Data Table 2). For example, 198 peaks were identified to the gene named *TRAPPC9,* which has the highest FE_G (476.4) but a low base coverage of 15.5%. In contrast, for microRNA gene *MIR182* with a FE_G of 7, only 3 peaks were identified, but the base coverage of these peaks to the whole gene of *MIR182* was 74.7% (Extended Data Table

2). With regard to this, the FE_G in this work is only an indicator of CDG-mapping confidence, but not of the degree of cisplatin damage to a gene.

Ingenuity Pathway Analysis (IPA) database was employed to perform genome-wide annotation on the mapped CDGs. The 16216 CDGs were input into the IPA data pool and matched 11952 protein encoding genes to the human genome (hg19), including 2121 enzymes, 1220 transcription regulators, 723 transporters, 591 kinases and 197 phosphatases (Fig. 2M), as well as 11 microRNA (miRNA) encoding genes of which the majority have relative low fold-enrichment as their sequences are much shorter than genomic DNA (Extended Data Table 2). Notably, among the 591 cisplatin-damaged kinase genes, 400 are protein kinase genes (PKGs, Extended Data Table 3), accounting for 77% of 518 reported putative protein kinase genes in the human genome[22]. Besides, there are 131 protein phosphatase genes (PPGs) among the 210 cisplatin-damaged phosphatase genes (Extended Data Table 4), accounting for 96% of the 136 reported protein phosphatases in human genome. Significantly, among the CDGs, 418 genes belong to the 628 prior therapeutic gene targets for cancers identified by the CRISPR/Cas9-based screening, 54 (13%) of which are targets of clinically-used drugs or candidates in preclinical development[27] (Extended Data Table 5). Furthermore, 62.5% (25) of the first group of 40 prior gene targets[27] are damaged by cisplatin, including 19 protein kinase genes (PKGs), e.g., the well-known drug targets *EGFR* and *MTOR* (Extended Data Table 5).

Mitochondrial DNA (mDNA) has been shown to be more susceptible to cisplatin attack than nDNA[6,12]. However, here we detected only 14 cisplatin-damaged mitochondrial genes (CDMGs) with low FE_G ranging from 1.5 to 2.4 (Extended Data Table 6). This difference between the previous and present work is likely ascribable to the fact that once HMGB1 is transferred outside the nucleus, it prefers to bind to circular mitochondrial DNA[28,29], which enabled the HMGB1a probe to capture more native mitochondrial DNA from the control samples in the absence of competitive cisplatin-crosslinked DNA, lowering the FE_G ($=\sum FE_P$) of CDMGs for which FE_P is the ratio of the read of an mDNA fragment in a positive sample to that in the control sample. However, considering the much lower G-density in mDNA (Fig. 2N), the possibility should not be excluded that it is the affinity of HMGB1a for native mitochondrial DNA that leads to a false positive report of the susceptibility of mDNA to cisplatin attack when the HMGB1a probe is used to enrich cisplatin-damaged DNA without use of blank controls[12].

It is notable that among the 16216 CDGs with FE_G >1.5, there are 36 pseudogenes (Extended Data Table 2). In general, pseudogenes are thought to be non-functional “junk genes”. However, increasing evidence argues that pseudogenes can act as key regulators at DNA, RNA or protein levels in various human diseases such as cancer[30,31]. Therefore, the biological implications of the cisplatin-damaged pseudogenes deserve further investigation.

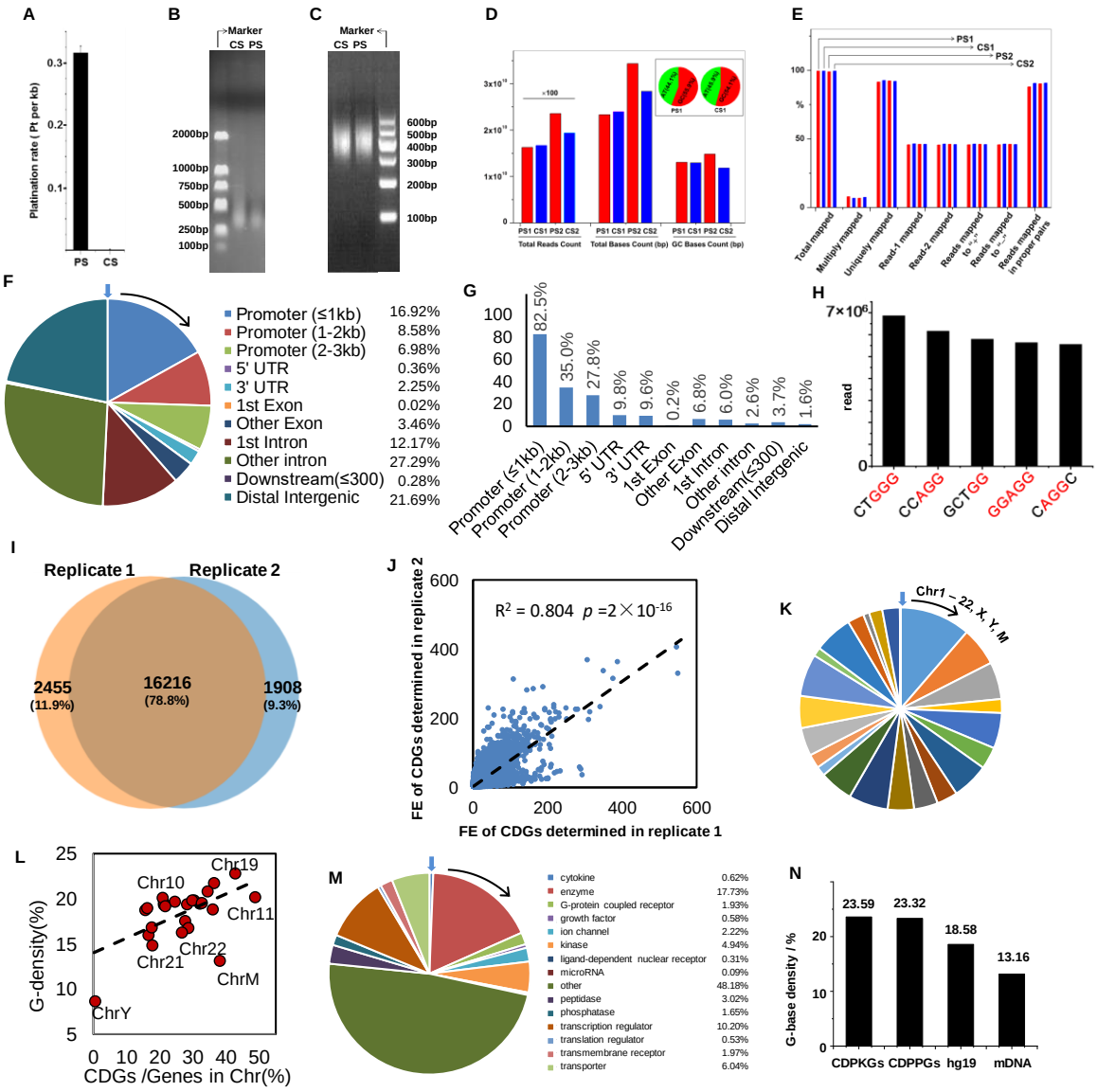

Fig. 2. Mapping of cisplatin damaged genes. **(A)** The platination level of DNA fragments extracted from positive sample (PS) and control sample (CS), determined by ICP-MS. **(B)** The size distribution of DNA fragments from positive sample (PS) and control sample (CS). **(C)** The size distribution of sequences in DNA libraries constructed from captured DNA fragments. **(D)** Total reads, total bases and GC bases mapped for positive samples and control samples. **(E)** Mapping rates counted for positive samples and control samples. **(F)** Distribution of cisplatin-damaged DNA fragments in genetic regions. **(G)** Ratio of the lengths of the cisplatin-damaged DNA fragments in specific genetic regions. **(H)** The five pentanucleotide motifs that were the most frequently detected in CDGs. **(I)** Venn Diagram of total cisplatin-damaged genes (CDGs) with FE_G >1.5 in two independent biological replicates. **(J)** Correlation analysis of the normalized fold-enrichment of CDGs in two replicates. **(K)** Distribution of CDGs over chromosomes. **(L)** Correlation of the relative G-density in the chromosome (y-axis) with the proportion of CDGs detected in a chromosome to total number of genes in human genome (x-axis). **(M)** Biological function classification of 11952 protein encoding genes cisplatin-damaged genes (CDGs) with FE_G >1.5. **(N)** Average percent of G bases in CDPKGs and CDPPGs, hg19 and mDNA.

Western Blot assays

To verify the genome-wide mapping results of CDGs, we performed classical Western Blot assays to measure the gene expression level of 14 functionally important genes with or without cisplatin exposure (Extended Data Table 7). These included 13 protein kinases and *SPAG9*, which plays a key role in a number of important signalling pathways, such as the upstream mediator of the MAPK signalling pathway and middle transducer of the PI3K signalling pathway (Extended Data Fig. 2A-B).

As shown in Fig. 3A, the expression levels of all selected genes decreased after cisplatin treatment. The changes in expression levels of 8 genes, including 7 protein kinase genes and *SPAG9*, were significant ($P < 0.05$) or very significant ($P < 0.01$) subject to cisplatin treatment (Fig. 3B). These data indicate that these genes are indeed damaged by cisplatin, strongly supporting the reliability and credibility of the gene mapping results described above. However, there is no simple correlation between the fold-enrichment of a specific CDG and base coverage (%), and fold-change in the expression level of the gene (Extended DATA Fig. 3 and Extended data Table 7), most probably due to complexity of the gene expression machinery that consists of DNA replication, transcription, translation and repair. This sophisticated system finely controls gene expression such that any alteration in this system can influence the expression of a specific gene in addition to the direct damage imposed by cisplatin on the gene. Another possible reason is that the expression level of a selected gene is too low to be characterized, e.g., PRKG1 in NCCIT cells and *EPHB1* in HeLa cells (Extended Data Fig. 4A). Additionally, the location of cisplatin induced lesions on genes is also an important factor in determining their inhibition potency.

It is notable that *SPAG9* (the sperm associated antigen 9) abundantly expresses C-jun-amino-terminal kinase-interacting protein 4 (JIP-4) in testicular haploid germ cells, and is essential for the development, migration and invasion of cancer[32]. JIP-4 as a scaffolding protein knits mitogen-activated protein kinases (MAPKs) and their transcription factors to activate specific signalling pathways[33]. These imply that cisplatin damage on *SPAG9* ($FE_G = 61$) may play a crucial role in the anticancer activity of cisplatin.

Interestingly, the full-length protein product (JIP-4) of *SPAG9* significantly decreased in abundance by 2.0-fold, and two subunits in band c&d were also decreased, but the two subunits visualized in band a&b showed pronounced increases in intensity in NCCIT cells upon the treatment of cisplatin (Fig. 3A-B). This implies that the cisplatin lesion more likely occurs in the non-encoding region and/or the linkage region of *SPAG9* than in the promotors of SPAG9 and the encoding region of its four subunits (Extended Data Fig. 4B). Indeed, when we further analysed the cisplatin-damaged sites on *SPAG9* (Extended Data Table 8), we found that (i) no cisplatin lesions were identified on the four promotor sequences; and (ii) cisplatin damaged the non-encoding regions, i.e., intron and intergenic regions, much more than the encoding regions of the gene, i.e., exons (Extended Data Fig. 4C). These make it possible that although the full-length JIP-4 protein decreased in abundance, one or two subunits of JIP4 increased in abundance. Nevertheless, the increase in abundance of subunits shown in band a&b (Fig. 3B) does not compensate the loss of full-length JIP-4.

Given that *SPAG9* specifically expresses JIP-4 in high abundance in testicular haploid germ cells, the cisplatin damage on it may account for its high cure rate in testicular cancer, which also deserves further investigation.

Therefore, we applied siRNA to silence the *SPAG9* gene (Fig. 3D), and found that silencing of *SPAG9* induced a similar ratio of early- and late-stage apoptosis in NCCIT cells as 12 μM cisplatin (Fig. 3E), though its potency to inhibit the growth of the NCCIT cell line was weaker than cisplatin (Fig. 3F). This confirmed that *SPAG9* plays an important role in the action of cisplatin as the upstream mediator of the MAPK pathway (Extended Data Fig. 2).

The expression of *MERTK* was the most strongly inhibited (FC = 32) due to cisplatin damage (Fig. 3A-B). This receptor tyrosine kinase, MERTK, phosphorylates Akt1-Y26, mediating Akt activation and survival signalling, which in turn drives oncogenesis and therapeutic resistance[34]. Thus, cisplatin damage to *MERTK* may be implicated in mechanism of action of cisplatin, also supporting that *MERTK* is a potential therapeutic target[34,35].

The expression of *MERTK*-related gene *AKT3* was inhibited 3.5-fold by cisplatin (Fig. 3A-B). *AKT3* encodes a member of the AKT serine/threonine protein kinase family. As a middle-stream regulator of IGF1 signalling, AKT3 plays a key role in signalling and regulating cell survival in insulin signalling, angiogenesis and tumour formation. Cisplatin-induced damage to this gene could trigger a series of downstream changes, inducing apoptosis and death of cancer cells[36].

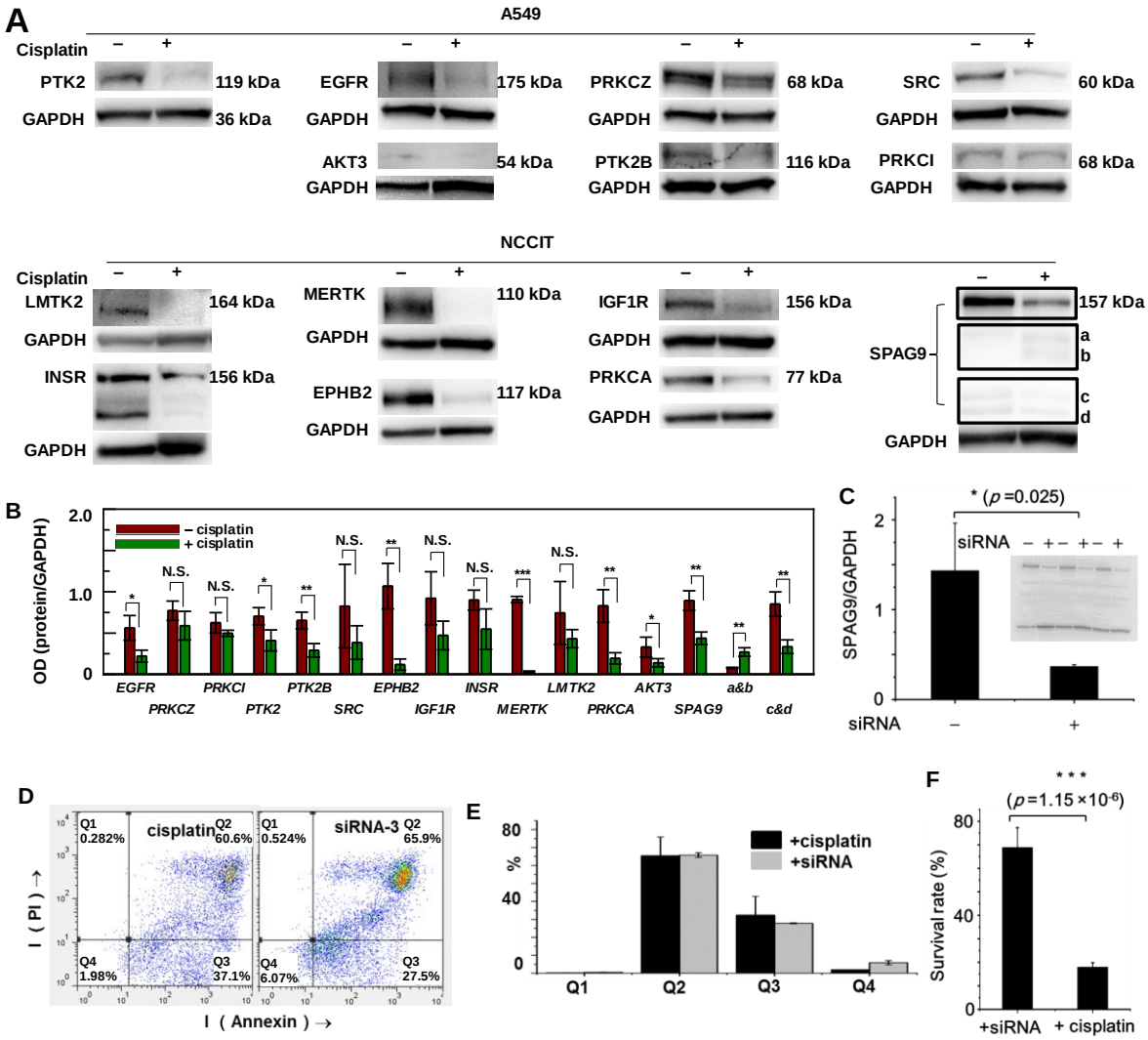

Fig. 3. Western Blot assays and siRNA silencing. **(A)** Western Blotting images of selected gene encoding proteins expressed in A549 and NCCIT cells with (+) and without (−) cisplatin treatment (12 μM). GAPDH was used as the internal reference. **(B)** Optical density (OD) ratio of a gene-encoded protein to GAPDH with (+) and without (-) cisplatin treatment. Two-tailed unpaired Student's t-test (n = 3), N.S. refers to not significant; * indicates $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. P_{EGFR}=0.0244, P_{PRKCZ}=0.2057, P_{PRKCI}=0.1615, P_{PTK2}=0.0366, P_{PTK2B}=0.0088, P_{SRC}=0.2343, P_{EPHB2}=0.0044, P_{IGF1R}=0.1000, P_{INSR}=0.0893, P_{MERTK}=2.71×10^{-6}, P_{LMTK2}=0.2453, P_{PRKCA}=0.0062, P_{AKT3}=0.0126, $P_{SPAG9\text{-full}}$=0.0049, $P_{SPAG9\text{-a\&b}}$=0.0030, $P_{SPAG9\text{-c\&d}}$=0.0061. **(C)** Western Blotting data for siRNA silencing of SPAG9. **(D)** Flow cytometric analysis of viability (Q1), early-stage apoptosis (Q2), late-stage apoptosis (Q3) and necrosis (Q4) for NCCIT cells subject to cisplatin treatment (12 μM) in comparison with siRNA silencing of *SPAG9*. **(E)** The quantitative analysis of the flow cytometric analysis. **(F)** Survival rate (%) of NCCIT cells subject to cisplatin treatment (12 μM) or siRNA silencing of *SPAG9*.

Proteomic analysis

Following the genome-wide mapping of cisplatin damaged gene loci, to address further the relationship between the cisplatin induced gene lesions and pharmacological functions and toxic side effects of the drug, we have completed an MS based global quantitative proteomics and phosphorproteomics study on the human testicular NCCIT cell line subjected to the treatment of cisplatin. Testicular cancer is commonly accepted to be the most sensitive solid tumor to cisplatin. After treating with cisplatin, the NCCIT cells were separated in to two groups, living cells, and living & dead cells, which were compared with drug free cells (control), respectively. This gave two comparison groups, L/C group and LD/C group, respectively. The proteins were extracted, digested to peptides by trypsin, separated and fractions collected by an reverse phase LC column following the program shown in Extended Data Fig. 5. The fractions were examined by LC-MS analysis equipped with an Orbitrap mass spectrometer. After the proteomics approach, the data were searched in Proteome Discoverer (PD), and found 4615 common proteins in three biological replicates (Fig. 4A). The Volcano plots exhibiting relative changes in protein abundance are shown in Fig. 4B and C, in which 350 proteins were found to be downregulated and 253 proteins were upregulated. In L/C group, 184 proteins were downregulated and 222 proteins were upregulated. As shown in Fig. 4E, more proteins up/down regulated fold change over 1.2, 1.5 and 2.0 times in DL/C groups than for the L/C, which suggests that more protein is affected in dead cells due to the damaged genes by cisplatin. We believe that compared to living cells, the dead cells were much worse influenced by cisplatin. Therefore, it is more reasonable to consider both living and dead cells in the proteomic level. In NCCIT cells treated with cisplatin, the differentially expressed proteins (DEPs) were further compared with the cisplatin damaged genes (CDGs) described above. As shown in Fig. 4D, either in the DL/C groups or in the L/C group, ca. 75% of the downregulated proteins is attributed to cisplatin induced damage on genes, suggesting that cisplatin induced gene lesions are the main factor modulating protein expression.

As mentioned above, a vast portion of protein kinase genes and protein phosphatase genes were found to be damaged by cisplatin (Extended Data Table 3 and 4). The proteomic data of L/C group was explored and 21 protein kinase and 10 protein phosphatases were found to be downregulated (p <0.05 and fold change <-1.2) and their abundance ratio were correlated with their CDGs fold enrichment (Fig. 4 E and F). For the analysis of correlation between cisplatin damaged genes (CDGs), L/C group would show more consistence, as the gene sequencing were carried out with living cells. This result suggests that the downregulation of protein kinase and protein phosphatase is largely due to the cisplatin damaged DNA (CDGS).

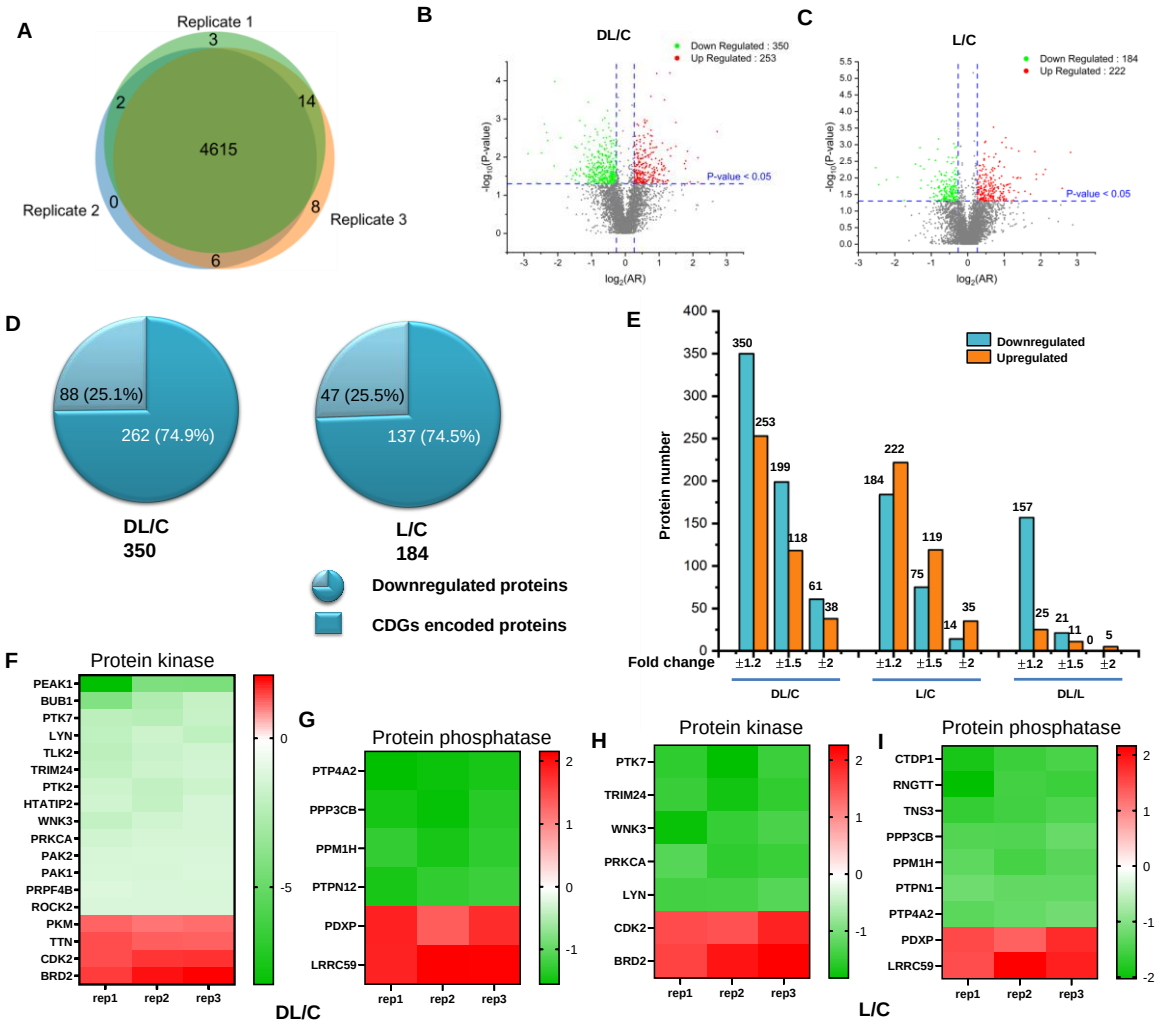

Fig. 4. Global proteomics analysis of NCCIT cells upon the treatment of cisplatin. (A) Venn diagram of total proteins identified in three biological replicates. (B, C) Volcano plots exhibit relative changes in protein abundance in (B) dead plus living cells (DL/C group) and (C) living cells only (L/C group) upon administration of cisplatin compared to the untreated cells (control), respectively. Red and green spots represent differentially expressed proteins (DEPs) with $p < 0.05$ and fold change > 1.2 (red) or < −1.2 (green). (C) Proportion of cisplatin damaged genes (CDGs) that correlated with downregulated proteins (fold change <−1.2) detected in dead & living cells and living cells compared to control cells (DL/C and L/C, respectively). (E) The number of DEPs detected between various groups of cells. Fold change is abundance ratio (AR) when > 1, and is −1/(AR) when <1. ((F-I) Heat map of protein kinases and protein phosphatases (fold change > 1.2 and <−1.2) in DL/C and L/C groups, respectively.

Phosphoproteomic analysis of NCCIT cells

As protein kinases and protein phosphatases play dominant roles in cancer cell proliferation and growth, and cisplatin damaged a large percent of genes of protein kinases and protein phosphatases, phosphoproteomic

analysis of NCCIT cells was also carried out. We firstly compared the application of two types of phosphopeptides enrichment strategy, ZrAs-Fe_3O_4 and TiO_2 functionalized beads, respectively. The results are shown in Fig. 5A and B. Using TiO_2 functionalized beads, 4210 phosphopeptides were detected, and using ZrAs-Fe_3O_4 functionalized beads, 6664 phosphopeptides were detected. When we used ZrAs-Fe_3O_4 and TiO_2 functionalized beads sequentially, 11575 phosphopeptides were detected. The results here reveal that the sequential use of ZrAs-Fe_3O_4 and TiO_2 functionalized microbeads achieve a higher enrichment efficiency of phosphopeptides, compared with the solo use of these two functionalized microbeads. Therefore, we used this method in the phosphoproteomic analysis for NCCIT cells treated with cisplatin. Similar to proteomic analysis, the phosphopeptides were firstly fractioned by an LC column, following the program in Extended Data Fig. 7A, before LC-MS analysis. After preliminary searching in the database, 11240 phosphopeptides, 2514 phosphorylated proteins out of 3448 proteins were observed in total. The distribution of phosphopeptides with the phosphorylated amino acid residues, and the different numbers of phosphorylation sites are shown in Extended Data Fig. 7B and 7C, and the numbers of the up/down regulated phosphopeptides are shown in Fig. 5C, which all suggest that our data is reliable for phosphoproteomic analysis. As shown in Fig. 5D, subjected to the treatment of cisplatin, similar or slightly higher number of phosphoproteins (295 and 306) in NCCIT cells were upregulated and downregulated with a fold-change of >1.2, or <-1.2, respectively, in dead & living cells compared to control cells (DL/C group). But in live cells compared to control cells (L/C group), much more phosphoproteins were downregulated (396) than upregulated (232) with a fold-change of >1.2, or <-1.2, respectively. The trend is the same for higher fold change such as >1.5, or <-1.5, and >2.0, or <-2.0. The downregulated phosphorylation level suggested substantial reprogramming of protein phosphorylation by cisplatin in the survived cells.

Motif analysis of significantly regulated phosphopeptides

Finally, the motif of the phosphopeptides were examined by the MotifeR algorithm, and the frequency of the phosphopeptides is shown in Extended Data Fig. 8. According to the sequence characteristics, the motifs shown in this picture were further grouped into two subgroups. Subgroup on the left comprises known kinase motifs, while subgroup on the right represents brand new phosphorylation motifs discovered in this work. These new phosphorylation motifs are very possibly due to the reprogramming by cisplatin.

Further bioinformatics analysis was carried out with String database. In DL/C group, the down regulated protein kinase (18) and protein phosphatases (6) are closely related to the up/down regulation of the phosphorylated proteins (601) (Fig. 5E). In L/C group, the down regulated protein kinase (7) and protein phosphatases (9) are closely related to the up/down regulation of the phosphorylated proteins (628) (Fig. 5F). This result provides solid evidence that cisplatin damaged significantly induced the reprogramming of protein phosphorylation.

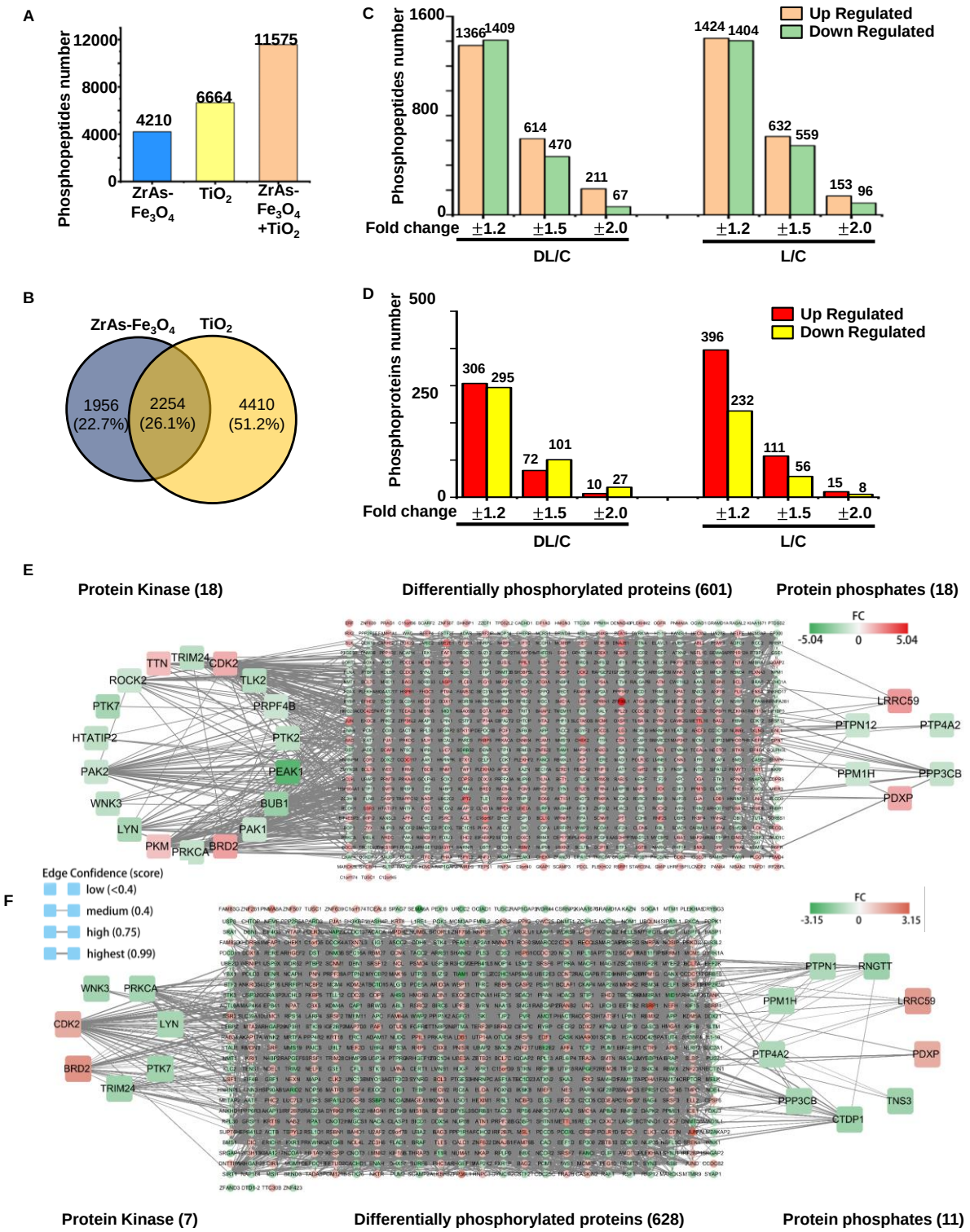

Fig. 5. Global phosphoproteomics analysis of NCCIT cells subjected to cisplatin treatment. (A) The number of phosphopeptides enriched by different methods. (B) Venn diagram of phosphopeptides enriched by ZrAs-Fe_3O_4 and TiO_2 functionalized microbeads. (C) and (D), the numbers of phosphopeptides (C) and phosphoproteins (D) with different fold changes for dead plus living cells vs control cells (DL/C group), and living cells vs control cells (L/C group) ($p < 0.05$) detected by combined enrichment of ZrAs-Fe_3O_4 and TiO_2

functionalized microbeads in NCCIT cells. (E-F) The correlation of down regulated protein kinase and protein phosphatases with the phosphorylated proteins. (E) DL/C group, (F) L/C group.

Bioinformatics analysis of significantly regulated proteins and phosphoproteins

The bioinformatics analysis for the significantly regulated proteins (SRPs) were further carried out by IPA, and the following results were obtained. The DEPs detected in DL/C group are shown in the heat map in Extended Data Fig. 6. The canonical signaling pathways for DL/C group and L/C group are shown in Extended Data Fig. 9A and 9B. By comparison of the data from DL/C and L/C group, we obtained the canonical signaling pathways for DL/L group (Fig. 9C). The DEPS induced by cisplatin significantly inhibits EIF2 signaling and processing of capped Intron-containing Pre-mRNA, but activated post-translational protein phosphorylation. Inhibition of EIF2 signaling pathway inhibits the transcription/translation of RNA, and the synthesis of proteins. Inhibition of the processing of capped Intron-containing Pre-mRNA also inhibition the expression of proteins. The activation of the post-translational protein phosphorylation is usually responsible to the signal of protein degradation. These cellular signals together result in inhibition of proliferation and death of NCCIT tumor cells, and very well explain the mechanisms of the action of cisplatin.

The signal transduction pathways of the significantly regulated phosphoproteins (SRPPs) induced by cisplatin are shown in Extended Data Fig. 10. For the DL/C group (Extended Data Fig. 10B), the SRPPs activated the G2/M DNA Damage Checkpoint Regulation pathway and ATM Signaling pathway (Fig. 6), of which both cause cell arrest on G2/M phase to enable cell apoptosis and death. It is notable that the phosphorylation of CDK1 and ATM proteins (not the intact form) were significantly upregulated, as examined by Western blot. For the L/C group (Extended Data Fig. 10A), the survived cells activated the processing of capped Intron-containing Pre-mRNA pathway and RNA polymerase II transcription, which may lead to resistance of cisplatin. These results are in good agreement with the pharmacological functions and toxic side effects of cisplatin in clinic. Collectively, we concluded that cisplatin activated the G2/M DNA damage checkpoint regulation and ATM signaling pathway by reprogramming protein phosphorylation, arresting the cells at G2/M phase.

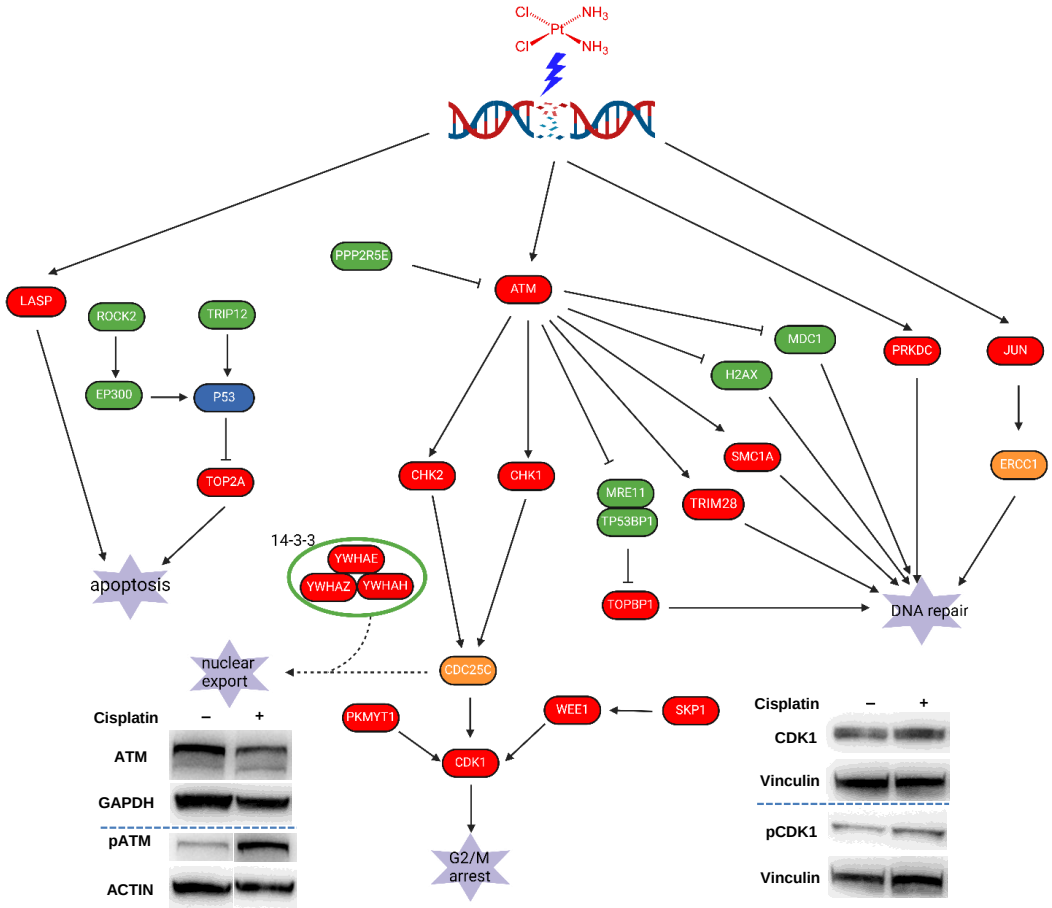

Fig. 6. Cisplatin disturbed G2/M DNA Damage Checkpoint Regulation pathway and ATM signaling pathway in significantly regulated phosphoproteins for DL/C group. Red: Observed upregulation; Green: Observed downregulation; Orange: Predicted upregulation; Blue: Predicted downregulation. Purple star: molecular functions. Solid arrow: Direct interaction; dotted arrow: indirect interaction. Insert: Western blot for the intact and phosphorylated CDK1 and ATM proteins.

DISCUSSION

Cisplatin is a genetically toxic anticancer drug, but its gene targets or precise gene loci are largely unknown, limiting the understanding of the cellular impact of DNA damage induced by the drug. In 2016, two methods, termed "cisplatin-seq"[12] and "damage-seq"[15], respectively, were developed back-to-back to map or locate the cisplatin damaged sites in genomic DNA. Both the methods achieved single-base (nucleotide) resolution mapping of cisplatination sites on specific genic regions such as promotors, transcription termination sequences and intergenic regions[12,15]. However, the precise genes damaged by cisplatin and their biological consequences were overlooked.

To address this issue, transcriptomics has been used in combination with the "damage-seq" method to map the cisplatin damaged gene loci across mouse organs[16]. However, due to the tissue-specificity of transcriptomics, only the genes transcribed in specific tissues of mouse, e.g., liver, lung and spleen, could be mapped for cisplatin lesions. In recent years, a number of studies have applied chemical genetic strategies, in particular chemical proteomic approaches to uncover gene targets of cisplatin, and have produced significant advances in dissecting its mechanism of action. However, similar to the transcriptomics approach, these studies do not fully reveal cisplatin-damaged gene loci throughout the whole genome because they focused on discovering gene targets of cisplatin *via* measuring the difference between gene expressions in a specific type of cancer cells or tumour tissues upon cisplatin exposure. For example, Jimenez and co-workers found that UGGT1, COL6A1 and MAP4 can be used as potential biomarkers for determining cisplatin response, by proteomic analysis of non-small cell lung cancer cells[37]. Wilmes et al. have used integrated omics techniques to study differentially-expressed proteins and related pathways for cisplatin nephrotoxicity[38].

In this work we have used the principle of forward chemical genetics[17,18], combining affinity separation and high throughput sequencing, proteomic analysis and phosphoproteomic analysis, to the best of our knowledge, for the first time to map cisplatin-damaged gene loci and their influences to the cellular signalling pathways. Importantly, by integration with bioinformatics analysis, our data give rise to two important and innovative findings. One is that among the 11952 identified cisplatin-damaged protein-encoding genes, 418 genes are included in the 628 prior gene targets for cancer treatment reported very recently[27]. The other one is that cisplatin attacks 400 protein kinase genes with fold-enrichment >1.5, covering 77% of consensual protein kinase genes in the human genome[22]. Moreover, we discovered that cisplatin also induced damage on 131 protein phosphatase genes, accounting for 96% of the 136 reported protein phosphatases in human genome. It is clear that cisplatin damaged genes significantly disturb the signalling of protein phosphorylation of cells.

Another key discovery was that mitochondrial genes were not the major targets of cisplatin as low fold enrichment of CDGs were observed. There were two possible reasons, one being that mitochondrial genes are largely short, and the other being the low density of guanine in mDNA (Extended Data Table 6). The result in our work is in consistent with the widely accepted knowledge that nuclear DNA is the major target of cisplatin [3,5]. As HMGB1 is also highly affinity to bent DNA, which is rich in mitochondrial, proper blank controls is a key factor in this work, to exclude the possibly that HMGB1 also binds to native bend DNA in mitochondria.

Protein kinases accompanied by phosphatases regulate protein phosphorylation, the most common and important protein post-translational modification, involved in many natural biochemical pathways and cellular

processes. Since abnormal protein phosphorylation is usually and closely related to cancer development, protein kinases have been major drug targets for cancer therapy[39,40]. During the first and second wave of intensive studies of molecularly-targeted drugs, a number of small molecule protein kinase inhibitors (PKIs), e.g. gefitinib, imatinib and dasatinib[41], have been developed and approved for clinical treatment of various cancers. Given our findings that protein kinase genes are highly susceptible to cisplatin attack, this work unambiguously demonstrates that cisplatin acts as a broad-spectrum protein kinase inhibitor by inducing lesions in protein kinase and protein phosphatase genes. The protein phosphorylation and de-phosphorylation were therefore significantly disturbed evidenced by the proteomic and phophoproteomic data. Combining the bioinformatic analysis of these data, cisplatin activates a series of important signalling pathways, such as ATM and G2/M DNA Damage Checkpoint Regulation pathways, which in turn lead to apoptosis and G2M phase arrest. The regulated phosphorylation of the proteins in these signal pathways such as ATM and CDK1 provided solid evidence for that. Taken together, cisplatin-induced damage on protein kinase and phosphatase genes genome-wide, implying that its pharmacological functions were exerted by reprogramming protein phosphorylation in cancer cells. These results also suggest that a strategy for the design of next-generation platinum anticancer drugs and/or drug delivery systems might involve minimizing non-specific attack on protein kinase genes of healthy cells[2,42]

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Materials and Methods

Escherichia coil

E. coli strain BL21 with HMGB1a-encoding plasmid (DE3) (Tian Gen Biotech, China) was cultivated in Luria-Bertani (LB) medium with 50 μg/mL kanamycin. A single colony picked from a fresh plate was used to inoculate 2YT medium (containing Bacto™ Peptone (16 mg/mL), Bacto™ Yeast Extract (10 mg/mL, Becton, Dickinson and company, USA), sodium chloride (5 mg/mL, Beijing Chemical Works, China) and kanamycin (50 μg/mL, Solarbio, China)) overnight at 37 °C and 180 rpm. Then the cells were transferred to a large volume of fresh 2YT medium at 37 °C and 180 rpm. When the OD_{600} value of the cells reached 0.5 – 1.0, IPTG (Sigma-Aldrich, USA) was added at a final concentration of 1 mM to induce gene expression. After incubation at 37 °C and 180 rpm for further 12 h, the cells were harvested and washed once with PBS (Solarbio, China), and frozen at -80°C for at least one hour for better sonication.

Cell lines

The A549 human non-small cell lung cancer cell line, HeLa cervical cancer cell line, HepG2 hepatoma cell line (National Infrastructure of Cell Line Resource, Beijing, China) and NCCIT testicular cancer cell line (Fukesai Biological Technology Co., Ltd, Nanjing, China) were individually grown in DMEM medium (Gibco) supplemented with 10% FBS (Gibco) and 1% penicillin and streptomycin (GE Healthcare) at 37 °C. When the cell density reached confluency, the culture medium was replaced by fresh DMEM in the absence or presence of cisplatin at various concentrations (50 μM for A549 and HepG2, 12 μM for NCCIT, 25 μM for HeLa) for 24 h. Then the cells were harvested and washed twice by PBS. Whole cell protein or genomic DNA were extracted from different cells with different extraction kits.

Expression and purification of HMGB1a

The cDNA of human HMGB1 domain A (HMGB1a, Extended Data Chart 1.) was synthesized by Proteintech (Rosemont, USA) and cloned into pET-28a plasmid. The resulting plasmid encoding a his_6-tag was transfected into BL21 (DE3) *E. coli* cells (Tian Gen Biotech, China). The cells were grown in a small volume of 2YT medium containing Bacto™ Peptone (16 mg/mL), Bacto™ Yeast Extract (10 mg/mL, Becton, Dickinson and company, USA), sodium chloride (5 mg/mL, Beijing Chemical Works, China) and kanamycin (50 μg/mL, Solarbio, China) overnight at 37 °C and 180 rpm. Then the cells were transferred to a large volume of fresh 2YT medium at 37 °C and 180 rpm. When the OD_{600} value of the cells reached 0.5 – 1.0, IPTG (Sigma-Aldrich, USA) was added at a final concentration of 1 mM to induce gene expression. After incubation at 37 °C and 180 rpm for further 12 h, the cells were harvested and washed once with PBS (Solarbio, China), and frozen at -80°C for at least one hour for better sonication. The deep frozen cells were resuspended in 10 mL cold lysis buffer (PBS and 5 mM imidazole, pH 7.4), and lysed by sonication for 20 min at 0 °C, followed by centrifugation for 30 min at 4 °C and 12000 rpm. The supernatant was purified by His Trap™ FF crude column (GE Healthcare) which was eluted first by 50 mM imidazole (Innochem), then by 300 mM imidazole in phosphate buffer. The eluted HMGB1a containing a his_6-tag was characterized by SDS-PAGE and mass spectrometry (Extended Data Fig. 11) .

Assembly and characterization of HMGB1a-functionalized affinity microprobes

To construct affinity microprobes for cisplatin-crosslinked DNA, 2.1 mg purified HMGB1a was incubated with 7.5 mg nickel-modified magnetic beads (MAg25K/IDA Ni, Enriching Biotechnology, Shanghai, China) suspended

in 1 mL PBS, and rotated for 1 h at 4 °C. The beads were then separated by magnetic force and washed twice with PBS for 10 min. As shown in Extended Data Fig. 12A, the absorbance of the supernatant over the wavelength range 250 – 300 nm significantly decreased due to loading of HMGB1a onto the magnetic beads. This indicated successful functionalization of the magnetic beads by HMGB1a.

Cell culture and genomic DNA extraction

The A549 human non-small cell lung cancer cells obtained from the Centre for Cell Resource of Peking Union Medical College Hospital were cultured in DMEM medium (Gibco) supplemented with 10% FBS (Gibco) and 1% penicillin and streptomycin (GE Healthcare) at 37 °C. When the cell density reached confluency, the culture medium was replaced by fresh DMEM containing 50 μM cisplatin (Beijing Ouhe Technology, China), and the cells were incubated at 37 °C for 24 h. Then the cells (~10^7) were harvested, and genomic DNA was extracted by whole genome extraction kit (Tian Gen Biotech). The DNA extracts were crushed into fragments between 250 to 750 bp with an ultrasonic breaker (Scientz-IID ultrasonic homogenizer, SCIENTZ, Ningbo, China). At the same time, a similar amount of cells without exposure to cisplatin were harvested for genomic DNA extract and fragmentation. The DNA fragments from the cells treated with cisplatin were considered as a positive sample and those from the cells without cisplatin treatment as a control sample for capture and sequencing of cisplatin damaged genes. An inductively coupled plasma-mass spectrometer (ICP-MS, Agilent 7700) was used to determine the Pt concentrations of the DNA fragments obtained from positive and control samples.

Capture of cisplatin-bound DNA fragments

The same amounts (74 μg) of DNA fragments gained from cells treated and untreated with cisplatin were individually re-dissolved in 10 mM Tris-HCl (pH 7.4, Beijing Biodee Biotechnology), and added into the HMGB1a-functionlized magnetic beads suspended in PBS, followed by rotation for 1 h at room temperature. The beads were then separated by magnetic force and washed twice with 1 mL 10 mM Tris-HCl. The captured HMGB1a-DNA complexes were eluted with 1 mL of buffer containing 20 mM sodium phosphate, 500 mM NaCl and 400 mM imidazole (pH 7.4) *via* rotation at room temperature for 40 min. As shown in Extended Data Fig. 12B, C, the absorbance of the supernatant over the wavelength range 250 – 300 nm significantly decreased subject to incubation with HMGB1a-functionlized magnetic beads, providing evidence for the capture of DNA fragments by the HMGB1a-based affinity microprobes.

Each eluted HMGB1a-DNA complex sample was centrifuged and lyophilized, and then re-dissolved in 500 μL deionized water, followed by addition of 500 μL mixture of phenol (Solarbio) – chloroform (Beijing Chemical Works) – isopropanol (Concord Technology, Tianjin) (25: 24: 1). The resulting mixture was shaken firstly for 10 s, and kept at room temperature for 2 min, then centrifuged at 12000 rpm and 4 °C for 7 min. The supernatant was mixed with 500 μL of a mixture of chloroform-isopropanol (24: 1), shaken again for 10 s, kept at room temperature for 2 min, and then centrifuged at 12000 rpm and 4 °C for 7 min. The isolated supernatant was mixed with 1500 μL pre-chilled absolute ethanol (Concord Technology, Tianjin) and 50 μL sodium acetate solution (3 M, Beijing Chemical Works), kept at −20 °C overnight, and then centrifuged at 12000 rpm and 4 °C for 7 min. The supernatant was carefully removed, and the residues were left at room temperature for drying. Finally, the purified DNA fragments were re-dissolved in an appropriate amount of TE solution (Solarbio) for sequencing.

NGS Sequencing and gene mapping

The next-generation-sequencing (NGS) of the captured and purified DNA fragments described above was performed by Shanghai Sangon Biotech Ltd. The DNA fragments were firstly characterized by 1% agarose gel electrophoresis for quality check, and then fragmented to about 500 bp by Covaris and recovered for DNA library construction. Library construction was performed following the instruction of NEB Next® Ultra™ DNA Library Prep Kit for Illumina® (https://international.neb.com/products/e7370-nebnext-ultra-dna-library-prep-kit-for-illumina#Protocols,%20Manuals%20&%20Usage). Since cisplatin binding to DNA fragments inhibits DNA replication and synthesis[3,5,25], the PCR expansion time was prolonged to 30 min. The constructed DNA libraries were characterized by a 2% agarose gel, and then each positive or control sample was sequenced by Illumina® providing at least 20 G of data.

The raw Illumina sequences were filtered for quality control by using Fastp(0.22.0) (fastp -q 15 -u 40 -n 5 -e 0 -l 35), and the clean reads were aligned to the reference human genome (hg19; National Centre for Biotechnology Information [NCBI]) by using the Burrows-Wheeler Aligner software program (0.7.17-r1188) with default parameter [bwa mem -M <genome> <R1> <R2>] to acquire SAM files. Then, Samtools (1.11) was used to sort out the SAM files to BAM files in which only uniquely mapped nonduplicate reads were retained. The Chip-sequencing enrichment regions were identified using the MACS2(2.1.2) program (Model-based Analysis, a Poisson-based method, with a P-value cutoff of 0.001 and Mfold values adjusted to build the model). Finally, the R-Chipseeker package was employed to annotate and visualize all peaks identified.

To calculate the fold-enrichment of a cisplatin damaged gene (CDG), we firstly calculated the fold-enrichment of all peaks mapped to the gene by using equation 2.

$$FE_P = \frac{R_{PS}/T_{PS}}{R_{CS}/T_{CS}} \quad \text{.....(2)}$$

Where R_{PS} and R_{CS} are read (tag) counts for a peak detected in a pair of positive sample (PS) and control sample (CS), respectively, and T_{PS} and T_{CS} are total read (tag) counts of all peaks detected in the pair of positive sample (PS) and control sample (CS), respectively. A peak was considered unreliable and then abandoned if it matched multiple genes. A peak with an fold-enrichment (FE_p) <1.5 was also taken as unreliable and discarded[43]. Thus, the fold-enrichment of a CDG is the sum of fold-enrichment of all peaks mapped to the gene, i.e.

$$FE_G = \sum_1^i (FE_P)_i \qquad (3)$$

Where i represents the number of peaks mapped for a gene in a positive sample/control sample pair.

Taking into account that cisplatin kills cells by attack on DNA, it might be expected that more cisplatin-damaged genes would be detected in the dead cells of positive samples. However, our sequencing data showed that there was no significant difference between the total numbers and fold-enrichments of cisplatin-damaged genes mapped from the DNA fragments collected from living cells alone and from living cells and dead cells together after 24 h of incubation with 50 μM cisplatin (data not shown). The other software and databases used for gene screening and localization are listed in Extended Data Table 9.

CCK-8 assay

The survival rates of *SPAG9*-selicenced and cisplatin-treated NCCIT cells were measured using the CCK-8 (Cell Counting Kit-8) assay (MedChemExpress, USA). Briefly, the NCCIT cells (1×10^3 cells/per wells) were plated at a density of 2000 cells per well in 100 μL of media into 96-well plates. After 24 h for adherence, the cells were exposed to 12 μM cisplatin for 24 h or transfected with the siRNA against *SPAG9* and grown for 48 h. Then, 10 μL CCK-8 was added into the medium and the cells were grown for further 4 h. Finally, the optical density (OD) for each well was measured using a microplate reader (SpectraMax M5 Molecular Devices Corporation) at the wavelength of 450 nm. The survival rate (SR, %) of cells was calculated based on the equation (1):

$$\mathrm{SR}(\%) = \frac{\mathrm{OD}_{\mathrm{sample}} - \mathrm{OD}_{\mathrm{blank}}}{\mathrm{OD}_{\mathrm{control}} - \mathrm{OD}_{\mathrm{blank}}} \times 100\% \qquad (1)$$

All reported values of SR were averages of three independent experiments and expressed as mean ± SD (standard deviation).

Flow cytometric analysis

The apoptosis rate of NCCIT cells subject to cisplatin treatment and *SPAG9* silencing was determined by Annexin V / propidium iodide (PI) double staining assay following the Apoptosis Detection Kit I protocols (BD Pharmingen, USA). The NCCIT cells (1×10^3 cells/well) were plated into a six-well plate. Twenty-four hours later, NCCIT cells were transfected with siRNA against *SPAG9* for 48 h. Another group of cells were treated with 12 μM cisplatin for 24 h. Then, the supernatant was removed, and cells were transferred to tubes and centrifuged at 1000 rpm for 3 min. Thereafter, the cells were re-suspended in 1× Annexin V binding buffer, and incubated with 5 μL Annexin-V and 5 μL PI conjugate for 15 min in the dark prior to FACS analysis. The FACS assays were performed on a Calibur flow cytometer (BD，Franklin Lakes, New Jersey, US), of which the FL1 channel was used to record the intensity of annexin V-FITC staining and FL2 channel to record the intensity of PI staining. The data were quantified by Sell Quest software (BD, Franklin Lakes, New Jersey, US).

Quantification of protein and DNA concentration

The concentrations of purified protein HMGB1a and extracted genomic DNA were determined by NanoDrop OneC (Thermo Fisher Scientific Inc.). The concentrations of whole cell proteins from different cells were determined using a BCA kit (Beyotime Biotech Inc.).

Western Blot assays

The A549 human non-small cell lung cancer cell line, HeLa cervical cancer cell line, HepG2 hepatoma cell line (National Infrastructure of Cell Line Resource, Beijing, China) and NCCIT testicular cancer cell line (Fukesai Biological Technology Co., Ltd, Nanjing, China) were individually grown in the presence and absence of cisplatin at various concentrations (50 μM for A549 and HepG2, 12 μM for NCCIT, 25 μM for HeLa) for 24 h. The cells were then harvested for extracting whole cell proteins using a total protein extraction kit (BestBio, China). The concentration of each protein extract was determined by standard BCA assay (Beyotime, China). To determine the change in expression level of a specific protein due to gene damage induced by cisplatin, the same amount of whole cell proteins extracted from a positive sample and a control sample were individually

boiled with the gel-loading buffer (4×LDS, Genscript, Nanjing, China), and loaded onto a 4 – 12% gradient SDS-PAGE gel (Genscript) for electrophoresis. The separated proteins were transferred to a PVDF membrane (Millipore, 0.2 μm). The membrane was blocked in 5% nonfat dry milk (Biofroxx) dissolved in 0.1% TBST, of which 1 L contained 2.4 g Tris base (Innochem), 8.8 g NaCl and 0.1% (v/v) Tween 20 (Beijing Topbio Science&Technology), at 4 °C overnight, and then incubated with respective primary antibodies (Abcam) in appropriate dilutions at room temperature for 2 h. Thereafter, the membranes were washed three times with 5% nonfat dry milk in 0.1% TBST for 30 min each time and incubated with horseradish peroxidase-conjugated secondary antibody (Abcam) at room temperature for 1 h. After washing twice with 5% nonfat dry milk in 0.1% TBST and once with 0.1% TBST, the protein bands were visualized by enhanced chemiluminescence (Beyotime, China), and the optical densities were determined using an image analyser (Image J).

Since these chosen genes are expressed at different levels in different types of cells, we selected four human cancer cell lines, A549 (non-small cell lung cancer cell line), NCCIT (testicular cancer cell line), HepG2 (hepatocellular cancer cell line) and HeLa (cervical cancer cell line) to perform Western Blot assays. Among the four cell lines, NCCIT is the most sensitive to cisplatin with an IC_{50} value of 5.8 μM (24 h treatment). The IC_{50} values for HeLa, A549 and HepG2 cells were ca. 8.2 μM (24 h), 10.1 μM (48 h), 22.4 μM (24 h)[44], respectively. Thus, we applied different doses of cisplatin to treat the cell lines, i.e. 50 μM for A549 and HepG2, 25 μM for HeLa and 12 μM for NCCIT.

For siRNA silencing of *SPAG9* experiments, the NCCIT testicular cancer cell line were transfected with siRNA against *SPAG9* (sequence 5'-3': rGrGrArGrCrArGrArUrUrUrArCrUrArGrGrArATT, Suzhou Biosyn Biotechnology Co., Ltd.), or not, for 48 h. The cells were then harvested for extracting whole cell proteins using a total protein extraction kit (BestBio, China). The concentration of each protein extract was determined by standard BCA assay (Beyotime, China). To determine the change in expression level of JIP-4 due to gene silencing by siRNA, the same amount of whole cell proteins extracted from a positive sample and a control sample were individually analysed by using the same method for Western Blot assays described above.

Calculation of fold-change of protein expression

We carried out three technical repetitions of Western Blot assays. The optical densities of protein bands were determined using an image analyser (Image J). The fold-change (FC) of a protein was calculated using equation 4.

$$\mathrm{FC} = \frac{\mathrm{OD_{PC}}/\mathrm{OD_{GC}}}{\mathrm{OD_{PN}}/\mathrm{OD_{GN}}} \qquad (4)$$

Where OD_{PC} and OD_{GC} are optical densities of a protein band and the GAPDH (the reference) band in a sample treated with cisplatin, and OD_{PN} and OD_{GN} are optical densities of the protein band and the GAPDH band in a sample without cisplatin treatment, respectively.

Cell culture and protein extraction

The NCCIT testicular cancer cell line obtained from Fukesai Biological Technology Co., Ltd were cultured in RPMI-1640 medium (Gibco) supplemented with 10% FBS (Gibco) and 1% penicillin and streptomycin (GE Healthcare) at 37°C. When the cell density reached confluency, the culture medium was replaced by fresh RPMI-1640 containing 12 μM cisplatin (Beijing Ouhe Technology, China), and the cells were incubated at 37 °C for 24 h. Cells without cisplatin treatment were set as C group. A group of cells were treated with cisplatin for 24 h and only live cells were collected as L/C group. Another group of cells were treated with cisplatin for 24 h and both live cells and dead cells were collected as DL/C group. The cells were harvested for extracting whole cell proteins using a total protein extraction kit (BestBio, China) supplemented with protease inhibitor, phosphatase inhibitor and 8 M urea. The concentration of each protein extract was determined by standard BCA assay (Beyotime, China).

Sample preparation

Reduce the disulfide bonds in the denatured proteins by adding DTT (J&K Scientific) to 300 μg (for phosphoproteomics, the amount of initial protein is 1 mg) of sample (group C, L and DL, three biological replicates) at a final concentration of 5 mM DTT, followed by incubation at 37 °C for 1 h in a Thermomixer. Then alkylate the reduced cysteine residues by adding IAA (J&K Scientific) to a final concentration of 10 mM IAA, followed by incubation for 45 min in the dark at 25 °C. Dilute the sample 1:3 (vol/vol) with 50 mM Tris-HCl (pH 8.0, Beijing BioDee Biological Technology Co., Ltd) to decrease the urea concentration to <2 M. Add trypsin (Sigma-Aldrich) in an enzyme/substrate ratio of 1:49 (wt/wt) for overnight digestion at 25 °C in the incubator shaker. Then quench the digestion reaction by acidifying the digestion mixture to a final concentration of 1% (vol/vol) formic acid (FA) using 100% (vol/vol) FA and dilute the samples with 0.1% (vol/vol) FA to a 1.5-mLvolume, followed by desalting by C18 cartridge (Waters Sep-Pak). The C18 cartridge was firstly activated by 1 mL of 100% acetonitrile (ACN, Fisher Chemical) and 1 mL of 0.1% FA/50% ACN sequentially, and then equilibrated by 1 mL of 0.1% trifluoroacetic acid (TFA, Alfa Aesar) for three times. The collected peptides were loaded into the equilibrated column, and washed by 1 mL of 0.1% TFA for three times and 1 mL of 1% FA, and then eluted by 1 mL of 0.1% FA/50% ACN and 1 mL of 0.1% FA/80% ACN sequentially. Finally, the desalted peptides were merged and dried in vacuum centrifuge for further use.

TMT labeling

Reconstitute the sample in 100 μL of 50 mM HEPES (pH 8.5) by mixing well. Remove a 5-μL aliquot for the peptide-level BCA estimation. On the basis of the BCA results at the peptide level, dilute the samples with 50 mM HEPES (pH 8.5) for a concentration of 1 μg/μL. Vortex the well to mix. Reconstitute each 0.8 mg TMT reagent (Thermo Fisher, for phosphoproteomics, the amount of TMT reagent is 5 mg) vial in 41 μL of anhydrous acetronitrile. Allow it to sit for 5 min. Vortex the reagent tubes briefly and spin down on a benchtop centrifuge at 1000 g at RT for 30 s. Add 41 μL of each reagent to the corresponding aliquot of peptides. Mix well and incubate at 25 °C for 1 h with shaking on a tabletop shaker set at 300 rpm. After 1 h, quench all individual TMT-labeling reactions by adding 8 μL of 5% (vol/vol) hydroxylamine (J&K Scientific), followed by incubation at RT for 15 min. Combine the volumes of each of the nine TMT-labeled samples in a 1.5 mL conical tube and add

0.1% FA to 1.5 mL. Then desalted this sample by using the method as described above. The desalted peptides were merged and dried in vacuum centrifuge for further use.

High-pH reversed-phase pre-separation of peptides

Prepare the mobile phases A and B required for pre-separation according to the literature[45]. The TMT-labeled and desalted peptide mixture was re-dissolved in 100 µL mobile phases A. Aliquot (95 µL) of each labeled peptide mixture was loaded into HPLC (Agilent Technologies 1260 infinity) with a C18 column (CAPCELL PAK MG II S-5, 4.6 150 mm, 5 µm, SHISEIDO), and eluted by basic (pH =10) mobile phases A (4.5 mM $HCOONH_4$ in 2% ACN) and B (4.5 mM $HCOONH_4$ in 90% ACN). The gradient started with 0% B until 7 min and continuously increased to 16% B at 13 min, 40% B at 73 min, 44% B at 77 min, 60% B at 82 min and kept until 96 min with a flow rate of 1 mL/min. The fractions were collected chronologically into twelve tubes from 3 min to 90 min, individually lyophilized and re-dissolved in 10 µL of 0.1% formic acid for NanoLC-MS/MS analysis.

Phosphopeptide enrichment

The twelve peptide solutions obtained by pre-separation were dried in vacuum centrifuge separately, and then continuously enriched by using zirconium arsenate magnetic beads (ZrAs-Fe_3O_4, a gift from) and a titanium dioxide enrichment kit (Thermo Fisher, https://www.thermofisher.cn/order/catalog/product/88303). In brief, the peptides were re-dissolved in 500 µL 50%ACN-1%TFA. Add 1.4 mg ZrAs-Fe_3O_4 to each tube to enrich phosphopeptides for 30 min. The beads were then separated by magnetic force and washed twice with 500 µL 50%ACN-1%TFA. The enriched phosphopeptides were eluted twice with 500 µL 2.5% ammonia at room temperature for 15 min. The supernatant solution and washing solution after enrichment with ZrAs-Fe_3O_4 beads are subsequently enriched with a titanium dioxide enrichment kit. The phosphopeptides enriched by the two methods in different fractions were mixed together and dried in vacuum centrifuge for further use, separately.

NanoLC-MS/MS analysis

The NanoLC-MS/MS detection system consisted of an Orbitrap Fusion Lumos mass spectrometer (Thermo Fisher Scientific) coupled to an EASY-nLC 1200 nanoHPLC system equipped with a Acclaim™ PepMap™ 100 pre-column (20 mm 75 µm, 3 µm) and a Acclaim™ PepMap™ RSLC C18 analytical column (150 mm 75 µm, 2 µm). The mobile phases for the NanoLC separation were 0.1% FA in H_2O (A) and in 80% ACN/20% H_2O (B), respectively, at a flow rate of 300 nL/min. We run a 110-min gradient to separate these peptides (0-7 min, 2-7% B; 7-69 min, 7-20% B; 69-90 min, 20-35% B; 90-95 min, 35-95% B; 95-99 min, 95% B; 99-101 min, 95-50% B; 101-105 min, 50% B; 105-107 min, 50-2% B; 107-110 min, 2% B). Aliquot (1 µL) of each HPLC fractions containing 0.1% formic acid was loaded, and elution from the analytical column was directly infused to the mass spectrometer for MS/MS analysis. The ion source type was set as NSI and the detector type was set as Orbitrap. For a full mass spectrometry survey scan, the target value was 4×10^5 and the scan ranged from 350 to 1,800 m/z at a resolution of 120,000 and a maximum injection time of 50 ms. For the MS2 scan, a duty cycle of 3 s was set with the top-speed mode. Only spectra with a charge state of 2–5 were selected for

fragmentation by collision induced dissociation with a normalized collision energy of 35%. The MS2 spectra were acquired in the orbitrap at a resolution of 15,000 with an AGC target of 5,000 and a maximum injection time of 22 ms. For the MS3 scan, the number of SPS precursor was set as 8 and the MS2 isolation window was set as 3 m/z. The target value was 5,000 and the scan ranged from 100 to 400 m/z at a resolution of 50,000 and a maximum injection time of 86 ms. Eight precursors were selected for fragmentation by higher-energy collision dissociation with a normalized collision energy of 65%.

The phosphopeptides were separated by a 120-min gradient (0-5 min, 2-7% B; 5-77 min, 7-20% B; 77-103 min, 20-35% B; 103-108 min, 35-95% B; 108-112 min, 95% B; 112-114 min, 95-50% B; 114-120 min, 50% B). The Orbitrap Fusion mass spectrometer was operated in the positive-ion mode at an ion transfer tube temperature of 320 °C. The positive-ion spray voltage was 2.3 kV. The full mass spectrometry survey scan resolution was set to 120,000 with an automated gain control (AGC) target of 4.1×10^5 for a scan range of 350–1,800 m/z and a maximum injection time of 110 ms. Only spectra with a charge state of 2–7 were selected for fragmentation by higher-energy collision dissociation with a normalized collision energy of 37%. The MS2 AGC target was set to 5,000 with a maximum injection time of 54 ms, and the dynamic exclusion was set to 20 s.

Proteomic Data Analysis

Acquired MS/MS spectra were analyzed by Proteome Discovers 2.2 (Thermo Fisher Scientific) to identify and quantify proteins based on Protein Database (Version 10/25/2017, 42252 sequences) download from Uniport database. The taxonomy was Rattus norvegicus, and taxon identifier was 10116. Sequest HT 2.2 search engine was used for peptide matching. Trypsin was selected as the proteolytic enzyme, and four missed cleavages sites were allowed. The static modifications were carbamidomethylating at cystine, TMT labeling at N-terminus of all peptides. The dynamic modifications were oxidation at methionine, TMT labeling at lysine, methylation at lysine/glutamine/arginine, +Pt/+Pt(NH_3) at histidine/aspartic acid/glutamic acid/ methionine, and phosphorylation at serine/threonine/tyrosine. The peptide mass tolerance was set to 10 ppm. The fragment ion tolerance was set to 0.02 Da. The quantitative results were normalized based on the abundance of total peptides in each set. Abundance ratios were calculated for each protein identified in all groups other than controls by comparing with value of each protein to that of the same protein in control group. The false discovery rates of the peptide-spectrum matches (PSMs) and proteins were set to less than 1%. The other settings are the defaults of the PD software. The phospho-proteome data were searched against the same database with Proteome Discovers 2.2 with similar parameters, except for the phosphorylation sites probability was set as >75%.

Bioinformatics Analysis

The proteins identified and quantified by MS/MS, of which false-discovery rates (FDRs) are <0.01, *p*-values <0.05 and abundance ratios (sample vs control) >1.2 or <0.833 (fold change >1.2 or <-1.2), were uploaded into the STRING (https://string-db.org) 10.0 data pool for Gene Ontology (GO) and protein-protein interaction (PPI) network analysis. The Uniport accession ID of the differentially expressed proteins (DEPs) in the DL/C group and L/C group were inputted into the list of gene name box, and Homo sapiens was selected for the analysis.

Ingenuity Pathway Analysis (IPA, QIAGEN)[46] was used to analyze the core pathways, diseases, toxicities and functions associated with the DEPs. IPA analysis maps each protein to the corresponding molecule in the Ingenuity Pathway Knowledge Base, which is available at the Ingenuity System's web site (http://www.ingenuity.com).

Statistical analysis

All data were analysed with Origin 8.0 and Microsoft offices excel 2010 software. Statistically significant differences were determined using unpaired t-tests, Student's t-tests, Fisher's exact test right-tailed, as applicable and according to test requirements. A two-sided P value < 0.05 was considered statistically significant. * indicates $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. No statistical methods were used to predetermine sample size. The experiments were not randomized and the investigators were not blinded to allocation during experiments and outcome assessment.

Phosphopeptide Motif Analysis

Phosphopeptide sequences were submitted to MotifeR algorithm[47] (https://www.omicsolution.org/wukong/motifeR/) for the identification of known and novel phosphorylation motifs present in our data set. Only confidently identified phosphorylation sites with site probability >75% were used in analysis. Sequences were firstly aligned with the background database (9606-Homo sapiens) and truncated into uniform length sequences for the following analysis. Sequences were centered on each phosphorylation site and extended to 15 amino acids (±7 residues). When the site is located in the N/C-terminal of the protein, the sequence was filled up with up to 15 amino acids with the required number of "X". The significance threshold was set to $P < 10^{-6}$ and the occurrence threshold was set as 20. This website can also generate logo-like representations to graphically display the identified motif. Moreover, according to the kinase-substrate information given in the literature, the upstream kinases of these significantly regulated phosphorylated peptides can also be given.

DATA AVAILABILITY

The raw sequencing data were deposited on Mendeley Data, and available on https://data.mendeley.com/datasets/k9sm56gj5y/1, https://data.mendeley.com/datasets/2tv7ntcgh6/1, https://data.mendeley.com/datasets/4zj3kz34c5/1, https://data.mendeley.com/datasets/dmyfkn7pnz/1, https://data.mendeley.com/datasets/dw3v4sm9nb/1, https://data.mendeley.com/datasets/xb6s7vzcwm/1, https://data.mendeley.com/datasets/75dyzmvbrz/1, https://data.mendeley.com/datasets/s7jbxvwf4z/1, and https://data.mendeley.com/datasets/fv6f9rpg5m/1.

The flow cytometry data contained in this article are deposited on FlowRespository with a Repository ID of FR-FCM-Z45H on https://flowrepository.org/experiments/4273.

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AUTHOR CONTRIBUTIONS

F.Y.W. and Y.Z. conceived the project. L.Y.Q., Y.Z.H, Q.L., Y.X. and T.T.F. carried out the expression of HMGB1a and construction of HMGB1a-functionilizaed microprobes. L.Y.Q., B.B. X., Y.Y.Z., F.F.J., X.J.L., P.J.S., Y.Z. and F.Y.W. analysed sequencing data. L.Y.Q., W.C.Y. Y.Z. and X.K.L. performed bioinformatics analysis and Western Blot assays. L.Y.Q., F.Y.W. P.J.S., Y.Z. and S.J.W. wrote and revised the manuscript with inputs from all authors.

CONFLICT OF INTEREST

We have documented selected cisplatin-damaged genes as potential drug targets and biomarkers for cancer therapy in a Chinese patent application.

ATGGGCAGCAGCCATCATCATCATCATCACAGCAGCGGCCTGGTGCCGCGCGGCAGCCATATGGCTAGCATGACTG
GTGGACAGCAAATGGGTCGCGGATCCCCTCGTGGTAAAATGAGCAGCTATGCATTTTTTGTTCAGACCTGTCGTGA
GGAACACAAAAAAAAGCATCCTGATGCAAGCGTGAACTTTAGCGAATTCAGCAAAAAATGTAGCGAACGCTGGAA
AACCATGAGCGCAAAAGAAAAAGGCAAATTTGAGGATATGGCCAAAGCAGATAAAGCCCGTTATGAACGTGAAAT
GAAAACCTATTAA

Extended Data Chart 1. The cDNA sequence of human HMGB1 domain A (HMGB1a)

Extended Data Table 1 Information for sequencing samples prepared from genomic DNA extracts of A549 cells with cisplatin treatment (positive sample: PS) or without cisplatin treatment (control sample: CS), statistical analysis of sequencing and rates of reads mapped to the human genome.

Replicate	1		2	
	Positive Sample	Control Sample	Positive Sample	Control Sample
DNA concentration (ng/μL)	17.1	15.7	15.1	17.1
Volume (μL)	260	260	200	200
Total Reads Count	162704730	166832042	235408404	194048372
Total Bases Count (bp)	23334767257	23950671824	34314608427	28416732137
Average Read Length (bp)	143.42	143.56	145.77	146.44
Q10 Bases Count (bp)	23334513721	23950417040	34314572665	28416702861
Q10 Bases Ratio (%)	100.00%	100.00%	100.00%	100.00%
Q20 Bases Count (bp)	23043629520	23642092904	33800146069	27994785627
Q20 Bases Ratio (%)	98.75%	98.71%	98.50%	98.52%
Q30 Bases Count (bp)	22389034358	22959532355	32465733778	26882783529
Q30 Bases Ratio (%)	95.95%	95.86%	94.61%	94.60%
N Bases Count (bp)	57152	58518	35762	29276
N Bases Ratio (%)	0.00%	0.00%	0.00%	0.00%
GC Bases Count (bp)	13042819189	12964880382	14818176354	11829771086
GC Bases Ratio (%)	55.89%	54.13%	43.18%	41.63%
Total reads	162704730(100.00%)	166832042(100.00%)	186590790(100.00%)	199928074(100.00%)
Total mapped	161812149(99.45%)	165872115(99.42%)	184756224(99.02%)	198808893(99.44%)
Multiple mapped	12953876(7.96%)	11386662(6.83%)	12622909(6.77%)	14897960(7.45%)
Uniquely mapped	148858273(91.49%)	154485453(92.60%)	172133315(92.25%)	183910933(91.99%)
Read-1 mapped	74517147(45.80%)	77310628(46.34%)	86135705(46.16%)	92029339(46.03%)
Read-2 mapped	74341126(45.69%)	77174825(46.26%)	85997610(46.09%)	91881594(45.96%)
Reads map to '+'	74429891(45.75%)	77242602(46.30%)	86075833(46.13%)	91975255(46.00%)
Reads map to '-'	74428382(45.74%)	77242851(46.30%)	86057482(46.12%)	91935678(45.98%)
Reads mapped in proper pairs	143215783(88.02%)	151046514(90.54%)	168403729(90.25%)	181266998(90.67%)

Extended Data Table 2 – 8 are separately provided in Excel format.

Extended Data Table 9. The software and databases used for gene screening and localization.

Software	Version	R package	Version
BBTools	37.48	ggplot2	2.2.1
R	3.4.2	reshape2	1.4.2
BLAST+	2.2.28	magrittr	1.5
Java	1.8.0_151		
picard	2.14.0		
htslib	1.6		
samtools	1.6		
macs14	1.4.2		
SnpEff	4.3s		
bedtools	2.26.0		