

磷酸化蛋白质组学联合蛋白质组学分析敲除维甲酸诱导蛋白16对人结肠癌细胞的影响

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Proteomics and Phosphoproteomics Analysis of Effect of Retinoic Acid-Induced Protein 16 Knockout on Human Colon Cancer Cells

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Abstract: Objective To analyze the differences in the expressions of the total and phosphorylated proteins in human colon cancer HCT116 cells after the knockout (KO) of retinoic acid-induced protein 16 (RAI16) and explore the possible mechanism and related signaling pathways affecting its protein function in HCT116 cells. **Methods** HCT116 KO and WT cell proteins were collected and extracted, and the protein extraction efficiency was detected via a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) experiment. After protein digestion, the peptides were labeled with TMT and analyzed via mass spectrometry. We used bioinformatics methods to analyze the identified differential proteins and differentially phosphorylated proteins by using GO, KEGG, and STRING databases. **Results** The results of SDS-PAGE showed no evident protein degradation. In addition, some key bands were significantly different between the experimental and control groups. A total of 147 up-regulated and 230 down-regulated differential proteins were screened in accordance with the conditions of $\text{Foldchange} \geq 1.5$ or $\text{Foldchange} \leq 1/1.5$ and $P < 0.05$. Meanwhile, 106 up-regulated and 217 down-regulated phosphorylation sites were screened. GO enrichment analysis revealed that the differential proteins were mainly enriched in the composition of nucleoplasm, nucleus and cytoplasm, RNA binding, cadherin and chromatin, DNA repair, RNA splicing, and positive regulation of DNA as template transcription. The results of KEGG enrichment indicated that the differential proteins were mainly enriched in nucleocytoplasmic transport, spliceosomes, cell cycle, cell-cell tight junctions, viral carcinogenesis, microRNAs in cancer, etc. The protein interaction network mainly focused on DDX17, NCL, EEF2, CDK1, SSRP1, and SMARCC1. The statistical findings unveiled the up-regulated changes in the two omics of SKP1, ORC1, and BAD and the down-regulated changes in RBL1, RB1, CDK1, CDC6, MCM4, TFDP1, CHD4, and SNW1. Moreover, the phosphorylation differences were more significant than the protein differences. **Conclusion** RAI16 plays the possible crucial role in multiple biological functions and signaling pathways through key proteins, such as SKP1, ORC1, RB1, and CDK1, which affect the cell cycle and thereby the occurrence and development of cancer.

Key words: Colon cancer; Retinoic acid-induced protein 16; HCT116 cells; Phosphoproteomics

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摘要: 目的 分析人结肠癌HCT116细胞敲除维甲酸诱导蛋白16(RAI16)后细胞内总蛋白及磷酸化蛋白质表达的差异, 探究RAI16影响HCT116细胞蛋白质功能的可能机制及相关信号通路。方法 收集并提取HCT116 KO和WT细胞蛋白, SDS-PAGE检验蛋白提取效果。利用胰蛋白酶酶解蛋白后, 标记肽段并进行质谱分析。对鉴定到的差异蛋白及差异磷酸化蛋白质

利用GO数据库、KEGG数据库和STRING数据库进行生物信息学分析。结果 SDS-PAGE结果提示蛋白无明显降解，且实验组与对照组部分关键条带有明显差异；按Foldchange ≥ 1.5 或Foldchange $\leq 1/1.5$ 且 $P < 0.05$ 为条件进行差异蛋白的筛选，共筛选出147个上调差异蛋白和230个下调差异蛋白；并筛选到106个上调磷酸化位点和217个下调磷酸化位点。将去本底差异磷酸化位点功能GO富集分析，发现差异蛋白主要在核质、细胞核及细胞质组成，RNA、钙黏着蛋白及染色质结合，DNA修复、RNA剪接及DNA为模板转录的正调控等多个方面有显著富集趋势。KEGG富集结果显示，差异蛋白主要在核质转运、剪接体、细胞周期、细胞间紧密连接、病毒致癌作用和癌症中的微小RNA等通路具有显著富集趋势。蛋白互作网络主要以DDX17、NCL、EEF2、CDK1、SSRP1和SMARCC1为核心蛋白质。统计发现，SKP1、ORC1和BAD等两组学差异变化均上调，且磷酸化差异变化比蛋白差异变化更显著，RBL1、RB1、CDK1、CDC6、MCM4、TFDP1、CHD4和SNW1等两组学差异变化均下调，且磷酸化差异变化比蛋白差异变化更显著。结论 RAI16可能通过SKP1、ORC1、RB1和CDK1等关键蛋白质在多方面生物功能和多条信号通路中发挥作用，影响细胞周期，进而影响癌症发生发展。

关键词：结肠癌；维甲酸诱导蛋白16；HCT116

细胞；磷酸化蛋白质组学

中图分类号：R730.2；R735.3*5

开放科学(资源服务)标识码(OSID):



0 引言

维甲酸诱导蛋白(Retinoic acid-induced protein, RAI)家族是一类由维甲酸诱导的蛋白质。同源分析显示RAI在人、大鼠、小鼠、兔子、斑马鱼中高度保守，提示RAI蛋白可能在细胞基本功能中发挥重要作用。目前已有许多文献报道了维甲酸诱导蛋白家族与人类的多种疾病相关。RAI1与胚胎神经发育、神经元分化、细胞生长和细胞周期调节有关，其突变可以导致Smith-Magenis综合征的发生^[1]。RAI2可作为多种恶性肿瘤的生物标志物，包括乳腺癌^[2-3]、胃癌^[4]、结直肠癌^[5]、前列腺癌^[6]、膀胱癌^[7]、肺腺癌^[8]、肝细胞癌^[9]等。据报道，RAI3可作为食管癌^[10]的生物标志物，并通过调节STAT3的磷酸化影响细胞增殖与分化^[11]。RAI14可以作为胃癌的一种预后生物标志物，会促进胃癌细胞生长和侵袭^[12]，而下调RAI14可通过STAT3通路抑制食管癌的进展^[13]。人类RAI16基因位于第8对染色体，有研究表明RAI16作为一种雄激素受体的靶基因，它参与前列腺癌细胞中雄激素受体信号转导的细胞生长^[14]，而测序结果提示RAI16在胸腺和免疫细胞中呈高表达，说明其可能与人体免疫系统存在一定关联。本课题组长期从事RAI16分子相关研

究，前期研究发现RAI16抑制结肠炎及结肠炎相关结直肠癌的进展^[15-16]。本研究以人结肠癌HCT116细胞敲除维甲酸诱导蛋白16后与原始HCT116细胞(HCT116 WT)进行蛋白质组学联合磷酸化蛋白质组学分析，从蛋白质功能、信号通路和蛋白质相互作用等3个层面探究敲除维甲酸诱导蛋白16对人结肠癌细胞的影响。

1 材料与方法

1.1 材料与试剂

人结肠癌细胞系HCT116由我室保存，RAI16敲除的HCT116细胞系(HCT116 KO)通过CRISPR/Cas9技术构建。IMAC磷肽富集试剂盒、BCA试剂盒和串联质谱标签(Tandem mass tag, TMT)标记试剂盒均购自美国Thermo公司，色谱级试剂乙腈购自美国Fisher Chemical公司，四乙基溴化铵(Tetraethylammonium bromide, TEAB)购自美国Sigma-Aldrich公司，二硫苏糖醇(Dithiothreitol, DTT)购自上海Adamas-beta公司，Q Exactive HF质谱仪和Easy-nLC 1200液相系统均购自美国Thermo有限公司，高pH分离液相色谱仪购自美国Agilent公司，eStain LG蛋白染色仪购自南京金斯瑞生物科技有限公司，全自动数码凝胶图像分析系统购自上海天能科技有限公司。

1.2 细胞裂解和蛋白质提取

将HCT116 KO(实验组)和HCT116 WT(对照组)细胞用预冷的PBS洗三遍，而后用干净的细胞刮棒收集细胞，并将含细胞的PBS转移至预冷的离心管内，离心去上清液。细胞沉淀用液氮充分研磨，取出适量至1.5 ml离心管中，加入裂解液(裂解液在使用前需添加蛋白酶抑制剂和磷酸酶抑制剂)，充分裂解后低温超声破碎。将裂解液4℃ 12 000 r/min离心10 min后取上清液。该上清即为样品的总蛋白溶液，测定其蛋白浓度并分装后于-80℃储存备用。

1.3 蛋白浓度测定

在干净的96孔板中分别加入0、1、2、4、8、12、16和20 μl 体积的BSA标准蛋白溶液，并用超纯水将每孔体积补充至20 μl 作为标准曲线。而后按照每孔2 μl 待测蛋白溶液和18 μl 超纯水的配比将待测蛋白溶液同样加入96孔板中，每个样品设置三个复孔。各孔内加入200 μl 显色液，37℃反应30 min后使用酶标仪进行吸光度值测定(波长562 nm)。根据标准蛋白溶液的已知浓度和吸光度值计算标准曲线，代入待测样本吸光度值求得其蛋白浓度值。

1.4 SDS-聚丙烯酰胺凝胶电泳

取10 μg 样品蛋白于12%SDS-PAGE中进行电泳分离蛋白,待各条带分离清楚后,用eStain LG蛋白染色仪对凝胶进行考马斯亮蓝染色,并将染色完成的凝胶用全自动数码凝胶图像分析系统成像。

1.5 胰蛋白酶酶解

将不同组样品标准化至同一浓度和体积,并加入DTT混匀,55 $^{\circ}\text{C}$ 孵育30 min。待溶液冷却至室温后加入碘乙酰胺并混匀,室温避光孵育15 min。在体系中加入丙酮以沉淀蛋白,-20 $^{\circ}\text{C}$ 孵育至少4 h。孵育结束后,溶液4 $^{\circ}\text{C}$ 12 000 r/min离心10 min去上清液,静置沉淀2~3 min以充分挥发丙酮。而后加入 NH_4HCO_3 复溶沉淀,并加入胰酶37 $^{\circ}\text{C}$ 酶解过夜。样品冻干后于-80 $^{\circ}\text{C}$ 保存。

1.6 肽段标记与除盐

用TEAB缓冲液溶解冻干样品,TMT试剂于室温解冻后加入无水乙腈振荡复溶,将TMT试剂与样品振荡混匀后室温孵育1 h。加入5%羟胺终止孵育,并用Sep-Pak C18 Cartridge将酶解得到的肽段进行脱盐。

1.7 磷酸化肽段富集

将冻干的肽段样品完全悬浮在200 μl 的结合/洗涤缓冲液中,再将混合液添加到已平衡好的离心柱中,树脂与样品混合孵育30 min。孵育完成后将色谱柱放入微量离心管中,1 000 g离心30 s后丢弃流通过液。利用结合/洗涤缓冲液和LC-MS级水洗脱色谱柱,最后用洗脱缓冲液洗脱色谱柱,立即在高速真空浓缩器中干燥洗脱液,以除去洗脱缓冲液,随即得到富集的磷酸化肽段。

1.8 数据预处理及统计学方法

原始数据采用Proteome Discoverer 2.4.1.15进行搜库分析后,对蛋白定性定量结果进行统计,而后利用常见的多个数据库(如GO、KEGG等)对鉴定的蛋白进行功能注释分析。筛选Score Sequest HT>0、unique peptide \geq 1,表达值不含空值的蛋白,对筛选到的蛋白进行中位数标准化及Log₂对数转换后得到可信蛋白。利用可信蛋白的表达量进行主成分分析(Principal component analysis, PCA)、相关性分析、欧氏距离的层次聚类分析(Hierarchical clustering analysis of sample Euclidean distance)后,通过*t*检验即*P*<0.05且差异性倍数(Fold change, FC) \leq 1/1.5或FC \geq 1.5为标准筛选差异蛋白,对筛选得到的差异蛋白进行GO分析、Pathway分析、互作分析三部分,同时针对差异比较组数据做火山图、表达模式聚类热图、venn分析等。

2 结果

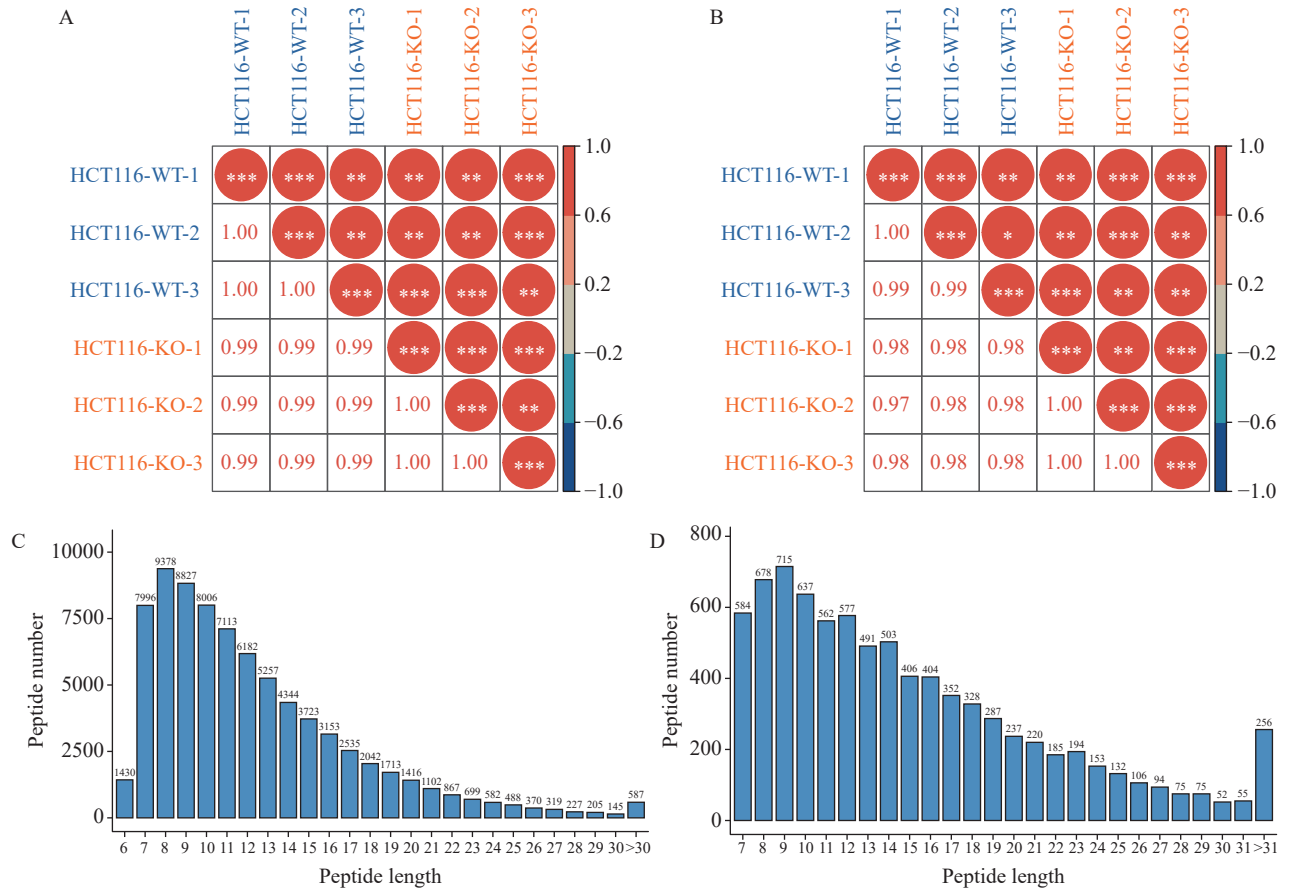
首先进行所有样本的质控评估,对实验组与对照组可信蛋白进行样品相关性分析,通过度量样品之间的相关程度,反映样品间的分组情况和重复性问题,结果发现全蛋白组(图1A)和磷酸化蛋白组(图1B)的实验组与对照组各自组内相关性高,重复性较好;全蛋白组(图1C)和磷酸化蛋白组(图1D)肽段长度分布的结果均显示酶切正常。

2.1 蛋白质组学技术分析RAI16对HCT116细胞的影响

2.1.1 HCT116 KO与WT细胞中差异蛋白表达分析为评估实验组与对照组间的蛋白表达差异性,拟用FC和经*t*检验得到的*P*值作为参考。按FC \geq 1.5或FC \leq 1/1.5且*P*<0.05为条件进行差异蛋白的筛选,共筛选出147个上调差异蛋白和230个下调差异蛋白(图2A),而对差异蛋白进行火山图分析,结果显示实验组与对照组蛋白差异明显(图2B)。

2.1.2 差异蛋白质功能的富集 差异性蛋白的功能富集主要通过GO分类和KEGG通路两个层面进行分析,检测差异蛋白是否在某些功能类型有显著性的富集趋势。差异蛋白在GO分类功能富集从生物进程、细胞组分和分子功能三个层面进行详细分析,阐释蛋白不同角度的生物学作用。筛选三个层面中*P*值最小的各10条绘制条形图,结果显示,差异蛋白主要分布于细胞分裂、血管生成及凋亡过程的负调控,细胞表面、胞液及细胞质核周区组成,ATP、酶及钙离子结合等多个方面(图3A)。分别选取GO上下调*P*值最小的10个条目绘制上下调对比图,结果显示,上调蛋白更多集中在上皮细胞分化、行为恐惧反应和组织再生等方面;下调蛋白更多集中在细胞分裂及核质、细胞质、细胞核的组成等方面(图3B)。

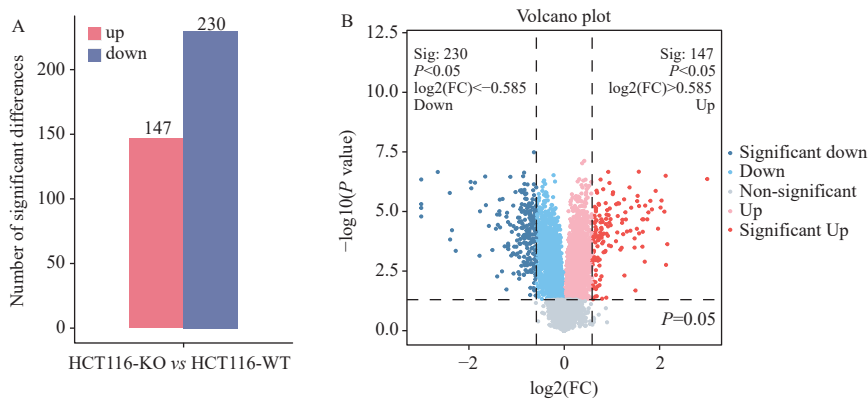
KEGG数据库从细胞过程、环境信息处理、遗传信息处理、人类疾病、新陈代谢、生物体系统、药物开发等7个方面对生物代谢通路进行研究。本研究通路富集结果表明,差异蛋白在癌症的发病途径、细胞周期、内质网中的蛋白加工过程、小细胞肺癌和癌症中的微小RNA等通路中有显著富集趋势(图4A)。分别选取KEGG上下调*P*值最小的10个通路绘制上下调对比图,结果显示,在癌症中的微小RNA、雌激素信号通路、金黄色葡萄球菌感染和视黄醇代谢等通路上调蛋白出现显著富集趋势,而癌症的发病途径、细胞周期、内质网中的蛋白加工过程和卵母细胞减数分裂等通路下调蛋白出现显著富集趋势;细胞外受体相互作用这条通路则是上下



A: correlation analysis of credible proteins between the experimental group and the control group; B: correlation analysis of credible phosphorylated proteins between the experimental group and the control group; C: histogram of peptide length distribution in protein samples; D: histogram of peptide length distribution in phosphorylated protein samples. *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$.

图1 蛋白质及磷酸化蛋白质样本质量控制评估

Figure 1 Quality control evaluation of protein and phosphorylated protein samples



A: screening results of differential proteins between the experimental and control groups; B: volcano map of up-regulated and down-regulated differential proteins.

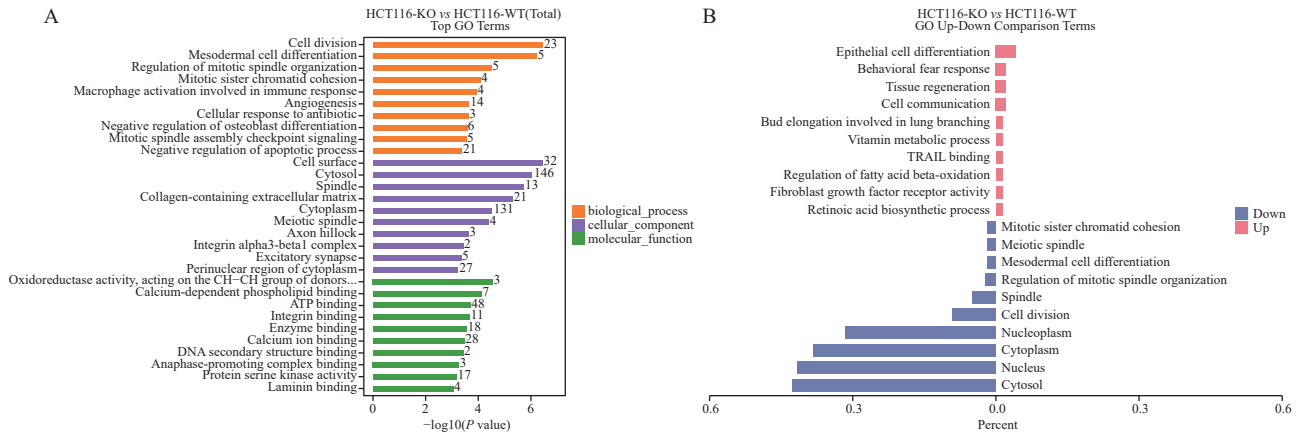
图2 差异蛋白分析结果

Figure 2 Differential protein analysis results

调蛋白均显著富集 (图4B)。

2.1.3 差异蛋白质互作网络分析获得关键蛋白质
 利用STRING数据库的蛋白质互作信息,选择其中本物种/近缘物种对差异蛋白进行互作关系分析,选取连接度排名前25的蛋白进行互作网络分析 (图5)。结果表明,整个蛋白互作网络主要以CCNB1、ALB、细胞周期蛋白依赖性激酶6 (Cyclin-dependent kinase 6, CDK6)、细胞周期蛋白依赖性激酶抑制剂

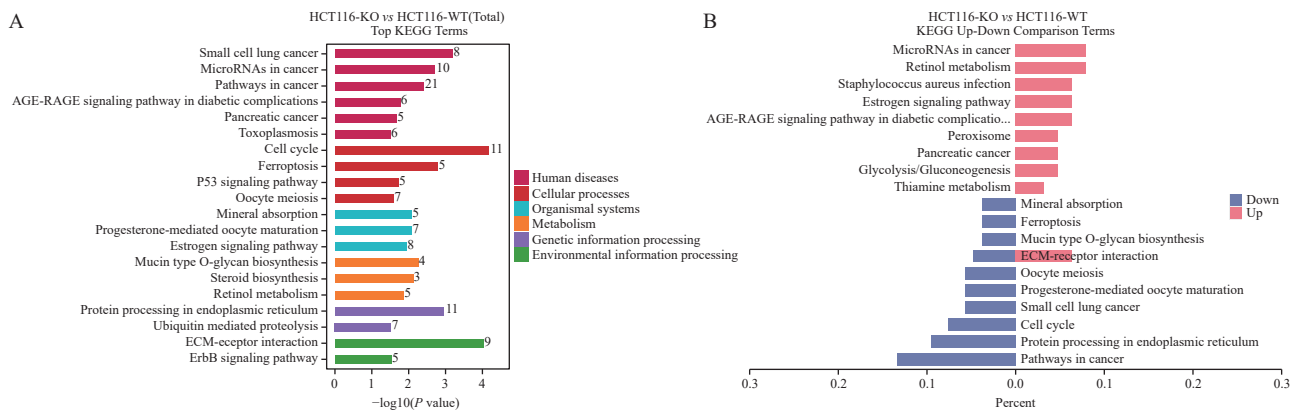
1 (Cyclin-dependent kinase inhibitor 1A, CDKN1A) 和notch受体1 (Notch receptor 1, NOTCH1) 为核心蛋白质。对差异蛋白质表达分析发现,信号转导子和转录激活子1 (Signal transducer and activator of transcription 1, STAT1)、细胞色素P450家族24亚家族A成员1 (Cytochrome P450 family 24 subfamily A member 1, CYP24A1)、死亡相关蛋白激酶1 (Death-associated protein kinase 1, DAPK1)、成



A: bar chart of GO enrichment analysis at the levels of biological process, cellular composition, and molecular function; B: comparison chart of up-regulation and down-regulation of differential proteins.

图3 差异蛋白功能GO富集分析

Figure 3 GO enrichment analysis of differential protein functions



A: enrichment of differential proteins in the KEGG pathway; B: comparison of up-regulation and down-regulation of differential proteins.

图4 差异蛋白功能KEGG富集分析

Figure 4 KEGG enrichment analysis of differential protein functions

纤维细胞生长因子受体2 (Fibroblast growth factor receptor 2, FGFR2)、SMAD家庭成员3 (SMAD family member 3, SMAD3) 和CDKN1A等蛋白表达上调显著, 整合素亚单位β1 (Integrin subunit beta 1, ITGB1)、CDK6、NOTCH1和RAD51重组酶 (RAD51 recombinase, RAD51) 等蛋白表达下调显著 (表1)。

2.2 磷酸化蛋白质组学技术分析RAI16对HCT116细胞的影响

2.2.1 RAI16对HCT116细胞中蛋白磷酸化修饰的影响

本研究共筛选出106个上调磷酸化位点和217个下调磷酸化位点 (图6A), 火山图分析结果显示实验组与对照组磷酸化位点差异明显 (图6B)。

2.2.2 差异磷酸化蛋白质功能的富集

GO富集分析结果表明, 差异磷酸化修饰位点对应蛋白主要分布于DNA修复、染色体分离的调节及DNA复制,

核浆、胞液及细胞核组成, RNA、钙黏着蛋白及染色质结合等多个方面 (图7A)。GO上下调对比图显示, 上调蛋白更多集中在细胞质及核斑点的组成、RNA剪接和细胞凋亡过程的正调控方面; 下调蛋白更多集中在DNA修复、钙黏蛋白结合和细胞分裂等方面; 而RNA及染色质的结合、核浆及细胞核的组成等方面均可富集到上下调蛋白 (图7B)。

KEGG通路富集结果表明, 差异磷酸化修饰位点对应蛋白在细胞周期、细胞间紧密连接、核质转运及剪接体的遗传信息处理、病毒致癌作用和癌症中的微小RNA等通路中有显著富集趋势 (图8A)。KEGG上下调对比图显示, 在核质转运、肌萎缩侧索硬化症、mRNA监测途径、内分泌等通路上调蛋白出现显著富集趋势, 而细胞周期、病毒致癌作用、卵母细胞减数分裂、细胞衰老等通路则是下调蛋白出现显著富集趋势; 剪接体及癌症中的微小RNA这两条通路则是上下调蛋白均显著富集 (图8B)。

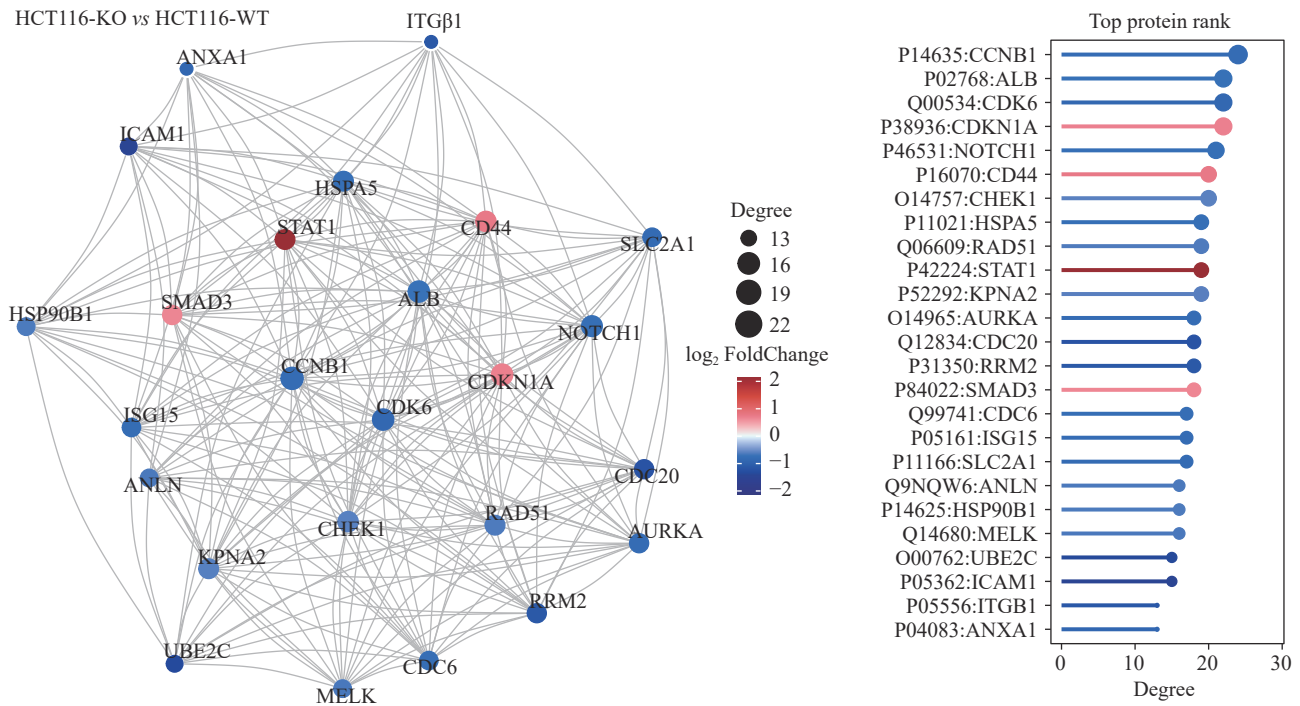


图5 差异蛋白互作网络

Figure 5 Differential protein interaction networks

表1 HCT116细胞敲除RAI16后差异表达显著的关键蛋白质

Table 1 Differentially expressed key proteins after RAI16 knockout in HCT116 cells

Gene name	Product	Regulation
STAT1	Signal transducer and activator of transcription 1-alpha/beta	Up
CYP24A1	1,25-dihydroxyvitamin D(3) 24-hydroxylase, mitochondrial	Up
ITGB1	Integrin beta-1	Down
DAPK1	Death-associated protein kinase 1	Up
FGFR2	Fibroblast growth factor receptor 2	Up
SMAD3	Mothers against decapentaplegic homolog 3	Up
CDK6	Cyclin-dependent kinase 6	Down
NOTCH1	Neurogenic locus notch homolog protein 1	Down
CDKN1A	Cyclin-dependent kinase inhibitor 1	Up
RAD51	DNA repair protein RAD51 homolog 1	Down

2.2.3 差异磷酸化蛋白质互作网络分析获得关键蛋白质 蛋白互作分析发现整个差异磷酸化蛋白互作网络主要以细胞周期蛋白依赖性激酶1 (Cyclin-dependent kinase 1, CDK1)、SSRP1、TRIM28、SNW1 (SNW domain containing 1) 和EIF4G1为核心蛋白质 (图9)。经过对差异磷酸化蛋白质表达分析发现, S期激酶相关蛋白1 (S-phase kinase-associated protein 1, SKP1) 和起源识别复合体亚基1 (Origin recognition complex subunit 1, ORC1) 等蛋

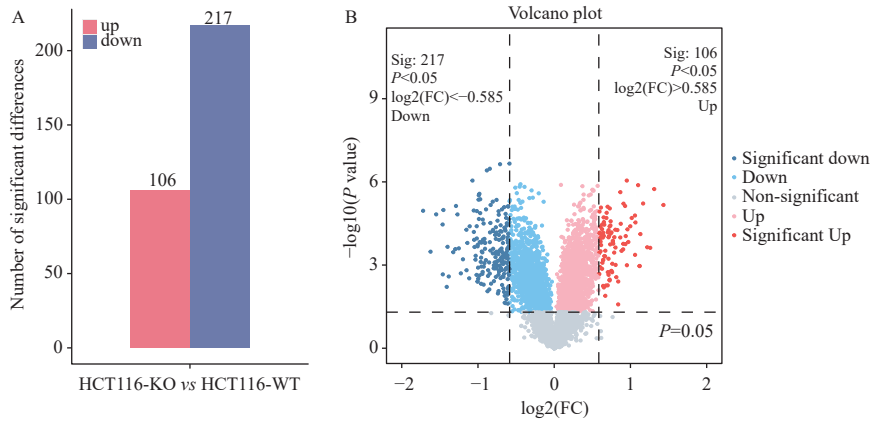
白表达上调显著, CDK1、细胞分裂周期20 (Cell division cycle 20, CDC20)、视网膜母细胞瘤转录辅助因子1 (Retinoblastoma transcriptional corepressor 1, RB1)、PTTG1姐妹染色单体分离调节器 (PTTG1 regulator of sister chromatid separation, PTTG1) 和视网膜母细胞瘤转录辅因子样1 (Retinoblastoma transcriptional corepressor like 1, RBL1) 等蛋白表达下调显著 (表2)。

2.3 蛋白质与磷酸化蛋白质组学技术联合分析RAI16对HCT116细胞的影响

蛋白质相互作用可以影响酶活性, 从而影响特定定位点的磷酸化修饰水平。在联合分析中去除本底蛋白可以提高分析特异性、敏感度和信噪比。

2.3.1 去本底蛋白修饰位点功能富集分析 将去本底差异磷酸化位点功能GO富集分析, 发现差异蛋白主要在核质、细胞核及细胞质组成, RNA、钙黏着蛋白及染色质结合, DNA修复、RNA剪接及DNA为模板转录的正调控等多个方面有显著富集趋势 (图10A)。KEGG富集结果显示, 差异蛋白主要在核质转运、剪接体、细胞周期、细胞间紧密连接、病毒致癌作用和癌症中的微小RNA等通路具有显著富集趋势 (图10B)。

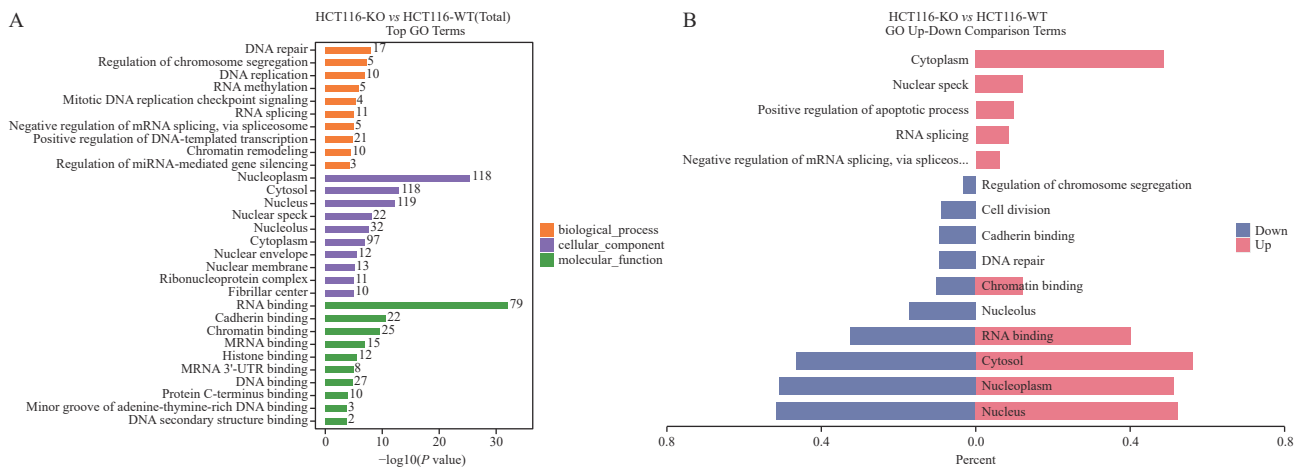
2.3.2 去本底蛋白修饰位点蛋白互作网络分析 对去本底蛋白修饰位点进行蛋白互作网络分析, 发现整个蛋白互作网络主要以DDX17、NCL、EEF2、CDK1、SSRP1和SMARCC1为核心蛋白质 (图11)。



A: screening results of differential phosphorylated proteins between the experimental and control groups; B: volcano map of up-regulated and down-regulated differential phosphorylated proteins.

图6 差异磷酸化蛋白分析结果

Figure 6 Differential phosphorylated protein analysis results



A: bar chart of GO enrichment analysis at the levels of biological process, cellular composition, and molecular function; B: comparison chart of up-regulation and down-regulation of differential phosphorylated proteins.

图7 差异磷酸化蛋白功能GO富集分析

Figure 7 GO enrichment analysis of differential phosphorylated protein functions

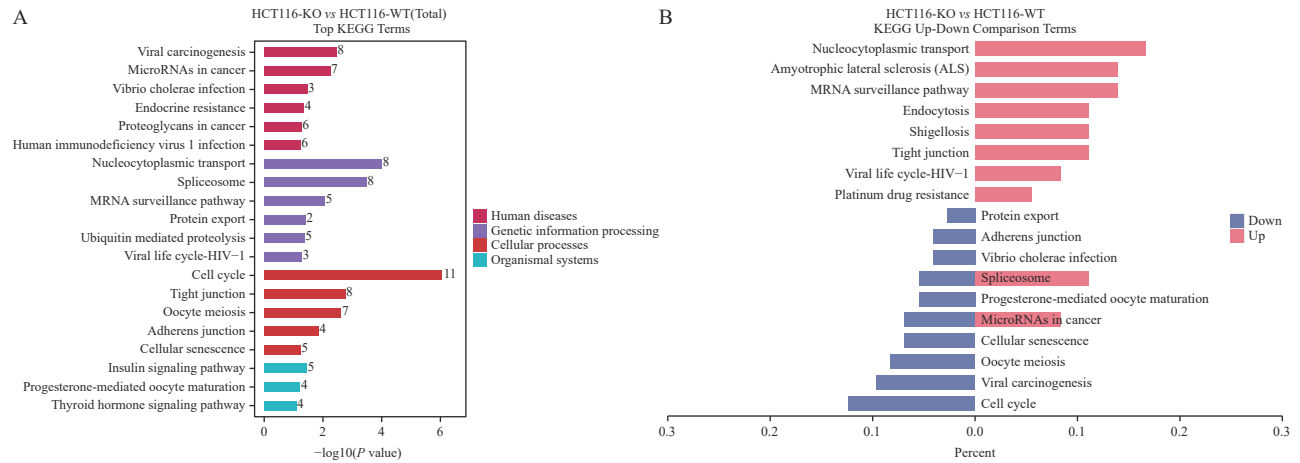
经过对去本底后差异修饰位点统计发现，SKP1、ORC1和BCL2相关细胞死亡激动剂（BCL2 associated agonist of cell death, BAD）等蛋白两组学差异变化均上调，且磷酸化差异变化比蛋白差异变化更显著，RBL1、RB1、CDK1、CDC6、微小染色体维持复合体4组分（Minichromosome maintenance complex component 4, MCM4）、转录因子Dp-1（Transcription factor Dp-1, TFDP1）、色域解旋酶DNA结合蛋白4（Chromodomain helicase DNA binding protein 4, CHD4）和SNW1等蛋白两组学差异变化均下调，且磷酸化差异变化比蛋白差异变化更显著（表3）。

2.3.3 激酶分析 激酶-底物富集分析是根据磷酸化差异位点，分析其上游可能存在的调控激酶。激酶活性预测结果提示，磷酸化激酶活性上调最显著的激酶依次为VRK2、VRK1、PAK1、CHEK1、CSNK2A1；下调的激酶依次为CDK2、CDK1、CDK4、PRKCD、MTOR、RPS6KA3、CDK6、CDK9（图12A）。而对激酶-底物数量进行统计发现，CDK1和2激酶的底物数量显著高于其他激酶（图12B）。

激酶-底物调控网络图分析发现该图主要以CDK1、CDK2和AKT1为核心，其对应底物数量较多，关系较为密切（图12C）。

3 讨论

作为E3泛素连接酶的核心组成成分，SKP1可以调节特定蛋白质底物的泛素化，从而在下游生物过程的调控中发挥关键作用^[17]。其异常表达可能会影响基因组和染色体的稳定性，进而诱发癌症^[18-19]。细胞周期的进展受细胞周期蛋白的顺序表达调节，而细胞周期蛋白A与CDK2的复合物可与ORC1特异性识别，进而控制DNA复制活性，影响细胞周期^[20]。ORC1作为唯一具有ATP酶功能的哺乳动物ORC亚基，可以募集CDC6、CDT1和MCM等蛋白，完成复制前复合物（pre-RC）的组装，启动DNA复制并调控其准确性^[21-23]。ORC1在大多数肿瘤中过表达，可作为诊断的新型生物标志物，并可能成为癌症的潜在治疗靶点^[24]。而MCM4还可参与构成MCM复合物，具有DNA解旋酶活性，是DNA复制的关



A: enrichment of differential phosphorylated proteins in the KEGG pathway; B: comparison of up-regulation and down-regulation of differential phosphorylated proteins.

图8 差异磷酸化蛋白功能KEGG富集分析

Figure 8 KEGG enrichment analysis of differential phosphorylated protein functions

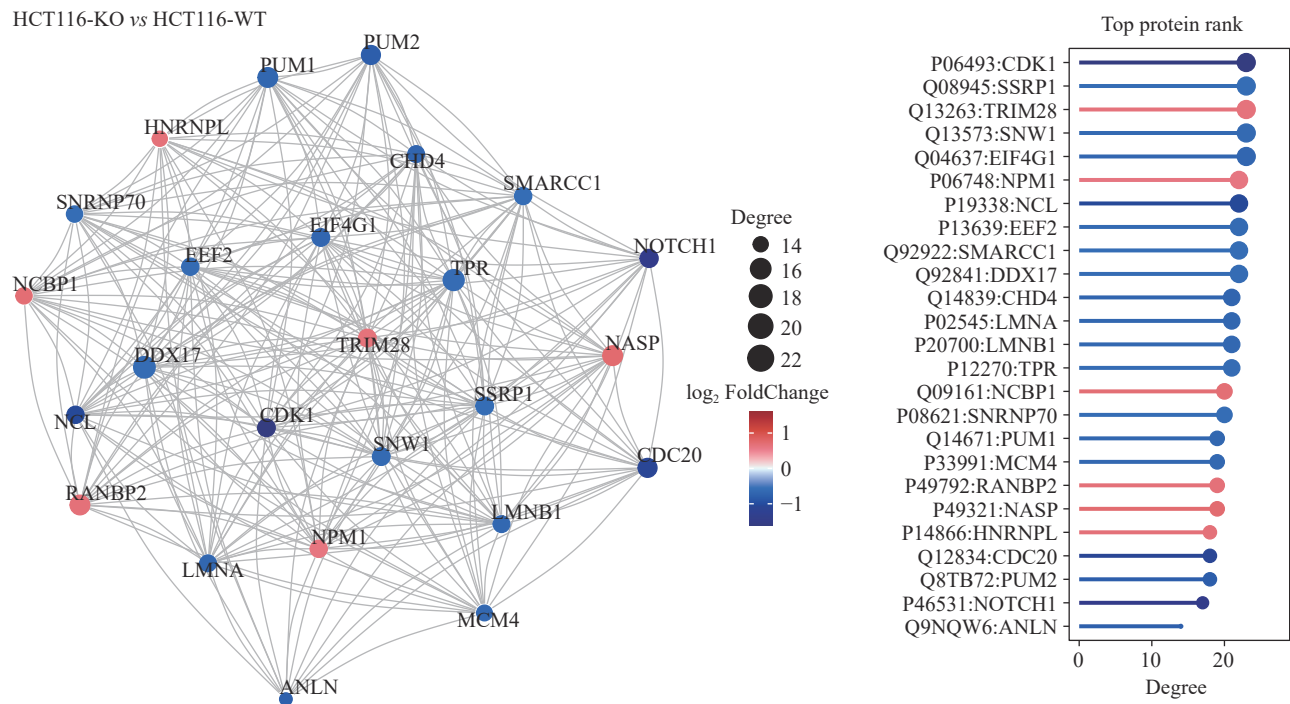


图9 差异磷酸化蛋白互作网络

Figure 9 Differential phosphorylated protein interaction networks

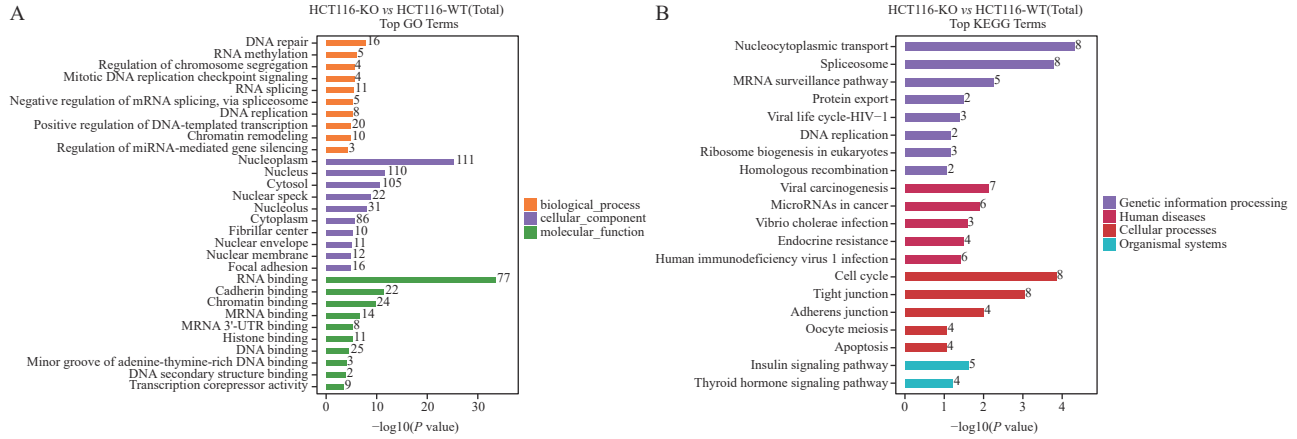
表2 HCT116细胞敲除RAI16后差异表达显著的关键磷酸化蛋白质

Table 2 Differentially expressed key phosphorylated proteins after RAI16 knockout in HCT116 cells

Gene name	Product	Regulation
CDK1	Cyclin-dependent kinase 1	Down
SKP1	S-phase kinase-associated protein 1	Up
ORC1	Origin recognition complex subunit 1	Up
CDC20	Cell division cycle protein 20 homolog	Down
RB1	Retinoblastoma-associated protein	Down
PTTG1	Securin	Down
RBL1	Retinoblastoma-like protein 1	Down

键调节因子，MCM4的缺失会导致DNA损伤和基因组不稳定，从而可能促进癌症的发展^[25-26]。

RB1和RBL1是细胞周期的重要调节剂，可受诸多CDK调控，具有调节染色质加工和修饰等功能，影响基因组稳定性^[27-28]。作为一种肿瘤抑制因子，其失调与前列腺癌、胶质母细胞瘤、视网膜下血管瘤和血管母细胞瘤等肿瘤的发生和进展有关^[29-30]。TFDP1编码转录因子E2F的异二聚体伴侣DP1，而E2F可通过激活参与细胞周期进程和DNA复制的一组基因来调节细胞增殖。E2F既可激活ARF和TAp73



A: GO classification; B: KEGG pathway.

图 10 去本底蛋白修饰位点功能富集分析

Figure 10 Functional enrichment analysis of modified sites in background-removal proteins

等肿瘤抑制基因发挥抗癌作用^[31]，又可通过上调CDK1的表达进而促进肿瘤细胞的生长、迁移或侵袭^[32]。CDK1是哺乳动物中唯一一对细胞周期进程至关重要的CDK，可与所有细胞周期蛋白结合，促进G₂/M期和G₁/S期的转变，以及G₁期的进展，并可作为癌症的潜在治疗靶点^[32]。CDC6是细胞周期调节的重要参与者，参与S期和M期的调节，并且它还是CDK1的关键抑制调节因子^[33]。CDC6还是一种关键的复制许可因子，在调节DNA复制过程中起着关键作用，也与肿瘤发生密切相关^[34]。CHD4是Mi-2/核小体重塑和脱乙酰酶复合物的的重要组成部分，可参与基因转录、DNA损伤修复、维持基因

组稳定性和染色质组装^[35-36]。CHD4对于体内染色质可及性的调节对于B细胞产生抗体至关重要，进而影响免疫系统发挥功能^[37]。BAD是Bcl-2家族的促凋亡蛋白，其表达可作为黑色素瘤、前列腺癌和乳腺癌等肿瘤进展的标志^[38]。作为剪接因子，SNW1可以参与DNA修复，直接影响有丝分裂^[39]。

综上，所述差异磷酸化蛋白质几乎一致地影响细胞周期，进而影响癌症发生发展。所筛选出的与RAI16相关具有研究价值的蛋白激酶、磷酸化事件和关键信号通路，为后续开展验证及功能性研究提供了理论依据。

利益冲突声明：

所有作者均声明不存在利益冲突。

HCT116-KO vs HCT116-WT

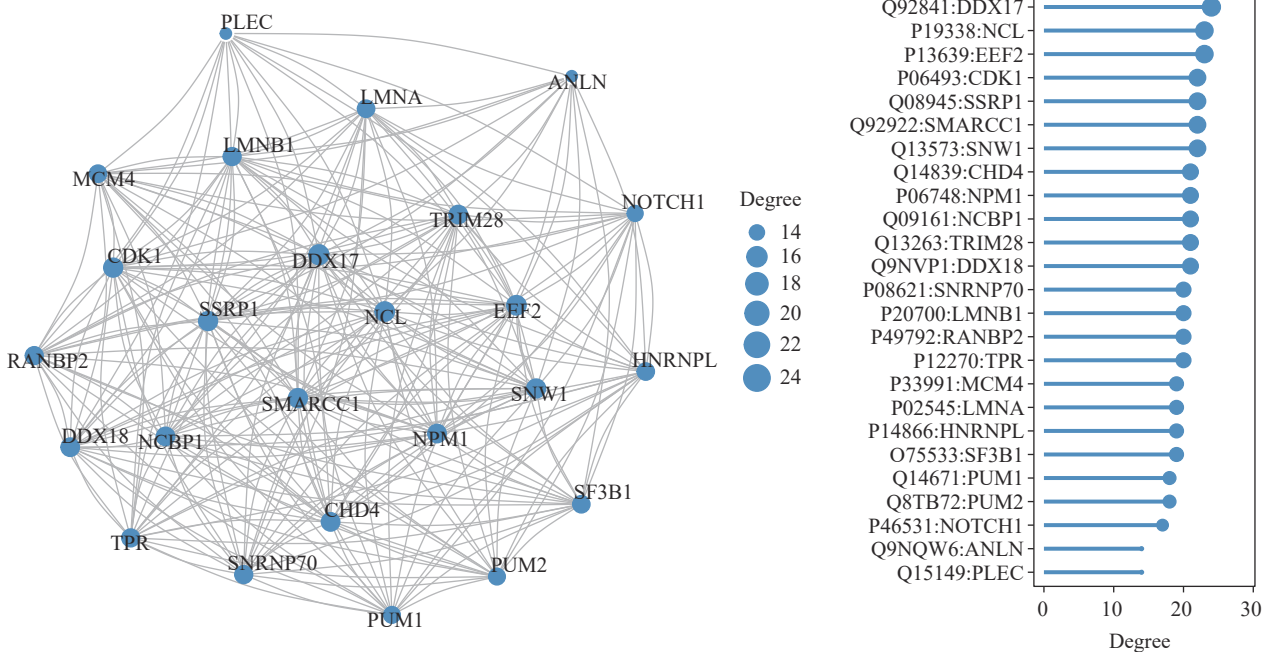


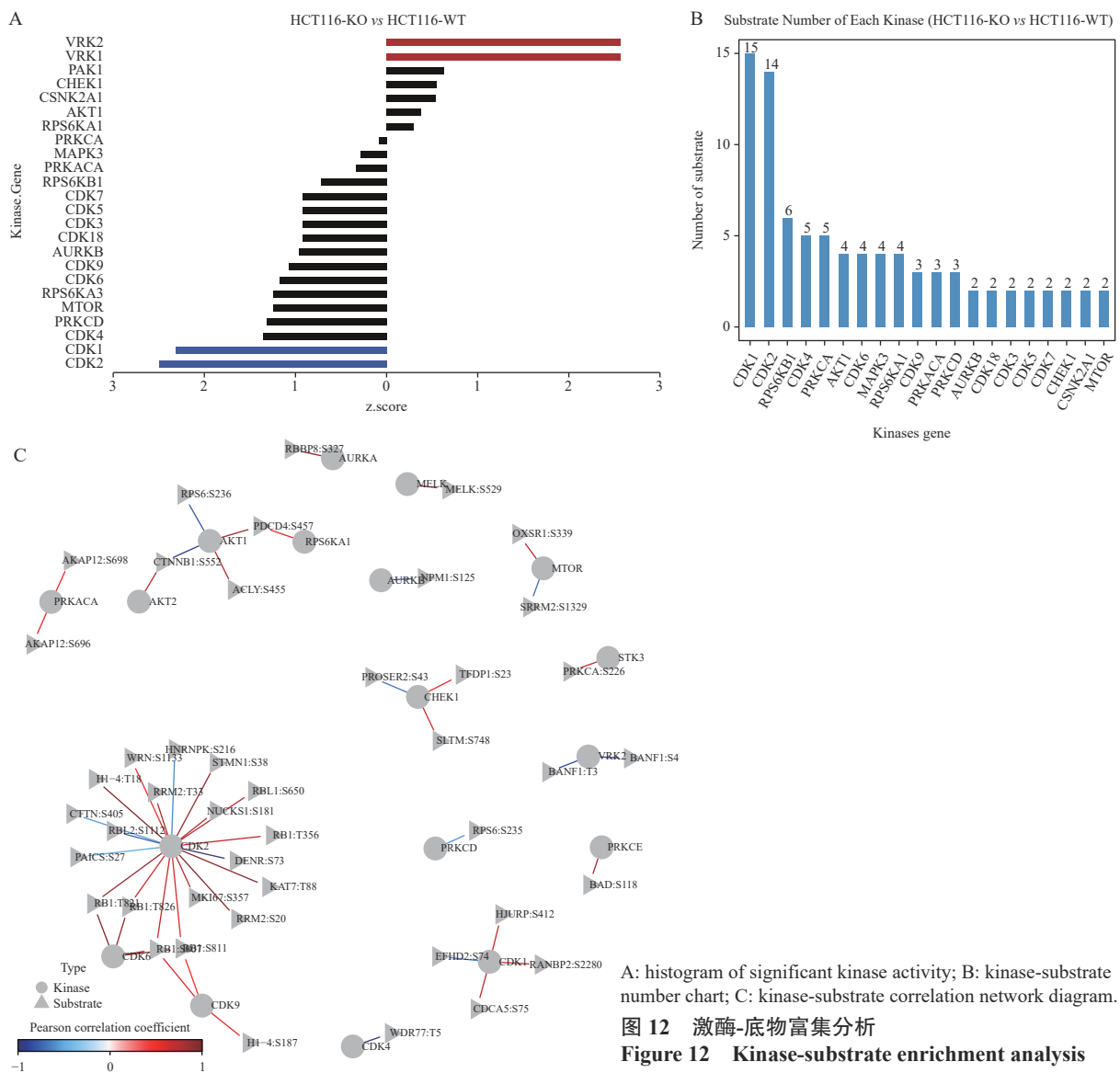
图 11 去本底蛋白修饰位点蛋白互作网络

Figure 11 Protein interaction network of modified sites in background-removal proteins

表 3 HCT116细胞敲除RAI16后差异表达显著的去本底蛋白修饰位点

Table 3 Differentially expressed key modified sites in background-removal proteins after RAI16 knockout in HCT116 cells

Gene name	class	Product	NEGG description
SKP1	Phospho-more up	S-phase kinase-associated protein 1	Modification-dependent protein catabolic process
ORC1	Phospho-more up	Origin recognition complex subunit 1	DNA replication
RBL1	Phospho-more down	Retinoblastoma-like protein 1	Regulation of cell cycle
RB1	Phospho-more down	Retinoblastoma-associated protein	Regulation of cell cycle
CDK1	Phospho-more down	Cyclin-dependent kinase 1	Cyclin-dependent protein serine/threonine kinase activity
CDC6	Phospho-more down	Cell division control protein 6 homolog	DNA replication initiation
MCM4	Phospho-more down	DNA replication licensing factor MCM4	DNA replication initiation
TFDP1	Phospho-more down	Transcription factor Dp-1	Cell cycle
CHD4	Phospho-more down	Chromodomain-helicase-DNA-binding protein 4	Peripheral T cell tolerance induction
BAD	Phospho-more up	Bcl2-associated agonist of cell death	Positive regulation of intrinsic apoptotic signaling pathway in response to osmotic stress
SNW1	Phospho-more down	SNW domain-containing protein 1	Generation of catalytic spliceosome for second transesterification step



A: histogram of significant kinase activity; B: kinase-substrate number chart; C: kinase-substrate correlation network diagram.
图 12 激酶-底物富集分析
Figure 12 Kinase-substrate enrichment analysis

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