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HDAC6通过介导FLOT2去乙酰化维持其在鼻咽癌中的稳定及促瘤作用

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［摘 要］**目的：**浮舰蛋白(flotillin-2，FLOT2)是典型的致瘤蛋白和潜在的肿瘤治疗靶点，但靶向FLOT2的干预策略仍未确定。翻译后修饰作为表观调控的重要方式，对蛋白质的活性、定位和稳定性等特性具有重要的调控作用，揭示蛋白质翻译后修饰的调控机制和功能是靶向治疗开发的一种有效手段。本研究旨在研究鼻咽癌中FLOT2赖氨酸乙酰化修饰的调控机制及其功能，为靶向FLOT2的肿瘤干预手段提供新思路。**方法：**利用PhosphoSitePlus数据库分析FLOT2的赖氨酸乙酰化位点，并构建赖氨酸乙酰化位点突变的FLOT2突变体[FLOT2(K211R)]；用组蛋白去乙酰化酶(histone deacetylases，HDAC)抑制剂曲古菌素A(trichostatin A，TSA)、Sirt家族去乙酰化酶抑制剂烟酰胺(nicotinamide，NAM)处理鼻咽癌细胞，TSA处理转染FLOT2突变体质粒的人胚肾细胞(human embryonic kidney，HEK)-293T细胞；利用免疫共沉淀(co-immunoprecipitation，Co-IP)检测FLOT2的总赖氨酸乙酰化水平以及特定赖氨酸(K)位点突变对FLOT2总赖氨酸乙酰化水平的影响。用蛋白质印迹法检测TSA处理未转染/转染FLOT2突变体质粒后的鼻咽癌细胞中FLOT2/FLAG-FLOT2的蛋白质表达，实时反转录聚合酶链反应(real-time reverse transcription PCR，real-time RT-PCR)检测TSA处理后鼻咽癌细胞中*FLOT2* mRNA的表达。用TSA分别联合MG132或氯喹(chloroquine，CQ)处理鼻咽癌细胞后，检测FLOT2的蛋白质表达。用放线菌酮(cycloheximide，CHX)分别处理已转染FLAG-FLOT2(WT)或FLAG-FLOT2(K211R)质粒的HEK-293T细胞，检测FLAG-FLOT2、FLOT2(K211R)的蛋白质表达水平以反映蛋白质的降解速率。通过BioGrid数据库查询FLOT2与HDAC6之间是否可能存在相互作用，并采用Co-IP验证。用FLAG-FLOT2(WT)/FLAG-FLOT2(K211R)质粒分别联合空白对照(Vector)/HDAC6质粒转染HEK-293T细胞，分为FLAG-FLOT2(WT)+Vector、FLAG-FLOT2(WT)+HDAC6、FLAG-FLOT2(K211R)+Vector、FLAG-FLOT2(K211R)+HDAC6共4组，分析K211R突变对FLOT2总赖氨酸乙酰化水平的影响。在6-0B细胞中，分别过表达FLOT2(WT)和FLOT2(K211R)，用细胞计数试剂盒-8(cell counting kit-8，CCK-8)、平板克隆形成和Transwell侵袭检测FLOT2乙酰化位点突变体的生物学功能。**结果：**PhosphoSitePlus数据库显示FLOT2的K211位点存在乙酰化修饰，Co-IP结果证实FLOT2蛋白存在明显的乙酰化修饰，且TSA可以显著上调FLOT2的总乙酰化修饰水平，而NAM则无此作用；K211位点突变后FLOT2的总赖氨酸乙酰化水平显著下降，且不受TSA影响。TSA下调鼻咽癌细胞中FLOT2的蛋白质表达水平，而不影响*FLOT2* mRNA的表达水平，也不影响转染FLAG-FLOT2(K211R)的鼻咽癌细胞中FLOT2(K211R)的蛋白质表达水平。FLOT2(K211R)的蛋白质降解速率显著慢于FLOT2(WT)的降解速率。蛋白酶体抑制剂MG132可以阻止TSA引起的FLOT2降解，溶酶体抑制剂CQ则无此功能。BioGrid数据库数据显示FLOT2与HDAC6可能存在相互

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作用，Co-IP结果证实FLOT2与HDAC6抗体可以相互共沉淀对方蛋白。在敲减*HDAC6*表达的鼻咽癌细胞中，FLOT2的总赖氨酸乙酰化水平显著提高；共转染HDAC6和FLAG-FLOT2(WT)可显著降低总赖氨酸乙酰化水平，而共转染HDAC6和FLAG-FLOT2(K211R)不影响总赖氨酸乙酰化水平。敲减*HDAC6*可以显著下调FLOT2的蛋白质水平而不影响其mRNA水平；MG132可以阻止敲减*HDAC6*引起的FLOT2降解。敲减*HDAC6*，FLOT2的降解速率显著加快。转染FLOT2(K211R)突变体的鼻咽癌细胞增殖速度和侵袭能力显著强于转染FLOT2(WT)的细胞。**结论：**FLOT2 K211位点存在乙酰化修饰，HDAC6通过介导FLOT2 K211的去乙酰化修饰抑制FLOT2经蛋白酶体途径降解，维持其在鼻咽癌中的稳定和促瘤功能。

[关键词] 浮舰蛋白2；赖氨酸乙酰化；鼻咽癌；组蛋白去乙酰化酶6

HDAC6-mediated deacetylation of FLOT2 maintains stability and tumorigenic function of FLOT2 in nasopharyngeal carcinoma

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ABSTRACT

Objective: Flotillin-2 (FLOT2) is a prototypical oncogenic and a potential target for cancer therapy. However, strategies for targeting FLOT2 remain undefined. Post-translational modifications are crucial for regulating protein stability, function, and localization. Understanding the mechanisms and roles of post-translational modifications is key to developing targeted therapies. This study aims to investigate the regulation and function of lysine acetylation of FLOT2 in nasopharyngeal carcinoma, providing new insights for targeting FLOT2 in cancer intervention.

Methods: The PhosphoSitePlus database was used to analyze the lysine acetylation sites of FLOT2, and a lysine acetylation site mutation of FLOT2 [FLOT2 (K211R)] was constructed. Nasopharyngeal carcinoma cells were treated with histone deacetylase (HDAC) inhibitor trichostatin A (TSA) and Sirt family deacetylase inhibitor nicotinamide (NAM). TSA-treated human embryonic kidney (HEK)-293T were transfected with FLOT2 mutant plasmids. Co-immunoprecipitation (Co-IP) was used to detect total acetylation levels of FLOT2 and the effects of specific lysine (K) site mutations on FLOT2 acetylation. Western blotting was used to detect FLOT2/FLAG-FLOT2 protein expression in TSA-treated nasopharyngeal carcinoma cells transfected with FLOT mutant plasmids, and real-time reverse transcription PCR (real-time RT-PCR) was used to detect *FLOT2* mRNA expression. Nasopharyngeal carcinoma cells were treated with TSA combined with MG132 or chloroquine (CQ) to analyze FLOT2 protein expression. Cycloheximide (CHX) was used to treat HEK-293T cells transfected with FLAG-FLOT2 (WT) or FLAG-FLOT2 (K211R) plasmids to assess protein degradation rates. The BioGrid database was used to identify potential interactions between FLOT2 and HDAC6, which were validated by Co-

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IP. HEK-293T cells were co-transfected with FLAG-FLOT2 (WT)/FLAG-FLOT2 (K211R) and Vector/HDAC6 plasmids, and grouped into FLAG-FLOT2 (WT) +Vector, FLAG-FLOT2 (WT) +HDAC6, FLAG-FLOT2 (K211R) +Vector, and FLAG-FLOT2 (K211R) + HDAC6 to analyze the impact of K211R mutation on total lysine acetylation levels. In 6-0B cells, overexpression of FLOT2 (WT) and FLOT2 (K211R) was performed, and the biological functions of FLOT2 acetylation site mutants were assessed using cell counting kit-8 (CCK-8), colony formation, and Transwell invasion assays.

Results: The PhosphoSitePlus database indicated that FLOT2 has an acetylation modification at the K211 site. Co-IP confirmed significant acetylation of FLOT2, with TSA significantly increasing overall FLOT2 acetylation levels, while NAM had no effect. Mutation at the K211 site significantly reduced overall FLOT2 acetylation, unaffected by TSA. TSA decreased FLOT2 protein expression in nasopharyngeal carcinoma cells without affecting *FLOT2* mRNA levels or FLOT2 (K211R) protein expression in transfected cells. The degradation rate of FLOT2 (K211R) protein was significantly slower than that of FLOT2 (WT). The proteasome inhibitor MG132 prevented TSA-induced FLOT2 degradation, while the lysosome inhibitor CQ did not. BioGrid data suggested a potential interaction between FLOT2 and HDAC6, confirmed by Co-IP. Knockdown of *HDAC6* in nasopharyngeal carcinoma cells significantly increased FLOT2 acetylation; co-transfection of HDAC6 and FLAG-FLOT2 (WT) significantly decreased total lysine acetylation levels, whereas co-transfection of HDAC6 and FLAG-FLOT2 (K211R) had no effect. Knockdown of *HDAC6* significantly reduced FLOT2 protein levels without affecting mRNA levels. MG132 prevented *HDAC6*-knockdown-induced FLOT2 degradation. Knockdown of *HDAC6* significantly accelerated FLOT2 degradation. Nasopharyngeal carcinoma cells transfected with FLOT2 (K211R) showed significantly higher proliferation and invasion than those transfected with FLOT2 (WT).

Conclusion: The K211 site of FLOT2 undergoes acetylation modification, and HDAC6 mediates deacetylation at this site, inhibiting proteasomal degradation of FLOT2 and maintaining its stability and tumor-promoting function in nasopharyngeal carcinoma.

KEY WORDS　flotillin-2; lysine acetylation; nasopharyngeal carcinoma; histone deacetylase 6

鼻咽癌是中国中南和东南沿海地区高发的头颈部恶性肿瘤，发病率和病死率均位居世界第一，严重威胁患者的生命健康[1-2]。早期鼻咽癌行单独放射治疗可实现临床治愈，但鼻咽癌的早期症状与鼻咽良性疾病症状界限模糊，多数患者确诊时已处于中晚期，目前以放化疗为基础的综合治疗效果有限，鉴定鼻咽癌的关键驱动因子是开发新治疗手段、提高临床治疗水平的有效途径[1-2]。

浮舰蛋白2(flotillin-2，FLOT2)是细胞膜脂筏结构的重要组成蛋白，在细胞内外物质转运和细胞信号转导中发挥重要作用[3]。研究[4-8]表明：FLOT2在鼻咽癌、胃癌、乳腺癌和黑色素瘤多种实体恶性肿瘤中高表达，通过与A型红细胞生成素肝配蛋白受体2(ephrin type-A receptor 2，EPHA2)、表皮生长因子受体(epidermal growth factor receptor，EGFR)等蛋白质相互作用，激活磷脂酰肌醇3激酶(phosphoinositide 3-kinase，PI3K)/蛋白激酶B(protein kinase B，AKT)、无翅型MMTV整合位点家族成员(wingless-type MMTV integration site family，WNT)、核因子κB(nuclear factor kappa-B，NF-κB)和转化生长因子-β(transforming growth factor-β，TGF-β)等信号通路，促进肿瘤的恶性增殖、侵袭和转移，是靶向治疗肿瘤的理想靶点。尽管部分微RNA(microRNA，miRNA)相关的转录后水平调控和反式调节蛋白相关的转录水平调控与FLOT2在肿瘤中的高表达相关[5, 9-10]，但上述调控方式的特异性仍待进一步验证，难以作为靶向FLOT2治疗研发的理论依据，探究FLOT2在肿瘤中高表达的机制仍是推动FLOT2转化研究的关键。

蛋白质的翻译后修饰是重要的表观调控机制之

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一。磷酸化、乙酰化、甲基化、糖基化和泛素化等修饰在调控蛋白质自身的稳定性、活性、亚细胞定位和寡聚化程度等方面发挥关键作用[11]。在肿瘤的发生和发展中，蛋白质的翻译后修饰是诸多肿瘤相关蛋白质异常活化的重要机制，这为肿瘤的靶向干预提供了理想的靶点[12]。如p53、Myc和EGFR等肿瘤分子的自身翻译后修饰是决定其活性、定位和稳定性等特性的关键因素，这为靶向干预肿瘤进展提供了有效的切入点[13]。既往研究[14]表明组蛋白等蛋白质的乙酰化修饰特征是鼻咽癌的潜在分子治疗靶点；组蛋白去乙酰化酶(histone deacetylase，HDAC)作为介导蛋白乙酰化调控的关键家族，通过调控不同底物的乙酰化水平在鼻咽癌的发生和发展中发挥重要作用[15-16]。本课题组前期的研究[7-8]证实FLOT2在鼻咽癌中高表达，并促进肿瘤细胞的增殖、侵袭及转移，是鼻咽癌的潜在干预靶点，但是目前暂无有效的干预策略。基于FLOT2的蛋白质翻译后修饰及其在FLOT2自身特性中的作用仍不明确，本研究在鼻咽癌中探究赖氨酸乙酰化修饰调控FLOT2自身稳定性的作用，以及其对鼻咽癌恶性表型的影响，旨在为靶向FLOT2干预肿瘤进展提供新思路。

1 材料与方法

1.1 材料

鼻咽癌细胞系5-8F、CNE2和6-10B，以及人胚肾细胞(human embryonic kidney，HEK)-293T细胞均由本课题组前期保存。RPMI 1640基础培养基和高糖DMEM培养基均购自生工生物工程(上海)股份有限公司。HDACs抑制剂曲古菌素A(trichostatin A，TSA)、Sirt家族去乙酰化酶抑制剂烟酰胺(nicotinamide，NAM)、蛋白酶体抑制剂MG132、溶酶体抑制剂氯喹(chloroquine，CQ)、抑制真核生物翻译的放线菌酮(cycloheximide，CHX)和细胞计数试剂盒-8(cell counting kit-8，CCK-8)均购自美国MCE公司。放射免疫沉淀法裂解液(radio- immunoprecipitation assay lysis buffer，RIPA)、蛋白抑制剂Cocktails和增强型化学发光试剂均购自苏州新赛美生物科技有限公司。AG RNAex Pro RNA提取试剂、Evo M-MLV反转录试剂盒(含去除gDNA试剂)和SYBR Green Pro Taq HS预混型qPCR试剂盒均购自湖南艾科瑞生物工程有限公司。抗赖氨酸乙酰化[pan-lysine(K) acetylation，pan-Kac]抗体和FLOT2(C42A3)兔单克隆抗体均购自美国CST公司。HDAC6兔单克隆抗体、GAPDH鼠单克隆抗体、Myc-Tag兔单克隆抗体和DDDDK (FLAG)-Tag兔单克隆抗体均购自武汉爱博泰克生物科技有限公司。Lipo8000转染试剂和兔IgG抗体均购自上海碧云天生物技术股份有限公司。Protein A/G磁珠购自上海翎因生物科技有限公司。pcDNA3.1-HDAC6及对照质粒为本课题组早期构建和保存。pLKO.1-shHDAC6-1/-2及shNC质粒委托北京擎科生物科技有限公司构建。慢病毒包装系统质粒pSPAX2和pMD2.G为本课题组前期引进并保存。pcDNA3.1-FLAG-FLOT2购自云舟生物科技(广州)股份有限公司。胎牛血清、2×Hieff Canace® AdvanceFast PCR Master Mix (With Dye)均购自翌圣生物科技(上海)股份有限公司。

1.2 方法

1.2.1 细胞培养和稳定细胞系的构建

5-8F、CNE2、6-10B和HEK-293T细胞用含10%胎牛血清的RPMI 1640培养基，于37 ℃，5% CO_2细胞培养箱中培养。参照本课题组已发表论文[6]，病毒包装(见1.2.4)完成后，利用病毒感染鼻咽癌细胞，加入含嘌呤霉素(2 μg/mL)的培养基进行培养，筛选获得稳定敲减*HDAC6*的鼻咽癌细胞系。

1.2.2 FLOT2乙酰化位点分析

利用PhosphoSitePlus数据库[17]，分析FLOT2的蛋白质翻译后修饰情况，发现其K211位点存在乙酰化修饰。进一步用美国国家医学图书馆国家生物技术信息中心(National Center for Biotechnology Information，NCBI)的GENE数据库分析FLOT2蛋白质K211位点在人、小鼠、大猩猩和狗等哺乳动物中的保守性情况。

1.2.3 突变体的构建

利用2×Hieff Canace® AdvanceFast PCR Master Mix (With Dye)，基于pcDNA3.1-FLAG-FLOT2进行突变，构建pcDNA3.1-FLAG-FLOT2(K211R)突变体。参照说明书设置PCR扩增体系。程序：预变性(98 ℃，30 s)后；进行变性(98 ℃，10 s)、退火(60 ℃，5 s)，延伸(72 ℃，60 s)，共31个循环，最后终延伸(72 ℃，2 min)。引物序列：正向为5'-AGACACCA-GGATTGCTGACTCTAAGCGAGCC-3'，反向为5'-GCAATCCTGGTGTCTGCCATGAACTTC-AC-3'。

1.2.4 质粒转染和慢病毒包装

参照文献[6]进行质粒转染：转染前24 h将细胞接种至6孔板或6 cm细胞培养皿，当细胞达到约80%的融合时，根据Lipo8000转染试剂说明书配制质粒和转染试剂的混合物(质粒和转染试剂比例为1 μg: 1.5 μL)，6孔板或6 cm细胞培养皿中加入的质粒[pcDNA3.1-FLAG-FLOT2/pcDNA3.1-FLAG-FLOT2 (K211R)/pcDNA3.1-Myc-HDAC6]总量分别为2.5 μg

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或8.0 μg。转染6 h后更换新鲜培养基。

慢病毒包装质粒配比为pLKO.1-shNC/shHDAC6-1/shHDAC6-2: pSPAX2: pMD2. G 为 9: 6: 3(10 cm 培养皿)，质粒和转染试剂配比同上，转染HEK-293T细胞，48 h后收集病毒悬液，经离心和0.45 μm滤器过滤后，用于感染鼻咽癌细胞。

1.2.5 蛋白质印迹法

参照文献[6]，用15 min离心RIPA冰上裂解细胞，经12 000 r/min收集上清蛋白液，利用二喹啉甲酸(bicinchonininc acid，BCA)法测定蛋白质浓度后，经5×SDS上样缓冲液变性，进行蛋白质印迹法实验，每孔上样为30 μg。经SDS聚丙烯酰胺凝胶电泳(SDS polyacrylamide gel electrophoresis，SDS-PAGE)分离、转膜、封闭和一抗(FLOT2、HDAC6、GAPDH、FLAG-tag抗体)孵育和二抗孵育后进行化学发光成像。

相关分组和处理：1)用TSA(1 μmol/L)分别处理鼻咽癌细胞(5-8F、CNE2)0、6、12和16 h后，检测FLOT2的蛋白质表达水平。2)用TSA(1 μmol/L)处理转染FLAG-FLOT2(K211R)的鼻咽癌细胞(5-8F、CNE2)0、6、12和16 h后，检测FLAG-FLOT2的蛋白质表达水平。3)用CHX(100 μmol/L)分别处理已转染FLAG-FLOT2(WT)或FLAG-FLOT2(K211R)质粒的HEK-293T细胞0、12、18 h后，检测FLAG-FLOT2、FLOT2(K211R)的蛋白质表达水平以反映蛋白质的降解速率(CHX蛋白降解实验)。4)用TSA(1 μmol/L)分别联合MG132(10 μmol/L)或CQ(50 μmol/L)处理鼻咽癌细胞(5-8F、CNE2)16 h后，检测FLOT2的蛋白质表达水平。5)敲减*HDAC*表达后，检测FLOT2的蛋白质表达水平，实验分shNC对照、shHDAC6-1、shHDAC6-2共3组。6)用MG132(10 μmol/L)分别处理敲减/未敲减*HDAC6*表达的鼻咽癌细胞16 h后，检测FLOT2、HDAC6的蛋白质表达水平。7)用CHX(100 μmol/L)分别处理敲减/未敲减*HDAC6*表达的鼻咽癌细胞0、12、18 h后，检测FLOT2、HDAC6的蛋白质表达水平以反映蛋白质的降解速率(CHX蛋白降解实验)。

1.2.6 免疫共沉淀

参照文献[6]进行免疫共沉淀(co-immunoprecipitation，Co-IP)：用500 μL RIPA裂解细胞，提取总蛋白质，取40 μL裂解液作为Input，剩余上清液加入2 μg相应抗体(FLOT2、HDAC6、FLAG-tag抗体)，在万向旋转仪上于4 ℃混匀4 h后，每管加入30 μL预处理的Protein A/G磁珠，再次混匀4 h后，用含吐温20的磷酸盐缓冲液(phosphate-buffered saline with tween 20，PBST)清洗3次，加入60 μL 1×SDS上样缓冲液洗脱磁珠结合的蛋白质，后续按蛋白质印迹法步骤进行。

相关分组和处理：1)鼻咽癌细胞(5-8F、CNE2)用TSA(5 μmol/L)或NAM(200 μmol/L)处理6 h后，采用Co-IP检测FLOT2的总赖氨酸乙酰化水平。2)FLAG-FLOT2(WT)或FLAG-FLOT2(K211R)质粒转染到HEK-293T细胞中，并用TSA(5 μmol/L)处理细胞6 h后，采用Co-IP检测K211突变对FLOT2的总赖氨酸乙酰化水平的影响。3)通过BioGrid数据库[18]查询FLOT2与HDAC6之间是否可能存在相互作用，并采用Co-IP实验验证，实验分为Input(细胞总蛋白裂解液)、IgG沉淀对照、FLOT2/HDAC6特异性抗体沉淀组。4)用MG132(10 μmol/L)分别处理敲减/未敲减*HDAC6*表达的鼻咽癌细胞(shNC、shHDAC6-1、shHDAC6-2)16 h后，检测敲减*HDAC6*对FLOT2蛋白质总赖氨酸乙酰化水平影响。5)用MG132(10 μmol/L)处理FLAG-FLOT2(WT)/FLAG-FLOT2(K211R)质粒分别联合空白对照(Vector)/HDAC6质粒转染的HEK-293T细胞[FLAG-FLOT2(WT)+Vector、FLAG-FLOT2(WT)+HDAC6、FLAG-FLOT2(K211R)+Vector、FLAG-FLOT2(K211R)+HDAC6]16 h后，检测FLOT2蛋白质的总赖氨酸乙酰化水平。

1.2.7 RNA提取和实时反转录聚合酶链反应

参照文献[6]进行RNA提取和实时反转录聚合酶链反应(real-time reverse transcription PCR，real-time RT-PCR)。*FLOT2*的正向引物为5'-TTGCTGACTCT-AAGCGAGCC-3'，反向引物为5'-TCCACGGCAATC-TGTTTCTTG-3'，产物大小178 bp；*HDAC6*的正向引物为5'-ACCCCAGTGTCCTCTATTTCTC-3'，反向引物为5'-CCTGGTTCCAAGGCACATTGA-3'，产物大小135 bp。

相关分组和处理：1)用TSA(1 μmol/L)分别处理鼻咽癌细胞(5-8F、CNE2)0、4、8和16 h后，检测*FLOT2*的mRNA表达水平；2)敲减*HDAC6*表达后，检测*FLOT2*的mRNA表达水平，实验分shNC、shHDAC6-1、shHDAC6-2共3组。

1.2.8 CCK-8实验

参照文献[6]进行CCK-8实验：按照1×10^3个/孔的密度将细胞接种于96孔板，间隔24 h(共4次)，每孔加入10 μL CCK-8试剂，继续培养1 h后，用酶标仪检测450 nm波长处细胞的吸光度值，根据4次的吸光度值绘制生长曲线。在鼻咽癌细胞6-0B细胞中，分别转染Vector、FLOT2(WT)、FLOT2(K211R)质粒，采用CCK-8实验检测FLOT2(K211R)突变体对鼻咽癌细胞生长的影响。

1.2.9 平板克隆形成实验

参照文献[7]进行平板克隆形成实验：按照1×10^3

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个/孔的密度将细胞接种于6孔板，每组设3个复孔，连续培养8 d后，细胞经甲醇固定、结晶紫染色、漂洗干燥后进行拍照，计算克隆数。分组同1.2.8。

1.2.10 Transwell侵袭实验

参照文献[7]进行Transwell侵袭实验：按照2.5×10^4个/孔的密度将细胞接种于Transwell上室(无血清基础培养基培养)，向Transwell下室加入750 μL含10%胎牛血清的完全培养基，连续培养24 h后，用甲醇固定细胞，结晶紫将细胞染色，棉签小心刮去小室内部未侵袭细胞，于倒置显微镜下进行成像拍照，取5个视野，计算侵袭的平均细胞数。分组同1.2.8。

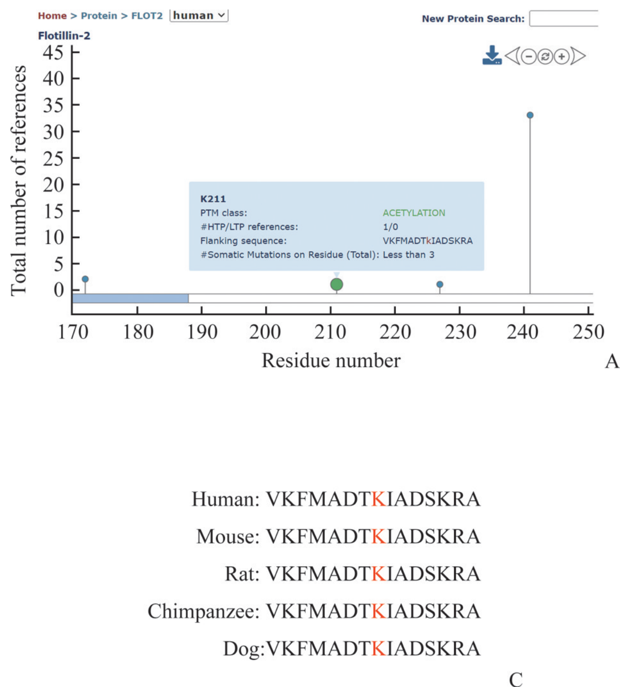
1.3 统计学处理

采用GraphPad Prism 8.0软件进行数据可视化和统计分析。计量资料以均数±标准差表示，2组之间比较采用两独立样本t检验(数据符合正态分布)，3组之间比较采用单因素方差分析。P<0.05为差异有统计学意义。

2 结　果

2.1 鼻咽癌细胞中FLOT2 K211位点存在乙酰化修饰

PhosphoSitePlus数据库数据显示FLOT2的K211位点存在乙酰化信号(图1A)，本研究进一步在鼻咽癌细胞中进行了验证。Co-IP结果显示：FLOT2蛋白存在明显的乙酰化修饰信号，且HDACs抑制剂TSA可以显著上调FLOT2的乙酰化修饰水平，而Sirt家族去乙酰化酶抑制剂NAM则无此作用(图1B)。序列保守性分析结果显示K211位点在人、小鼠、大鼠、大猩猩和狗中的具有高度的保守性(图1C)，进一步提示K211是FLOT2的乙酰化修饰位点。基于此，本研究构建了K211位点突变的突变体质粒(K211R)，分析其对FLOT2乙酰化修饰的影响。Co-IP结果显示：K211突变后，FLOT2的总赖氨酸乙酰化水平显著下降，且TSA处理无法恢复其乙酰化水平(图1D)。



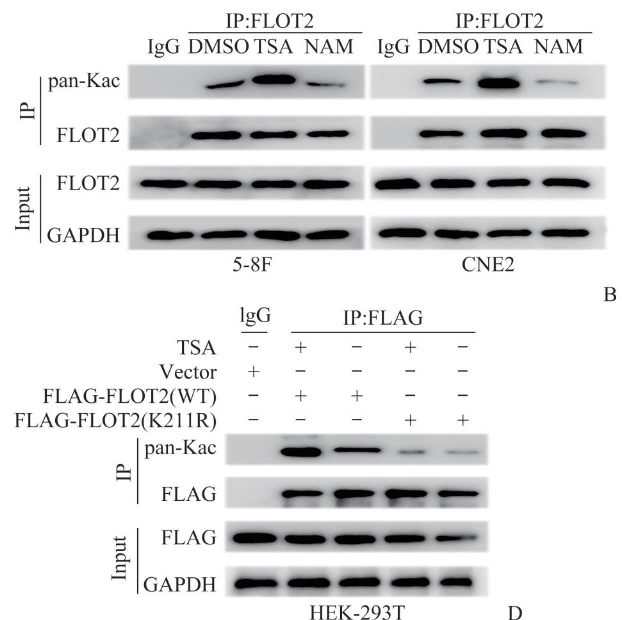


图1　FLOT2 K211位点存在乙酰化修饰

Figure 1　Acetylation modifications at the FLOT2 K211 site

A: PhosphoSitePlus database shows acetylation signal at K211 of FLOT2. B: 5-8F and CNE2 cells were treated with TSA (5 μmol/L) and NAM (200 μmol/L) for 6 hours, and Co-IP was conducted to measure the overall lysine acetylation level (pan-Kac) of FLOT2. C: Conservation analysis of the K211 site of FLOT2 across species includes human, mouse, rat, gorilla, and dog. D: FLAG-FLOT2 (WT) and FLAG-FLOT2 (K211R) plasmids were transfected into HEK-293T cells, and cells were treated with TSA (5 μmol/L) or left untreated for 6 hours. Co-IP was performed to assess the effect of K211 mutation on the pan-Kac level of FLOT2. FLOT2: Flotillin-2; TSA: Trichostatin A; Co-IP: Co-immunoprecipitation; WT: Wild type; pan-Kac: Pan-lysine (k) acetylation.

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2.2 K211位点的乙酰化修饰负调控FLOT2在鼻咽癌中的稳定性

蛋白质印迹法结果显示：TSA呈时间依赖性的方式下调鼻咽癌细胞(5-8F、CNE2)中FLOT2的蛋白质表达水平(图2A)，但不影响转染了FLAG-FLOT2(K211R)的鼻咽癌细胞(5-8F、CNE2)中FLOT2(K211R)的蛋白质表达水平(图2B)。Real-time PCR结果显示TSA处理不影响鼻咽癌细胞(5-8F、CNE2)中*FLOT2*的mRNA水平(P>0.05，图2C)，提示FLOT2 K211位点的乙酰化修饰促进自身蛋白降解。CHX蛋白降解实验结果显示：FLOT2(K211R)的蛋白质降解速率显著慢于FLOT2(WT)的降解速率(图2D)。蛋白质印迹法结果显示：蛋白酶体抑制剂MG132可以阻止TSA引起的FLOT2降解，溶酶体抑制剂CQ则无此功能(图2E)。这表明FLOT2乙酰化修饰促进自身经蛋白酶体途径降解。

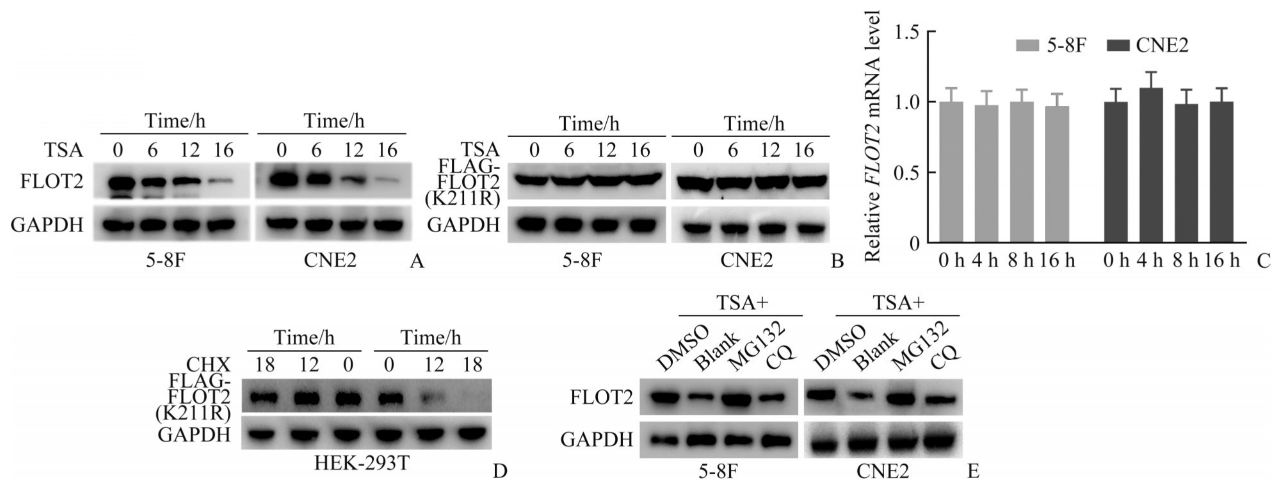

图2　K211位点的乙酰化修饰促进FLOT2蛋白质经蛋白酶体途径降解

Figure 2　K211 site acetylation modification promotes FLOT2 protein degradation via the proteasome pathway

A and B: 5-8F and CNE2 cells transfected without (A) or with (B) FLAG-FLOT2 (K211R) were treated with TSA (1 μmol/L) for 0, 6, 12, and 16 hours. Western blotting was conducted to measure the protein levels of FLOT2 (A) and FLAG-FLOT2 (K211R). C: 5-8F and CNE2 cells were treated with TSA (1 μmol/L) for 0, 4, 8, and 16 hours. Real-time RT-PCR was performed to assess the mRNA expression level of *FLOT2*. D: FLAG-FLOT2 (WT) and FLAG-FLOT2 (K211R) plasmids were transfected into HEK-293T cells, and cells were treated with CHX (100 μmol/L) for 0, 12, and 18 hours. Western blotting was conducted to measure the levels of FLAG-FLOT2 (Lane 1−3) and FLAG-FLOT2 (K211R) (Lane 4−6). E: 5-8F and CNE2 cells were treated with TSA (1 μmol/L) alone or in combination with MG132/CQ for 16 hours, and Western blotting was performed to measure the protein levels of FLOT2. FLOT2: Flotillin-2; CHX: Cycloheximide; TSA: Trichostatin A; DMSO: Dimethyl sulfoxide; CQ: Chloroquine.

2.3 HDAC6介导鼻咽癌细胞中FLOT2 K211位点的去乙酰化修饰并维持其稳定

BioGrid数据库数据显示FLOT2与HDAC6可能存在相互作用(图3A)。Co-IP结果显示：FLOT2与HDAC6抗体可以相互共沉淀对方蛋白(图3B和3C)；在敲减*HDAC6*表达(shHDAC6-1、shHDAC6-2组)的鼻咽癌细胞5-8F和CNE2中，FLOT2的总赖氨酸乙酰化水平显著升高(图3D)；FLAG-FLOT2(WT)+HDAC6组的FLOT2(WT)总赖氨酸乙酰化水平显著低于FLAG-FLOT2(WT)+Vector组，但是FLAG-FLOT2(K211R)+HDAC6组的FLOT2(K211R)总赖氨酸乙酰化水平与FLAG-FLOT2(K211R)+Vector组差异不明显(图3E)，共转染HDAC6可显著降低FLOT2(WT)的总赖氨酸乙酰化水平，而并不影响FLOT2(K211R)的水平。这表明HDAC6介导FLOT2 K211位点的去乙酰化修饰。

蛋白质印迹法(图4A)和real-time PCR(图4B)结果显示：敲减*HDAC6*显著下调FLOT2的蛋白质水平而不影响其mRNA水平。蛋白酶体抑制剂MG132阻止敲减*HDAC6*引起的FLOT2降解(图4C)。CHX蛋白降解实验结果显示：敲减*HDAC6*，FLOT2的降解速率显著加快(图4D)。

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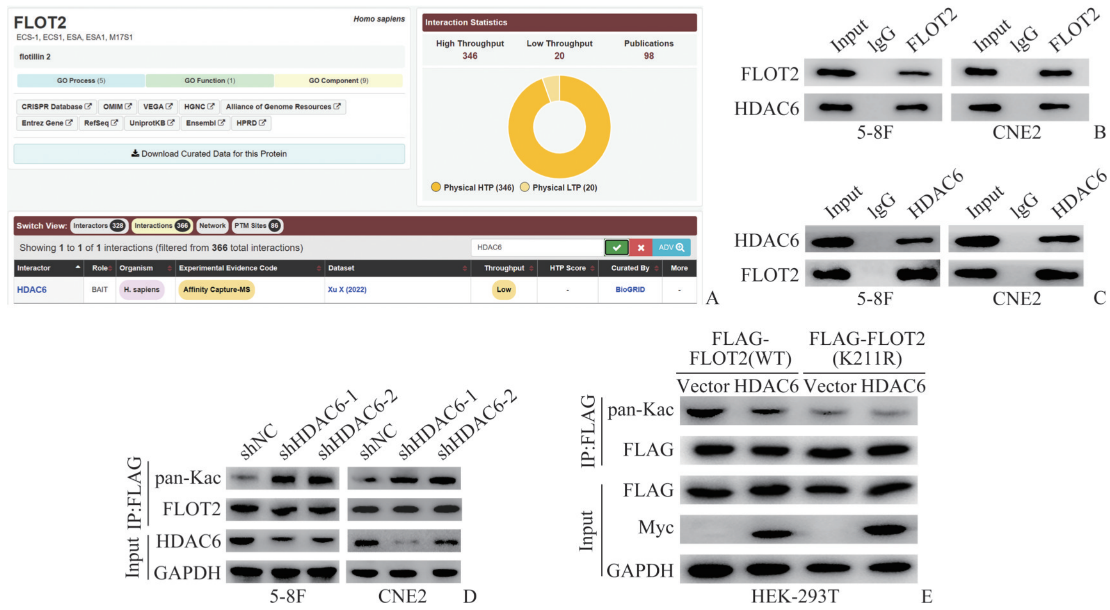

图3 HDAC6介导鼻咽癌细胞中FLOT2的去乙酰化

Figure 3 HDAC6 mediates deacetylation of FLOT2 in nasopharyngeal carcinoma cells

A: BioGrid data shows the interaction between FLOT2 and HDAC6. B and C: Co-IP was performed using FLOT2 and HDAC6 antibodies to detect the interaction between HDAC6 and FLOT2 in 5-8F and CNE2 cells. D: 5-8F and CNE2 cells with or without *HDAC*-knockdown (shNC, shHDAC6-1, and shHDAC6-2) were treated with MG132 (10 μmol/L) for 16 hours, and Co-IP was conducted to assess the effect of *HDAC6* knockdown on the overall lysine acetylation level of FLOT2. E: HEK-293T cells co-transfected with FLAG-FLOT2 (WT)/FLAG-FLOT2 (K211R) and vector/HDAC6 plasmids were treated with MG132 (10 μmol/L) for 16 hours, Co-IP was performed to detect the overall lysine acetylation level of FLOT2. HDAC6: Histone deacetylase 6; FLOT2: Flotillin-2; Co-IP: Co-immunoprecipitation; WT: Wild type; pan-Kac: Pan-lysine (k) acetylation.

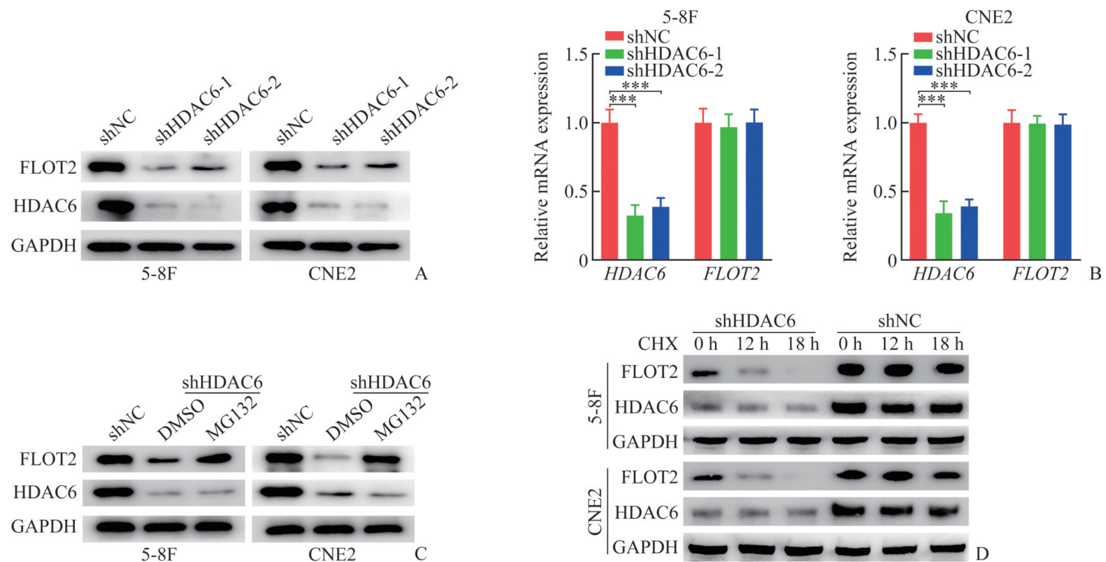

图4 HDAC6维持鼻咽癌细胞中FLOT2的稳定性

Figure 4 HDAC6 maintains FLOT2 stabilization in nasopharyngeal carcinoma cells

A: Western blotting was performed to detect the levels of FLOT2 and HDAC6 in nasopharyngeal carcinoma cells with or without *HDAC6*-knockdown (shNC, shHDAC6-1, and shHDAC6-2). B: Real-time RT-PCR was conducted to measure the expression levels of *FLOT2* and *HDAC6* in nasopharyngeal carcinoma cells with or without *HDAC6*-knockdown (shNC, shHDAC6-1, and shHDAC6-2). C: Nasopharyngeal carcinoma cells with or without *HDAC6*-knockdown (shNC and shHDAC6) were treated with MG132 (10 μmol/L) for 16 hours, and Western blotting was performed to detect the levels of FLOT2 and HDAC6. D: Nasopharyngeal carcinoma cells with or without *HDAC6*-knockdown (shNC and shHDAC6) were treated with CHX (100 μmol/L), and Western blotting was conducted to measure the levels of FLOT2 and HDAC6 at 0, 12, and 18 hours. ***P<0.001. HDAC6: Histone deacetylase 6; FLOT2: Flotillin-2; CHX: Cycloheximide; NC: Negative control.

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2.4 FLOT2(K211R)促进鼻咽癌细胞的体外恶性表型

蛋白质印迹法结果显示：FLOT2(WT)/FLOT2(K211R)在6-0B细胞中过表达成功(图5A)。转染FLOT2(K211R)突变体的6-0B细胞生长速度(图5B和5C)和侵袭能力(图5D)均显著强于转染FLOT2(WT)的细胞(均P<0.001)。

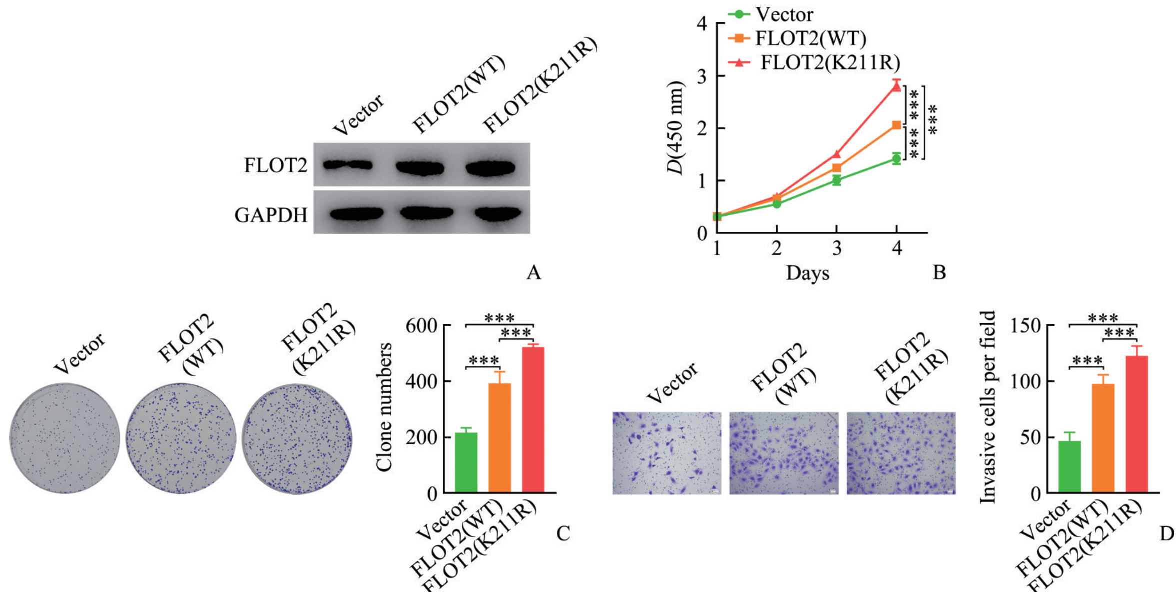

图5　FLOT2(K211R)在体外促进鼻咽癌细胞的恶性表型

Figure 5　FLOT2 (K211R) promotes the malignant phenotype of nasopharyngeal carcinoma cells in vitro

6-10B cells were transfected with FLOT2 (WT) and FLOT2 (K211R). A: Western blotting was performed to detect the expression of FLOT2. B and C: CCK-8 (B) and plate colony formation assays (C) were conducted to assess cell proliferation. D: Transwell invasion assay was performed to evaluate cell invasion ability. ***P<0.001. FLOT2: Flotillin-2; WT: Wild type; CCK-8: Cell counting kit-8.

3 讨　论

FLOT2广泛的促瘤功能已被证实，深入研究FLOT2在肿瘤中异常表达的机制，是推进靶向FLOT2干预新手段研发的理论需求[19]。MiRNA可通过结合*FLOT2* mRNA促进其降解而抑制肿瘤恶性表型[9-10]。TBL1X则可结合FLOT2的启动子区域促进其转录，促进鼻咽癌恶性进展[5]。然而，上述调控与FLOT2虽然存在因果关系，但是特异性不强，作为抑制FLOT2靶点的策略可行性不高。本研究从蛋白质翻译后修饰的角度切入，探究FLOT2乙酰化修饰的机制、生物学意义和功能，进一步证实FLOT2 K211位点存在乙酰化修饰，K211乙酰化修饰水平升高，则FLOT2的稳定性降低；HDAC6介导FLOT2的去乙酰化修饰，维持其在鼻咽癌中的高表达和促瘤功能，为以靶向FLOT2乙酰化修饰调控作为抑制鼻咽癌的新策略提供了可行思路。

赖氨酸残基是翻译后修饰最丰富的氨基酸残基之一，可发生乙酰化、巴豆酰化、乳酸化、琥珀酰化、丙酰化、丁酰化、丙二酰化、戊二酰化、2-羟基异丁酰化和3-羟基丁酰化乙酰化等多种酰化修饰和甲基化修饰等[20]。乙酰化修饰是目前研究最为广泛的赖氨酸修饰类型，是组蛋白调控染色体结构和转录活性调控的重要途径[20]。非组蛋白的赖氨酸乙酰化修饰对于自身特性和功能同样具有重要作用[20]。在肿瘤中，P53，MYC等关键肿瘤相关蛋白质在不同位点的乙酰化动态修饰，是应对不同环境，有序调控转录活性、DNA结合能力和相互作用蛋白谱等特性的重要方式[21-22]。此外，乙酰化修饰与蛋白质的稳定性密切相关，其调控具有两面性。一方面，赖氨酸乙酰化修饰可以拮抗相应位点的泛素化修饰，进而促进蛋白质的稳定；另一方面，赖氨酸乙酰化修饰也可以促进自身蛋白质与Hsc70等降解途径相关分子的结合，进而促进自身蛋白质降解[23-24]。本研究证实FLOT2的K211位点存在明显的乙酰化信号，并发现突变此位点(K211R)可以显著增强鼻咽癌细胞中FLOT2的稳定性，进一步增强FLOT2的促瘤功能，表明K211位点的乙酰化修饰可促进FLOT2降解，进

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而抑制其促瘤功能。

乙酰转移酶和组蛋白去乙酰化酶蛋白负责动态调控赖氨酸乙酰化修饰，其中已明确的乙酰转移酶包括GNAT家族、MYST家族、P300/CBP家族，以及ATAT1、ESCO1/2和HAT1等，去乙酰化酶包括Zn^{2+}依赖的HDACs家族成员(HDAC1~11)和NAD^{+}依赖的Sirtuins家族成员(Sirt1~7)[25]。相较于乙酰转移酶，去乙酰化酶在肿瘤中的表达和功能被更广泛和深入地研究，其通过介导底物蛋白质的去乙酰化修饰或作为转录复合体的组成部分，调控底物蛋白质的稳定性、亚细胞定位、酶活性及DNA的转录活性等，在肿瘤的恶性增殖、侵袭和转移，以及治疗抵抗等过程中发挥促进或抑制的作用[26-27]。在鼻咽癌中，HDAC-1/2/4/7/9及Sirt2直接发挥促瘤作用或介导上游促瘤因子的功能[16, 28-32]；Sirt6则通过抑制NK-κB活性和Snail的表达诱导鼻咽癌细胞凋亡，抑制其侵袭和转移[33-34]。本课题组前期的研究[35]发现：丁酸钠通过抑制HDAC6，下调EGFR/AKT/mTOR活性而诱导鼻咽癌细胞自噬性凋亡。同时，Hsp90抑制剂AT13387通过下调HDAC6，促进α-微管蛋白的乙酰化和稳定性，进而抑制鼻咽癌细胞的迁移[36]。本研究进一步证实FLOT2是HDAC6的新底物，HDAC6可以介导FLOT2的去乙酰化，抑制FLOT2降解，维持其在鼻咽癌中的稳定。

本研究初步证实了FLOT2的K211位点的乙酰化修饰促进自身蛋白质的降解，HDAC6可以介导FLOT2的去乙酰化修饰，维持FLOT2在鼻咽癌中的稳定。本研究仍存在一些局限：1)未能揭示FLOT2乙酰化促进自身降解的具体机制，即FLOT2乙酰化的生物学意义是促进自身与泛素连接酶等负调控因子的结合，抑或是促进自身与去泛素化酶等正调控因子的解离。2)未能揭示HDAC6在鼻咽癌中的功能是否依赖其FLOT2的去乙酰化。这些问题都是我们后续研究的重点方向。

总之，本研究揭示了HDAC6通过介导FLOT2去乙酰化修饰维持FLOT2在鼻咽癌中稳定的新机制，为靶向FLOT2的鼻咽癌治疗新手段提供了理想的切入点。

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