

circUSP25 编码的线粒体蛋白促进结肠癌细胞增殖及迁移

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摘要:目的 探索 circUSP25 编码蛋白的生物学功能及其潜在机制。方法 通过 circRNADb 和 circBank 数据库预测 circUSP25 的编码潜能;采用 Sanger 测序验证 circUSP25 的剪接位点;构建 circUSP25-Flag 野生型及起始密码子 ATG 突变载体,转染结肠癌细胞 SW620 和 HCT116,应用 Western blotting、免疫沉淀、质谱及免疫荧光鉴定 circUSP25 编码的蛋白及其亚细胞定位;运用 TargetP-2.0 数据库预测蛋白的信号肽;采用 CCK-8 和 Transwell 小室迁移实验评估该蛋白对结肠癌细胞增殖及迁移的影响;通过免疫共沉淀及质谱筛选其相互作用蛋白,免疫荧光验证其与候选蛋白 PHB2 的共定位关系;应用 CCK-8 和 Transwell 小室迁移实验检测敲低 PHB2 对 circUSP25 编码蛋白促进增殖及迁移作用的回复。结果 circUSP25 由 USP25 基因第 2、3 外显子背向剪接形成,含有编码 121 个氨基酸的开放阅读框。Western blotting 及质谱鉴定证实 circUSP25 可编码蛋白,该蛋白定位于结肠癌细胞的线粒体。TargetP-2.0 预测显示其氨基端 1-32aa 内含有线粒体转运肽。过表达 circUSP25 编码蛋白可显著促进结肠癌细胞增殖及迁移($P<0.05$)。免疫共沉淀联合质谱鉴定发现该蛋白可与线粒体蛋白 PHB2 等结合,免疫荧光证实二者在线粒体存在共定位,功能挽救实验表明敲低 PHB2 可以回复 circUSP25 编码蛋白对结肠癌细胞增殖及迁移的作用。结论 circUSP25 编码的蛋白定位于线粒体,可能通过与线粒体蛋白相互作用促进结肠癌细胞增殖与迁移。

关键词:结肠癌;环状 RNA;circUSP25;hsa_circ_0001178;线粒体蛋白

The mitochondrial protein encoded by circUSP25 promotes colon cancer cell proliferation and migration

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Abstract: Objective To investigate the regulatory role of the protein encoded by circUSP25 in proliferation and migration of colon cancer cells. **Methods** The coding potential of circUSP25 was predicted using circRNADb and circBank databases, and the splice junction was validated by Sanger sequencing. circUSP25-Flag wild-type vector and mutant vector with start codon ATG mutation were constructed and transfected into SW620 and HCT116 colon cancer cells, and the expression of the encoded protein and its subcellular localization were analyzed using Western blotting, immunoprecipitation, mass spectrometry, and immunofluorescence staining. The signal peptide was predicted using TargetP-2.0. The effects of this protein on proliferation and migration of colon cancer cells were evaluated using CCK8 and Transwell migration assays. The interacting proteins were screened by co-immunoprecipitation coupled with mass spectrometry, and its co-localization with the candidate protein PHB2 was verified by immunofluorescence staining. The rescue effect of PHB2 knockdown on the activity of circUSP25-encoded protein for promoting colon cancer cell proliferation and migration were observed. **Results** circUSP25 was formed by back-splicing of exons 2 and 3 of the parental gene USP25 and contained an open reading frame encoding 121 amino acids. circUSP25 encodes a protein localizing to the mitochondria of colon cancer cells, and its N-terminal 1-32 amino acids contain a mitochondrial transit peptide. Overexpression of the circUSP25-encoded protein significantly promoted colon cancer cell proliferation and migration. Co-immunoprecipitation coupled with mass spectrometry revealed that this protein binds to the mitochondrial proteins such as PHB2, and they co-localize in the mitochondria. Functional rescue experiments showed that PHB2 knockdown reversed the pro-proliferative and pro-migratory effects of this circUSP25-encoded protein. **Conclusion** The circUSP25-encoded protein localizes to the mitochondria, and its overexpression promotes colon cancer cell proliferation and migration possibly by interacting with mitochondrial proteins.

Keywords: colon cancer; circular RNA; circUSP25; hsa_circ_0001178; mitochondrial proteins

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结直肠癌是全球常见的恶性肿瘤之一,其发病率和死亡率分别位于第3位和第2位^[1]。尽管诊疗技术不断进步,但部分患者仍面临复发和转移的风险,其潜在的分子机制尚未完全阐明。深入探索结直肠癌发生发展的调控网络,对于发现新的诊断标志物和治疗靶点具有重要意义。

传统观点认为,蛋白主要来源于 mRNA 的翻译。然而,随着高通量测序和核糖体印记分析技术的发展,

越来越多的研究发现,被注释为非编码RNA的转录本同样具备编码潜能,其翻译产生的多肽或蛋白质在肿瘤演进中扮演着重要角色^[2-6]。环状RNA是一类具有共价闭合环状结构的非编码RNA,具有稳定性高的特征,大量研究表明环状RNA在多种肿瘤恶性进展中发挥关键作用^[7-9]。机制上,早期研究主要集中于环状RNA作为微小RNA(miRNA)“海绵”或与蛋白结合而发挥功能^[7,8]。近年来,环状RNA的编码能力被逐渐揭示,为理解肿瘤调控机制提供了新视角^[10-18]。环状RNA由于缺乏5'端帽子结构,其翻译起始依赖于内部核糖体进入位点或m6A修饰介导的非经典途径^[19,20]。

环状RNA hsa_circ_0001178源自亲本基因 USP25 的第2、3外显子,根据环状RNA命名规范^[21],我们将其命名为circUSP25(2,3),简称circUSP25。已有研究表明,circUSP25在结直肠癌组织中表达上调,并可通过吸附miR-382/587/616上调EZB1表达,进而促进肿瘤细胞转移^[22]。值得注意的是, Van等^[23]通过核糖体印记测序发现circUSP25能与核糖体结合,这提示circUSP25可能具有编码蛋白的潜力,但目前尚无相关实验报道。本研究旨在验证circUSP25的编码能力,鉴定其翻译产物的亚细胞定位及生物学功能,并初步探索其分子机制,以期揭示结直肠癌恶性进展的机制提供新思路。

1 材料和方法

1.1 人结肠癌细胞株及培养条件

本研究所用人源结肠腺癌细胞系SW620和HCT116均购自森贝伽生物科技有限公司,分别采用DMEM(生航)和McCoy's 5A培养基(森贝伽),并添加10%胎牛血清(森贝伽)。所有细胞均在恒温37℃、含5% CO₂的培养箱中常规培养与传代,选取处于对数生长期的细胞进行实验。

1.2 小干扰RNA(siRNA)、载体构建与细胞转染

针对PHB2的siRNA由苏州吉玛基因股份有限公司设计并合成,siRNA序列:GGUCCAGUACCCCAUUAUTT。circUSP25-Flag及其突变体circUSP25-Flag-Mut过表达载体均由广州睿博生物科技有限公司完成构建。首先采用基因合成技术获得circUSP25目的片段,并通过定点突变方法将其翻译起始密码子ATG突变为TAA。进一步将获得的野生型与突变型circUSP25序列分别克隆至环状RNA真核表达载体pCD5-ciR(吉赛)中,从而成功构建相应的过表达质粒。所有构建的载体均经Sanger测序鉴定,结果证实插入序列及突变位点完全正确。细胞转染操作使用Lipofectamine 3000转染试剂(Thermo Fisher),按照试剂说明进行siRNA及质粒的转染。

1.3 Western blotting

circUSP25-Flag、circUSP25-Flag-Mut及对照载体分别转染SW620和HCT116细胞。转染后48 h,应用RIPA裂解液(弗德)裂解细胞,离心后收集总蛋白,取等量蛋白进行SDS-PAGE凝胶电泳分离,应用湿转系统将蛋白转移至PVDF膜(Millipore),并置于快速封闭液(新赛美)中室温孵育封闭。封闭结束后,分别与以下一抗在4℃条件下孵育过夜:anti-Flag(1:1000, ImmunoWay)和anti-β-Tubulin(1:2000, Proteintech)。次日,膜经充分洗涤后,加入辣根过氧化物酶标记的山羊抗兔/小鼠二抗(1:5000, 弗德),室温继续孵育1h。ECL超敏发光液(弗德)进行化学发光,应用化学发光成像系统采集并记录蛋白信号图像。

1.4 免疫共沉淀与质谱鉴定

参照之前发表文章中的方法进行^[8]。应用裂解缓冲液提取细胞总蛋白,将细胞裂解液与Flag抗体(ImmunoWay)或对照IgG(ABclonal)混合,于4℃翻转孵育2 h。随后向混合物中加入约20 μL琼脂糖珠(Santa Cruz),置于4℃继续孵育过夜。充分洗涤并收集琼脂糖珠后,洗脱结合蛋白。洗脱产物经SDS-PAGE凝胶电泳分离,并采用快速银染试剂盒(碧云天)进行染色。最后,将差异蛋白条带切下,由微纳菲生物技术有限公司(深圳)进行质谱分析。

1.5 细胞免疫荧光检测

SW620及HCT116细胞接种于共聚焦培养小皿,培养24 h,根据实验需要可进行线粒体染色,细胞经预热的Mito-Tracker探针(200 nmol/L,碧云天)于37℃孵育20 min,随后以4%多聚甲醛固定10 min,0.2% Triton X-100处理15 min增强通透性,并用山羊血清封闭30 min,之后加入一抗anti-Flag(1:200, ImmunoWay)与anti-PHB2(1:200, Proteintech),4℃孵育过夜,次日加入DyLight488/DyLight647标记的羊抗鼠/兔二抗(1:200, ImmunoWay),室温避光孵育1 h,DAPI染色细胞核,激光共聚焦显微镜下观察并采集图像。

1.6 CCK-8检测

细胞转染质粒24 h后收集,调整细胞密度后以1×10³/孔、100 μL培养基接种于96孔板,边缘孔加无菌PBS。培养至相应时间点,加入10 μL/孔CCK-8溶液(恩晶),置于细胞培养箱中继续孵育2 h,应用酶标仪检测吸光度A_{450nm},分别于第1、2、3、4天进行检测。

1.7 Transwell小室迁移实验

HCT116细胞转染质粒24 h后收集细胞,以无血清培养基重悬并计数。在Transwell小室下室加入含10%胎牛血清的完全培养基,上室接种3×10⁴个细胞。迁移48 h后取出小室,用4%多聚甲醛固定,吉姆萨染色。在显微镜下随机选取5~7个200倍视野采集图像,计数细胞数。

1.8 统计学分析

使用 GraphPad Prism 9.5.1 软件进行 Student *t* 检验, 计量资料以均数±标准差表示, 以 $P<0.05$ 为差异具有统计学意义。

2 结果

2.1 环状RNA circUSP25 具有编码蛋白的开放阅读框

Sanger测序表明 circUSP25 是 USP25 在转录后由第2、3外显子背向剪接形成的环状RNA(图1A)。应用

circRNADb^[24] 和 circBank^[25] 数据库搜索, 发现 circUSP25 含有编码 121 个氨基酸的开放阅读框 (ORF), ORF 从第 128 bp 起始密码子 ATG 开始, 跨 2 次剪切位点后在第 47 bp 终止密码子 TAA 结束(图1B)。本研究在 circUSP25 终止密码子 TAA 前插入 Flag 序列, 克隆入 pCD5-ciR 载体, 构建 circUSP25-Flag 载体, 再突变 circUSP25-Flag 的起始密码子 ATG 为 TAA 构建 circUSP25-Flag-Mut 载体(图1C、D)。

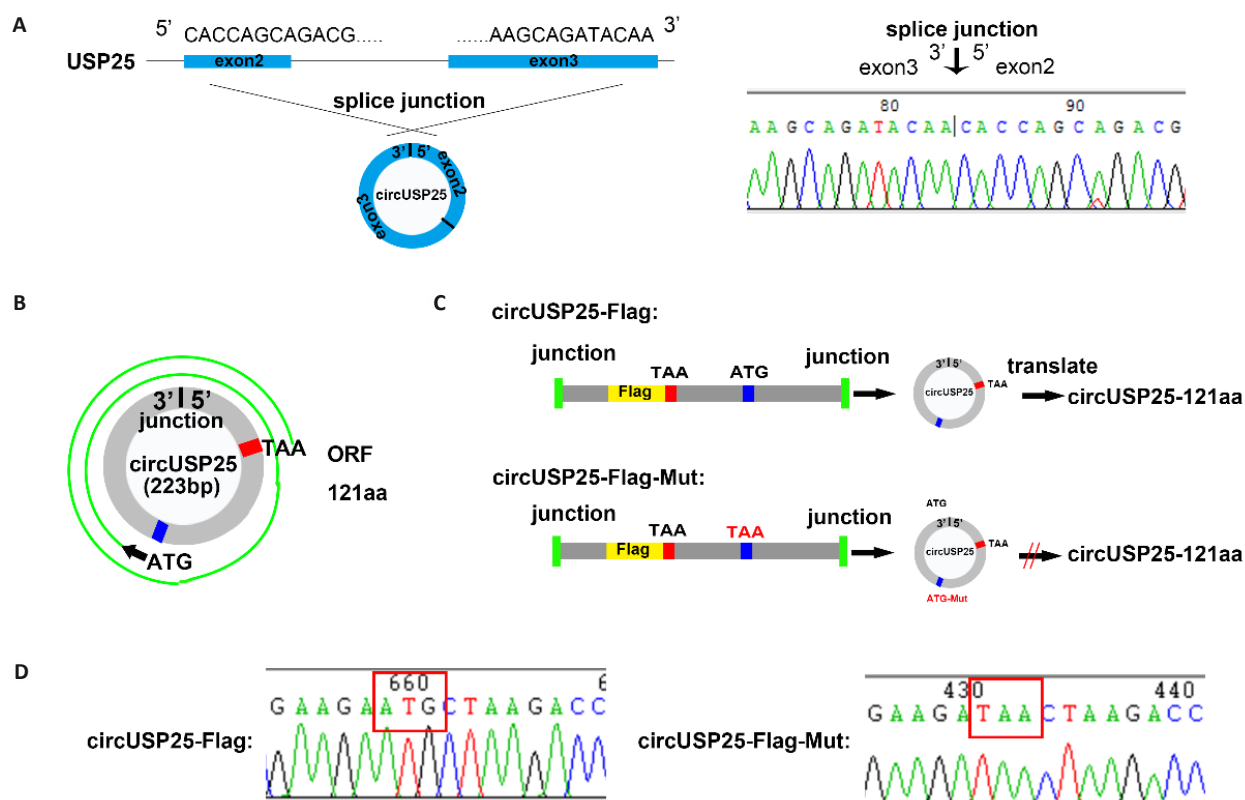


图1 环状RNA circUSP25 开放阅读框的预测

Fig.1 Prediction of the open reading frame (ORF) of circUSP25. A: Schematic diagram of the formation of circUSP25, which is generated from back-splicing of exons 2 and 3 of the parental gene USP25. Sanger sequencing confirms that the splice junction sequence of circUSP25 is completely consistent with the head-to-tail splicing of exons 2 and 3 of USP25. B: The ORF of circUSP25 (the green line). C: Schematic diagrams of the wild-type circUSP25-Flag vector containing the translation start codon ATG and the mutant circUSP25-Flag-Mut vector with ATG mutation. D: Sanger sequencing of circUSP25-Flag and circUSP25-Flag-Mut vectors confirms the correct sequences of the translation start codon ATG and its mutation.

2.2 circUSP25 编码蛋白的鉴定

Western blotting 显示只有 circUSP25-Flag 载体可表达出蛋白, 而 ATG 突变的载体不能翻译出蛋白(图2A), 进一步应用免疫沉淀技术富集 circUSP25 翻译的蛋白, 通过电泳分离、银染、质谱, 在 16 000 D 左右的条带中鉴定到属于 circUSP25 编码蛋白的肽段(图2B), 免疫荧光显示 circUSP25 翻译的蛋白位于结肠癌细胞的细胞质(图2C)。

2.3 circUSP25 编码的蛋白定位于结肠癌细胞的线粒体

本研究应用 TargetP-2.0^[26] 数据库分析 circUSP25

编码蛋白的信号肽, 发现蛋白的氨基端 1-32aa 内含有线粒体转运肽(图3A), 1-32aa 是 circUSP25 编码的蛋白所特有的, 其亲本基因 USP25 编码的蛋白没有线粒体转运肽。免疫荧光显示 circUSP25 编码的蛋白定位在结肠癌细胞的线粒体(图3B)。

2.4 circUSP25 编码的蛋白促进结肠癌细胞增殖和迁移

在 SW620 和 HCT116 细胞中过表达 circUSP25-Flag, CCK-8 实验表明 circUSP25-Flag 过表达能够促进结肠癌细胞的增殖 ($P<0.05$, 图4A), Transwell 小室表明过表达 circUSP25-Flag 能够促进结肠癌细胞迁移

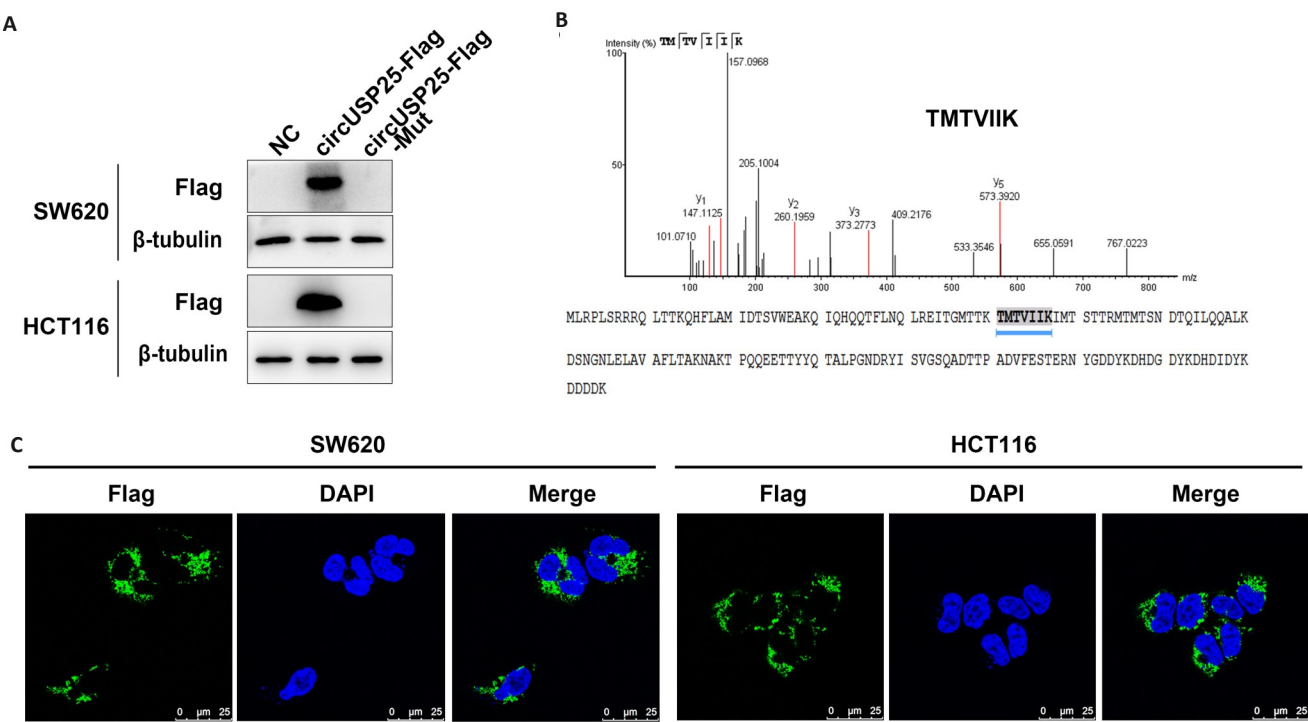


图2 circUSP25 编码蛋白的鉴定

Fig.2 Identification of the protein encoded by circUSP25. **A**: Western blotting showing that the circUSP25-Flag vector translates the protein, while the ATG-mutated vector fails to translate. **B**: Immunoprecipitation coupled with mass spectrometry identifies peptides corresponding to the protein encoded by circUSP25-Flag. **C**: Immunofluorescence staining reveals that the protein encoded by circUSP25-Flag localizes to the cytoplasm of colon cancer cells (Scale bar=25 μm).

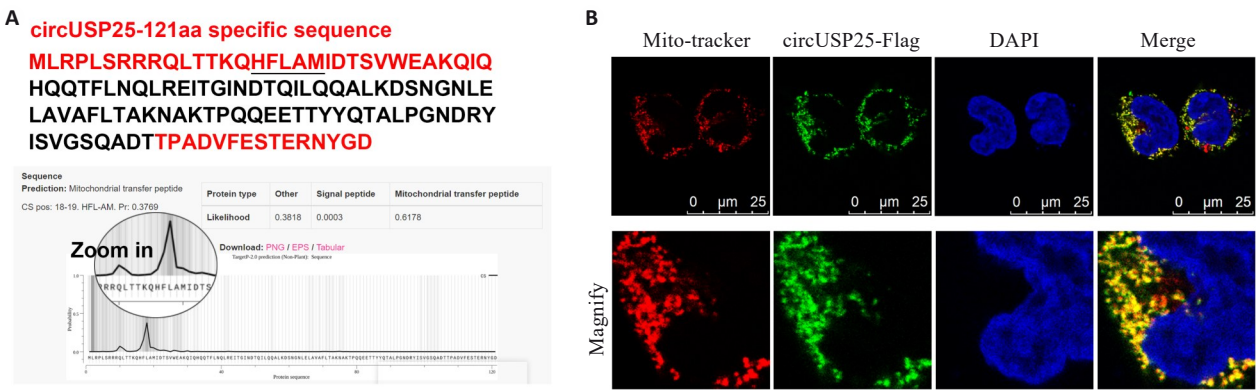


图3 circUSP25 编码的蛋白定位于结肠癌细胞的线粒体

Fig.3 The protein encoded by circUSP25 localizes to the mitochondria of colon cancer cells. **A**: TargetP-2.0 database prediction shows that the N-terminal 1-32 amino acids of circUSP25-encoded protein contain a mitochondrial transit peptide. **B**: Immunofluorescence staining demonstrates that circUSP25-encoded protein localizes to the mitochondria of colon cancer cells.

($P<0.001$,图4B)。

2.5 circUSP25 编码的蛋白与线粒体蛋白结合

应用免疫共沉淀将 circUSP25 编码蛋白所结合的蛋白富集下来,银染后对 35 000 左右的差异条带进行质谱鉴定,共鉴定到 138 个蛋白,其中相对分子质量大小符合的有 15 个蛋白,按照可信度和唯一肽段数量从高到低排列后,发现排在前 4 位的有 3 个是线粒体蛋白(图 5A~C)。免疫荧光也表明 circUSP25 编码的蛋白与 PHB2 在线粒体共定位(图 5D)。CCK-8 和 Transwell 迁移实验表明,敲低 PHB2 可回复 circUSP25 对结肠癌

细胞增殖及迁移的促进作用(图 6A、B)。

3 讨论

本研究发现环状 RNA circUSP25 能够编码一个定位于线粒体的新蛋白。功能实验表明,该蛋白促进结肠癌细胞增殖与迁移。进一步的机制探索发现, circUSP25 编码的蛋白可与 PHB2 等线粒体蛋白结合,提示其可能通过影响线粒体功能来发挥促癌作用。

首先,本研究证实了 circUSP25 的编码能力。通过构建带有 Flag 标签的起始密码子野生型及突变载体,

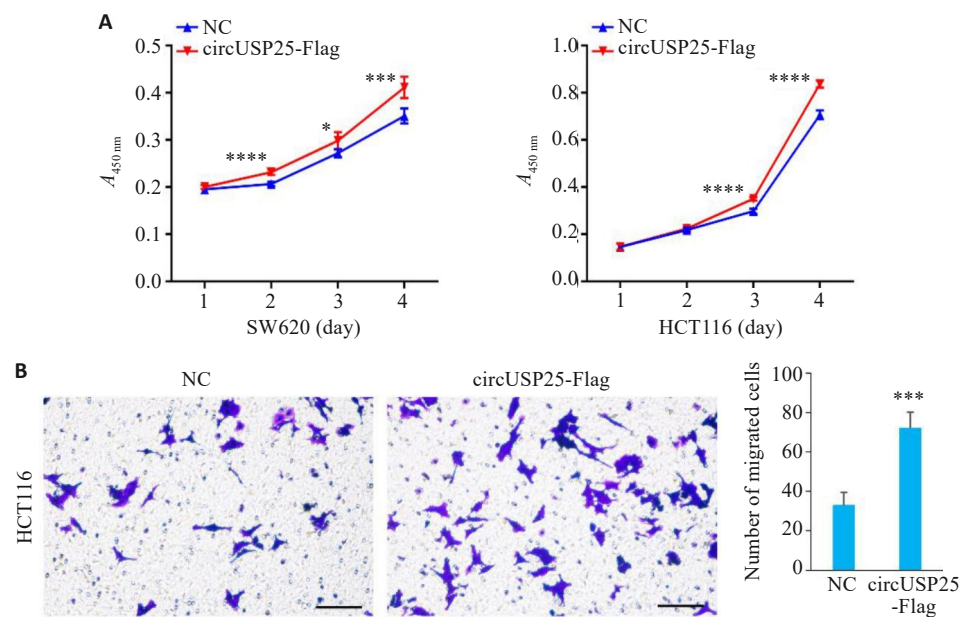


图4 circUSP25 编码的蛋白促进结肠癌细胞增殖和迁移

Fig. 4 The protein encoded by circUSP25 promotes proliferation and migration of colon cancer cells. **A**: CCK8 assays showing that circUSP25-Flag transfection promotes proliferation of colon cancer cells. **B**: Transwell assays showing that transfection with circUSP25-Flag enhances migration of colon cancer cells (Scale bar=100 μ m). * P <0.05, *** P <0.001, **** P <0.0001 vs NC group.

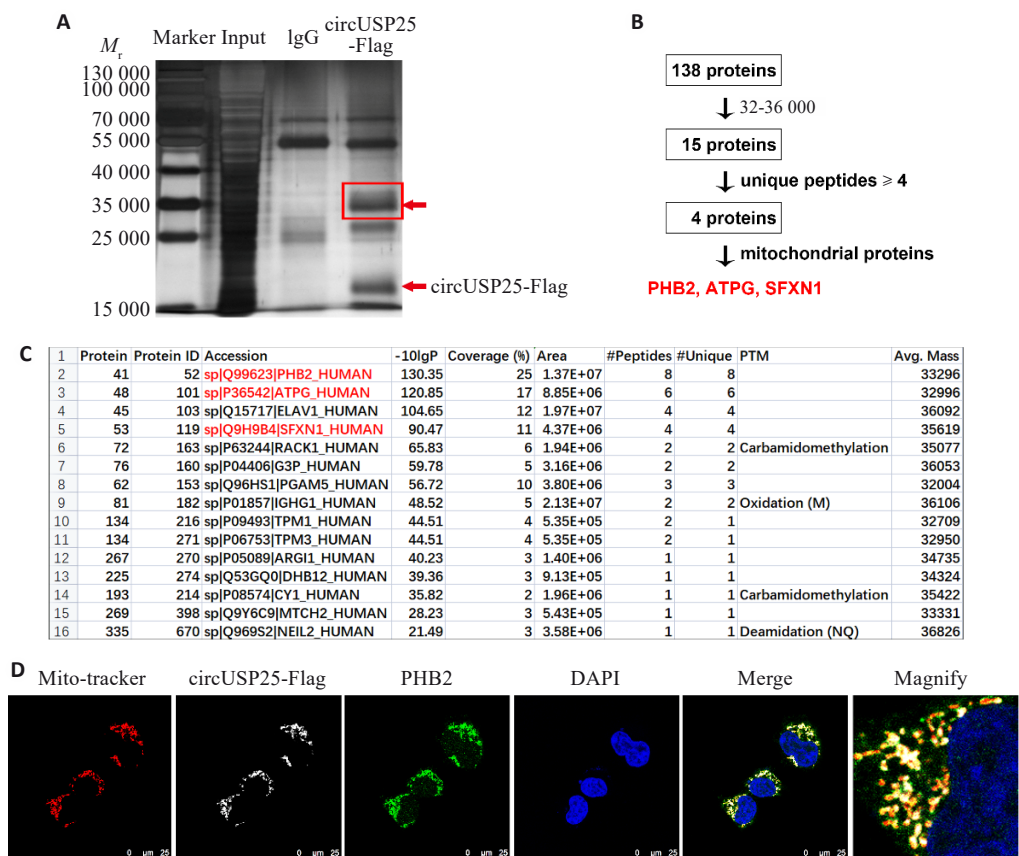


图5 circUSP25 编码的蛋白与线粒体蛋白结合

Fig. 5 The protein encoded by circUSP25 binds to mitochondrial proteins. **A**: Co-immunoprecipitation, electrophoretic separation, and silver nitrate staining reveal a differential band near 35 kD, identified subsequently by mass spectrometry as proteins interacting with the circUSP25-encoded protein. **B**: Mass spectrometry identifies 138 proteins, among which 15 match the expected molecular weight range. Ranked by confidence and the number of unique peptides, 3 of the top 4 proteins are mitochondrial proteins. **C**: Top 15 proteins matching the molecular weight range ranked by confidence and the number of unique peptides, with mitochondrial proteins highlighted in red. **D**: Immunofluorescence staining showing co-localization of the circUSP25-encoded protein with PHB2 in the mitochondria.

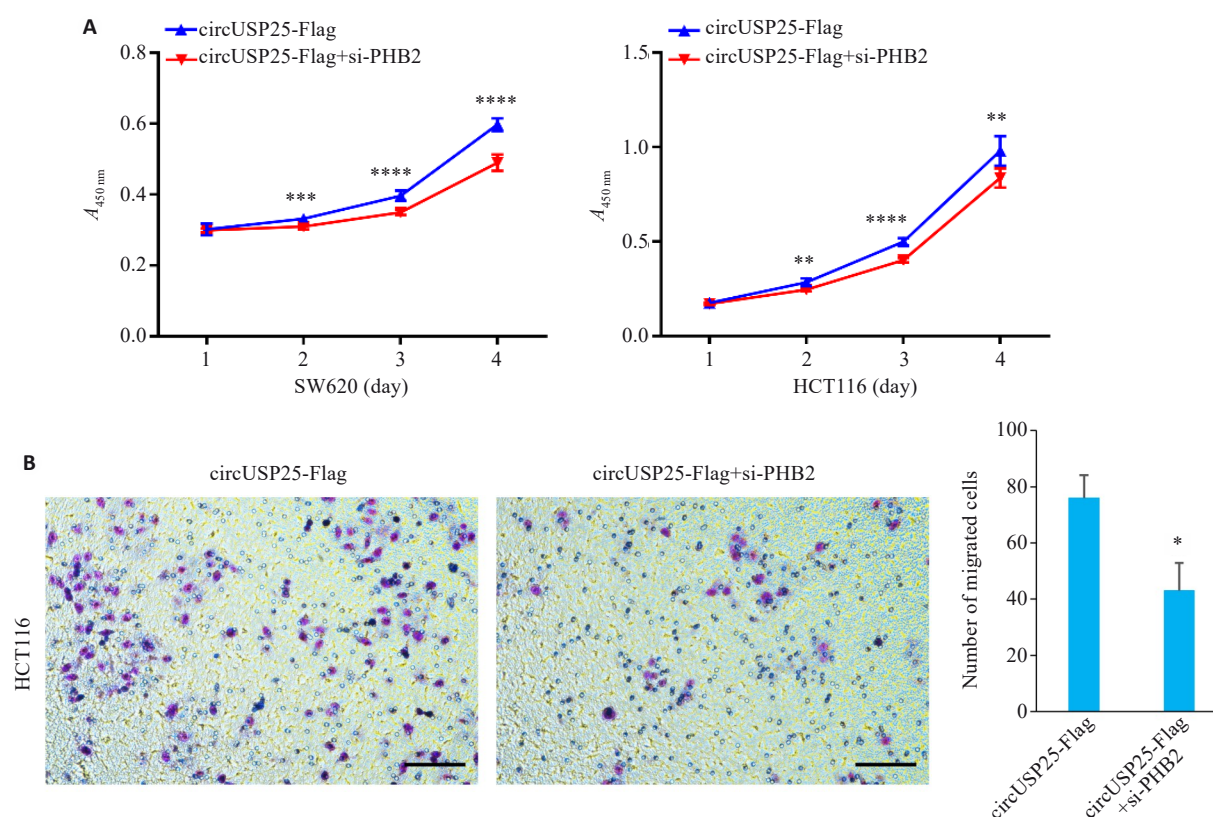


图6 敲低 PHB2 可回复 circUSP25 编码蛋白对结肠癌细胞增殖和迁移的影响

Fig. 6 Knockdown of PHB2 reverses the effects of the circUSP25-encoded protein on colon cancer cell proliferation and migration. **A:** CCK8 assays showing that PHB2 knockdown reverses the proliferation-promoting effect of the circUSP25-encoded protein. **B:** Transwell assays showing that PHB2 knockdown reverses the migration-promoting effect of the circUSP25-encoded protein (Scale bars=100 μm). * $P<0.05$, ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$ vs circUSP25-Flag.

Western blotting 结果显示仅野生型 circUSP25 可成功表达 Flag 标签蛋白,而突变载体则丧失翻译能力,质谱鉴定进一步验证了该蛋白肽段的存在。这一发现不仅验证了核糖体印记测序的预测结果^[23],也拓展了对 circUSP25 在结肠直肠癌中作用机制的认识。既往研究仅揭示了 circUSP25 作为 miRNA“海绵”的非编码调控功能^[22],而本研究首次证明其具有编码能力,这提示 circUSP25 可能通过编码蛋白和非编码 RNA 双重途径参与肿瘤恶性进展过程。其次,本研究明确了该蛋白的亚细胞定位及功能。生物信息学预测及免疫荧光实验证实, circUSP25 编码的蛋白定位于结肠癌细胞的线粒体,这与亲本基因 USP25 编码的蛋白定位不同,提示该环状 RNA 翻译了一个具有独特定位的新蛋白。CCK8 和 Transwell 实验结果显示,过表达该蛋白能够显著促进结肠癌细胞的增殖和迁移,表明其在线粒体定位可能与其促癌功能密切相关。

在机制方面,免疫共沉淀联合质谱鉴定发现, circUSP25 编码的蛋白能够与线粒体蛋白 PHB2 等结合。PHB2 作为阻抑素蛋白家族成员,其功能具有多样性。一方面,PHB2 定位于线粒体内膜,参与维持线粒体嵴形态、调控细胞增殖与凋亡,在多种肿瘤中发挥促癌

作用^[27-30];另一方面,PHB2 也是介导线粒体自噬的关键受体,其功能异常可能导致受损线粒体堆积,从而影响细胞稳态^[31]。因此, circUSP25 编码蛋白与 PHB2 的结合,可能通过调控 PHB2 的稳定性、亚细胞定位或线粒体自噬受体功能,进而影响线粒体代谢与细胞命运,这为本研究发现的促增殖和迁移表型提供了潜在的分子解释。然而,本研究仍存在一定的局限性。首先,我们虽鉴定到与 circUSP25 编码蛋白相互作用的线粒体蛋白,但其具体的调控机制,例如如何影响 PHB2 的功能或线粒体稳态,仍需进一步深入探索。其次,本研究主要依赖于外源性过表达实验,后续需定制特异性抗体,检测结肠癌临床样本及细胞系中该蛋白的内源性表达水平,并结合体内动物模型进一步验证其促癌功能。

综上所述,本研究揭示了 circUSP25 可编码定位于线粒体的新蛋白,该蛋白可能通过与线粒体蛋白 PHB2 等相互作用,促进结肠癌细胞的生长与迁移。这一发现揭示了 circUSP25 在结肠癌恶性进展中的新功能,为理解环状 RNA 的多重调控机制提供了新视角,也为结肠癌的诊断与治疗提供了潜在的新靶点。

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