WDR91-WDR81复合体调控内吞体-溶酶体运输及神经发育

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摘要 WD40重复序列蛋白在真核生物中广泛存在, 并参与胞内运输、信号转导、凋亡发生、转录调控等生命过程. 通过以秀丽隐杆线虫(*Caenorhabditis elegans*)为模式动物进行遗传筛选, 本课题组发现了两个新基因, 其人类同源基因编码WD40重复序列蛋白WDR91和WDR81, 然而此前对这两种蛋白的功能知之甚少. 本课题组后续工作发现, WDR91和WDR81形成复合体, 作为晚期内吞体的特征性小GTP酶Rab7的效应因子被招募到内吞体上, 与III型磷脂酰肌醇-3-激酶(PI3K)复合体的Beclin 1亚基相互作用, 抑制内吞体上磷脂酰肌醇3-磷酸(PtdIns3P)的合成, 使早期内吞体向晚期内吞体转化, 从而确保内吞体-溶酶体运输得以实现. WDR91-WDR81复合体与神经发育紧密相关, 具体表现在特异性敲除WDR91的小鼠神经突起生长受损, 而WDR81通过内吞体SARA-TGF β 信号通路影响成体神经发生. 此外, WDR81还可作为衔接蛋白结合p62和LC3C, 以不依赖于WDR91-WDR81复合体的形式促进蛋白聚集体的选择性自噬. 这些发现为揭示WDR91和WDR81相关的神经系统疾病致病机制提供了重要理论依据.

关键词 WDR91-WDR81复合体, 内吞体转化, Rab7, 神经发育, 选择性自噬

WD40重复序列蛋白作为真核生物中的蛋白大家族, 其特征是包含约40个氨基酸并终止于色氨酸和天冬氨酸二肽(WD)的重复序列^[1,2], 该序列折叠形成的 β 螺旋桨结构为蛋白之间的相互作用及多种蛋白进行组装提供了反应平台^[3,4]. 目前发现, WD40重复序列蛋白在胞内运输、信号转导、凋亡发生、转录调控等生命过程中发挥着重要功能, 并且WD40重复序列蛋白突变与多种人类疾病密切相关^[4,5]. 本课题

组充分运用突变体筛选和基因克隆方法, 在模式动物秀丽隐杆线虫(*Caenorhabditis elegans*)中发现了两个新基因 $sorf-1$ 和 $sorf-2$, 其人类同源基因分别编码WD40重复序列蛋白WDR91和WDR81. 本文将从蛋白的发现、早晚期内吞体转化、小GTP酶Rab7效应因子、神经元生长、成体神经发生和选择性自噬六个方面介绍WDR91和WDR81的功能及相关作用机制.

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1 WDR91和WDR81的发现

模式动物秀丽隐杆线虫有六个类巨噬细胞, 此类细胞的内吞体和溶酶体体积较大, 利用微分干涉显微镜(DIC)观察时, 内吞体显示出凹坑状结构, 溶酶体则显示出纽扣状结构. 缺失HOPS/CORVET复合体核心亚基*vps-18*的突变体出现内吞体之间以及内吞体与溶酶体之间的融合障碍, 在DIC显微镜下该突变体类巨噬细胞中内吞体和溶酶体呈现出体积变小的颗粒状结构^[6]. 以*vps-18*突变体为背景进行遗传筛选, 获得了恢复*vps-18*突变体表型的基因*ZK563.5*和*F52C9.1*. 为了显示*ZK563.5*和*F52C9.1*基因本身对内吞体和溶酶体的影响, 使用GFP::RAB-5或RME-8::GFP^[7]标记早期内吞体, 使用LMP-1::GFP标记晚期内吞体和溶酶体, 结果显示*ZK563.5*和*F52C9.1*两个基因单突变体的内吞体和溶酶体异常增大, 说明这两个基因均可抑制胞内囊泡融合, 因此*ZK563.5*和*F52C9.1*分别被命名为*sorf-1* (suppressor of organelle fusion 1)和*sorf-2* (suppressor of organelle fusion 2). SORF-1和SORF-2两种蛋白进化保守, 从线虫到人类都有其同源蛋白. SORF-1与人源蛋白WDR91的N端有32%的氨基酸序列同源性, 而且该同源区域都并未鉴定出已知结构域. SORF-2则与人源蛋白WDR81有20%的氨基酸序列同源性, 并且两者都含有BEACH结构域和WD40重复序列结构域(图1).

2 WDR91-WDR81 复合体调控早晚期内吞体转化

内吞体是真核细胞中膜包裹的结构, 通常分为早期内吞体(early endosome, EE)、晚期内吞体(late endosome, LE)和循环内吞体(recycling endosome, RE). 不同内吞体可通过特征性的小GTP酶(Rab)和磷脂酰肌醇磷酸(phosphoinositide, PI)进行区分^[8]. 三种内吞体与胞内其他细胞器形成动态的网络系统, 共同维持细胞的正常生命活动. 细胞内吞的外部物质或内质网合成的蛋白可通过内吞体-溶酶体运输途径进行降解, 并且相应过程伴随着早晚期内吞体的转化^[9]. 目前认为, 早晚期内吞体转化的特征是内吞体膜表面特有的Rab和PI的转变, 即Rab5被Rab7取代^[10], PtdIns(3)P消失而PtdIns(3,5)P₂出现^[11~13](图2). 有研究基本揭示了Rab5

转换为Rab7的相关机制: Mon1和Ccz1形成的复合体可定位到早期内吞体膜表面, 一方面使Rab5的鸟苷酸交换因子(guanine nucleotide exchange factors, GEF) Rabex-5从早期内吞体替换下来, 阻止GTP活化形式的Rab5的产生, 另一方面其作为Rab7的GEF蛋白, 促进GTP活化形式Rab7的生成^[14,15]. 另外, 活化形式的Rab7还可招募Rab5的GTP酶激活蛋白(GTPase-activating protein, GAP)TBC-2, 进一步抑制Rab5的活性^[16,17]. 而对于内吞体膜表面的PtdIns(3)P水平, 研究认为可通过PI3K复合体的合成作用和myotubularin/MTMR家族的降解作用进行调控^[11,18~21], 其中III型PI3K复合体作为PtdIns(3)P合成最主要的来源, 包含三个核心亚基, VPS34作为催化亚基具有激酶活性, 而Beclin1和VPS15作为调节亚基. 虽然Rab5和Rab7都能与III型PI3K复合体结合^[20,21], 但PtdIns(3)P在早晚期内吞体的水平差异如何被精确调节不甚明了.

由于*sorf-1*和*sorf-2*突变体具有异常增大的早期内吞体, 接下来观察了PtdIns(3)P结合蛋白2×FYVE和PtdIns(3)P结合因子EEA-1^[22,23]在类巨噬细胞的定位情况, 结果显示*sorf-1*和*sorf-2*突变体早期内吞体膜上2×FYVE和EEA-1的信号显著增强, 说明SORF-1和SORF-2缺失导致内吞体膜表面的PtdIns(3)P水平升高. 另外, 利用活细胞成像实时监测早期内吞体标志物2×FYVE或RAB-5和晚期内吞体标志物RAB-7的动态变化以及红色荧光基团Texas Red交联的BSA内吞进入类巨噬细胞的运输过程^[7]. 结果表明, SORF-1和SORF-2缺失阻碍早晚期内吞体的转化, 并阻断向溶酶体方向的运输过程. 为了探究SORF-1和SORF-2在内吞体-溶酶体运输途径的调控机制, 干扰了*sorf-1*和*sorf-2*突变体中RAB的转化过程, 如引入*rab-7*和*sand-1*基因突变以阻碍RAB转化^[15~17]. 结果显示, PtdIns(3)P很难从早期内吞体上清除, 而敲降VPS34水平能有效缓解早期内吞体异常持续融合的现象, 进而说明PtdIns(3)P累积导致早期内吞体过度融合而使早晚期内吞体转化滞后. 后续的工作主要针对SORF-1和SORF-2如何影响PtdIns(3)P而进行深入研究, 揭示了SORF-1和SORF-2对早晚期内吞体转化的调控机制^[24], 即SORF-1和SORF-2形成蛋白复合体, 并与III型PI3K复合体BEC-1/Beclin1亚基相互作用, 抑制VPS34亚基的激酶活性, 使早晚期内吞体转化过程中PtdIns(3)P的产生受到控制.

图 1 秀丽线虫SORF-1和SORF-2与人类WDR91和WDR81蛋白结构对比示意图。SORF-1和SORF-2的人类同源蛋白分别为WDR91和WDR81, BEACH结构域和WD40重复序列结构域分别用红色和绿色表示

Figure 1 Schematic comparison of *Caenorhabditis elegans* SORF-1 and SORF-2 with human WDR91 and WDR81. The mammalian homologs of SORF-1 and SORF-2 are WDR91 and WDR81, respectively. The BEACH domains are indicated in red and WD40 repeats are indicated in green

图 2 早晚期内吞体转化过程。早晚期内吞体转化过程伴随着早期内吞体膜表面特有的Rab5和PtdIns(3)P转变为晚期内吞体膜表面特有的Rab7和PtdIns(3,5)P₂

Figure 2 Conversion from early to late endosomes. Conversion from early to late endosomes is accompanied by switching of early endosome, specific Rab5 and PtdIns(3)P, to late endosome-specific Rab7 and PtdIns(3,5)P₂

研究还发现, SORF-1和SORF-2的同源蛋白WDR91和WDR81通过相似的作用机制调控内吞体膜上PtdIns(3)P水平。在敲除WDR91和WDR81的HeLa细胞(KO-91和KO-81细胞)中, 不仅早期内吞体体积显著变大, 内吞体膜上PtdIns(3)P水平升高, 而且表皮生长因子受体(epidermal growth factor receptor, EGFR)的降解过程严重受阻。利用蛋白质相互作用实验技术, 研究证实WDR91和WDR81形成复合体^[25], 通过与Beclin1互作抑制III型PI3K复合体VPS34亚基活性以调控早期内吞体上PtdIns(3)P水平, 从而影响内吞体-溶酶体运输途径中早晚期内吞体转化过程^[24]。

3 WDR91是小GTP酶Rab7的效应因子

通过分析蛋白结构发现, SORF-1只与WDR91的N端具有很高的同源性, WDR91蛋白的C端多出一个含

WD40重复序列的结构域, 这提示SORF-1和WDR91功能很可能不尽相同。有意思的是, SORF-1蛋白结构并没有磷脂结合结构域, 但SORF-1蛋白能特异结合PtdIns(3)P并且两者之间的结合作用与PtdIns(3)P的浓度正相关, 这预示着SORF-1很可能能够感知早期内吞体膜上PtdIns(3)P水平的变化, 在特定时间被招募到内吞体膜上发挥作用, 实现对PtdIns(3)P的精准调控。然而, 研究未发现WDR91具有结合PtdIns(3)P的能力, 其如何被招募到内吞体不得而知。

鉴于在细胞中过表达WDR91可引发Rab7阳性囊泡结构的聚集, 且WDR91定位于这些囊泡上。接下来, 蛋白相互作用实验显示, WDR91不与Rab5和Rab9结合, 而特定地与Rab7相互作用, 并且是与GTP结合活化形式的Rab7互作, 即WDR91可作为小GTP酶Rab7的效应因子。进一步研究表明, WDR91通过其C端的WD40重复序列与活化形式的Rab7结合, 也就是说WDR91与活化形式Rab7的结合促使WDR91-WDR81复合体被招募到内吞体发挥作用(图3)。此外, 采用截断突变筛选法和甘氨酸突变筛选法, 同时结合Phyre2服务器对WDR91蛋白结构建模、预测和分析的数据^[26], 找到了WDR91的WD40重复序列结构域中与Rab7结合的关键位点F570, N571和H572, 并发现WDR91的N端159~163区段对于WDR91与Rab7之间的特异结合起着重要的作用^[27]。

4 WDR91作为神经元生长的必需因子

神经元是高度极化的细胞, 正常神经元通常拥有一个细长的轴突和多个较短的树突, 轴突的延伸和树

图 3 内吞体上WDR91-WDR81复合体的功能示意图。WDR91-WDR81复合体通过与活性形式的Rab7结合而被招募到内吞体上,特异地抑制PI3K复合物的活性,降低PtdIns(3)P的生成

Figure 3 Mechanistic model of WDR91-WDR81 complex on the endosomes. WDR91-WDR81 complex is recruited to endosomes by interacting with the active form of Rab7, and it specifically inhibits phosphatidylinositol 3-kinase activity to reduce the generation of PtdIns(3)P

突的分支作为神经元生长的形态学特征^[28]。神经元生长是神经发育的核心事件之一,不仅与突触连接的形成以及神经环路的建立息息相关,也为大脑行使学习与记忆等正常功能提供了基础^[29,30]。

由于内吞体-溶酶体运输途径对于维持正常神经发育至关重要^[31~33],因此本实验室着手探究WDR91对神经元生长的影响。首先,制备了*Wdr91*全身敲除小鼠,然而该小鼠在出生几个小时之内死亡。其次,制备了第三个外显子前后携带方向相同LoxP位点的*Wdr91*小鼠(*WDR91^{ff}*),通过与Nestin基因启动子驱动的Cre小鼠(Nestin-Cre)杂交获得脑组织特异性敲除*Wdr91*的小鼠(*WDR91^{ff}* cKO)。值得注意的是,*Wdr91*条件性敲除小鼠刚出生时与野生型小鼠在全身和脑部重量以及存活率方面无差异,但出生3~4周左右,*Wdr91*条件性敲除小鼠全身和脑部重量明显减小,并且存活率显著降低。体外培养原代海马神经元的结果显示,缺失WDR91的神经元存在大量异常增大的早、晚期内吞体,并且神经元突起的长度和复杂度均显著降低。最后,在WDR91缺失的神经元中重新表达野生型WDR91和突变型WDR91,结果显示与Rab7结合有缺陷的突变体WDR91(3A)和WDR91(Δ5)无法恢复内吞体增大及神经突起生长阻遏的表型。以上结果反映出WDR91在神经元突起生长中发挥不可或缺的作用^[27,34]。

5 WDR81影响成体神经发生

前期研究发现,WDR81的突变与CAMRQ2综合征相关,患者出现小脑萎缩、胼胝体形态异常,临床症状为小脑性共济失调、四肢行走和智力低下^[35~37]。海马成体神经发生对维持大脑认知功能起着重要作用^[38~41],为了揭示WDR81在神经发育中的功能及在神经系统疾病中的致病机制,本实验室研究了WDR81对海马成体神经发生的影响。

由于WDR81在脑组织丰富表达^[35],制备了第二个与第四个外显子前后携带方向相同LoxP位点的*Wdr81*小鼠(*WDR81^{ff}*)。从*WDR81^{ff}*小鼠分离培养的成体神经干/祖细胞(aNPCs)可通过感染CreGFP的慢病毒使WDR81表达量下调,但此后BrdU标记的增殖细胞^[42]数量和Tuj1标记的未成熟神经细胞^[43]数量减少,这提示WDR81缺失抑制aNPCs的增殖和神经分化。接下来,为了研究在体情况下WDR81对神经发生的作用,将*WDR81^{ff}*小鼠与三苯氧胺(TAM)诱导Nestin基因启动子驱动的Cre小鼠(Nestin-CreERT2)以及Rosa26-stop-DTR-2A-EGFP小鼠杂交获得了一种诱导条件性敲除*Wdr81*的小鼠(*Wdr81* cKO)。注射TAM后,*Wdr81* cKO小鼠海马齿状回结构可通过GFP的荧光指示敲除WDR81的细胞。而细胞谱系特异分子标记的结果显示,注射TAM一天之后*Wdr81* cKO小鼠各谱系的细胞数量与对照小鼠相比并无差别,注射TAM七天开始,海马齿状回结构中类型2的神经干细胞、瞬时扩增祖细胞、成神经细胞、未成熟神经细胞和成熟神经细胞的数量与对照小鼠相比均明显变化,说明WDR81缺失严重影响海马结构的神经发生。与此同时,将*WDR81^{ff}*小鼠与Nestin-CreERT2小鼠杂交获得了另一种诱导条件性敲除*Wdr81*小鼠(*Wdr81* cKO),该小鼠注射TAM五天之后海马依赖性学习行为出现障碍,反映出海马神经发生对于学习行为的重要性。

与前期结论一致,WDR81缺失的aNPCs中III型PI3K复合体VPS34激酶活性增强,导致PtdIns(3)P水平升高。有趣的是,利用III型PI3K复合体阻断剂3-MA^[44]不仅能够有效恢复*Wdr81* cKO小鼠aNPCs增殖和分化的缺陷,而且能够改善小鼠海马依赖性学习行为。由于内吞体介导运输的信号分子受到PtdIns(3)P水平的调控^[45],检测了结合PtdIns(3)P并参与信号转导的蛋白,如SARA,FGD1和Mtmr3等蛋白^[46],发现了aNPCs中SARA蛋白在增大内吞体上富集,并且SARA蛋白招募

的Smad2^[47]磷酸化水平(*p*-Smad2)及其下游靶向基因*p15*、*p57*和*p21*^[48,49]转录和翻译水平增加,意味着SARA蛋白依赖的TGFβ信号通路被持续激活^[50]。此外,降低SARA的表达量或者使用I/II型TGFβ受体激酶抑制剂LY2109761阻断TGFβ信号^[51]能明显改善WDR81缺失导致的海马神经发生障碍。上述研究结果从多方面确证,WDR81通过内吞体SARA-TGFβ信号通路调控海马成体神经发生^[52]。

6 WDR81不依赖于WDR91-WDR81复合体参与选择性自噬

WDR81亚细胞定位的结果显示,WDR81除了与WDR91形成复合体定位于内吞体外^[24],还与泛素标记的蛋白共定位。由于蛋白酶体抑制剂MG132和蛋白质合成抑制剂嘌呤霉素(puromycin)能够诱导胞内产生大量泛素标记的蛋白聚集体^[53-55],发现WDR81与MG132和puromycin诱导形成的蛋白聚集体都存在很好的共定位,这进一步证实了WDR81亚细胞定位的结果。此外,自噬受体p62和K63偶联的泛素化蛋白(K63-Ub)与选择性自噬途径紧密相关^[56-59],在降低WDR81表达量的情况下,细胞内p62和K63-Ub发生累积,并且MG132处理之后能进一步增加p62和泛素化蛋白的水平,这些结果表明WDR81帮助蛋白聚合体通过选择性自噬途径而被清除。

利用电子显微镜观察敲除WDR81的HeLa细胞(KO-81细胞)发现,自噬体和自噬溶酶体的总量与对照HeLa细胞并无差异,然而KO-81细胞存在更多无膜包裹或部分膜包裹的蛋白结构,这提示WDR81缺失阻碍蛋白聚集体发展形成自噬体。后续研究发现,WDR81不与参与过氧化物酶体自噬的NBR1和参与异体吞噬的NDP52等其他自噬受体分子^[60,61]共定位,而特异与p62共定位,并且WDR81通过其含有BEACH结构域的N端与p62含有PB1结构域的N端相互作用。另外,KO-81细胞中累积的泛素化蛋白以及K63偶联的泛素化蛋白并不与p62结合,而在WDR81存在的情况下,p62与K63偶联的泛素化蛋白之间的相互作用显著增强,这

些结果说明WDR81可通过与p62结合促进自噬受体分子对泛素化蛋白聚集体的识别。与此同时,研究还发现,WDR81不与LC3家族中LC3A和LC3B结合,而特异与LC3C相互作用,并且WDR81的BEACH结构域中两个经典LIR motif(W544/L547和S577/Y578/V581)和LC3C疏水区域的W位点(K32/F33)和L位点(L64/F69)^[62]对于WDR81与LC3C的结合起到关键作用。正常细胞敲降LC3C水平后,泛素化蛋白和p62水平显著增加,且泛素化蛋白与p62和WDR81共定位形成很大的聚集体,这些结果表明,WDR81还可通过与LC3C互作促进泛素化蛋白聚集体被自噬途径清除。

类似地,将WDR81^{ff}小鼠与Nestin基因启动子驱动的Cre小鼠(Nestin-Cre)杂交获得了脑组织特异性敲除Wdr81的小鼠(WDR81^{ff} cKO)。令人惊奇的是,脑组织特异性敲除Wdr81小鼠并无明显的大脑发育异常,但泛素化蛋白和p62聚集体在皮层和纹状体神经元中明显累积,且新生小鼠死亡率极高。免疫组学结果显示,正常小鼠脑组织的WDR81主要分布于NeuN标记的神经细胞而非GFAP标记的星形胶质细胞,而脑组织特异性敲除Wdr81小鼠中p62聚集体同样集中于神经元而非星形胶质细胞。以上结果进一步证实,WDR81作为选择性自噬过程的调控因子,对于神经元中蛋白聚集体的清除发挥着重要作用^[63]。

7 总结

综上所述,本实验室近期的研究工作关注两种WD40重复序列蛋白WDR91和WDR81,分别揭示了两种蛋白在早晚期内吞体转化,包括神经突起生长和成体神经发生的神经发育以及选择性自噬中的功能及调控机制,相关结果有利于进一步发掘WDR91-WDR81复合体在溶酶体发生及其介导的降解过程、蛋白分选再利用运输途径,神经突触形成及其传导功能等方面不可替代的作用,为解析WDR91缺失引发的神经发育障碍和WDR81突变导致的CAMRQ2综合征提供线索,为改善WDR91和WDR81异常造成的生理病理缺陷提供有益指导。

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WDR91-WDR81 complex-dependent endolysosomal trafficking and neural development

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WD40-repeat proteins participate in diverse cellular processes, such as intracellular trafficking, signal transduction, apoptosis, and transcriptional regulation. From genetic screens of the model organism *Caenorhabditis elegans*, we identified two novel genes that encode homologs of mammalian WDR91 and WDR81, two WD40-repeat proteins with previously unknown functions. Our study revealed that WDR91 and WDR81 form a complex that functions as an effector of the late endosome-specific Rab GTPase Rab7. The WDR91-WDR81 complex is recruited to the endosomes, where these proteins interact with Beclin 1, a subunit of class III PI3K complex, thereby inhibiting the endosomal synthesis of early endosome-specific PtdIns3P and allowing the conversion of early endosomes to late endosomes, ensuring the delivery of the endosomal cargos to the lysosomes. WDR91 or WDR81 loss impairs neurogenesis and development, possibly by affecting endosome-dependent signaling. Furthermore, WDR81 plays a critical role in aggrephagy by interacting with p62 and LC3C, distinct from its role in endosomal trafficking. Taken together, these findings provide important mechanistic insights into WDR91- and WDR81-related neurodevelopmental disorders.

WDR91-WDR81 complex, endosome conversion, Rab7, neural development, selective autophagy

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