

E3泛素连接酶基因CG4911敲除和功能的初步研究

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摘要 蛋白质的泛素化是一种重要的翻译后修饰过程, 参与调控细胞周期、基因转录、信号转导、炎症反应和干细胞的维持等过程。泛素连接酶E3(ubiquitin ligase)是泛素化过程中关键酶。但许多E3基因在发育中的功能和作用机制还不明确。该研究以黑腹果蝇为模式动物, 研究泛素连接酶家族一个重要基因CG4911的功能及分子机制。获得CG4911基因敲除果蝇, CG4911敲除果蝇纯合子可活。原位杂交结果显示, CG4911在胚胎发育早期表达。通过构建CG4911-pUAST-3HA重组子转染Hela细胞, 确定CG4911定位于细胞质中, 其表达并无修饰作用, 并且过表达基因CG4911可导致背板发育缺陷。该研究首次获得了CG4911基因敲除果蝇和CG4911转基因果蝇, 并初步探索了F-box基因CG4911的功能, 为进一步阐明泛素连接酶的功能及分子机制提供了科学依据。

关键词 泛素连接酶基因; RNA干扰; 原位杂交; 果蝇

泛素-蛋白酶体系(ubiquitin-proteasome system, UPS)是真核细胞内选择性降解蛋白的主要途径, 参与了多种细胞进程的调控, 包括调控细胞周期、基因转录、信号转导、炎症反应和干细胞的维持等^[1-2]。待降解的蛋白在一系列酶的作用下被泛素化, 这些酶包括泛素活化酶E1^[2](ubiquitin-activating enzymes)、泛素偶联酶E2(ubiquitin-conjugating enzymes)和特异性识别蛋白底物的泛素连接酶E3(ubiquitin ligase)。

作为特异性识别底物的泛素酶E3广泛存在于细胞中, 并在进化过程中保守。根据E3与E2连接序列结构域的不同, E3可以分为3大类: E6-AP羧基结构域同源结构域类E3(homologous to E6-AP carboxyl terminus, HECT)、RING环指结构域类E3(really interesting new gene, RING)、U-box结构域类E3。F-box蛋白是一种适配蛋白, 是由大约50个氨基酸组成的介导蛋白之间相互作用的结构域, 属于RING环指结构域类E3。F-box与Skp、Cullin一起构成一个多蛋白的E3复合体SCF^[3]。大多数的SCF参与调控细胞周期进程、基因转录、信号转导、DNA修复等生物学进程^[4-7]。大量的研究数据表明, 当SCF异常时可导致增殖失控、基因稳定性下降, 引发肿瘤等疾病^[4]。目前, 在果蝇基因组中共检测到33个F-box基因, 其中大部分基因的生物功能尚不明确。

果蝇基因CG4911位于果蝇三号染色体左臂, 编

码450个氨基酸, 属于F-box结构域家族, 与人类基因FBXO33^[8]同源。体外细胞实验证实, FBXO33被认为与Skp-1/Cul1一起参与转录因子YB-1的泛素化过程^[9], YB-1在细胞周期调控中起着重要作用。在果蝇中, 胚胎发生早期CG4911表达明显, 定位于原肠胚的头部褶皱、腹鳍褶皱、后部和中肠胚内陷^[8]。对于基因CG4911的功能目前未见报道, 本课题首次获得了CG4911敲除果蝇和CG4911转基因果蝇株, 检测了基因CG4911在胚胎和3龄幼虫翅膀成虫盘中的表达模式, 对基因CG4911的功能进行了初步的探索, 为进一步研究基因CG4911的功能提供了依据。

1 材料与方 法

1.1 果蝇品系

CG4911^{V45141}w[1118];P{GD11468v45141/TM3,y[1]w[67c23];P{w[+mC]=GSV6}GS15004/TM3,Sb[1]Ser[1]购自Vienna *Drosophila* RNAi Center。

Act-gal4-UAS-dcr²/Cyo, Tub-gal4-UAS-dcr²/Tm3, MS1096-gal4-UAS-dcr²/Cyo, Vg-gal4-UAS-dcr²/Cyo, En-gal4-UAS-dcr²/Cyo, AP-gal4-UAS-dcr²/Cyo。

收稿日期: 2012-01-21 接受日期: 2012-02-27

国家重大科技计划专项课题(No.2011CB943900)、温州医学院人才启动项目(No.QTJ08012)、温州医学院科研基金重大项目(No.XNK07005)、温州市科技局计划项目(No.Y20110154)和温州医学院眼视光学院、医院内课题(No.YNKT201113)资助项目

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1.2 成虫RNAi表型分析

取 *CG4911*^{V45141}w[1118];P{GD11468}jyinv45141/TM3雄蝇分别与Act-gal4-UAS-dcr²/Cyo, Tub-gal4-UAS-dcr²/Tm3, MS1096-gal4-UAS-dcr²/Cyo, Vg-gal4-UAS-dcr²/Cyo, En-gal4-UAS-dcr²/Cyo, AP-gal4-UAS-dcr²/Cyo雌蝇杂交, 放入25 °C的恒温培养箱中培养。在F1代果蝇羽化前移除培养管中的亲代果蝇。F1代果蝇羽化后观察其表型。

1.3 重组子的构建

以野生型果蝇w1118 cDNA为模板, 利用PCR的方法(phusion high fidelity PCR master mix, finnzyme)扩增出*CG4911*片段, 将片段纯化, 分别连接至pUAST-V5载体、pUAST-3HA载体, 得到*CG4911*-pUAST-V5和*CG4911*-pUAST-HA重组子。所得重组子用于果蝇胚胎显微注射和细胞转染。

1.4 获得突变体果蝇

利用果蝇胚胎显微注射方法将*CG4911*-pUAST-V5与Δ-3辅助质粒混合液共同注射至野生型果蝇w1118胚胎后脊部, 通过筛选、平衡及定位, 获得转基因*CG4911*-pUAST-V5果蝇。利用遗传方法, 借助P-element跳跃, 用果蝇株y[1]w[67c23];P{w[+mC]=GSV6}GS15004/TM3, Sb[1]Ser[1]进行*CG4911*基因敲除, 获得*CG4911*敲除突变体果蝇。

1.5 免疫荧光及Western blot方法检测CG4911在Hela细胞中的定位和表达情况

1.5.1 免疫荧光 将*CG4911*-pUAST-3HA与PM1-gal4质粒共转染到Hela细胞中。所用试剂盒为Effectene Transfection Reagent(QIAGEN), 方法详见试剂盒说明书。培养48 h后收集细胞, 按照文献[10]方法进行固定和免疫荧光染色。制片后, 于激光共聚焦显微镜下观察细胞。使用的抗体为mouse anti-histone 3H(1:200), 购自Santa Cruz公司; mouse anti-HA(1:200)、荧光偶联二抗均购自Invitrogen公司。

1.5.2 Western blot 将质粒*CG4911*-pUAST-3HA及pUAST-3HA分别同PM1-gal4共转染Hela细胞, 方法详见试剂盒说明书。按照文献[11]方法进行Western blot检测。使用的一抗为mouse anti-HA(1:2 000)、mouse anti-actin(1:5 000), 购自Abmart公司; 二抗为IR Dye 700 DX-goat anti-mouse(1:15 000), 购自Rockland公司。

1.6 原位杂交技术检测CG4911基因表达模式

取约500只野生型雌雄果蝇于葡萄汁平板培养

瓶中培养18 h, 收集包含各时期的胚胎。取野生型果蝇、AP-gal4诱导的*CG4911*过表达果蝇的三龄幼虫, 解剖翅膀成虫盘。以基因组为模板, PCR获得单独条带(T7-CG4911-Prob-S: 5'-TAA TAC GAC TCA CTA TAG GGA GAG TCC AAG GCT TCG GCG TTC ATC-3', T3-CG4911-Prob-A: 5'-AAT TAA CCC TCA CTA AAG GGA GAC TAA AGG CTA TGG GAA AAC CAC AGG-3')。将PCR产物纯化, 以此为模板体外合成正义及反义探针, 按照文献[12]方法进行原位杂交检测。合成探针所需试剂Fluorescein RNA Labeling Mix、T7 RNA polymerase、T3 RNA polymerase以及anti-fluorescein-AP抗体均购自罗氏公司。使用NBT/BCIP液显色。

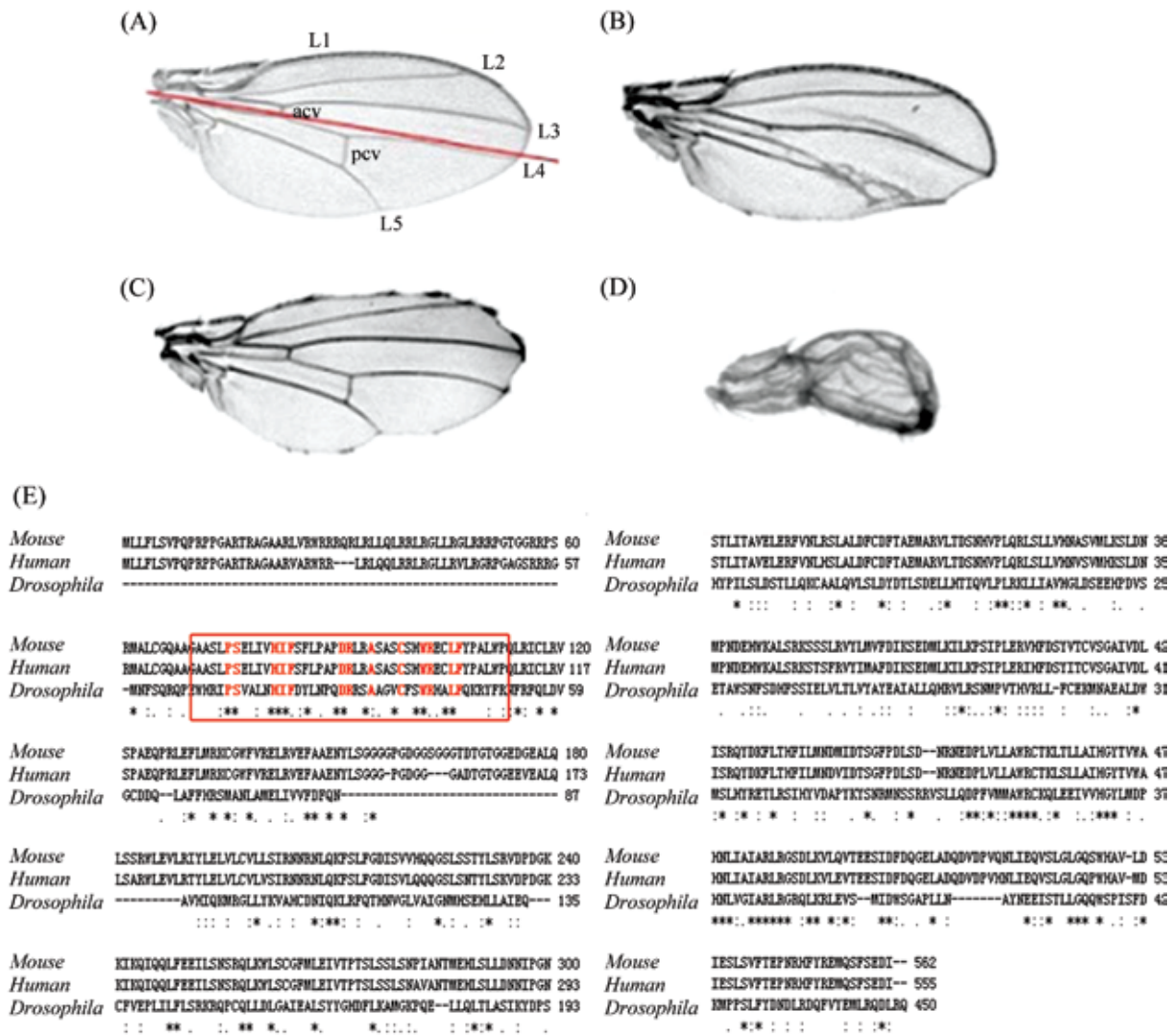
2 结果

2.1 RNAi沉默CG4911表达可影响果蝇翅膀发育

为研究*E3*基因功能, 本实验组对果蝇中200多个具有RING、HECT等E3典型结构域的基因进行RNAi筛选。通过观察果蝇成虫的翅膀和眼的表型缺陷作为初步的筛选标准。果蝇的RNAi由gal4-UAS^[13]系统控制, gal4可以驱动UAS表达使得连接于UAS后RNAi序列过表达从而沉默靶基因的表达。基因*CG4911*是筛选的基因之一。基因*CG4911*编码蛋白含有*F-box*结构域(图1E, 红色方框内), 在进化过程中保守, 在果蝇、斑马鱼、非洲爪蟾、小鼠、人类中都发现其同源基因, 其与人类的同源基因*FBXO33*蛋白序列的同源性为40.78%(图1E)。观察杂交F1代果蝇的表现型发现, 当用Act-gal4、Tub-gal4在所有组织中沉默*CG4911*表达果蝇不能存活, 进一步观察发现这些果蝇都在蛹初期死亡。而在翅膀中选择性沉默基因*CG4911*的果蝇En-gal4-UAS-dcr2/*CG4911* RNAi、MS1096-gal4-UAS-dcr2/*CG4911* RNAi和Vg-gal4-UAS-dcr2/*CG4911* RNAi则个体能够发育成熟, 但翅膀出现水泡样突起(图1B)、边缘缺刻(图1C)、翅膀严重皱缩(图1D)等严重翅膀缺陷。

2.2 基因CG4911的表达模式

基因的表达的时间性和基因功能密切相关, 为进一步深入研究基因*CG4911*的功能, 我们对基因*CG4911*在胚胎发育过程中和3龄幼虫翅膀成虫盘的表达情况使用原位杂交的方法进行检测。实验结果表明*CG4911*为母源性表达。其表达主要集中在胚



A: 野生型果蝇翅膀, 红线标注的为前后部分界线; B: Engrailed(En)特异性表达在翅膀后部, 因此, *En-gal4*诱导翅膀后部的基因*CG4911*沉默, 成虫翅膀后部出现囊泡, L4、aCV、pCV翅脉缺失; C: *Vg-gal4*诱导的*CG4911*沉默, 导致翅膀边缘出现缺口; D: *MS1096-gal4*诱导的*CG4911*沉默使得整个翅膀皱缩; E: 小鼠*Fbxo33*、人类*FBXO33*和果蝇*CG4911*基因编码蛋白的同源性比对, “*”标注蛋白序列在3个物种中完全相同, “:”标注蛋白序列在两个物种中相同。

A: wide-type of *Drosophila* wing, anterior and posterior boundary was marked by red line; B: Engrailed (En) expressed on the posterior of wings. Vesicles were found with L4, aCV, pCV veins missing on the posterior of adult wing; C: expression of *CG4911* was induced by *Vg-gal4*. Notch was found surrounding the wing's boundary; D: expression of *CG4911* was induced by *MS1096-gal4*. Wings were crumple; E: protein sequence alignment among the predicted *CG4911* product, mouse *Fbxo33* and the human *FBXO33*, “*” marked that protein sequence was identical in the three species, “:” labeled that protein sequence was identical in the two species.

图1 果蝇基因*CG4911*沉默后成虫翅膀表型及*CG4911*与小鼠、人类同源蛋白同源性比对

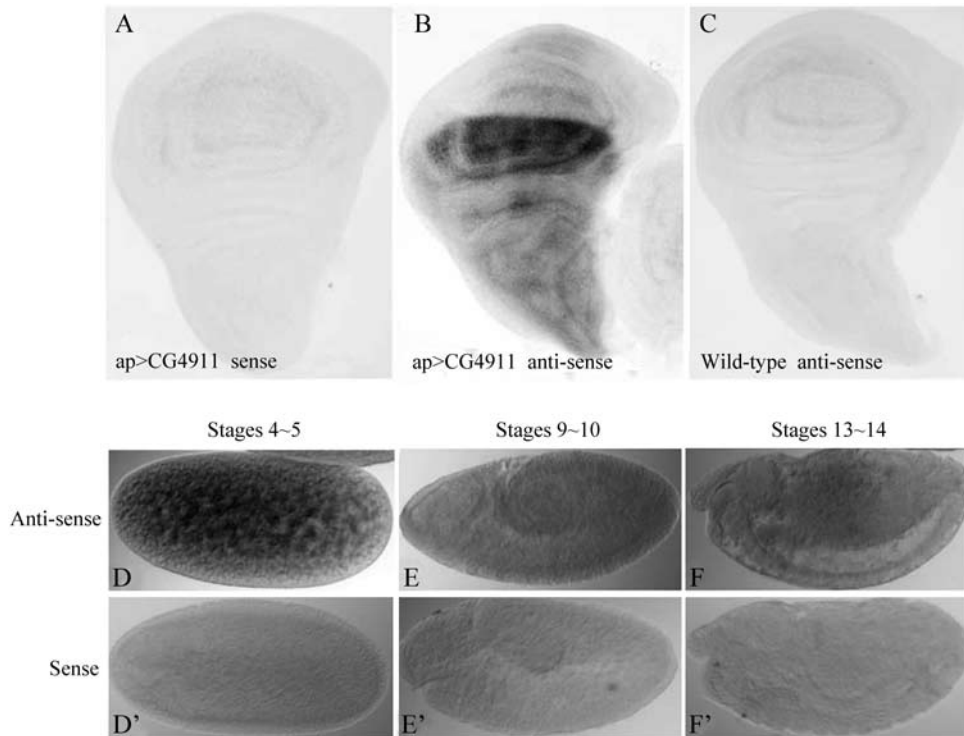
Fig.1 *CG4911* RNAi created critical wing defects and protein sequences alignment among the *CG4911* product, mouse *Fbxo33* and the human *FBXO33*

胎发育的早期4~5期, 且分布于胚胎各个部位。随着发育的进行逐渐减弱, 到14期以后表达量很低(图2D~图2F)。在3龄幼虫翅膀成虫盘中*CG4911*表达量低(图2A~图2C)。FlyAtlas对*CG4911*在果蝇各发育过程和成熟组织中的分布情况分析显示, *CG4911*除在卵巢中高表达外在其他组织中表达量都很低。推

测*CG4911*可能在胚胎发育早期起作用, 从而影响个体发育。

2.3 基因*CG4911*编码的蛋白定位于细胞质且不被修饰

RNAi结果提示, 基因*CG4911*在果蝇翅膀发育过程中起作用, *CG4911*沉默后会致果蝇翅膀的发



A~C: 原位杂交检测*CG4911*在3龄幼虫翅膀成虫盘中的表达。A: 使用阴性探针检测AP-gal4诱导*CG4911*的过表达, 在果蝇3龄幼虫翅膀成虫盘中未能检测到*CG4911*的表达; B: 使用阳性探针检测AP-gal4诱导*CG4911*过表达, 果蝇3龄幼虫翅膀成虫盘中*CG4911*表达于翅膀成虫盘背部; C: 使用阳性探针检测野生型果蝇3龄幼虫翅膀成虫盘, *CG4911*的表达量很低; D~F: 原位杂交检测*CG4911*在果蝇胚胎发育过程中的表达。D: 使用阳性探针检测, 在胚胎4~5期可见*CG4911*的表达分布于胚胎各个部位; E: 使用阳性探针检测, 在胚胎9~10期*CG4911*的表达大量减少, 表达量很低; F: 使用阳性和阴性探针检测在胚胎13~14期, *CG4911*的表达与9~10期类似, 难以检测到基因的表达; D'~F'分别以阴性探针为对照, 检测不到相应时期*CG4911*在胚胎中的表达。

A~C: the expression pattern of *CG4911* in wing discs. A: negative probe was used to detect the expression of *CG4911* in AP-gal4 induced *CG4911* over-expression wing discs. No signal can be detected; B: positive probe was used to detect the expression pattern of *CG4911* in AP-gal4 induced *CG4911* over-expression wing discs. Strong signal was detected on dorsal part; C: positive probe was used to detect the expression pattern of *CG4911* in wide-type fly's wing discs. No signal was detected; D~F: the expression pattern of *CG4911* in embryo; D: positive probe was used to detect the expression pattern of *CG4911* in embryo stage 4~5. *CG4911* expressed in almost all the embryo; E: positive probe was used to detect the expression pattern of *CG4911* in embryo stage 9~10. The expression of *CG4911* dramatic decline. F: positive probe were used to detect the expression pattern of *CG4911* in embryo stage 13~14. The similar result with F is found; D'~F': negative probe was introduced to detect the expression of *CG4911* in the corresponding stages as control. No signal can be detected.

图2 *CG4911*在胚胎和3龄幼虫成虫盘中的表达模式

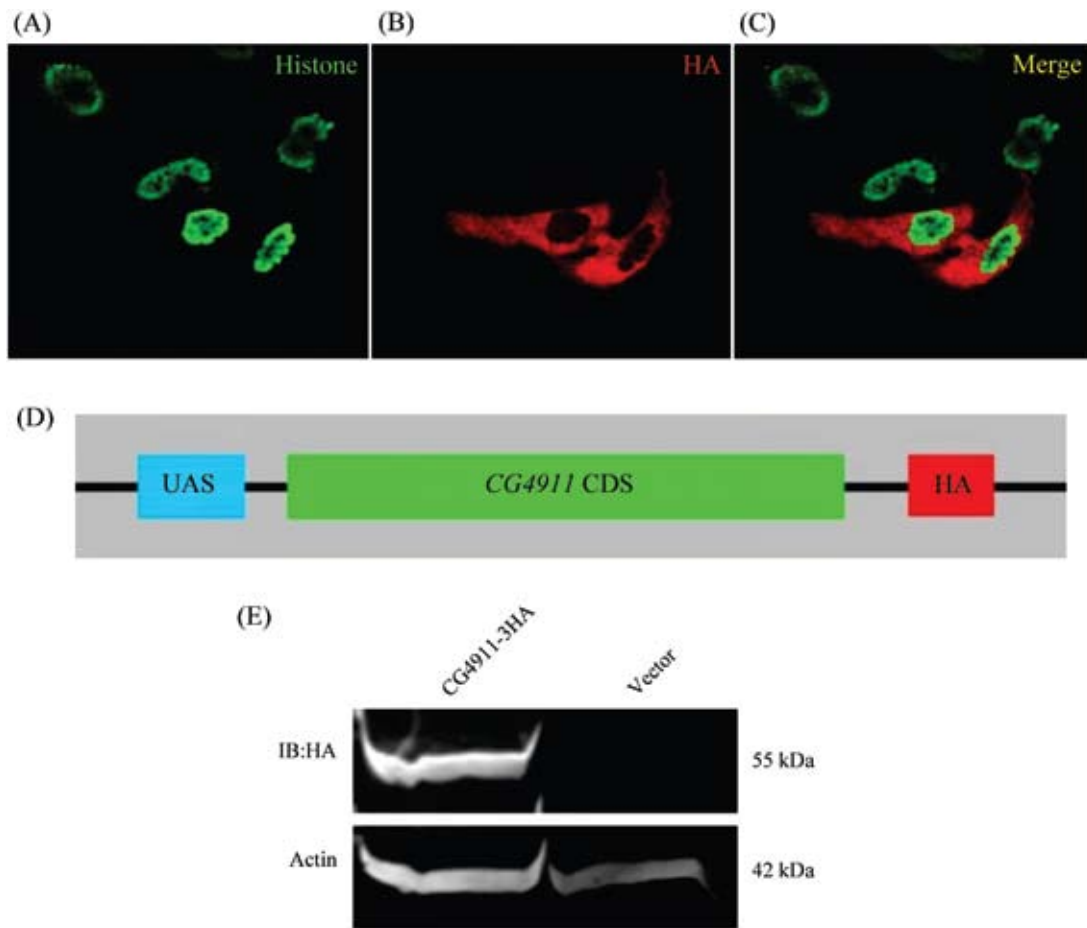
Fig.2 The expression pattern of *CG4911* in embryo and wing discs

育障碍。为了进一步研究*CG4911*的基因功能, 我们构建了包括基因*CG4911*全序列cDNA的*CG4911*-pUAST-3HA重组子(图2D)。基因的表达定位与基因的功能密切相关。*CG4911*-pUAST-3HA重组子转染Hela细胞后, 免疫荧光染色以H3标记细胞核(图3A), HA标记*CG4911*的表达位置(图3B), 结果显示*CG4911*在整个细胞质中表达。细胞质是蛋白合成和降解的主要场所, 也是多数泛素化相关蛋白酶发挥功能的位置, 因此*CG4911*广泛分布于细胞质中以发挥其泛素连接酶催化泛素化底物的效用。同时, Western blot的结果显示, 在Hela细胞中

过表达*CG4911*-pUAST-3HA重组子, 以HA为标记检测*CG4911*的表达仅有一条单一条带(图3E), 显示*CG4911*在Hela细胞中不会被修饰。

2.4 基因*CG4911*基因敲除果蝇纯合子可活

鉴于RNAi存在一定的脱靶(off-target)几率, 获得*CG4911*基因敲除果蝇对于进一步研究基因功能十分必要, 本课题组利用P-element跳跃的方法对基因*CG4911*进行敲除。P-element位于*atg*上游(图4) 61 bp处, P-element跳跃后共获得两个突变果蝇株。PCR和基因测序结果验证确定基因*CG4911*已被敲除。突变体敲除部分示意图如图4(红色方框部分)。

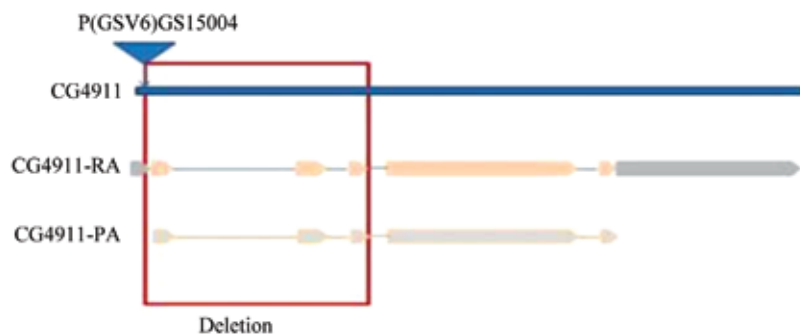


A: H3免疫荧光染色标记细胞核; B: HA标签标记*CG4911*的表达位置, 染色结果显示*CG4911*定位在细胞质中; D: *CG4911*-pUAST-3HA重组子构建示意图, 插入片段为*CG4911* cDNA全长; E: 以空载体pUAST-3HA为对照, Western blot检测*CG4911*-pUAST-3HA在HeLa细胞中过表达成单一一条带。

A: H3 tag showed the nucleus of HeLa cell; B: HA tag staining illustrated *CG4911* localized in cytoplasm; D: the construction *CG4911*-pUAST-3HA was used to transfect the HeLa cell. *CG4911* full length cDNA was inserted into pUAST-HA vector; E: *CG4911*-pUAST-3HA over-expression in HeLa cells. Western blot showed a single band of 55 kDa.

图3 检测*CG4911*在HeLa细胞中的定位及表达

Fig.3 The localization and expression of *CG4911* detected by immunofluorescence staining in HeLa cell



*CG4911*敲除区域示意图, 敲除所用的P-element位于基因*CG4911*的5'UTR区, 红色方框内为基因被敲除的序列, 包括部分的5'UTR和第1至第3个外显子。

CG4911 locus structure showed transposon insertion site, P-element was inserted in 5'UTR of *CG4911*. The deletion (marked by red box) is about 1 Kb which included part of 5'UTR and the first three exons.

图4 *CG4911*敲除区域示意图

Fig.4 *CG4911* locus structure

然而, 我们发现*CG4911*敲除果蝇纯合子可活, 并未见任何翅膀缺陷表型。

2.5 过表达基因*CG4911*导致背板发育缺陷

RNAi和基因敲除都基于Loss-of-function的分析, 鉴于*CG4911*基因敲除以后果蝇纯合子可活且没有明显可见的表型缺陷, 我们构建了*CG4911*-pUAST-V5和*CG4911*-pUAST-3HA转基因果蝇株, 并利用UAS-gal4体系诱导基因*CG4911*在果蝇体内过表达。En-gal4、Wg-gal4诱导的*CG4911*过表达未见明显表型缺陷。AP-gal4诱导的*CG4911*过表达可见背板和后部胸廓鬃毛的缺失以及后部胸廓畸形(图5箭头所示), 以及翅膀面积减小(图5C)。统计发现外显率达100%, 进一步观察可发现背板畸形的严重程度有所

不同, 轻微缺陷仅有背部鬃毛的缺失, 严重缺陷则是鬃毛缺失伴随后部胸廓变短。其中表型严重和轻微的比率约为2/3。EGFR(epidermal growth factor receptor)^[14]、Dpp(decapentaplegic)^[15]、Wingless^[16]等都与果蝇背板的发育密切相关。有关*CG4911*在背板发育过程中的功能尚待进一步研究。

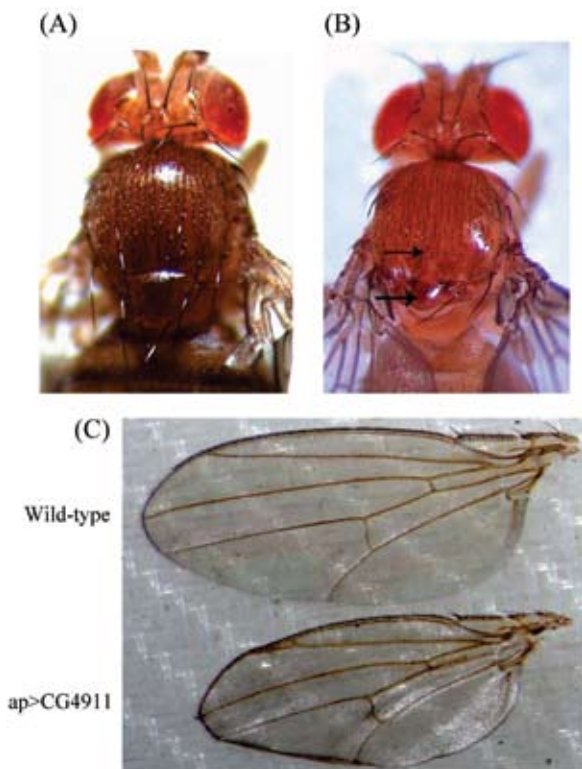
3 讨论

*CG4911*作为在生物进化过程中保守的基因, 编码的F-box蛋白在果蝇、斑马鱼、小鼠、灵长类、人类中都发现其同源基因。但关于这个基因的功能研究目前鲜见报道。在这篇文章中, 我们首次获得了*CG4911*基因敲除果蝇, 确定了*CG4911*在细胞中的定位及表达情况, 获得*CG4911*转基因果蝇。检测了*CG4911*在胚胎发育期的表达模式。本课题组的工作为进一步揭示*CG4911*的功能奠定了基础。

RNAi作为一种强大的研究基因功能的工具为基因研究提供了很大的便利, 但也存在一定的脱靶效应。在本实验中, RNAi结果显示*CG4911*沉默后果蝇表现为严重的翅膀发育缺陷, 但在我们随后的基因敲除突变体果蝇中并没有观察到一致的表型缺陷, 组织免疫荧光染色也证实*CG4911*并未影响翅膀发育关键基因*Wingless*及下游靶基因的表达。基于以上, 可以判定RNAi结果中观察到翅膀缺陷表型为脱靶效应引起。然而, 转基因果蝇过表达基因*CG4911*发现其成虫背板存在发育缺陷, 可见基因*CG4911*在果蝇发育过程中确实有重要作用。

*CG4911*胚胎及翅膀成虫原位杂交结果表明, *CG4911*表达在胚胎发育的早期。FlyAtlas对*CG4911*在果蝇各发育过程和成熟组织中的分布情况分析显示, *CG4911*除在卵巢中高表达外在其他组织中表达量都很低。推测*CG4911*基因的表达可能会影响个体的早期发育, 包括卵子的数量及成熟率, 以及卵巢的发育等, 本课题组将继续进行深入研究。

F-box蛋白是一种适配蛋白, 可以特异性的标识蛋白底物使其被泛素化从而被蛋白酶体降解。F-box首先在酵母中发现并被报道在细胞周期调控中起重要作用^[17-18]。*CG4911*基因敲除果蝇纯合子可活, 并未见明显的表型缺陷。然而, 过表达*CG4911*可观察到背板和翅膀的表型缺陷。可能原因为F-box常被认为有多个作用底物。一个F-box可以作用于多个蛋白底物, 如Skp2的底物包括P27^[19]、P21和P57^[20], 同



A: AP-gal4成虫的背板可见6根鬃毛, 在后部的胸廓可见4根鬃毛; B: AP-gal4诱导的基因*CG4911*过表达, 果蝇成虫背板和后部胸廓鬃毛缺失, 后部胸廓变短(箭头所示), 显示为背板发育缺陷; C: AP-gal4诱导的*CG4911*过表达, 果蝇翅膀变小。

A: six bristles on notum and four bristles on posterior thorax were showed on AP-gal4 adult fly; B: the bristles of notum and posterior thorax were missing in AP-gal4 induced *CG4911* over-expression adult fly (arrow), meanwhile the posterior thorax was shorter than AP-gal4 adult fly's (arrow); C: the wings of AP-gal4 induced *CG4911* over-expression adult fly were smaller than wild-type's.

图5 AP-gal4诱导的基因*CG4911*过表达引起背板发育缺陷
Fig.5 Notum defects were caused by AP-gal4 induced over-expression of *CG4911* in adult fly

时一个底物也可以被多个F-box识别而被泛素化,如细胞周期蛋白cyclin E可以被Skp2和Fbxw7识别^[21-22]。*CG4911*可能与其他F-box蛋白共同作用调控其底物的降解过程,当*CG4911*不表达时,其他*F-box*基因可以替代其作用。另外,本实验的关注重点放在了*CG4911*对果蝇翅膀发育的研究上,对于果蝇的其他方面如神经系统、消化系统、生殖系统、干细胞维持、生命周期等尚待进一步研究,以揭示基因*CG4911*的生物学功能和作用的分子机制。

*CG4911*的人类同源基因*FBXO33*被证实是哺乳动物SCF-E3泛素连接酶的F-box部分,作用于多功能调控因子YB-1的蛋白酶体降解过程,负调控YB-1的激活^[9]。不同于其他F-box蛋白的作用模式^[17-18],*FBXO33*是首次发现的与底物通过N末端连接的F-box蛋白,并且*FBXO33*与YB-1连接不依赖于YB-1的磷酸化^[9]。因此,探索*FBXO33*的靶基因和研究其基因功能对于揭示泛素连接酶作用的分子机制非常重要。但目前对于*FBXO33*的研究仅限于体外实验,对于小鼠同源基因*Fbxo33*的研究鲜有文献报道,*CG4911*敲除果蝇的获得为研究*FBXO33*基因在体内的作用机制提供了基础。

本实验通过RNAi和体内实验首次报道了一个在进化上保守的基因*CG4911*的初步研究结果,获得了*CG4911*敲除果蝇株、*CG4911*转基因果蝇株,明确了*CG4911*表达在细胞质中,检测了*CG4911*在胚胎发育期的表达模式。为进一步研究基因*CG4911*的功能继而研究其人类同源基因*FBXO33*的功能具有重要作用。

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E3 Ubiquitin Ligase Gene *CG4911* Gene Knock-out and Function Analysis

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Abstract Protein ubiquitination is an important post-translational modification process involved in cell cycle regulation, gene transcription, signal transduction, inflammation and stem cell maintenance. Ubiquitin ligase E3 is an essential enzyme and plays important role in ubiquitin-proteasome process. However, little is known about its function and molecular mechanism of E3 ligase during development. In this study, *Drosophila melanogaster* was used as a model to study the function and molecular mechanism of gene *CG4911*, which is one of the important ubiquitin ligases genes. We have generated mutant for *CG4911* and construct *CG4911*-pUAST-V5 vector as well as its transgenic lines. The result showed that *CG4911* mutant is viable. The protein of *CG4911* localized in the cytoplasm and there was no modification happened with *CG4911* when expressed in Hela cells. Furthermore, over expression of *CG4911* leads to the notum developmental defects. In summary, this is the first time to get *CG4911* knock out mutant and *CG4911* transgenic fly. Also, it's the first time to uncover a novel F-box gene *CG4911* gene function in developmental contexts. Our studies provide basis to further clarify the function of ubiquitin ligases and molecular mechanisms.

Key words ubiquitin E3 ligase; RNAi; *in situ* hybridization; *Drosophila melanogaster*

Received: January 21, 2012 Accepted: February 27, 2012

This work was supported by the National Basic Research Program of China (No.2011CB943900), the Research Foundation for Advanced Talents of Wenzhou Medical College (No.QTJ08012), the Wenzhou Municipal Science and Technology Program (No.Y20110154) and the Research Foundation of Ophthalmology and Optometry, Wenzhou Medical College (No.YNKT201113)

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