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AMPK γ is Required for Maintaining Epithelial Cell Structure and Polarity

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AMP-activated protein kinase (AMPK), a heterotrimeric complex comprising a catalytic α subunit and regulatory β and γ subunits, has been primarily studied as a major metabolic regulator in various organisms, but recent genetic studies discover its novel physiological functions. The first animal model with no functional AMPK γ subunit gene was generated by using *Drosophila* genetics. *AMPK γ* null flies demonstrated lethality with severe defects in cuticle formation. Further histological analysis found that deletion of *AMPK γ* causes severe defects in cell polarity in embryo epithelia. The phosphorylation of nonmuscle myosin regulatory light chain (MRLC), a critical regulator of epithelial cell polarity, was also diminished in *AMPK γ* null embryo epithelia. These defects in AMPK γ mutant epithelia were successfully restored by over-expression of *AMPK γ*. Collectively, these results suggested that AMPK γ is a critical cell polarity regulator in metazoan development.

Key words : *Drosophila*, AMPK γ, MRLC, epithelia, cell polarity

Introduction

AMP-activated protein kinase (AMPK), a heterotrimeric complex comprising a catalytic α subunit and regulatory β and γ subunits, is well conserved from yeast (*Saccharomyces cerevisiae*), worm (*Caenorhabditis elegans*) and fruit fly (*Drosophila*) to human [2,6,8]. During metabolic stress, when cellular AMP:ATP ratios rise, AMPK senses increased AMP level with its cystathionine beta-synthase (CBS) domains in its regulatory γ subunit and is activated by phosphorylation of Thr172 in the activation loop of its catalytic α subunit [2,6,8]. This activated AMPK down-regulates ATP-consuming anabolic pathways, and up-regulates ATP-generating catabolic pathways to maintain energy homeostasis in the cell [2,6,8]. Although the biochemical characteristics of AMPK were extensively studied by cell line-based studies, there were few genetic data on *in vivo* function of metazoan AMPK, due to the existence of multiple AMPK subunit isoforms encoded by different genes [8]. Because *Drosophila* has no redundancy in AMPK subunit genes [13], AMPK signaling was successfully nullified in the *Drosophila* system [10,12]. All *AMPK α* -null mutant flies are lethal and fail to develop to adulthood even in the presence of sufficient nutrients [10,12]. Surprisingly, loss of *AMPK α* induces disruption of cell polarity accompanying with disorganized actin cytoskeleton in embryonic and wing epithelial cells [10,12]. These abnormalities in epithelial cell polarity are highly similar to those of the mutants of LKB1, the upstream kinase of AMPK [10]. Moreover, constitutive activation of AMPK restores these defects in *LKB1*-null mutants, demonstrating AMPK as a novel regulator of cell polarity [10]. These genetic studies using *Drosophila* successfully discovered novel physiological functions of AMPK, and also provide valuable tools to dissect its *in vivo* signaling mechanisms.

In this report, the first *AMPK γ* null *Drosophila* mutant was generated and characterized. The deletion of *AMPK γ* induced lethality and the severe defects in cuticle formation. Further analysis showed that *AMPK γ* has an important role in maintaining epithelial cell polarity. These data strongly suggest that *AMPK γ* is critical for *in vivo* AMPK signaling.

Materials and Methods

Fly Strains

The *G5100* fly line with a P-element in the *AMPK γ* locus was obtained from GenExel (Taejon, Korea). The deletion mutants were generated from P-element excision experiments. To generate the over-expression lines for *AMPK γ*, a HA-tagged entire *AMPK γ V* open reading

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frame was subcloned into pUAST vector. The fly lines for FLP-DFS (autosomal flipase-dominant female sterile) technique and *hs*-GAL4 were obtained from the Bloomington Stock Center (Bloomington, IN, USA).

Production of AMPK γ null embryos

The germ line clones of *AMPK* γ^{D39} were generated using the autosomal FLP-DFS technique. In detail, 82AFRT *AMPK* γ^{D39}/TM6B females were crossed with *yw hs*-FLP/Y;; 82AFRT P[w^{+}, ovo^{D1}] males. Their progeny larvae were heat-shocked for 2 hr at 37°C at the first instar larval stage. *yw hs*-FLP;; 82AFRT P[w^{+}, ovo^{D1}]/82AFRT *AMPK* γ^{D39} females (3 day-old) were selected and crossed with *AMPK* γ^{D39}/TM3 GFP males to obtain *AMPK* γ^{D39} null embryos. To produce *AMPK* γ null embryos expressing *AMPK* γ, *yw hs*-FLP;; 82AFRT P[w^{+}, ovo^{D1}]/82AFRT *AMPK* γ^{D39} females were crossed with *hs-Gal4 UAS-AMPK* γ */CyO Act-GFP*; *AMPK* γ^{D39}/TM3 GFP males. For expression of *UAS-AMPK* γ in *AMPK* γ null embryos, eggs were collected and aged at 30°C.

Cuticle preparation

For the cuticle preparations, embryos were collected and dechorinated as previously described [10]. Dechorinated embryos were immersed in a solution containing acetic acid and glycerol at a 3:1 ratio and incubated overnight at 65°C. Embryos were then mounted in Hoyer's medium and incubated 24 hr at 65°C.

Immunostaining

I used anti-phospho MRLC (1:50, Cell Signaling Technology, Danvers, MA, USA), anti-aPKC (1:1,000, Santa Cruz Biotechnology, Santa Cruz, CA, USA), and anti-Discs large (4F3, 1:200, DSHB, Iowa City, IA, USA) antibodies as primary antibodies. Texas red and fluorescein isothiocyanate (FITC)-conjugated secondary antibodies (Molecular Probes, Eugene, OR, USA) were used at a 1:200 dilution. DNA was visualized by DAPI (Sigma, St. Louis, MO, USA). *Drosophila* tissues were fixed in 4% formaldehyde for 5 min. After the standard immunostaining procedures [10], tissues were observed with a laser scanning confocal microscope LSM700 (Carl Zeiss, Göttingen, Germany).

Results and Discussion

Drosophila AMPK γ subunit is highly homologous to its mammalian counterparts and *Saccharomyces cerevisiae* SNF4, especially in its CBS domains [17]. *Drosophila* has 6 AMPK γ subunit isoforms encoded by a single gene (17, Fig. 1), but the null mutant which nullified the expression of all

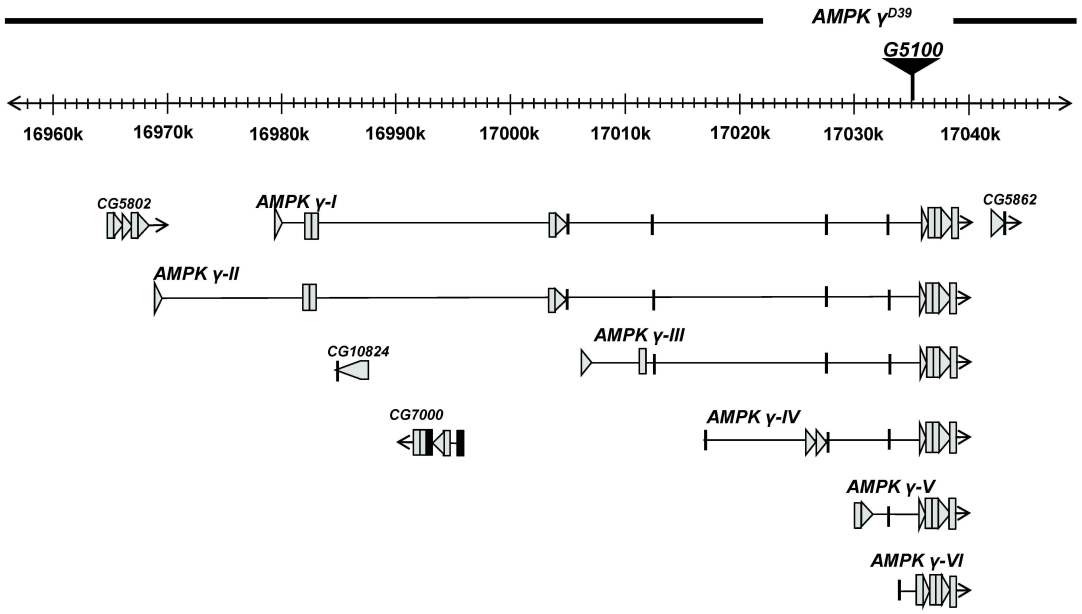

Fig. 1. Genomic map of *AMPK* γ. P-element insertion (triangle), exons (rectangles and arrow heads) and introns (lines) are shown. *AMPK* γ^{D39} contains an about 17 kb deletion encoding whole CBS domains.

AMPK γ isoforms was not available. From an extensive searching of the GenExel library (~20,000 independent EP lines), we isolated *AMPK γ* ^{G5100} (*G5100*), an EP line with a P-element insertion near exons encoding the CBS domains shared by all AMPK γ subunit isoforms (Fig. 1). Subsequently, I have generated an *AMPK γ* deficient line, *AMPK γ* ^{D39} by imprecise excision of the P-element from *G5100*. PCR-based molecular analyses demonstrated that the exons containing the CBS domains were totally deleted in this mutant (Fig. 1). RT-PCR clearly demonstrated that *AMPK γ* ^{D39} is a genuine null allele (data not shown).

This null mutant displayed a larval lethality, demonstrating that *AMPK γ* is essential to complete development. Then, I investigated role of AMPK γ in early development by generating germ line clones (GLC) of *AMPK γ* null mutants to eliminate the maternal effect. Interestingly, *AMPK γ* ^{D39} null embryos completely failed to hatch, demonstrating that *AMPK γ* is indispensable for the completion of embryogenesis. Extensive examination of *AMPK γ* mutant embryos revealed almost complete loss of the cuticle structure (Fig. 2).

Because the structure of embryonic cuticle highly reflects the organization of underlying epidermis that secretes it, I supposed that the epithelial cell structures of *AMPK γ* mutant embryos would be also severely impaired. Wild-type *Drosophila* embryonic epithelia contain two distinct membrane domains-an apically localized cell-cell adhesive junction known as zonula adherens (ZA) and a more basal junctional complex known as septate junction (SJ) [9]. However, in *AMPK γ* mutant embryos, localization of atypical PKC (aPKC), a component of the apical complex which regulates the formation of ZA [9], was found severely disrupted (Fig. 3). Discs-large (Dlg), normally localizing at or below SJ [9], was also mislocalized in *AMPK γ* mutant embryos (Fig. 3). When *AMPK γ* was re-introduced in *AMPK γ* mutants using GAL4-UAS system, the defected epithelial structures and mislocalized polarity determinants were successfully restored (Fig. 3). These results strongly supported that *AMPK γ* is critical for maintaining epithelial structures in *Drosophila* development.

In previous reports, extensive biochemical and genetic analyses demonstrated that AMPK regulates cell polarity by phosphorylating myosin regulatory light chain (MRLC; also known as MLC2), a critical molecule for cell polarity establishment [3,7,10,15]. The regulatory phosphorylation site of MRLC is directly phosphorylated by activated AMPK *in vitro* and *in vivo* [10]. After this phosphorylation, MRLC induces the actin cytoskeleton structural change which has a critical role in the regulation of cell polarity [10]. To test the role of *AMPK γ* in *in vivo* MRLC phosphorylation, *AMPK γ* mutant embryos were stained with phospho-specific MRLC antibodies. Although phosphorylated MRLC was specifically localized to apical region of wild-type epithelia, the deletion of *AMPK γ* almost completely suppressed MRLC phosphorylation (Fig. 4). Moreover, over-expression of *AMPK γ* completely restored MRLC phosphorylation in the *AMPK γ* null epithelia (Fig. 4). Collectively, these data demonstrated that

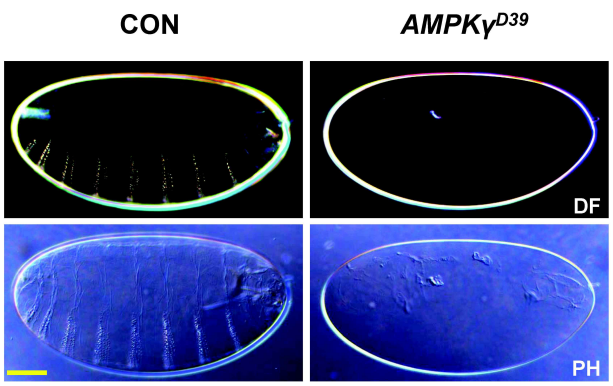

Fig. 2. Cuticle formation defects in *AMPK γ* null embryos. Wild type (Con) and *AMPK γ* null (*AMPK γ* ^{D39}) embryo cuticles were analyzed by dark field (DF) and phase contrast (PH) microscopy. Yellow scale bar: 50 μm.

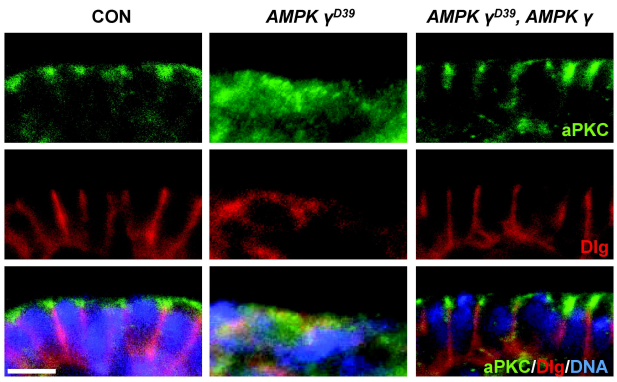

Fig. 3. AMPK γ is required for maintaining epithelial cell polarity. Epithelia of wild type (Con), *AMPK γ* null (*AMPK γ* ^{D39}), and *AMPK γ* null expressing *AMPK γ* (*AMPK γ* ^{D39}, *AMPK γ*) were stained with anti-aPKC antibody (aPKC, green), anti-Dlg antibody (Dlg, red) and DAPI (DNA, blue). White scale bar: 5 μm.

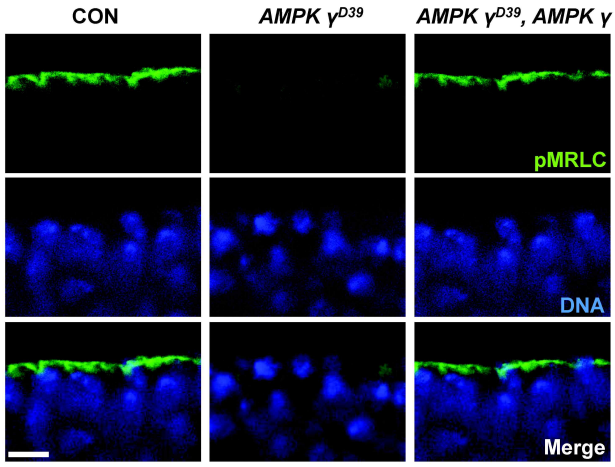

Fig. 4. Loss of MRLC phosphorylation in *AMPK γ* null epithelia. Wild type (Con), *AMPK γ* null (*AMPK γ* ^{D39}), and *AMPK γ* null expressing *AMPK γ* (*AMPK γ* ^{D39}, *AMPK γ*) embryo epithelia were stained with anti-phospho MRLC antibody (pMRLC, green) and DAPI (DNA, blue). White scale bar: 5 μm.

AMPK γ is essential for *in vivo* MRLC phosphorylation, suggesting the critical role of AMPK γ in AMPK-mediated cell polarity regulation.

In genetic analyses during decades, mutations in *AMPK γ* isoforms induce various symptoms in various animals. An autosomal dominant mutation in *AMPK γ 3* induces a dramatic increase in skeletal muscle glycogen content in pigs [11]. After this discovery, several groups identified AMPK γ 2 gene mutations associated with familial cardiac hypertrophy [1,5]. The most patients with these γ 2 mutations also demonstrated severe defects in electrical conductance, similar to the conduction abnormalities observed in Wolff-Parkinson-White syndrome [1,5]. In addition, a deletion of first exon of an AMPK γ isoform induced progressive neurodegeneration and neuronal cell death in *Drosophila* [17]. Because loss of cell polarity is strongly correlated with more aggressive and invasive growth of malignant cells [16], the cell polarity controlling roles of AMPK γ suggest that AMPK γ mediates the tumor suppressing function. A small scale case study shows that metformin, an AMPK activating anti-diabetic drug, reduces the risk of cancer in diabetic patients [4]. Moreover, metformin suppresses carcinogen-induced cancers in hamsters [14]. These data support the tumor suppressing role of AMPK, and raise the possibility that metformin and other AMPK activating agents can be used for the treatment of AMPK-related cancers. Collectively, the *AMPK γ* mutant and *AMPK γ* transgenic models generated in this study will provide valuable tools and insights into investigating various AMPK γ-related diseases and abnormalities.

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초록 : AMPK γ 유전자의 표피세포극성 유지기능 규명

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AMPK는 catalytic α subunit과 regulatory β 및 γ subunit으로 구성된 인산화 효소로, 그 동안 생체 내 중요 대사 조절자로써 연구되어 왔으나, 최근 유전학 연구를 통해 지금까지 밝혀지지 아니한 새로운 생체기능을 가짐이 밝혀졌다. 본 연구에서 초파리 유전학 기법을 활용하여 AMPK γ subunit 유전자가 결손된 모델 초파리를 제작 하여 연구한 결과, AMPK γ 유전자 결손 시 초파리 embryo의 표피형성이 심각하게 저해됨을 발견하였고, 조직학적 실험을 통해 표피세포의 극성이 AMPK γ 유전자 결손 초파리에서 손상되어 있음을 확인하였다. 또한 세포극성을 조절하는 중요 분자인 MRLC의 인산화 또한 AMPK γ 유전자 결손 시 저해되었으며, AMPK γ 유전자 재도입 시 MRLC인산화와 표피세포의 극성이 모두 회복됨이 확인되어, 초파리 표피세포의 극성유지에 AMPK γ 유전자가 필수적 임을 확인하였다.