LKB1 and AMPK maintain epithelial cell polarity under energetic stress

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LKB1 is mutated in both familial and spontaneous tumors, and acts as a master kinase that activates the PAR-1 polarity kinase and the adenosine 5’-monophosphate-activated kinase (AMPK). This has led to the hypothesis that LKB1 acts as a tumor suppressor because it is required to maintain cell polarity and growth control through PAR-1 and AMPK, respectively. However, the genetic analysis of LKB1–AMPK signaling in vertebrates has been complicated by the existence of multiple redundant AMPK subunits. We describe the identification of mutations in the single Drosophila melanogaster AMPK catalytic subunit AMPKα. Surprisingly, ampkα mutant epithelial cells lose their polarity and overproliferate under energetic stress. LKB1 is required in vivo for AMPK activation, and lkb1 mutations cause similar energetic stress–dependent phenotypes to ampkα mutations. Furthermore, lkb1 phenotypes are rescued by a phosphomimetic version of AMPKα. LKB1 signals through AMPK to coordinate epithelial polarity and proliferation with cellular energetic status. This might underlie the tumor suppressor function of LKB1.

Results and discussion

AMPK contains three protein subunits, α, β, and γ, which form a heterotrimer. The α subunit (AMPKα) encodes a highly conserved serine/threonine kinase, and the other subunits are regulatory. From a D. melanogaster forward genetic screen for mutants affecting larval neuronal dendrite development (Medina et al., 2006), we identified several lethal mutations in AMPKα. The ethylmethanesulfonate mutants, ampkα1 and ampkα2, contain a
AMPK has been shown to play important roles in the regulation of energy metabolism and cell polarity. In Drosophila melanogaster, AMPK mutations result in a phenotype where cells round up and lose their apical–basal polarity, which is consistent with the absence of an actin phenotype (Fig. 2 A). Under energetic stress conditions, however, ampka+ mutant cells show a more severe phenotype, in which the cells round up and lose their cortical localization, as do the lateral and apical domains. Apical markers, such as atypical PKC (aPKC) and Crumbs (Crb), lose their cortical localization. A considerable proportion of ampka+ mutant clones show a more severe phenotype, in which the cells round up and lose their apical–basal polarity, which is consistent with the absence of an actin phenotype (Fig. 2 B). This indicates that Baz is not in a complex with aPKC in columnar follicle cells, but is instead associated with the adherens junctions, as has recently been described in the D. melanogaster embryo and in neuroepithelial cells of the Zebrafish neural tube (Harris and Peifer, 2005; Afonso and Henrique, 2006).

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oxidase subunit; therefore, mutants have reduced intracellular ATP concentrations to levels sufficient to maintain cell survival and growth, but not cell division (Mandal et al., 2005). This cell cycle block is believed to require AMPK activation. In agreement with a role for Tend in cell cycle progression, we did not see clones bigger than four to six cells under energetic starvation conditions (Fig. 2 D). In contrast to \textit{ampk} mutant cells, however, \textit{tend} mutant cells showed no polarity defects, ruling out the possibility that the \textit{ampk} phenotype is a secondary effect of low ATP levels. We also tested the effect of specific nutrient starvation by feeding flies only glucose, but these conditions did not induce any polarity phenotypes in \textit{ampk} mutant cells (Fig. 2 E). Thus, AMPK is specifically required to maintain epithelial polarity and growth control under conditions of energetic stress.

Because our results indicate that \textit{ampk} plays a role in epithelial polarity, we assessed whether the localization of the protein itself is polarized. We also examined LKB1 localization, as it is a potential regulator of AMPK. Transgenic wild-type fusion proteins for both AMPK and LKB1 rescue lethal null mutants to viability, and should therefore mimic the localizations of the endogenous proteins. LKB1-GFP is mainly found at the apical and lateral cortex of the follicle cells, and is absent from the basal domain (Fig. 3 A). This basal exclusion is surprising, as cortical localization of LKB1 requires its membrane targeting by prenylation of a conserved CAAX motif (Martin and St Johnston, 2003). This suggests that the lipid composition of the basal domain is different from the rest of the plasma membrane and/or that LKB1 posttranslational modifications are asymmetrically controlled. In contrast, mCherry-AMPK\(\alpha\) does not show any enrichment or asymmetric localization at the plasma membrane, and it is found distributed throughout the...
cytoplasm, but absent from the nucleus (Fig. 3 A). The localization of LKB1 suggests that AMPK could be activated specifically at the apical and lateral cortices of the cells. To test this hypothesis, we used an antibody against the LKB1 phosphorylation site of AMPK (phospho-T184). The immunostaining is reduced to background levels in both ampka and lkb1 mutant clones. This confirms the specificity of the antibody and indicates that LKB1 is the principle AMPK kinase in these cells (Fig. 3 B). In wild-type cells, PhosphoT184-AMPK is found diffusely in the cytoplasm (Fig. 3 B). The effect of AMPK on apical–basal polarity is therefore not related to a polarized distribution of the kinase or its localized activation by LKB1.

Because LKB1 activates AMPK, we wondered if similar phenotypes could be observed in lkb1 mutant cells. lkb1 clones can lead to severe polarity defects in follicle cells in normally fed flies (Martin and St Johnston, 2003). However, these defects are observed only in large clones that are induced in the stem cells that give rise to the follicular epithelium, whereas small lkb1 mutant clones, which are induced after the formation of the epithelium, have no effect on follicle cell polarity or the organization of the actin cytoskeleton (n = 24; Fig. 4 A). This suggests that LKB1 is required for the establishment of epithelial polarity in well-fed flies, but not for its maintenance, as is the case for PAR-1 (Doerflinger et al., 2003). In contrast, under conditions of glucose starvation, small lkb1 clones that were induced after the formation of the follicular epithelium show a fully penetrant polarity phenotype (100%; n = 21). Under these conditions, we observed a loss of the polarized localization of Dlg, aPKC, Crb, and Cora (Fig. 4 A). However, Baz distribution is usually not affected by lkb1 loss of function (unpublished data). Dg extends laterally and occasionally localizes to the apical domain (Fig. 4 A). The actin cytoskeleton is also disturbed, with more F-actin apically and a decreased density of stress fibers on the basal side. Finally, large lkb1 clones lose their epithelial organization completely and overproliferate to form small neoplasms (Fig. 4, B and C). Thus, lkb1 mutant cells exhibit identical phenotypes to ampka mutant cells under low-energy conditions.

Because lkb1 and ampka mutant clones lead to very similar polarity defects and LKB1 phosphorylates AMPKα, we wondered if a constitutively active form of AMPKα could rescue the lkb1 phenotype. Therefore, we generated transgenic lines carrying a UAS-AMPKα construct, in which Threonine184 is replaced by an aspartate, which should mimic the activating phosphorylation of AMPKα by LKB1 (Lizcano et al., 2004). The expression of UAS-AMPKα-T184D in lkb1 mutant clones fully rescues their starvation-dependent polarity and overproliferation phenotype (n = 37), whereas the Gal4 driver alone has no effect (Fig. 4 A). Furthermore, AMPKα-T184D–expressing mutant clones also have a normal actin cytoskeleton (100%; n = 20; Fig. 5). Thus, the phosphomimetic version of AMPKα completely rescues the lkb1 mutant phenotype under conditions of energetic stress.

The recovery of null mutations in ampka has allowed the first in vivo analysis of AMPK function in a multicellular organism, which has revealed an unexpected role for the kinase in the maintenance of epithelial polarity, but only under conditions of energetic stress. This implies that at least one of the pathways that normally maintain cell polarity cannot function when cellular energy levels are too low, and that AMPK activation compensates for this defect.

A surprising feature of the ampka polarity phenotype is that it has opposite effects on the actin cytoskeleton and the cortical polarity cues. In mildly affected clones, basal actin is strongly reduced, with a corresponding increase in the amount of apical actin. In contrast, mutant clones show an expansion of the basal markers into the lateral and apical regions, as well as a loss of lateral and apical markers. Thus, the effects on actin may be independent of other polarity defects, suggesting that AMPK acts through different pathways to regulate actin and cortical polarity in opposite ways.

It is unclear how AMPK regulates the actin cytoskeleton, but it is possible that it acts on only one side of the cell and that the reciprocal changes on the other are caused by a change in the concentration of free G-actin or an actin nucleator, as has been shown for abl mutants during cellularization (Grevengoed et al., 2003). For example, loss of AMPK could increase actin polymerization apically, thereby depleting the pool of free actin that can polymerize basally. Alternatively, ampka mutants may
prevent the formation of basal actin stress fibers, and thus increase the concentration of free actin, which enhances apical actin polymerization.

The cortical polarity defects of ampkα mutant clones also suggest a reciprocal relationship between the basal and apical/lateral membrane domains because the basal domain, marked by Dg, is dramatically expanded, whereas the determinants for the lateral domain (Dlg) and the apical domain (aPKC and Crb) disappear from the cortex. This suggests that there is some form of mutual antagonism between the basal and lateral domains that maintains a sharp boundary between them, as has been described for apical and lateral domains through the inhibition of phosphorylation of Baz (PAR-3) by lateral PAR-1, and of Dlg by apical aPKC (Benton and St Johnston, 2003; Goode et al., 2005). Loss of this repression might provide an alternative explanation for the overgrowth of ampkα mutant clones.

Although the molecular pathways involved remain to be elucidated, our results demonstrate that ampkα mutant cells lose their polarity under low-energy conditions and overproliferate to give rise to tumorous phenotypes. The activation of AMPK depends on its phosphorylation, and the tumor suppressor function of LKB1, and loss of LKB1 produces a similar loss of apical and lateral markers. Thus, the novel functions of AMPK revealed in this work may provide a basis for the tumor suppressor function of LKB1.

**Construction of AMPKα transgenes**

The wild-type AMPKα transgene was cloned into the pUAST vector (Brand and Perrimon, 1993) as an EcoRI-BglII fragment of an EST, corresponding to an AMPKα-RA transcript (www.flybase.org). The mCherry-AMPKα fusion protein was made using a mCherry construct provided by R. Tsien, University of California, San Diego, San Diego, CA at the N terminus fused in-frame to AMPKα into the pUAST vector. The UAS-mCherry-AMPKα transgene rescues viability and fertility when expressed by Ubiquitin-Gal4 in either ampkα or ampkα mutants. The phosphomimetic activated form of AMPKα (AMPKα T184D) was made by PCR amplification of mouse genomic DNA, and the gene product (AMPKα; CG3051; NM_057965) was discovered that had mutations in all three alleles.

**Fly stocks and crosses**

AMPKα alleles were recombined with FRT101 for mitotic recombination. Other mutant stocks used were FRT82B, lkb14A4-2 and FRT82B, tend. UAS:AMPKα and UAS:GFP:UAS:AMPKα T184D were expressed in all cells using the Cy2-Gal4 driver. Flip-out experiments were performed by crossing FRT82B, lkb14A4-2 and FRT82B, tend. UAS:AMPKα and UAS:AMPKα T184D to y, w, hs:flp; tub-FRT101,FRT101,FRT101, UAS:GAL4; UAS:GAL4 and heat-shocking pupae. For rescue experiments, two independent stocks were established and crossed together: UAS:AMPKα T184D and UAS:AMPKα and END; FRT82B, UbilnsGFP and y, w, hs:flp; da:Gal4, FRT82B, lkb14A4-2/FRT101,TM3, Scb.

**Starvation conditions and clone induction**

Adult flies were placed in vials containing “normal” D. melanogaster food media (5% glucose, 5% yeast extract, 3.5% wheat flour, and agar 0.8%), energetic starvation medium (1% yeast extract, 3.5% wheat flour, and agar 0.8%).
or specific nutrient-starvation medium (5% glucose and agar 0.8%). Clones were induced by heat-shocking adult females at 37°C for 2 h on two consecutive days. Females were dissected 2 d after the last heat shock.

Staining and imaging procedures

Immunofluorescence on ovaries was performed using standard procedures. Primary antibodies were used as follows: rat anti-DEC1 (1:1,000; Oda et al., 1994); mouse anti-Crb (1:50; Developmental Studies Hybridoma Bank); Guinea pig anti-Cora (1:2,000; Fehon et al., 1994); rabbit anti-aPKC (1:500; Sigma-Aldrich); rat anti-Baz (1:500; Wodarz et al., 1999), mouse anti-Dig (1:50; Developmental Studies Hybridoma Bank); rabbit anti-Og (1:1,000; Deng et al., 2003); and rabbit anti-phosphoT385-AMPK (1:100; Cell Signaling Technology). Actin staining was performed with a 0.1% (wt/vol) Triton X-100 (Sigma) in 0.1 M (vol/vol) buffered glycerol (Sigma-Aldrich) with a 2-μm optical slice and LSM Imaging software (Carl Zeiss Microimaging, Inc.). Images were resized and cropped with Photoshop (Adobe), and imported into Illustrator (Adobe) for labels and arrangement.

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