JNK pathway mediates apoptotic cell death induced by tumor suppressor LKB1 in *Drosophila*

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Abstract

Although recent progresses have unveiled the diverse *in vivo* functions of LKB1, detailed molecular mechanisms governing these processes still remain enigmatic. Here, we showed that *Drosophila* LKB1 negatively regulates organ growth by caspase-dependent apoptosis, without affecting cell size and cell cycle progression. Through genetic screening for LKB1 modifiers, we discovered the JNK pathway as a novel component of LKB1 signaling; the JNK pathway was activated by LKB1 and mediated the LKB1-dependent apoptosis. Consistently, *LKB1*-null mutant was defective in embryonic apoptosis and displayed a drastic hyperplasia in the central nervous system; these phenotypes were fully rescued by ectopic JNK activation as well as wild-type LKB1 expression. Furthermore, inhibition of LKB1 resulted in epithelial morphogenesis failure, which was associated with a decrease in JNK activity. Collectively, our studies unprecedentedly elucidate JNK as the downstream mediator of the LKB1-dependent apoptosis, and provide a new paradigm for understanding the diverse LKB1 functions *in vivo*.
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Abbreviations: JNK, c-Jun N-terminal kinase; PJS, Peutz–Jegher syndrome; TSC, tuberous sclerosis complex

Introduction

Tumor suppressor LKB1 is a serine/threonine kinase that is heterozygotically mutated in the germ line of Peutz–Jegher syndrome (PJS) patients.[1,2] PJS patients have a highly increased risk of malignant tumors, especially in the gastro-intestinal tract, breast, uterine cervix, and ovary.[3–5] Many of these tumors acquire somatic mutations in the remaining wild-type allele of *LKB1*.[6,7] Moreover, various sporadic cancers are also associated with the loss of LKB1, implicating the general role of LKB1 in tumor suppression.[8–10] As point mutations of LKB1 found in PJS mostly reside in the highly conserved kinase domain, it is very likely that the kinase activity of LKB1 is essential for its tumor suppressive function.[11,12]

Since the discovery of LKB1 in 1998, various functions of LKB1 molecule were suggested to be responsible for its tumor-suppressing activity — such as inhibition of cell cycle progression, cell growth retardation, apoptotic cell death, and cell polarity control.[11,13] The LKB1 protein was originally characterized as a cell cycle inhibitor, and its putative downstream targets such as Brg1 and p21 were suggested to mediate LKB1-dependent cell cycle arrest.[14–16] It has been recently proposed that LKB1 also regulates cellular growth by controlling another tumor suppressor tuberous sclerosis complex (TSC) via the AMP kinase (AMPK)-dependent pathway.[17,18] In addition, studies showing the absence of apoptosis in PJS polyps revealed that LKB1 is an inducer of apoptosis *in vivo*.[19] Another tumor suppressor p53, the first identified *in vitro* substrate of LKB1, was proposed as a mediator of this apoptosis.[19,20] Finally, LKB1 was shown to be necessary for the polarization of intestinal epithelial cells and *Drosophila* ovary cells, demonstrating another important function of LKB1 in regulating cell structure.[21,22]

Although considerable progress has been made to characterize the *in vivo* function of LKB1, only a limited amount of information concerning its molecular mechanisms has been found in detail; the major biological pathway responsible for the tumor suppressive function of LKB1 remains to be clarified.

The c-Jun N-terminal kinase (JNK) is a member of the mitogen-activated protein kinase (MAPK) family that mediates various cellular responses including programmed cell death, epithelial sheet movement, and planar polarity.[23] The activity of JNK is tightly regulated by reversible phosphorylation, which is stimulated by a sequential cascade of protein kinases and inhibited by JNK-specific phosphatases. Activation of the JNK pathway is required for the release of cytochrome *c* from the mitochondria and the subsequent activation of the caspase cascade.[24] Therefore, abrogation of the JNK signaling pathway causes various defects in developmental or stress-induced apoptosis.[25,26] In addition to controlling apoptosis, regulation of epithelial morphogenesis is another well-known function of the JNK signaling pathway.[27] Depletion of JNK activity results in various epithelial defects, including those of dorsal closure and planar polarity in *Drosophila*, and of the optic and neuronal system in mice.[28–32]

Since JNK is required for apoptosis and epithelial organization, disruption of the JNK signaling pathway is thought to be a prerequisite of tumorigenesis by enabling tumor cells to evade programmed cell death and to acquire a metastatic potential.[33] Indeed, *JNK1* and *JNK2* are both required for the suppression of oncogenic transformation and tumorigenesis.[34] Loss of *JNK3*, which is selectively expressed in neuronal cells, is also closely associated with human brain tumors.[35] Furthermore, the *Mkk4* gene, which encodes an

upstream kinase of JNK, has been identified as a tumor suppressor gene and a metastasis suppressor gene.[36,37]

In this paper, we are the first to show a genetic connection between tumor suppressor LKB1 and the JNK signaling pathway. Our results indicate that LKB1 activates the JNK pathway *in vivo* and that the JNK pathway mediates the LKB1-dependent apoptotic cell death.

Results

LKB1 negatively regulates compartment size

Using various Gal4 drivers, we were able to achieve a high-level expression of both wild-type and kinase-dead LKB1 in the specific region of various tissues: the posterior region of the eye imaginal disc by the *glass_multiple_reporter*-Gal4 driver (*gmr*-Gal4; Figure 1a, b), the dorsal region of the wing disc by the *apterous*-Gal4 driver (*ap*-Gal4; Figure 1c, d), and the central pouch region of the wing disc by the *ms1096*-Gal4 driver (Figure 1e, f). The expression levels of the LKB1 protein were determined by immunostaining using *Drosophila* LKB1-specific antisera. Eye-specific expression of wild-type LKB1 by the *gmr*-Gal4 driver induced a slight reduction in overall eye size (Figure 1g, h), and this reduction became severer when

the LKB1 expression level was elevated (Figure 1j). However, the kinase-dead LKB1 overexpression did not induce this specific phenotype (Figure 1i), showing that the kinase activity of LKB1 is important in inducing tissue-size reduction. The dorsal region-specific expression of wild-type and kinase-dead LKB1 by the *ap*-Gal4 driver displayed more dramatic phenotypes. As *Drosophila* wing discs are composed of two layers of tissues, dorsal and ventral, alterations in size of only the dorsal layer give rise to bent-up or bent-down wing phenotypes.[38] Although the dorsal tissue-specific expression of kinase-dead LKB1 did not induce any alterations (Figure 1k, m), wild-type LKB1 induced a dramatic bent-up wing phenotype (Figure 1l, n) in a dose-dependent manner. Consistently, the *ms1096*-Gal4-driven expression of wild-type LKB1 dose-dependently reduced the overall wing size (Figure 1o, p, r), although kinase-dead LKB1 did not (Figure 1q). Therefore, we concluded that *Drosophila* LKB1 negatively regulates the tissue and organ size in a kinase activity-dependent manner.

LKB1 reduces clone size but not cell size

To examine the effect of LKB1 in individual cells, we performed clonal analyses by inducing LKB1 overexpression
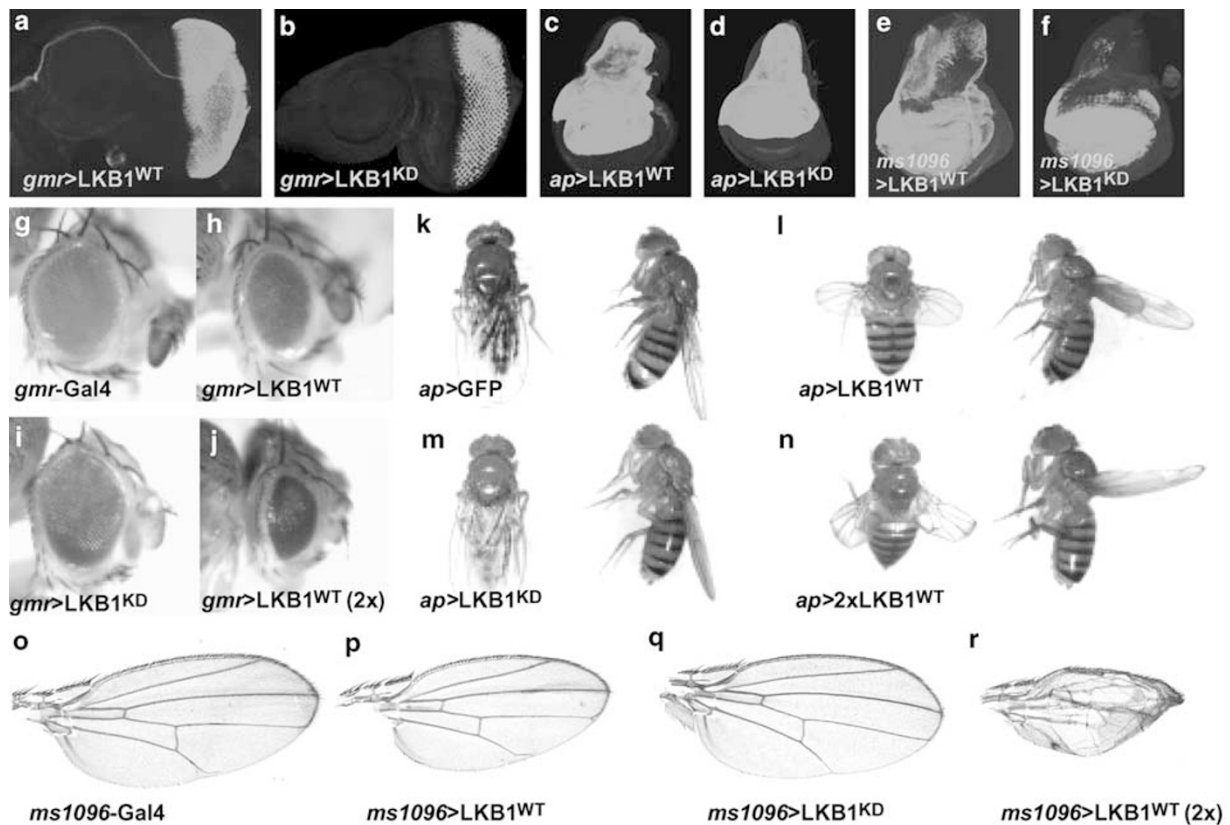
Figure 1 LKB1 reduces organ size in a kinase activity-dependent manner. (**a–f**) Tissue-specific expression of *Drosophila* LKB1. *gmr*-Gal4- (**a**, **b**), *ap*-Gal4- (**c**, **d**), or *ms1096*-Gal4- (**e**, **f**) driven expression of wild-type LKB1 (LKB1^WT) (**a**, **c**, **e**) and kinase-dead LKB1 (LKB1^KD) (**b**, **d**, **f**) was visualized using anti-LKB1 antisera. No specific signals were observed in wild-type imaginal discs (data not shown). (**g–j**) Adult fly eyes expressing LKB1 transgenes under the control of *gmr*-Gal4. Genotypes are: *gmr*-Gal4/ + (**g**), *gmr*-Gal4/UAS-LKB1^WT (**h**), *gmr*-Gal4/UAS-LKB1^KD (**i**), and *gmr*-Gal4 UAS-LKB1^WT/*gmr*-Gal4 UAS-LKB1^WT (**j**). (**k–n**) Dorsal (left) and lateral (right) views of flies expressing transgenes under the control of *ap*-Gal4. Genotypes are: *ap*-Gal4/UAS-GFP (**k**), *ap*-Gal4/UAS-LKB1^WT (**l**), *ap*-Gal4/UAS-LKB1^KD (**m**), and *ap*-Gal4 UAS-LKB1^WT/UAS-LKB1^WT (**n**). (**o–r**) Adult fly wings expressing transgenes under the control of *ms1096*-Gal4. Genotypes are: *ms1096*-Gal4/Y (**o**), *ms1096*-Gal4 UAS-LKB1^WT/Y (**p**), *ms1096*-Gal4/Y; UAS-LKB1^KD/ + (**q**), and *ms1096*-Gal4 UAS-LKB1^WT/*ms1096*-Gal4 UAS-LKB1^WT (**r**)

in a specific subset of cells within wing imaginal discs. Compared to the control clones expressing only GFP (Figure 2a, d), the LKB1-expressing clones expanded very poorly (Figure 2b, e). Similarly, overexpression of the TSC complex, another well-known tumor suppressor, also decreased the clone size, although with a milder severity than the over-expression of LKB1 (Figure 2c, f).[39]

Recent mammalian studies suggested that LKB1 nega-tively regulates TOR signaling via the AMPK-TSC path-way.[17,18] Since reduction in cell size is a typical outcome of TSC overexpression, we attempted to test whether the LKB1 overexpression phenotype results from cell size reduc-tion.[39,40] As expected, the overexpression clones of TSC (Figure 2i) displayed a dramatic reduction in wing marginal bristle size. However, surprisingly, although LKB1 reduced clone size more severely than did TSC, LKB1 expression did not cause any alterations in the bristle size (Figure 2g, h). Consistently, in contrast to TSC (Figure 2j, l), LKB1 again failed to reduce the bristle size when expressed by the *ms1096*-Gal4 driver (Figure 2j, k). Furthermore, LKB1 over-expression in the clones of endoreplicating tissues, such as the salivary gland and fat body, altered neither the cell size nor the DNA content (data not shown), confirming that the tumor-suppressing activity of LKB1 is unrelated to the negative

regulation of cell growth control. Therefore, we concluded there to be no connection between LKB1 and TSC in the context of cell growth regulation in *Drosophila*.

LKB1 induces apoptotic cell death but not cell cycle arrest

Since the LKB1-dependent reduction of tissue size appro-priately reflected the tumor-suppressing activity of LKB1, we assessed whether this tissue-size reduction is caused by cell cycle arrest or apoptotic cell death, both of which have been proposed as major cellular processes underlying the tumor-suppressing function of LKB1.[11] To check the G1–S cell cycle progression of LKB1-expressing tissues, we performed the bromodeoxyuridine (BrdU) incorporation assay, which speci-fically labels S-phase cells. Although LKB1 was highly expressed in these tissues (Figure 1), no significant altera-tions in the S-phase cell number were detected (Figure 3b, d and e) with respect to the control tissues (Figure 3a, c). Similarly, there was no change in the antiphosphospecific histone 3 (PH3) antibody staining pattern between the wild-type (Figure 3f and h) and the LKB1-expressing tissues (Figure 3g, i and j), demonstrating that G2–M cell cycle transition was also unaffected. However, when the equivalent
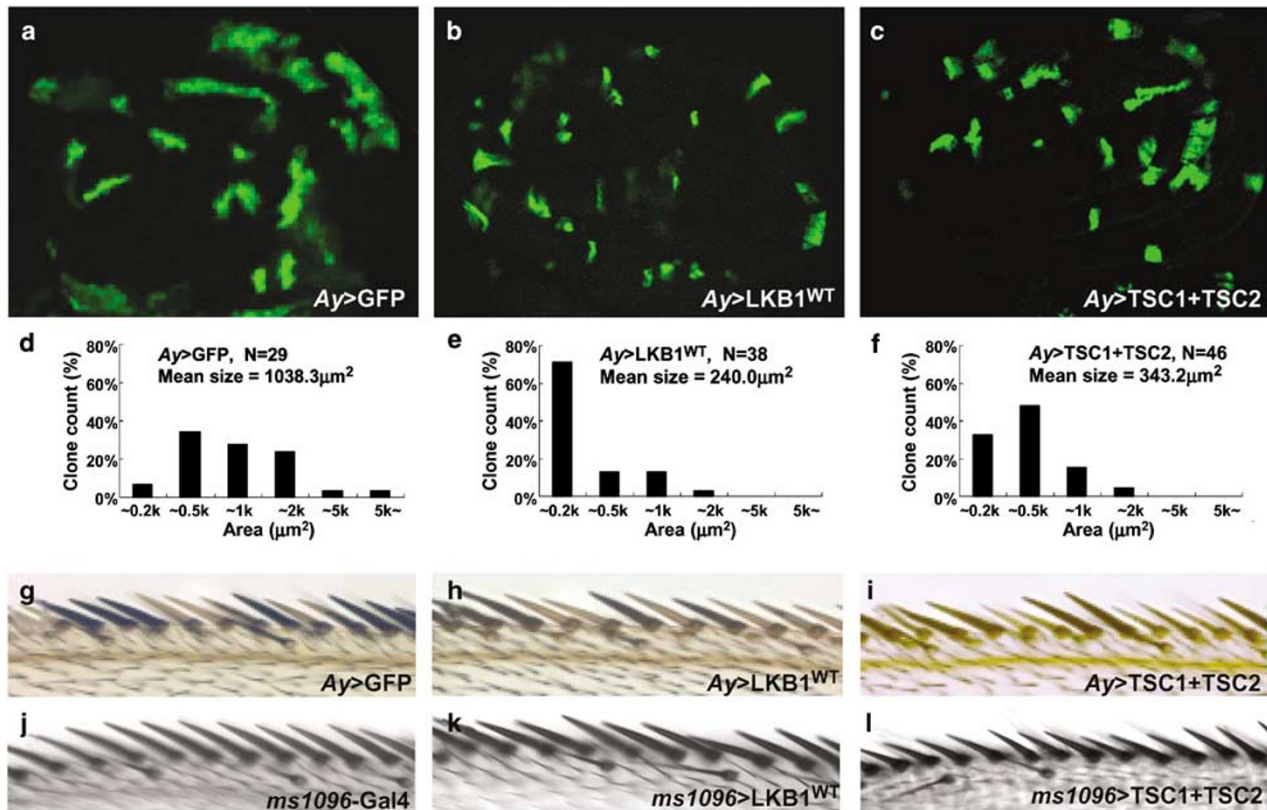
Figure 2 LKB1 reduces clone size without affecting cell size. (**a–c**) Flipped-out clones expressing either GFP alone (**a**); GFP and LKB1 (**b**); and GFP, TSC1, and TSC2 (**c**) were photographed 70 h after clone induction. (**d–f**) Distributions of clonal areas ($k = 1000~\mu m^2$) in flipped-out clones expressing GFP alone (**d**); GFP and LKB1 (**e**); and GFP, TSC1, and TSC2 (**f**) were presented as bar graphs. Numbers of clones examined and means of clone sizes are indicated in the graphs. (**g–i**) Wing marginal bristle sizes were examined in *yellow*-negative clones expressing GFP alone (**g**); GFP and LKB1 (**h**); and GFP, TSC1, and TSC2 (**i**). (**j–l**) Wing marginal bristles expressing transgenes under the control of *ms1096*-Gal4. Genotypes: *y w hs*-FLP/ + ; *Ay*-Gal4 UAS-GFP/ + (**a, d, g**), *y w hs*-FLP/ + ; *Ay*-Gal4 UAS-GFP/UAS-LKB1[WT] (**b, e, h**), *y w hs*-FLP/ + ; *Ay*-Gal4 UAS-GFP/UAS-TSC1 UAS-TSC2 (**c, f, i**), *ms1096*-Gal4/Y (**j**), *ms1096*-Gal4 UAS-LKB1[WT]/Y (**k**), and *ms1096*-Gal4/Y; UAS-TSC1 UAS-TSC2/ + (**l**)
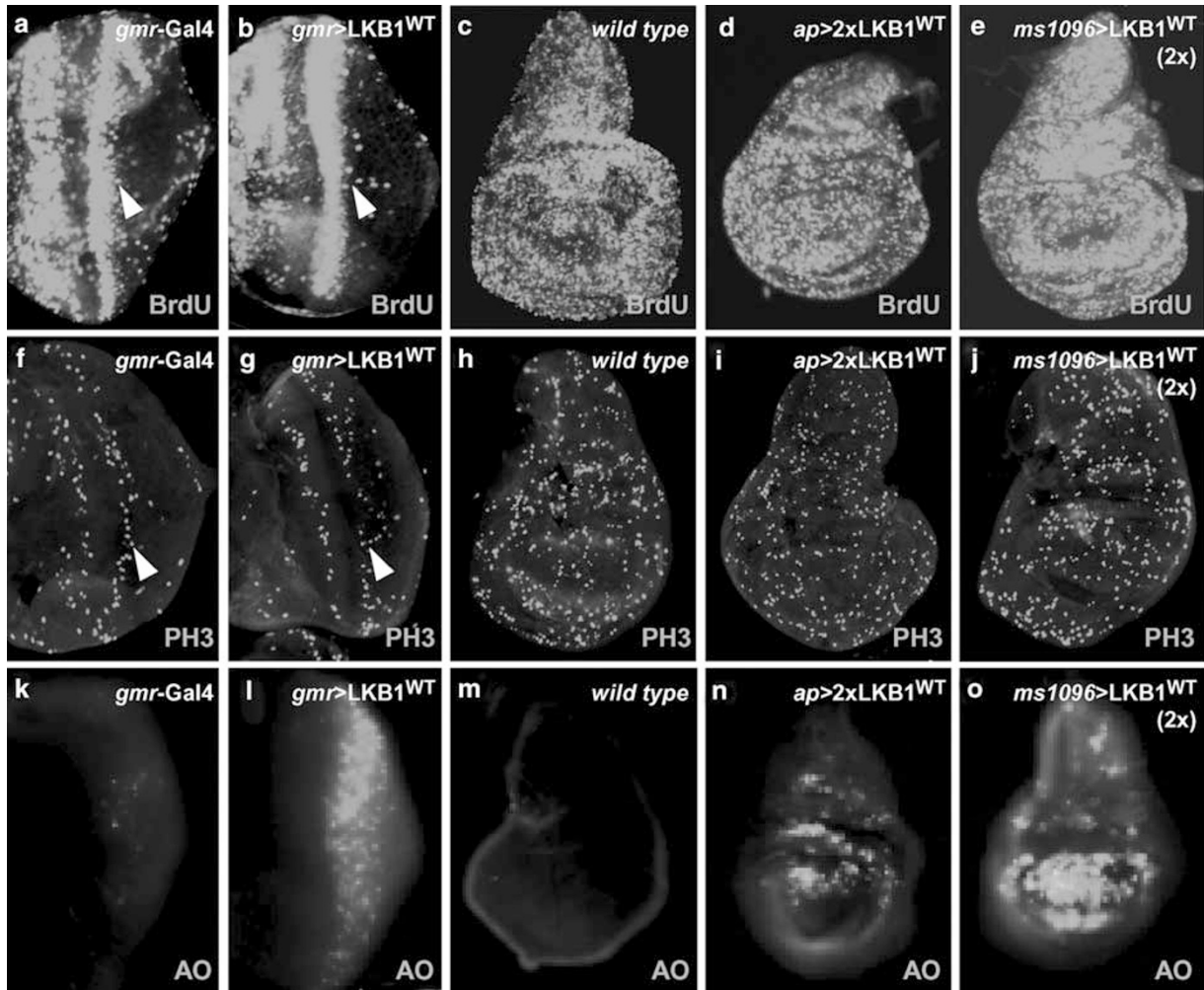
Figure 3 LKB1 promotes cell death without affecting cell cycle progression. (**a–e**) Eye discs (**a**, **b**) and wing discs (**c–e**) of indicated genotypes were analyzed by BrdU staining. Spots indicate S-phase cells with DNA replication. A white arrowhead indicates the normal position of the second mitotic wave. (**f–j**) Eye discs (**f**, **g**) and wing discs (**h–j**) of indicated genotypes were analyzed by antiphosphospecific histone 3 (PH3) antibody staining. Spots indicate mitotic cells with condensed chromosomes. A white arrowhead indicates the normal position of the second mitotic wave. (**k–o**) Eye discs (**k**, **l**) and wing discs (**m–o**) of indicated genotypes were analyzed by acridine orange (AO) staining. Spots indicate apoptotic cells with disrupted membrane integrity. The regions of extensive apoptotic signals (**l**, **n**, **o**) largely overlap with the regions of LKB1 expression (Figure 1a, **c** and **e**)

tissues were subjected to acridine orange (AO) staining, which specifically labels the dying cells with disrupted membrane integrity, they displayed prominent death signals (Figure 3l, n and o) compared to the controls (Figure 3k, m), specifically in the region of LKB1 overexpression (Figure 1a, c and e). These results strongly implied that the LKB1-induced organ size reduction is caused by apoptotic cell death, not by cell cycle arrest. Furthermore, TUNEL staining of the eye (Figure 4a, b) and wing discs (data not shown) also confirmed the existence of extensive apoptosis in the LKB1-expressing cells. Therefore, we concluded LKB1 to be a potent apoptosis instigator in *Drosophila*.

LKB1-induced apoptosis is caspase dependent but p53 independent

It has been reported that mammalian LKB1 physically associates with p53, and that *Drosophila* p53 is involved in the cell death process.[19,41–43] Therefore, we questioned whether LKB1-dependent apoptosis requires p53 activity. When a dominant-negative p53 was expressed by the *gmr*-Gal4 driver, the ionizing radiation-dependent apoptosis — which is fully dependent on p53 – was specifically suppressed in the *gmr*-expressing region (Figure 4c, d), demonstrating that the dominant-negative p53 is sufficient to inhibit endogenous p53 activity. However, the LKB1-induced apoptosis (Figure 3l) was not suppressed at all by the dominant-negative p53 (Figure 4e), while *Drosophila* inhibitor of apoptosis (DIAP) completely suppressed the LKB1-induced apoptosis (Figure 4f). Interestingly, even when the LKB1-induced apoptosis was completely blocked by DIAP, the BrdU (Figure 4h) and PH3 (Figure 4i) staining patterns were not significantly deviated from the wild-type patterns (Figure 3a, f), further confirming that LKB1 does not affect cell cycle progression. Since DIAP directly blocks caspase activity, we deduced the LKB1-induced apoptosis to be caspase depen-
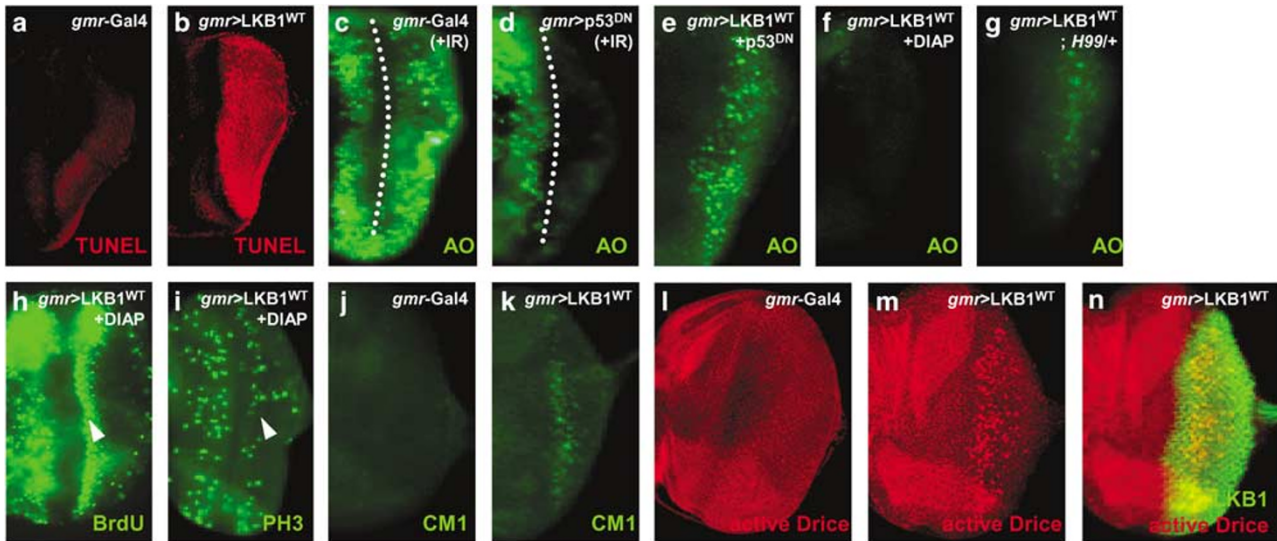
Figure 4 LKB1-induced apoptosis is caspase dependent but p53 independent. (**a**, **b**) Eye discs of indicated genotypes were analyzed by TUNEL staining. Red spots indicate DNA fragmentation in apoptotic cells. (**c**, **d**) Eye discs were analyzed by AO staining. Prior to dissection, third instar larvae were challenged by gamma-irradiation at 4000 rads (+IR), and incubated 4 h at 25°C. Note the absence of apoptosis after the morphogenetic furrow (dotted line) of *gmr*-Gal4/ + ; UAS-p53^{DN}/ + eye discs (**d**), compared to the control *gmr*-Gal4/ + eye discs (**c**). (**e**, **f**, **g**) Eye discs from *gmr*-Gal4 UAS-LKB1^{WT}/ + flies with either UAS-p53^{DN} (**e**), UAS-DIAP (**f**), or *Df(3L)H99* (**g**) were analyzed by AO staining. (**h**, **i**) Eye discs of UAS-DIAP/ + ; *gmr*-Gal4 UAS-LKB1^{WT}/ + were analyzed by BrdU (**h**) and PH3 (**i**) staining. (**j-n**) Eye discs of indicated genotypes were analyzed by immunostaining using antiactive caspase-3 (CM1; green spots in **j** and **k**), antiactive Drice (active Drice; red spots in **l–n**), and anti-LKB1 (LKB1; green area in **n**) antibodies

dent but p53 independent.[44] Consistently, extensive caspase activation was observed in the LKB1-overexpressed region (Figure 4n), detected by either CM1 antibody (Figure 4j, k) or antiactive Drice antibody (Figure 4l–n).

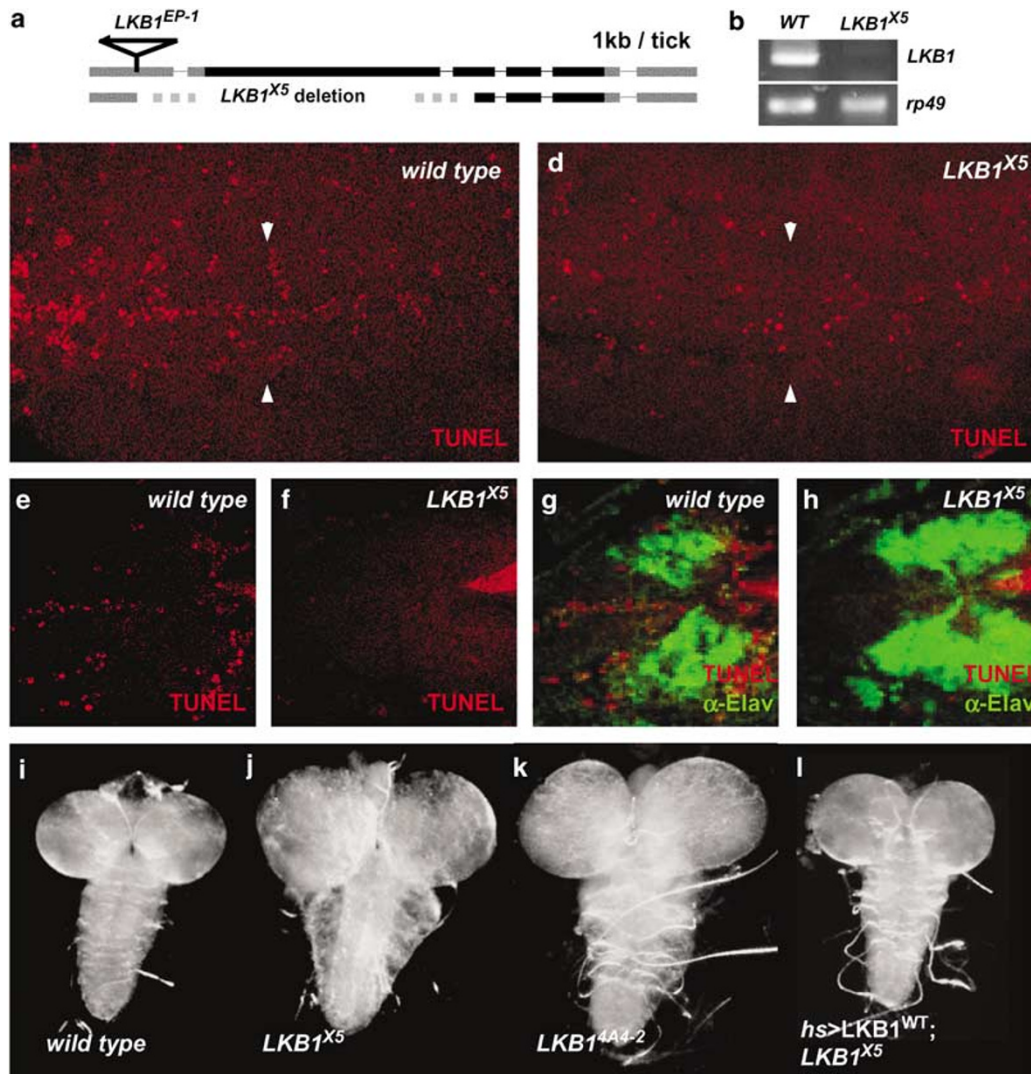## Endogenous LKB1 is required for developmental apoptosis

Next, we questioned whether endogenous LKB1 is required for developmental apoptosis and subsequent control of organ size in *Drosophila*. In order to address this, we generated an *LKB1*-null mutant allele, *LKB1^{X5}* with a specific deletion in the *LKB1* locus (76 208–77 847th nucleotides of AE003701; Figure 5a), leading to the complete loss of *LKB1* transcripts (Figure 5b). Every *LKB1^{X5}* mutant, as well as the previously described *LKB1^{4A4-2}* mutant, died before mid-pupal stage.[22] Transgenic expression of wild-type LKB1 using the *hs*-Gal4 driver successfully rescued the lethality of *LKB1^{X5}* showing the authenticity of *LKB1^{X5}*-null allele (data not shown).

To substantiate whether the endogenous LKB1 is required for apoptosis, we performed TUNEL staining of wild-type and *LKB1*-null embryos. As the nerve cord condensed (embryonic stage 16), prominent cell death was observed throughout the central nervous system of wild-type embryos (Figure 5c, e).[45] However, in *LKB1^{X5}* homozygous embryos, significantly fewer apoptotic cells were observed both in the ventral nerve cord (Figure 5d) and the embryonic head (Figure 5f) region, which was completely reversed by transgenic LKB1^{WT} expression (data not shown). Moreover, when stained with anti-Elav antibody, which recognizes all differentiated neurons, *LKB1^{X5}* homozygous embryos showed a dramatically increased mass of Elav-positive cells in the brain (Figure 5h) compared to that in wild type (Figure 5g).

As some *LKB1*-null animals escaped their embryonic lethality and further developed into pupal stages, we questioned whether *LKB1*-null mutant shows brain hyperplasia during larval stages as in the *Apaf-1* null mutants.[46,47] Hence, we dissected the brain lobes and ventral ganglion from the wandering third instar larvae of wild-type and *LKB1*-null mutants. Strikingly, in *LKB1^{X5}* homozygous larvae, the brain hemispheres and ventral ganglion were dramatically enlarged (Figure 5j) compared to those in wild type (Figure 5i), and this phenotype was consistently observed in *LKB1^{4A4-2}* mutants (Figure 5k). Furthermore, ectopic expression of LKB1^{WT} successfully suppressed this big-brain phenotype (Figure 5l), confirming that the brain hyperplasia is indeed due to the absence of LKB1 activity. Therefore, we concluded that endogenous LKB1 regulates organ size of the central nervous system by inducing developmental apoptosis during embryogenesis.

Grim, *reaper*, and *hid* are located downstream of LKB1

Apoptotic defects and neuronal hyperplasia of *LKB1*-null mutants are strikingly similar to the phenotypes of other *Drosophila* mutants deficient in apoptosis-inducing genes, such as *grim*, *reaper*, and *hid*.[48] Since the activation of these apoptosis instigators also induces extensive caspase activation, we speculated that these genes are involved in LKB1-dependent apoptosis. Interestingly, we found that the dosage reduction of *grim*, *reaper*, and *hid* genes alleviates the LKB1-induced apoptosis (Figure 4g). However, the dosage reduction of LKB1 did not affect apoptosis induced by *reaper* and *hid* (Supplementary Figure 2a, b), implicating that LKB1 exists upstream of these genes.

Figure 5 Endogenous LKB1 is required for embryonic neuronal apoptosis. (**a**) Genomic organization of *LKB1* locus. The P-element insertion site of original *LKB1*[EP−1] allele (triangle) and the deletion site of the *LKB1*[X5] allele were presented (dotted line). Thick line (gray: noncoding region, black: coding region) indicates exons of *LKB1* gene, and thin line indicates introns of *LKB1* gene. (**b**) Absence of *LKB1* message in *LKB1*[X5]-null larvae. Extensive RT-PCR ($\sim$40 cycles) could not detect any *LKB1* signals from *LKB1*[X5] larvae, while a strong *LKB1* signal was detected in w^{1118} larvae. *rp49* was used as a loading control (bottom panel). (**c–f**) Reduced apoptosis in *LKB1*[X5] mutant. Embryos were stained with TUNEL (red spots) to detect relative levels of apoptosis. Ventral nerve cords (**c**, **d**; between the arrowheads) and brains (**e**, **f**) of comparably staged w^{1118} (**c**, **e**) and *LKB1*[X5] (**d**, **f**) embryos (stage 16) were stained. (**g**, **h**) Embryos were simultaneously stained by anti-Elav antibody (green area) to visualize neurons of (**e**) and (**f**). Note the expansion of Elav-stained area (green in **h**) onto the normally apoptosis-occurring region (red spots in **g**). (**i–l**) CNS dissections from third instar larvae of indicated genotypes. Quantification of brain phenotypes is presented in Supplementary Figure 3 as a bar graph

LKB1 is dispensable for p53-dependent apoptosis

Although *LKB1*-null embryos displayed severe defects in developmental apoptosis, they showed a prominent cell death signal after the irradiation-induced DNA damage (Supplementary Figure 1b) as in the wild type (Supplementary Figure 1a), indicating that LKB1 is not required for the irradiation-dependent cell death machinery that is fully dependent on p53 activity (Supplementary Figure 1c).[41–43] Furthermore, ectopic apoptosis induced by p53 overexpression was not affected by LKB1 dosage reduction (Supplementary Figure 2e). Therefore, taken together with previous data showing that p53 is dispensable for LKB1-dependent apoptosis (Figure 4c–e), we

concluded that LKB1 is dispensable for the p53-dependent cell death in *Drosophila*.

LKB1 genetically interacts with the JNK pathway

The remarkable contribution of LKB1 on apoptotic cell death prompted us to conduct a genetic modifier screening that was designed to search for additional components mediating the LKB1-induced apoptosis. By crossing *ms1096*>LKB1[WT] flies with $\sim$600 independent EP/UAS lines, we obtained the F$_1$ progenies with wing-specific coexpression of a random gene and LKB1 (Figure 6a). These progenies were screened for modification of the *ms1096*>LKB1[WT] phenotype. Through

this method, three independent UAS lines, UAS-Misshapen (Msn, *Drosophila* NIK), UAS-Hemipterous (Hep, *Drosophila* MKK7), and UAS-Basket (Bsk, *Drosophila* JNK), were identified as candidates. Although the sole expression of each candidate gene resulted in mild or undetectable phenotypic changes (Figure 6b–e), simultaneous expression of the candidate genes with LKB1 strongly enhanced the $ms1096>$LKB1^{WT} phenotype with a severe disruption in the structure and size of the wing blades (Figure 6g–j). Interestingly, all of the candidates we identified – NIK, MKK7, and JNK – fell into a single signaling module, namely the JNK signaling pathway. Moreover, partial inactivation of the *puckered* (*puc*) gene, a JNK-specific dual-specificity phosphatase, dramatically enhanced the LKB1-induced small wing phenotype (Figure 6f, k), further confirming the connection between LKB1 and the JNK signaling pathway.[49] Since the JNK

signaling pathway regulates apoptosis both in the mammalian and *Drosophila* systems, we strongly suspected the JNK signaling pathway to be involved in the LKB1-induced apoptosis.[24–26]

LKB1 activates the JNK pathway

To examine whether the JNK pathway is activated by LKB1, we first induced transient expression of *Drosophila* LKB1 by the *hs*-Gal4 driver (Figure 7a, fourth panel) in third instar larvae, and determined the biochemical status of JNK by immunoblot analyses. As shown in Figure 7a, the amount of activated JNK protein was significantly increased by the transgenic expression of LKB1 (Figure 7a, first panel), while the total amount of JNK protein remained unchanged (Figure 7a, second panel). Moreover, an electrophoretically retarded JNK band, corresponding to a highly phosphorylated and activated form of the protein, was observed in the same blot with longer exposure (Figure 7a, third panel). This biochemical evidence strongly implied JNK to be a physiological target of LKB1.

For further confirmation, we next attempted to test whether JNK activity is modulated by the tissue-specific expression of LKB1. For this purpose, we adopted the *puc*-LacZ reporter system to measure the activity of JNK signaling *in vivo*.[26,29,50] *puc* is transcriptionally induced by JNK, forming a negative feedback loop of the JNK-dependent signaling pathway.[49] As expected, eye-specific (Figure 7b, c) and wing-specific (Figure 7d, e) expression of LKB1 induced a robust expression of the *puc*-LacZ reporter, indicating that JNK signaling is strongly activated by LKB1. Furthermore, immunohistochemistry using antiactive JNK antibody clearly confirmed that JNK activation occurred in the region of LKB1 overexpression (Figure 7f, g). All of these results consistently supported that LKB1 is an activator of JNK.

## JNK is indispensable for LKB1-induced apoptosis

We next questioned whether the activation of JNK is absolutely required for the LKB1-dependent apoptosis. To fully inhibit JNK activity, we utilized a strong *hep*^{r75} mutant allele, which was previously reported to have a minimal JNK activity in developing larval imaginal discs.[29] Since Hep is the *Drosophila* homologue of mammalian MKK7, a well-known upstream kinase of JNK, we speculated that LKB1 would not activate JNK without Hep activity. As expected, in the *hep*^{r75} mutant background, the *gmr*-Gal4 (Figure 8a, b) and the *ap*-Gal4 (Figure 8c, d)-driven overexpression of LKB1 could not provoke JNK activation at all, implicating the requirement of Hep for the LKB1-dependent JNK activation. More importantly, in the *hep*^{r75} mutant background, the LKB1-induced apoptosis in eye imaginal discs was also completely suppressed (Figure 8e, f). This clearly demonstrated that the JNK pathway is indispensable for the LKB1-induced apoptosis. Consistently, a partial downregulation of JNK activity by expressing kinase-dead JNK modestly suppressed the adult phenotype induced by the *ap*-Gal4-driven LKB1 overexpression (Figure 8g, h). Collectively, we concluded that LKB1 activates JNK via the Hep-dependent pathway, and this
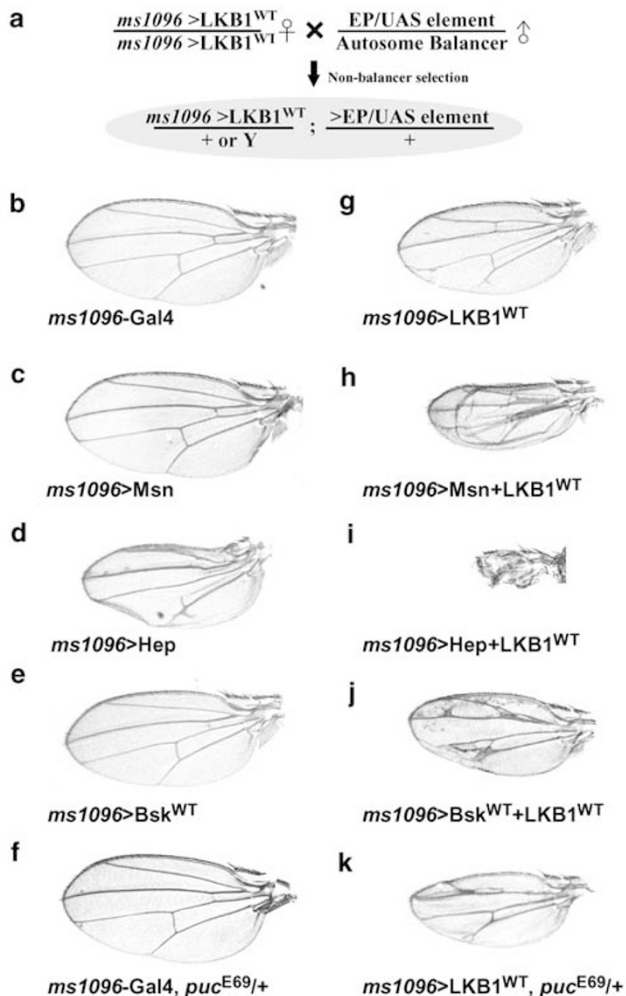
Figure 6 The JNK pathway is involved in LKB1-induced organ size reduction. (**a**) Schematic representation of a strategy for screening genetic modifiers of LKB1. (**b–k**) Adult fly wings expressing transgenes under the control of the *ms1096*-Gal4 driver. Genotypes are *ms1096*-Gal4/Y (**b**), *ms1096*-Gal4/Y; UAS-Msn/ + (**c**), *ms1096*-Gal4/Y; UAS-Hep/ + (**d**), *ms1096*-Gal4/Y; UAS-Bsk^{WT}/ + (**e**), *ms1096*-Gal4/Y; *puc*^{E69}/ + (**f**), *ms1096*-Gal4 UAS-LKB1^{WT}/Y (**g**), *ms1096*-Gal4 UAS-LKB1^{WT}/Y; UAS-Msn/ + (**h**), *ms1096*-Gal4 UAS-LKB1^{WT}/Y; UAS-Hep/ + (**i**), *ms1096*-Gal4 UAS-LKB1^{WT}/Y; UAS-Bsk^{WT}/ + (**j**), and *ms1096*-Gal4 UAS-LKB1^{WT}/Y; *puc*^{E69}/ + (**k**)
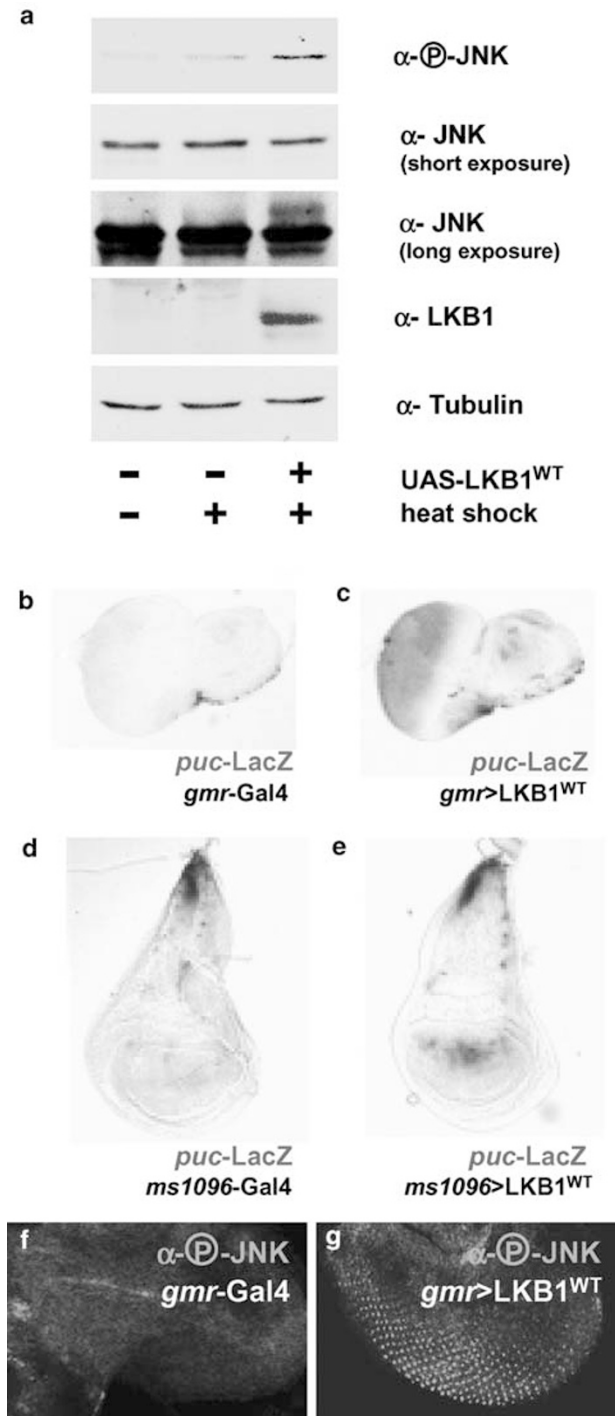
Figure 7 LKB1 activates the JNK pathway. (**a**) JNK phosphorylation induced by LKB1. Third instar larvae of *hs*-Gal4/+ (second lane) and *hs*-Gal4 UAS-LKB1^{WT}/+ (third lane), which were heat-shock treated (+) as described in Materials and Methods, as well as untreated *hs*-Gal4/+ (first lane) control (−) were subjected to immunoblot analyses with the indicated antibodies: antiphosphospecific JNK antibody for the first panel, anti-JNK antibody for the second and third panels, anti-LKB1 antibody for the fourth panel, and anti-tubulin antibody for the fifth panel. The third panel is a longer exposure of the second panel. (**b-e**) X-gal staining of *gmr*-Gal4/+; *puc*-LacZ/+ (**b**), and *gmr*-Gal4 UAS-LKB1^{WT}/+; *puc*-LacZ/+ (**c**) eye discs, and *ms1096*-Gal4/Y; *puc*-LacZ/+ (**d**) and *ms1096*-Gal4 UAS-LKB1^{WT}/Y; *puc*-LacZ/+ (**e**) wing discs. (**f, g**) Antiphosphospecific JNK antibody staining of the eye discs from *gmr*-Gal4 (**f**) and *gmr*-Gal4 UAS-LKB1^{WT} (**g**)
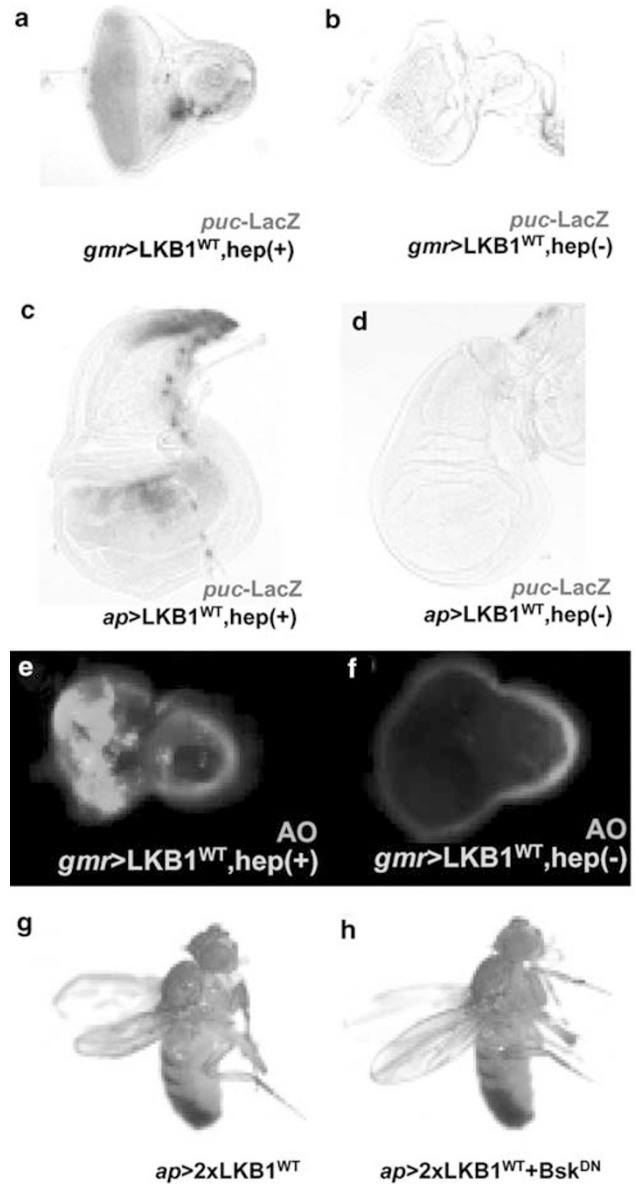
Figure 8 The JNK pathway is required for LKB1-dependent apoptosis. (**a–f**) Hep is required for LKB1-dependent JNK activation and apoptosis. X-gal staining (**a–d**) and acridine orange (AO) staining (**e, f**) of *gmr*-Gal4 UAS-LKB1^{WT}/+; *puc*-LacZ/+ (**a, e**) and *hep*^{r75}/Y; *gmr*-Gal4 UAS-LKB1^{WT}/+; *puc*-LacZ/+ (**b, f**) eye discs, and *ap*-Gal4 UAS-LKB1^{WT}/+; *puc*-LacZ/+ (**c**) and *hep*^{r75}/Y; *ap*-Gal4 UAS-LKB1^{WT}/+; *puc*-LacZ/+ (**d**) wing discs. (**g, h**) Lateral views of the flies expressing transgenes under the control of *ap*-Gal4. Genotypes are +/Y; *ap*-Gal4 UAS-LKB1^{WT}/+; UAS-LKB1^{WT}/+ (**g**), UAS-Bsk^{DN}/Y; *ap*-Gal4 UAS-LKB1^{WT}/+; UAS-LKB1^{WT}/+ (**h**)

activation is essential for the LKB1-dependent apoptosis in *Drosophila*.

JNK is involved in LKB1-dependent neuronal apoptosis

With JNK shown to be essential for the LKB1-dependent apoptosis, we next attempted to test the relationship between

JNK and embryonic neuronal apoptosis, which is dependent on endogenous LKB1 activity (Figure 5). First, we examined whether JNK activity is associated with the embryonic apoptosis by costaining wild-type embryos with antiphosphospecific JNK antibody and TUNEL reaction. Interestingly, we observed many cells possessing both phospho-JNK and TUNEL signals, implicating the correlation between JNK activation and apoptotic cell death in embryonic neuronal cells (Figure 9a–c). Moreover, LKB1-null embryos did not show any prominent phospho-JNK signals compared to wild type (Figure 9d, e), showing that LKB1 is required for inducing JNK activation during embryogenesis. Furthermore, ectopic activation of JNK signaling by Hep overexpression restored embryonic apoptosis in LKB1-null embryos (Figure 9f, g), and completely rescued the big-brain phenotype of LKB1-null larvae (Figure 9h, i), while Hep overexpression itself did not

induce any effects on wild-type brain size (Figure 9j). Therefore, we concluded that induction of the JNK-dependent apoptosis is one of the crucial functions of endogenous LKB1 during embryonic neuronal development in *Drosophila*.

LKB1 is not essential for Eiger and TRAF1-dependent apoptosis

As Eiger (*Drosophila* TNF ligand) and TRAF1 (*Drosophila* TNF receptor-associated factor) have been shown to induce JNK-dependent apoptosis, we questioned whether LKB1 is involved in the Eiger/TRAF1-dependent apoptotic pathway.[50–52] Therefore, we tested genetic interaction between LKB1 and these genes. However, dosage reduction of LKB1 did not affect Eiger- or TRAF1-induced apoptosis at all
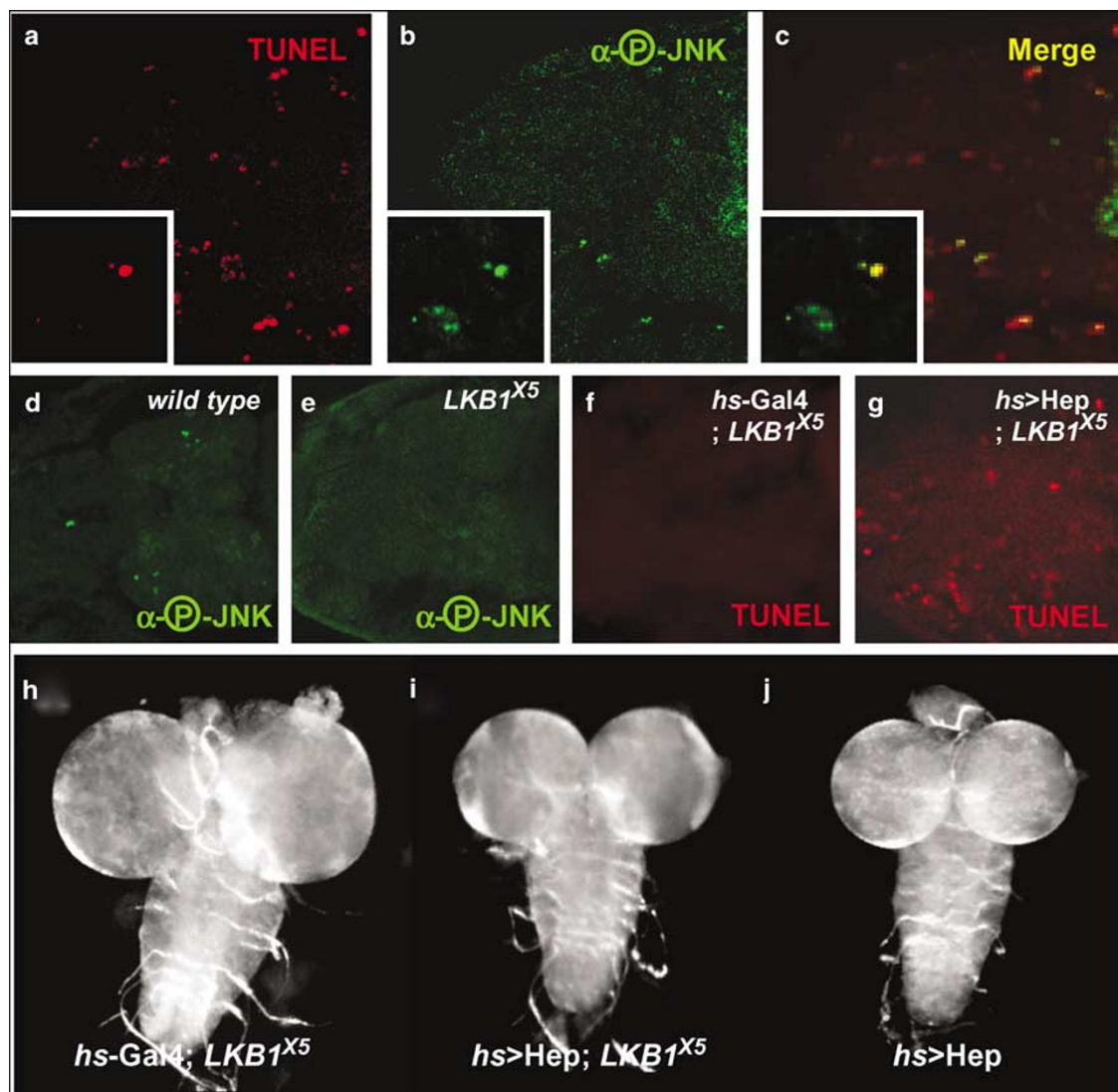
Figure 9 The JNK pathway is involved in the apoptosis caused by endogenous LKB1. (**a–c**) TUNEL (**a**, **c**; red spots) and antiphoshospecific JNK antibody staining (**b**, **c**; green spots) were simultaneously conducted in *w*[1118] embryonic head. Magnified views of representative region are presented as insets. (**d**, **e**) Antiphosphospecific JNK antibody staining of *w*[1118] (**d**) and LKB1[X5] (**e**) embryos. (**f**, **g**) Embryonic apoptosis of control LKB1[X5] (**f**) was rescued by transgenic expression of Hep (**g**). Embryos were stained with TUNEL (red spots) to detect relative levels of apoptosis. (**h–j**) CNS dissections from third instar larvae of indicated genotypes. For transgenic expressions (**f–j**), heat-shock has been treated for 9–12 h AEL at 37°C. Quantification of brain phenotypes is presented in Supplementary Figure 3 as a bar graph

(Supplementary Figure 2c, d and data not shown). Moreover, coexpression of these genes with LKB1 did not result in synergistic phenotype enhancement in contrast to other signaling components of the JNK pathway as shown in Figure 6 (data not shown). Furthermore, null mutants of eiger and TRAF1 displayed distinct phenotypes from LKB1-null mutants.[50,51] Therefore, we concluded there to be no direct involvement of LKB1 in the Eiger/TRAF1-dependent apoptotic pathway.

LKB1 genetically interacts with JNK during thoracic morphogenesis

Finally, since the JNK pathway is involved in the regulation of cytoskeletal structures, we determined whether LKB1 is involved in the JNK-dependent cell structure control. To achieve this, we examined whether the LKB1 knockdown causes thoracic development defects. When we reduced LKB1 expression in the thorax region by a complementary RNA hairpin corresponding to LKB1 mRNA (LKBi), thoracic morphogenesis was significantly disrupted with bristle and scutellum formation defects (Supplementary Figure 4a–d).[53] The same phenotypes were consistently observed with comparable penetrances (Supplementary Figure 4e) when dsRNAi was generated by other approaches such as usage of the hairpin construct with the inversed orientation (Inv) and of the SympUAST vector inducing symmetrical transcription to generate dsRNAi (Sym).[53,54] Therefore, we concluded that LKB1 is involved in the thoracic morphogenetic processes.

To further relate these thoracic phenotypes of the LKB1 loss-of-function flies with the JNK pathway, we performed additional genetic analyses. Specifically, expression of Puc (Supplementary Figure 4f) or kinase-dead JNK (Supplementary Figure 4h) in the thorax by the ap-Gal4 driver led to a mild but significant thorax-open phenotype as previously described.[30,50] Intriguingly, RNAi-mediated LKB1 inhibition further enhanced these thorax phenotypes (Supplementary Figure 4g, i), supporting that the endogenous functions of LKB1 and JNK are tightly related. Moreover, pnr-Gal4-induced LKBi expression, which inhibited LKB1 activity at the dorsal-most region of the thorax, modestly suppressed the puc-LacZ reporter activity and ultimately resulted in a thorax-open phenotype (Supplementary Figure 4j, k), implicating that LKB1 participates in the JNK-dependent thorax closure process.

LKB1 controls JNK-dependent embryonic dorsal closure

As LKB1 was involved in the JNK-dependent thoracic morphogenesis (Supplementary Figure 4), we tested whether downregulation of LKB1 activity could result in embryonic dorsal closure defects, which is a more typical outcome of reduced JNK activity in vivo. When dsRNAi corresponding to the coding region of LKB1 mRNA (dsLKBi) was directly injected into wild-type Drosophila embryos, various dorsal-open phenotypes were induced in hatched (Supplementary Figure 5b, c) or unhatched (Supplementary Figure 5d, e) first-instar larvae. These phenotypes were also observed in a

small portion ($\sim$10%) of $LKB1^{X5}$ homozygotes (Supplementary Figure 5f, g), demonstrating the involvement of LKB1 in embryonic dorsal closure. Furthermore, transheterozygotic combinations between $LKB1^{X5}$ and basket (bsk^1 and bsk^2) allele induced significant dorsal open phenotypes (Supplementary Figure 5h, i) in unhatched first instar larvae. In addition, LKB1-null mutant displayed a substantial reduction of dpp transcription specifically in the dorsal-most region (Supplementary Figure 5j, k), which is dependent on the JNK activity and required for epithelial sheet movement.[27]These results strongly implicated a functional interaction between LKB1 and JNK during embryonic development.

Discussion

Tumor suppressor LKB1 is a unique serine/threonine kinase with no close relatives in the mammalian genome. Likewise, only a single LKB1 orthologue exists in the Drosophila genome, implying well-conserved functional characteristics between the mammalian and Drosophila LKB1.[22,55] Using the Drosophila model system, we discovered a novel functional connection between LKB1 and the JNK pathway: the JNK pathway is present downstream of the LKB1-dependent signaling pathway and controls apoptosis and organ size in response to LKB1 activities.

Tumor suppressor LKB1 is a potent apoptosis instigator

Previous studies on classical tumor suppressors in the Drosophila model system showed that hyperactivation of each tumor suppressor negatively regulates organ size by cell size reduction, cell cycle inhibition, or cell death promotion.[39,42,56] This study presented that tumor suppressor LKB1 also negatively regulates organ size in Drosophila in a kinase activity-dependent manner (Figure 1). Recent reports on the TSC tumor suppressor suggested that LKB1 suppresses TOR activity via the TSC complex, ultimately resulting in inhibition of cell growth and proliferation.[17,18] Likewise, we observed that TSC overexpression dramatically reduces cell size in Drosophila clonal overexpression analyses (Figure 2). However, unexpectedly, LKB1 overexpression did not induce any cell size reduction (Figure 2), in stark contrast to the case of TSC. Moreover, LKB1 overexpression in the clones of endoreplicating tissues altered neither cell size nor DNA content (data not shown), confirming that the tumor-suppressing activity of LKB1 is not related to the negative regulation of cell growth. Therefore, we concluded there to be no physiological connection between LKB1 and TSC in the context of cell growth control in Drosophila.

However, our histological analyses clearly demonstrated that the LKB1-overexpressing tissues suffer caspase-dependent programmed cell death, without apparent alteration in G1/S and G2/M cell cycle transition (Figure 3). The cell death induced by LKB1 overexpression displayed the most typical phenotypes of apoptosis, such as membrane integrity disruption, DNA fragmentation, and caspase activation (Figure 4). Furthermore, endogenous LKB1 was physiologically required for normal embryonic apoptosis, and also

essential for the size control of the central nervous system (Figure 5). Consistent with our results, LKB1 was reported to be highly expressed in apoptotic cells of the small intestine in humans, and PJS polyps that lack the functional LKB1 protein possessed fewer apoptotic cells than in adjacent normal tissues.[19] Collectively, these results strongly suggested that instigation of apoptosis is a critical function of tumor suppressor LKB1.

Surprisingly, in contradiction to previous research done with the mammalian system, the LKB1-induced apoptosis in *Drosophila* was a p53-independent event, as it was not blocked by the expression of a dominant-negative version of p53 (Figure 4). Moreover, apoptosis induced by DNA damage or p53 overexpression was also unaffected by downregulation of LKB1 activity (Supplementary Figure 1 and 2). Furthermore, diagnostic symptoms and major tumor types of PJS patients are highly distinct from those of Li–Fraumeni syndrome patients.[11] Therefore, it is very likely that LKB1 and p53 are indirectly related as they both have caspase activation as a common downstream signaling event.[41]

LKB1 functionally interacts with JNK

Genetic screening using the *Drosophila* model system enabled us to discover the JNK pathway as a downstream effector of the LKB1-dependent signaling pathway. Through simple genetic screening, we analyzed more than 500 genes to interact with LKB1, which covers various signal transduction pathways including the Ras/ERK, JNK, PI3K, TOR, Wnt, TGF-beta, and NF-κB pathways (data not shown). Among them, some components of the JNK signaling pathway showed outstanding genetic interactions with LKB1, strongly enhancing the tissue-size reduction phenotype of LKB1 (Figure 6). This result implied the specific involvement of the JNK pathway in LKB1-dependent signal transduction, prompting us to conduct additional studies concerning the relationship between LKB1 and the JNK signaling pathway.

Our biochemical and histological analyses clearly demonstrated that JNK can be activated by LKB1 overexpression (Figure 7). As the kinase-dead mutant of LKB1 did not induce this JNK activation (Figure 1 and data not shown), this phenomenon seems to be a direct consequence of LKB1 phosphotransferase activity. Moreover, blockage of the JNK signaling pathway strongly suppressed the LKB1-induced apoptosis, as well as the tissue-size reduction (Figure 8). Furthermore, the activation of JNK signaling was shown to be involved in the physiological apoptosis induced by endogenous LKB1 (Figure 9). Therefore, we concluded that the JNK pathway acts as a downstream effector of LKB1 by mediating the LKB1-induced apoptosis. Accordingly, any signals upregulating the kinase activity of LKB1 would also activate the JNK pathway, ultimately leading to apoptosis in the cell. In fact, some microtubule-disrupting agents that induce the LKB1-dependent cell death also activate the JNK pathway to promote apoptosis.[19]

Another interesting point of our research is that inhibition of LKB1 results in defective epithelial morphogenesis, which is associated with low JNK activity (Supplementary Figure 4 and 5). Several previous studies already demonstrated the requirement of LKB1 and JNK for epithelial integrity and polarity, both in the mammalian and *Drosophila* systems.[21,22,27,57] Consistently, we observed that inhibition of LKB1 activity during development caused various morphogenesis defects including malformation of adult thoraxes and failure in embryonic dorsal closure. Furthermore, we also showed that LKB1 is required for maintaining adequate JNK activity during embryonic and larval development. Since loss of tissue integrity is a common feature of PJS polyps and cancer, the role of LKB1 in epithelial organization seems to be also important for mediating its tumor-suppressing activity.

Here, we demonstrated that the JNK pathway is involved in the LKB1-dependent apoptosis and epithelial morphogenesis in *Drosophila*. Since deregulation of both LKB1 and the JNK pathways is highly implicated in cancer development, the novel connection between LKB1 and the JNK signaling pathway provides a new direction for future studies in better understanding the complex functions of tumor suppressor LKB1.

Materials and Methods

Fly stocks and genetics

Full-length *Drosophila* LKB1 (GM01569, IMAGE), as well as its kinase-dead form (K201I), was cloned into the pUAST vector prior to injection. dsRNAi transgenes were constructed against 519–1104 base pairs of LKB1 coding sequence, as previously described.[53,54] For direct RNAi microinjection, dsRNAi against 971–1479 base pairs of LKB1 was synthesized and microinjected into embryos as described.[53] The Gal4 lines used in this study are *Ay*-Gal4,[58] *ap*-Gal4,[38] *gmr*-Gal4,[41] *ms1096*-Gal4,[41] *hs*-Gal4,[50] and *pnr*-Gal4.[30] Other stocks used in this study, UAS-Hep, UAS-Bsk^WT, and UAS-Bsk^DN were gifts from Dr Mlodzik; *puc^E69*, which is also called *puc*-LacZ, was a gift from Dr Adachi-Yamada; UAS-DIAP was a gift from Dr Hay; UAS-Puc was a gift from Dr Peifer; *LKB1^{4A4−2}* was a gift from Dr St Johnston; *dpp*-LacZ was a gift from Dr Kim; UAS-Eiger was obtained from Genexel Inc.; and *bsk^1*, *bsk^2*, *hep^{r75}* Df(3L)H99, UAS-Reaper, *gmr*-Hid, *Ay* > GFP (*Ay*-Gal4 UAS-GFP), UAS-GFP, and UAS-Msn were obtained from the Bloomington *Drosophila* Stock Center. All flies were grown on standard cornmeal–yeast–agar medium at 25°C.

LKB1^{EP−1} line was isolated from the GenExel's EP collection and subjected to imprecise excision as described.[41] Among the ∼800 excised allele, we found *LKB1^{X5}* using PCR-based approaches. The molecular lesion of *LKB1^{X5}* was determined by sequencing the genomic PCR product.

Clonal expression studies were performed using the *Ay*-Gal4 system.[58] In this system, clones in larval tissues were marked by GFP expression and clones in adult wing bristles were marked by a yellow color. Clone induction was conducted 80 h after egg-laying (AEL) and larval wing discs were analyzed 150 h AEL.

Antibodies

C-terminal protein (amino acids 468–567) of *Drosophila* LKB1 was used to generate anti-LKB1 mouse antisera. Antiphosphospecific JNK (Promega), anti-JNK (Santa-Cruz), antiactive human caspase-3 (CM1, Cell Signaling), antiactive Drice (a gift from Dr Hay), antiphosphospecific histone H3 (Upstate Laboratories), anti-tubulin E7, anti-LacZ JIE7, anti-Elav 9F8A9, and anti-BrdU G3G4 antibodies (DSHB, Iowa) were used for immunoblot and histological analyses.

Immunoblot

The third instar larvae of *hs*-Gal4/ + (control) and *hs* > LKB1[WT]/ + were heat-shocked at 37°C for 30 min in a water bath and allowed to recover at 25°C for 2 h before processing. The larvae were homogenized as previously described.[38] Proteins in the denatured supernatant were immunoblotted with the indicated antibodies after separation in sodium dodecyl sulfate-polyacrylamide gel.

Histology

Adult wing blades were mounted in 50% Canadian balsam (Sigma) in methyl salicylate (Fischer). GFP visualization,[50] BrdU incorporation assay,[59] AO staining,[41] TUNEL *in situ*,[50] and X-Gal staining[50] were performed as described. For the selection of *LKB1*[X5] homozygous embryos, we utilized TM3-LacZ and TM3-GFP balancers.[38] For the cuticle preparations, dechorionated embryos or larvae were immersed in a solution containing acetic acid and glycerol at a 3 : 1 ratio and incubated 3 h at 65°C. Embryos and larvae were then mounted in Hoyer's medium and incubated 24 h at 65°C.

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Supplementary Information accompanies the paper on Cell Death and Differentiation website (http://www.nature.com/cdd)