

AMP-activated protein kinase negatively regulates FcεRI-mediated mast cell signaling and anaphylaxis in mice

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Background: Aggregation of FcεRI activates a cascade of signaling events leading to mast cell activation, followed by inhibitory signals that turn off the activating signals. However, the overall view of negative signals in mast cells is still incomplete. Although AMP-activated protein kinase (AMPK), which is generally known as a regulator of energy metabolism, is also associated with anti-inflammation, little is known about the role of AMPK in mast cells.

Objectives: We investigated the role of AMPK and its regulatory mechanism in mast cells.

Method: The roles of AMPK in FcεRI-dependent activation of bone marrow–derived mast cells (BMMCs) were evaluated by using chemical agents, small interfering RNAs (siRNAs), or adenovirus that modulated the activity or expression of AMPK signaling components. In addition, *AMPKα2*^{−/−} mice were used to verify the role of AMPK in anaphylactic models.

Results: FcεRI signaling and associated effector functions in BMMCs were suppressed by the AMPK activator 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) and were conversely augmented by siRNA knockdown of AMPKα2 or liver kinase B1 (LKB1), an upstream kinase of AMPK. Furthermore, *AMPKα2* deficiency led to increased FcεRI-mediated BMMC activation and anaphylaxis that were insensitive to AICAR, whereas enforced expression of AMPKα2 in *AMPKα2*^{−/−} BMMCs reversed the hypersensitive FcεRI

signaling to normal levels. Pharmacologic inhibition or siRNA knockdown of Fyn mimicked AMPK activation, suggesting that Fyn counterregulates the LKB1-AMPK axis. Mechanistically, Fyn controlled AMPK activity by regulating LKB1 localization. **Conclusions:** The Fyn-regulated LKB1-AMPK axis acts as a novel inhibitory module for mast cell activation, which points to AMPK activators as therapeutic drugs for allergic diseases. (J Allergy Clin Immunol 2013;■■■:■■■-■■■.)

Key words: Mast cell, AMP-activated protein kinase, liver kinase B1, Fyn, anaphylaxis

Allergen-induced aggregation of FcεRI on mast cells triggers a cascade of activating signals leading to release of various mediators.^{1–3} After FcεRI aggregation, spleen tyrosine kinase (Syk)–mediated activation of phospholipase Cγ (PLCγ) and phosphoinositide 3-kinase promotes Ca²⁺ influx, degranulation, lipid mediator production, and activation of signaling pathways, including mitogen-activated protein kinases (MAPKs) and nuclear factor κB (NF-κB), which regulate cytokine synthesis.^{1–3} These activation signals are counterbalanced by negative signals that allow mast cells to return to their basal state.⁴ Although several surface receptors and associated tyrosine phosphatases negatively regulate FcεRI signaling,^{4,5} the overall view of negative signals in mast cells is still incomplete.

Fyn, an Src family tyrosine kinase, has been implicated in FcεRI signaling, although its actions in mast cells are controversial.^{6–9} Bone marrow–derived mast cells (BMMCs) from *Fyn*^{−/−} mice show decreased FcεRI signaling through attenuated GRB2-associated-binding protein 2 (Gab2) and Akt or Syk activation.^{6–8} In contrast, Fyn small interfering RNA (siRNA)–treated BMMCs show minimal impairment of FcεRI signaling, suggesting that Fyn has a minor role.⁹ These discrepancies probably arise because transient knockdown might avoid developmental changes in compensatory signals that often occur as a result of permanent knockout, or alternatively, Fyn might affect some unidentified signals with effects on mast cells that vary according to the experimental settings. Several studies have implicated Fyn in insulin signaling,^{10,11} suggesting that Fyn bridges metabolic and immune responses. Notably, pharmacologic or genetic inhibition of Fyn leads to increased energy expenditure through a previously unrecognized mechanism involving AMP-activated protein kinase (AMPK), an energy state sensor.¹²

AMPK, which has 1 catalytic subunit α (α1 or α2) plus 2 regulatory subunits β (β1 or β2) and γ (γ1–γ3), is activated when the AMP/ATP ratio increases in nutritional deficiency.^{13,14} AMPK responds to metabolic requirements by stimulating energy production and inhibiting energy consumption. Mounting evidence suggests that reduced AMPK activity is associated with inflammation, whereas increased AMPK activity inhibits obesity and inflammation induced by distinct insults.^{15,16} Liver kinase B1

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Abbreviations used

ACC:	Acetyl-CoA carboxylase
AICAR:	Activator 5-aminoimidazole-4-carboxamide-1- β -D-ribofuranoside
AMPK:	AMP-activated protein kinase
β -Hex:	β -Hexosaminidase
BMMC:	Bone marrow–derived mast cell
CA-AMPK:	Catalytically active AMPK α 2
ERK:	Extracellular signal-regulated kinase
Gab2:	GRB2-associated-binding protein 2
HSA:	Human serum albumin
IKK:	I κ B kinase
JNK:	c-Jun N-terminal kinase
LKB1:	Liver kinase B1
LTC ₄ :	Leukotriene C ₄
MAPK:	Mitogen-activated protein kinase
mTOR:	Mammalian target of rapamycin
NF- κ B:	Nuclear factor κ B
PCA:	Passive cutaneous anaphylaxis
PGD ₂ :	Prostaglandin D ₂
PLC γ :	Phospholipase C γ
PSA:	Passive systemic anaphylaxis
siRNA:	Small interfering RNA
Syk:	Spleen tyrosine kinase
WT:	Wild-type

(LKB1) is a master AMPK kinase regulated by phosphorylation, acetylation, association into a ternary complex, and subcellular translocation.^{17,18} Interestingly, Fyn directly phosphorylates LKB1, resulting in sequestration of LKB1 in the nucleus and thereby reduced phosphorylation of AMPK,¹² whereas AMPK phosphorylates Fyn.¹⁹ Furthermore, activation of sirtuin1 or ATM kinase by AMPK stimulates LKB1, which in turn activates AMPK,^{20,21} suggesting complex, positive-feedback cross-talk among Fyn, LKB1, and AMPK. However, the regulatory role of AMPK in mast cells is currently unknown.

Herein, using pharmacologic, molecular, and genetic approaches, we provide evidence that the Fyn-regulated LKB1-AMPK axis inhibits Fc ϵ RI signaling. *AMPK α 2*^{-/-} BMMCs were hypersensitive to Fc ϵ RI stimulation, whereas mice treated with a Fyn inhibitor or an AMPK activator were protected from passive cutaneous anaphylaxis (PCA) or passive systemic anaphylaxis (PSA). Fyn blocked cytosolic retention of LKB1, thereby shutting off the inhibitory AMPK signaling. Collectively, our findings add AMPK to a growing list of negative regulators of mast cell activation, reveal a novel regulatory role of Fyn in the context of AMPK signaling, and highlight the LKB1-AMPK pathway as a potential drug target aimed at control of mast cell responsiveness and allergy-associated traits.

METHODS**Mice**

AMPK α 2^{-/-} mice²² were bred under pathogen-free conditions in the Asan Institute for Life Science. BALB/cJ, C57BL/6 (HyoChang Science, Daegu, Korea), or *AMPK α 2*^{-/-} mice on the C57BL/6 background were maintained in a laminar airflow cabinet with standard diet (Purina, Seoul, Korea) and water at a temperature of 22°C $\pm$ 1°C and a humidity of 55% $\pm$ 10% with a 12-hour/12-hour (light/dark) cycle. All animal experiments were approved by the Institutional Animal Care and Use Committees of Yeungnam University and Tokyo Metropolitan Institute of Medical Science.

Statistical analysis

Data calculation and statistical analysis were performed with GraphPad Prism 3-5.0 software (GraphPad Software, La Jolla, Calif). The statistical significance of differences between 2 groups was determined with the unpaired Student *t* test, and multiple comparisons were analyzed by using 1-way ANOVA. All data are presented as means $\pm$ SEMs. Differences were considered statistically significant at a *P* value of less than .05.

Other procedures

For further information, see the **Methods** section in this article's Online Repository at www.jacionline.org.

RESULTS**Pharmacologic Fyn inhibition and AMPK activation similarly dampen mast cell activation both *in vitro* and *in vivo***

Although Fyn seems to participate in mast cell activation in cooperation with Gab2 and Syk,^{23,24} results of different studies have varied.⁶⁻⁹ To investigate whether Fyn is required for Fc ϵ RI-mediated signaling, we treated BMMCs with the Fyn inhibitor SU6656 and stimulated with IgE/antigen. SU6656 effectively blocked Fc ϵ RI-stimulated phosphorylation of PLC γ 1, extracellular signal-regulated kinase (ERK) 1/2, c-Jun N-terminal kinase (JNK), and I κ B kinase (IKK; Fig 1, A), as well as Fyn (see Fig E1 in this article's Online Repository at www.jacionline.org). Consistently, Fc ϵ RI-induced exocytosis of β -hexosaminidase (β -Hex; Fig 1, B), generation of leukotriene C₄ (LTC₄; Fig 1, C) and prostaglandin D₂ (PGD₂; Fig 1, D), secretion of TNF- α (Fig 1, E) and IL-6 (Fig 1, F), and increase in intracellular Ca²⁺ levels (Fig 1, G) were all markedly reduced by SU6656. However, SU6656 did not prevent the antigen-induced phosphorylation of Gab2 and the downstream Akt and p38 (Fig 1, A), suggesting that Fyn is segregated from the Gab2-mediated pathway in this setting. Thus although our results suggest a physiologic role of Fyn in mast cell activation, they are not entirely identical to those of previous studies with Fyn knock-out^{6,7} or knockdown.⁹ Because of this inconsistency, we investigated whether Fyn might affect another signaling pathway that could be responsible for the effects of SU6656 on mast cells.

It has recently been shown that *Fyn*^{-/-} mice display increased AMPK activity accompanied by increased energy expenditure and better insulin sensitivity.²⁵ We found that treatment of BMMCs with SU6656 significantly increased the phosphorylation of AMPK, its upstream kinase LKB1, and its downstream target acetyl-CoA carboxylase (ACC), without any significant effect on their total protein levels (Fig 1, A). This effect of SU6656 was evident even in the absence of antigen (see Fig E2, A, in this article's Online Repository at www.jacionline.org), and Fc ϵ RI stimulation *per se* did not increase the basal phosphorylation of LKB1, AMPK, and ACC (Fig 1, A). These results suggest that rapid reduction of Fyn activity is sufficient to enhance AMPK activity. SU6656 also reduced the antigen-induced phosphorylation of mammalian target of rapamycin (mTOR) and its target, S6K (Fig 1, A), which act in the nutrition-sensing pathway that is negatively regulated by AMPK.²⁶

Therefore we next assessed the effect of pharmacologic activation of AMPK using activator 5-aminoimidazole-4-carboxamide-1- β -D-ribofuranoside (AICAR), an AMPK activator, on Fc ϵ RI signals. In AICAR-treated BMMCs there were significant increases in the phosphorylation of LKB1, AMPK, and ACC (Fig 1, A), even in the absence of antigen (see Fig E2, A).

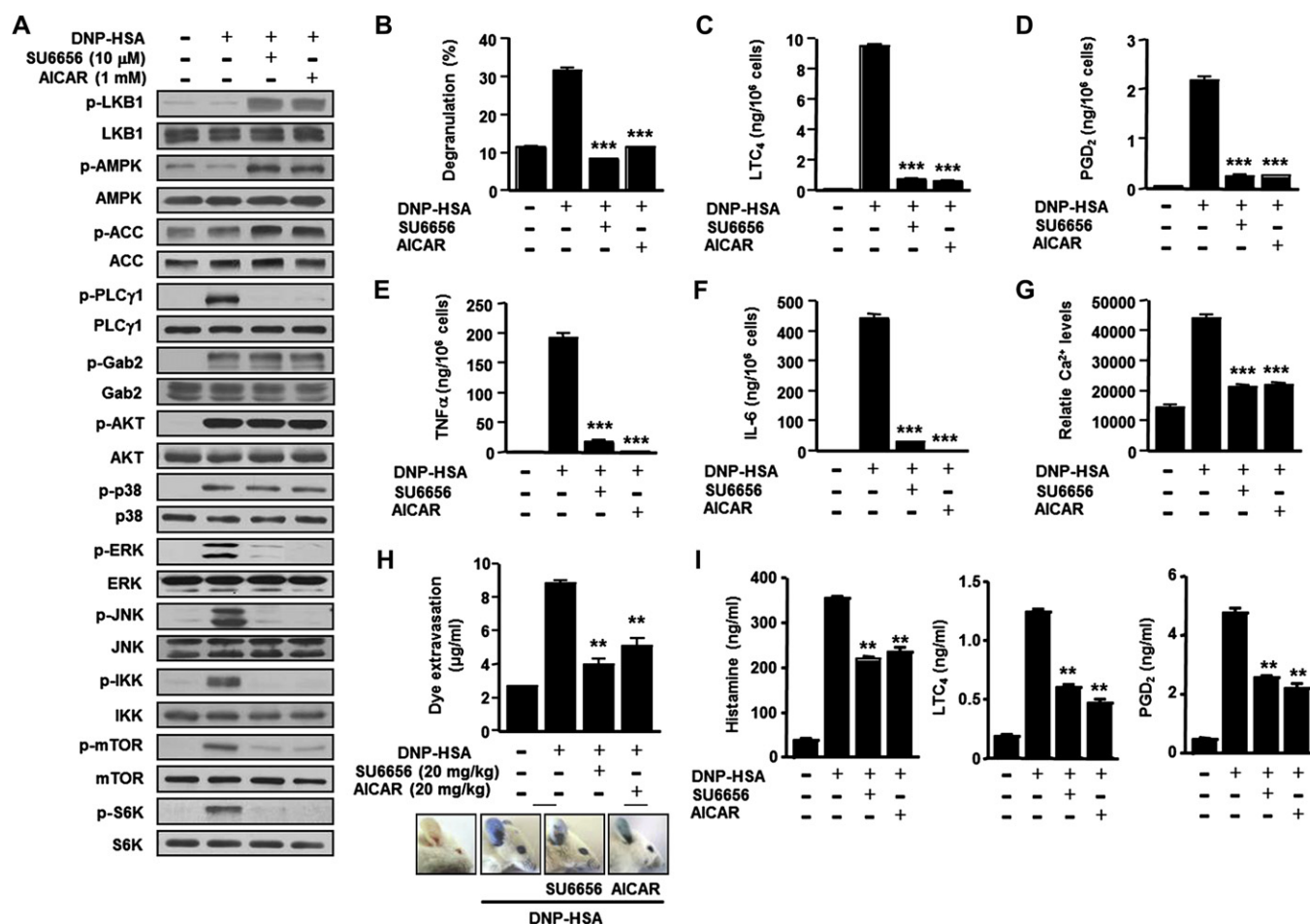


FIG 1. SU6656 or AICAR inhibits IgE/antigen-induced mast cell activation. **A-G**, Effect of SU6656 or AICAR on phosphorylation (*p*-) of signaling molecules (15 minutes; Fig 1, **A**); release of β -Hex (15 minutes; Fig 1, **B**), LTC₄ (15 minutes; Fig 1, **C**), PGD₂ (7 hours; Fig 1, **D**), and cytokines (6 hours; Fig 1, **E** and **F**); and influx of Ca²⁺ (5 minutes; Fig 1, **G**) in IgE/antigen-activated BMMCs (*n* = 3). ****P* < .001 versus without SU6656 or AICAR. **H** and **I**, Effects of SU6656 or AICAR on IgE/antigen-induced PCA (Fig 1, **H**) and PSA (Fig 1, **I**; *n* = 5). ***P* < .01 versus without SU6656 or AICAR. The bottom panel in Fig 1, **H**, shows representative photos of ears.

Strikingly, AICAR also attenuated the Fc ϵ RI-dependent phosphorylation of PLC γ 1, ERK, JNK, IKK, mTOR, and S6K but not that of Gab2, Akt, and p38 (Fig 1, **A**), suggesting that AMPK ameliorates the core, if not all, Fc ϵ RI signaling. Consistently, AICAR markedly reduced Fc ϵ RI-mediated degranulation (Fig 1, **B**), generation of LTC₄ (Fig 1, **C**) and PGD₂ (Fig 1, **D**), secretion of TNF- α (Fig 1, **E**) and IL-6 (Fig 1, **F**), and increase in intracellular Ca²⁺ levels (Fig 1, **G**). Importantly, the overall effects of AICAR on BMMCs mimicked those of SU6656, implying a functional link between Fyn inhibition and AMPK activation. However, AICAR did not affect the Fc ϵ RI-induced phosphorylation of Fyn (see Fig E1), suggesting that AMPK activation is located downstream of Fyn inhibition. Neither AICAR nor SU6656 affected Fc ϵ RI signaling components under basal conditions, whereas both agents inhibited the IgE/antigen-induced Fc ϵ RI signals and effector functions in a dose-dependent manner (see the Results section and Fig E2, *B-E*, in this article's Online Repository at www.jacionline.org), suggesting that the Fyn-regulated AMPK activity might alter a threshold for the Fc ϵ RI-mediated activation of mast cells.

To investigate the potential contribution of Fyn and AMPK to mast cell functions *in vivo*, we examined mast cell-dependent

anaphylactic reactions in mice. The PCA reaction, as evaluated by means of dye extravasation in antigen-challenged ears, was significantly reduced in mice treated with SU6656 or AICAR (Fig 1, **H**). Likewise, the PSA reaction, as evaluated by increased serum levels of histamine, LTC₄, and PGD₂, were markedly decreased by SU6656 or AICAR (Fig 1, **I**). Thus in agreement with the *in vitro* results using BMMCs, the *in vivo* manifestations of anaphylaxis can be dampened by pharmacologic inhibition of Fyn or activation of AMPK.

Knockdown of Fyn, AMPK α 2, and LKB1 reveals a Fyn-regulated LKB1/AMPK inhibitory module in BMMCs

Although SU6656 is generally used as a Fyn inhibitor, it can also inhibit other Src family kinases, such as Lyn.²⁷ To circumvent this, siRNA targeting Fyn was used to verify the role of this kinase in mast cell signaling. Treatment of BMMCs with Fyn-specific siRNA effectively, if not solely, reduced the expression of Fyn compared with that seen in cells treated with control siRNA, whereas the activity and expression level of Lyn were not altered (Fig 2, **A**). The Fyn siRNA increased the phosphorylation

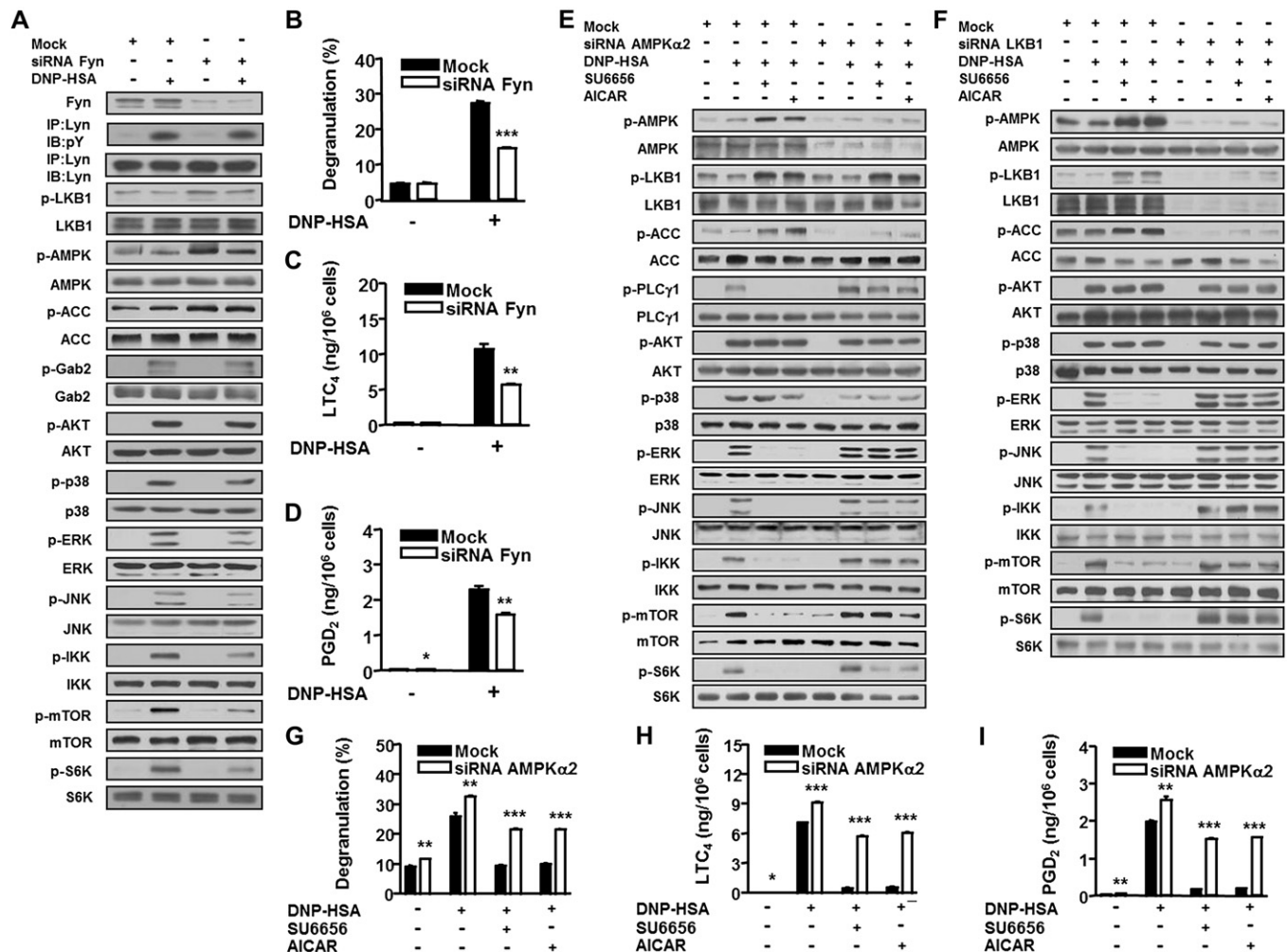


FIG 2. Effects of Fyn, AMPK α 2, or LKB1 knockdown on IgE/antigen-activated BMMCs. BMMCs were treated with siRNA for Fyn (A-D), AMPK α 2 (E and G-I), LKB1 (F), or control (Mock) for 48 hours. Then IgE/antigen-induced phosphorylation of signaling molecules (Fig 2, A, E, and F) and release of β -Hex (Fig 2, B and G), LTC₄ (Fig 2, C and H), and PGD₂ (Fig 2, D and I) were evaluated (n = 3). **P* < .05, ***P* < .01, and ****P* < .001 versus control for each treatment.

of LKB1, AMPK, and ACC independently of Fc ϵ RI stimulation (Fig 2, A, and see Fig E3 in this article's Online Repository at www.jacionline.org), confirming that activation of the LKB1/AMPK/ACC axis solely relies on depletion of Fyn activity. The Fyn siRNA-induced phosphorylation of LKB1/AMPK/ACC was significantly reduced by antigen stimulation (Fig 2, A, and see Fig E3), underscoring that Fc ϵ RI signaling counteracts the Fyn-regulated AMPK pathway. Additionally, the Fyn siRNA noticeably reduced the phosphorylation of ERK, JNK, and IKK, as well as mTOR and S6K, but not the phosphorylation of Gab2, Akt, or p38 (Fig 2, A, and see Fig E3). Furthermore, degranulation and eicosanoid generation were also reduced by the Fyn siRNA (Fig 2, B-D). Thus the effects of the Fyn siRNA essentially recapitulated those of the Fyn inhibitor SU6656. Notably, SU6656 also inhibited the Fc ϵ RI-induced phosphorylation of Lyn (see Fig E1). However, siRNA knockdown of Lyn did not affect the phosphorylation of Fyn, LKB1, AMPK, and ACC, even though it reduced the Fc ϵ RI-induced activation of ERK, JNK, p38, and IKK (see the Results section and Fig E4 in this article's Online Repository at www.jacionline.org). Furthermore, AICAR did not affect the phosphorylation of Lyn, suggesting that post-Fc ϵ RI

activation of Lyn occurs independently of AMPK. Likewise, siRNA silencing of Syk also did not affect the activation of AMPK (see the Results section and Fig E5 in this article's Online Repository at www.jacionline.org). Thus these results substantiate the specific role of Fyn in AMPK signaling.

A similar approach was used to confirm and extend our understanding of the role of AMPK in the regulation of mast cells. To this end, BMMCs were treated with an AMPK α 2-specific siRNA, which efficiently, if not solely, reduced AMPK α 2 expression (Fig 2, E). The knockdown of AMPK α 2 decreased SU6656- or AICAR-dependent phosphorylation of AMPK and ACC, without altering phosphorylation of upstream LKB1 (Fig 2, E). Fc ϵ RI-stimulated phosphorylation of PLC γ 1, ERK, JNK, IKK, mTOR, and S6K was suppressed by SU6656 or AICAR in BMMCs transfected with control siRNA, whereas siRNA silencing of AMPK α 2 rendered these phosphorylation events rather insensitive to SU6656 or AICAR (Fig 2, E). In contrast, phosphorylation of Akt and p38 remained unaffected by SU6656 or AICAR, irrespective of AMPK α 2 siRNA treatment. Similarly, silencing of LKB1 by LKB1-specific siRNA greatly, if not completely, reduced the phosphorylation of AMPK and ACC by SU6656 or AICAR and

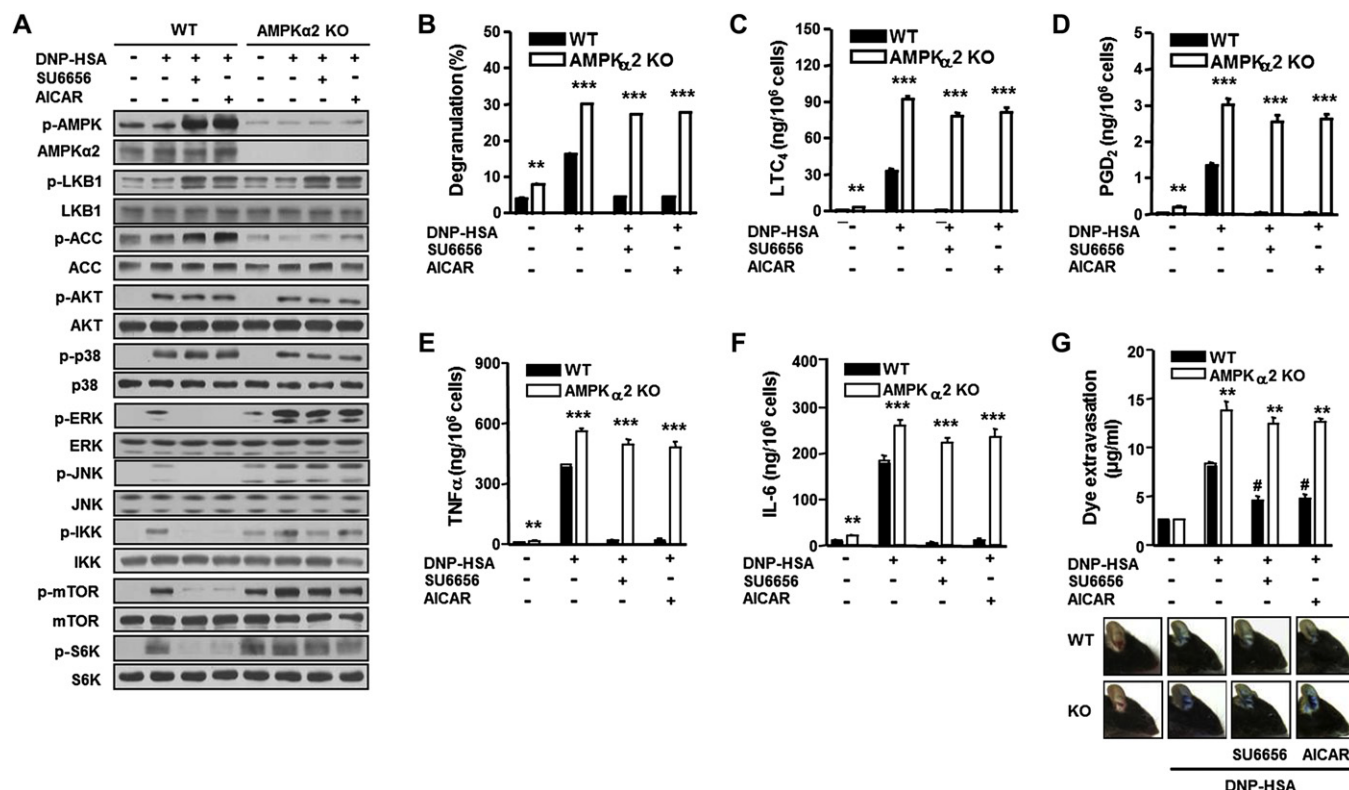


FIG 3. Gene ablation of *AMPKα2* augments FcεRI-mediated signals in mast cells. **A–F**, IgE/antigen-induced phosphorylation (*p*-) of signaling molecules (Fig 3, A) and release of β-Hex (Fig 3, B), LTC₄ (Fig 3, C), PGD₂ (Fig 3, D), and cytokines (Fig 3, E and F) in WT and *AMPKα2*^{−/−} BMMCs with or without SU6656 or AICAR (n = 3). ***P* < .01 and ****P* < .001 versus WT for each treatment. **G**, IgE/antigen-induced PCA in WT and knock-out (KO) mice with or without SU6656 or AICAR (n = 5). #*P* < .01 versus without SU6656 or AICAR and ****P* < .01 versus WT for each treatment. The bottom panel in Fig 3, G, shows representative photos of ears.

cancelled out the inhibitory effects of these agents on the phosphorylation of ERK, JNK, IKK, mTOR, and S6K, whereas the phosphorylation of Akt and p38 was again unaffected (Fig 2, F). Consistently, FcεRI-stimulated releases of β-Hex and eicosanoids were reduced to their basal levels by SU6656 or AICAR in control siRNA-treated cells, whereas these effector functions were not blunted by either of these agents in *AMPKα2* siRNA-treated cells (Fig 2, G–I). Even without addition of SU6656 or AICAR, degranulation and eicosanoid generation were slightly but significantly greater in *AMPKα2*-silenced cells than in control cells (Fig 2, G–I), which is consistent with a tendency for modest increase in the FcεRI-induced phosphorylation of PLCγ1, ERK, JNK, IKK, mTOR, and S6K in cells treated with *AMPKα2* siRNA compared with those treated with control siRNA (Fig 2, E). Thus activation of the LKB1/AMPK pathway has a negative effect on FcεRI signaling and is triggered by reduction in Fyn activity.

AMPKα2 deficiency leads to increased IgE/antigen-dependent activation of mast cells

To gain more solid evidence for the role of AMPK in mast cell activation, we used BMMCs from *AMPKα2*-null mice.²² The absence of *AMPKα2* did not influence the development of BMMCs (see the Results section and Fig E6 in this article's Online Repository at www.jacionline.org). As expected, no *AMPKα2* protein was detected in *AMPKα2*^{−/−} BMMCs (Fig 3, A). Residual weak expression of phosphorylated AMPK and phosphorylated

ACC in *AMPKα2*^{−/−} BMMCs might reflect the presence of *AMPKα1* (data not shown), which appeared to play a minor role in ACC phosphorylation. LKB1, an upstream AMPK kinase, was unaffected by *AMPKα2* deficiency (Fig 3, A). In agreement with the experiments using *AMPKα2* siRNA, significant increases in the IgE/antigen-induced phosphorylation of ERK, JNK, IKK, mTOR, and S6K, but not the phosphorylation of Akt and p38, were seen in *AMPKα2*-deficient BMMCs compared with that seen in wild-type (WT) BMMCs (Fig 3, A). Even without stimulation, the phosphorylation of ERK, JNK, IKK, mTOR, and S6K, but not Akt and p38, was increased in *AMPKα2*^{−/−} BMMCs to levels similar to those seen in antigen-stimulated WT BMMCs, indicating that *AMPKα2* plays a role in protecting these signaling components from unwanted activation in a basal state. Accordingly, IgE/antigen-dependent and even spontaneous degranulation (Fig 3, B), eicosanoid generation (Fig 3, C and D), and cytokine secretion (Fig 3, E and F) were greater in *AMPKα2*^{−/−} BMMCs than in WT BMMCs. In contrast to WT BMMCs, neither phosphorylation of ERK, JNK, IKK, mTOR, and S6K (Fig 3, A) nor effector functions (Fig 3, B–F) were significantly affected by SU6656 or AICAR in *AMPKα2*^{−/−} BMMCs. Moreover, adenoviral transfer of catalytically active *AMPKα2* (CA-AMPK) reversed FcεRI-mediated hyperresponses to normal levels in *AMPKα2*^{−/−} BMMCs (see the Results section and Fig E7 in this article's Online Repository at www.jacionline.org). Collectively, our findings demonstrate that AMPK serves as a negative-regulatory component to protect mast cells from

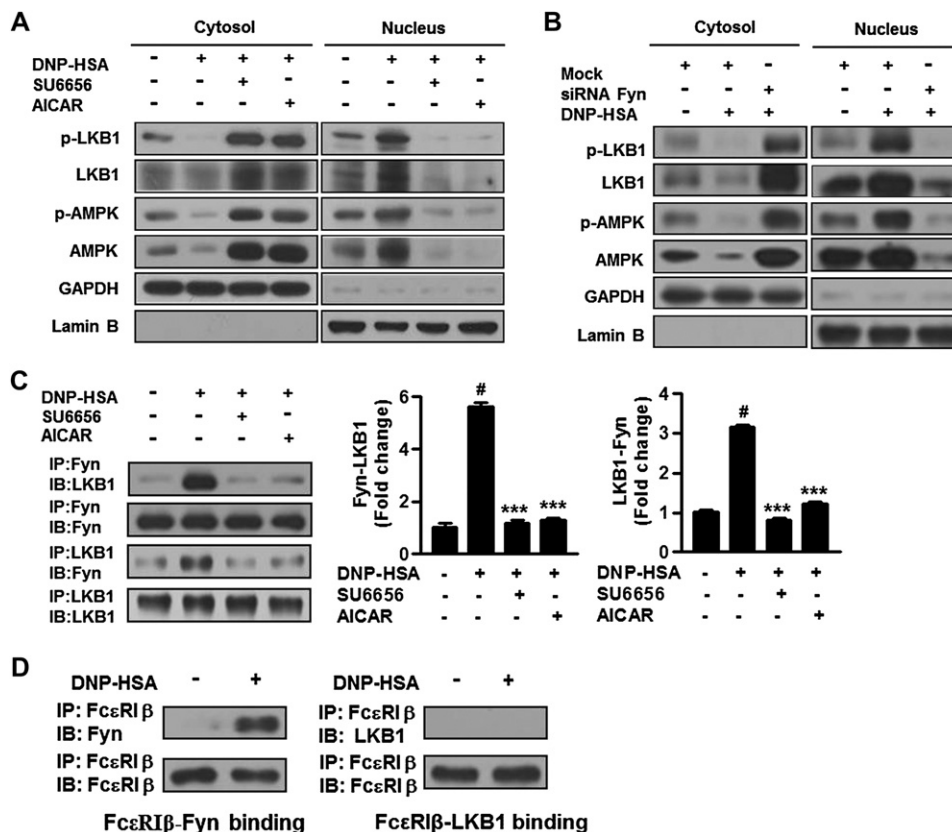


FIG 4. SU6656 or AICAR induces translocation of LKB1 from the nucleus to the cytosol. **A** and **B**, BMMCs were treated with SU6656 or AICAR (Fig 4, A) and Fyn or mock siRNA (Fig 4, B) for 48 hours, treated with or without DNP-HAS for 15 minutes, and then subjected to immunoblotting of signaling molecules in the cytosol and nucleus, with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and lamin B as respective fraction markers. **C**, Cell extracts were immunoprecipitated with anti-Fyn or anti-LKB1 antibody and immunoblotted with anti-LKB1 or anti-Fyn antibody. The relative ratios of Fyn to LKB1 levels (or *vice versa*) were determined by means of scanning densitometry, with the value for nonstimulated cells being regarded as 100% ($n = 3$). $\#P < .001$ versus without DNP-HSA and $***P < .001$ versus without SU6656 or AICAR. **D**, Cell lysates were subjected to immunoprecipitation with anti-FcεRIβ antibody and immunoblotted with anti-Fyn or anti-LKB1 antibody.

hyperactivation and that Fyn contributes to the release of FcεRI signaling from inhibitory AMPK.

To definitively prove the physiologic importance of AMPK in mast cells *in vivo*, we assessed IgE/antigen-dependent PCA in *AMPKα2*^{-/-} mice in comparison with WT mice on the C57BL/6 background. Consistent with the increased activation of *AMPKα2*^{-/-} BMMCs *in vitro*, *AMPKα2*^{-/-} mice had more advanced edema than WT mice (Fig 3, G). Moreover, in contrast to WT mice, in which the PCA reaction was blunted by SU6656 or AICAR, the enhanced anaphylaxis in *AMPKα2*^{-/-} mice was refractory to these agents (Fig 3, G). These data suggest the *in vivo* relevance of the antiallergic role of AMPK. Furthermore, adoptive transfer of WT or *AMPKα2*^{-/-} BMMCs into mast cell-deficient *Kit*^{W-sh/W-sh} mice verified the contribution of AMPKα2 in mast cells to the suppression of PCA and the sensitivity to SU6656 or AICAR (see the Results section and Fig E8 in this article's Online Repository at www.jacionline.org).

Fyn regulates the subcellular localization of LKB1

Fyn has been reported to regulate the intracellular distribution of LKB1 in muscle cells, where Fyn-phosphorylated LKB1 is sequestered into the nucleus and thereby spatially dissociated

from the substrate AMPK, whereas inhibition of Fyn results in cytosolic retention of LKB1, allowing it access to AMPK.¹² To assess whether Fyn regulates LKB1 localization in mast cells, we examined the effect of SU6656 or AICAR on the subcellular localization of LKB1. Under normal conditions, LKB1 and AMPK (both total and phosphorylated forms) were present in both the nuclear and cytosolic fractions, whereas after IgE/antigen activation, their levels in cytosol were decreased, with concomitant increases in the nucleus (Fig 4, A), implying FcεRI-induced translocation of the LKB1/AMPK from the cytosol to the nucleus. After treatment with SU6656, total and phosphorylated LKB1 and AMPK were mostly redistributed to the cytosol of IgE/antigen-activated cells (Fig 4, A). A similar result was obtained when the cells were treated with Fyn siRNA, which allowed cytosolic retention of total and phosphorylated LKB1 and AMPK (Fig 4, B). Moreover, Fyn was coimmunoprecipitated with LKB1 (and *vice versa*), even in resting cells, and this Fyn-LKB1 interaction was significantly enhanced by IgE/antigen stimulation and reversed by SU6656 (Fig 4, C). These results suggest that FcεRI-activated Fyn interacts with the LKB1/AMPK signaling complex and sequesters it into the nucleus, whereas the inhibition of Fyn activity results in cytosolic retention of LKB1 and thereby activation of AMPK in the cytosol. There

was a significant increase in the association of FcεRIβ with Fyn, whereas that with LKB1 was completely absent, in BMMCs stimulated with IgE/antigen (Fig 4, D), suggesting that LKB1 interacts with a pool of Fyn that is dissociated from the FcεRI complex after antigen activation. AICAR also exerted effects similar to SU6656 or Fyn siRNA on the cytosolic retention of LKB1 and AMPK (Fig 4, A) and on Fyn-LKB1 interaction (Fig 4, C). Thus AMPK, once activated, facilitates upstream Fyn-LKB1 interaction and LKB1 translocation, revealing a feed-forward amplification loop. ATM kinase might be one of the intermediates that links AMPK to upstream LKB1 (see the Results section and Fig E9 in this article's Online Repository at www.jacionline.org).

DISCUSSION

Clues to the functional link between Fyn and AMPK have been provided by findings of increased AMPK activity in *Fyn*-null mice²⁵ and the requirement of Fyn for sustained AMPK activity through control of the subcellular distribution of LKB1 in muscle cells.¹² In this study we have demonstrated an unexplored role for AMPK as a negative regulator of mast cell function. AMPK activity negatively regulates the basal level of phosphorylation in the FcεRI signaling cascade, thus setting up a threshold for the FcεRI-mediated activation of mast cells.

Mice lacking AMPKα2 displayed increased susceptibility to antigen-induced anaphylaxis *in vivo*, and BMMCs from these animals showed greater antigen-induced and even spontaneous MAPK and NF-κB activation, which correlated with enhanced effector functions. Moreover, the mast cell-related phenotypes observed in *AMPKα2*^{-/-} mice were recapitulated in *in vitro* studies by using siRNA knockdown, adenovirus-directed overexpression, or AICAR-induced activation of AMPKα2 or its upstream LKB1.

Although the role of Fyn in mast cells has remained a mystery because of different results from knockout or knockdown studies,⁶⁻⁹ our study now highlights that one role of Fyn is to counteract the LKB1/AMPK-mediated inhibition of FcεRI signaling by sequestering the LKB1/AMPK complex into the nucleus, thereby preventing AMPK from gaining access to its cytosolic targets. Overall, our findings emphasize that Fyn-regulated LKB1-AMPK signaling is of fundamental importance in the constitutive control of FcεRI-activated signals in mast cells. The regulatory mode of AMPK in mast cells is thus different from that in T cells, where triggering of the T-cell receptor results in rapid activation of AMPK through the calcium/calmodulin-dependent protein kinase kinase (Ca²⁺-CaMKK)-dependent pathway.²⁸

There have been several articles describing the beneficial effects of AICAR on mast cell degranulation and associated allergic diseases,^{29,30} although these studies have not provided the molecular mechanisms for the regulatory action of AMPK. Our study is the first to have used a genetic approach to comprehensively demonstrate the function of AMPK in mast cells. Several studies have pointed out the regulatory roles of AMPK in immune and metabolic systems. For instance, coordinated metabolic regulation of T-cell homeostasis involves AMPK, even though it is not always critical for adoptive immune responses.³¹ AMPK deficiency equates to defective fatty acid oxidation and promotes excessive inflammation in T cells through augmented mTOR activation.^{32,33} Downregulation of AMPK or expression of dominant-negative AMPK in macrophages exacerbates TNF-α, IL-6, and COX-2 expression in response to LPS, which might stem from the inhibition of NF-κB.³⁴ These proinflammatory responses are conversely

attenuated by introduction of CA-AMPK or pharmacologic activation by AICAR.³⁵ Aortic endothelial cells from *AMPKα2*^{-/-} mice show higher expression of endoplasmic reticulum stress markers and an increased intracellular Ca²⁺ level, both being abolished by adenoviral overexpression of CA-AMPK.³⁶ Moreover, AMPK phosphorylates IKK, leading to its inhibition *in vitro*.³⁷ These lines of evidence further highlight AMPK as a critical regulator connecting metabolism and immunity.

On the basis of our present findings, it can be speculated that reduced expression or activation of AMPK might be a contributing factor to an increased sensitivity to allergens. Evidence is now mounting that obesity is a risk factor for allergy and that asthmatic patients who are obese are likely to have more severe asthma than those who are not obese.^{38,39} The link between obesity and asthma is unlikely to be simply explained by enhancement of the "classical" forms of airway inflammation resulting from the systemic inflammatory effects of obesity itself but might involve other mechanisms.³⁸ Our present study points to a causal relationship between obesity and allergy in that the reduced AMPK activity in obese subjects could contribute to increased susceptibility to mast cell activation and thereby to allergy.

In conclusion, our present study has revealed a novel pathway that negatively regulates FcεRI-mediated activation of mast cells by AMPK. From this standpoint, agents that can activate AMPK might be useful for treatment of not only metabolic syndrome but also allergic diseases.

We thank Dr Benoit Viollet (INSERM U567) for providing *AMPKα2*^{-/-} mice.

Key messages

- AMPK suppresses FcεRI signaling in mast cells and thereby anaphylaxis.
- The LKB1/AMPK axis is counterregulated by Fyn kinase.
- AMPK, which is generally known as a metabolic controller, represents a potential drug target aimed at control of mast cell responsiveness and allergy-associated traits.

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METHODS

Culture and activation of BMMCs

Preparation, culture, and activation of BMMCs were performed as described previously.^{E1} Briefly, BMMCs were cultured in RPMI 1640 medium (Thermo Fisher Scientific, Waltham, Mass) containing 10% FBS, 100 U/mL penicillin (Thermo Fisher Scientific), 10 mmol/L HEPES (Sigma-Aldrich, St Louis, Mo), 100 μ mol/L MEM nonessential amino acid solution (Invitrogen, Grand Island, NY), and 20% pokeweed mitogen spleen cell-conditioned medium as a source of IL-3. For cell stimulation, 10⁶ cells/mL were sensitized overnight with 500 ng/mL anti-DNP IgE (Sigma-Aldrich) and then stimulated for appropriate periods with 100 ng/mL DNP-human serum albumin (HSA; Sigma-Aldrich). When the effects of 10 μ mol/L SU6656 (Calbiochem, San Diego, Calif), 1 mmol/L AICAR (Calbiochem), and 10 μ mol/L KU-55933 (Calbiochem) were examined, they were added 1 hour, 5 hours, and 1 hour, respectively, before the addition of DNP-HSA. Degranulation was evaluated by measuring β -Hex release, as described previously.^{E1} PGD₂, LTC₄, IL-6, and TNF- α were quantified with respective immunoassay kits (Cayman Chemicals, [Ann Arbor, Mich] for eicosanoids and R&D Systems [Minneapolis, Minn] for cytokines).

Local and systemic anaphylaxis in mice

PCA and PSA were elicited, as described previously.^{E1} Briefly, for PCA, 80 ng of mouse anti-DNP IgE was intradermally injected into one ear of 7-week-old male mice. After 24 hours, the mice were challenged intravenously with 60 ng of DNP-HSA after intraperitoneal administration of 20 mg/kg SU6656 or AICAR. Evans blue was extracted overnight at 63°C with formamide and measured based on absorbance at 630 nm. For PSA, all injections were performed intraperitoneally in a final volume of 100 μ L per injection. Mice were injected with 2 μ g of DNP IgE and challenged intravenously 24 hours later with 2 mg of DNP-HSA in 100 μ L of saline after intraperitoneal administration of 20 mg/kg SU6656 or AICAR for 1 hour, and blood was collected by means of cardiac puncture 5 minutes after antigen challenge. Concentrations of serum histamine were determined by using a histamine assay kit (Cayman Chemicals). Serum LTC₄ and PGD₂ levels were determined, as described above.

Western blotting

Procedures for Western blotting were described in detail previously.^{E1} Primary antibodies used were as follows. Antibodies against phosphorylated forms of LKB1 (Ser428), AMPK α (Thr172), ACC (Ser79), mTOR (Ser2448), ERK1/2, p38, JNK, Akt (Ser473), IKK α/β , PLC γ 1 (Tyr783), and Gab2 (Ser159) and those against total LKB1, AMPK α , ACC, mTOR, S6K, Akt, ERK1/2, JNK, p38, and Gab2 were from Cell Signaling Technology (Danvers, Mass). Antibodies against phosphorylated forms of S6K, IKK α/β , PLC γ 1, LAT, Syk, Fyn, Lyn, glyceraldehyde-3-phosphate dehydrogenase, and lamin B were from Santa Cruz Biotechnology (Santa Cruz, Calif). Anti-phosphotyrosine antibody was from Millipore (Temecula, Calif), and anti-AMPK α 2 antibody was from Abcam (Cambridge, Mass). Preparation of nuclear and cytosolic extracts was described previously.^{E1}

Immunoprecipitation

Cell lysates were collected with modified lysis buffer (0.1% Nonidet P-40, 50 mmol/L HEPES [pH 7.0], 250 mmol/L NaCl, 5 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride, and 0.5 mmol/L dithiothreitol). Total cell lysate (1 mg of protein equivalent) was incubated with anti-Fyn, anti-LKB1, anti-Syk, or anti-LAT antibodies for 2 hours at 4°C, and the immunocomplexes were precipitated with 20 μ L of protein A-Sepharose. These precipitates were extensively washed 3 times with ice-cold lysis buffer. Either these precipitates or total cell lysates were subjected to SDS-PAGE and immunoblotted with corresponding antibodies.

Measurement of intracellular Ca²⁺ levels

Intracellular Ca²⁺ levels were determined with the FluoForte Calcium Assay Kit (Enzo Life Sciences, Ann Arbor, Mich), as described previously.^{E2} Briefly, BMMCs were preincubated with FluoForte Dye-Loading Solution for 1 hour at room temperature. After washing the dye from the cell surface

with HBSS, the cells (5×10^4) were seeded into 96-well microplates. Then the cells were pretreated with SU6656 and AICAR for 1 hour before adding DNP-HSA. Fluorescence was measured with a fluorometric imaging plate reader at an excitation of 485 nm and an emission of 520 nm on a BMG Labtechnologies FLUOStar OPITIMA plate reader (Offenburg, Germany).

Transfection of siRNA

For experiments with siRNAs, the SMARTpool for mouse LKB1 (L-100539-01-0020), AMPK α 2 (L-100623-00-0020), Fyn (L-040112-00-0020), and Syk (L-041084-00-0020) was obtained from Dharmacon (Lafayette, Colo). Nonspecific siRNA (ONTARGETplus siCONTROL Non-Targeting Pool, D-001810-10-20) was used as a control. BMMCs were cultured for 16 hours in serum-free media and were transfected with a DharmaFECT transfection reagent (Dharmacon), according to the manufacturer's protocol, in 12-well plates (100 nmol/L siRNA per well). After 48 hours, BMMCs were sensitized with IgE with or without pretreatment with SU6656 or AICAR and then stimulated with DNP-HSA, as described above.

Adenoviral infection

The CA-AMPK adenoviral vector was constructed from a rat cDNA encoding residues 1 to 312 of AMPK α 2 and bearing a mutation of Thr¹⁷² to aspartic acid (T172D), as described previously.^{E3} BMMCs were infected with 100 multiplicity of infection of adenoviruses 1 day before the experiments.

Adoptive transfer of BMMCs into mast cell-deficient mice

Mast cell-deficient *Kit^{W-sh/W-sh}* mice were obtained from Jackson Laboratories (Sacramento, Calif). BMMCs (10⁶ per mouse) were intradermally injected into 5-week-old male *Kit^{W-sh/W-sh}* mice. After 6 weeks, the mice were subjected to IgE/antigen-induced PCA, as described above. Quantitative PCR of mast cell markers was performed with the TaqMan Gene Expression Assay System (Applied Biosystems, Foster City, Calif) on the ABI7700 Real Time PCR device (Applied Biosystems). The probe/primer sets used were Mm00456104_m1 for *Hdc*, Mm00487636_g1 for *Mcpt4*, mm00487645_m1 for *Mcpt6*, and Mm00483940_m1 for *Cpa3*. Quantitative PCR for *Gapdh* using a primer pair of 5'-ccttgagatcaacagctaccag-3' and 5'-cgccgtctacctccaccac-3' with Power SYBR Green PCR system (Applied Biosystems) was used as a control.

RESULTS

Dose-response experiments with SU6656 and AICAR

To determine whether the doses of SU6656 (10 μ mol/L) and AICAR (1 mmol/L) used in most of the *in vitro* studies were appropriate, we examined the dose-dependent effects of the compounds on phosphorylation events and effector functions in Fc ϵ RI-stimulated BMMCs. Increased phosphorylation of LKB1, AMPK, and ACC; decreased phosphorylation of PLC γ 1, ERK, JNK, p38, IKK, mTOR, and S6K; and suppressed release of β -Hex, LTC₄, and PGD₂ occurred in accordance with the incremental concentrations of SU6656 (Fig E1, C and D) and AICAR (Fig E1, E and F), with maximal effects at 10 μ mol/L and 1 mmol/L, respectively.

Neither Lyn nor Syk is involved in AMPK activation

Expression of Lyn was reduced by knockdown with a Lyn-specific siRNA to investigate the effect of reduced Lyn activity on mast cell signaling. Whereas Lyn siRNA did not substantially affect the IgE/antigen-induced phosphorylation of Fyn, mTOR, and S6K, it markedly reduced the phosphorylation of all MAPKs and IKK, modestly increased the phosphorylation of Gab2 and Akt (Fig E4, A), slightly reduced degranulation (Fig E4, B), and slightly augmented LTC₄ generation (Fig E4, C). Thus Lyn kinase can exert positive and negative effects on mast cell signals^{E4,E5} that are independent of Fyn kinase. Importantly, the basal,

FcεRI-independent phosphorylation of LKB1, AMPK, and ACC was barely affected by Lyn siRNA (Fig E4, A).

We then attempted to knockdown Syk to ascertain whether this master kinase could contribute to AMPK activation. As expected,^{E6} partial silencing of Syk expression in BMMCs by siRNA reduced the FcεRI-elicited phosphorylation of ERK, JNK, p38, IKK, and Akt (Fig E5, A) and attendant degranulation and eicosanoid synthesis (Fig E5, B-D). However, the Syk knockdown had no apparent effect on LKB1, AMPK, or ACC or on mTOR and S6K (Fig E5, A). These results suggest that the LKB1/AMPK-dependent pathway is regulated independently of the Lyn- or Syk-dependent pathway and that the ablation of Fyn dominantly influences the AMPK-mediated suppression of FcεRI signals.

Development of mast cells is unaffected by AMPKα2 deficiency

We examined whether the absence of AMPKα2 would affect the development of mast cells. Cell-surface expression of FcεRI and c-Kit (Fig E6, A), proliferation (data not shown), and expression of mast cell markers, including *Hdc* (histidine decarboxylase), *Mcpt4* (tryptase), and *Cpa3* (carboxypeptidase), before and after coculture with fibroblasts (a procedure that facilitates mast cell maturation^{E7}; Fig E6, B) did not differ significantly between WT and *AMPKα2*^{-/-} BMMCs, indicating that the absence of AMPKα2 did not profoundly affect the development and maturation of mast cells *in vitro*.

Adenoviral restoration of AMPKα2 reverses the hyperresponses in *AMPKα2*^{-/-} BMMCs

We examined whether overexpression of AMPKα2 could reverse the hyperactivation of *AMPKα2*-null BMMCs. WT and *AMPKα2*^{-/-} BMMCs were infected with c-myc–harboring adenovirus (myc-Ad-control) or those carrying a constitutively active AMPK mutant (myc-Ad-CA-AMPK). Successful transfection was confirmed by the expression of c-myc in both myc-Ad-control- and myc-Ad-CA-AMPK-infected cells and by the increase of phosphorylated AMPK and phosphorylated ACC in Ad-CA-AMPK-infected but not Ad-control-infected cells (Fig E7, A). Adenovirus did not affect basal phosphorylation and the quantity of all analyzed effectors (Fig E7, B). The total AMPKα2 protein level was not substantially increased in myc-Ad-CA-AMPK-infected cells, indicating that the presence of only a small fraction of CA-AMPK might be sufficient to drive the downstream signals.

The adenoviral transfer of CA-AMPK and not the control decreased the FcεRI-induced physiologic activation and hyperactivation of ERK, JNK, IKK, mTOR, and S6K, but not Akt or p38, in WT and *AMPKα2*^{-/-} BMMCs, respectively (Fig E7, A). Likewise, infection with Ad-CA-AMPK, but not Ad-control, significantly reduced FcεRI-mediated degranulation and eicosanoid generation (Fig E7, C-E), which paralleled the decreased phosphorylation of the signaling components (Fig E7, A), in *AMPKα2*^{-/-} BMMCs. These results indicate that restoration of AMPKα2 can reverse FcεRI-mediated hyperresponses in *AMPKα2*^{-/-} mice.

AMPK in mast cells is important for suppression of anaphylaxis *in vivo*

Considering that AMPK is widely distributed *in vivo*, the augmented PCA response observed in systemic *AMPKα2*^{-/-} mice (Fig 3, G) might not necessarily distinguish whether

AMPK is acting in mast cells, microenvironments, or both. Mast cell–deficient *Kit*^{W-sh/W-sh} mice were engrafted with WT or *AMPKα2*^{-/-} BMMCs to address this issue. After this reconstitution, the expression levels of the mast cell markers *Hdc*, *Mcpt4*, *Mcpt6*, and *Cpa3* in the ears of mice receiving *AMPKα2*^{-/-} BMMCs were similar to those of mice receiving WT BMMCs (Fig E8, A), confirming that AMPKα2 deficiency in mast cells does not affect their tissue residence and maturation. The PCA-induced edema was elicited in both WT BMMC–transferred and *AMPKα2*^{-/-} BMMC–transferred mice in comparison with that seen in the parent *Kit*^{W-sh/W-sh} mice, which were insensitive to antigen challenge (Fig E8, B). AICAR reversed the anaphylactic response to almost the basal level in mice that had received WT BMMCs, whereas it did not affect the response in mice that had received *AMPKα2*^{-/-} BMMCs, underscoring the contribution of AMPK in mast cells to AICAR sensitivity. However, unlike the systemic AMPKα2 deficiency (Fig 3, G), the levels of edema in mice receiving WT BMMCs and those receiving *AMPKα2*^{-/-} BMMCs were comparable (Fig E8, B), indicating that the augmented PCA response observed in systemic *AMPKα2*^{-/-} mice might involve the role of this kinase in both mast cell–intrinsic and mast cell–extrinsic locations.

Given that the activation of AMPK is influenced by nutritional status, we next performed PCA in these mast cell–reconstituted mice after overnight fasting, a condition that is known to activate AMPK. In WT BMMC–reconstituted mice the edema under fasting conditions was less than that under feeding conditions and was insensitive to AICAR (Fig E8, B), probably because AMPK had already been activated by nutrition deprivation. In contrast, in mice reconstituted with *AMPKα2*^{-/-} BMMCs, PCA remained high and AICAR insensitive, even after fasting (Fig E8, B). These results suggest that physiologic (nutrition deprivation) or pharmacologic (AICAR) activation of AMPK in mast cells negatively regulates FcεRI-dependent mast cell activation and the attendant anaphylactic response *in vivo*.

ATM kinase might link AMPK to upstream LKB1

The finding that AICAR suppresses LKB1 phosphorylation suggests that once AMPK is activated, it facilitates upstream Fyn–LKB1 interaction and LKB1 translocation. KU-55933, an inhibitor of ATM kinase that is activated by AMPK-directed phosphorylation and then phosphorylates and activates LKB1,^{E8} counteracted the AICAR-induced phosphorylation of LKB1 and AMPK (Fig E9), suggesting that ATM might be one of the intermediates that links AMPK to upstream LKB1. However, neither AICAR nor KU-55933 affected FcεRI-induced tyrosine phosphorylation of Fyn (Fig E9), indicating that the LKB1/AMPK activation loop through ATM occurs downstream of receptor-proximal Fyn activation.

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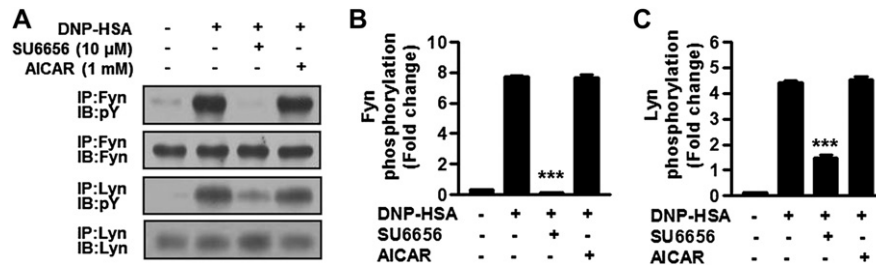


FIG E1. SU6656, but not AICAR, blocks Fyn and Lyn phosphorylation. BMMCs were sensitized with IgE with or without 10 μ M/L SU6656 or 1 mM/L AICAR pretreatment and then stimulated with DNP-HSA for 15 minutes. **A**, Immunoprecipitates of Fyn and Lyn were analyzed by means of immunoblotting with anti-phosphotyrosine antibody, followed by reprobing with anti-Fyn and anti-Lyn antibody, respectively. **B**, Immunoblot; **IP**, immunoprecipitation. **B** and **C**, The relative ratios of phosphorylated to total Fyn (Fig E1, **B**) and Lyn (Fig E1, **C**) were determined by using scanning densitometry ($n = 3$). *** $P < .001$ versus without SU6656 or AICAR.

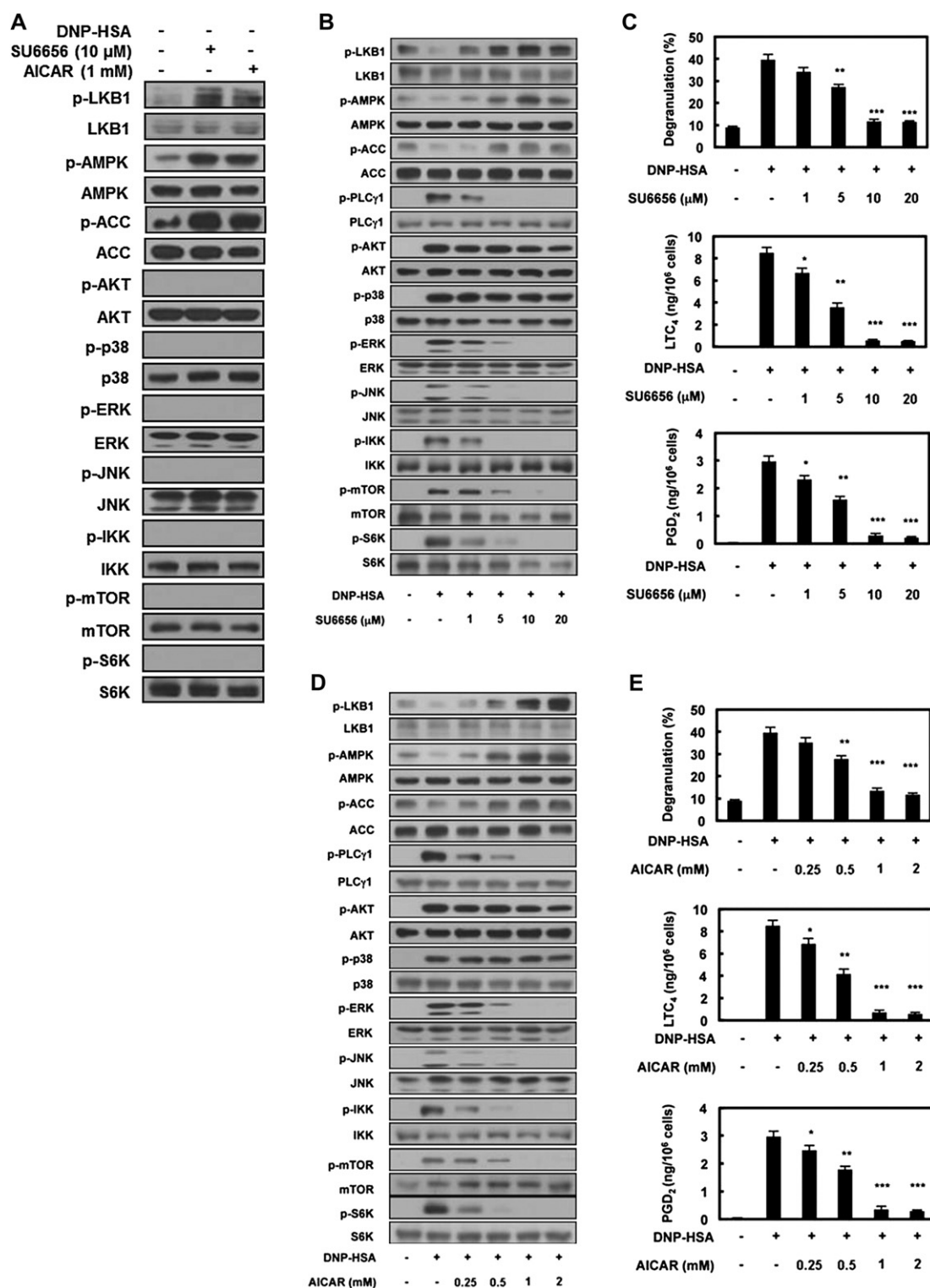


FIG E2. SU6656 or AICAR dose-dependently inhibits IgE/antigen-activated mast cell signals. **A**, BMMCs were treated with 10 μ M/L SU6656, 1 mM/L AICAR, or vehicle for 1 hour without antigen stimulation, and the phosphorylation and expression of signaling molecules were analyzed by means of immunoblotting with indicated antibodies. A representative result of 3 independent experiments is shown. **B-E**, BMMCs pretreated for 1 hour with various doses of SU6656 (Fig E2, **B** and **C**) or AICAR (Fig E2, **D** and **E**) were stimulated with IgE/antigen for 15 minutes and then analyzed for phosphorylation of signaling molecules (Fig E2, **B** and **D**) and for effector functions, including β -Hex, LTC₄, and PGD₂ release ($n = 3$). * $P < .05$, ** $P < .01$, and *** $P < .001$ versus without the compounds (Fig E2, **C** and **E**).

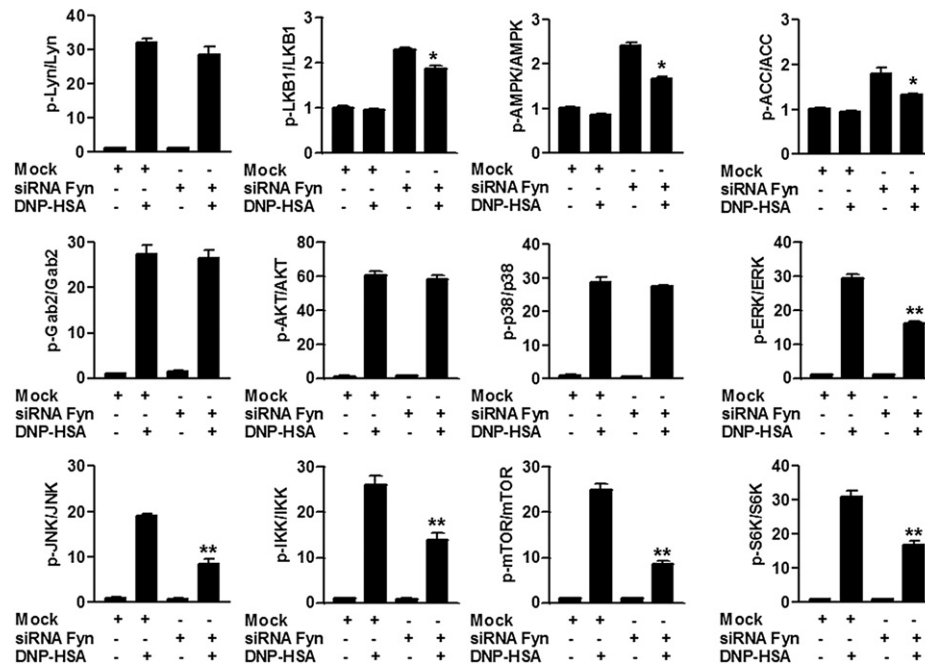


FIG E3. Densitometric analysis of the effects of Fyn knockdown on phosphorylation of signaling molecules in BMMCs. BMMCs were treated with siRNA for Fyn or control (*Mock*) for 48 hours. Then IgE/antigen-induced phosphorylation of signaling molecules was evaluated (see Fig 2, A). The relative ratios of phosphorylated and total proteins were determined by means of scanning densitometry ($n = 3$). * $P < .05$ versus siRNA Fyn-treated cells without antigen and ** $P < .01$ versus antigen-stimulated mock-treated cells.

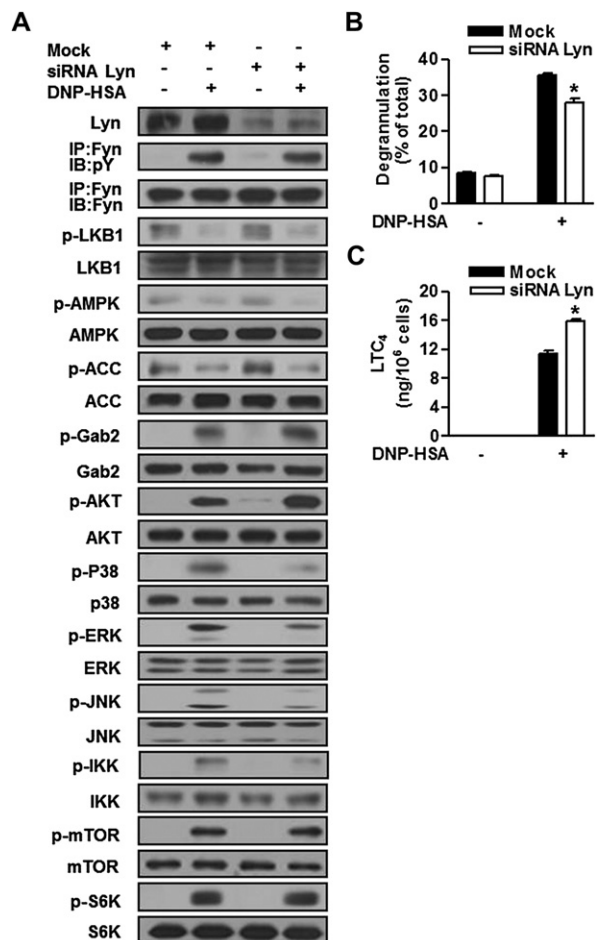


FIG E4. Knockdown of Lyn does not affect the LKB1/AMPK axis in BMMCs. BMMCs were treated with siRNA for Lyn or control (*Mock*) for 48 hours. Then IgE/antigen-induced phosphorylation of signaling molecules (**A**) and release of β -Hex (**B**) and LTC₄ (**C**) were evaluated ($n = 3$). * $P < .05$ versus antigen-stimulated mock-treated cells.

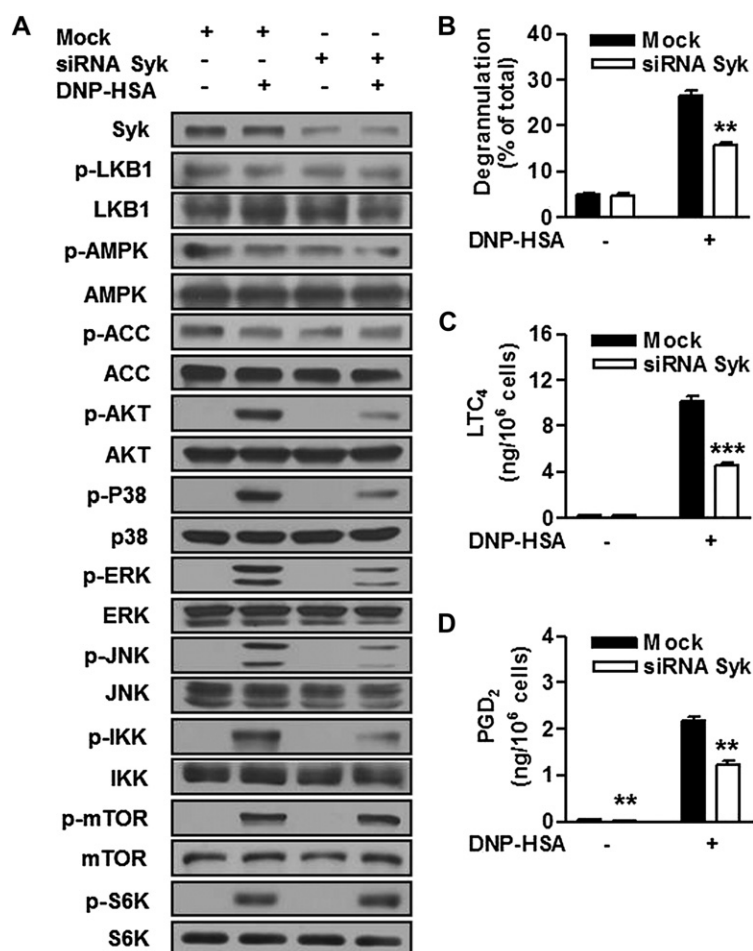


FIG E5. Knockdown of Syk does not affect the LKB1/AMPK axis in BMMCs. **A**, BMMCs were treated with siRNA specific for Syk or control (*Mock*) for 48 hours. Then IgE/antigen-induced phosphorylation of signaling molecules (**A**) and release of β -Hex (**B**), LTC₄ (**C**), and PGD₂ (**D**) were evaluated ($n = 3$). ** $P < .01$ and *** $P < .001$ versus control for each treatment.

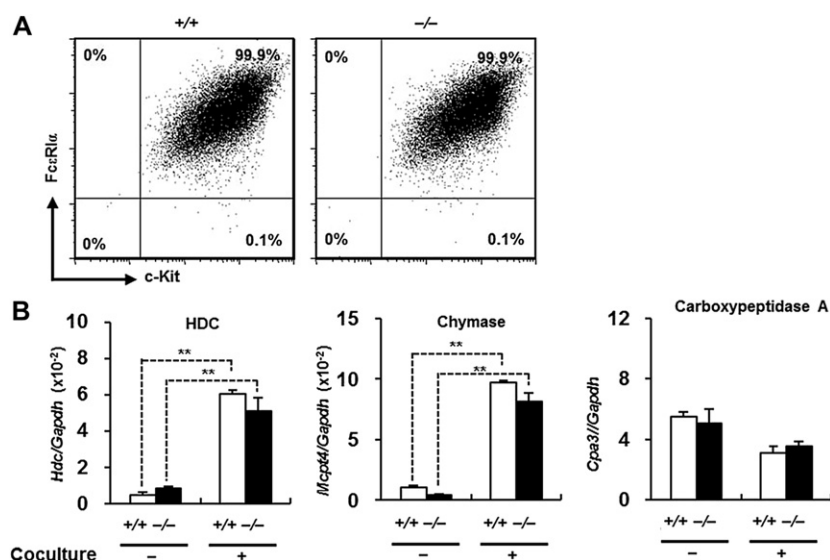


FIG E6. Absence of AMPK α 2 does not affect mast cell differentiation. **A**, BMMCs from WT and AMPK α 2^{-/-} mice were analyzed by means of flow cytometry with fluorescein isothiocyanate-labeled anti-Kit antibody (clone 2B8, BD Biosciences, San Jose, Calif) and phycoerythrin-labeled anti-FcεRIα antibody (clone MAR-I, eBioscience, San Diego, Calif) by using a FACSARIA III (BD Biosciences) with FlowJo (TreeStar, Ashland, Ore) software. **B**, WT and AMPK α 2^{-/-} BMMCs were cocultured with Swiss 3T3 fibroblasts in the presence of stem cell factor for 4 days to allow their maturation.^{E5} Then the cells with (+) or without (-) coculture were analyzed for mRNA expression of histidine decarboxylase (*HDC*), chymase (mast cell protease 4), and carboxypeptidase A by using real-time PCR (n = 6). ***P* < .01.

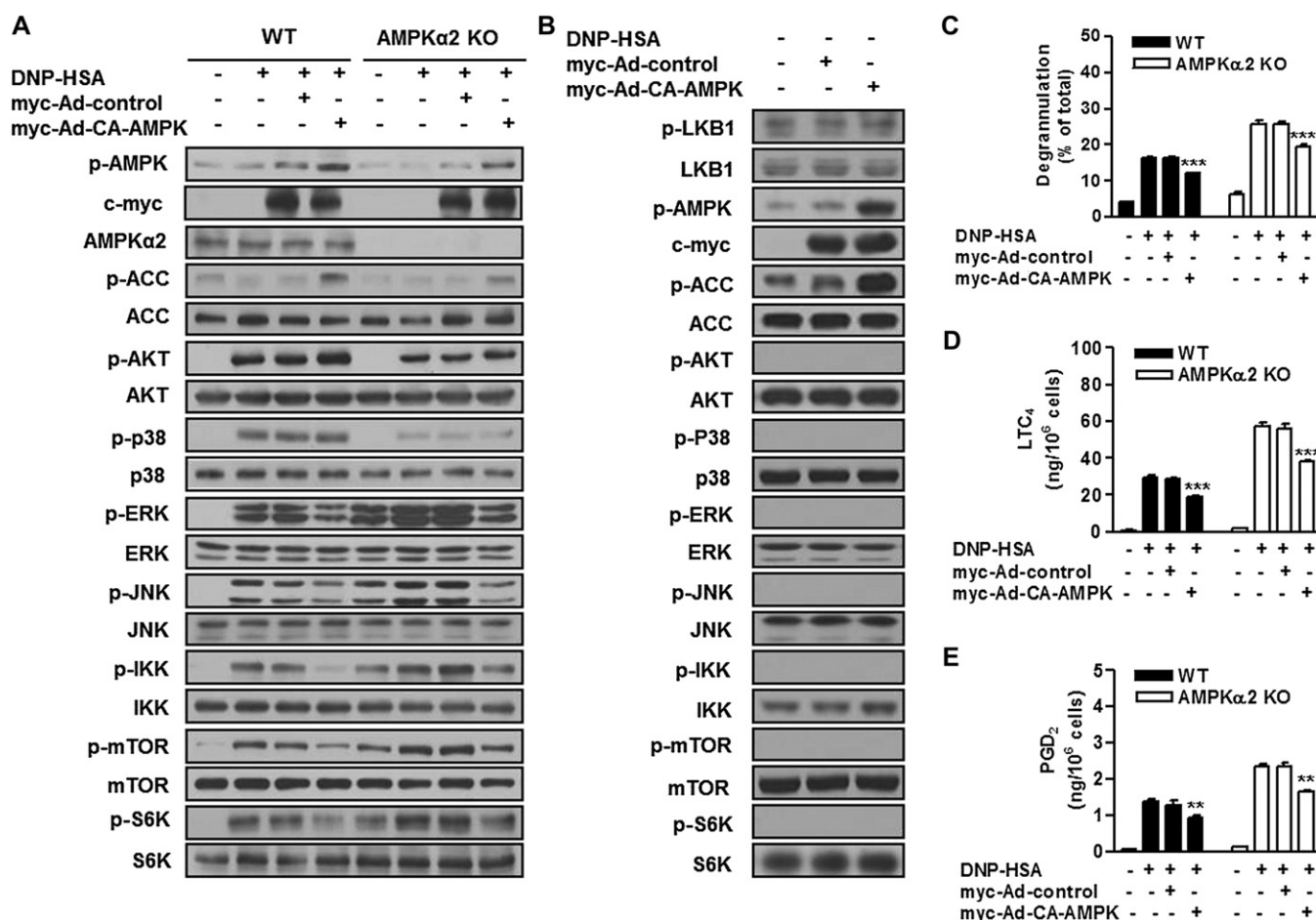


FIG E7. Adenoviral overexpression of CA-AMPK alleviates FcεRI-mediated mast cell signals in BMMCs isolated from WT and *AMPKα2*^{-/-} mice. **A**, Adenoviruses carrying myc-Ad-CA-AMPK or myc-Ad-control were used to infect WT and *AMPKα2*^{-/-} BMMCs. Then IgE/antigen-induced phosphorylation of signaling molecules was examined. **B**, Adenovirus alone did not affect the phosphorylation and expression levels of signaling molecules. Adenovirus infection was detected by using anti-c-myc antibody. Data in Fig E7, A and B, are representative of at least 3 independent experiments. **C-E**, In the experiments shown in Fig E7, C, release of β-Hex (Fig E7, C), LTC₄ (Fig E7, D), and PGD₂ (Fig E7, E) were evaluated (n = 3). **P < .01 and ***P < .001 versus Ad-control-transfected cells. KO, Knockout.

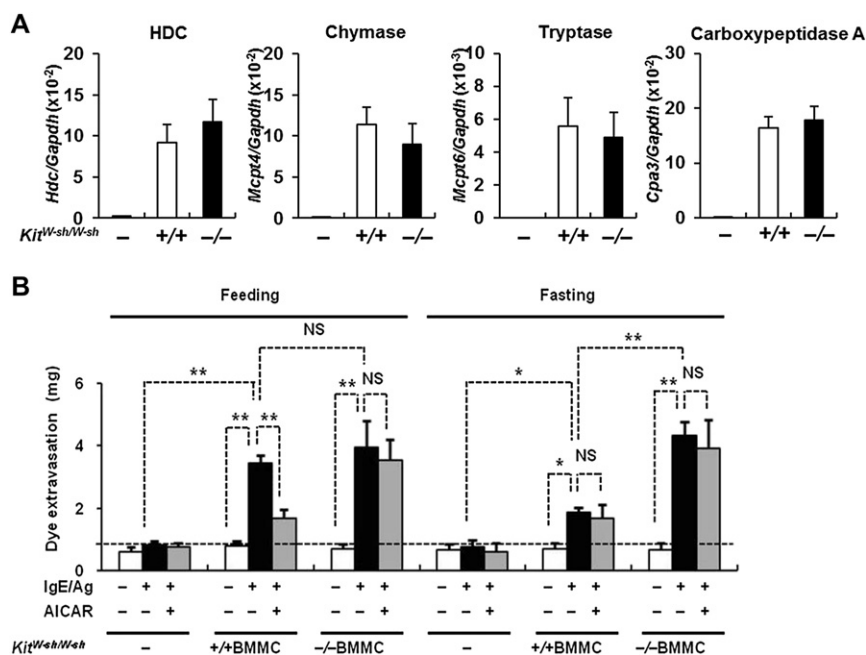


FIG E8. Lack of AMPK α 2 in mast cells significantly increases anaphylaxis in mice. **A**, Expression of mRNAs for histidine decarboxylase (*HDC*), chymase, tryptase (mast cell protease 6), and carboxypeptidase A in the ears of *Kit^{W-sh/W-sh}* mice reconstituted with WT and AMPK α 2^{-/-} BMMCs ($n = 6$). **B**, *Kit^{W-sh/W-sh}* mice with or without transfer of WT (+/+) or AMPK α 2^{-/-} (-/-) BMMCs were fed *ad libitum* (feeding) or fasted for 24 hours (fasting) and then subjected to PCA with or without AICAR treatment ($n = 6$). * $P < .05$ and ** $P < .01$. NS, Not significant.

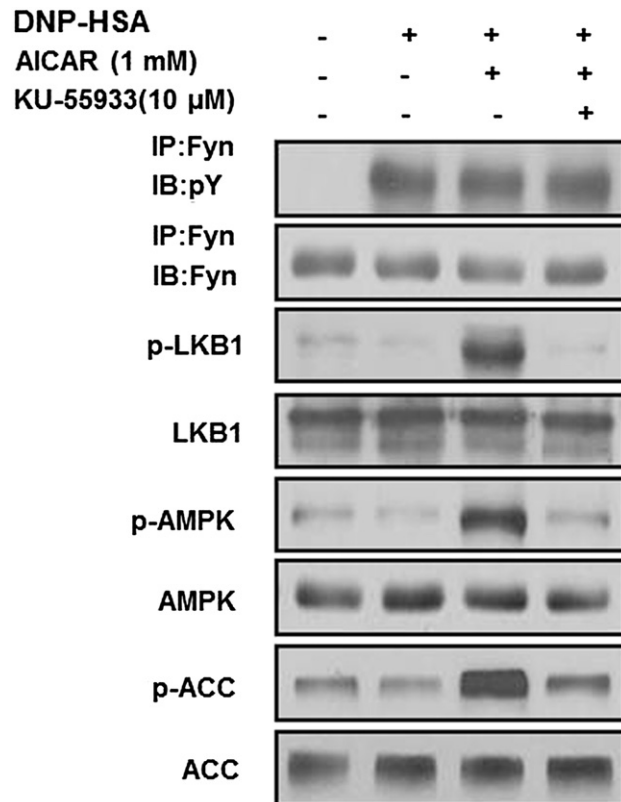


FIG E9. Effects of AICAR and KU-55933 (ATM inhibitor) on the AMPK pathway. IgE-sensitized BMMCs with or without AICAR or KU-55933 were stimulated with DNP-HSA for 15 minutes and taken for immunoblotting. Immunoprecipitates with anti-Fyn antibody were analyzed by means of immunoblotting with anti-phosphotyrosine antibody, followed by anti-Fyn antibody.