Myc-induced AMPK-phospho p53 pathway activates Bak to sensitize mitochondrial apoptosis

Anni I. Nieminen1,3, Vilja M. Eskelinen4,5, Heidi M. Haikala6, Topi A. Tervonen4, Yan Yan4, Johanna I. Partanen7, and Juha Klefström2,8

*Translational Cancer Biology Research Program and Institute of Biomedicine, and 2Institute of Biotechnology, Biomedicum Helsinki, University of Helsinki, 00014, Helsinki, Finland

Edited by Karen H. Vousden, The Beatson Institute for Cancer Research, Glasgow, United Kingdom, and accepted by the Editorial Board March 21, 2013 (received for review May 23, 2012)

Oncogenic transcription factor Myc deregulates the cell cycle and simultaneously reprograms cellular metabolism to meet the biosynthetic and bioenergetic needs of proliferation. Myc also sensitizes cells to mitochondria-dependent apoptosis. Although metabolic reprogramming has been circumstantially connected to vulnerability to apoptosis, the connecting molecular pathways have remained poorly defined. Here, we show that Myc-induced altered glutamine metabolism involves ATP depletion and activation of the energy sensor AMP-activated protein kinase (AMPK), which induces stabilizing phosphorylation of p53 at Ser15. Under influence of Myc, AMPK-stabilized tumor suppressor protein p53 accumulates in the mitochondria and interacts with the protein complex comprised of B-cell lymphoma 2 (Bcl-2) antagonist/killer (Bak) and Bcl2-like 1 (Bcl-xL). Mitochondrial p53 induces conformational activation of proapoptotic Bak without disrupting the Bak–Bcl-xL interaction. Further liberation of Bak specifically from the p53-activated Bak–Bcl-xL complex leads to spontaneous oligomerization of Bak and apoptosis. Thus, Myc-induced metabolic changes are coupled via AMPK and phospho-p53 to the mitochondrial apoptosis effector Bak, demonstrating a cell-intrinsic mechanism to counteract uncontrolled proliferation.


The authors declare no conflict of interest.

This article is a PNAS Direct Submission. K.H.V. is a guest editor invited by the Editorial Board.

Frequently available online through the PNAS open access option.

1A.I.N. and V.M.E. contributed equally to this work.

2To whom correspondence should be addressed. E-mail: juha.klefstrom@helsinki.fi.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1208530110/-/DCSupplemental.

www.pnas.org/cgi/doi/10.1073/pnas.1208530110

PNAS Early Edition | 1 of 10

Healthy cells coordinate the cell cycle and growth according to the supply of nutrients by slowing down the cell cycle under low supply. Oncogenic transcription factor Myc deregulates cell cycle independently of the extracellular nutrient status, and such cells rapidly succumb to apoptosis if deprived of glucose or, particularly, of glutamine (1–3). The strict dependence of the viability of Myc overexpressing cells on excess glucose and glutamine has been attributed to Myc-induced metabolic reprogramming (metabolic transformation), which fuels autonomous patterns of cell proliferation and renders cells “addicted” to reprogrammed metabolic pathways (4–6). However, the mechanisms coupling metabolic reprogramming and apoptotic machinery are poorly understood.

Glucose and glutamine are the main nutrients that cultured mammalian cells catabolize for energy production and biosynthesis (7). Myc induces up-regulation of proteins responsible for glucose uptake and glycolysis (6, 8). Moreover, Myc stimulates glutamine uptake and metabolism through regulation of glutamine transporters and glutaminase (GLS) (3, 9). Deregulated Myc can divert glucose away from mitochondrial metabolism and reprogram metabolic metabolism toward using glutamine as the main oxidizable substrate for maintenance of the tricarboxylic acid cycle and ATP production (3, 9). In addition to providing carbon for bioenergetic reactions, glutamine is also the obligate donor of nitrogen in biosynthesis of nucleic acids and the primary source of nitrogen for the synthesis of nonessential amino acids (4). These changes in protein expression and nutrient utilization set the stage for Myc-induced reprogramming of cell metabolism, which on the whole is seen as a molecular adaptation to the high proliferation rate of cancerous cells. A fundamental feature of cancer cell-associated metabolic programs is inefficient ATP production (7), and also Myc-mediated metabolic reprogramming has been associated with depletion of cellular ATP levels (2). B-cell CLL/Lymphoma 2 (Bcl-2) and Bcl2-like 1 (Bcl-xL) suppress the glutamine starvation-mediated apoptosis, which indicates involvement of the mitochondrial apoptosis pathway (2, 3).

The cellular energy status is sensed by AMP-activated protein kinase (AMPK), which monitors shifts in the cellular AMP/ATP ratio. The kinase activating signals come from declining ATP and increasing AMP and ADP levels, resulting in the AMP and ADP bound active heterotrimeric AMPK complex (10–12). In normal physiological conditions, active AMPK dynamically reprograms ATP generating and consuming processes so as to restore ATP levels in the cell (5). However, the classical view that AMPK acts only as a biochemical toggle switch between ATP-consuming anabolic and ATP-consuming catabolic processes is changing as recent findings have provided evidence that AMPK also coordinates cell proliferation with availability of carbon sources and participates in apoptosis (13, 14). Under low glucose conditions, AMPK induces phosphorylation of tumor suppressor protein p53 at serine 15, which mediates AMPK-dependent cell cycle arrest (13). However, in the absence of tumor cells, low glucose induces AMPK and p53-dependent apoptosis (14).

In addition to nutrient deprivation, Myc renders cells sensitive to the apoptotic action of death receptor ligands and diverse stress signals such as limited growth factor supply or hypoxia (15–17). Therefore, it is believed that Myc sensitizes cells to apoptosis via some general mechanism, which promiscuously renders cells sensitive to different triggers of apoptosis. Bcl-2 and Bcl-xL efficiently inhibit Myc-induced apoptosis, which mechanism is often crucial for progression of Myc-driven tumorigenesis (16). Bcl-2 and Bcl-xL together with myeloid cell leukemia sequence 1 (Mcl-1) and BCL2-related protein A (A1) form a group of antiapoptotic Bcl-2 family members, which antagonize proapoptotic multidomain Bcl-2 family members and BCL2-antagonist/killer (Bak) and Bcl-2-associated X protein (Bax). In healthy cells, Bak and Bax exist as globular monomeric proteins comprised of nine α-helices buried in the protein structure. Apoptotic stimuli induce a series of conformational rearrangements, which expose the N terminus (including α1 helix), the Bcl-2 homology domain 3 (BH3) domain (α2), and, specifically in Bax, the mitochondrial outer membrane anchoring domain (α9) (18). Exposed BH3 domain binds to the hydrophobic surface groove on another member, which produces Bak or Bax
homodimers. These homodimers have competence to further assemble into pore-forming oligomers, which contributes to mitochondrial outer membrane permeabilization (MOMP) and leads to lethal activation of effector caspases (18, 19). However, the BH3:groove interaction can also take place with antiapoptotic Bcl-2 family members, which blocks apoptosis by preventing formation of Bak and Bax homooligomers (18). Implementation of Myc-induced apoptosis requires Bak and Bax although the specific requirement for effector may vary from one cell type to another (20–22). The third category of Bcl-2 family proteins includes proapoptotic BH3-only family members such as BH3 interacting domain death agonist (Bid), Bcl-2–like 11 (Bim), and Bcl-2–binding component 3 (Puma), which are activators of Bak and Bax. The activators generally act by neutralizing antiapoptotic Bcl-2 proteins via BH3:groove interactions, and, in addition, a subset of BH3-only proteins can directly activate Bak and Bax (23–25).

Besides the Bcl-2 family, a central element in the Myc-induced apoptotic program and roadblock for Myc-induced tumorigenesis is the tumor suppressor protein p53 (17). Multifunctional p53 has different ways to engage into Myc-driven apoptotic programs. For example, in lymphoid cells, Myc transcriptionally up-regulates tumor suppressor protein ARF to induce E3 ubiquitin-protein ligase Mdm2 (HDM2 in humans) routed p53 stabilization (26). Further downstream, p53 can mediate apoptosis via transcription-dependent mechanisms, which involve up-regulation of proapoptotic proteins, or transcription-independent mechanisms, and both pathways can interact with the mitochondrial

---

**Fig. 1.** Bcl-xL directly controls conformational activation of Bak. (A and B) Bcl-xL silencing induces conformational activation of Bak. MCF10A cells were transduced with control or Bcl-xL targeted lentiviral shRNA (shbcl-xL) and immunostained on coverslips with N-terminal Bak antibody (Ab-1). (A) Immunofluorescence (IF) images demonstrating N-terminal exposure of Bak. (B) Quantification of the cells with conformationally active (N terminus exposed) Bak. Cells were treated as in A. (C) Overexpression of Bcl-xL blocks Myc-induced conformational activation of Bak in MCF10A-MycER cells. (C) IF images of cells expressing activated Myc targeted lentiviral shRNA (shbcl-xL) and immunostained on coverslips with N-terminal Bak antibody (Ab-1). (D) Western blot analysis shows expression of Bak and Bcl-xL, in breast cancer cell lines. (E) ABT-737 induces conformational Bak activation in breast cancer cell lines. Cells were treated with ABT-737 for 24 h and quantitated as in B. (F) Formation of Bak and Bax oligomers in apoptosis sensitizing and inducing conditions. Cells were treated as in E and lysed. Proteins were chemically cross-linked with BMH, and the mitochondria-enriched heavy membrane fractions were isolated and analyzed by Western blotting using antibodies for Bak and Bax. For quantitative cell analyses, at least 200 cells were scored per each treatment. The graphs represent mean ± SD of at least three separate experiments. *P < 0.05.
apoptosis pathway (27). The transcription-independent p53 pathways of apoptosis have been attributed to translocation of stabilized p53 to mitochondria, where p53 can directly interact with Bak, Bax, and antiapoptotic Bcl-2 family members (28, 29).

Acute Myc activation induces conformational activation of Bak but not morphological apoptosis in nontransformed human mammary epithelial MCF10A cells (20). Nonetheless, Bak is required for the apoptotic function of Myc in these cells. Here, we establish that conformational activation of Bak is a specific marker of the sensitized mitochondrial apoptosis pathway. Our investigations of the molecular underpinnings mediating Myc-induced conformational activation of Bak show that deregulation of Myc changes the cellular ADP/ATP ratio and leads to AMPK activation, which induces ser15 phosphorylation and mitochondrial accumulation of p53. Mitochondrial phospho-p53 functionally reorganizes the Bak–Bcl-xL complex and activates Bak. This study exposes a surprisingly direct molecular pathway coupling Myc-induced metabolic reprogramming via AMPK and p53 to mitochondrial apoptotic machinery, thus defining a framework for understanding tumor cell vulnerabilities in the context of transformation-specific metabolic alterations.

Results

Conformationally Altered Bak Is a Distinct Marker of Apoptosis Sensitized Cells. Activation of Myc sensitizes mammary epithelial cells to apoptosis, which occurs concomitantly with N-terminal exposure of Bak in the mitochondria (Fig. 1 and Fig. S1) (20). Bak is also a central mediator of Myc-dependent apoptosis in these cells (Fig. S1 A–G) (20). We wished to determine whether antiapoptotic Bcl-2 family members control the conformational status of Bak and thereby silenced Bcl-xL, Mcl-1, and A1 in MCF10A cells expressing a conditionally active, tamoxifen inducible Myc fusion protein (MycER) (Fig. S1H). Silencing of Bcl-xL, but not the two other proteins, induced spontaneous Bak activation independently of Myc (Fig. 1 A and B). We also found that overexpression of Bcl-xL prevents the Myc-induced conformational activation of Bak (Fig. 1 C and D). To determine whether Bcl-xL controls conformational status of Bak via physical interaction, we neutralized Bcl-xL with ABT-737. ABT-737 is a pharmacological small molecule that blocks the BH3-binding surface groove in Bcl-2, Bcl-xL, and Bcl2-like 2 (Bcl-w) (but not in Mcl-1) (30). Like Myc, ABT-737 also induced conformational activation of Bak and sensitized cells to death receptor pathway inducing TNF-related apoptosis inducing ligand (TRAIL) (Fig. 1E). We observed conformational activation of Bak only in the cell populations undergoing morphological apoptosis, which association we have reported earlier (20). ABT-737 generally promoted conformational activation of Bak in a wide variety of cell lines, including breast cancer cell lines and other epithelial cell types (Fig. 1 F and G and Fig. S1I; and see Fig. 7 and Fig. S3 C and D). We asked whether the Myc-induced conformational activation of Bak is a discrete step from oligomerization of Bak, which generates the pore forming activity and MOMP (18). In 1,6-bismaleimidohexane (BMH) cross-linking

Fig. 2. Myc induces cytosolic and mitochondrial accumulation of Ser15 phosphorylated p53. (A) Immunoprecipitation (co-IP) analysis of interactions between Bak and Bcl-xL or Mcl-1 in MCF10A-MycER cells. Cells were treated as in Fig. 1E, and polyclonal Bak antibody (H-211) was used for pulldowns. IgG, antibody only control. (B) p53 protein levels in cell fractions. MCF10A-MycER cells were treated as in Fig. 1E and lysed, and cell fractions were analyzed by Western blotting. Antibodies for CoxIV (mitochondria), Lamin B (nuclear envelope), and tubulin (cytosol) verify sample purity. (C) p-p53 (S15) in total lysate and mitochondrial fractions. MCF10A-MycER cells were treated as in Fig. 1E. Actin (cytosol) and Bak (mitochondria) are sample controls. (D) Cytosolic expression of Ser-18 phosphorylated p53 in the mouse mammary gland. Representative images of immunohistochemically stained tissue sections from control mice and WAP-Myc mice exposed to two sequential pregnancies. (E and F) Percentages of epithelial cells exhibiting phosphor-Ser18 or Ki-67 positive immunostaining in the samples. Images representing samples from 4 or 11 (n) separate mice were analyzed blindly. Cells with clear cytosolic staining pattern were scored whereas cells exhibiting unspecific apical border/luminal staining were excluded from analyses. ***P < 0.0005.
analysis, we observed prominent formation of high-order oligomers of Bak and Bax in the cell populations induced to undergo apoptosis (Fig. 1H). However, neither Myc nor ABT-737 alone was sufficient to trigger Bak or Bax oligomerization (Fig. 1H).

The results establish Bcl-xL-controlled conformational Bak activation as a distinct marker of the sensitized mitochondrial apoptosis pathway, contrasting with conformational Bak activation present only in dying populations of mammary epithelial cells.

**Myc Induces Stabilization and Mitochondrial Accumulation of p53.**

The results raised the possibility that Myc-induced conformational activation of Bak involves dissociation of the Bak–Bcl-xL interaction or down modulation of Bcl-xL expression. However, Myc activation did not alter Bcl-xL expression or Bak–Bcl-xL interaction in MCF10A cells (Fig. 2A). As expected, ABT-737 dissociated Bak from Bcl-xL but not from Mcl-1 (Fig. 2A). We concluded that Myc activates Bak via an alternative mechanism acting upstream of the Bak–Bcl-xL interaction.

Bak has around 30 interaction partners [Protein Interaction Network Analysis (PINA)] including p53, which is an established mediator of Myc-dependent apoptosis. We found that activation of p53 significantly elevates p53 levels in the mitochondria and cytosol (Fig. 2B). Myc did not affect p53 mRNA levels (Fig. S1J), but the Myc-induced mitochondrial p53 was strongly phosphorylated at the N-terminal Ser15 site (Fig. 2C). Phosphorylation of this N-terminal site prevents p53 from interacting with its negative regulator Mdm2, therefore stabilizing p53 (31, 32).

---

**Fig. 3.** Mitochondrial accumulation of p53 induces conformational activation of Bak. (A) Nutlin-3a induces stabilization of p53 in MCF10A cells. Cells were treated for 24 h with indicated concentrations of Nutlin-3a and analyzed by Western blotting. (B) Western blot showing p53 levels in the mitochondrial and cytosolic cell fractions. MCF10A cells were treated with 10 μM Nutlin-3a for 24 h before preparation of cell fractions. Bak and COXIV are markers of the mitochondrial fractions, and GAPDH is a marker of the cytosolic fraction. (C) Quantification of Bak and Bax activation in the cells treated with 20 μM Nutlin-3a for 24 h. Cells were scored as in Fig. 1. (D) Status of Bak-Bcl-xL interaction in MCF10A cells treated with 1 μM ABT-737 or 10 μM Nutlin-3a for 24 h. Co-IP analysis was performed as in Fig. 2A. (E) DNA damage, but not Myc, induces nucleoplasmic accumulation of Ser15 phosphorylated p53. p-p53 (Ser15) antibody also recognizes nucleolar p53, which generates some unspecific background. (F) Quantification of the cells with nucleoplasmic p53. MCF10A-MycER cells were treated for 24 h with 4-OHT, 1 μM ABT-737, 10 μM Etoposide, or 0.1 μg/ml Doxorubicin. Cells were scored as in Fig. 1. (G) Quantification of the cells with conformationally active Bak or Bax. MCF10A-MycER cells were treated and scored as above. (H) DNA damage, but not Myc, induces up-regulation of the p53 target p21. Western blot analysis of p21 levels in MCF10A cells after 24 h Myc activation or Doxorubicin treatment. (I) Nutlin-3a and Etoposide but not Myc induce up-regulation of the p53 target genes GADD45 and TIGAR. Myc was activated for 24 h or cells were treated with 10 μM Nutlin-3a or Etoposide for 24 h before qPCR analysis of mRNA levels. SD represent values from three different experiments. (J) Western blot analysis of MCF10A-MycER and MCF10A p53null-MycER cells demonstrating lack of p53 and expression of MycER. (K) Lenti-virally delivered wild-type p53 and p53<sup>WT</sup> transcription-deficient mutant rescue Myc’s ability to activate Bak in p53null cells. Cells were infected with lentiviruses expressing p53<sup>WT</sup> or p53<sup>Del</sup> and allowed to grow for 48 h, and, subsequently, Myc was activated for 24 h. Cells with active Bak were scored as in Fig. 1. **P < 0.005.
We further investigated whether Myc might also induce stabilization phosphorylation of p53 in the epithelial cells of mouse mammary gland. Tissue sections were obtained for analyses from a transgenic mouse strain harboring whey acidic protein (WAP)-controlled MYC allele (33). In this strain, lactogenic hormones activate WAP-Myc in luminal epithelial cells during late pregnancy, and the allele remains active thereafter (34). Stimulation of WAP-Myc by multiple rounds of pregnancies leads to development of hyperplasia in all glands and solitary adenocarcinomas, generally appearing in one or two glands (33, 35). Phospho-Ser18 p53 (Ser18 is equal to human Ser15) antibody was validated for use in immunohistochemistry (Fig. S1 K and L), and tumor-free (hyperplastic) and tumor-bearing mammary glands from parous mice were analyzed for phospho-Ser18 p53 status. Samples from the control wild-type mice were negative for cytosolic phospho-Ser18 p53 staining. In contrast, tumor-free WAP-Myc glands widely expressed cytosolic phospho-p53 (Fig. 2 D and E). Surprisingly, mammary tumors derived from the same mice showed no staining with phospho-p53 antibody. The differences in phospho-p53 status are not attributable to the proliferative rate or the proliferation of interactions between p53 and Bax, Bak, or Bcl-x.

Mitochondrial Accumulation of p53 Activates Bak. We next asked whether a chemical stabilization of p53, in the absence of oncogene signaling, would be sufficient to induce conformational Bak activation. A small molecule Nutlin-3a prevents Mdm2 from recognizing p53 and consequently stabilizes p53 (36). Addition of Nutlin-3a to MCF10A cells induced Ser15 phosphorylation as well as mitochondrial, cytosolic (Fig. 3 A and B), and nuclear accumulation of p53 (Fig. S2 A–C). In addition, Nutlin-3a induced up-regulation of transcriptional p53 targets (Fig. 3 F). Nutlin-3a also induced conformational activation of Bak (Fig. 3C and Fig. S2 D and E), like Myc, without disrupting the Bak–Bcl-xL complex (Fig. 3D).

DNA damage induces a well-defined pathway for p53 stabilization via DNA-activated protein kinase (DNA-PK), Ataxia telangiectasia mutated kinase (ATM), ataxia telangiectasia and Rad3 related (ATR), and checkpoint kinase 2 (CHK2). These DNA damage response kinases phosphorylate N-terminal sites of p53 following by dissociation of p53 from Mdm2, nuclear accumulation of p53, and induction of repair and cell cycle regulatory genes (32). We investigated whether the DNA damage-induced nuclear p53 pathway induces conformational activation of Bak. Administration of DNA-damaging drugs Etoposide or Doxorubicin led to a nucleoplasmic accumulation of Ser15 phosphorylated p53 [hereafter, p-p53 (S15)], up-regulation of mRNA encoding GADD45 (growth arrest and DNA-damage–inducible) and mRNA for p53-induced glycosylation and apoptosis regulator TIGAR and accumulation of cyclin-dependent kinase inhibitor protein p21, all of which are established p53 targets (Fig. 3 E, F, H, I, and Fig. S2F). In addition to nuclear p53 response, DNA damage also induced mitochondrial accumulation of p53 (Fig. S2G). Nonetheless, DNA damage failed to induce conformational Bak activation (Fig. 3G). Notably, activation of Myc did not induce nucleoplasmic accumulation of p53 or p-p53 (S15) (Fig. 3 E and F and Fig. S2F). Furthermore, active Myc did not lead to up-regulation of any of the examined transcriptional targets of p53 (Fig. 3 H and I). These results altogether establish a tight link between the mitochondrial accumulation of p53 and conformational activation of Bak but also demonstrate that mitochondrial accumulation of p53 does not autonomously activate Bak.

We next asked whether p53 is required for Myc-induced Bak activation and found that siRNA-mediated silencing of p53 reduces Myc’s ability to activate Bak in several cell types (Fig. S2 H–J). Furthermore, Myc was completely unable to induce conformational activation of Bak in MCF10A cells rendered p53null by zinc finger nuclease approach (MCF10A p53null-MycER cells; Fig. 3 J and K). We reconstituted wild-type (WT) p53 expression in MCF10A p53null-MycER cells and found that WT p53 rescues Myc’s ability to induce conformational Bak activation (Fig. 3K). Interestingly, p53null transactivation-deficient mutant also rescued Myc-induced Bak activation (Fig. 3K). The results show that transactivation-deficient p53 can convey Myc’s apoptotic signal to Bak.

**Myc-Induced Mitochondrial Accumulation of p53 Promotes Apoptosis by Functionally Reorganizing the Bak–Bcl-xL Complex.** The patterns of interactions between p53 and Bak, Bak, or Bcl-xL have been

![Image](https://example.com/image.png)

**Fig. 4.** Activation of Myc rearranges interaction of p53 with Bak-Bcl-xL complex. (A and B) Activation of Myc leads to close proximity colocalization of p53 with conformationally active Bak. Proximity Ligation Assay (PLA) was performed with rabbit p53 antibody (FL-393) and N-terminal Bak antibody (Ab-1). (A) IF images of MCF10A-MycER cells treated as in Fig. 1E and subjected to PLA assay. (B) Quantification of PLA spots (close proximity sites) scored per cell. (C and D) Effects of Myc, ABT-737, or apoptotic conditions (Myc+TRAIL) to the interaction between p53 and Bcl-xL in MCF10A-MycER cells. Cells were treated as in Fig. 1E. Co-IP analysis was performed using (C) polyclonal p53 antibody (Ab-1) and (D) monoclonal p53 antibody (Bp53-12) for Bcl-xL, pulldown. (E) ABT-737 selectively induces apoptosis in the cells expressing active Myc or treated with Nutlin-3a. Cells were pretreated for 24 h with 4-OHT or Nutlin-3a followed by 1 h treatment with 10 μM ABT-737. Cells were stained for active Bax and scored as in Fig. 1. *P < 0.05.
intensively studied by cell biological, biophysical, and structural approaches. In contrast to relatively stable interaction between p53 and Bcl-xL, it is widely held that p53 interacts with Bak and Bak via transient molecular interaction (28, 37–40). However, unstable protein interactions are difficult to detect by methods requiring cell lysis, such as coimmunoprecipitation (co-IP) analysis. Therefore, we analyzed the physical proximity of p53 and Bak in situ by using quantitative proximity ligation assay (PLA). PLA revealed a colocalization of N-terminally exposed Bak and p53 in the cells with active Myc (Fig. 4A and B). The colocalization signal was strongly enhanced in apoptotic conditions.

p53 has higher affinity for Bcl-xL than for Bak (40), and the endogenous p53–Bcl-xL interaction has been detected by co-IP analysis in various cells (37). Also in mammary epithelial cells, p53 coimmunoprecipitated with Bcl-xL (Fig. 4C). Surprisingly, activation of Myc significantly diminished the interaction between p53 and Bcl-xL, and ABT-737 fully disrupted the complex (Fig. 4C). Moreover, TRAIL-induced apoptosis in sensitized cells led to dissociation of p53 from Bcl-xL. The results were corroborated with three different p53 antibodies used in co-IP analysis (Fig. 4C and D and Fig. S2 K and L). Taken together, our results suggest that activation of Myc alters the steady-state interactions of p53 with Bak and Bcl-xL. In mammary epithelial cells, p53 acquires a proximal position next to conformationally activated Bak and becomes less tightly bound to Bcl-xL.

The described action of p53 is reminiscent of allosteric regulation because p53 seems to promote conformational activation of Bak outside of the active site (BH3-groove). To determine whether p53-induced Bak activation in fact promotes apoptosis, we disrupted the BH3-groove interaction by ABT-737 in the absence or presence of conditions inducing mitochondrial accumulation of p53. Strikingly, ABT-737 treatment induced activation of Bak, which indicates full activation of the mitochondrial apoptosis pathway, only in the cells with active Myc or treated with Nutlin (Fig. 4E). The results suggest that mitochondrial accumulation of p53 promotes the apoptotic properties of Bak even when it stays bound to Bcl-xL. A schematic model for the altered interactions of p53 with the Bak–Bcl-xL complex and functional consequences is shown in Fig. S3N.

**Myc Induces p53-Bak Pathway via AMPK.** To further elucidate the mechanisms by which Myc induces p53 stabilization and conformational Bak activation, we used chemical inhibitors targeting most of the kinases linked to Ser15 phosphorylation of p53 (41). The compounds were analyzed for their ability to perturb Myc-induced Bak activation and p53 stabilization. None of the inhibitors targeting DNA damage response kinases could prevent activation of Bak (Fig. 5A). However, we found that compound C, an ATP competitive inhibitor of AMPK (42), specifically prevents the Myc-induced activation of Bak (Fig. 5A and B). Inhibition of AMPK, phospho Acetyl-CoA carboxylase (p-ACCSer79) serving as a marker of inhibition (see below), also prevented Myc-induced Ser15 phosphorylation of p53 and consequent stabilization and accumulation of p53 in the cytosol and mitochondria (Fig. 5C and D). Pharmacological AMPK activation with an AMP analog aminoimidazole carboxamide ribonucleotide (AICAR) induced Ser15 phosphorylation of p53 (Fig. 5C), establishing that active AMPK does phosphorylate p53 at Ser15 in mammary epithelial cells. These and earlier (Fig. 3) findings suggest that Myc does not induce p53 and Bak activation via cellular DNA damage response but, instead, via AMPK activation-coupled metabolic effects.

*Fig. 5.* Myc-induced stabilization of p53 and conformational activation of Bak is dependent on AMPK. (A) Effects of indicated kinase inhibitors on Myc-induced conformational activation of Bak. MCF10A-MycER cells were pretreated for 4 h with DMSO carrier or indicated inhibitors before Myc activation. Cells were then incubated for a further 24 h before analysis. Kinase inhibitors were titrated to sublethal concentration, which was 1 μM except for AMPK inhibitor (compound C; 10 μM) and Chk2 inhibitor (500 nM). Cells with active Bak were scored from IF images. (B) Representative IF images showing effect of AMPK inhibitor compound C. (C) Effect of AMPK inhibition on Myc-induced Ser15 phosphorylation of p53. Western blot analysis shows levels of p53 and p-p53 (S15) in MCF10A-MycER cells after indicated treatments. Phospho Ser-79 ACC is a biomarker of AMPK activity. Cells were pretreated for 4 h with 10 μM AMPK inhibitor followed by 24 h further treatment with 100 nM 4-OHT to activate Myc or 200 nM AICAR to ectopically activate AMPK. Actin is a loading control. (D) Effect of AMPK inhibition on Myc-induced accumulation of Ser15 phosphorylated mitochondrial p53. Cells were treated as in C followed by isolation of mitochondria-enriched fractions and analysis by Western blotting. Bak is a sample control. **P < 0.005.
promoting the activating phosphorylation of Thr172 in the activation loop of AMPK, whereas ATP has the opposite effect (11). Therefore, we explored whether Myc alters the cellular energy status. Activation of Myc decreased cellular ATP levels and concomitantly increased ADP levels (Fig. 6D). This change in ADP/ATP ratio was coincident with prominent increase in Ser15 phosphorylation of p53 and activating Thr-172 phosphorylation of the catalytic subunit AMPKα (Fig. 6C). Active AMPK inhibits anabolic reactions, for example repressing de novo synthesis of fatty acids by inhibitory phosphorylation of the rate-limiting enzyme Acetyl-CoA carboxylase (ACC). Therefore, phospho ACC is a widely used biomarker of AMPK activity (43). In keeping with the Myc-induced AMPK activity in epithelial cells, we detected inactivating Ser79 phosphorylation of ACC (Fig. 6C). Moreover, we determined phospho ACC status in the mammary glands of WAP-Myc mice and found that the Myc-induced hyperplastic mammary epithelium was strongly positive for phospho-Ser79 ACC (Fig. 6D). The control glands from parous mice were mainly negative for phospho ACC (Fig. 6D). The Myc-induced adenocarcinomas were scored positive although the staining intensity was weaker than in the hyperplasia. The results consistently demonstrate Myc-dependent AMPK pathway activation in vitro and in vivo.

We determined whether Myc-induced AMPK signaling affects the glutaminolytic pathway because mitochondrial glutaminolysis fuels Myc-driven cell growth and proliferation programs. As reported earlier in other cell types, also in mammary epithelial cells Myc elevated the expression of GLS and rendered cells addicted to glutamine (Fig. 6E and F). Interestingly, the Myc-induced elevated GLS expression was completely dependent on AMPK activity (Fig. 6E), suggesting that AMPK activity controls Myc’s ability to reprogram metabolic pathways.

Next, we investigated whether AMPK contributes to the onset of mitochondrial apoptosis. We induced Myc-dependent apoptosis with TRAIL or DNA damaging agents and observed that chemical inhibition of AMPK efficiently blocks conformational activation of active Bak and Bax in the treated cells as scored in Fig. 1. *P < 0.05, **P < 0.005.

---

Fig. 6. Myc-induced metabolic reprogramming is coupled to apoptotic sensitization via AMPK. (A) Effect of Myc activation on cellular ATP levels. MCF10A cells were grown in normal culture medium followed by change of fresh medium and 24 h or 48 h Myc activation. Alternatively, cells were deprived of EGF and insulin for 20 h followed by 4 h Myc activation. (B) ADP/ATP ratio change in MCF10A cells after 24 h Myc activation. (C) Myc induces activation of the AMPK pathway. Myc was activated with 4-OHT in MCF10A-H-ErbB2 cells for 24 h, and both cytosolic fractions and total cell lysates were analyzed by Western blotting using indicated phospho-specific antibodies. The status of activating T172 phosphorylation of AMPK, phospho ACC, and phospho-p53 was analyzed. (D) Immunohistochemical analysis of ACC phosphorylation at Ser79 in histological samples described in Fig. 2D. (E) Myc-induced GLS up-regulation is dependent on AMPK activity. Cells were treated as in Fig. 5C, and the mitochondria-enriched fractions were analyzed for expression of GLS. CoxIV is a sample control. (F) Myc-induced addiction to glutamine metabolism. Myc was preactivated for 24 h followed by 24 h glutamine withdrawal in the presence of Myc activity. Apoptosis was measured with Caspase-Glo 3/7 kit. (G–I) Effect of AMPK inhibition on Myc induced activation of Bak and Bax. (G) IF images of cells pretreated for 4 h with 10 μM Compound C followed by combined activation of Myc and TRAIL pathway. Cells were immunostained for Bak and Bax. (H) Quantification of active Bax (apoptosis) in the cells treated as in G. (I) Quantification of active caspase-3 (apoptosis) in the cells treated for 4 h with 10 μM Compound C followed by 4 h treatment with 100 μM Etoposide. SD represent values from three different experiments. (J) Activation of AMPK with compound A-769662 or AICAR induces Ser15 phosphorylation of p53. (K) Active AMPK sensitizes cells to apoptosis. Cells were treated for 24 h with 1 μM A769662 to activate AMPK. For induction of apoptosis, sensitized cells were further incubated for 2 h with 50 ng/mL TRAIL. Conformational activation of Bak and Bax in the treated cells was scored as in Fig. 1. *P < 0.05, **P < 0.005.
Bak and apoptosis (Fig. 6 G–I). We also asked whether a pharmacological activation of AMPK would be sufficient to induce mitochondrial accumulation of p53 and conformational activation of Bak. For these experiments, we used AICAR and compound A769662, the latter of which is a specific and direct pharmacological activator of AMPK (44). Administration of AICAR or A769662 to mammary epithelial cells induced activation of the AMPK pathway, stabilizing Ser15 phosphorylation of p53, and conformational activation of Bak (Figs. 6J and K and Fig. S2 N–Q). Moreover, when AICAR or A769662 was added together with TRAIL, the combination treatment synergistically induced apoptosis (Fig. 6 K and Fig. S2 P and R).

Finally, to determine how generally Myc triggers activation of the AMPK pathway, phospho p53, and Bak, we introduced MycER into several different wild-type p53-expressing epithelial cell lines, including nonmammary epithelial types. Fig. 7 and Fig. S3.4–M summarize the overall impact of Myc on AMPK, p53, and Bak effectors in nine different epithelial cell lines, in a fibroblast cell line, and in the mammary gland. We found that Myc triggers activation of both AMPK and p53 in most (7/10) of the examined cell lines. Myc-induced conformational activation of Bak was detected in half of the cell lines (5/10), and Nutlin was slightly more efficient, inducing Bak activation in 8 cell lines.

In summary, our data reveal AMPK as a central mediator of the oncogene-induced reprogramming of metabolic and apoptotic machineries (Fig. 8).

Discussion

Myc-induced up-regulation of GLS and further addiction to the glutaminolytic pathway are hallmarks of Myc-induced metabolic transformation, which adapts cells to meet the bioenergetic and biosynthetic demands of increased cell proliferation. Here, we show that Myc-induced metabolic transformation of mammary epithelial cells is accompanied by cellular ATP depletion, change in the ADP/ATP ratio, and activation of AMPK. We find that AMPK activity is needed to maintain the Myc-induced GLS expression, implying that AMPK activity may contribute to the viability of transformed cells. Paradoxically, Myc-induced AMPK activity is also particularly important for the onset of the apoptotic cell program, which involves AMPK-mediated phosphorylation of p53 at Ser15, stabilization and mitochondrial accumulation of p53, altered interactions between phospho-p53 and the Bak–Bcl-xL complex, and conformational activation of Bak. Therefore, Myc-induced AMPK activity has a surprising dual role in regulating the prosurvival glutaminolytic pathway and mitochondrial apoptotic signaling (Fig. 8).

Previous results have demonstrated that, in primary fibroblast culture, glucose deprivation-induced AMPK activity mediates Ser15 phosphorylation and consequent stabilization of p53, leading to cell cycle arrest (13). These observations have given rise to the concept that AMPK-p53 signaling mediates a metabolic cell cycle checkpoint, which halts the cell cycle in response to low extracellular carbon supply (13). In human tumor cells, glucose deprivation also triggers AMPK activation, which induces p53-dependent cell death (14). Importantly, these observations and indications that even transient decreases in ATP concentration can trigger apoptosis (45, 46) have suggested that AMPK also mediates apoptosis in response to metabolic stress. However, currently there are no general frameworks defining the molecular pathways and biological or pathological context for metabolic stress-induced AMPK activation, p53 stabilization, and consequent apoptotic response.

The apoptotic activity of p53 has often been associated with signaling cascades triggered by extensive cell damage, for example, DNA damage or mitotic catastrophe (32, 47). However, our findings reveal that the metabolic induction of p53-dependent apoptosis uses an alternative, direct molecular pathway involving AMPK-mediated Ser15 phosphorylation and mitochondrial accumulation of p53 tethered to conformational activation of Bak. We note that, although our results demonstrate Ser15 phosphorylation mediated stabilization of p53, they do not exclude the possibility that other posttranslational mechanisms contributed to the mitochondrial accumulation of p53.

The AMPK-dependent apoptotic mechanism may in extreme circumstances control physiological cell death, for example, if ATP levels fall critically low. However, this form of apoptosis may be rare in healthy cells where AMPK activity can quickly replenish ATP levels by inhibiting anabolic reactions and activating catabolic reactions (10, 43). Oncogenic Myc deregulates cell cycle independently of the nutrient availability, and dividing cells require constant anabolic metabolism, at the expense of ATP production,

### Table: AMPK-Mediated Bak Activation

<table>
<thead>
<tr>
<th>Tissue origin</th>
<th>Cell line</th>
<th>Myc-induced effect</th>
<th>(a) apoptotic sensitivity</th>
<th>Bak activation</th>
<th>AMPK activation (pACO)</th>
<th>p-p53S15 phosphorylation</th>
<th>(b) ABT-737-induced Bak activation</th>
<th>(b) Nutlin-induced Bak activation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breast</td>
<td>MCF10A</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>n/d</td>
<td>n/d</td>
</tr>
<tr>
<td></td>
<td>MCF12A</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>HMEC-hT</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Meckel's</td>
<td>MCF-7</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Retinal</td>
<td>ZR-75-30</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Keratinocyte</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>HepG2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>HeLa</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>RPE</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Fibroblast</td>
<td>MRCS-hT</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

(a) + indicates > 30% increase in act. Bax or caspase 3/7 activity; Figs S1 C and G, S3 A–B
(b) + > 10% cells act. Bak positive. ABT-737 induced in all cell lines >30% Bak activation. Figs 1D, 3C and S3 C–D
(c) Fig. S3 E–M, n/d = not determined. Keratinocytes have very low p53 level due to presence of HPV.

1 MCF7 cells are lacking caspase-3. Apoptosis quantified with active Bax (Fig. S1 G) 2 Myc sensitizes ZR-75-30 cells to autophagic or necrotic cell death (Fig. S3 B) 3 Myc-dependent sensitization of MRCS-hT cells to TRAIL-induced apoptosis described in (1).

4 Myc activation by lactogenic hormones in 3D cultures of WAP-Myc mouse mammary epithelial cells renders cells sensitive to apoptosis (2). WAP-Myc mammary glands express p-ACC (Fig. 6 D) and p-p53 (Fig. 2 D).

Fig. 7. Myc-induced activation of AMPK, p53, and Bak in multiple cell lines.
complex is fi

Fig. S3

A model: Myc-induced altered metabolism activates AMPK and p53, which sensitizes the mitochondrial apoptosis pathway. Activation of Myc promotes metabolic transformation, which adapts cells to meet the biosynthetic and bioenergetic requirements of rapid cell proliferation. Metabolic transformation decreases cellular ATP levels, activating the cellular energy sensor AMPK. AMPK promotes catabolic mode of metabolism (phospho-ACC) and contributes to establishment of glucolipidic pathway (GLS). These AMPK-mediated processes release carbon for bioenergetic reactions and provide glutamine-derived nitrogen for biosynthesis of nucleic acids and nonessential amino acids (4). However, AMPK also induces phosphorylation of p53 at Ser15, which stabilizes the protein and leads to mitochondrial accumulation of p53. At the mitochondrial surface, phospho-p53 adjoins and rearranges the Bak-Bcl-xL complex (Fig. S3N), resulting in conformational activation of Bak and apoptotic sensitization. This mechanism may act as a falsafe mechanism against cells with an inability to recover from energy stress, for example, due to oncogene-enforced cell cycle progression.

Anaplerosis for macromolecule synthesis and production of biomass (48).

Therefore, Myc-promoted anabolic metabolism accompanied by low ATP content may lead to chronic AMPK activation, which we observed in the mammary glands of WAP-Myc mice. It is therefore conceivable that AMPK-induced Ser15 phosphorylation, stabilization, and mitochondrial accumulation of p53 is a transient and relatively harmless event for healthy cells but will compromise the long-term viability of transformed cells by apoptotic sensitization. Therefore, we propose that the apoptosis sensitizing AMPK-p53-Bak pathway may act as an intrinsic tumor suppressor mechanism and may constitute an important module in the metabolic tumor suppressor network involving AMPK and its regulators (43).

The results show that Myc-induced mitochondrial accumulation of p53 promotes the apoptotic properties of Bak without disrupting the BH3-groove interaction between Bak and Bcl-xL. Instead, according to our study, the critical sensitization step involves phospho-p53-mediated conformational activation of Bak, which occurs in molecular arrangement allowing close proximity of these proteins. A schematic model for apoptosis-sensitizing interactions between p53 and Bak-Bcl-xL complex is presented in Fig. S3N. We also show that mitochondrial accumulation of phospho p53 cooperates with dissociation of the BH3-groove interaction in induction of apoptosis. Therefore, it appears that the p53-induced molecular rearrangement of the Bak–Bcl-xL complex activates not only conformationally but also functionally apoptotic properties of Bak. Earlier investigations have shown that, in systems involving recombiant proteins and synthetic membranes or cell fractions, recombinant p53 induces N-terminal exposure and oligomerization of Bak and Bax, as well as cytochrome c release (29, 49). These studies have demonstrated an interaction between Bak and p53, and the results have advocated a model that p53 acts in a similar manner as BH3-only proteins, which liberate apoptotic effectors from the anti-apoptotic Bcl-2 family members. However, the model has been criticized because available structural studies suggest that p53 interacts with the acidic “underside” of Bcl-xL that is a separate domain from the hydrophobic binding groove formed by BH1, BH2, and BH3 (50). Our studies describe that the status of mitochondrial p53 is functionally coupled to the apoptotic properties of the Bak–Bcl-xL complex already before the events inducing liberation of Bak from Bcl-xL. Therefore, we suggest an allosteric type of activation mechanism for the Bak–Bcl-xL complex, which would enable cooperative interactions between Bcl-2 family members and nonmembers such as p53 (Fig. S3N). We stress that the present observations in mammary epithelial cells do not preclude the possibility that in other cell types p53 induces apoptosis more directly than via described sensitization mechanism.

The present studies give mechanistic insight into the synthetic lethal interaction of Myc with a wide variety of different apoptotic pathways, a concept that has been perceived as an alternative to direct therapeutic targeting of Myc in cancer. We and others have observed over the past decades that Myc remarkably sensitizes cells to proapoptotic signals, which do not cause any obvious acute damage to the cells. However, many signals that are synthetically lethal with Myc activate BH3-only proteins, for example, death receptor signaling (Bid), growth factor deprivation (Bad), and targeted drugs (Bim) (15, 16). Because deregulation of Myc induces metabolic transformation almost universally in cells, we believe that AMPK activation and downstream mitochondria-targeted p53 signaling form an important axis for apoptotic sensitization. The allosteric activation model for p53-mediated sensitization of the Bak–Bcl-xL complex (Fig. S3N) provides a plausible framework to explain apoptotic cooperation between AMPK-mitochondrial p53 signaling and diverse BH3-only proteins in induction of apoptosis. However, our results also show evidence that Myc does not exclusively sensitizes cells to apoptosis via Bak in all cell types. Myc may activate in some cells to redundant mechanisms involving Bax or other apoptotic effector proteins (21). The role of Myc-induced AMPK activity in such apoptotic responses, which are not coupled to conformational activation of Bax, remains to be clarified.

Finally, frequent deregulation of Myc expression (51) and relatively low mutation rate in p53 (52), as well as the expression of cytosolic wild-type p53, have been observed in invasive breast cancer (53), warranting future studies on predicted patterns of metabolic drug sensitivities in in vivo mammary tumor progression models.

Materials and Methods

Biological Materials. The cells, mouse strains, and protocols are described in SI Materials and Methods. All experiments involving animals were approved by the National Animal Ethics Committee of Finland. The mice were maintained according to the protocols of the Experimental Animal Committee of the University of Helsinki.

Reagents and Antibodies. A complete list of genetic reagents, chemicals, and antibodies used in this study is provided in SI Materials and Methods. Protocols for shRNA design, cloning, lentiviral production, and transduction have been described previously by Nieminen et al. (28) and are available from the Biomedicum Functional Genomics Unit. The validated shRNA sequences are listed in SI Materials and Methods.

Genetic Analyses. Protocols and sequences for siRNA-mediated gene silencing, quantitative PCR, and site-directed mutagenesis are described in SI Materials and Methods.

Immunofluorescence Microscopy and Histological Procedures. Cultured cells were grown and treated on coverslips and fixed in 4% (vol/vol) paraformaldehyde. For immunostainings with conformational-specific antibodies, cells were permeabilized with Saponin (20). Otherwise, Triton-X permeabilization protocols were used. Fixed cells or paraffin-embedded, 3-μm-thick tissue sections were immunostained as described in SI Materials and Methods: Imaging was performed with microscopes and facilities (SI Materials and Methods) provided by the Biomedicum Imaging Unit of the University of Helsinki.

In Situ Proximity Ligation Assay. The assay was performed using the Duolink kit (Olink Bioscience) according to the manufacturer’s protocol.

Mitochondria-enriched fractions and light membrane/cytosolic fractions were isolated by using the Mitochondria/Cytosol Fractionation Kit (Biovision).
Cells were collected into ice-cold PBS, pelleted, and homogenized by using Dounce homogenizer. Nuclear and cytosolic fractions were separated with the Nuclear/Cytoplasm Fractionation Kit (Biovision).

**Chemical Cross-Linking.** Total lysates were cross-linked with the cysteine cross-linking reagent BMH, followed by isolation of mitochondria-enriched cell fractions (SI Materials and Methods) for analyses.

**Coimmunoprecipitation Assays.** Cells were lysed in 2% (wt/vol) CHAPS lysis buffer, and immunoprecipitation was performed as described in SI Materials and Methods using the Catch and Release v2.0 Reversible Immunoprecipitation System kit (Millipore).

**Metabolic Assays.** Cellular ATP and ADP levels were analyzed in cells seeded on 96-well plates with the luminescence-based EnzymLight kit (ELDT-100; BioAssay Systems) according to the manufacturer’s protocol. Glutamine dependence of cells was analyzed by seeding cells to glutamine-free MCDB 170 (Media USA). For controls, media were resupplemented with 2 mM l-glutamine.

**ACKNOWLEDGMENTS.** We thank Abbott Laboratories for ABT-737; Dr. O. Monni for expert help with assays, members of the J.K. laboratory for critical comments on the manuscript; and T. Neejärvi, T. Inkinen, and T. Välämaä for technical support. The Biomedicum Imaging Unit and Biomedical Functional Genomics Unit are acknowledged for core services. This work was supported by the Academy of Finland, the Finnish Funding Agency for Technology and Innovation (TEKES), the Sigrid Juselius Foundation, the Finnish Cancer Organizations, the Biomedicum Foundation, the Emil Aaltonen Foundation, the Ida Montini Foundation, the Orion-Farmos Research Foundation, the Paolo Foundation, and the University of Helsinki. The research leading to these results has received support from the Innovative Medicines Initiative Joint Undertaking under Grant agreement no. 115188, resources of which are composed of financial contribution from the European Union’s Seventh Framework Programme (FP7/2007–2013) and the European Federation of Pharmaceutical Industries and Associations in kind contribution. A.I.N. is a graduate student at Helsinki Biomedical Graduate School.
Supporting Information

Nieminen et al. 10.1073/pnas.1208530110

SI Materials and Methods

Cells, Genetic Manipulations, and Transgenic Mice. Human mammary epithelial cell lines MCF10A-MycER (MycER is a tamoxifen inducible Myc fusion protein) and MCF10A-MycER+Bcl2-like 1 (Bel-xl) and fibroblast cell line MRC5-hT-MycER have been described previously by Nieminen et al. (1). pBabe-MycER, which encodes MycER, was retrospectively introduced into the following cell lines in this study: breast cancer MCF7, mammary nontransformed MCF12A, human mammary nontransformed HMEC-hT, hepatocellular carcinoma HepG2, cervical cancer HeLa, skin keratinocyte C1102 KERTr (Keratinocyte), Retinal Pigmented Epithelial (RPE), breast cancer ZR-75-30 and MCF10Ap53null cells. All these cell lines express wild-type tumor suppressor protein p53 (http://p53.free.fr). The cells were transfected as described earlier (1) and selected with antibiotics. Expression of MycER in the cells was verified after selection. The cell lines used in this study were obtained from American Type Culture Collection (ATCC) except for RPE cells, which were a kind gift from Emmy Verschuren [Institute for Molecular Medicine FINN, University of Helsinki] and MCF10Ap53null cells, which were from Sigma. Mammary epithelial MCF10A cells were cultured in epithelial cell basal media MCDB 170 (US Biological) supplemented with insulin 5 μg/mL, Bovine Pituitary Extract (BPE) 70 μg/mL, Hydrocortisone 0.5 μM, Epidermal Growth Factor 5 ng/mL, Human Transferrin 5 μg/mL, and Isoproterenol 0.01 μM. MCF12A cells (ATCC) were cultured in DMEM/F-12 (Gibco) supplemented with 10% (vol/vol) FCS, epidermal growth factor 20 ng/mL, hydrocortisone 1 μg/mL, insulin 10 μg/mL, human transferrin 5 μg/mL, transferrin 10 μg/mL, glutamine, and antibiotics. Mammary breast adenocarcinoma Sk-B3 cells were grown in McCoy's 5a media (Lonza), DMAB-MB-361 and DMAB-MB-436 in Leibovitz media (Sigma), BT-474, HeLa, HepG2, and MRC-5-hT cells in DMEM, ZR-75-30 cells in RPMI medium 1640 (Lonza), RPE cells in DMEM/F-12 (Gibco), and MCF7 in EMEM (Lonza) all supplemented with 10% FCS, glutamine, and antibiotics. MDA-MB-436 and MCF7 media had 10 μg/mL insulin. CCD 1102 KERTr (Keratinocyte) cells were cultured with Keratinocyte-SFM Medium (Kit) with l-Glutamine, EGF, and BPE (Gibco).

siRNA Transfections. The 3’ Alexa Fluor546 siRNAs were ordered from Qiagen and transfected into the cells by using HiPerFect transfection reagent (Qiagen). Cells were plated on 24-well plates as a semiconfluent culture and next day transfected with siRNA. Validated siRNA sequences are listed below.

Animals. Wild-type inbred FVB mice (Harlan Laboratories) and transgenic mice expressing whey acidic protein (WAP) promoter directed Myc (WAP-Myc) [FVB.Cg-Tg[WapMyc]212Bri/J] were from The Jackson Laboratory. The crosses and procedures to obtain tumors and tumor histopathology have been described previously by Partanen et al (2). In short, to obtain WAP-Myc females, males containing WAP-Myc allele were crossed with FVB females, and the offspring were genotyped at the age of 3 wk. To induce WAP-Myc-mediated tumorigenesis, females with WAP-Myc allele were exposed to two sequential pregnancies (2). The license for the experiments ESLH-2008–10886/Ym23 was granted by the State Provincial Office of Southern Finland. The license for strain maintenance in the Laboratory Animal Center is KEK-075.

Genetic Reagents. All shRNAs targeting Bel-2 family members were cloned in pDSL_hpU6IGH lentivector (American Type Culture Collection). McI-1 cDNA was cloned in pLenti/VS-V5-DEST vector (Invitrogen). The constructs were lentivirally transduced to MCF10A-MycER cells followed by antibiotic selection. Virus production and transduction protocols are available from the Biomedical Functional Genomics Unit.

The shRNA mRNA target sequences of B-cell CLL/lymphoma 2 (Bel-2)-family MCF10A cells are as follows:

myeloid cell leukemia sequence 1 (Mcl-1): 5’-UUCGAUG-CAGCUUUCUUGTGT-3’
A1:5’-UAUCUAAUUCAACAGUCUGTT-3’
Bcl-xL:5’-UUCAACUACCUGUAAAGCTT-3’
The siRNA mRNA target sequences are as follows:
p53: 5’-AAGGAAATTTTGGTGTGGAGT-3’
Bak: 5’-AAGCGAAGTCTTTGCGCTTCTC-3’

Quantitative RT-PCR. RNA was isolated by RNeasy kit (Qiagen) from cells transfected with siRNA and grown on six-well plates for 72 h after treatment, and mRNA levels were analyzed by using the Light Cycler 480 II instrument (Roche) using Universal ProbeLibrary (no. 43 tggggcag) probes (Roche Applied Science) and LightCycler 480 Probes Master mix and PCR grade H2O (Roche Applied Science). To analyze the gene silencing activity of siRNA constructs, the expression level of the gene of interest was compared with that of a housekeeping gene (β-actin) and normalized to control siRNA transduced control. Control siRNA samples were tested in technical duplicates and the gene of interest siRNA samples in technical triplicates. The analysis was performed by the Biomedical Functional Genomics Unit.

Site-Directed Mutagenesis. Expression vectors for mutagenesis were pLenti6/VS-p53_WT p53 and pLenti6/VS-p53 R175H (Bernard Futschek laboratory plasmids, Addgene: plasmids 22945 and 22936, ref. 3). Mutagenesis was performed with the QuickChange Site-Directed Mutagenesis kit (Agilent Technologies) according to the manufacturer’s instructions. QS mutation (L22Q and W23S) was introduced with the following primers: sense 5’-agtcaggaacctg-3’ and antisense 5’-gtttgctacgaagatggagttc-3’. The presence of the mutations in the vector was confirmed by sequencing.

Antibodies. N-terminal BCL2-antagonist/killer (Bak) antibody Ab-1, tumor suppressor protein p53 Ab-1 (Calbiochem), Acetyl-CoA carboxylase (ACC), p-ACC Ser79, AMP-activated protein kinase (AMPK), AMPKThr172, BCL2-associated agonist of cell death (Bad), N-terminal BCL2-associated X protein (Bax), Bel-2, BCL2-like 1 (Bim), BCL2-related protein A1 (Bfl-1/A1), BCL2-related ovarian killer (Bok), active caspase-3, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Histone 3 (H3), cyclin-dependent kinase inhibitor protein p21, BCL2 binding component 3 (Puma), p-p53Ser15, (Cell Signaling), BCL2-like 1 (Bel-2L), (BD Biosciences), N-terminal Bax (6A7, Treviron), β-actin, β-tubulin, Bak H-211, heat shock 70 kDa protein (Hsp70), nuclear envelope marker Lamin B, McI-1, proliferating cell nuclear antigen (PCNA), p53 Bp33-12, p53 FL-393 (Santa Cruz Biotechnology), α-actin (Sigma), Cytochrome c (BD Pharmingen),

Nieminen et al. www.pnas.org/cgi/content/short/1208530110

1 of 8
β-tubulin, glutaminase (Abcam), antigen identified by monoclonal antibody Ki67 (Novocorsa laboratory), cytochrome c oxidase subunit IV (COXIV) (Molecular Probes), Myc 9E10 (Covance).

Apoptosis Assay. Cells were seeded on 96-well plates and treated, and apoptosis was measured using the Caspase-Glo 3/7 luminescence kit. Luminescence (RLU) was measured using a VICTORX3 (PerkinElmer) plate reader.

Reagents. The following reagents were used in the experiments: 4-Hydroxytamoxifen, aminooimidazole carboxamide ribonucleotide (AICAR) (WB: Cell Signaling, IF: Sigma), Etoposide, Doxorubicin, checkpoint kinase 2 (Chk2) inhibitor II (Sigma), mitogen-activated protein kinase (MEK) inhibitor U0126 (Promega), Nutlin-3a (Alexis Biochemicals), phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) inhibitor Ly294002 (Cell Signaling), mitogen-activated protein kinase 1 (ERK1) inhibitor II, ataxia telangiectasia mutated (ATM) inhibitor CGK-733 (Santa Cruz), AMPK inhibitor Compound C, mitogen-activated protein kinase (p38) inhibitor SB203580, protein kinase, DNA-activated (DNA-PK) inhibitor C401, AMPK activator A769662 (Biovision), and recombinant human TNF-related apoptosis inducing ligand (TRAIL) (R&D Systems).

Chemical Cross-Linking. Cells were collected from 15-cm plates into ice-cold PBS and pelleted by centrifugation at 1,500 rpm (Heraeus Megafuge 1.0 R) for 10 min. The cell pellet was resuspended in SCEB buffer (200 mM sucrose, 10 mM Hepes pH 7.4, 50 mM KCl, 5 mM EGTA, 5 mM MgCl₂) containing DTT, PMSF, and cytochalasin B and incubated for 30 min before homogenization with Dounce homogenizer. The unbroken cells and debris were removed by centrifugation at 2,000 rpm (Heraeus Megafuge 1.0 R) for 10 min at +4 °C. Subsequently, the supernatant was subjected to a cross-linking assay. BMH (1,6-bismaleimidohexane) was added to a final concentration of 1 mM, and lysates were incubated for 30 min at room temperature on constant mixing. After quenching the reaction with DTT, the mitochondria-enriched heavy membrane fraction was pelleted at 13,000 rpm (Heraeus Megafuge 1.0 R) for 10 min at +4 °C. The cell pellet was resuspended in SCEB buffer containing 10% (vol/vol) normal goat serum (Gibco) in buffer containing 10% (vol/vol) normal goat serum (Gibco) in mammalian histological buffer [0.1% (wt/vol) BSA, 0.2% (vol/vol) Triton-X (Sigma), 0.05% (vol/vol) Tween 20] for 30 min followed by 1 h incubation with primary antibody and secondary antibody using standard avidin–biotin complex detection with 3,3-diaminobenzidine (Vector Laboratories), and counterstaining with hematoxyline (Thermo Scientific). Sections were dehydrated and mounted with Vectastain permanent mounting medium (Vector Laboratories). Microscopic analyses were performed using Zeiss Axiosplan fluorescence microscopes and either Plan-Neofluor 20x or 40x objectives, appropriate filters for Alexa 488, 546, or Hoechst and Zeiss AxioCam HRc color camera. The (Heraeus Pico17) immunohistochemistry (IHC) samples were analyzed and photographed with a Leica DM LB microscope and software Studio-Lite 1.0.

Fig. S1. (A–G) siRNA mediated silencing of Bak prevents Myc-induced apoptosis. (H) validation of shRNAs and (I) ABT-737-induced conformational activation of Bak. (J) Analysis of p53 mRNA levels. (K and L) Phospho-p53 antibody validation for IHC. (M) Ki-67 analysis of mammary tissue and tumors. (A) Validation of Bak silencing in MCF10A-MycER cells. Cells were transfected with siRNAs, and 72 h later RNA was isolated. Bak mRNA levels in the control and Bak siRNA transfected cells were analyzed by qRT-PCR, using LightCycler 480 II (Roche). (B) Active Bak protein is absent in the cell populations transfected with Bak-targeted siRNA. MCF10A-MycER cells were exposed to 24 h Myc activation followed by 2 h 50 ng/mL of TRAIL treatment. Cells with active Bak were scored from coverslips immunostained with N-terminal Bak antibody. (C) Analysis of active Bax (apoptosis) in the Bak siRNA transfected MCF10A-MycER cells. The cells were treated and analyzed as in B with the exception that coverslips were immunostained with N-terminal Bax antibody. (D) Myc induces conformational activation of Bak. Legend continued on following page
of Bak in MCF7 cells. MCF7-MycER cells were treated for 24 h with 4-hydroxytamoxifen (4-OHT) to activate Myc and analyzed for activation of Bak as above. (E) qRT-PCR and Western Blot validation of siRNA mediated silencing of Bak in MCF7-MycER cells. Cells were analyzed as in A. (F) Active Bak protein is absent in the MCF7 cell populations transfected with Bak-targeted siRNA. Experiments were performed as in B. (G) Analysis of active Bax (apoptosis) in the Bak siRNA transfected MCF7-MycER cells. Experiments were performed as in C. In the graphs, SD represents at least two separate experiments. (H) Western blot analysis of lentiviral shRNA-mediated silencing of Mcl-1, A1, and Bcl-xL. MCF10A-MycER cells were transduced with pDSL_hpUGIH lentiviruses expressing indicated shRNAs followed by antibiotic selection of the cells. (I) ABT-737-induced conformational activation of Bak. IF images of breast cancer cell lines treated with 1 μM ABT-737. Cells were immunostained for active Bak. (J) qRT-PCR analysis of p53 mRNA levels in MCF10A-MycER cells with or without active Myc. RNA was extracted from the cells treated for 24 h with 4-OHT or carrier, and p53 mRNA was analyzed by qRT-PCR, using LightCycler 480 II (Roche). The graph represents normalized values (fold change) and SD of three separate experiments. (K) Validation of p-p53 (Ser15) antibody for IHC on mouse mammary gland tissue. (L) Detection of cytosolic p53 by p-p53 (Ser15) antibody in IHC. Lactating human breast epithelial cells have been documented to express cytosolic p53 (1). In this study, we observe that the epithelial cells of murine lactating glands exhibit specific cytosolic staining of phospho-p53. Similar cytosolic staining was observed in hyperplastic mammary glands of WAP-Myc mice but not in the tumors or controls. Notably, the luminal space of alveoli is occasionally filled with secretory material, which unspecifically cross-reacts with many antibodies (2D, S1L, S1M). However, the unspecific staining was restricted to luminal area and therefore did not compromise the detection of cytosolic p53.

(M) Representative images of Ki67 (proliferation marker) staining on mouse mammary tissue and tumors.

Fig. S2. (A and B) Nutlin-3a and DNA damage induces nuclear p53 accumulation. (C–E) Nutlin-3a stabilizes p53 and activates Bak in MCF-7 breast cancer cells. (F and G) Myc, Nutlin-3a, and DNA damage-induced p53 accumulation in the mitochondria, cytosol, and nuclei. (H–J) siRNA-mediated silencing of p53 inhibits Myc's ability to activate Bak. (K and L) Coimmunoprecipitation analysis of interactions between p53, Bak, and Bcl-xL. (M) Effect of Myc activation on ADP levels. (N and O) Both A-769662- and AICAR-induced AMPK activates Bak. (P) AICAR, similar to A-769662 (Fig. 6K), sensitizes cells to apoptosis. (Q) AICAR-induced Bak activation in MCF7 cells. (R) AMPK activation sensitizes HeLa, HepG2, and RPE cells to apoptosis. (A) Immunoﬂuorescence images show nucleoplasmic accumulation of p53 in MCF10A cells treated for 24 h with 20 μM Nutlin-3a. (B) Western blot shows increase of phospho p53 levels in the nuclei of MCF10A cells after treatment with Nutlin-3a or 10 μM Etoposide. Nuclear fractions were analyzed for p-p53Ser15. Tubulin is used as a marker of cytosolic fraction. (C) Western blot analysis demonstrating Nutlin-induced stabilization of p53 in MCF7 cells. Cells were treated for 24 h with 10 μM Nutlin, and the levels of phospho p53 (p-p53Ser15) were analyzed. (D) Nutlin-3a-induced Bak activation in MCF7 cells. Quantification of the MCF7 cells expressing active Bak after 24 h Nutlin treatment. Cells were immunostained with N-terminal Bak antibody and scored as in Fig. S1. The results in the graph represent two separate experiments. (E) Legend continued on following page
Representative immunofluorescence image of MCF7 cells expressing active Bak. (F) Nuclear accumulation of phospho-p53 in MCF10A-MycER cells in response to 24 h Myc activation or treatment with 1 μM A-769662 (control—AMPK activating compound, see also Fig. 8) or 0.1 μM Doxorubicin. H3 is a purity control for nuclear fractions and tubulin for cytosolic fractions. (G) Mitochondrial accumulation of phospho p53 in MCF10A-MycER cells in response to Myc activation or Nutlin or Doxorubicin treatment. Mitochondrial and cytosolic fractions and whole cell lysates were prepared from MCF10A cells treated for 24 h as in B and F and analyzed by Western blotting. COXIV was used as a purity control for mitochondrial fractions. (H) siRNA-mediated silencing of p53 in MCF7-MycER and HeLa-MycER cells. Cell lysates from control and p53 siRNA transfected cells were analyzed for protein expression by Western blotting (Upper) and for mRNA expression by qRT-PCR (Lower). (I) Immunofluorescence images show decrease in Myc-induced Bak activation after p53 silencing. MCF7-MycER cells were transfected with control or p53 siRNAs before assays. (J) Quantification of Myc-induced Bak activation in MCF7-MycER and HeLa-MycER cells transduced with p53 siRNAs. Representative results are shown. (K) Coimmunoprecipitation analysis of p53 interaction with Bcl-xL using polyclonal FL-393 p53 antibody (Santa Cruz). p53 pull down reduced amount of Bcl-xL in lysates representing MCF10A-MycER cells treated with 4-OHT or ABT-737, corroborating results in Fig. 4D and E. (L) Coimmunoprecipitation analysis of p53 or Bak interaction with Bcl-xL using polyclonal Ab-1 p53 antibody (Calbiochem) and polyclonal H-211 Bak antibody (Santa Cruz). Both Bak and p53 pull down reduced the amount of Bcl-xL in lysates representing human mammary epithelial MCF12A cells treated with ABT-737. This finding confirmed results in Figs. 2A and 4C and D. (M) Effect of Myc activation on cellular ADP levels in MCF10A-MycER cells. The cells were grown in normal culture medium followed by change of fresh medium and 24 h Myc activation. ADP levels were quantitated as described in Materials and Methods. (N and O) Representative immunofluorescence images of Bak activation in MCF10A cells induced by 1 μM A769662 or 5 mM AICAR. (P) AICAR-induced sensitization to apoptosis. MCF10A cells were treated for 24 h with 5 mM AICAR before fixation, immunostaining, and scoring. (Q) AICAR-induced Bak activation in MCF7 cells. The cells were treated for 24 h with 5 mM AICAR before fixation, immunostaining, and scoring. (R) AMPK activation sensitizes HeLa-MycER, HepG2-MycER, and RPE-MycER cells to apoptosis. Cells were treated for 24 h with 1 μM A769662 to activate AMPK. For induction of apoptosis, sensitized cells were further incubated for 2 h with 50 ng/mL TRAIL. Apoptosis was measured with the Caspase-Glo 3/7 kit. The graphs represent ±SD of three separate experiments.
Fig. S3. Myc-induced activation of AMPK, p53, and Bak in multiple cell lines and a model to explain p53-dependent priming of the mitochondrial pathway. (A) Myc-dependent sensitization of the cells to TRAIL-induced apoptosis. The data demonstrates the presence of a conditional proapoptotic Myc in the cells. (B) Myc and TRAIL kill breast cancer ZR-75–30 cells via autophagic or necrotic form of cell death. TRAIL induces autophagic-type vacuolization in the cells but the cells remain viable. Activation of Myc in the presence of TRAIL leads to detachment of the cells and loss of viability via caspase-independent mechanisms. (C) Quantification of Myc-induced Bak activity. (D) Quantification of Nutlin-induced Bak activity. (E–M) Myc-induced activation of AMPK pathway (pACCSer79) and Ser-15 phosphorylation of p53. Myc was activated for 24 h or 72 h before lysing the cells for Western blot analysis. (N1–N4) An allosteric activation model for p53-mediated sensitization to mitochondrial apoptosis. Classically, the central activation step of the mitochondrial pathway has been attributed to pharmacologically (ABT-737) or biologically (BH3-only proteins) induced dissociation of the BH3-groove interaction between Bak (or Bax) and Bcl-xL. This event

Legend continued on following page
liberates Bak, which can proceed to form pore-forming homo-oligomers. The present data argue that mechanisms converting Bak into an oligomerization competent conformer also involve p53-mediated activation of Bak, which occurs when Bak is still bound to Bcl-xL. p53 acts as an allosteric activator of Bak rather than a BH3-only domain-like antagonist of Bcl-xL. According to the model (N1), in mammary epithelial cells, Bak, Bcl-xL, and p53 form an inactive protein complex on the mitochondrial surface. In the inactive complex, p53 and Bak bind Bcl-xL via distinct interaction sites (1), Bak adopts closed conformation, and Bak is sequestered via BH3:groove interaction by Bcl-xL. (N2) Chemical dissociation of Bak from the inactive protein complex is not sufficient to trigger autonomous oligomerization of Bak. (N3) Mitochondrial accumulation of Ser15 phosphorylated, stabilized p53 leads to conformational activation of Bak within the Bak-Bcl-xL complex. In the “activated” complex, p53 resides in close proximity to open Bak conformer and has reduced affinity to Bcl-xL. (N4) Exposure of p53-activated Bak-Bcl-xL complex to chemicals or proteins that disrupt the BH3:groove interaction leads to liberation and spontaneous oligomerization of Bak. The allosteric activation model suggests that p53-induced functional activation of Bak is an independent event of the dissociation of BH3:groove interaction, which may explain synthetic lethal interactions of the Myc-p53 pathway with BH3-only signaling. Circed P denotes phosphorylation, and double arrows mark reversible steps in the pathway.