Ionizing radiation induces ataxia telangiectasia mutated kinase (ATM)-mediated phosphorylation of LKB1/STK11 at Thr-366

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The serine/threonine protein kinase LKB1 functions as a tumour suppressor, and mutations in this enzyme lead to the inherited Peutz–Jeghers cancer syndrome. We previously found that LKB1 was phosphorylated at Thr-366 *in vivo*, a residue conserved in mammalian, *Xenopus* and *Drosophila* LKB1, located on a C-terminal non-catalytic moiety of the enzyme. Mutation of Thr-366 to Ala or Asp partially inhibited the ability of LKB1 to suppress growth of G361 melanoma cells, but did not affect LKB1 activity *in vitro* or LKB1 localization *in vivo*. As a first step in exploring the role of this phosphorylation further, we have generated a phosphospecific antibody specifically recognizing LKB1 phosphorylated at Thr-366 and demonstrate that exposure of cells to ionizing radiation (IR) induced a marked phosphorylation of LKB1 at Thr-366 in the nucleus. Thr-366 lies in an optimal phosphorylation motif for the phosphoinositide 3-kinase-like kinases DNA-dependent protein kinase (DNA-PK), ataxia telangiectasia mutated kinase (ATM) and ataxia telangiectasia-related kinase (ATR), which function as sensors for DNA damage in cells and mediate cellular responses to DNA damage. We demonstrate that both DNA-PK and ATM efficiently phosphorylate LKB1 at Thr-366 *in vitro* and provide evidence that ATM mediates this phosphorylation *in vivo*. This is based on the finding that LKB1 is not phosphorylated in a cell line lacking ATM in response to IR, and that agents which induce cellular responses via ATR in preference to ATM poorly induce phosphorylation of LKB1 at Thr-366. These observations provide the first link between ATM and LKB1 and suggest that ATM could regulate LKB1.

Key words: ataxia telangiectasia-related kinase (ATR), DNA-dependent protein kinase (DNA-PK), Peutz–Jeghers syndrome, phosphopeptide mapping.

INTRODUCTION

Peutz–Jeghers syndrome (PJS) [1,2] is an autosomal dominantly inherited disorder that predisposes to a wide spectrum of benign and malignant tumours [3,4]. Most patients with PJS possess germ-line mutations in a widely expressed protein kinase termed LKB1 [1] (also known as STK11 [2]). The overexpression of LKB1 in a number of tumour cells suppresses growth by inducing a $G_{(1)}$ cell-cycle block [5–7]. Further evidence that mutations in LKB1 cause PJS come from the recent observations that mice lacking a single copy of the LKB1 allele develop multiple tumours similar to those found in PJS patients [8–12]. Taken together these results provide compelling evidence that LKB1 functions as a tumour-suppressor protein kinase *in vivo*. As catalytically inactive mutants of LKB1 are unable to suppress cell growth, it is likely that the catalytic activity of LKB1 is required for its growth-suppression function [7], but thus far no physiological LKB1 substrates have been identified.

The mechanism by which LKB1 is regulated *in vivo* is not known, and many groups have attempted to address this question by identifying proteins that interact with LKB1. These studies have revealed that LKB1 interacts with p53 [13], the Brahma-related gene-1 protein [14] and a novel protein termed LKB1-interacting protein-1 [15]. The results of these studies indicate that these interactions may contribute to LKB1 tumour-suppressor function but further work is required to establish the physiological importance of these in regulating LKB1. We have focused on determining whether LKB1 is regulated by phosphorylation and have demonstrated that LKB1 over-expressed in HEK-293 cells is phosphorylated at four residues (Ser-31, Ser-325, Thr-366 and Ser-431) and that *in vitro* LKB1 autophosphorylates at Thr-336 and to lower extent at Thr-366 [16]. To investigate the relevance of these phosphorylations, the sites were mutated to Ala to prevent phosphorylation or to acidic residues to mimic phosphorylation. How these mutations affected LKB1 activity *in vitro*, the ability of LKB1 to suppress cell growth and LKB1 cellular localization was determined. None of the mutations affected the *in vitro* kinase activity or cellular localization of LKB1, but mutation of Ser-431 to either Ala or Asp or Thr-336 to Glu prevented LKB1 from inhibiting the

Abbreviations used: ATM, ataxia telangiectasia mutated kinase; ATR, ataxia telangiectasia-related kinase; DNA-PK, DNA-dependent protein kinase; DTT, dithiothreitol; EGF, epidermal growth factor; GST, glutathione S-transferase; IGF1, insulin-like growth factor 1; IR, ionizing radiation; KD-LKB1, kinase-dead LKB1; LPA, lipophosphatidic acid; MALDI-TOF, matrix-assisted laser-desorption ionization–time-of-flight; MMS, methyl methanesulphonate; PI 3-kinase, phosphoinositide 3-kinase; PJS, Peutz–Jeghers syndrome; PKA, cAMP-dependent protein kinase; PKB, protein kinase B; RSK, p90 ribosomal S6 protein kinase; WT-LKB1, wild-type LKB1; YFP, yellow fluorescent protein.

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growth of G361 melanoma cells [16,17]. Mutation of Thr-366 to either Ala or Asp also partially reduced the ability of LKB1 to suppress growth of G361 cells, whereas mutation of Ser-31 or Ser-325 had no effect [16]. These results indicate that phosphorylation of LKB1 at Thr-336, Thr-366 and Ser-431 could regulate its activity *in vivo*, but the mechanism by which this is achieved is unclear.

Ser-431, located five residues from the C-terminal end of the protein, is phosphorylated *in vivo* by p90 ribosomal S6 protein kinase (RSK) [17] and the cAMP-dependent protein kinase (PKA) [17,18], in response to stimuli that activate these enzymes. However, the identity of the enzymes that phosphorylate LKB1 at Ser-31, Ser-325, Thr-336 and Thr-366 *in vivo* has not been investigated. In this study we demonstrate that exposure of cells to ionizing radiation (IR) induces a marked phosphorylation of both wild-type and a catalytically inactive mutant of LKB1 at Thr-366. Thr-366 lies in an optimal phosphorylation motif for the phosphoinositide 3-kinase (PI 3-kinase)-like protein kinases DNA-dependent protein kinase (DNA-PK), ataxia telangiectasia mutated kinase (ATM) and ataxia telangiectasia-related kinase (ATR), which play key roles in regulating the DNA-damage responses in cells [19–21]. We demonstrate that both ATM and DNA-PK phosphorylate LKB1 at Thr-366 *in vitro* and provide evidence that ATM mediates this phosphorylation *in vivo*.

EXPERIMENTAL

Materials

Protease-inhibitor cocktail tablets were from Roche. Foetal bovine serum, Eagle's minimum essential medium and tissue-culture reagents were from BioWhittaker Europe. Microcystin-LR was from Life Technologies, tetracyclin-free foetal bovine serum was from Hy-Clone, and DMSO, forskolin, PMA, lipophosphatidic acid (LPA), methyl methanesulphonate (MMS) and dimethyl pimelimidate were from Sigma. Epidermal growth factor (EGF), insulin-like growth factor 1 (IGF1) and precast 4–12 % Bis-Tris gradient SDS/polyacrylamide gels were from Invitrogen. Dynabeads M-450 conjugated to goat anti-mouse IgG were from Dynal. All the peptides used in this study were synthesized by Dr G. Blomberg (University of Bristol, Bristol, U.K.). Insulin was from Novo Nordisk. Calf thymus DNA was purchased from Aldrich. The catalytic subunit of DNA-PK and the Ku70/80 complex were purified from human placenta as described previously [22].

Antibodies

Antibodies recognizing LKB1, the phosphospecific antibodies recognizing LKB1 phosphorylated at Ser-431 (S431-P), Thr-336 (T336-P) and Thr-366 (T366-P) have been described previously [16]. The phosphospecific antibody recognizing LKB1 phosphorylated at Ser-31 (S31-P) was raised in rabbit against the peptide TFIHRIDSTEVIYQP (corresponding to the residues 24–36 of mouse LKB1), in which the underlined residue is phosphoserine. The phosphospecific antibody recognizing LKB1 phosphorylated at Ser-325 (S325-P) was raised in rabbit against the peptide ALVPIPPSTPDTKDRW (corresponding to the residues 318–333 of mouse LKB1), in which the underlined residue is phosphoserine. Both antibodies were affinity purified on CH-Sepharose covalently coupled to the phosphorylated peptides, then passed through a column of CH-Sepharose coupled to the non-phosphorylated peptides. Antibodies that did not bind to the latter column were selected. S31-P, when immunoblotted in the presence of the dephospho-peptide, recognized the wild-type LKB1 (WT-LKB1), but not LKB1[S31A], phosphorylated *in vitro* by AMP kinase, which we have shown is able to phosphorylate this residue (G. P. Sapkota, unpublished work), which lies in a perfect consensus sequence for phosphorylation by this kinase [23]. Moreover, the ability of the S31-P antibody to recognize LKB1 phosphorylated by AMP kinase was lost when the immunoblots were carried out in the presence of the phospho-peptide used to raise this antibody (results not shown). The specificity of the S325-P antibody was investigated by performing immunoblots in the presence of the dephospho-peptide, of WT-LKB1 and LKB1[S325A], phosphorylated *in vitro* by CDK5, which is capable of phosphorylating Ser-325 (results not shown), which is preceded by a Pro residue [16]. S325-P strongly recognized WT-LKB1 but not LKB1[S325A] phosphorylated by CDK5 and the phospho-peptide used to raise this antibody prevented recognition of WT-LKB1 phosphorylated at Ser-325 (results not shown). Antibody recognizing Chk1 was raised in sheep against glutathione S-transferase (GST)-Chk1 protein [24]. The phosphospecific antibodies recognizing Chk1 phosphorylated at Ser-345 (S345-P antibody) and protein kinase B (PKB) phosphorylated at Ser-473 (S473-P antibody) were from Cell Signalling. Antibody recognizing PKBα was raised in sheep against the pleckstrin homology domain [25]. Mouse monoclonal antibody recognizing ATM was generated as described previously [26]. Antibody recognizing the FLAG epitope tag (Flag-M2) was from Sigma.

General methods and buffers

Restriction-enzyme digests, DNA ligations, site-directed mutagenesis and other recombinant DNA procedures were performed using standard protocols. All DNA constructs were verified by DNA sequencing, which was performed by The Sequencing Service, School of Life Sciences, University of Dundee, Dundee, Scotland, U.K., using DYEnamic ET terminator chemistry (Amersham Biosciences) on Applied Biosystems automated DNA sequencers. Buffer A was 50 mM Tris/HCl, pH 7.5, 0.1 mM EGTA, 0.27 M sucrose and 0.1 % (v/v) 2-mercaptoethanol. Buffer B was 50 mM Tris/HCl, pH 7.5, and 0.1 mM EGTA. Buffer C was 50 mM Tris/HCl, pH 7.5, 1 mM EGTA, 1 mM EDTA, 1 % (w/v) Triton-X 100, 1 mM sodium orthovanadate, 50 mM NaF, 5 mM sodium pyrophosphate, 0.27 M sucrose, 1 μM microcystin-LR, 0.1 % (v/v) 2-mercaptoethanol and 'complete' proteinase inhibitor cocktail (one tablet per 25 ml). Buffer D was 20 mM Hepes, pH 7.4, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 0.2 % Tween, 20 mM NaF, 2 mM $NaVO_4$, 1 mM dithiothreitol (DTT), 10 mM β-glycerol-phosphate, 1 μM microcystin-LR and 'complete' proteinase inhibitor cocktail (one tablet per 25 ml). SDS sample buffer was 250 mM Tris/HCl, pH 6.8, 10 % (w/v) SDS, 50 % (v/v) glycerol and 5 % (v/v) 2-mercaptoethanol. Expression of wild-type and mutant forms of N-terminally tagged GST mouse LKB1 in *Escherichia coli* and HEK-293 cells were described previously [17]. Cells were exposed to UV irradiation in the absence of medium using the UV cross-linker XL-1500 (Spectronics Corporation) or IR in the presence of medium using a caesium source irradiator (IBL 437C; CIS UK). Calf thymus DNA was sheared by sonication as described previously [27].

Cell culture, stimulation and cell lysis

Rat-2, HEK-293 and G361 cells were cultured and lysed as described previously [17]. AT2211JE-T [28] stably expressing ATM (hereafter termed $ATM^{+/+}$) or empty vector AT2211JE-FT cells (hereafter called $ATM^{-/-}$) were cultured on 10 cm-diameter dishes in Dulbecco's modified Eagle's medium supplemented

with 15 % foetal bovine serum, 2 mM L-glutamine and 0.1 mg/ml hygromycin B. In the experiments described in Figure 6 (see below), $ATM^{+/+}$ and $ATM^{-/-}$ cells were transfected using the Effectene transfection reagent (Qiagen) according to the manufacturer's protocol, with a vector encoding wild-type LKB1 with an N-terminal yellow fluorescent protein (YFP) epitope tag [16]. Where indicated, the cells were exposed to the indicated doses of IR in the presence of media or UV irradiation in the absence of media, which was replaced following irradiation. The cells were then left at 37 °C for the times indicated prior to lysis. For all experiments cells were cultured on 10 cm-diameter dishes and, unless otherwise indicated, lysed in 1 ml of ice-cold buffer C. Lysates were clarified by centrifugation at 4 °C for 5 min at 16000 ***g***. Protein concentrations were determined using the Bradford method and BSA was employed as the standard.

Construction of HeLa stable cell lines

HeLa cells expressing the Tet-ON tetracycline repressor were purchased from Invitrogen and cultured in Eagle's minimal essential medium supplemented with 10 % (v/v) tetracycline-free foetal bovine serum in the absence of zeocin. The cells were transfected using the Effectene transfection reagent with the pcDNA4/TO vector (Invitrogen) encoding the wild-type or the indicated mutants of FLAG-epitope-tagged LKB1. Post-transfection (48 h), cells expressing LKB1 were selected by addition of 100 μg/ml zeocin. Single colonies that grew under these conditions were isolated and subsequently expanded. To induce the expression of LKB1, the cells were cultured for 24 h in the presence or absence of 1 μg/ml tetracycline, lysed and the lysates immunoblotted with the LKB1 antibody. Colonies in which tetracycline induced a $>$20-fold increase in LKB1 expression in 24 h were selected.

Immunoprecipitation of endogenous LKB1 from Rat-2 cells

The polyclonal LKB1 antibody raised against GST-LKB1 (1 mg) was covalently coupled to Protein G–Sepharose (1 ml) using dimethyl pimelimidate [29]. For these experiments Rat-2 cells were lysed in buffer C lacking 2-mercaptoethanol, and 4 mg of clarified lysate was incubated for 60 min at 4 °C with the LKB1–Protein G–Sepahrose conjugate (5 μl). The immunoprecipitates were washed twice with 1 ml of buffer C (lacking 2-mercaptoethanol) containing 0.5 M NaCl, washed twice with buffer B and resuspended in SDS sample buffer, which did not contain 2-mercaptoethanol, for immunoblot analysis.

Protein kinase assays

For the ATM assays shown in Figure 6, cells were lysed by sonication in buffer D and ATM immunoprecipitated as described previously [26]. ATM assays were performed in 50 μl reaction volumes containing 1 μg of kinase-dead LKB1 (KD-LKB1) or KD-LKB1[T366A], 100 μM [γ-^{32}P]ATP (500 c.p.m./pmol), 10 mM Hepes, pH 7.4, 50 mM NaCl, 10 mM $MgCl_2$, 10 mM $MnCl_2$, 1 mM DTT, 10 mM β-glycerol-phosphate and 1 μM microcystin-LR. The assays were carried out for 1 h at 30 °C and terminated by the addition of 13 μl of SDS sample buffer. For non-specific antibody immunoprecipitations, the Flag-M2 monoclonal antibody was used rather than the monoclonal ATM antibody.

In order to assay DNA-PK, 1 μg of KD-LKB1 or KD-LKB1[T366A] was incubated at 30 °C for 30 min in a 50 μl reaction mixture, containing 50 mM Hepes, pH 7.5, 50 mM KCl, 10 mM $MgCl_2$, 0.2 mM EDTA, 1 mM DTT and 100 μM [γ-^{32}P]ATP (500 c.p.m./pmol) with the indicated amounts of catalytic subunit of DNA-PK and 3-fold less (by mass) of purified Ku70/80 complex in the presence or absence of 10 ng of calf thymus DNA, conditions that have been employed previously to assay DNA-PK [30]. The reactions were terminated by the addition of 13 μl of SDS sample buffer.

Phosphopeptide sequence analysis

In order to map the DNA-PK phosphorylation site in LKB1 for the experiments shown in Figure 4 (see below), 2 μg of KD-LKB1 or KD-LKB1[T366A] was phosphorylated with DNA-PK in a 50 μl reaction as described above except that the specific radioactivity of the [γ-^{32}P]ATP was 5000 c.p.m./pmol. The reactions were terminated by adding SDS (1 %, w/v, final concentration) and DTT (10 mM, final concentration). LKB1 was alkylated with 4-vinylpyridine, subjected to electrophoresis on a polyacrylamide gel and digested with trypsin as described previously for LKB1 phosphorylated with RSK [17]. The tryptic peptides were analysed by C_{18} chromatography as described in the legend to Figure 4. The major eluting ^{32}P-labelled peptide was analysed by matrix-assisted laser-desorption ionization–time-of-flight (MALDI-TOF) MS on a PerSeptive Biosystems Elite-STR mass spectrometer using α-cyanocinnamic acid as the matrix. Spectra were obtained in both linear and reflector modes. This peptide was also subjected to solid-phase Edman degradation of the peptide coupled to Sequelon-AA membrane (Milligen) as described previously [31], on an Applied Biosystems 476A sequenator.

Immunoblotting

For blots of total cell lysates 20 μg of protein was used. Samples were subjected to electrophoresis in an SDS/polyacrylamide gel (4–12 % gel) and transferred to nitrocellulose according to manufacturer's protocol. Only for ATM transfers, the gel was soaked for 15 min in transfer buffer containing 0.02 % SDS (w/v) and the transfer was performed for 16 h at 30 V in transfer buffer containing 0.02 % SDS (w/v). The membranes were blocked for 1 h in 50 mM Tris/HCl, pH 7.5, 0.15 M NaCl, 0.5 % (v/v) Tween and 10 % (w/v) skimmed milk. The membranes were then incubated in 50 mM Tris/HCl, pH 7.5, 0.15 M NaCl, 0.5 % (v/v) Tween and either 10 % (w/v) skimmed milk (sheep antibodies) or 5 % BSA (rabbit and mouse antibodies) for 7 h at 4 °C in the presence of 0.5–1 μg/ml antibody. Immunoblotting with the S31-P, S325-P, T336-P, T366-P and S431-P antibodies (0.5 μg/ml) was carried out as above, except that non-phosphorylated peptides (10 μg/ml) corresponding to the antigen used to raise the antibodies were included. For experiments using Chk1(S345-P), PKB(S473-P) and ATM antibodies, we used a 1000-fold dilution of the stock antibodies, and for PKB and Chk1 antibodies were employed at 1 μg/ml. Detection was performed using horseradish peroxidase-conjugated secondary antibodies and the enhanced chemiluminescence reagent (Amersham Bioscience).

Immunostaining of HeLa cells with the LKB1 T366-P antibody

HeLa cells stably expressing WT-LKB1 or mutant LKB1[T366A] were cultured as above but on sterile glass coverslips (13 mm diameter; BDH). After exposure to 0 or 20 Gy of IR, cells were left for 30 min at 37 °C, fixed in freshly prepared 4 % (v/v) paraformaldehyde in 60 mM Pipes, 25 mM Hepes, 10 mM EGTA and 2 mM $MgSO_4$, pH 7.0, for 10 min at room tem-

perature. After washing twice in PBS containing 0.1 % (v/v) Tween-20 (PBS-T), the cells were permeablized for 5 min at room temperature in PBS containing 1 % (w/v) Triton X-100. The cells were then washed twice in PBS-T and incubated for 30 min at room temperature in PBS-T containing 0.5 % (v/v) fish skin gelatin (Sigma), which was also used to dilute the rabbit polyclonal T366-P antibody and the FITC-conjugated anti-rabbit IgG antibody (Molecular Probes). The cells were then incubated for 1 h at room temperature in T366-P (1 μg/ml) in the presence of the dephospho form of the peptide (10 μg/ml) used to raise the antibody. After three 5 min washes in PBS-T, the cells were incubated for 30 min at room temperature in FITC-conjugated anti-rabbit IgG antibody (1:500 dilution). The cells were washed three times for 5 min each in PBS-T, once in PBS-T containing 0.3 μg/ml 4′,6′-diamidino-2-phenylindole (DAPI; Sigma) for nuclear staining, and once again in PBS-T. The cells were mounted on to slides using MOWIOL and imaged on a Leica microscope (Improvision), and the data were analysed using Openlab software.

RESULTS

IR induces phosphorylation of LKB1 at Thr-366

In order to investigate whether extracellular stimuli induced phosphorylation of LKB1 at Ser-31, Ser-325, Thr-336 or Thr-366 we generated phosphospecific antibodies that recognize these phosphorylation sites. The specificity of the antibodies recognizing Thr-336 (T336-P) and Thr-366 (T366-P) was established previously [16], whereas the approach used to demonstrate the specificity of the phosphospecific antibodies recognizing Ser-31 (S31-P) and Ser-325 (S325-P) is described in the Experimental section. To study phosphorylation of LKB1 at these residues, we employed a Hela cell line that lacks endogenous expression of LKB1, in which we stably expressed wild-type or mutant forms of LKB1 at a level that is about 20-fold higher than that of the endogenous LKB1 expressed in the Rat-2 cell line used in our previous study [17] (Figure 1, top panel). HeLa cells expressing WT-LKB1 were stimulated with agonists that activate PI 3-kinase (insulin and IGF1), extracellular signal-related protein kinases ERK1/ERK2 (EGF and PMA), PKA (forskolin) and stress, as well as treatments which activate DNA-structure-dependent checkpoints (IR, UV, H_2O_2, MMS, hydroxyurea) and G-protein-coupled receptors (LPA). Lysates derived from these cells were immunoblotted with the S31-P, S325-P, T336-P, T366-P and S431-P antibodies. No agonist employed stimulated significant phosphorylation of LKB1 at Ser-31, Ser-325 or Thr-336 (results not shown). In contrast, IR and to a much lesser extent UV, hydroxyurea and MMS, stimulated phosphorylation of LKB1 at Thr-366 (Figure 1, second panel). Other agonists tested did not promote significant Thr-366 phosphorylation. As expected [17], agonists that activate RSK (EGF and PMA) and PKA (forskolin) induced phosphorylation of LKB1 at Ser-431 (Figure 1, second panel). As a control we verified that IR, UV, hydroxyurea and MMS stimulated the phosphorylation of Chk1 at Ser-345, a reaction mediated by the PI 3-kinase-like enzymes ATR and ATM that are activated in response to these treatments [24,32–34]. Moreover, insulin, IGF1 and EGF, which did not induce phosphorylation of LKB1 at Thr-366, did stimulate the phosphorylation of PKB at Ser-473, a known downstream response of these agonists [35]. In Figure 1 (third panel), we demonstrate that IR stimulation induced a rapid phosphorylation of LKB1 at Thr-366, which was maximal within 15 min and then slowly declined to near basal levels over an 8 h period. Notably, the time course of IR-induced phosphorylation and subsequent dephosphorylation of Chk1 closely mirrored that of LKB1 at Thr-366.

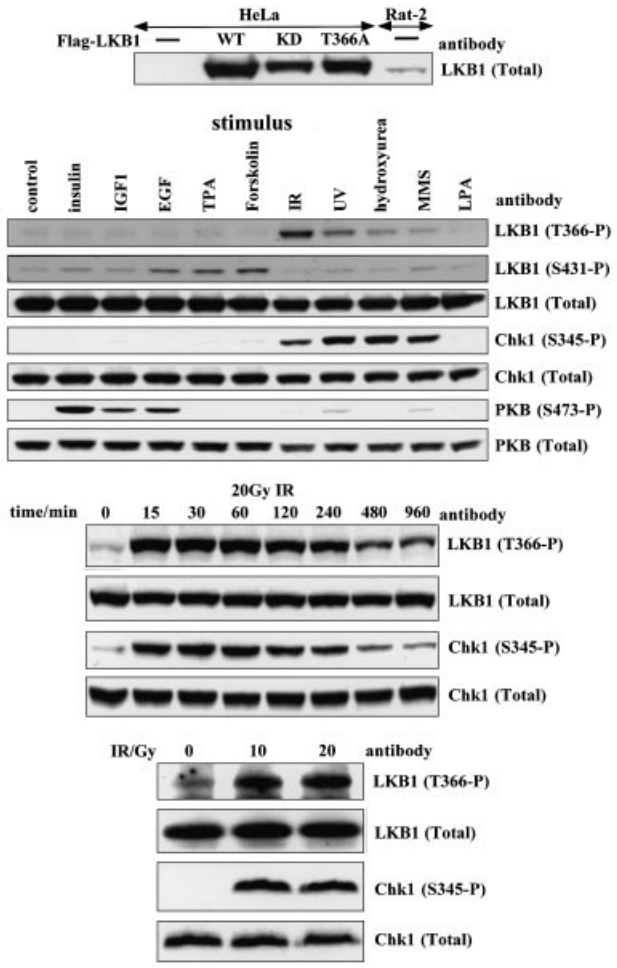

Figure 1 IR induces phosphorylation of LKB1 at Thr-366

Top panel: 20 μg of cell lysate derived from control HeLa cells transfected with the empty pcDNA4/TO vector (—), or HeLa cells stably expressing WT-LKB1 (WT), KD-LKB1 (KD) or mutant LKB1[T366A] (T366A), or Rat-2 cells were immunoblotted with an antibody recognizing LKB1. The HeLa cells were treated with 1 μg/ml tetracycline for 24 h prior to lysis. Second panel: HeLa cells stably expressing WT-LKB1 cultured with 1 μg/ml tetracycline for 24 h prior to lysis, were stimulated with insulin (100 nM, 10 min), IGF1 (10 ng/ml, 10 min), EGF (100 ng/ml, 10 min), PMA (400 ng/ml, 10 min), forskolin (20 μM, 10 min), IR (20 Gy, 30 min), UV (200 J/m^2, 30 min), hydroxyurea (2 mM, 2 h), MMS (0.01 %, v/v, 2 h) or LPA (1 μg/ml, 20 min). For the control, insulin, IGF1, EGF, PMA forskolin and LPA stimulations the cells were deprived of serum for 16 h prior to stimulation. Cell lysate (20 μg) was immunoblotted with the indicated antibodies as described in the Experimental section. Third panel: as above except cells were exposed to 20 Gy of IR and left for the indicated times prior to cell lysis. For the 0 min time point the cells were not irradiated. Bottom panel: Rat-2 cells cultured in the presence of serum were exposed to 0, 10 or 20 Gy of IR, left for 30 min at 37 °C, lysed and the endogenous LKB1 immunoprecipitated from lysates as described in the Experimental section. The immunoprecipitates were immunoblotted with LKB1 T366-P or total antibody. Total Rat-2 cell lysates (20 μg) were also immunoblotted with the Chk1 S345-P or total antibodies. Similar results were obtained in two (top and bottom panels) and three (middle panels) separate experiments.

We next investigated whether endogenous LKB1 in Rat-2 cells was phosphorylated at Thr-366 in response to IR. In Figure 1 (bottom panel) we demonstrate that exposure of Rat-2 cells to 10 or 20 Gy of IR induced a marked phosphorylation of endogenous LKB1 at Thr-366 as well as phosphorylation of Chk1 at Ser-345.

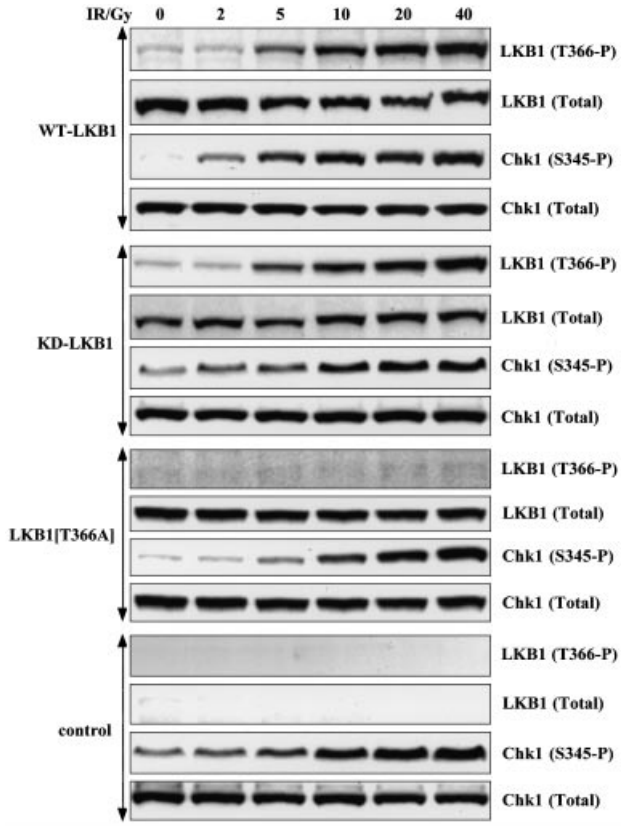

Figure 2 IR induces phosphorylation of KD-LKB1 at Thr-366

HeLa cells stably expressing WT-LKB1 (top panel), KD-LKB1 (second panel), LKB1[T366A] (third panel) or control HeLa cells transfected with empty pcDNA4/TO vector (bottom panel) were cultured with 1 μg/ml tetracycline for 24 h prior to lysis. The cells were exposed to the indicated dose of IR and lysed after 30 min. Total cell lysate (20 μg) was immunoblotted with the indicated antibodies. Similar results were obtained in three separate experiments.

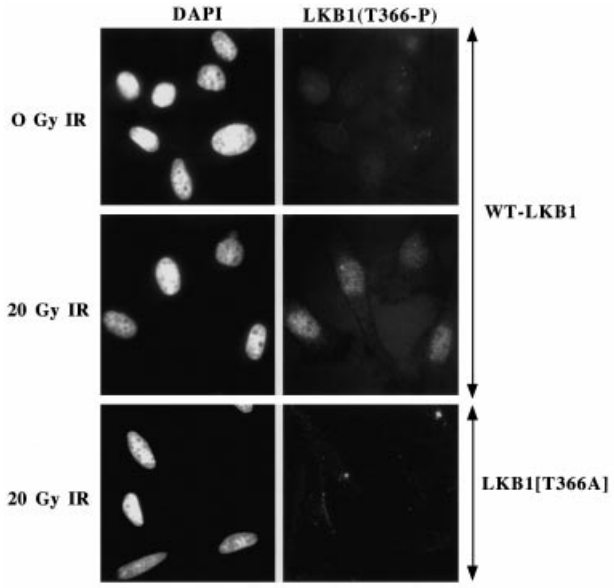

Figure 3 LKB1 phosphorylated at Thr-366 is located in the nucleus

HeLa cells stably expressing WT-LKB1 or LKB1[T366A] were cultured with 1 μg/ml tetracycline for 24 h. The cells were then left untreated or exposed to 20 Gy of IR and after 30 min fixed in 4% (v/v) paraformaldehyde, permeablized and immunostained with the T366-P antibody and stained with 4′,6′-diamidino-2-phenylindole (DAPI) to visualize nuclei as described in the Experimental section. The cells were examined using a Leica microscope.

Phosphorylation of LKB1 at Thr-366 induced by IR is not mediated by autophosphorylation

As discussed in the Introduction, LKB1 autophosphorylates itself weakly at Thr-366 *in vitro* [16]. To determine whether, in cells, IR was inducing phosphorylation of LKB1 at Thr-366 by an upstream kinase or through an autophosphorylation reaction, we generated a HeLa cell line that stably expresses KD-LKB1 and verified whether IR stimulated the phosphorylation of Thr-366 in these cells. We show in Figure 2 that WT-LKB1 and KD-LKB1 are similarly phosphorylated in HeLa cells exposed to increasing doses of IR. As a control to establish further the specificity of the T366-P antibody, we show that this antibody does not recognize the mutant LKB1[T366A] stably expressed in IR-exposed HeLa cells (Figure 2, third panel), which is expressed at the same level as WT-LKB1 (Figure 1, top panel). Moreover, the T366-P antibody does not recognize any protein in the parental HeLa cell line, which does not express LKB1 following IR exposure (Figure 2, bottom panel).

LKB1 phosphorylated at Thr-366 is nuclear

To investigate the location of LKB1 phosphorylated at Thr-366, we performed immunofluorescence studies on HeLa cells stably expressing WT-LKB1, employing the T366-P antibody. In non-IR-exposed cells, a low level of nuclear immunofluorescence was detected (Figure 3, top panels), which was markedly increased following exposure of cells to 20 Gy of IR for 30 min (Figure 3, middle panels). In the majority of IR-exposed cells, the staining of LKB1 phosphorylated at Thr-366 appeared entirely nuclear, and only a minority of cells displayed a low level of cytosolic staining. Consistent with the LKB1 T366-P phosphospecific antibody recognizing only phosphorylated LKB1, this antibody did not detect a significant signal in the nuclei of IR-stimulated HeLa cells stably expressing mutant LKB1[T366A] (Figure 3, bottom panels) or control HeLa cells not expressing LKB1 (results not shown). Moreover, in IR-treated HeLa cells stably expressing WT-LKB1, the phosphopeptide used to raise the T366-P antibody, but not the dephosphopeptide, completely blocked staining (results not shown).

DNA-PK and ATM phosphorylate LKB1 *in vitro* at Thr-366

In human, mouse and *Xenopus* LKB1, Thr-366 lies in the sequence Tyr-Thr-Gln-Asp and in *Drosophila* it lies in Gly-Thr-Gln-Glu [16]. It is well known that the PI 3-kinase-like protein kinases, such as DNA-PK, ATM and ATR, that are stimulated by IR and other DNA-damaging agents and which are also present in the cell nucleus, preferentially phosphorylate Thr/Ser residues that are followed by a Gln residue [19,21,36–39]. We therefore tested whether DNA-PK (Figure 4) and ATM (Figure 5) could phosphorylate LKB1 at Thr-366 *in vitro*. KD-LKB1 purified from HEK-293 cells was incubated in the presence or absence of the DNA-PK catalytic subunit, Ku70/80 complex, sheared calf thymus DNA and [γ-^{32}P]ATP [22]. KD-LKB1 only became significantly phosphorylated by DNA-PK catalytic subunit, when both Ku70/80 complex and sheared calf thymus DNA were included in the reaction (Figure 4, top left panel). Mutation of Thr-366 to Ala markedly inhibited LKB1 phosphorylation by DNA-PK, indicating that Thr-366 was the

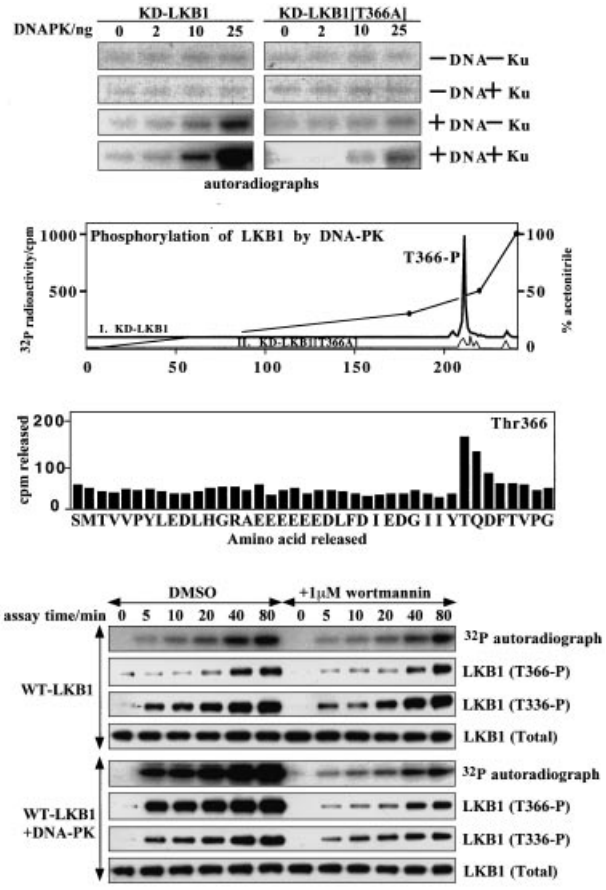

Figure 4 Phosphorylation of LKB1 at Thr-366 by DNA-PK

Top panel: a 50 μl reaction was set up as described in the Experimental section containing 1 μg of KD-LKB1 or KD-LKB1[T366A], the indicated amounts of catalytic subunit of DNA-PK, Mg[γ-^{32}P]ATP in the presence or absence of calf thymus DNA (DNA; 10 ng) or Ku70/80 subunit (Ku; a 3-fold less by mass than the amount of DNA-PK catalytic subunit). After 30 min of incubation, SDS sample buffer was added and the reactions were subjected to electrophoresis on polyacrylamide gel and transferred to a nitrocellulose membrane. The membranes were stained with Ponceau S and the bands corresponding to LKB1 were autoradiographed. Second panel: KD-LKB1 or KD-LKB1[T366A] (2 μg) was incubated for 1 h in the presence of Mg[γ-^{32}P]ATP, DNA-PK (50 ng), calf thymus DNA (10 ng) and Ku70/80 (17 ng) and electrophoresed on a polyacrylamide gel which was autoradiographed. The ^{32}P-labelled LKB1 was digested with trypsin and the resulting ^{32}P-labelled peptides were chromatographed on a Vydac 218TP54 C_{18} column (Separations Group, Hesperia, CA, U.S.A.) equilibrated in 0.1% (v/v) trifluoroacetic acid in water. The column developed with a linear acetonitrile gradient (diagonal line) at a flow rate of 0.8 ml/min, and fractions of 0.4 ml were collected; approx. 80% of the radioactivity applied to the column was recovered in the fractions. Third panel: the major ^{32}P-labelled peptide derived from the KD-LKB1 sample was covalently coupled to a Sequelon arylamine membrane and analysed on an Applied Biosystems 476A sequenator. ^{32}P radioactivity was measured after each cycle of Edman degradation. Bottom panel: WT-LKB1 was incubated in the presence or absence of DNA-PK or 1 μM wortmannin employing the conditions used in the top panel, for the times indicated. The reactions were terminated by addition of SDS sample buffer. A proportion (90%) of each sample was used to analyse LKB1 phosphorylation as described in the top panel. Of each reaction, 1% (containing 10 ng of LKB1) was also electrophoresed and immunoblotted with the indicated antibodies. Similar results were obtained in two (top and bottom panels) and three (middle panels) separate experiments.

major site of phosphorylation (Figure 4, top right panel). This was also verified by quantitative tryptic phosphopeptide mass analysis (Figure 4, second panel). LKB1 phosphorylated by DNA-PK *in vitro* to approx. 0.2 mol of [^{32}P]phosphate/mol of LKB1 was digested with trypsin and the resulting tryptic peptides resolved on a C_{18} column. A single major late-eluting ^{32}P-labelled peptide was recovered, whose mass was determined by MALDI-TOF MS as 6658.5, which is close to the expected mass (6657.1) of the LKB1 peptide comprising residues 334–392 phosphorylated at a single residue and comprising an oxidized Met. Solid-phase Edman sequencing of this peptide resulted in a single release of ^{32}P radioactivity at the 33rd cycle of Edman sequencing, confirming that this peptide is phosphorylated at the position equivalent to Thr-366 (Figure 4, third panel). As expected, this ^{32}P-labelled peptide was not present when the LKB1[T366A] mutant was phosphorylated by DNA-PK (Figure 4, second panel). As discussed above, we previously observed that WT-LKB1, but not KD-LKB1, is able to weakly phosphorylate itself at Thr-366 when incubated with MnATP *in vitro* [16]. In Figure 4 (bottom panel), we demonstrate that the rate at which DNA-PK phosphorylates WT-LKB1 is vastly higher than the rate at which WT-LKB1 becomes phosphorylated at Thr-366 in the absence of DNA-PK. Furthermore, the phosphorylation of LKB1 at Thr-366 by DNA-PK is largely prevented by 1 μM wortmannin, an inhibitor of all PI 3-kinase-like enzymes, but wortmannin does not affect the low level of autophosphorylation of LKB1 at Thr-366 that occurs in the absence of DNA-PK (Figure 4, bottom panel). We also assessed the effect that phosphorylation of LKB1 at Thr-366 by DNA-PK had on the ability of LKB1 to phosphorylate itself at its principal autophosphorylation site (Thr-336). As shown in Figure 4 (bottom panel), the phosphorylation of LKB1 at Thr-366 by DNA-PK does not significantly affect the rate at which LKB1 phosphorylates itself at Thr-336.

ATM was immunoprecipitated from melanoma G361 cells, which express relatively high levels of this enzyme, an approach previously employed to isolate ATM for *in vitro* kinase assays [26,38]. ATM immunoprecipitates significantly phosphorylated KD-LKB1, but not KD-LKB1[T366A] (Figure 5). Immunoblotting with the T366-P antibody also confirmed that ATM phosphorylated LKB1 at Thr-366. As a control, we demonstrate that a non-specific antibody failed to immunoprecipitate an activity from G361 cells that phosphorylated LKB1. Moreover, we show that ATM immunoprecipitations with the ATM antibody, employing cell lysates derived from ATM$^{-/-}$ cells [28], were not associated with an activity that phosphorylated LKB1 (Figure 5). The immunoprecipitates were also immunoblotted with an ATM antibody to confirm the presence of ATM in the immunoprecipitates derived from G361 cells (Figure 5).

Evidence that ATM mediates IR-induced phosphorylation of LKB1 at Thr-366

We next expressed LKB1 in the well-characterized ATM-proficient (ATM$^{+/+}$) and ATM-deficient (ATM$^{-/-}$) AT2211JE-FT cell line [28]. We show in Figure 6 that IR induced phosphorylation of LKB1 at Thr-366 in ATM$^{+/+}$ but not ATM$^{-/-}$ cells. We also exposed these cells to UV irradiation and MMS, a DNA-methylating agent, which unlike IR is thought to engage checkpoint responses mediated by ATR rather than ATM and DNA-PK [40,41]. Consistent with LKB1 being phosphorylated by ATM, in the ATM$^{+/+}$ cells, UV only stimulated Thr-366 phosphorylation to a small extent, whereas MMS failed to promote LKB1 phosphorylation. In ATM$^{+/+}$ cells IR, UV and MMS stimulated phosphorylation of Chk1 at Ser-345, but in ATM$^{-/-}$ cells IR barely induced Chk1 phosphorylation, suggesting that under these conditions Chk1 is phosphorylated by ATM. As expected, the ATR-activating treatments UV and MMS stimulated phosphorylation of Chk1 to a level similar to that observed in ATM$^{+/+}$ cells (Figure 6).

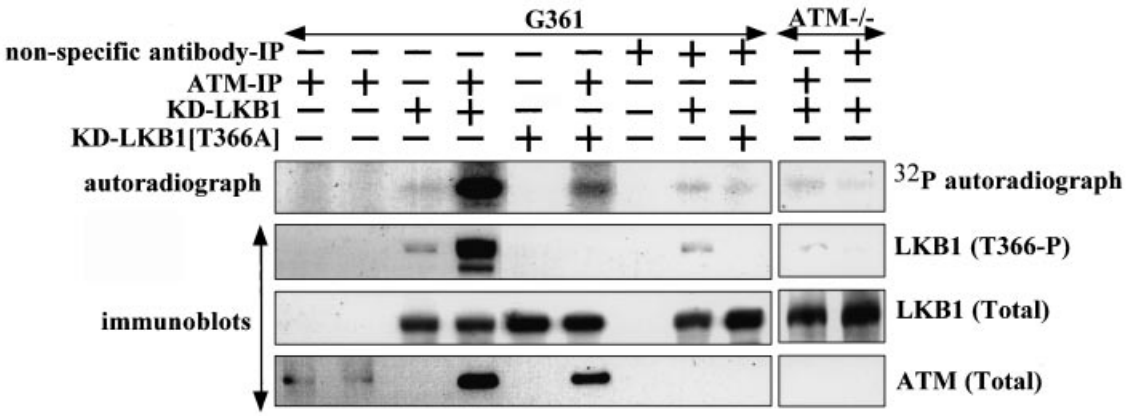

Figure 5 Phosphorylation of LKB1 at Thr-366 by ATM

KD-LKB1 or KD-LKB1[T366A] (1 μg) was incubated with Mg/Mn[γ-^{32}P]ATP in the presence or absence of the indicated immunoprecipitates as described in the Experimental section. After 1 h, the reactions were terminated by addition of SDS sample buffer. Each reaction (80%) was subjected to electrophoresis on a polyacrylamide gel and transferred to a nitrocellulose membrane. The membranes were stained with Ponceau S and the bands corresponding to LKB1 were autoradiographed. Then 2% of each reaction (containing 20 ng of LKB1) was also electrophoresed and immunoblotted with the indicated antibodies. Similar results were obtained in three separate experiments.

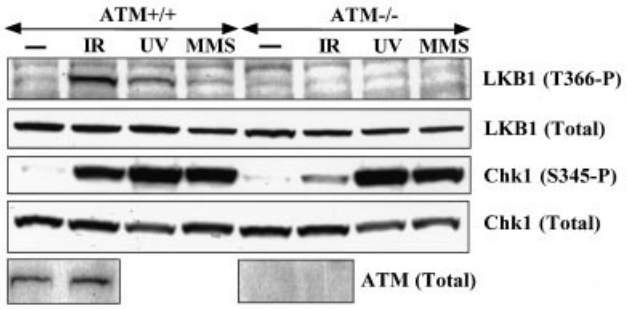

Figure 6 Evidence that ATM phosphorylates LKB1 at Thr-366 *in vivo*

ATM-proficient (ATM$^{+/+}$) and ATM-deficient (ATM$^{-/-}$) AT2211JE-FT cell lines were transfected with a construct coding for YFP-LKB1 to visualize transfected cells. Post-transfection (36 h) the cells were left untreated or exposed to IR (20 Gy, 30 min), UV (200 J/m^2, 30 min) or MMS (0.01%, v/v, 2 h). The cells were lysed and 20 μg of cell lysate was electrophoresed and subsequently immunoblotted with the indicated antibodies. Similar results were obtained in three separate experiments.

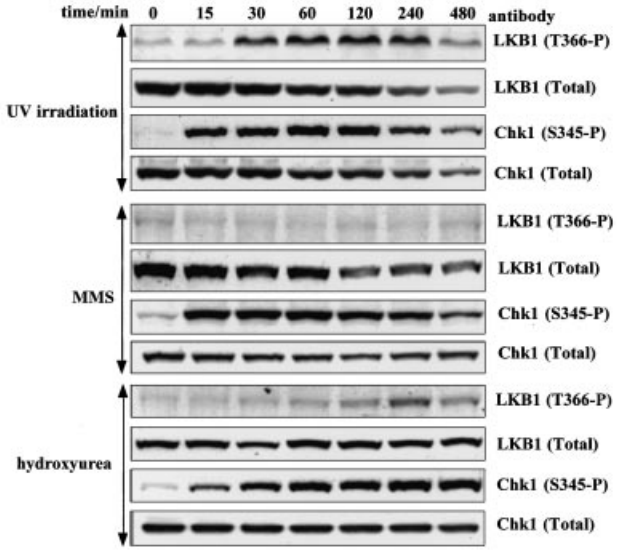

Figure 7 Evidence that LKB1 is a poor substrate for ATR

HeLa cells stably expressing WT-LKB1 were cultured with 1 μg/ml tetracycline for 24 h prior to lysis. The cells were exposed to UV (200 J/m^2), MMS (0.01%, v/v) or hydroxyurea (2 mM) for the times indicated. Total cell lysate (20 μg) was immunoblotted with the indicated antibodies. Similar results were obtained in two (MMS and hydroxyurea) and four (UV) separate experiments.

We also investigated the effects of stimulating the HeLa cell line stably expressing WT-LKB1 with UV irradiation, MMS and hydroxyurea, an inhibitor of DNA replication, which also preferentially activates ATR [42]. UV irradiation induced a moderate phosphorylation of LKB1 compared with IR, and this phosphorylation occurred at a later time point (compare Figure 1, bottom panel, and Figure 7, top panel). In contrast, UV irradiation stimulated maximal phosphorylation of Chk1 within 15 min (Figure 7, top panel). The ATR activators MMS (Figure 7, middle panel) and hydroxyurea (Figure 7, bottom panel) failed to stimulate significant phosphorylation of LKB1 at Thr-366, whereas they markedly promoted Chk1 phosphorylation within 15 min. It should be noted that we consistently observed that after 2 h of UV irradiation, and to a lesser extent MMS treatment, the levels of LKB1 protein were also reduced. The molecular basis for this later observation was not investigated further.

DISCUSSION

The results of this study provide the first link between two protein kinases, ATM and LKB1, that are mutated in inherited cancer syndromes and are both implicated in regulating cell division. Mutations in ATM cause ataxia telangiectasia, an autosomal recessive disorder characterized by cancer predisposition, immunodeficiency and radiation sensitivity [43,44]. Recent evidence establishes that ATM together with the related

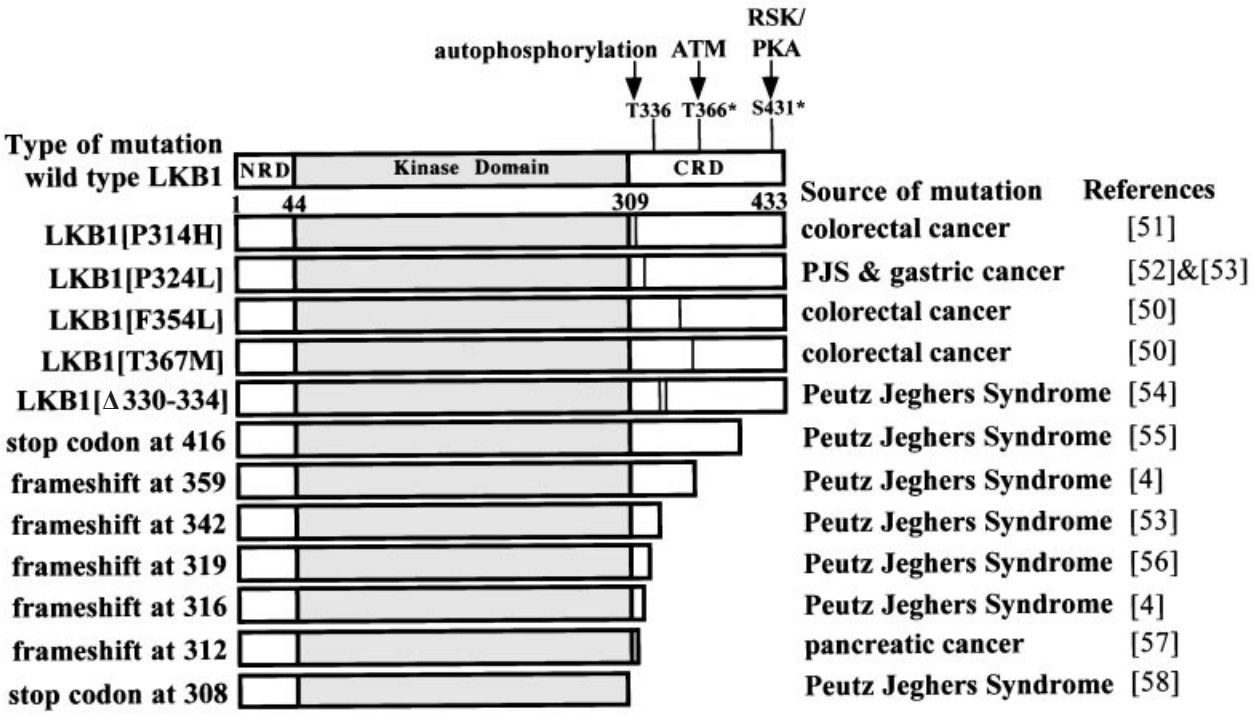

Figure 8 C-terminal mutants of LKB1 found in human cancer

The 12 different mutations of LKB1 that have thus far been identified that only affect the C-terminal non-catalytic domain of LKB1 are illustrated. The locations of the Thr-336, Thr-366 and Ser-431 phosphorylation sites are indicated. Asterisks on Thr-366 and Ser-431 denote numbering based on mouse LKB1 sequence that has been employed throughout the text, which correspond to Thr-363 and Ser-428 in human LKB1 respectively. The numbering of the mutations found in human cancers is based on the human sequences. NRD, N-terminal regulatory domain; CRD, C-terminal regulatory domain.

enzymes DNA-PK and ATR function as master sensors of aberrant DNA structures, ensuring that when these structures are detected cell-cycle progression is delayed until the structures are resolved [19,20]. Aberrant DNA structures engage DNA-PK, ATM and ATR, resulting in these enzymes phosphorylating proteins that regulate DNA-damage responses such as p53 [26], Chk1 [24,33], Chk2 [45] and interferon regulatory protein-3 [41].

A key feature that has enabled the identification of several substrates of these PI 3-kinase-like enzymes has been the characterization of the key determinants of their substrate specificity. DNA-PK and ATM appear to preferentially phosphorylate Ser/Thr residues preceded by a Gln residue [19,21,36,37,39]. Using oriented peptide library approaches, the substrate specificity requirements of ATM have been further refined to Hyd-Thr/Ser-Gln-Glu/Asp, where Hyd comprises a hydrophobic amino acid and the underlined residue is phosphorylated [21,38,39]. The residues surrounding Thr-366 of mammalian and *Xenopus* LKB1 are Tyr-Thr-Gln-Asp, which match perfectly with the predicted optimal consensus motif for ATM. Consistent with ATM phosphorylating LKB1 at Thr-366 *in vivo*, we find that IR stimulation, which generates double-stranded breaks in DNA, thereby activating ATM, induces phosphorylation of LKB1 at Thr-366 (Figures 1 and 2), and this does not occur in ATM-deficient cells (Figure 6). We also show that LKB1 phosphorylated by ATM is located in the nucleus (Figure 3), which would be expected as ATM is present in the nucleus [46] and LKB1 is also predominantly localized in the nucleus of HeLa cells [6,16,47]. We also expressed YFP-tagged LKB1 in HeLa cells and did not observe any change in the nuclear localization pattern of LKB1 following IR stimulation of cells (results not shown). The time course and dose dependence of LKB1 phosphorylation at Thr-366 closely parallels that of phosphorylation of Chk1 at Ser-345 (Figures 1 and 2) in IR-treated cells. Chk1 has been shown to be phosphorylated [24,48] and activated [49] in response to IR, and this phosphorylation is reduced or absent in $ATM^{-/-}$ cells [24]. Taken together, these data and the results presented in Figure 6 suggest that Chk1 is a direct substrate for ATM. Moreover, exposure of cells to UV radiation, hydroxyurea and MMS, treatments that engage ATR rather than ATM, induce little or no phosphorylation of LKB1, while still promoting marked Chk1 phosphorylation (Figures 6 and 7).

The key next stage of this project would be to establish the mechanism by which phosphorylation of LKB1 at Thr-366 regulates the function of this enzyme. Mutation of Thr-366 to Ala or Glu does not affect the cellular localization of LKB1 or the rate at which LKB1 phosphorylates the p53 protein *in vitro* or phosphorylates itself at Thr-336 [16]. Consistent with the notion that phosphorylation of LKB1 at Thr-366 does not directly regulate LKB1 kinase activity, we demonstrate in Figure 4 (bottom panel) that maximal phosphorylation of LKB1 at Thr-366 by DNA-PK does not affect the rate at which LKB1 autophosphorylates at Thr-336. However, phosphorylation of LKB1 at Thr-366 may have some role in enabling LKB1 to suppress cell growth, as LKB1[T366A] and LKB1[T366E] mutants in G361 cells were 3-fold less able to suppress cell growth compared with WT-LKB1 [16].

The function of the non-catalytic domain of LKB1, in which Thr-366 is located, is not known. We have shown that an LKB1

mutant encompassing residues 1–343, lacking most of the C-terminal non-catalytic domain, possesses similar *in vitro* kinase activity as full-length LKB1, but was unable to suppress cell growth (G.P.S. and A.K., unpublished work). These findings indicate that this region of LKB1 is essential for its ability to suppress cell growth. We have analysed the literature on LKB1 mutations found in human cancers and show in Figure 8 that 12 different mutations have thus far been identified, which only affect the C-terminal non-catalytic domain. To our knowledge the ability of these mutants to affect cell growth has not yet been analysed. Interestingly, one of the mutations identified in a PJS patient converts a Thr located four amino acids C-terminal to Thr-366 into a Met residue [50]. It would be interesting to explore whether this PJS mutation affects phosphorylation of Thr-366 by LKB1 or the transmission of the effect that Thr-366 phosphorylation has on LKB1 function. In future studies, it will be essential to determine the role that phosphorylation of LKB1 at Thr-366 plays and whether this has a role in regulating cellular response to DNA damage. Indeed, to achieve this it may be necessary to generate a knock-in mutation of LKB1 in mice or cells that prevents or mimics phosphorylation of this site. One would also need to identify the mechanism by which the non-catalytic region of LKB1 participates in regulating LKB1 function.

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