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Pharmacological inhibition of Polo-like kinase 1 (PLK1) by BI-2536 decreases the viability and survival of hamartin and tuberin deficient cells via induction of apoptosis and attenuation of autophagy

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Abbreviations: LAM, Lymphangioleiomyomatosis; MEF, mouse embryonic fibroblasts, mTORC1, mechanistic target of rapamycin complex 1; PLK1, polo-like kinase 1; TSC, Tuberous Sclerosis Complex.

The mechanistic target of rapamycin complex 1 (mTORC1) increases translation, cell size and angiogenesis, and inhibits autophagy. mTORC1 is negatively regulated by hamartin and tuberin, the protein products of the tumor suppressors *TSC1* and *TSC2* that are mutated in Tuberous Sclerosis Complex (TSC) and sporadic Lymphangioleiomyomatosis (LAM). Hamartin interacts with the centrosomal and mitotic kinase polo-like kinase 1 (PLK1). Hamartin and tuberin deficient cells have abnormalities in centrosome duplication, mitotic progression, and cytokinesis, suggesting that the hamartin/tuberin heterodimer and mTORC1 signaling are involved in centrosome biology and mitosis. Here we report that PLK1 protein levels are increased in hamartin and tuberin deficient cells and LAM patient-derived specimens, and that this increase is rapamycin-sensitive. Pharmacological inhibition of PLK1 by the small-molecule inhibitor BI-2536 significantly decreased the viability and clonogenic survival of hamartin and tuberin deficient cells, which was associated with increased apoptosis. BI-2536 increased p62, LC3B-I and GFP-LC3 punctae, and inhibited HBSS-induced degradation of p62, suggesting that PLK1 inhibition attenuates autophagy. Finally, PLK1 inhibition repressed the expression and protein levels of key autophagy genes and proteins and the protein levels of Bcl$^-$2 family members, suggesting that PLK1 regulates both autophagic and apoptotic responses. Taken together, our data point toward a previously unrecognized role of PLK1 on the survival of cells with mTORC1 hyperactivation, and the potential use of PLK1 inhibitors as novel therapeutics for tumors with dysregulated mTORC1 signaling, including TSC and LAM.

Introduction

Mutations in the tumor suppressor genes *TSC1* and *TSC2* cause Tuberous Sclerosis Complex (TSC) and sporadic Lymphangioleiomyomatosis (LAM). TSC is a syndrome of neurocutaneous origin characterized by hamartomatous growth of benign tumors. LAM is caused by diffuse smooth muscle cell infiltration and proliferation in the lung parenchyma. Hamartin and tuberin, the protein products of *TSC1* and *TSC2*, respectively, form a functional heterodimer that negatively regulates cell growth via tuberin's GTPase Activating Protein activity toward the small GTPase Rheb, which in turn activates the mechanistic target of rapamycin complex 1 (mTORC1). Upon growth factor stimulation, the hamartin/tuberin complex is subjected to inhibitory phosphorylation by multiple kinases, including AKT/PKB, ERK1/2, and RSK1. Under oxidative/bioenergetic stress or

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hypoxia the hamartin/tuberin heterodimer is positively regulated by AMPK and REDD1, respectively (reviewed in refs.[1-4]).

Since the primary signaling defect in TSC and LAM tumors is hyperactivation of mTORC1, rapamycin and rapamycin analogs have been proposed as a treatment option for these diseases. Rapamycin, or its analogs, effectively reduce subependymal giant astrocytoma and renal angiomyolipoma volume, and improve pulmonary function.[5-8] However, upon cessation of therapy angiomyolipomas rapidly re-grow and pulmonary function deteriorates,[7,8] suggesting that long-term treatment with these drugs, with potentially unknown adverse effects, may be necessary to control TSC and LAM lesions.

Macroautophagy (hereafter termed simply autophagy) is an evolutionary conserved catabolic mechanism whose signaling events have only lately been thoroughly delineated. Autophagy is negatively regulated by mTORC1 and positively regulated by AMPK via phosphorylation of the ULK1/ULK2 initiation complex (reviewed in refs.[9,10]). Upon nutrient starvation (mTORC1 inhibition) or bioenergetic/oxidative stress (AMPK activation) autophagy is promoted. In tumor cells autophagy can contribute to either their survival or their death depending on stage of tumorigenesis, growth conditions, and presence of cellular stress (for a review see ref.[11]). Perturbations in autophagy in hamartin and tuberin deficient cells, which have decreased autophagy due to mTORC1 hyperactivation, either via genetic inactivation of autophagy genes or pharmacological inhibition of autophagolysosomal content degradation leads to decreased cell survival and tumorigenesis.[12]

Previously we found that hamartin interacts with the polo-like kinase 1 (PLK1), and that silencing of PLK1 causes a significant decrease in phosphorylation of the mTORC1 effectors p70S6K, ribosomal protein S6, and 4E-BP1, suggesting that PLK1 positively regulates mTORC1.[13] PLK1 has been implicated in regulation of centrosome maturation and duplication, mitotic entry, anaphase progression, cytokinesis, and re-entry to the cell cycle after DNA damage repair (for a review see ref. [14]). Expression of PLK1 is increased in several cancer cells and tumors, including breast, pancreatic, colorectal, and prostate.[15-19] Inhibition of PLK1 by small-molecule inhibitors, RNAi, or hypomorphic shRNA variants, leads to prolonged G2/M arrest, mitotic spindle defects and mitotic catastrophe, and sensitizes cancer cells to apoptosis.[20-24] Several PLK1 small-molecule inhibitors are currently in phase I/II clinical trials for use in oncology (for a review, see ref.[25])

Here we report that hamartin and tuberin deficient cells have increased PLK1 protein levels, which is rescued by ectopic expression of hamartin or tuberin. This increase in PLK1 protein levels is rapamycin-sensitive. Additionally, PLK1 expression correlates with phosphorylation of ribosomal protein S6 in LAM patient-derived tumor specimens. Inhibition of PLK1 using the small-molecule inhibitor BI-2536 significantly decreased the viability and anchorage-dependent clonogenic survival of hamartin and tuberin deficient cells, and induced apoptosis, compared to controls. Unexpectedly, BI-2536 strongly attenuated autophagy, measured by steady-state p62 and LC3B-I protein levels and number of GFP-LC3 punctae, and by amino acid starvation-induced p62 degradation. Additionally, in tuberin deficient LAM patient-derived cells BI-2536 modified the expression and protein levels of autophagy and Bcl-2 family members.

Results

Hamartin and tuberin deficient cells have increased PLK1 protein levels

We previously showed that *Tsc1*$^{-/-}$ mouse embryonic fibroblasts (MEF) have increased PLK1 protein levels, compared to *Tsc1*$^{+/+}$, both in asynchronous cell populations and in cells arrested in G2/M with nocodazole.[13] We confirmed the increased PLK1 protein levels in *Tsc1*$^{-/-}$ MEF, compared to *Tsc1*$^{+/-}$, and in *Tsc2*$^{-/-}$/*Tp53*$^{-/-}$ MEF, compared to *Tsc2*$^{+/+}$/*Tp53*$^{-/-}$ (**Fig. 1A**). Stable retroviral transduction of hamartin or tuberin in *Tsc1*$^{-/-}$ MEF and tuberin deficient ELT3 cells, respectively, resulted in decreased PLK1 protein levels, compared to isogenic cell lines stably transduced with empty vector (**Fig. 1B**). To determine whether this increase in PLK1 protein levels is evident in human tumors with mTORC1 hyperactivation, PLK1 immunohistochemical staining was performed in 5 LAM-derived lung specimens. In all cases, PLK1 immunostaining correlated

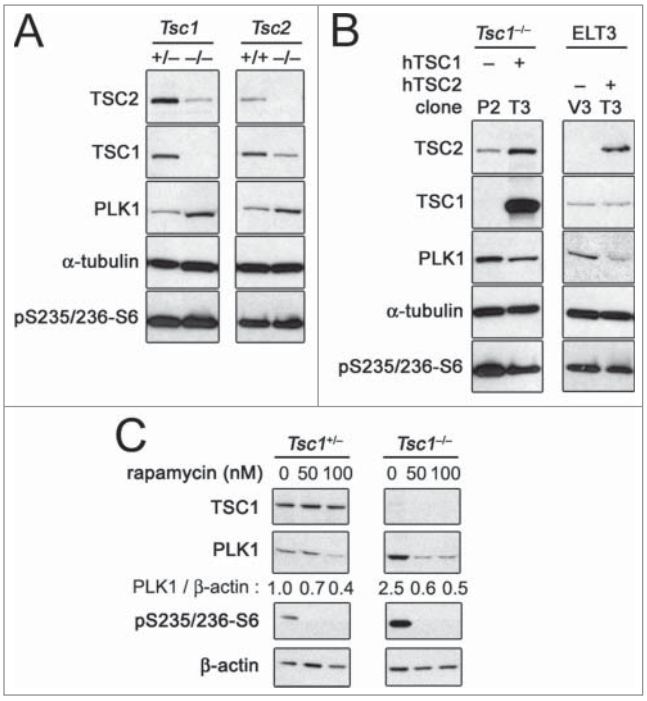

Figure 1. PLK1 protein levels are increased in hamartin and tuberin deficient cells and are rapamycin-sensitive. (**A**) Lysates from exponentially growing *Tsc1*$^{+/-}$, *Tsc1*$^{-/-}$, *Tsc2*$^{+/+}$/*Tp53*$^{-/-}$ and *Tsc2*$^{-/-}$/*Tp53*$^{-/-}$ MEF were immunoblotted with the indicated antibodies. (**B**) Lysates from *Tsc1*$^{-/-}$ MEF transduced with vector (208-P2) or TSC1 (208-T3), and from tuberin-deficient ELT3 cells transduced with vector (ELT3-V3) or TSC2 (ELT3-T3) were immunoblotted with the indicated antibodies. (**C**) Lysates from *Tsc1*$^{+/-}$ and *Tsc1*$^{-/-}$ MEF treated for 24 hours with 0, 50 or 100 nM rapamycin were immunoblotted with the indicated antibodies. PLK1 protein levels normalized to β-actin and to vehicle-treated *Tsc1*$^{+/-}$ MEF are indicated below the PLK1 immunoblots.

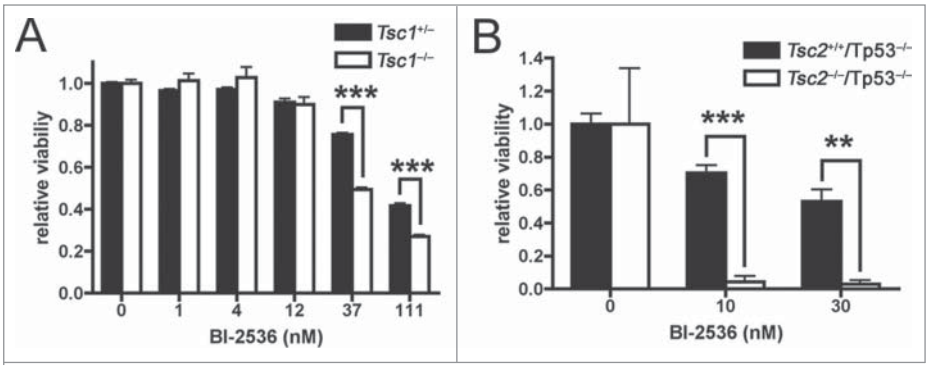

Figure 2. Pharmacological inhibition of PLK1 decreases the viability of hamartin and tuberin deficient cells. (**A**) *Tsc1*$^{+/-}$ and *Tsc1*$^{-/-}$ MEF and (**B**) *Tsc2*$^{+/+}$/*Tp53*$^{-/-}$ and *Tsc2*$^{-/-}$/*Tp53*$^{-/-}$ MEF, were treated with BI-2536 for 4 days, viability was measured by MTT conversion, and normalized to vehicle-treated controls ($n = 8$; ** indicates $P < 0.01$; *** indicates $P < 0.001$).

with increased immunoreactivity to pS235/236-S6 in adjacent sections (**Fig. S1**). Rapamycin treatment resulted in a significant decrease of PLK1 protein levels in *Tsc1*$^{-/-}$ MEF (**Fig. 1C**), and to a lesser extent in *Tsc1*$^{+/-}$ MEF. Similar results were obtained with stable isogenic clones of *Tsc1*$^{-/-}$ MEF and ELT3 cells (**Fig. S2**). These data demonstrate that loss of hamartin or tuberin results in increased PLK1 protein levels in a rapamycin-sensitive manner, and that mTORC1 hyperactivation in LAM specimens is associated with increased PLK1.

Inhibition of PLK1 decreases the viability and clonogenic survival of hamartin and tuberin deficient cells

Increased PLK1 expression has been reported in several forms of cancer. PLK1 small molecule inhibitors preferentially sensitize cancer cells to apoptosis, compared to normal cells, and are currently in clinical trials for oncology. To determine whether PLK1 inhibition decreases the viability of hamartin and tuberin deficient cells, MTT assays were performed after treatment with the PLK1 inhibitor BI-2536.[24] BI-2536 induced a significant decrease of viability in *Tsc1*$^{-/-}$ and *Tsc2*$^{-/-}$/*Tp53*$^{-/-}$ MEF, compared to control cells, in a dose dependent manner (**Fig. 2**). Similarly, BI-2536 resulted in decreased viability of hamartin deficient 208-P2 MEF, compared to hamartin re-expressing 208-T3 isogenic cells (**Fig. S3**).

To determine whether PLK1 inhibition decreases the survival of hamartin and tuberin deficient cells, clonogenic assays were performed after treatment with BI-2536 for 72 hours. Consistent with the results showing that PLK1 inhibition decreases the viability of hamartin and tuberin deficient cells, BI-2536 caused a significant decrease in anchorage-dependent clonogenic survival of *Tsc1*$^{-/-}$ MEF, compared to *Tsc1*$^{+/-}$ (**Fig. 3A**), and in HeLa cells transduced with TSC1 or TSC2 shRNA, compared to control shRNA (**Fig. 3B**). Similarly, BI-2536 significantly decreased the survival of hamartin deficient 208-P2 MEF, compared to hamartin re-expressing 208-T3 (**Fig. S4A**). A significant reduction of clonogenic survival was also observed in 208-P2 cells treated with compound 1, another PLK1 inhibitor,[23] compared to 208-T3 (**Fig. S4B**).

Collectively, these data demonstrate that PLK1 inhibitors decrease the viability and anchorage-dependent clonogenic survival of hamartin and tuberin deficient cells.

Inhibition of PLK1 induces apoptosis and attenuates autophagy in hamartin and tuberin deficient cells

Treatment of hamartin deficient 208-P2 MEF with 30 nM BI-2536 for 72 hours resulted in a profound growth inhibition and induction of morphological changes reminiscent of apoptosis (**Fig. 4A**). On the other hand, BI-2536

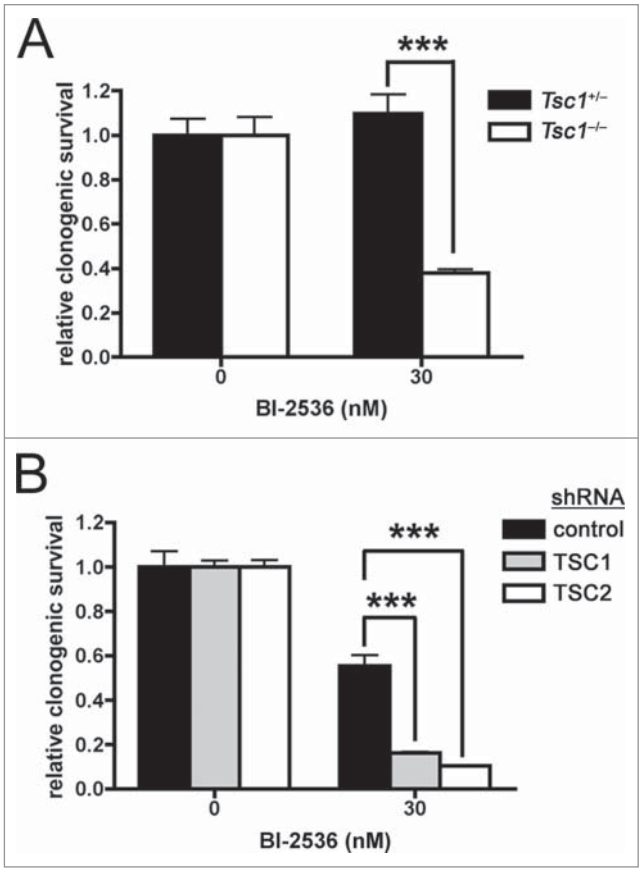

Figure 3. Pharmacological inhibition of PLK1 decreases the clonogenic survival of hamartin and tuberin deficient cells. (**A**) *Tsc1*$^{+/-}$ and *Tsc1*$^{-/-}$ MEFs were treated with 30 nM BI-2536 for 3 days, followed by clonogenic survival assay. (**B**) HeLa cells after shRNA-mediated silencing of TSC1 or TSC2 were treated with 30 nM BI-2536 for 24 hours, followed by clonogenic survival assay. For both panels $n = 3$, and *** indicates $P < 0.001$.

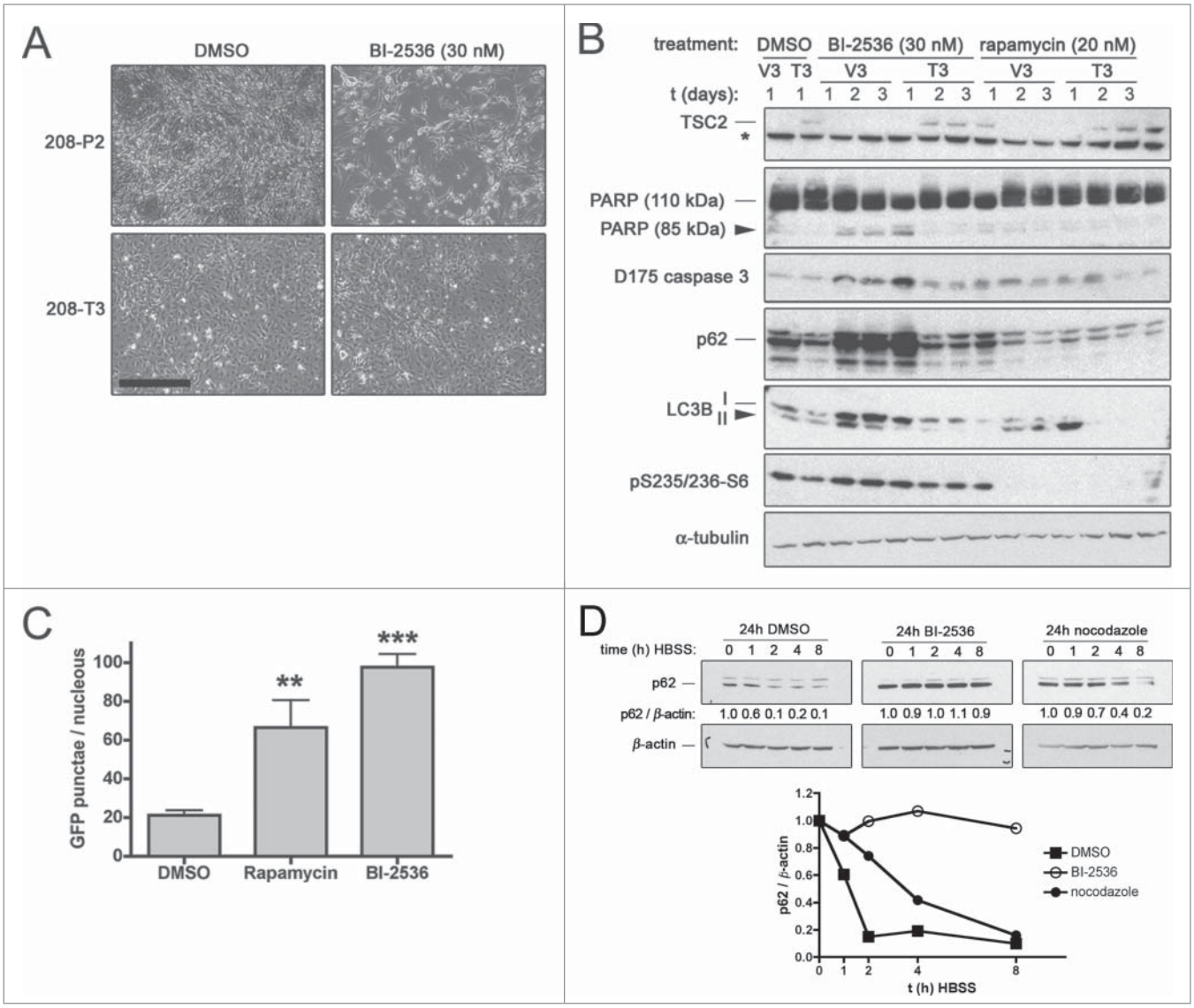

Figure 4. Pharmacological inhibition of PLK1 induces apoptosis in hamartin and tuberin deficient cells and attenuates autophagy. (**A**) Phase contrast micrographs of hamartin deficient 208-P2 MEF and hamartin re-expressing 208-T3 MEF treated with vehicle control (DMSO) or 30 nM BI-2536 for 3 days. Scale bar 400 μm. (**B**) Tuberin deficient ELT3-V3 and tuberin re-expressing ELT3-T3 cells were treated with vehicle control (DMSO), 30 nM BI-2536, or 20 nM rapamycin for 1, 2 and 3 days, and lysates were immunoblotted with the indicated antibodies. Asterisk (*) on tuberin immunoblot indicates a non-specific band identified on rat species with the anti-tuberin antibody used, and was previously described.[50] (**C**) WI38 primary fibroblasts ectopically expressing GFP-LC3 were treated with vehicle control (DMSO), 20 nM rapamycin, or 100 nM BI-2536 for 24 hours, fixed, and the number of GFP-LC3 punctae per nucleus was quantified from epi-fluorescence digital micrographs. $n \geq 9$, ** and *** indicate $P < 0.01$ and $P < 0.001$, respectively, compared to DMSO. (**D**) HEK293 cells were pre-treated for 24 hours with 0.1% v/v DMSO, or 100 nM BI-2536, or 70 ng/ml nocodazole, media were removed, cell

had little effect on growth of hamartin re-expressing 208-T3 cells. To determine whether the BI-2536-induced decrease in viability and survival of hamartin and tuberin deficient cells is attributed to apoptosis, cells were treated with BI-2536 for 1-3 days and lysates were immunoblotted for poly (ADP-ribose) polymerase (PARP) and D175-caspase 3 (cleaved caspase 3). BI-2536 strongly induced the cleaved forms of PARP and caspase 3 in tuberin deficient ELT3-V3 cells at 1, 2, and 3 days of treatment, compared to tuberin re-expressing ELT3-T3 cells (**Fig. 4B**). Rapamycin treatment had no effect on induction of these apoptotic markers. Similar results were observed in hamartin deficient 208-P2 MEF, compared to hamartin re-expressing 208-T3, and in *Tsc1*$^{-/-}$ MEF, compared to *Tsc1*$^{+/-}$ (**Fig. S5A and S5B**, respectively). These results suggest that PLK1 inhibition by BI-2536 induces apoptosis preferentially in hamartin and tuberin null cells.

Since mTORC1 is a negative regulator of autophagy, we examined the effects of PLK1 inhibition on conversion of LC3B-I to LC3B-II and levels of p62 (SQSTM1), an autophagosome cargo protein. Treatment of tuberin deficient ELT3-V3 cells with BI-2536 resulted in a strong induction of LC3B-I and inhibition of its conversion to LC3B-II (**Fig. 4B**), whereas rapamycin induced the formation of LC3B-II as expected. A significant increase in p62 protein levels was observed in cells treated with

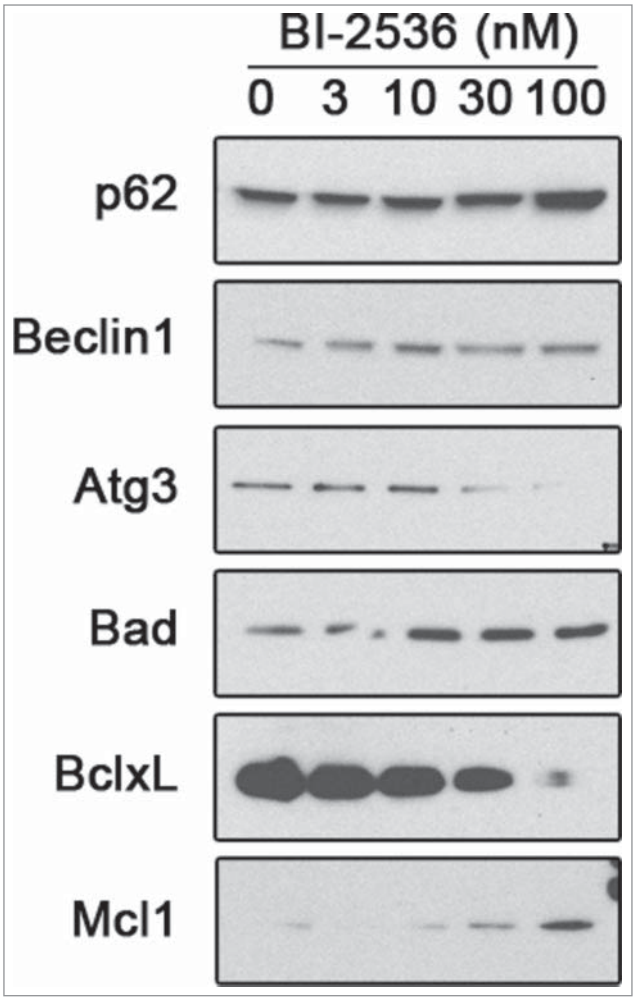

Figure 5. Pharmacological inhibition of PLK1 alters protein levels of autophagy and Bcl-2 family member proteins. 621-101 cells were treated for 24 hours with the indicated concentrations of BI-2536, and the lysates were immunoblotted with the indicated antibodies.

BI-2536, opposed to the expected and observed decrease in p62 after rapamycin treatment. These effects of BI-2536 were observed also in tuberin re-expressing ELT3-T3 cells, but to a lesser extent compared to tuberin deficient ELT3-V3. Similar results were observed in hamartin deficient 208-P2 MEF, compared to hamartin re-expressing 208-T3, and in *Tsc1*$^{-/-}$ MEF, compared to *Tsc1*$^{+/-}$ (**Fig. S5A and S5B**, respectively). BI-2536 treatment of WI38 primary fibroblasts expressing GFP-LC3 induced an increase in the number of GFP-LC3 punctae (**Fig. 4C**), compared to vehicle control and similar to rapamycin treatment, indicating that BI-2536 either increases the formation or blocks the degradation of autophagosomes. Finally, to determine whether PLK1 inhibition affects processing of autophagosome cargo proteins, HEK293 cells were pre-treated for 24 hours with BI-2536 followed by amino acid starvation for up to 8 hours. BI-2536 pre-treatment resulted in a significant inhibition of amino acid starvation-induced p62 degradation (**Fig. 4D**), whereas in vehicle control pre-treated cells amino acid starvation caused degradation of p62 as expected. Similarly to vehicle control, in nocodazole pre-treated cells, a control for prometaphase arrest, amino acid starvation caused degradation of p62. Collectively, these data suggest that inhibition of PLK1 by BI-2536 attenuates processing of autophagosomal cargo proteins via a mechanism probably involving LC3B-I to LC3B-II conversion. However, formation of autophagosomes may not be affected by BI-2536.

Inhibition of PLK1 alters the protein levels and expression of autophagy and Bcl-2 family member proteins and genes

To determine whether pharmacological inhibition of PLK1 affects additional proteins in the autophagic pathway, immortalized tuberin-deficient cells derived from the renal angiomyolipoma of a LAM patient (termed 621-101) were treated with increasing concentrations of BI-2536 for 24 hours, and immunoblotted with antibodies against autophagy proteins. p62 protein levels were increased in a dose-dependent manner, confirming our previous findings (**Fig. 5**). Beclin1 protein levels also increased in a dose-dependent manner, but to a much lesser degree than p62. Finally, Atg3 protein levels significantly decreased in response to BI-2536 in a dose-dependent manner. Global gene expression profiling showed that in 621-101 cells BI-2536 down-regulated the expression of *ATG16L2* and *ATG9B*, and up-regulated the expression of *ATG16L1* (**Table 1**).

Since there is strong evidence of crosstalk between the autophagy and apoptosis pathways, we screened for changes in the protein levels of Bcl-2 pro-apoptosis and pro-survival family members in response to BI-2536 treatment in 621-101 cells. BI-2536 caused a dose-dependent increase in protein levels of pro-apoptotic Bad, which contrasted a decrease in pro-survival Bcl-xL (**Fig. 5**). Protein levels of Mcl-1 increased in response to BI-2536 treatment.

Table 1. Autophagy-related genes differentially regulated by BI-2536. 621-101 cells were treated in triplicate with 100 nM BI-2536 or DMSO (vehicle control) for 24 hours, and subjected to whole-genome expression analysis. A total of 3,240 probes (1,743 up- and 1,497 downregulated) corresponding to 3,007 unique genes (1,560 up- and 1,447 down-regulated) were differentially regulated by BI-2536.

Regulation (BI-2536 vs. control)	Gene Symbol	Description	FC Absolute	P-value
down	ATG16L2	Homo sapiens ATG16 autophagy related 16-like 2 (S. cerevisiae) (ATG16L2), mRNA [NM_033388]	1.86	2.3E-04
down	ATG9B	Homo sapiens ATG9 autophagy related 9 homolog B (S. cerevisiae) (ATG9B), mRNA [NM_173681]	1.66	2.8E-03
up	ATG16L1	Homo sapiens ATG16 autophagy related 16-like 1 (S. cerevisiae) (ATG16L1), transcript variant 1, mRNA [NM_030803]	1.71	1.2E-02

Discussion

Advances in understanding the molecular pathology underlying Tuberous Sclerosis Complex (TSC) and sporadic Lymphangioleiomyomatosis (LAM) led to the identification of the mechanistic target of rapamycin complex 1 (mTORC1) as a critical effector of the hamartin/tuberin heterodimer. Since mutations in *TSC1* or *TSC2* cause hyperactivation of mTORC1, rapamycin and analogs have been proposed and are being used as therapeutic agents for TSC and LAM. Despite the initial enthusiasm, new data demonstrating tumor re-growth and pulmonary function decline after cessation of therapy suggest that rapamycin and analoges may not provide a permanent cure for these diseases, and that life-long treatment with these drugs, with thus far unknown adverse effects, may be needed to keep lesions under control. At least 2 potential mechanisms could lead to the minimal cytotoxicity of rapamycin, which has been reported in multiple TSC and LAM cell culture and *in vivo* animal models; these could involve (a) negative feedback from p70S6K to IRS1/2 and mTORC2 that can activate the pro-survival PI3K/AKT pathway upstream of mTORC1,[26,27] and (b) activation of the pro-survival mechanism of autophagy downstream of mTORC1 (for a review see ref. [10]). It is therefore essential to identify new druggable targets interacting with components of the mTORC1 pathway and to evaluate whether their pharmacological inhibition leads to an apoptotic response in TSC and LAM cell lines and tumors.

The hamartin/tuberin heterodimer physically and functionally interacts with components of a centrosomal and mitotic network of proteins, namely CDK1/cyclin B, PLK1, PLK2 and TACC3,[13,28-32] to regulate centrosome biology and mitotic progression. Here we report increased PLK1 protein level in hamartin and tuberin deficient cells, which is rescued by hamartin or tuberin re-expression and is rapamycin-sensitive, suggesting a positive correlation between mTORC1 and PLK1 activation. The latter is further demonstrated by our finding of positive immunoreactivity for PLK1 in LAM-derived lung specimens with mTORC1 hyperactivation.

Aberrant regulation of PLK1 has been reported for multiple cancers, including colorectal, gastric, hepatic, breast, ovarian, lung, and leukemias and lymphomas (reviewed in refs. [25,33]), and PLK1 small-molecule inhibitors are currently under clinical investigation for oncology. In this study, we provide evidence for the first time that pharmacological inhibition of PLK1 by BI-2536 leads to decreased viability and survival of hamartin and tuberin deficient cells. Interestingly, $Tsc2^{-/-}/Tp53^{-/-}$ MEF are more sensitive to BI-2536, compared to $Tsc1^{-/-}$, probably due to genetic inactivation of the p53 tumor suppressor in the former. Furthermore, BI-2536 induced apoptosis preferentially in hamartin and tuberin deficient cells, consistent with previous reports for other cancer cell lines, and rapamycin co-treatment increased BI-2536-induced apoptotic response in hamartin and tuberin deficient cells, but not in hamartin and tuberin expressing cells (data not shown). With the exception of simvastatin (alone or in combination with rapamycin),[34,35] other experimental therapeutics for TSC and LAM fail to induce apoptosis in hamartin and tuberin deficient cell lines, and in the corresponding *in vivo* animal models.

Despite having increased endoplasmic reticulum stress,[36,37] a positive regulator of autophagy,[38] hamartin and tuberin deficient cells have decreased autophagy due to mTORC1 hyperactivation. Additionally, these cells have increased levels of reactive oxygen species.[39] The increased endoplasmic reticulum stress and reactive oxygen species levels could potentially be exploited to sensitize hamartin and tuberin deficient cells by autophagy inhibitors. Indeed, inhibition of autophagy in tuberin null cells and tumors, either via genetic inactivation of *Atg5*, *Beclin1*, and *p62*, or by chloroquine, leads to decreased cell survival both *in vitro* and *in vivo*.[12] However, perturbing autophagy in the tuberin null cells, either *in vitro* or *in vivo,* is not associated with increased apoptosis. Our data show that BI-2536 led to attenuation of autophagy, measured by accumulation of p62 and LC3B-I at steady state and by stabilization of p62 after amino acid starvation. Secondly, BI-2536 caused a significant increase in GFP-LC3 punctae. Together these data support that under PLK1 inhibition conditions autophagosomes form but are not processed for lysosomal fusion and/or lysosomal degradation. In LAM patient-derived cells BI-2536 repressed *ATG9B* and induced *ATG16L1* gene expression, and decreased Atg3 protein levels, key molecules involved in autophagy. Atg9 has been implicated in the formation of the phagophore assembly site, a localized entity for the formation of the isolation membrane, and in recruitment of small vesicles and membrane remodeling machinery to this site (reviewed in ref. [40]). Atg16 is part of the first ubiquitin-like conjugation systems (Atg12-Atg5-Atg16) that acts as an E3 ligase for LC3 lipidation. Finally, Atg3 is an E2-like enzyme of the second ubiquitin-like conjugation system, which conjugates a phosphatidylethanolamine (PE) moiety on LC3. The consorted action of these 2 conjugation systems results in the recruitment of LC3-PE (termed LC3-II) to the autophagosome membrane. The decrease in *ATG9B* expression and Atg3 protein levels by BI-2536 point toward a role of PLK1 in the regulation of these 2 critical processes for autophagic flux (formation of the phagophores assembly site and LC3 lipidation), whereas the increase in *ATG16L1* expression by BI-2536 could represent a compensatory mechanism for maintenance of autophagic flux. Finally, *ATG16L2* expression was repressed by BI-2536. Although the role of *ATG16L2* in autophagy remains unclear, it does not seem to be a major contributor for the function of the Atg12-Atg5-Atg16 complex.[41] Collectively, our data support a role for PLK1 in a mechanism regulating autophagy. To date, such a role for PLK1 has not been reported, the exact molecular mechanism remains unknown, and warrants further investigation.

Increased autophagy can lead to cell death,[42-44] and crosstalk between autophagy and apoptosis has been reported through multiple ways including the interactions between Beclin1/Vps32 and Bcl^-2/Bcl^-xL and between Atg12 and Mcl^-1 (for a review, see ref. [45]). Here we report that PLK1 inhibition by BI-2536 resulted in increased protein levels of pro-apoptotic Bad and decrease of pro-survival Bcl^-xL. Unexpectedly, Mcl^-1 protein levels, another pro-survival molecule, were increased by BI-2536. Since Mcl^-1 forms a heterodimer with Atg12, it is possible that this increase in Mcl^-1 reflects a response to the attenuated autophagy induced by BI-2536.

Although the contribution of cellular senescence to TSC and LAM pathogenesis has not been adequately addressed, TSC patient-derived stromal angiofibroma cells exhibit strong senescent phenotypes, such as senescence-associated β-galactosidase staining, low proliferative capacity, increased cell size, and bi-nucleated cells,[46] the latter in accordance with our previous report of increased frequency of bi-nucleate cells in hamartin and tuberin null cell cultures.[32] Prolonged hyperactivation of the PI3K/mTORC1 signaling pathway during $p21^{Waf1/Cip1}$- or $p16^{Ink4a/Arf}$-induced cell cycle arrest may lead to cellular senescence through increased cyclin D1 levels,[47] and rapamycin preserves replicative/regeneration potential, and restricts senescence. Therefore, rapamycin treatment of tumors with hyperactive mTORC1, such as in TSC and LAM, may result in cell cycle re-entry of senescent tumor cells, and could be an additional factor contributing to tumor re-growth upon cessation of rapamycin treatment. In young primary human cells inhibition of PLK1 by siRNA decreased their replicative potential and induced senescence-associated phenotypes in a p53-dependent manner, whereas ectopic expression of PLK1 in old primary cells increased their replicative potential.[48] However, very little is known about the role of PLK1 in modulating senescence in tumor cells. These apparently opposing effects of mTORC1 and PLK1 inhibition on cellular senescence are worth investigating further, since manipulations of cell cycle re-entry have the potential to enhance or diminish the cancer therapeutic efficacy of PLK1 inhibitors.

It is now well established that PLK1 plays a critical role in the initiation of cytokinesis.[49] Our finding that hamartin and tuberin deficient cells are sensitive to PLK1 inhibition can be explained based on our previous findings that these cells have aberrant mitoses, including bi-nucleated cells and cytokinetict failure,[32] compared to hamartin and tuberin expressing cells. Thus, PLK1 inhibitors targeting 2 independent cellular processes that are aberrantly regulated in hamartin and tuberin deficient cells (autophagy and mitosis) may be superior to agents targeting each process alone.

In summary, we propose that the increased protein levels of PLK1 in hamartin and tuberin deficient cells and tumors augment their survival. By exploiting these molecular mechanisms, tumors with mTORC1 hyperactivation could be sensitized to apoptosis with concomitant inhibition of autophagy, which is considered a survival mechanism in tumor cells. Whether TSC, LAM, or other tumor syndromes with aberrant mTORC1 activation are good candidates for treatment with PLK1 small-molecule inhibitors, either alone or in combination with rapamycin, remains to be determined in the corresponding animal models.

Materials and Methods

Cell lines and chemicals

$Tsc1^{+/-}$ (clone 207), $Tsc1^{-/-}$ (clone 208), $Tsc2^{+/+}/Tp53^{-/-}$ (clone 118) and $Tsc2^{-/-}/Tp53^{-/-}$ (clone 110) MEF were cultured in Dulbecco's Modified Eagle's Medium containing 4.5 mg/ml D-glucose, 2 mM L-glutamine, and 110 mg/l sodium pyruvate (DMEM, Cellgro 10-013), supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 100 μM non-essential amino acids (Cellgro 25–025) and 10% v/v fetal bovine serum (FBS, Gemini Bio-Products 100–106). Isogenic stable clones of $Tsc1^{-/-}$ MEF and ELT3 cells transduced with empty vector pMSCVneo (208-P2 and ELT3-V3), or TSC1/pMSCVneo (208-T3), or TSC2/pMSCVneo (ELT3-T3) were previously described,[13,50] and were cultured in the above media with the addition of 100 μg/ml G418 (Invitrogen). Angiomyolipoma-derived tuberin-deficient 621-101 cells (a gift of Dr. Elizabeth P. Henske) were cultured in DMEM/F12 (Sigma-Aldrich D8437) supplemented with 50 nM Na_2SeO_3 (Sigma-Aldrich S9133), 1.6 μM $FeSO_4$ (Fisher Scientific I146), 25 μg/ml insulin (Invitrogen A11382IJ), 0.2 μM hydrocortisone (Sigma-Aldrich H4001), 10 μg/ml transferrin (Sigma-Aldrich T0665), 1 nM triiodothyronine (Sigma-Aldrich T2752), 10 μU/ml vasopressin (Sigma-Aldrich V0377), 10 nM cholesterol (Sigma-Aldrich C3045), 20 ng/ml epidermal growth factor (Becton Dickinson 354001), 1 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% FBS. HeLa cells were obtained from ATCC and cultured in DMEM supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% v/v FBS. GFP-LC3-expressing WI38 fibroblasts (a gift of Dr. Christian Sell) were cultured in MEM containing 1 mg/ml D-glucose and 1 mM L-glutamine (Cellgro 10-010), supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, MEM vitamin solution (Cellgro 25-020), and 10% v/v FBS.

Hank's Buffered Salt Solution (HBSS) was formulated with 137 mM NaCl, 5.37 mM KCl, 5.55 mM glucose, 0.3 mM Na_2HPO_4, 0.4 mM KH_2PO_4, 1.73 mM $CaCl_2$, 2.33 mM $MgCl_2$, and 4.2 mM $NaHCO_3$.

Rapamycin (Enzo Life Sciences BML-A275), BI-2536 (Axon Medchem 1129), compound 1 (GW843682X, Sigma-Aldrich G2171), and nocodazole (Sigma-Aldrich M1404) were dissolved in DMSO (Sigma-Aldrich D2650) and stored in aliquots at −20°C. All other chemicals were from Sigma-Aldrich, unless otherwise specified.

RNAi-mediated gene silencing

Human TSC1 (TRCN0000039734), TSC2 (TRCN0000040178) and non-targeting (SHC002) shRNA vectors were purchased from Sigma-Aldrich. Lentiviral particles were produced in 293T cells using the ViraPower Expression System (Invitrogen). HeLa cell monolayers (2×10^5 per 35 mm plate) were overlaid with 1 ml lentiviral supernatant for 16 hours, which was replaced with fresh media for 48 hours, and cells were selected for 4 days with 2 μg/ml puromycin (Invitrogen) prior to use in consecutive experiments.

Viability and clonogenic survival assays

For viability assays cells were plated in 96-well plates 16 hours prior to treatments. Cells were treated with inhibitors for 72-96 hours. On the day of assay, cells were incubated for 4 hours with 500 μg/ml thiazolyl blue tetrazolium bromide (MTT, Sigma-Aldrich M5655) dissolved in phosphate-buffered saline (PBS, Invitrogen 21600), lysed with an equal volume of 0.04 N HCl in isopropanol for 90 min, and optical densities were measured at 590 nm using a reference of 650 nm in an Emax plate reader (Molecular Devices).

For clonogenic survival assays, cells were plated in 6-well plates for 16 hours, treated with inhibitors for 24–72 hours,

trypsinized, living cells were counted by exclusion with trypan blue (Sigma-Aldrich T8154), and 300 viable cells were plated in 100 mm plates in triplicate. Colonies formed within 7-14 days after plating, were stained with 0.1% w/v crystal violet (Sigma-Aldrich C3886) in 50% v/v methanol and 50% v/v 2x PBS, destained with tap water, air-dried, and counted manually by an investigator blinded to the experimental conditions.

Western immunoblotting and antibodies

Cells were lysed in PTY buffer (50 mM HEPES, 50 mM NaCl, 5 mM EDTA, 1% Triton X-100, 50 mM NaF, 10 mM $Na_4P_2O_7$, 1 mM Na_3O_4V, 10 μg/ml phenylmethanesulfonyl fluoride) supplemented with protease and phosphatase inhibitors (Sigma-Aldrich P8340, P0044, and P5726). Protein concentration was measured with the BCA method (Fisher Scientific PI-23225). Cell lysates (5–20 μg) were resolved in 4-12% Bis-Tris NuPAGE or 18% Tris-Glycine Novex gels (Invitrogen) and transferred on Immobilon-FL PVDF membranes (Millipore). Primary antibodies used were against tuberin (Santa Cruz Biotechnologies SC-893); hamartin and PLK1 (Invitrogen 37-0400 and 33-1700, respectively); pS235/236-ribosomal protein S6 (2211), D175-caspase 3 (9661), PARP (9542), LC3B (3868), Beclin 1 (3495), Atg3 (3415), Bcl-xL (2764), Bad (9239), and Mcl-1 (5453) (all from Cell Signaling); and α-tubulin, β-actin, and p62/SQSTM1 (Sigma-Aldrich T6557, A5441, and P0067, respectively). HRP-conjugate secondary antibodies were goat anti-rabbit and goat anti-mouse (Bio-Rad). Chemiluminescence reaction was performed with SuperSignal WestPico substrate (Fisher Scientific PtdIns-34077) and was detected on X-ray film (ISCBioexpress) developed in an automated X-ray film processor. ImageJ (NIH) was used for densitometric quantitation of digital images from scanned X-ray films.

GFP-LC3 punctae quantitation

WI38 primary fibroblasts expressing GFP-LC3 were grown on 22 × 22 mm coverglasses in 6-well plates, treated with agents, washed twice with PBS, fixed with 4% v/v paraformaldehyde (Electron Microscopy Sciences) in PBS for 20 min in the dark, washed twice with PBS, permeabilized with 0.2% v/v Triton-X100 in PBS, and the DNA was counterstained with 1 μg/ml 4′, 6-diamidino-2-phenylindole dihydrochlroride. Coverglasses were mounted on microscopy slides using Fluoro-Gel (Electron Microscopy Sciences). Digital fluorescence micrographs were acquired with a Provis AX70 epi-fluorescence microscope (Olympus) equipped with a 100x oil-immersion objective and a Retiga EXiFast1394 camera (QImaging), using iVIsion software (BioVision Technologies). GFP-LC3 punctae were automatically counted using ImageJ particle analysis tool after thresholding.

Gene expression analysis

621-101 cells were treated for 24 hours with 100 nM BI-2536, or DMSO as a vehicle control, and total RNA was extracted using Trizol (Invitrogen) according to the manufacturer instructions. RNA amplification, labeling, hybridization, and data acquisition and analyses were performed by ArrayStar Inc.. (Rockville MD). Briefly, RNA was amplified and transcribed into fluorescently-labeled cRNA using Quick Amp Labeling (Agilent), hybridized on a 4 × 44k Whole Human Genome oligo microarray (Agilent), array data were acquired using scanner G2505C (Agilent), and analyzed by Feature Extraction software version 11.0.1.1 (Agilent). Quantile normalization and subsequent data processing were performed using GeneSpring GX v11.5.1 (Agilent). Differentially expressed genes with statistical significance were identified through Volcano Plot filtering with default threshold fold change ≥ 1.5 and $P < 0.05$.

Statistical analysis and graphing

Statistical analyses and graphing was performed using Prism v4.0 (GraphPad). Statistical significance was determined by the unpaired 2-tailed Student's *t*-test. Error bars in graphs represent the standard error of means.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Material

Supplemental data for this article can be accessed on the publisher's website.

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