The Pim protein kinases regulate energy metabolism and cell growth

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The serine/threonine Pim kinases are overexpressed in solid cancers and hematologic malignancies and promote cell growth and survival. Here, we find that a novel Pim kinase inhibitor, SMI-4a, or Pim-1 siRNA blocked the rapamycin-sensitive mammalian target of rapamycin (mTORC1) activity by stimulating the phosphorylation and thus activating the mTORC1 negative regulator AMP-dependent protein kinase (AMPK). Mouse embryonic fibroblasts (MEFs) deficient for all three Pim kinases [triple knockout (TKO) MEFs] demonstrated activated AMPK driven by elevated ratios of AMP:ATP relative to wild-type MEFs. Consistent with these findings, TKO MEFs were found to grow slowly in culture and have decreased rates of protein synthesis secondary to a diminished amount of 5′-cap–dependent translation. Pim-3 expression alone in TKO MEFs was sufficient to reverse AMPK activation, increase protein synthesis, and drive MEF growth similar to wild type. Pim-3 expression was found to markedly increase the protein levels of both c-Myc and the peroxisome proliferator-activated receptor gamma coactivator 1α (PGC-1α), enzymes capable of regulating glycolysis and mitochondrial biogenesis, which were diminished in TKO MEFs. Overexpression of PGC-1α in TKO MEFs elevated ATP levels and inhibited the activation of AMPK. These results demonstrate the Pim kinase-mediated control of energy metabolism and thus regulation of AMPK activity. We identify an important role for Pim-3 in modulating c-Myc and PGC-1α protein levels and cell growth.

LKB1 | mitochondria | mTOR | 4EBP1

The Pim serine/threonine kinases include three isoforms, Pim-1, Pim-2, and Pim-3, that are implicated in the growth and progression of hematological malignancies, prostate cancer, and, in the case of Pim-3, in precancerous and cancerous lesions of the pancreas, liver, colon, and stomach (1–5). Pim-1 and Pim-2 have been shown to cooperate with c-Myc in inducing lymphoma (6), and prostate cancer (7), and in the absence of Pim-1 and Pim-2, Pim-3 is activated in c-Myc-induced lymphomas (8). The mechanisms suggested to explain this Pim–Myc synergism include Pim-mediated stabilization of c-Myc protein (9) and regulation of gene transcription via Pim-1 phosphorylation of histone H3 at active sites of c-Myc transcription (10). Other Pim kinase substrates that suggest these enzymes play a role in cell cycle progression and antiapoptosis include BAD, Bcl-2, Bcl-xL (11, 12), $p27^{Kip1}$ (13), and Cdc25A (14).

Recently, Pim kinases have been suggested to promote the activity of the rapamycin-sensitive mammalian target of rapamycin (mTORC1) (15–17). mTORC1 is a serine/threonine kinase that regulates cell growth and metabolism (18). The mTORC1 complex, composed of mTOR, raptor, GβL, and PRAS40, promotes protein synthesis by phosphorylating 4EBP1, thus stimulating its dissociation from the translational regulator eukaryotic initiation factor 4E (eIF4E) (17) allowing for cap-dependent translation. mTORC1 activity is regulated by a cascade of enzymes including LKB1, AMP-dependent protein kinase (AMPK), and TSC1 and 2 (19). AMPK senses the cellular energy status and is activated via LKB1-mediated phosphorylation when there is a decline in ATP levels and concomitant rise in AMP levels; i.e., high AMP:ATP ratio (20). Activated AMPK down-regulates the energetically demanding process of protein synthesis by inhibiting mTORC1 activity through phosphorylating TSC2 and raptor (20). The mechanisms by which Pim kinase stimulates mTORC1 appear complex and include 4EBP1, eIF4E (16, 21–23), and PRAS40 phosphorylation (15).

Because of the importance of the Pim kinase signal transduction pathway in the progression of various cancers, multiple groups have developed small-molecule inhibitors of this kinase family (24–28). We have identified unique benzylidene-thiazolidine-2-4-diones (23, 29) that inhibit Pim kinase activity in vitro at nanomolar concentrations, and in culture induce apoptosis of human leukemic cells (30) and synerigize with rapamycin to downregulate 4EBP1 phosphorylation and inhibit cell growth (29). Taking advantage of these inhibitors, siRNA, and genetically engineered Pim-deficient cells, we have discovered a unique role for Pim-3 in regulating mTORC1 activity through modulation of ATP levels by the induction of c-Myc and the transcriptional coactivator and master regulator of mitochondrial biogenesis peroxisome proliferator-activated receptor gamma coactivator 1α (PGC-1α).

Results

Pim Kinase Negatively Regulates AMPK. To examine the mechanisms by which Pim kinase can regulate the mTORC1 pathway, the human erythroleukemia cell line K562 was incubated with the thiazolidinedione Pim kinase inhibitor SMI-4a (23), and the phosphorylation of AMPK was studied. AMPK activation results in the phosphorylation of raptor and TSC2 and thus inhibits mTORC1 activity (20, 31). Pim kinase inhibition with SMI-4a induced the activation of AMPK as determined by phosphorylation of AMPKα at Thr172, and the AMPK targets acetyl-CoA carboxylase (ACC) at Ser79 and raptor at Ser792 and inhibition of mTORC1 activity as determined by decreased phosphorylation of the mTORC1 targets S6K and 4EBP1 (Fig. 1 *A* and *B*). Additionally, knockdown of Pim-1 levels with a targeted siRNA increased AMPK phosphorylation (Fig. 1*C*), suggesting that Pim-1 negatively regulates the phosphorylation of this enzyme. Because the LKB1 kinase is known to activate AMPK via phosphorylation at Thr172 (32) and loss of LKB1 activity is frequently associated with the transformed phenotype (32), we examined the ability of SMI-4a and SMI-16a, another Pim kinase inhibitor, (29) to regulate AMPK phosphorylation in a panel of LKB1-containing (H358, H661) and deficient (H23, H460, A549) lung cancer cell

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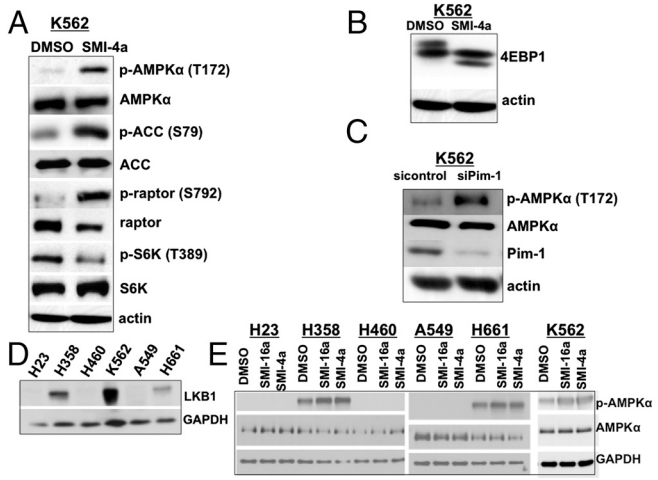

Fig. 1. Pim kinase inhibition activates AMPK. (*A* and *B*) K562 cells were treated with DMSO or SMI-4a (5 μM) for 1 h in the absence of serum, and lysates were probed for the indicated proteins by Western blotting. (*C*) K562 cells were transfected with scrambled siRNA (sicontrol) or Pim-1 siRNA (siPim-1), and 48 h later lysates were probed for the indicated proteins by Western blotting. (*D*) Western blot for LKB1 levels in lung cancer cell lines and the leukemia cell line K562. (*E*) Lung and leukemia cells were treated with DMSO, SMI-4a, or SMI-16a (5 μM) for 1 h in the absence of serum, and lysates were probed for the indicated proteins by Western blotting.

lines along with the LKB1-positive K562 cell line (Fig. 1*D*). These results demonstrate that the Pim kinase inhibitors SMI-4a and SMI-16a required LKB1 to stimulate AMPK activity (Fig. 1*E*).

To further confirm that Pim kinase regulates the activation of AMPK, we generated mouse embryonic fibroblasts (MEFs) deficient for Pim-1, -2, and -3 [triple knockout (TKO)] (33), and wild-type (WT) littermate control MEFs. Consistent with both the siRNA and small-molecule inhibition of Pim kinase activity, TKO MEFs had significantly higher AMPK phosphorylation compared to WT MEFs (Fig. 2*A*). To determine the contribution of each Pim isoform to AMPK activation, TKO MEFs were transduced with Pim-1, -2, or -3 lentiviruses. Although each Pim isoform reduced p-AMPK levels, Pim-3 showed the greatest effect (Fig. S1*A*) leading us to focus on elucidating the unique role of Pim-3. To confirm this result, we generated MEFs deficient in Pim-1 and Pim-2 (Pim-$1^{-/-}$, -$2^{-/-}$, -$3^{+/+}$) but expressing Pim-3, and demonstrated that these cells showed less activated AMPK than TKO MEFs (Fig. 2*A*). As AMPK activation is regulated by increased AMP, we measured the levels of AMP and ATP and found that AMPK phosphorylation correlates with the cellular AMP:ATP ratio in these knockout MEFs (Fig. 2*B*). Growth curves of these MEFs demonstrated a further correlation between proliferation, AMP:ATP ratio, and AMPK phosphorylation status (Fig. 2*C*) with the TKO MEFs showing the slowest growth rate. Similar results were obtained with immortalized WT or TKO MEFs transduced with empty vector or a lentivirus expressing Pim-3 (Fig. S1*B*).

Because activation of AMPK leads to inhibition of mTORC1 activity (31), we measured the level of protein synthesis in each of the MEFs. Labeling of MEFs with ^{35}S-methionine and measuring newly synthesized protein demonstrated, as predicted, that TKO MEFs when compared to WT have lower rates of protein synthesis (approximately 58% relative to WT). Expression of Pim-3 in the TKO cells increased protein synthesis from 58% (TKO) to 83% relative to WT (Fig. 2*D*). Consistent with this result, we found that in TKO MEFs the cap-dependent but not internal ribosome entry site (IRES)-dependent translational activity is reduced (Fig. 2*E*). Cap-dependent translation depends on the mTORC1-mediated release of 4EBP1 from eIF4E and the for-

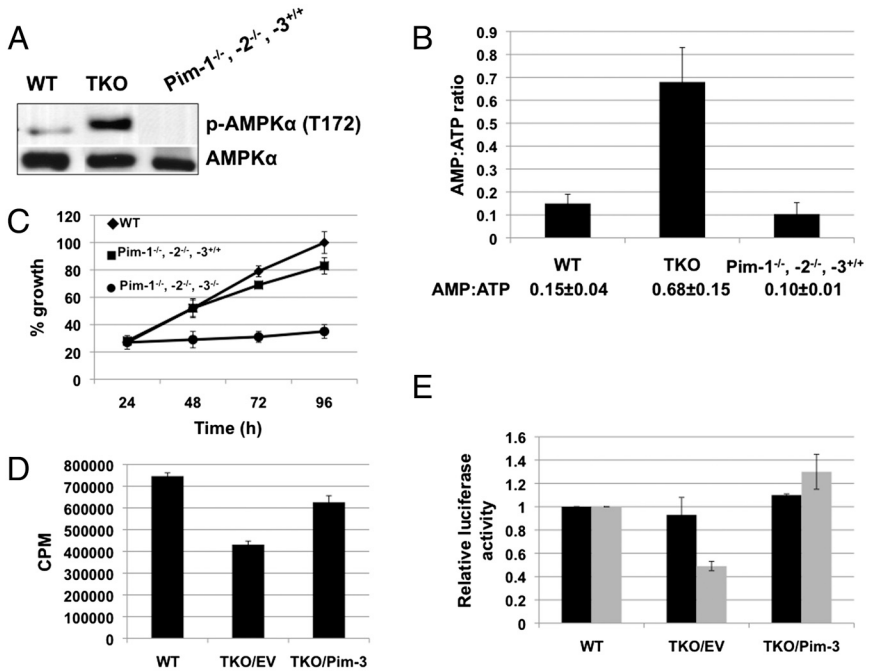

Fig. 2. Knockout of Pim kinase isoforms inhibits protein synthesis and cell growth. (*A*) Lysates were prepared from the different MEF cell lines and probed for the indicated proteins by Western blotting. (*B*) AMP:ATP ratios were determined by HPLC as described in *Materials and Methods*. Values are the average of three independent experiments, and the standard deviation from the mean is shown. (*C*) Growth curve of MEFs as determined by MTT assay. Percentage values are relative to the growth of WT MEFs at the 96 h time point (100%). The data points are the average of three independent measurements, and the standard deviation from the mean is shown. (*D*) ^{35}S-methionine incorporation into WT and TKO MEFs expressing empty vector (TKO/EV) or Pim-3 (TKO/Pim-3). Values (cpm/mg protein) are the average of three independent measurements, and the standard deviation for the mean is shown. (*E*) Cap-dependent (gray bars) and IRES-dependent (black bars) translation in MEFs as measured by Renilla and Firefly luciferase activities, respectively. As described in *Materials and Methods*, MEFs were infected with a virus expressing cap- and IRES-driven luciferase constructs. Values are the ratio of luciferase activity relative to WT and are the average of three independent measurements with the standard deviation from the mean shown.

mation of the eIF4F complex. Using m^7-GTP beads in a pull-down assay (34), we found that in TKO MEFs eIF4E is highly bound to 4EBP1, whereas eIF4G binding is lost and thus the ability of eIF4E to promote translation is inhibited (Fig. S1*C*). These results demonstrate that reduced Pim kinase activity correlates with increased AMP:ATP ratio, activation of AMPK, inhibition of mTORC1 activity, and reduced overall protein synthesis.

Pim-3 Regulates c-Myc Levels. In comparison to TKO MEFs, Pim-3–expressing cells demonstrated increased 5′-cap-dependent protein synthesis and growth similar to WT MEFs. Because the levels of the c-Myc protein are controlled by 5′-cap-dependent transcription, and c-Myc is important in the regulation of both cell growth and overall cellular metabolism, we examined the levels of c-Myc in MEFs from each genotype. Western blots demonstrate that the expression of Pim-3 either in primary MEFs (Fig. 3*A*) or after transduction into TKO MEFs (Fig. 3*B*) markedly increased c-Myc protein. Similar to p-AMPK (Fig. S1*A*), neither Pim-1 nor Pim-2 was able to induce the significant levels of c-Myc protein observed with Pim-3 overexpression in TKO MEFs (Fig. 3*B*). Furthermore, expression of Pim-1 or Pim-2 in Pim-3–only MEFs led to a decrease in c-Myc protein levels (Fig. S1*D*), suggesting the possibility that the Pim kinases could compete for substrates or interact directly. Because of the marked difference in c-Myc protein levels in MEFs containing Pim-3 only, we tested two additional MEF cell lines generated from different embryos of the same genotype and again observed increased c-Myc protein levels (Fig. S2*B*). To determine whether the increased c-Myc level in Pim-3–expressing cells is unique to MEFs in culture, we measured the c-Myc levels in spleen lysates of 4-mo-old WT, TKO, and Pim-$1^{-/-}$, -$2^{-/-}$, -$3^{+/+}$ mice and again found the highest level of c-Myc protein in the Pim-3–only genotype (Fig. S2*C*). Because deletion of one Pim might grossly elevate the level of another, in this case Pim-3, we measured the level of Pim-3 mRNA in the different MEF genotypes but did not find a significant difference (Fig. S2*A*). These results suggested that Pim-3 might modulate c-Myc translation.

To determine the effect of Pim-3 on c-Myc translation, we treated MEFs with cycloheximide until c-Myc protein was completely degraded, washed out the cycloheximide, and then monitored by Western blotting the rate of increase in c-Myc protein over time. To make this comparison possible, 2.5 times more protein from TKO MEFs was loaded on these SDS gels. TKO MEFs showed a delay in protein synthesis with only 53 and 43% as much c-Myc protein synthesis in the first 15 min when compared to WT and TKO/Pim-3 MEFS, respectively (Fig. 3*C*). This result is consistent with the reduced protein synthesis in the TKO MEFs (Fig. 2*D*). Because the overall translational efficiency in cells is reflected by changes in the polysome/monosome ratio, we determined the polysome profile using cytosolic extracts of WT, TKO, and TKO/Pim-3 cells. TKO cells showed a significant reduction in heavy polysomes and a corresponding increase in free ribosome subunits (Fig. 3*D Upper*). However, expression of Pim-3 in TKO cells resulted in a significant increase in heavy polysomes. c-Myc mRNA showed redistribution from heavy toward lighter polysomes and monosomes and free subunits in TKO cells relative to WT (Fig. 3*D Lower*). This shift was reversed by Pim-3 expression, suggesting that Pim-3 is capable of controlling the translation of c-Myc mRNA (Fig. 3*D Lower*). Because Pim-1 and -2 have been shown to increase the stability of c-Myc (9), we examined changes in c-Myc protein stability in TKO and TKO/Pim-3 MEFs after cycloheximide treatment but found no significant difference in c-Myc half-life in the Pim-3–containing cells (Fig. S2*D*). Finally, we found that the expression of c-Myc in TKO MEFs led to a decrease in AMPK activation (Fig. S2*E*) consistent with the ability of c-Myc to stimulate cell growth.

Pim-3 and c-Myc Regulate PGC-1α Levels. The differences in the growth rate between the TKO and Pim-3 only MEFs could possibly be explained by the Pim-3–mediated increased c-Myc because the latter is known to control multiple factors that regulate cell growth and metabolism (35, 36). Therefore, we compared the growth rate of TKO MEFs stably expressing empty vector, Pim-3, or c-Myc to WT MEFs. The TKO/c-Myc MEFs were able to

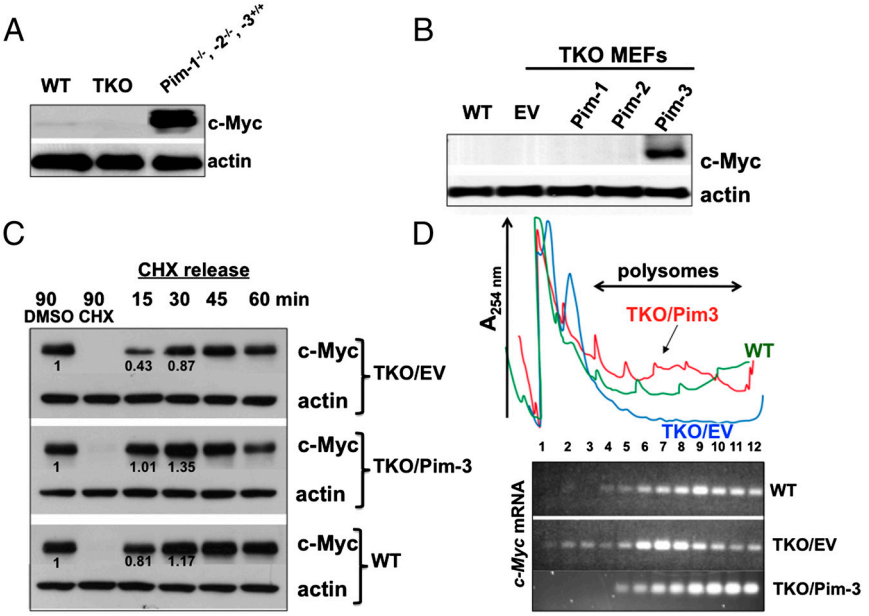

Fig. 3. Pim-3 elevates c-Myc levels. (*A*) c-Myc protein levels in each MEF genotype as determined by Western blotting. (*B*) TKO MEFs were infected with empty vector (EV), Pim-1, -2, or -3 lentiviruses, and 48 h later lysates were probed for c-Myc levels and compared to WT MEFs. (*C*) TKO MEFs expressing EV or Pim-3 were treated for 90 min with cycloheximide (CHX, 10 μM), the media replaced, and lysates probed for c-Myc levels by Western blotting. Densitometry analysis was performed, and the values at the 15 min time point relative to DMSO are shown. To obtain a relatively equal amount of c-Myc protein at the 90 min time point with DMSO, ~2.5-fold more TKO/EV protein lysate was loaded relative to TKO/Pim-3. (*D*) Ribosome fractions of WT and TKO MEFs expressing EV or Pim-3 were prepared by sucrose gradient (see *Materials and Methods*), and the level of c-Myc mRNA associated with each fraction was determined by PCR.

grow in the absence of Pim kinases but did not reach the same density in 96 h (Fig. 4*A*). The shRNA-mediated knockdown of c-Myc in TKO/Pim-3 MEFs did not completely inhibit cell proliferation (Fig. 4*B*). Together, these results suggest that Pim-3 and c-Myc do not have completely overlapping biologic activities. To understand how Pim-3 decreases the AMP:ATP ratio and inhibits AMPK phosphorylation, we measured the levels of PGC-1α. PGC-1α activates a wide variety of transcription factors that result in increased mitochondrial biogenesis and oxidative phosphorylation (37). Increased expression of PGC-1α can lead to elevations in ATP levels (38), whereas PGC-1α knockout leads to reduced ATP levels in murine hearts (39). PGC-1α expression and PGC-1α–dependent gene expression are induced by chemical activation of AMPK, and AMPK directly phosphorylates PGC-1α, leading to increased transcriptional activity (40–42). We found that the levels of PGC-1α mRNA and protein were greatly reduced in TKO MEFs, highest in Pim-3–only MEFs, and intermediate in WT cells (Fig. 4*C* and *D*). To examine the contributions of Pim-3 and c-Myc in regulating PGC-1α levels, we infected TKO MEFs with lentiviruses expressing c-Myc or Pim-3 and found that Pim-3 induced marked increases in PGC-1α mRNA (12-fold) and protein; the effect of c-Myc alone was a 4-fold increase in mRNA, and the increase in protein was quantitated at only 10% that of Pim-3 (Fig. 4*D*, *E*).

The above results suggest that the increased AMP:ATP ratio in TKO MEFs may be attributed to low ATP levels due to decreased PGC-1α protein, thus leading to AMPK activation. To examine whether overexpression of PGC-1α in TKO MEFs was sufficient to reduce p-AMPK by increasing the level of cellular ATP, we transduced TKO MEFs with a lentivirus expressing PGC-1α. Western blots and biochemical analysis demonstrate that PGC-1α expression in TKO MEFs decreased the level of p-AMPK (Fig. 5*A*) and increased the levels of ATP (Fig. 5*B*), leading to decreased 4EBP1 binding to eIF4E while increasing eIF4G association with the eIF4E protein (Fig. S3). In contrast, PGC-1α expression in TKO MEFs showed little effect on c-Myc levels (Fig. 5*A*). Thus, Pim-3, by controlling the levels of both c-Myc and PGC-1α, is able to impact on AMPK phosphorylation, mTORC1 activity, 5′-cap-dependent translation, and ultimately cell growth (Fig. 5*C*).

Discussion

The combined approach of genetic knockout, RNAi, and small-molecule inhibition implicate the Pim kinases in regulating the AMP:ATP ratio and energy metabolism. These effects lead to the modulation of the mTORC1 pathway by AMPK and the control of cell growth. In leukemic cells, the pan-Pim kinase inhibitor SMI-4a stimulated the phosphorylation and activation of AMPK, whereas in TKO MEFs the ratio of AMP:ATP was markedly increased and AMPK was activated. Because AMPK is a negative regulator of mTORC1, we found in leukemic cells treated with SMI-4a and in TKO MEFs that mTORC1 activity is inhibited and cap-dependent translation is significantly decreased. In MEFs, the expression of Pim-3 alone could reverse these processes, lowering the AMP:ATP ratio, decreasing the activation of AMPK, and increasing cap-dependent translation, all resulting in cellular growth rates comparable to WT MEFs. The differences between the TKO and Pim-3–only MEFs could be explained in part by the Pim-3–mediated increased c-Myc because the latter controls multiple transcription factors that regulate cell growth and metabolism (35, 36). Infection of TKO MEFS with a lentivirus expressing c-Myc increased the growth of these cells but did not duplicate the growth curve of Pim-3–expressing MEFs.

In muscle and fat tissue, the ability of activated AMPK to maintain an energy balance is achieved in part by stimulating PGC-1α (41). The ability of PGC-1α to coactivate multiple transcription factors makes this protein a master regulator of mitochondrial biogenesis (43). Considering this link between

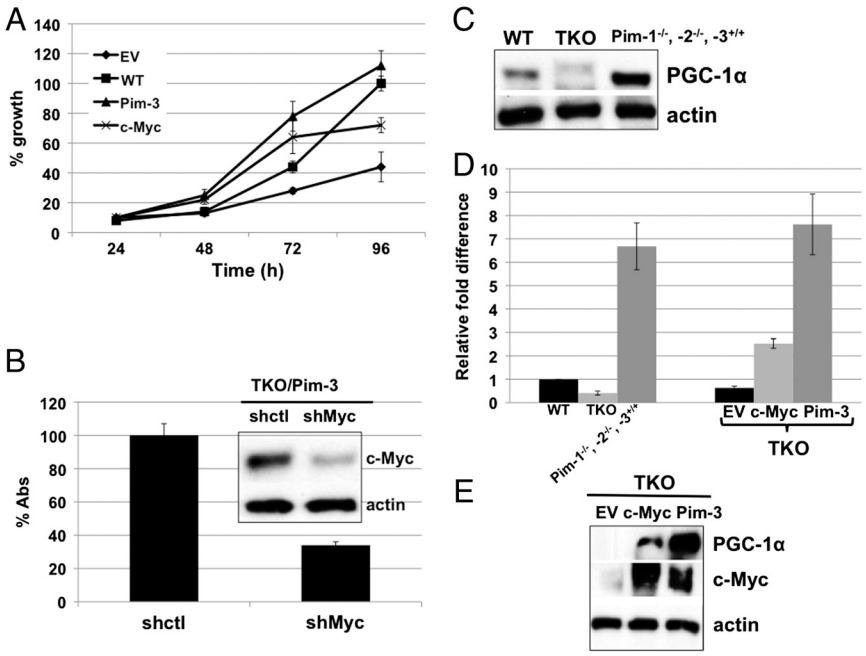

Fig. 4. Pim-3 and c-Myc affect PGC-1α levels. (*A*) Growth curve of TKO MEFs expressing empty vector (EV), c-Myc, or Pim-3 as determined by an MTT assay. Percentage values are relative to the value of WT MEFs at the 96 h time point (100%). The data points are the average of three independent measurements, and the standard deviation from the mean is shown. (*B*) TKO/Pim-3 MEFs were infected with nontargeting shRNA (shctl) or c-Myc targeting shRNA (shMyc) lentiviruses. Equal numbers of shctl and shMyc cells were plated 48 h postinfection, and after an additional 72 h viability was determined by an MTT assay and represented as a percent absorbance (%Abs) with shctl set at 100%. The data points are the average of three independent measurements, and the standard deviation from the mean is shown. (*Inset*) Lysates were prepared at 120 h postinfection and probed for the indicated proteins by Western blotting. (*C*) PGC-1α protein levels in MEFs as determined by Western blotting. (*D*) PGC-1α mRNA levels in primary MEFs (WT, TKO, Pim-1$^{-/-}$, -2$^{-/-}$, -3$^{+/+}$) or TKO MEFs infected with EV, c-Myc, or Pim-3 lentiviruses as determined by QT-PCR 48 h after infection. Values are the average of three independent measurements, and the standard deviation from the mean is shown. (*E*) PGC-1α protein levels as determined by Western blotting in TKO MEFs 48 h postinfection with EV, c-Myc, or Pim-3.

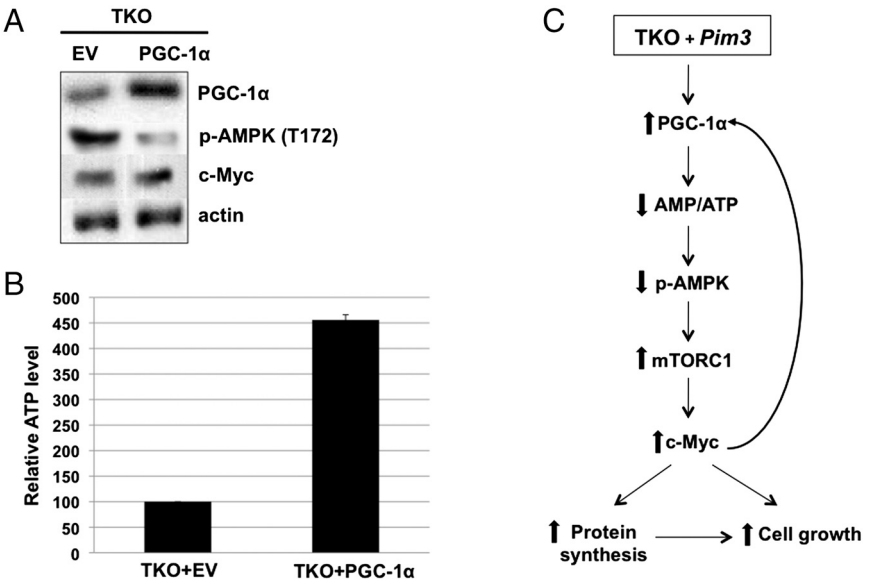

Fig. 5. Expression of PGC-1α restores the AMP:ATP ratio in TKO MEFs. (*A*) PGC-1α overexpression in TKO MEFs reduces AMPK activation. Lysates were prepared from TKO MEFs 48 h after transduction with empty vector (EV) or PGC-1α lentiviruses, and protein levels compared by Western blotting. (*B*) ATP levels determined in lysates from Fig. 5*A* as described in *Materials and Methods*. Values are the average of three independent measurements, and the standard deviation from the mean is shown. (*C*) Schematic summary of biologic changes observed in TKO MEFs expressing Pim-3.

AMPK and PGC-1α in the sensing and regulation of the cell's energy status, the levels of PGC-1α were investigated and found to be significantly lower in TKO MEFs. In comparison, Pim-3–containing MEFs showed increased levels of PGC-1α relative to WT. Therefore, in the case of the TKO MEFs, chronic AMPK activation coupled with drastically reduced levels of PGC-1α protein resulted in an elevated AMP:ATP ratio. Accordingly, infection of TKO MEFs with a lentivirus expressing PGC-1α was shown to increase ATP levels and decrease AMPK activation. The increased PGC-1α levels in Pim-3–only MEFs cannot be attributed solely to increased c-Myc because TKO/c-Myc MEFs showed lower levels of PGC-1α mRNA and protein relative to TKO/Pim-3 MEFs. This suggests the possibility that Pim-3 and c-Myc could cooperate in regulating PGC-1α levels in MEFs. This cooperation may extend beyond transcription/translation because PGC-1α levels and activity are regulated by multiple posttranslational mechanisms (37).

Pim-3 is the least-studied kinase of the Pim family; however, it has been linked to the development and progression of colon and pancreatic cancers (2–4, 44). Despite the high sequence identity and overlapping substrate specificity of the Pim kinases, Pim-3 expression alone is shown to overcome at least some of the defects found in the loss of both Pim-1 and Pim-2, including growth rate. Additionally, the knockout of Pim-1 and -2 and the expression of Pim-3 only led to a marked increase in c-Myc protein relative to WT MEFs. The observation that the transduction of Pim-1 or -2 into MEFs containing Pim-3 suppressed c-Myc levels suggested the possibility that individual Pim isoforms may regulate each other either directly or through substrate competition. This poses the question of whether Pim isoforms either individually or acting in concert regulate different biological processes and under what cellular circumstances. The question of the activity of Pim isoforms is of importance to the design of small-molecule inhibitors targeting these kinases and their use in the treatment of diseases, including cancer. Both Pim-1 and -2 are known to enhance c-Myc–induced transformation (6, 12) and phosphorylate and stabilize c-Myc protein, leading to increased transcriptional activity (9). In the MEFs used in this study, Pim-3 expression alone enhanced cap-dependent translation, increased c-Myc levels without changing the protein's stability, and increased the cell growth rate. Because elevated levels of both Pim-3 and c-Myc are found in gastrointestinal cancers, our results suggest the possibility that Pim-3 might enhance the growth of these tumor cells in part by regulating c-Myc levels, thus highlighting the potential utility of Pim-3 targeted inhibitors.

Materials and Methods

Cell Culture. MEFs were derived from 14.5-d-old embryos and were genotyped as described (45). For stable cell lines, TKO MEFs were transduced with lentiviruses encoding empty vector, PIM-1, Pim-2, Pim-3, or c-Myc and selected with puromycin (4 μg/mL).

Construction of Lentiviral Vectors. The open reading frames of PIM-1 (human, 33 kDa isoform), PIM-2 (mouse), Pim-3 (mouse), c-Myc (mouse), and PGC-1α (human, a gift from Young-In Chi, Department of Molecular and Cellular Biochemistry, University of Kentucky, Lexington, KY) were amplified by PCR from full-length cDNA clones and subcloned into the *AgeI-MluI* sites of pLex-MCS lentiviral vector (Open Biosystems). Methods for preparation of lentiviral stocks are detailed in *SI Materials and Methods*.

Quantitative RT-PCR (QT-PCR). Total RNA was isolated from MEFs using the RNeasy kit (Qiagen) according to the manufacturer's protocol. The first-strand cDNA was synthesized using Superscript first-strand synthesis kit and Oligo (dT) primer (Invitrogen).

Biochemical Analysis. K562 cells were transfected with scrambled siRNA or siPim-1 (ON-TARGETplus SMARTpool, Thermo Scientific) using Lipofectamine™2000 (Invitrogen) according to the manufacturer's protocol, and 48 h posttransfection lysates were prepared. Cell growth was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. ATP, ADP, and AMP were measured by HPLC as described previously (46), and ATP was also measured using the ATP Bioluminescence Assay Kit HS II (Roche) with 10^5 cells. eIF4E was captured on m^7-GTP sepharose (GE Lifesciences) from WT and TKO MEFs lysate and bound 4EBP1 and eIF4G determined by Western blotting.

^{35}S-Methionine Incorporation. Cells were serum starved for 1 h in methionine-free medium (Invitrogen), followed by labeling with 100 mCi of ^{35}S-methionine/mL. Lysates and labeled proteins were precipitated with trichloroacetic acid on glass microfiber filters (Whatman) using vacuum filtration, and ^{35}S-incorporation was counted.

Cap- vs. IRES-Dependent Translation. A bicistronic retroviral vector, pMSCV/rLuc-pol IRES-fLuc (a gift from Peter B. Bitterman, Department of Medicine, University of Minnesota, Minneapolis, MN), was used to produce viral particles for infecting WT, TKO and TKO/Pim-3 MEFs. Cells were collected

48 hr postinfection and Renilla/Firefly luciferase activities were quantified using the dual-luciferase reporter assay system (Promega) and a luminometer according to the manufacturer's instructions.

Polysome Profile Analysis. Sucrose density gradient centrifugation was employed to separate the ribosome fractions as described previously (47). c-Myc mRNA level in each fraction was measured by PCR.

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