Targeting cap-dependent translation blocks converging survival signals by AKT and PIM kinases in lymphoma

Jonathan H. Schatz,[1,4] Elisa Oricchio,[1] Andrew L. Wolfe,[1,7] Man Jiang,[1] Irina Linkov,[5] Jocelyn Maragulia,[4] Weiji Shi,[6] Zhigang Zhang,[6] Vinagolu K. Rajasekhar,[2] Nen C. Pagano,[3] John A. Porco Jr.,[8] Julie Teruya-Feldstein,[5] Neal Rosen,[3,4] Andrew D. Zelenetz,[4] Jerry Pelletier,[9,10] and Hans-Guido Wendel[1]

[1]Cancer Biology and Genetics Program, [2]Stem Cell Center and Developmental Biology Program, and [3]Program in Molecular Pharmacology, Sloan-Kettering Institute for Cancer Research, New York, NY 10065
[4]Department of Medicine, [5]Department of Pathology, and [6]Department of Biostatistics and Epidemiology, Memorial Sloan-Kettering Cancer Center, New York, NY 10065
[7]Weill Cornell Graduate School of Medical Science, New York, NY 10065
[8]Department of Chemistry, Center for Chemical Methodology and Library Development, Boston University, Boston, MA 02215
[9]Department of Biochemistry and [10]Rosalind and Morris Goodman Cancer Center, McGill University, Montreal, Quebec H3G 1Y6, Canada

New anticancer drugs that target oncogenic signaling molecules have greatly improved the treatment of certain cancers. However, resistance to targeted therapeutics is a major clinical problem and the redundancy of oncogenic signaling pathways provides back-up mechanisms that allow cancer cells to escape. For example, the AKT and PIM kinases produce parallel oncogenic signals and share many molecular targets, including activators of cap-dependent translation. Here, we show that PIM kinase expression can affect the clinical outcome of lymphoma chemotherapy. We observe the same in animal lymphoma models. Whereas chemoresistance caused by *AKT* is readily reversed with rapamycin, *PIM*-mediated resistance is refractory to mTORC1 inhibition. However, both *PIM*- and *AKT*-expressing lymphomas depend on cap-dependent translation, and genetic or pharmacological blockade of the translation initiation complex is highly effective against these tumors. The therapeutic effect of blocking cap-dependent translation is mediated, at least in part, by decreased production of short-lived oncoproteins including c-MYC, Cyclin D1, MCL1, and the PIM1/2 kinases themselves. Hence, targeting the convergence of oncogenic survival signals on translation initiation is an effective alternative to combinations of kinase inhibitors.

CORRESPONDENCE
Hans-Guido Wendel:
Wendelh@mskcc.org

Abbreviations used: ABC, activated B cell; CLL/SLL, chronic lymphocytic leukemia/small lymphocytic lymphoma; DLBCL, diffuse large B cell lymphoma; FL, follicular lymphoma; GC, germinal center; HPC, hematopoietic progenitor cell; MCL, mantle cell lymphoma; NHL, non-Hodgkin's lymphoma; OS, overall survival; shRNA, small hairpin RNA; TMA, tissue microarray; TTE, time to event.

Molecular signaling pathways are promising targets in cancer therapy, but resistance often thwarts clinical success. Acquired mutations of drug targets, feedback activation of oncogenic signals, and redundant signaling pathways are important causes of resistance, and cocktails of multiple inhibitors are considered one potential solution (Sawyers, 2007). For example, the rapamycin analogues (rapalogs) are potent inhibitors of mTORC1 with promising antitumor activity against some cancers (Dancey, 2010). mTORC1 blockade by rapamycin interferes with the activation of cap-dependent translation and exploits a cancer cell's dependence on increased translation of certain oncoproteins (Wendel et al., 2007; Sonenberg and Hinnebusch, 2009). In animal models, rapamycin dramatically enhances the effectiveness of DNA-damaging chemotherapy (Wendel et al., 2004). However, in clinical trials in non-Hodgkin's lymphoma (NHL), rapalogs have failed to show durable clinical benefit for most patients (Dancey et al., 2009; Hess et al., 2009; Smith et al., 2010). The causes are ill-understood, and new insight should enable better therapies.

Multiple oncogenic signaling pathways cause aberrant activation of protein translation in cancer cells, including RAS, PI3K–AKT, MAPK, and the PIM kinases (Sonenberg and Hinnebusch, 2009). The PIM kinases were identified in a genetic screen. They promote cell growth and survival and share many targets, including regulators of protein translation, with the better studied AKT/PKB kinases (Nawijn et al., 2011). PIM kinases are induced by cytokine signals and, unlike AKT do not require posttranslational modifications for activity (Fox et al., 2003). Activation of cap-dependent translation via derepression of the translation factor eIF4E is a critical output of both AKT and PIM signaling in cancer (Wendel et al., 2004; Hammerman et al., 2005). *PIM1* and *PIM2* are widely expressed in cancer; *PIM3* is restricted to certain solid tumors (Nawijn et al., 2011). Accordingly, PIM inhibitors have been developed, but clinical trials were terminated early because of cardiac toxicity (Morwick, 2010). Our study explores the clinical impact of *PIM1/2* expression in NHL, and we demonstrate that inhibition of cap-dependent translation is an effective therapy alternative to combinations of kinase inhibitors.

RESULTS AND DISCUSSION
PIM1 and PIM2 are widely expressed in NHL and affect the outcome of follicular lymphoma (FL)

We found widespread expression of *PIM1* and *PIM2* across multiple subtypes of NHL. Immunohistochemical staining of tissue microarrays (TMA) reveals that PIM1 is expressed in 87% of mantle cell lymphomas (MCL; Hsi et al., 2008), 76% of chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL; Chen et al., 2009), and 48 and 42% of diffuse large B cell lymphoma (DLBCL) and FL, respectively. PIM2 is detected in 42% of DLBCL and between 24% and 30% of FL, MCL, and CLL/SLL (Fig. 1, A–E; and Table S1). Similarly, *PIM1/2* mRNA levels are highly expressed in the activated B cell (ABC) type, rather than the germinal center (GC) type of DLBCL (Alizadeh et al., 2000; Rosenwald et al., 2003; Basso et al., 2005; Lenz et al., 2008; unpublished data). PIM2 is abundantly expressed across a panel of human lymphoma cell lines, whereas PIM1 is coexpressed in some, and immunoblots on mouse pro–B cells and Eμ–*Myc* lymphomas confirm PIM1/2 induction by cytokine signals (Fox et al., 2003; unpublished data).

PIM expression affects the outcome of therapy in follicular lymphoma patients. First, we analyzed pretreatment follicular lymphoma samples from 66 patients treated at Memorial Sloan-Kettering Cancer Center (MSKCC) between 1984 and 2000 (Table S2). All but five of these patients received chemotherapy, including doxorubicin in 61% of patients. In this cohort, time to event (TTE) and overall survival (OS) were significantly better for patients whose tumors were PIM–negative (PIM−, no PIM1 or PIM2) compared with patients whose tumors were PIM–positive (PIM+, PIM1, PIM2, or both; P = 0.0113 for TTE, P = 0.0372 for OS for PIM+ vs. PIM− tumors). The mean age was 60.9 and 52.6 yr for the groups, respectively; however, age alone did not explain the difference in outcome (P = 0.13; Fig. 1, F and G;

and Table S2). The same analyses of 116 DLBCL patients treated between 1989 and 2008 showed differences that did not reach statistical significance in OS (P = 0.1678) or TTE (P = 0.4461; Fig. 1, H and I; and Table S3). Similarly, another group recently reported association of PIM2 with outcome in DLBCL (Gómez-Abad et al., 2011). All but three of the DLBCL patients were treated with upfront chemotherapy, including doxorubicin in 88% of patients. Statistical analyses for each PIM kinase analyzed as a single variable or coexpression of PIM1/2 in FL and DLBCL are available in Table S4 and Table S5.

PIM promotes the development of drug–resistant lymphomas in vivo

To study the function of PIM kinase activity in lymphomas, we modeled its effects in murine models of aggressive pre–B cell (Adams et al., 1985) and indolent follicular lymphoma (Egle et al., 2004). In brief, we used adoptive transfer of Eμ-*Myc* or VavP-*Bcl2* transgenic hematopoietic progenitor cells (HPCs; Wendel et al., 2004) expressing *AKT, Pim2,* or vector into lethally irradiated, syngeneic wild-type recipients and monitored the animals for lymphomas (Fig. 2 A). *PIM1* and *PIM2* are highly homologous, therefore we did not examine *PIM1* separately (Nawijn et al., 2011). Both *Pim2* (*n* = 12; P < 0.0001) and *AKT* (*n* = 30; P < 0.0001) accelerated disease onset compared with controls (*n* = 64; P = 0.1209 *Pim2* vs. *AKT*; Fig. 2 A; Verbeek et al., 1991; Wendel et al., 2004). Immunoblotting confirmed expression of AKT and Pim2 and translational activation by both kinases as indicated by increased phosphorylation of 4E-BP1 and ribosomal protein S6 (Fig. 2 C). Histopathology and surface marker analysis revealed that *Pim2*- and *AKT*-expressing tumors were indistinguishable from aggressive pre–B cell lymphomas (Fig. 2 B and unpublished data). The VavP-*Bcl2* model is a genetically and pathologically accurate model of FL, and both *Pim2* (P < 0.0001) and *AKT* (P = 0.0292) accelerated development compared with vector of a slowly proliferating B cell lymphoma with splenic involvement and increased peripheral lymphocyte counts (unpublished data). Hence, *Pim2* and *AKT* activate protein translation and promote lymphomagenesis in mouse models of aggressive and indolent lymphoma.

Next, we examined how PIM and AKT affect treatment responses in vivo. In brief, we transplanted aggressive Eμ-*Myc* lymphomas with defined genetic alterations into nonirradiated recipients, and then treated with 10 mg/kg doxorubicin once lymphomas had developed (Wendel et al., 2004). A side-by-side comparison of chemosensitive Eμ-*Myc/Arf*$^{-/-}$ tumors (control; *n* = 44) with Eμ-*Myc/Pim2* (*n* = 6), or Eμ-*Myc/AKT* (*n* = 30) lymphomas, revealed early relapse and shortened survival with *Pim2*- and *AKT*-expressing tumors (Fig. 2 D; P = 0.0145 *Pim2* vs. *Arf*−, P < 0.0001 *AKT* vs. *Arf*−). Rapamycin alone had little effect on any tumor (Fig. 2 E). However, combinations of rapamycin with doxorubicin caused dramatic responses in *AKT* lymphomas, but had no effect on *Pim2*-expressing tumors (*Pim2, n* = 13; *AKT, n* = 21; control, *n* = 28; P < 0.0001, *Pim* vs. *AKT*; Fig. 2 F). Hence, chemoresistance caused by *AKT* but not by *Pim2* is readily reversed by mTORC1 inhibition.

PIM–expressing lymphomas remain dependent on eIF4E and cap-dependent translation

We examined how PIM bypasses mTORC1 inhibition in rapamycin-sensitive Eµ-*Myc*/*Tsc2*$^{-/-}$ lymphomas (Mills et al., 2008). TSC2 is the Rheb GTPase-activating protein and acts as a negative regulator of mTORC1 activation by Rheb (Tee et al., 2003; Mavrakis et al., 2008). Accordingly, tumors arising in *Tsc2* deficient animals show an mTORC1-dependent and rapamycin-sensitive activation of cap-dependent translation. *Pim2* expression in Eµ-*Myc*/*Tsc2*$^{-/-}$ cells abrogates rapamycin sensitivity, and in mixed populations of parental and *Pim2*/*GFP*-expressing Eµ-*Myc*/*Tsc2*$^{-/-}$ cells the *Pim2*/*GFP* cells

are rapidly enriched under rapamycin treatment (Fig. 3, A and inset). Pim2 causes partially rapamycin-insensitive increases in the phosphorylation of 4E-BP1, eIF4E, and Bad, whereas S6 phosphorylation remains sensitive to rapamycin (Fig. 3 B). The cap-binding protein eIF4E is the rate-limiting factor in cap-dependent translation that is activated by phosphorylation of its inhibitor 4E-BP1 and can be further enhanced by direct eIF4E (S209) phosphorylation (Wendel et al., 2007; Sonenberg and Hinnebusch, 2009). Profiles of ribosome loading on mRNAs (polysome profiles) indicate the efficiency of protein translation. Polysome profiles on parental and Pim2-expressing *EµMyc*/*Tsc2*$^{-/-}$ lymphoma cells reveal a partially rapamycin-refractory increase of protein translation in Pim-expressing lymphomas (Fig. 3 C). Accordingly, both Pim and direct expression of eIF4E protect against rapamycin and have a similar effect in cells treated with the TOR kinase inhibitors PP-242 and Torin1 (Feldman et al., 2009; Thoreen et al., 2009; Fig. 3 D). By comparison, a small hairpin RNA (shRNA) against BAD showed no protective effect during rapamycin treatment (unpublished data). To examine whether PIM-expressing tumors remained dependent on cap-dependent translation, we tested the antiproliferative effects of a constitutively active inhibitor of eIF4E (*4E-BP1-4A*) that acts downstream from mTORC1 (Rong et al., 2008). Surprisingly, parental Eµ-*Myc*/*Tsc2*$^{-/-}$ lymphomas and *Pim2* expressing Eµ-*Myc*/*Tsc2*$^{-/-}$ cells were equally sensitive to direct inhibition of eIF4E and cells expressing 4E-BP1/GFP were rapidly depleted from a mixed population, but had little effect in nontransformed cells (Fig. 3 E and unpublished data). Hence, PIM2 readily bypasses mTORC1 inhibition, but is unable to protect lymphoma cells from the effects of direct translation inhibition.
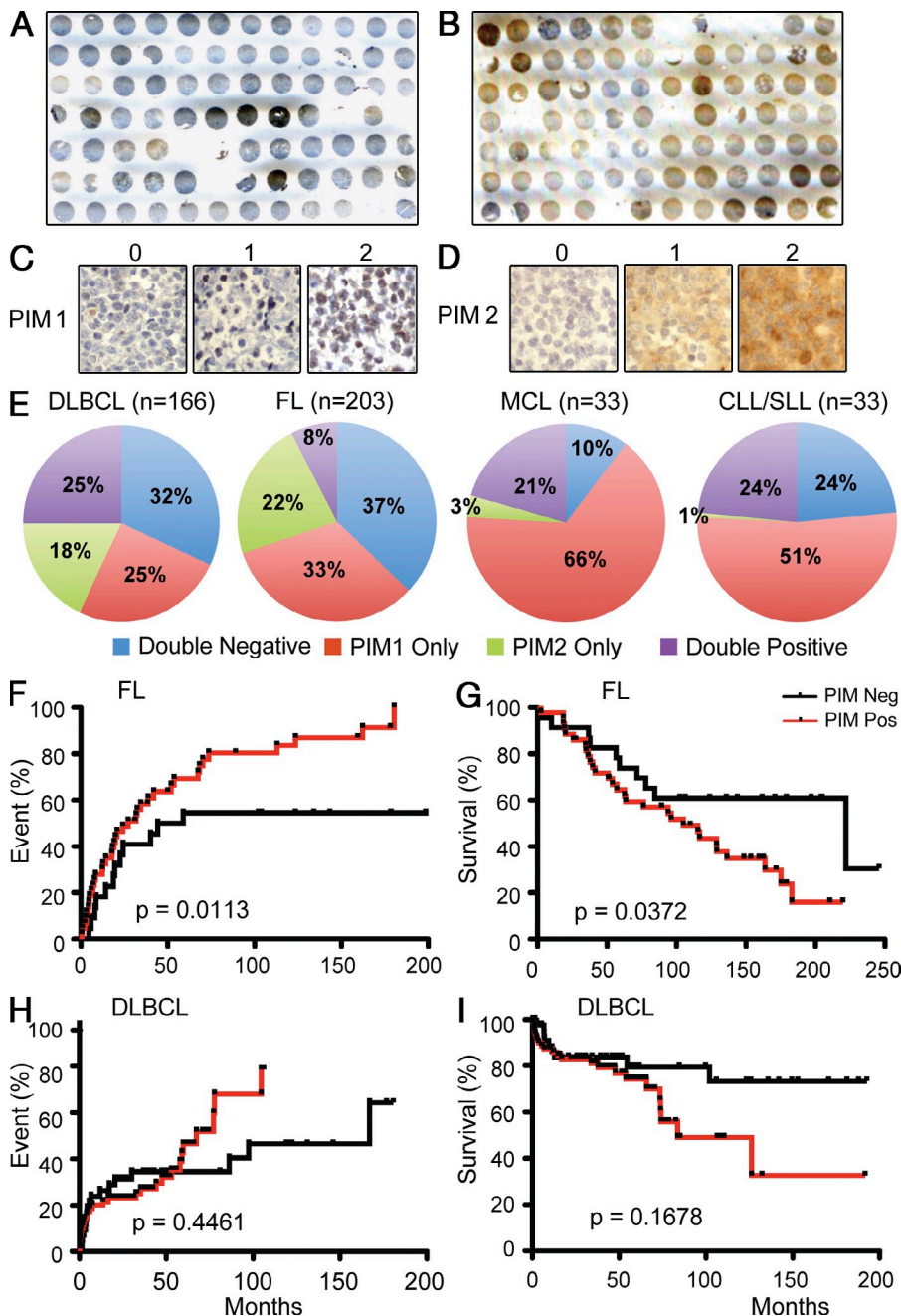

Figure 1.   PIM kinase expression affects the outcome of lymphoma therapy. (A and B) DLBCL TMAs stained for PIM1 (A) and PIM2 (B). (C and D) Representative tumor cores for each PIM histology score (0–2). (E) Pie graphs showing breakdown of PIM1/2 TMA scores by disease; see also Table S1. (F) TTE analysis after primary therapy in follicular lymphoma (*n* = 66). (G) OS analysis from date of diagnosis in follicular lymphoma. (H and I) TTE (H) and OS (I) in DLBCL (*n* = 116).

Silvestrol is a small molecule inhibitor of cap–dependent translation

Silvestrol was identified in a screen for inhibitors of eIF4A, the RNA helicase component of the translation initiation complex that is thought to unwind an mRNA's 5′UTR (Bordeleau et al., 2008). Consistent with our genetic data using a constitutive *4E-BP1* construct, we found that *Pim2* is unable to protect Eμ-*Myc*/*Tsc2*$^{-/-}$ cells from silvestrol alone or in combination with rapamycin (Figs. 4, A and B). Silvestrol kills parental and *Pim2*-expressing Eμ-*Myc*/*Tsc2*$^{-/-}$ cells at nanomolar concentrations in vitro, but is inactive against 3T3 fibroblasts and *Myc*/*Bcl2* lymphomas tumors that arise in the

absence of translational activation (Wendel et al., 2004; Fig. 4 C). Moreover, silvestrol is also far superior to two recently developed PIM inhibitors in human lymphoma cells. In brief, we tested SGI-1776, the only PIM inhibitor that has entered clinical trials (biochemical IC$_{50}$ for PIM1, 15 nM; PIM2, 363 nM), and SGI-1773 (biochemical IC50 for PIM1, 2 nM; PIM2, 43 nM); both drugs were developed and supplied to us by SuperGen Inc. (Morwick, 2010). The PIM kinase inhibitors induced cell death in various human lymphoma cells at concentrations between 1–10 μM; in comparison, silvestrol had the same cell kill at 1–10 nM (Fig. 4 D). In animals, silvestrol was able to reverse *Pim2*-mediated rapamycin resistance
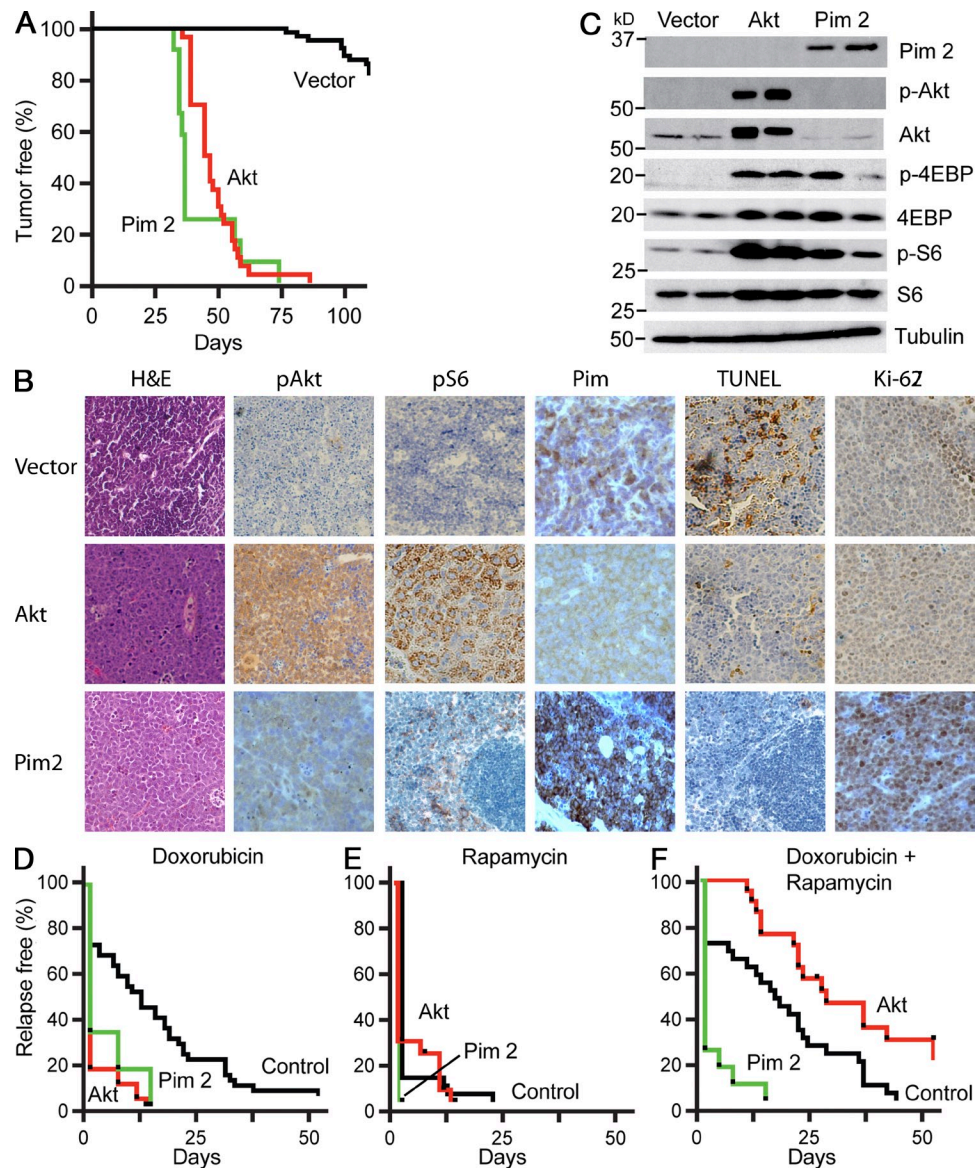

Figure 2. Pim2 and AKT in a mouse lymphoma model. (A) Eμ-*Myc* HPCs expressing *Pim2*, *AKT*, or vector were transplanted into lethally irradiated syngeneic wild-type mice. Tumor onset in *Pim2* (green; *n* = 12), *AKT* (red; *n* = 30) and vector (black; *n* = 64 recipients). (B) Histological and immunohistochemical analyses of indicated Eμ-*Myc* lymphomas. Bar, 20 μm. (C) Immunoblot analyses of indicated Eμ-*Myc* lymphomas. (D–F) Time to relapse in animals bearing Eμ-*Myc*/*Arf*$^{-/-}$ (control, black line), Eμ-*Myc*/*Pim2* (green), and Eμ-*Myc*/*AKT* (red) lymphomas treated with doxorubicin (D; control, *n* = 44; *Pim2*, *n* = 6; *AKT*, *n* = 30) or rapamycin (E; control, *n* = 27; *Pim2*, *n* = 7; *AKT*, *n* = 18) or a combination of both drugs (F; control, *n* = 28; *Pim2*, *n* = 13; *AKT*, *n* = 21).

and did not cause overt toxicity at an effective dose (0.2 mg/kg, d1–7), consistent with published silvestrol toxicity studies, showing no major adverse effects at this dose and duration of treatment (Cencic et al., 2009). In brief, animals bearing parental *Tsc2*-deficient tumors cells (*n* = 9) remained relapse free for up to 3 wk after rapamycin, whereas Eμ-*Myc*/*Tsc2*$^{-/-}$/*Pim2* lymphomas (*n* = 9) showed no response or relapsed early (P = 0.0006; Fig. 4 E). The addition of silvestrol to rapamycin treatment restored rapamycin sensitivity, and Eμ-*Myc*/*Tsc2*$^{-/-}$*Pim2* tumor–bearing animals remained relapse free for as long as sensitive controls (P = 0.7219; Fig. 4 E). Hence, the translation inhibitor silvestrol has good activity active against human lymphoma cells and can overcome PIM-mediated resistance in vivo.

Translation is required to maintain expression of oncoproteins including c-MYC and PIM

In cancer the activation of cap-dependent protein translation by AKT or PIM ensures the expression of short-lived

oncoproteins including c-MYC, MCL1 and Cyclin D1 (Sonenberg and Hinnebusch, 2009). Treatment of PIM-expressing human lymphoma cells with the PIM inhibitor SGI-1773 (10 μM) somewhat reduced Cyclin D1, but had no effect on c-MYC or MCL1 (Fig. 4 F). In contrast, silvestrol (10 nM) caused almost complete loss of Cyclin D1, c-MYC, and MCL1. Moreover, silvestrol completely ablated the expression of both PIM1 and PIM2 kinases (Fig. 4 F). Silvestrol had similar effects on PIM expression in DoHH2 and Su-DHL-10 (Fig. 4 G). This is consistent with the known short half-life of PIM1 and PIM2 and indicates that PIM expression is controlled, at least in part, by cap-dependent translation (Hoover et al., 1997). This dual effect of translation inhibition on PIM and its downstream targets likely accounts for silvestrol's dramatic activity against mouse and human lymphomas.

Our study provides new insight into oncogenic kinases in human lymphoma. The constitutively active PIM1 and PIM2 kinases are abundantly expressed across several subtypes of NHL, and in follicular lymphoma, PIM positivity identifies patients at risk of early relapse and shortened survival and who may require specific treatment. Similarly, in DLBCL, PIM1/2 expression is associated with the prognostically unfavorable ABC subtype (Alizadeh et al., 2000; Rosenwald et al., 2003; Wright et al., 2003; Basso et al., 2005; Poulsen et al., 2005; Lenz et al., 2008; Gómez-Abad et al., 2011). Although clinical data on the effect of PIM expression on rapalog treatment are not yet available, our data and other evidence indicate that neither rapalogs nor the newer TOR-kinase inhibitors will be
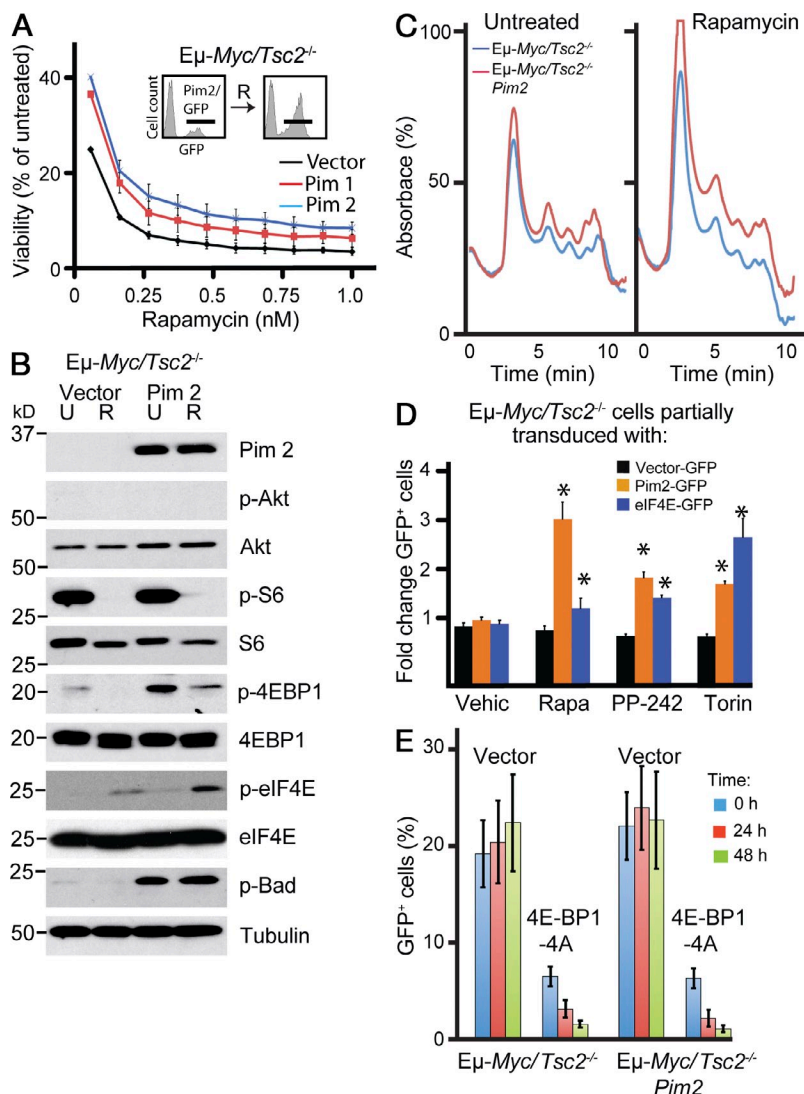

Figure 3. PIM confers resistance to mTOR inhibition, but not to genetic blockade of cap-dependent translation. (A) Cell viability in vitro comparing rapamycin-sensitive Eμ-*Myc*/*Tsc2*$^{-/-}$ lymphomas expressing vector-GFP, *Pim1*-GFP, or *Pim2*-GFP under rapamycin treatment. (inset) Enrichment of the *Pim2*-GFP–expressing subpopulation of Eμ-*Myc*/*Tsc2*$^{-/-}$ cells upon rapamycin exposure in vitro. (B) Immunoblot on lysates of Eμ-*Myc*/*Tsc2*$^{-/-}$/vector or Eμ-*Myc*/*Tsc2*$^{-/-}$/*Pim2* cells treated with vehicle (U) or rapamycin (R), and probed for the indicated proteins. (C) Polyribosome profiles generated from untreated and rapamycin-treated Eμ-*Myc*/*Tsc2*$^{-/-}$ and Eμ-*Myc*/*Tsc2*$^{-/-}$/*Pim2* tumors, indicating the ability of PIM2 to stimulate translation in a partially rapamycin-resistant manner (absorbance at 254 nm). (D) Enrichment of populations Eμ-*Myc*/*Tsc2*$^{-/-}$ cells expressing vector-GFP (black), *Pim2*-GFP (orange), and *eIF4E*-GFP (blue) and treated with rapamycin or the TOR-kinase inhibitors PP-242 and torin (mean fold change and SEM of 5 separate experiments; * indicates significance [P < 0.05] vs. vector). (E) Enrichment or loss of subpopulations of Eμ-*Myc*/*Tsc2*$^{-/-}$ and Eμ-*Myc*/*Tsc2*$^{-/-}$/*Pim2* cells engineered to express vector encoding GFP or a constitutively active inhibitor of eIF4E (*4E-BP1-4A*-GFP) during culture in vitro (mean results and SEM of three separate experiments).

active against PIM-expressing tumors (Fox et al., 2003; Hammerman et al., 2005). PIM kinase inhibitors are under development, and to date only SGI-1776 has entered phase I evaluation. However, its efficacy against multiple tumors and lymphoma was limited, and the trial was terminated because of cardiac toxicity (SuperGen press release November 10, 2010). Hence, PIM expression is a significant clinical problem in lymphoma and a new therapeutic strategy is needed.

We identify a therapeutic strategy that is highly effective against PIM-expressing lymphomas. Both the AKT and PIM kinases control regulators of cap-dependent translation (Sonenberg and Hinnebusch, 2009). Both kinases can limit the effectiveness of chemotherapy, and although the effects of AKT are readily reversed by blocking mTORC1 and translation with rapamycin (Wendel et al., 2004), PIM-expressing tumors remain refractory and are able to maintain translation in an mTORC1-independent manner. However, *PIM*-expressing tumor cells continue to depend on translational activation, and they are therefore sensitive to small molecules that directly target the translation initiation complex downstream from mTORC1. For example, silvestrol, an inhibitor of the eIF4A RNA helicase (Bordeleau et al., 2008), is highly
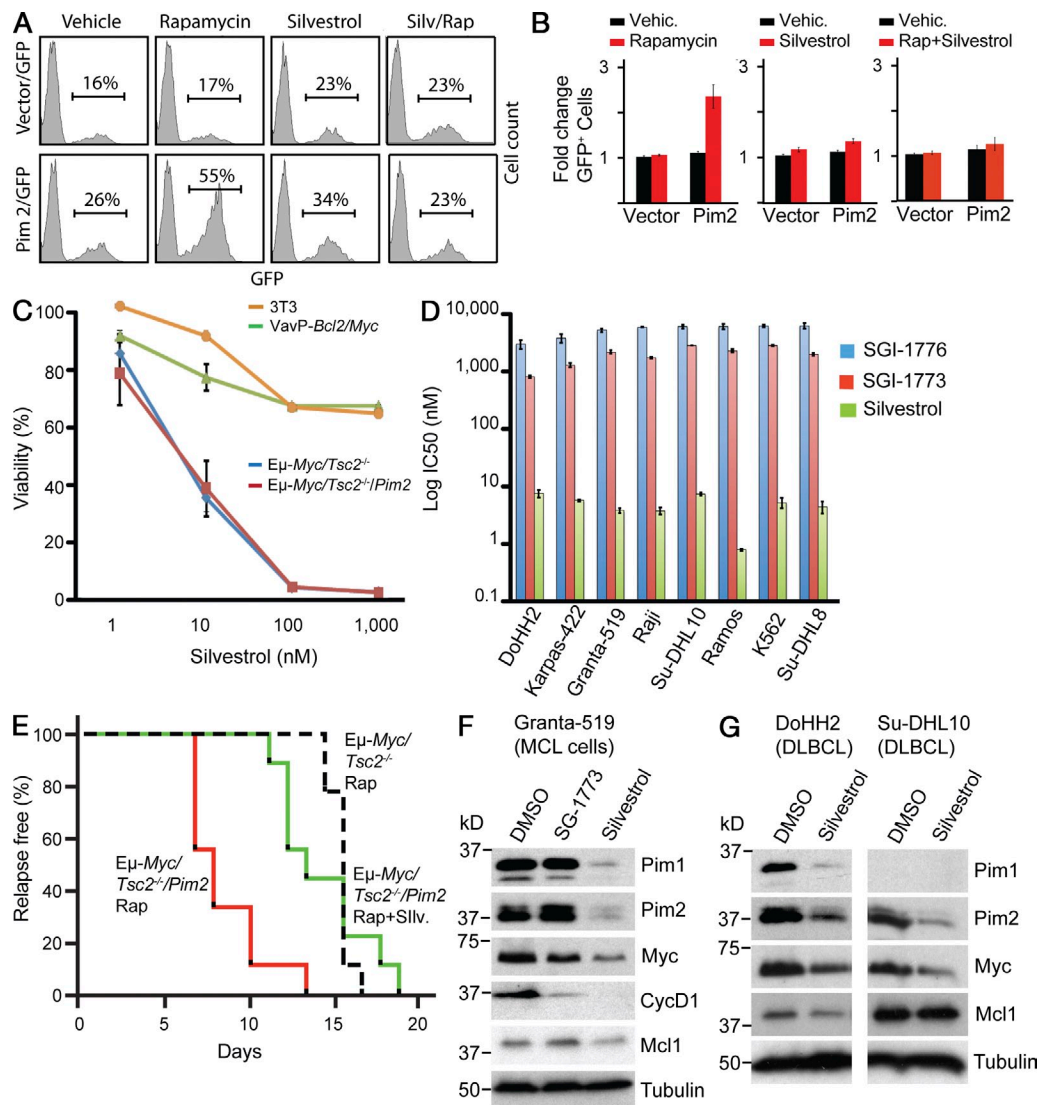

Figure 4. The eIF4A helicase inhibitor silvestrol is active against mouse and human lymphomas irrespective of PIM expression. (A) Representative flow cytometry plots showing enrichment of subpopulations of *Pim2*-GFP–expressing Eμ-*Myc/Tsc2*$^{-/-}$ upon treatment with vehicle, silvestrol, rapamycin, or silvestrol and rapamycin in vitro. (B) Cumulative analysis of three separate experiments showing mean and SEM. (C) Eμ-*Myc/Tsc2*$^{-/-}$ and Eμ-*Myc/Tsc2*$^{-/-}$/*Pim2* cells, or 3T3 fibroblasts or VavP-*Bcl2/Myc* tumor cells were treated with indicated concentrations of silvestrol. Viability was assessed after 24 h (mean and SEM of 4 separate assays per cell line). (D) Comparison of cell death induced by silvestrol or two PIM kinase inhibitors (SGI-1776, SGI-1773) in a panel of human lymphoma cells (mean of three separate assessments and SEM). (E) Time to relapse in animals bearing Eμ-*Myc/Tsc2*$^{-/-}$/*Pim2* tumors that were treated with rapamycin (red; $n = 9$) or rapamycin and silvestrol (green; $n = 9$), or mice bearing parental Eμ-*Myc/Tsc2*$^{-/-}$ tumors treated with rapamycin (black dotted line; $n = 9$). (F and G) Immunoblot on human lymphoma cells Granta-519 (F) or DoHH2 and Su-DHL-10 (G) treated with vehicle (DMSO), the PIM inhibitor SG-1776, or silvestrol.

effective against PIM-expressing human and mouse lymphoma cells and far superior to current PIM kinase inhibitors. Therapeutic blockade of translation affects several short-lived oncoproteins, including the PIM1/2 kinases and c-MYC, MCL1, and Cyclin D1. Silvestrol does not cause the feedback activation of upstream signaling molecules that has been seen upon rapamycin treatment (O'Reilly et al., 2006). In summary, PIM kinase expression adversely affects outcomes in NHL, and targeting the translation of oncoproteins like PIM and c-Myc effectively disables this critical output of converging oncogenic pathways.

MATERIALS AND METHODS

TMAs. TMAs were constructed from paraffin-embedded tumor cores of 452 NHL patients treated at MSKCC since the mid-1980s (173 DLBCL, 205 FL, 37 MCL, and 37 CLL/SLL). Use of tissue samples was approved by the Institutional Review Board and the Human Biospecimen Utilization Committee. All cancer biopsies were evaluated at MSKCC, and the histological diagnosis was based on hematoxylin and eosin (H&E) staining. TMAs were constructed, stained, and scored as previously described (Hedvat et al., 2002) with antibodies against Pim1 and Pim2 (Cell Signaling Technology). Pim1 polyclonal antibody staining was performed at 1:100 dilution using the manufacturer's protocol, with secondary staining by OmniMap DAB anti-Rb Detection kit (Ventana). Pim2 monoclonal antibody staining was performed manually at 1:100 dilution in citric acid, pH 6, with rabbit secondary antibody and finished with DAB (3,3′-Diaminobenzidine). All TMA scoring was performed by an expert lymphoma hematopathologist.

Clinical data and analyses. Under MSKCC IRB waiver approval, clinical data were collected on patients whose tumors appear on the DLBCL and FL TMAs. Of the FL cases, we identified 66 whose disease required treatment, whose specimen on the TMA was from before their initial therapy, and for whom treatment data and Pim scores were available. These cases were subjected to Kaplan-Meier TTE and OS analyses from initiation of therapy and date of diagnosis, respectively. Events were defined as progression of disease, death, or secondary malignancy. Log-rank analysis was used to compare groups. The same analyses were performed on 116 DLBCL patients with available treatment data and whose biopsy sample on the TMA was from before initial therapy. PIM+ versus PIM− patient groups were compared for age, sex, and additional clinical variables listed in Tables S1 and S2 based on data availability. χ^2 or fisher's exact test was used to compare categorical variables and Wilcoxon rank-sum test was used to compare continuous variables.

Mouse lymphoma generation and analysis. All animal experiments were approved by the MSKCC Institutional Animal Care and Use Committee in compliance with the U.S. Department of Health and Human Services Guide for the Care and Use of Laboratory Animals. The Eμ-*Myc* model of aggressive lymphoma (Adams et al., 1985) and the VavP-*Bcl2* model of follicular lymphoma (Egle et al., 2004) were adapted to the transplantation approach using retrovirally transduced HPCs (Wendel et al., 2004). In brief, we isolated HPCs from the fetal livers of day 13.5–14.5 transgenic embryos and infected them with retroviral constructs coexpressing GFP and murine *Pim2* or constitutively active myristoylated *AKT* using the MSCV-IRES-GFP vector. The HPCs were then transplanted into syngeneic wild-type C57/B6 recipient animals after sublethal irradiation (same day 4.5G + 4.5G). We then tracked animals for tumor onset by observation, palpation, and blood smear evaluation. Disease onset data were subjected to Kaplan-Meier analysis and the log-rank (Mantel-Cox) test for statistical significance. H&E, Ki67, TUNEL, phospho–AKT, phospho-4E-BP1, phospho-S6, Pim2, and surface marker analysis were previously described (Mavrakis et al., 2008). Eμ-*Myc*/*Tsc2*$^{-/-}$ lymphomas are generated by crossing Eμ-*Myc*$^{+/-}$ mice to *Tsc2*$^{+/-}$ mice (Mills et al., 2008). Double heterozygous offspring generate B cell tumors because of loss of heterozygosity at the *Tsc2* locus, resulting in tumors that can be cultured ex vivo.

In vivo treatment studies. Treatment studies with doxorubicin and/or rapamycin were as previously described (Wendel et al., 2004; Mavrakis et al., 2008). In brief, 10^6 primary lymphoma cells were injected into the tail vein of 10–12-wk-old female C57BL/6 mice. Upon the formation of well-palpable tumors, the animals were treated with rapamycin (LC Laboratories; 4 mg/kg, i.p.), doxorubicin (Sigma-Aldrich; 10 mg/kg, i.p.), or a combination of both. Eμ-*Myc*/*Arf*$^{-/-}$ tumors, which are homogeneous in respect to *p53* status, were used as controls where indicated. For treatment studies with Eμ-*Myc*/*Tsc2*$^{-/-}$ tumor cells, 10–12-wk-old female C57BL/6 mice were injected with 250,000 tumor cells. Rapamycin was given as above, and silvestrol was dosed as previously described (Bordeleau et al., 2008), given at 0.2 mg/kg daily for 7 d. After treatment, the mice were monitored by palpation and blood smears stained with Giemsa (Thermo Fisher Scientific). Tumor-free and OS data were analyzed in the Kaplan-Meier format using the log-rank (Mantel-Cox) test for statistical significance.

Cell culture, competition, and viability assays. Eμ-*Myc*/*Tsc2*$^{-/-}$ and Eμ-*Myc*/*p53*$^{-/-}$ tumor cells were cultured in B cell media (1:1 DMEM/IMDM, with 10% fetal bovine serum, penicillin/streptomycin, and L-glutamine) on feeder layers consisting of irradiated NIH-3T3 cells. Competition assays used the MSCV-IRES-GFP vector ± the indicated genes (*Pim1*, *Pim2*, and *eIF4E*) or the shRNA vector MLP (Mavrakis et al., 2008) for shBad (see below). GFP expression was assessed through FACS analysis (Guava EasyCyte; Millipore). Experiments were repeated three or more times and averaged based on fold change in the percentage of GFP$^+$ cells before and after treatment with drug or vehicle. In competition time point experiments, cells were treated with drug or vehicle on day 0 for 24 h and tracked for GFP expression daily. Human lymphoma cell lines were cultured in RPMI-1640 or DME supplemented with 10% fetal bovine serum, penicillin/streptomycin, and L-glutamine. Cell viability was assessed with CellTiter-Glo reagent (Promega). IC$_{50}$ values were determined from viability curves and represent a mean value from 3–4 curves per cell line. The *4E-BP1*-4A (in MSCV-IRES-GFP) vector was a gift from the laboratory of N. Rosen (Sloan-Kettering Institute, New York, NY) and was sequence confirmed to contain mutation to alanine at residues T37, T46, S65, and T70. Cytokine stimulation was performed for 6 or 12 h with 400 pg/ml recombinant mouse IL-3 (Fitzgerald Industries) and 10 ng/ml recombinant mouse IL-6 (Fitzgerald Industries). Puromycin selections were performed for 2 d at a concentration of 2 μg/ml.

In vitro treatment studies. Rapamycin (LC Laboratories) was dissolved in ethanol vehicle and stored as 10 mM stock solution protected from light at −20°C. It was diluted in ice-cold ethanol before use at the indicated concentrations in the results and compared with 1:1,000 ethanol-treated vehicle controls. Silvestrol was stored as 10-mM stock solution in DMSO at −80°C and diluted in DMSO before use at the indicated concentrations in the results. SGI-1773 and SGI-1776 were provided by SuperGen Inc. and were stored as 10-mM stock solutions in DMSO at −20°C. Comparisons for silvestrol and the Pim-kinase inhibitors were to 1:1,000 DMSO-treated vehicle controls. For detecting drug effects by immunoblot, cells were treated with 10 nM rapamycin for 4 h, 10 nM silvestrol for 24 h, or 10 μM SGI-1773 for 24 h.

Polysomal profiling. Sucrose density gradient centrifugation was used to separate the ribosome fractions. 15 min before collection, cycloheximide (100 μg/ml) was added to the culture medium. Cells were washed in ice-cold PBS containing 100 μg/ml cycloheximide and harvested. Cell pellets were resuspended in polysome lysis buffer (5 mM Tris-HCl, pH 7.5, 2.5 mM MgCl$_2$, 1.5 mM KCl, 2 mM DTT, 0.5% Triton X-100, 0.5% sodium deoxycholate, 100 μg/ml cycloheximide, RNAsin inhibitor, and protease and phosphatase inhibitors). Cells were incubated on ice for 15 min, and then centrifuged at 10,000 g for 10 min at 4°C. The supernatant (2 mg of protein) was layered on a prechilled 10–50% linear sucrose gradient prepared in 5 mM Tris-HCl, pH 7.5, 2.5 mM MgCl$_2$, and 1.5 mM KCl, and then centrifuged in a Beckman SW41Ti rotor at 35,000 rpm for 2.5 h at 4°C. Gradients were fractionated while monitoring absorbance continuously at 254 nm with a

JEM

Density Gradient Fractionation System (Teledyne ISCO). Curves were recorded by PeakTrak software (Teledyne Isco) in parallel. Data were replotted in Excel (Microsoft).

Western blot analysis. Immunoblots were performed from whole-cell lysates. In brief, 20–50 µg of protein/sample were resolved on SDS-PAGE gels and transferred to Immobilon-P membranes (Millipore). Antibodies were against human PIM1, human PIM2, AKT, phospho-AKT (S473), ribosomal protein S6, phospho–rpS6 (S240/244), 4E-BP1, phospho-4EBP1 (S65), eIF4E, phospho–eIF4E (S209), BAD, phospho-BAD (S112), phospho-MDM2 (S166), CyclinD1 (Cell Signaling Technology); mouse Pim1, mouse Pim2, c-*Myc* (Santa Cruz Biotechnology); MCL1 (Rockland Immunochemicals); and α–tubulin (Sigma-Aldrich).

shRNA gene knockdown. RNAi knockdown of mouse *Bad* was performed as previously described (Mavrakis et al., 2008) using the GFP-coexpressing, puromycin-selectable shRNA vector MLP. Three potential shRNAs were generated and tested by infecting FL5-12 cells with them or empty MLP vector, purification through puromycin selection, and immunoblotting protein lysates for Bad protein levels. Sequences of the hairpins were as follows: #1, 5′-TGCTGTTGACAGTGAGCGACAG-GAAGACGCTAGTGCTACATAGTGAAGCCACAGATGTATGTAG-CACTAGCGTCTTCCTGCTGCCTACTGCCTCGGA–3′; #2, 5′-TGCT-GTTGACAGTGAGCGAAAGACGCTAGTGCTACAGATATAGTGA-AGCCACAGATGTATATCTGTAGCACTAGCGTCTTCTGCCTAC-TGCCTCGGA-3′; #3, 5′-TGCTGTTGACAGTGAGCGACTGCAA-CACAGATGCGACAAATAGTGAAGCCACAGATGTATTTGTCGC-ATCTGTGTTGCAGCTGCCTACTGCCTCGGA-3′.

Online supplemental material. Table S1 shows complete TMA scoring. Tables S2 and S3 show clinical characteristics of analyzed FL and DLBCL patients, respectively. Tables S4 and S5 show statistical analyses of FL and DLBCL patients, respectively, by PIM1 and PIM2 expression. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20110846/DC1.

REFERENCES

Adams, J.M., A.W. Harris, C.A. Pinkert, L.M. Corcoran, W.S. Alexander, S. Cory, R.D. Palmiter, and R.L. Brinster. 1985. The c-myc oncogene driven by immunoglobulin enhancers induces lymphoid malignancy in transgenic mice. *Nature.* 318:533–538. doi:10.1038/318533a0

Alizadeh, A.A., M.B. Eisen, R.E. Davis, C. Ma, I.S. Lossos, A. Rosenwald, J.C. Boldrick, H. Sabet, T. Tran, X. Yu, et al. 2000. Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling. *Nature.* 403:503–511. doi:10.1038/35000501

Basso, K., A.A. Margolin, G. Stolovitzky, U. Klein, R. Dalla-Favera, and A. Califano. 2005. Reverse engineering of regulatory networks in human B cells. *Nat. Genet.* 37:382–390. doi:10.1038/ng1532

Bordeleau, M.E., F. Robert, B. Gerard, L. Lindqvist, S.M. Chen, H.G. Wendel, B. Brem, H. Greger, S.W. Lowe, J.A. Porco Jr., and J. Pelletier. 2008. Therapeutic suppression of translation initiation modulates chemosensitivity in a mouse lymphoma model. *J. Clin. Invest.* 118:2651–2660.

Cencic, R., M. Carrier, G. Galicia-Vázquez, M.E. Bordeleau, R. Sukarieh, A. Bourdeau, B. Brem, J.G. Teodoro, H. Greger, M.L. Tremblay, et al. 2009. Antitumor activity and mechanism of action of the cyclopenta[b]benzofuran, silvestrol. *PLoS ONE.* 4:e5223. doi:10.1371/journal.pone.0005223

Chen, L.S., S. Redkar, D. Bearss, W.G. Wierda, and V. Gandhi. 2009. Pim kinase inhibitor, SGI-1776, induces apoptosis in chronic lymphocytic leukemia cells. *Blood.* 114:4150–4157. doi:10.1182/blood-2009-03-212852

Dancey, J. 2010. mTOR signaling and drug development in cancer. *Nat Rev Clin Oncol.* 7:209–219. doi:10.1038/nrclinonc.2010.21

Dancey, J.E., R. Curiel, and J. Purvis. 2009. Evaluating temsirolimus activity in multiple tumors: a review of clinical trials. *Semin. Oncol.* 36(Suppl 3): S46–S58. doi:10.1053/j.seminoncol.2009.10.010

Egle, A., A.W. Harris, M.L. Bath, L. O'Reilly, and S. Cory. 2004. VavP-Bcl2 transgenic mice develop follicular lymphoma preceded by germinal center hyperplasia. *Blood.* 103:2276–2283. doi:10.1182/blood-2003-07-2469

Feldman, M.E., B. Apsel, A. Uotila, R. Loewith, Z.A. Knight, D. Ruggero, and K.M. Shokat. 2009. Active-site inhibitors of mTOR target rapamycin-resistant outputs of mTORC1 and mTORC2. *PLoS Biol.* 7:e38. doi:10.1371/journal.pbio.1000038

Fox, C.J., P.S. Hammerman, R.M. Cinalli, S.R. Master, L.A. Chodosh, and C.B. Thompson. 2003. The serine/threonine kinase Pim-2 is a transcriptionally regulated apoptotic inhibitor. *Genes Dev.* 17:1841–1854. doi:10.1101/gad .1105003

Gómez-Abad, C., H. Pisonero, C. Blanco-Aparicio, G. Roncador, J. Bischoff, and M. Piris. 2011. PIM inhibition as a rational therapeutic approach in diffuse large B-cell lymphoma. *Ann. Oncol.* 22:iv136–iv137.

Hammerman, P.S., C.J. Fox, M.J. Birnbaum, and C.B. Thompson. 2005. Pim and Akt oncogenes are independent regulators of hematopoietic cell growth and survival. *Blood.* 105:4477–4483. doi:10.1182/blood-2004-09-3706

Hedvat, C.V., A. Hegde, R.S. Chaganti, B. Chen, J. Qin, D.A. Filippa, S.D. Nimer, and J. Teruya-Feldstein. 2002. Application of tissue microarray technology to the study of non-Hodgkin's and Hodgkin's lymphoma. *Hum. Pathol.* 33:968–974. doi:10.1053/hupa.2002.127438

Hess, G., R. Herbrecht, J. Romaguera, G. Verhoef, M. Crump, C. Gisselbrecht, A. Laurell, F. Offner, A. Strahs, A. Berkenblit, et al. 2009. Phase III study to evaluate temsirolimus compared with investigator's choice therapy for the treatment of relapsed or refractory mantle cell lymphoma. *J. Clin. Oncol.* 27:3822–3829. doi:10.1200/JCO.2008.20.7977

Hoover, D.S., D.G. Wingett, J. Zhang, R. Reeves, and N.S. Magnuson. 1997. Pim-1 protein expression is regulated by its 5′-untranslated region and translation initiation factor elF-4E. *Cell Growth Differ.* 8:1371–1380.

Hsi, E.D., S.H. Jung, R. Lai, J.L. Johnson, J.R. Cook, D. Jones, S. Devos, B.D. Cheson, L.E. Damon, and J. Said. 2008. Ki67 and PIM1 expression predict outcome in mantle cell lymphoma treated with high dose therapy, stem cell transplantation and rituximab: a Cancer and Leukemia Group B 59909 correlative science study. *Leuk. Lymphoma.* 49:2081–2090. doi:10.1080/10428190802419640

Lenz, G., G. Wright, S.S. Dave, W. Xiao, J. Powell, H. Zhao, W. Xu, B. Tan, N. Goldschmidt, J. Iqbal, et al; Lymphoma/Leukemia Molecular Profiling Project. 2008. Stromal gene signatures in large-B-cell lymphomas. *N. Engl. J. Med.* 359:2313–2323. doi:10.1056/NEJMoa0802885

Mavrakis, K.J., H. Zhu, R.L. Silva, J.R. Mills, J. Teruya-Feldstein, S.W. Lowe, W. Tam, J. Pelletier, and H.G. Wendel. 2008. Tumorigenic activity and therapeutic inhibition of Rheb GTPase. *Genes Dev.* 22:2178–2188. doi:10.1101/gad.1690808

Mavrakis, K.J., A.L. Wolfe, E. Oricchio, T. Palomero, K. de Keersmaecker, K. McJunkin, J. Zuber, T. James, A.A. Khan, C.S. Leslie, et al. 2010. Genome-wide RNA-mediated interference screen identifies miR-19 targets in

Notch–induced T-cell acute lymphoblastic leukaemia. *Nat. Cell Biol.* 12:372–379. doi:10.1038/ncb2037

Mills, J.R., Y. Hippo, F. Robert, S.M. Chen, A. Malina, C.J. Lin, U. Trojahn, H.G. Wendel, A. Charest, R.T. Bronson, et al. 2008. mTORC1 promotes survival through translational control of Mcl-1. *Proc. Natl. Acad. Sci. USA.* 105:10853–10858. doi:10.1073/pnas.0804821105

Morwick, T. 2010. Pim kinase inhibitors: a survey of the patent literature. *Expert Opin Ther Pat.* 20:193–212. doi:10.1517/13543770903496442

Nawijn, M.C., A. Alendar, and A. Berns. 2011. For better or for worse: the role of Pim oncogenes in tumorigenesis. *Nat. Rev. Cancer.* 11:23–34. doi:10.1038/nrc2986

O'Reilly, K.E., F. Rojo, Q.B. She, D. Solit, G.B. Mills, D. Smith, H. Lane, F. Hofmann, D.J. Hicklin, D.L. Ludwig, et al. 2006. mTOR inhibition induces upstream receptor tyrosine kinase signaling and activates Akt. *Cancer Res.* 66:1500–1508. doi:10.1158/0008-5472.CAN-05-2925

Poulsen, C.B., R. Borup, F.C. Nielsen, N. Borregaard, M. Hansen, K. Grønbaek, M.B. Møller, and E. Ralfkiaer. 2005. Microarray-based classification of diffuse large B-cell lymphoma. *Eur. J. Haematol.* 74:453–465. doi:10.1111/j.1600-0609.2005.00429.x

Rong, L., M. Livingstone, R. Sukarieh, E. Petroulakis, A.C. Gingras, K. Crosby, B. Smith, R.D. Polakiewicz, J. Pelletier, M.A. Ferraiuolo, and N. Sonenberg. 2008. Control of eIF4E cellular localization by eIF4E-binding proteins, 4E-BPs. *RNA.* 14:1318–1327. doi:10.1261/rna.950608

Rosenwald, A., G. Wright, K. Leroy, X. Yu, P. Gaulard, R.D. Gascoyne, W.C. Chan, T. Zhao, C. Haioun, T.C. Greiner, et al. 2003. Molecular diagnosis of primary mediastinal B cell lymphoma identifies a clinically favorable subgroup of diffuse large B cell lymphoma related to Hodgkin lymphoma. *J. Exp. Med.* 198:851–862. doi:10.1084/jem.20031074

Sawyers, C.L. 2007. Cancer: mixing cocktails. *Nature.* 449:993–996. doi:10.1038/449993a

Smith, S.M., K. van Besien, T. Karrison, J. Dancey, P. McLaughlin, A. Younes, S. Smith, P. Stiff, E. Lester, S. Modi, et al. 2010. Temsirolimus has activity in non-mantle cell non-Hodgkin's lymphoma subtypes: The University of Chicago phase II consortium. *J. Clin. Oncol.* 28:4740–4746. doi:10.1200/JCO.2010.29.2813

Sonenberg, N., and A.G. Hinnebusch. 2009. Regulation of translation initiation in eukaryotes: mechanisms and biological targets. *Cell.* 136:731–745. doi:10.1016/j.cell.2009.01.042

Tee, A.R., B.D. Manning, P.P. Roux, L.C. Cantley, and J. Blenis. 2003. Tuberous sclerosis complex gene products, Tuberin and Hamartin, control mTOR signaling by acting as a GTPase-activating protein complex toward Rheb. *Curr. Biol.* 13:1259–1268. doi:10.1016/S0960-9822(03)00506-2

Thoreen, C.C., S.A. Kang, J.W. Chang, Q. Liu, J. Zhang, Y. Gao, L.J. Reichling, T. Sim, D.M. Sabatini, and N.S. Gray. 2009. An ATP-competitive mammalian target of rapamycin inhibitor reveals rapamycin-resistant functions of mTORC1. *J. Biol. Chem.* 284:8023–8032. doi:10.1074/jbc.M900301200

Verbeek, S., M. van Lohuizen, M. van der Valk, J. Domen, G. Kraal, and A. Berns. 1991. Mice bearing the E mu-myc and E mu-pim-1 transgenes develop pre–B-cell leukemia prenatally. *Mol. Cell. Biol.* 11:1176–1179.

Wendel, H.G., E. De Stanchina, J.S. Fridman, A. Malina, S. Ray, S. Kogan, C. Cordon-Cardo, J. Pelletier, and S.W. Lowe. 2004. Survival signalling by Akt and eIF4E in oncogenesis and cancer therapy. *Nature.* 428:332–337. doi:10.1038/nature02369

Wendel, H.G., R.L. Silva, A. Malina, J.R. Mills, H. Zhu, T. Ueda, R. Watanabe-Fukunaga, R. Fukunaga, J. Teruya-Feldstein, J. Pelletier, and S.W. Lowe. 2007. Dissecting eIF4E action in tumorigenesis. *Genes Dev.* 21:3232–3237. doi:10.1101/gad.1604407

Wright, G., B. Tan, A. Rosenwald, E.H. Hurt, A. Wiestner, and L.M. Staudt. 2003. A gene expression-based method to diagnose clinically distinct subgroups of diffuse large B cell lymphoma. *Proc. Natl. Acad. Sci. USA.* 100:9991–9996. doi:10.1073/pnas.1732008100