of intra-Golgi transport. Other tethers should identify other subpopulations, allowing us to map the flow patterns of resident, cargo, and recycling molecules. The intersections will dictate the nature and extent of the modifications to the transiting cargo, which should help us understand the functioning of the Golgi in different cells, tissues, and organisms.

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Phosphorylation and Regulation of Akt/PKB by the Rictor-mTOR Complex

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Deregulation of Akt/protein kinase B (PKB) is implicated in the pathogenesis of cancer and diabetes. Akt/PKB activation requires the phosphorylation of Thr^{308} in the activation loop by the phosphoinositide-dependent kinase 1 (PDK1) and Ser^{473} within the carboxyl-terminal hydrophobic motif by an unknown kinase. We show that in *Drosophila* and human cells the target of rapamycin (TOR) kinase and its associated protein rictor are necessary for Ser^{473} phosphorylation and that a reduction in rictor or mammalian TOR (mTOR) expression inhibited an Akt/PKB effector. The rictor-mTOR complex directly phosphorylated Akt/PKB on Ser^{473} in vitro and facilitated Thr^{308} phosphorylation by PDK1. Rictor-mTOR may serve as a drug target in tumors that have lost the expression of PTEN, a tumor suppressor that opposes Akt/PKB activation.

The Akt/PKB kinase is a well-characterized effector of phosphoinositide 3-kinase (PI3K), and its deregulation plays important roles in the pathogenesis of human cancers. PI3K is necessary for the activation of Akt/PKB, and current models suggest that phosphatidylinositol-3,4,5-triphosphates produced upon growth factor stimulation recruit Akt/PKB to the plasma membrane by binding to its N-terminal pleckstrin homology (PH) domain. At the membrane, Akt/PKB is phosphorylated on two key residues: Thr^{308} (T308) of the activation loop by PDK1 (*1*, *2*) and Ser^{473} (S473) in the hydrophobic motif of the C-terminal tail by a kinase whose identity has been elusive. The role of S473 phosphorylation is controversial, but there is an emerging view that it precedes the phosphorylation of T308 and is important for the recognition and activation of Akt/PKB by PDK1 (*3*–*5*).

The molecular identity of the S473 kinase (S473K), at times referred to as "PDK2" or the "hydrophobic motif (HM) kinase," has been hotly debated for many years. Several candidate S473Ks have been proposed, including PDK1 (*6*), integrin-linked kinase (ILK) (*7*), Akt/PKB itself (*8*), and, most recently, DNA-PK$_{cs}$ (*9*). Many lines of evidence argue that neither PDK1, ILK, nor Akt/PKB is the physiological S473K (*10*–*12*), and for several reasons, DNA-PK$_{cs}$ is also unlikely to have this function. There is no *Drosophila* ortholog of DNA-PK$_{cs}$ (*13*), and, thus, if DNA-PK$_{cs}$ is a physiological S473K in mammals, a distinct kinase must play that role in flies even though all other core components of the pathway (e.g., PI3K, Akt/PKB, PDK1, and PTEN) are well conserved. Moreover, it has not been shown that DNA-PK$_{cs}$ phosphorylates full-length Akt/PKB, and DNA-PK$_{cs}$ null mice (*14*) do not suffer the growth retardation or insulin signaling defects associated with Akt1/PKB1 (*15*, *16*) or Akt2/PKB2 (*17*) null mice, respectively.

Mammalian TOR (mTOR) is a large protein kinase that exists in two distinct complexes within cells: one that contains mTOR, GβL, and raptor (*18*–*21*) and another containing mTOR, GβL, and rictor (*21*, *22*). The raptor-containing complex is sensitive to the drug rapamycin and regulates cell growth, in part by phosphorylating the hydrophobic motif of S6K1 (*23*), a member of the same family of kinases to which Akt/PKB belongs. The rictor-containing complex does not appear to be rapamycin-sensitive, and its cellular function is just beginning to be understood (*22*). Despite its structural similarity to S6K1, Akt/PKB phosphorylation is not sensitive to acute rapamycin treatment, and thus mTOR has not previously been considered as the S473K.

We used RNA interference (RNAi) in cultured *Drosophila* cells to determine the role of TOR pathway components in the phosphorylation of the hydrophobic motif sites of *Drosophila* Akt/PKB (dAKT/dPKB) and S6K (dS6K). In mammals and *Drosophila*, S6K suppresses signaling through the PI3K/Akt pathway so that inhibition of S6K boosts Akt/PKB phosphorylation (*24*–*26*). Knockdown of dS6K or dRaptor expression with double-stranded RNAs (dsRNAs) inhibited the phosphorylation and activity of dS6K and increased the phosphorylation of dAkt/dPKB (Fig. 1A). Despite reducing dS6K phosphorylation to the same extent as did dRaptor dsRNA, the dTOR dsRNA failed to increase dAkt/dPKB phosphorylation and,

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surprisingly, decreased it by a small amount (Fig. 1A). The contrasting effects on dAkt/dPKB phosphorylation by the dTOR and dRaptor dsRNAs suggest that dTOR has an unexpected positive role in dAkt/dPKB signaling that is not shared with dRaptor and that dTOR is required for the increase in dAkt/dPKB phosphorylation caused by dS6K inhibition. Consistent with the dRaptor-independent role for dTOR in dAkt/dPKB phosphorylation, a knockdown of dRictor reduced dAkt/dPKB phosphorylation (Fig. 1A).

Because basal dAkt/dPKB phosphorylation is low in *Drosophila* Kc_{167} cells (Fig. 1A), we verified the roles of dRictor and dTOR in cells in which dAkt/dPKB phosphorylation was enhanced by decreasing the expression of dPTEN, the negative regulator of the PI3K/Akt pathway (Fig. 1B). Knockdown of dS6K or dRaptor expression in dPTEN-depleted cells further boosted dAkt/dPKB phosphorylation. In contrast, knockdown of dRictor expression almost completely prevented the dramatic increase in dAkt/dPKB phosphorylation caused by a dPTEN knockdown, whereas the knockdown of dTOR expression caused a slightly smaller suppression (Fig. 1B). Also, dRictor and dTOR were required for the increase in phosphorylation of dAkt/dPKB caused by a knockdown in the expression of dRaptor (fig. S1).

Our results in *Drosophila* cells suggest that dTOR and dRictor have a shared positive role in the phosphorylation of the hydrophobic motif site of dAkt/dPKB. This finding was unexpected, because previously (*18*) we observed no decrease in the phosphorylation of the hydrophobic motif site of Akt/PKB after reducing mTOR expression in human cells with small interfering RNAs (siRNAs). In retrospect, however, these experiments were undertaken when RNAi-mediated knockdowns of expression in mammalian cells were relatively inefficient. Here, with the use of a lentiviral short hairpin RNA (shRNA) expression system that robustly suppresses gene expression (*22*), we obtained results in human cell lines analogous to those in *Drosophila* cells (Fig. 2A). In human HT-29 colon and A549 lung cancer cells, knockdown of rictor or mTOR expression using two different sets of shRNAs decreased phosphorylation of both S473 and T308 of Akt/PKB. Mammalian cells may try to compensate for the effects of the rictor and mTOR knockdowns by boosting Akt/PKB expression (Fig. 2A). The decrease in T308 phosphorylation is consistent with the importance of S473 phosphorylation for T308 phosphorylation (*3*) and with the fact that the $Ser^{473} \rightarrow Asp^{473}$ mutant of Akt/PKB is a better substrate than the wild-type protein for T308 phosphorylation by PDK1 (*27*). Knockdown of raptor expression increased the phosphorylation of both S473 and T308 despite reducing Akt/PKB expression. Knockdown of rictor or mTOR expression also decreased S473 phosphorylation in HeLa and HEK-293T cells, two human cell lines that, like A549 and HT-29 cells, contain wild-type PTEN (Fig. 2B). In addition, the knockdowns also decreased S473 phosphorylation in the PTEN-null PC-3 prostate cancer cell line (Fig. 2B), a result reminiscent of that in *Drosophila* cells with reduced dPTEN expression (Fig. 1B). Furthermore, the knockdowns decreased S473 phosphorylation in M059J glioblastoma cells that are null for DNA-PK_{cs}, a proposed S473K

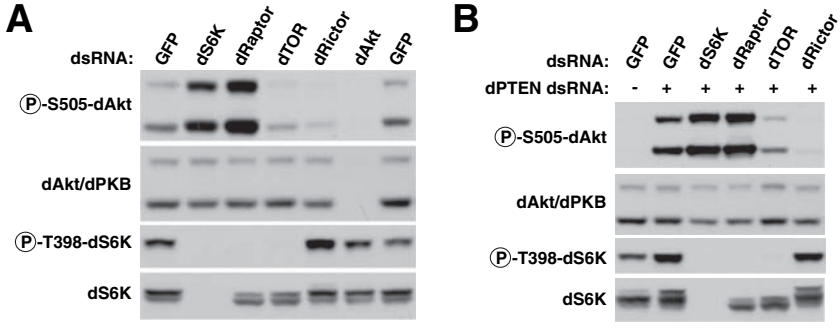

Fig. 1. *Drosophila* rictor and TOR positively regulate the phosphorylation of the hydrophobic motif site of dAkt/dPKB. (**A**) dsRNAs corresponding to the genes for the indicated proteins were transfected into Kc_{167} *Drosophila* cells (*30*). A dsRNA corresponding to green fluorescent protein (GFP) served as a negative control. After 4 days, lysates were prepared and analyzed by immunoblotting (*30*) for amounts of phospho- (P) and total dAkt/dPKB and dS6K. (**B**) dsRNAs corresponding to the genes for the indicated proteins were transfected into Kc_{167} *Drosophila* cells with (+) or without (–) a dsRNA for dPTEN, and samples were analyzed as in (A).

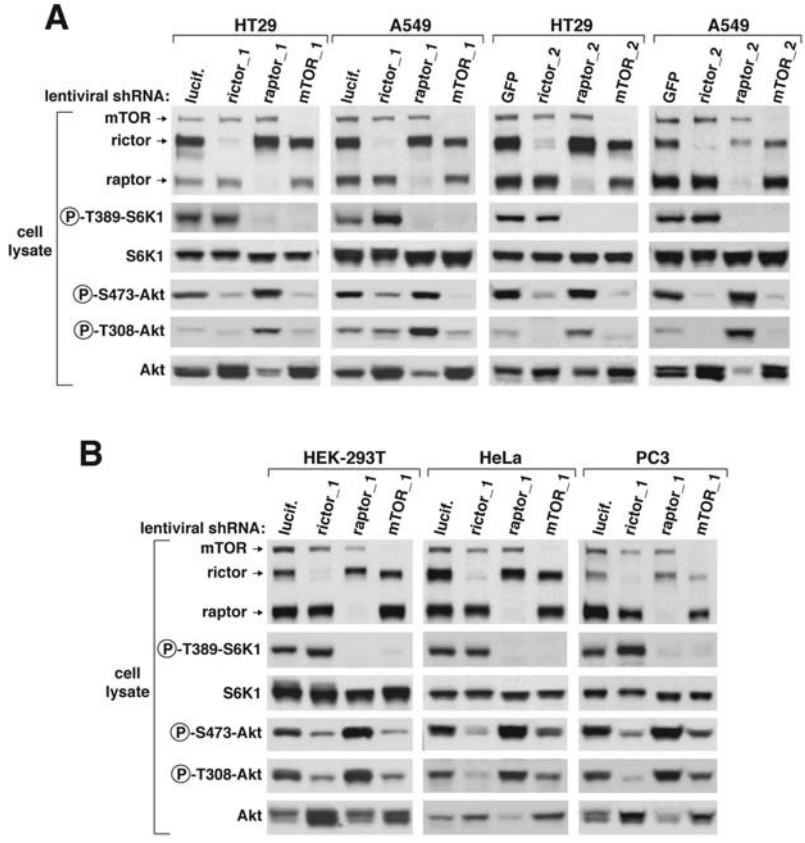

Fig. 2. Rictor and mTOR but not raptor positively regulate the phosphorylation of S473 and T308 of Akt/PKB in a variety of human cancer cell lines. (**A**) Immunoblotting was used to analyze the total amounts and phosphorylation states of the indicated proteins in two different sets of HT29 and A549 cell lines with stable decreases in rictor, raptor, or mTOR expression (*30*). Lentiviruses were used to express control shRNAs targeting luciferase or GFP or shRNAs targeting rictor, raptor, or mTOR (two distinct shRNAs per gene). (**B**) HEK-293T, HeLa, and PC3 cell lines with stable decreases in rictor, raptor, or mTOR expression were analyzed as in (A).

candidate (*9*) (fig. S2). Thus, in six distinct human cell lines, rictor and mTOR but not raptor are necessary for the phosphorylation of the hydrophobic motif of Akt/PKB.

Because the rictor and mTOR knockdowns inhibit phosphorylation events critical for Akt/PKB activity, they should affect Akt/PKB-regulated effectors. In HeLa cells, a reduction in the expression of rictor or mTOR but not raptor decreased phosphorylation of AFX (Foxo4a) (fig. S3), a forkhead family transcription factor that is a direct substrate of Akt/PKB (*28*). Because the raptor-mTOR complex directly phosphorylates the hydrophobic motif site of S6K1 (*23*), we determined whether rictor-mTOR has an analogous function for Akt/PKB. Rictor-mTOR complexes isolated from HEK-293T or HeLa phosphorylated S473 but not T308 of full-length, wild-type Akt/PKB in vitro (Fig. 3A). Immunoprecipitates of raptor, the ataxia telagiectasia mutated (ATM) protein, or protein kinase C α (PKCα) did not phosphorylate either site, and Akt/PKB did not autophosphorylate S473 (Fig. 3A). Importantly, the raptor immunoprecipitates also contain mTOR but did not phosphorylate Akt/PKB (Fig. 3A), suggesting that for mTOR to phosphorylate Akt/PKB, it must be bound to rictor and that raptor cannot substitute. This lack of phosphorylation holds even in the raptor immunoprecipitates isolated from HEK-293T cells that contain as much mTOR as the rictor immunoprecipitates (Fig. 3A). Consistent with a key role for rictor, mTOR immunoprecipitates prepared from the rictor knockdown cells did not phosphorylate Akt/PKB despite containing a similar amount of mTOR as the controls (Fig. 3B). To verify that mTOR is the S473K in the rictor immunoprecipitates, we prepared immunoprecipitates from control cells and from two different lines of mTOR knockdown cells. Although rictor levels were equivalent in all the immunoprecipitates, only those prepared from cells expressing mTOR phosphorylated Akt/PKB in vitro (Fig. 3B). Both the LY294002 and wortmannin mTOR kinase inhibitors blocked the in vitro phosphorylation of Akt/PKB by rictor-mTOR (Fig. 3C), and LY294002 acted at concentrations that inhibit S473 phosphorylation in cells (*3*). Staurosporine, an inhibitor of Akt/PKB kinase activity (*12*), did not decrease the phosphorylation of Akt/PKB by rictor-mTOR. Thus, in vitro the rictor-mTOR complex phosphorylates S473 of Akt/PKB in a rictor- and mTOR-dependent fashion and

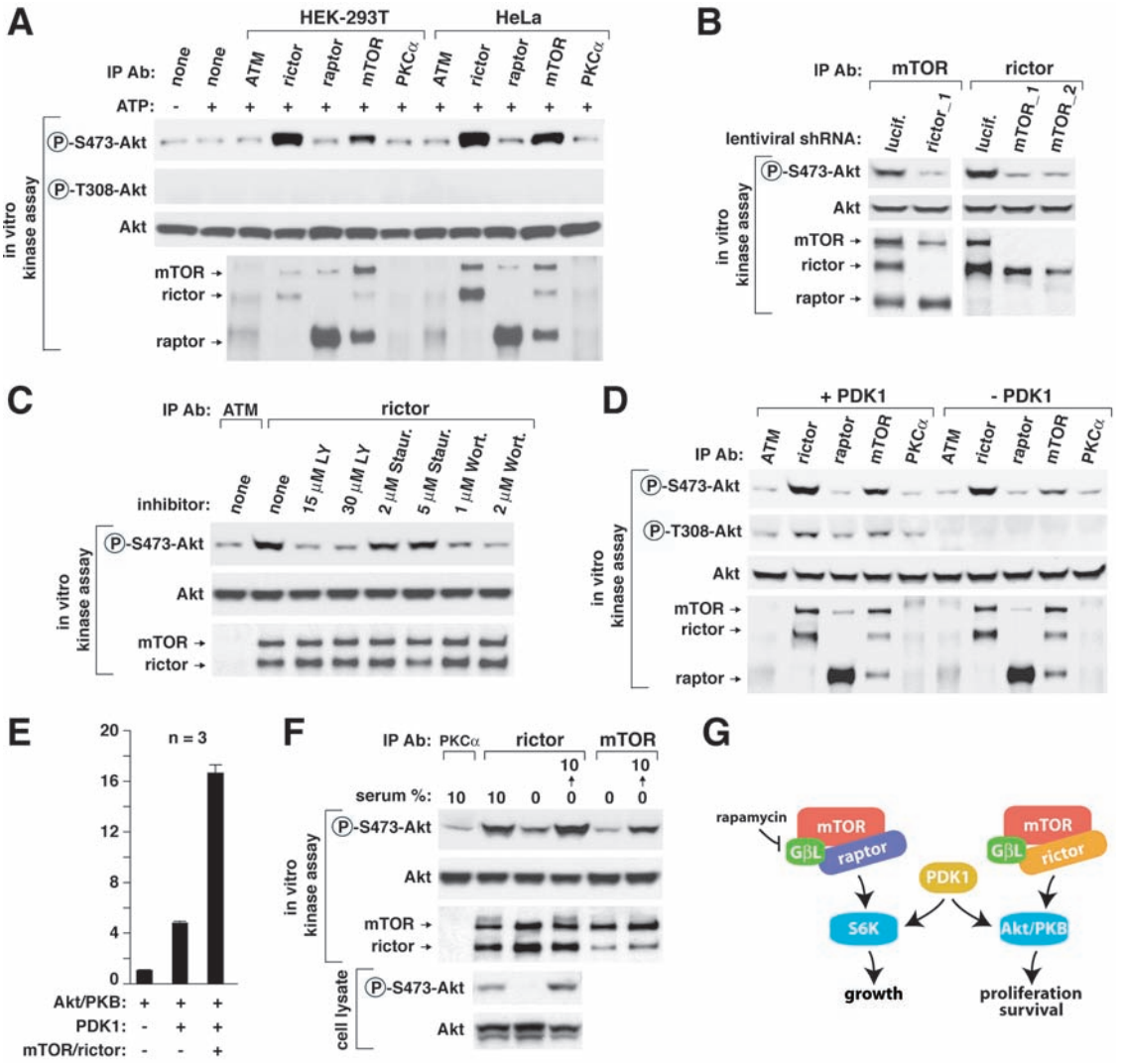

Fig. 3. The rictor-mTOR complex phosphorylates Akt/PKB on S473 in a rictor- and mTOR-dependent fashion and facilitates phosphorylation of T308 by PDK1. (**A**) Immunoprecipitates prepared from lysates of HEK-293T or HeLa cells with the indicated antibodies were used in kinase assays with full-length, wild-type Akt1/PKB1 as the substrate (*30*). Immunoblotting was used to detect the phosphorylation of Akt/PKB at S473 or T308 and the amounts of Akt/PKB, rictor, mTOR, or raptor in the kinase assays (*30*). Adenosine triphosphate was omitted from one sample to determine whether Akt/PKB authophosphorylation contributes to S473 phosphorylation. (**B**) Kinase assays were performed as in (A) with the use of cell lines with stable reductions in the expression of rictor (right) or mTOR (left), respectively. (**C**) Kinase assays containing the indicated concentrations of LY294002 (LY), staurosporine (Staur.), or wortmannin (Wort.) were performed as in (A). (**D**) The prior phosphorylation of S473 of Akt/PKB by rictor-mTOR increases the subsequent phosphorylation of T308 by PDK1. Assays were performed as in (A) with the use of immunoprecipitates from HeLa cells except that after incubation with the indicated immunoprecipitates, 100 ng of PDK1 (+PDK1) was added to half the samples for an additional 20-min incubation (*30*). Samples were analyzed with immunoblotting for the indicated phosphorylation states and protein levels. (**E**) The kinase activity of Akt/PKB after its phosphorylation with PDK1 or with rictor-mTOR followed by PDK1. (**F**) Kinase assays were performed as in (A) with the use of immunoprecipitates isolated from HeLa cells cultured for 24 hours in media containing 10% or 0% serum or from serum-deprived cells stimulated with 10% serum for 30 min. (**G**) Schematic diagram of the role of rictor-mTOR in Akt/PKB activation.

with a drug sensitivity profile consistent with mTOR being the phosphorylating kinase.

To determine whether the phosphorylation of Akt/PKB on S473 by rictor-mTOR activates Akt/PKB activity, we first used rictor-mTOR to phosphorylate Akt/PKB on S473 and then added PDK1 to the assay to phosphorylate T308. Prior phosphorylation of Akt/PKB on S473 boosted subsequent phosphorylation by PDK1 of T308 (Fig. 3D), consistent with the importance of S473 phosphorylation for T308 phosphorylation (*3*, *4*) and with the inhibitory effects of the rictor and mTOR knockdowns on T308 phosphorylation (Fig. 2AB). After phosphorylation with rictor-mTOR and PDK1, Akt1/PKB1 had about four- to fivefold more activity than after phosphorylation with PDK1 alone (Fig. 3E), confirming the important role of S473 in fully activating Akt/PKB. Because growth factors control the phosphorylation of Akt/PKB on S473, we determined whether the concentration of serum in the cell media regulated the in vitro kinase activity of rictor-mTOR toward Akt/PKB. Rictor-mTOR had decreased activity in HeLa cells deprived of serum and was reactivated by serum stimulation for 30 min (Fig. 3F), indicating that modulation of the intrinsic kinase activity of rictor-mTOR may be a mechanism for regulating S473 phosphorylation.

Our results indicate that the rictor-mTOR complex is a hydrophobic motif kinase for Akt/PKB (Fig. 3G). Rictor-TOR has essential roles in Akt/PKB hydrophobic motif site phosphorylation in *Drosophila* and human cells and in vitro phosphorylates full-length, wild-type Akt/PKB in a serum-sensitive fashion. No other proposed hydrophobic motif kinase has been shown to fulfill all these criteria. With hindsight, we do see clues in the literature to the important role of mTOR in Akt/PKB activation. Prolonged but not acute treatment of certain human cells with rapamycin partially inhibits Akt/PKB phosphorylation (*29*), and our findings provide a possible rationale to explain these results. Although rapamycin does not bind to a preformed rictor-mTOR complex (*22*), during long-term rapamycin treatment the drug should eventually sequester many of the newly synthesized mTOR molecules within cells. Thus, as the rictor-mTOR complex turns over, rapamycin may interfere with its reassembly or over time become part of the new complexes. It is reasonable to expect then that prolonged rapamycin treatment may partially inhibit rictor-mTOR activity, which would explain why rapamycin is particularly effective at suppressing the proliferation of tumor cells with hyperactive Akt/PKB. The PI3K/Akt pathway is frequently deregulated in human cancers that have lost the expression of the PTEN tumor suppressor gene, and our findings suggest that direct inhibitors of mTOR-rictor should strongly suppress Akt/PKB activity. Thus, the rictor-mTOR complex, like its raptor-mTOR sibling, may be a valuable drug target.

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30. Materials and methods are presented as supporting material on *Science* Online.

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Obligate Role of Anti-Apoptotic MCL-1 in the Survival of Hematopoietic Stem Cells

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Apoptosis is important in controlling hematopoietic stem cell (HSC) numbers. However, the specific BCL-2 family member(s) that regulate HSC homeostasis are not precisely defined. We tested myeloid leukemia–1 (MCL-1) as an attractive candidate that is highly expressed in HSCs and regulated by growth factor signals. Inducible deletion of *Mcl-1* in mice resulted in ablation of bone marrow. This resulted in the loss of early bone marrow progenitor populations, including HSCs. Moreover, growth factors including stem cell factor increased transcription of the *Mcl-1* gene and required MCL-1 to augment survival of purified bone marrow progenitors. Deletion of *Mcl-1* in other tissues, including liver, did not impair survival. Thus, MCL-1 is a critical and specific regulator essential for ensuring the homeostasis of early hematopoietic progenitors.

Hematopoietic stem cells (HSCs), which give rise to all cells of hematopoietic lineages, can undergo several developmental fates. These include self-renewal or differentiation into multipotent progenitors that give rise to mature hematopoietic cells (*1*). This process is tightly controlled and regulated by apoptosis (*2*). Constitutive expression of human *BCL-2* in vivo under the control of the mouse major histocompatibility class I promoter causes accumulation of HSCs and enhances their ability to form colonies in vitro and to reconstitute the hematopoietic system of lethally irradiated recipients (*3*). Thus, apoptosis appears to be an important mechanism for regulating HSC numbers, but whether one or

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