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**CDKN1B/p27 regulates autophagy via the control of Ragulator and MTOR activity
in amino acid-deprived cells**

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Abstract

The tumor suppressor CDKN1B/p27^{Kip1} binds to and inhibits cyclin-CDK complexes in the nucleus, inducing cell cycle arrest. However, when in the cytoplasm, CDKN1B may promote tumorigenesis. Notably, cytoplasmic CDKN1B was reported to promote macroautophagy/autophagy in response to nutrient shortage by a previously unknown mechanism. In our recent work, we found that during prolonged amino acid starvation, CDKN1B promotes autophagy via an MTORC1-dependent pathway. A fraction of CDKN1B translocates to lysosomes, where it interacts with the Ragulator subunit LAMTOR1, preventing Ragulator assembly, which is required for MTORC1 activation in response to amino acids. Therefore, CDKN1B represses MTORC1 activity, leading to nuclear translocation of the transcription factor TFEB and activation of lysosomal function, enhancing starvation-induced autophagy flux and apoptosis. In contrast, cells lacking CDKN1B survive starvation despite reduced autophagy, due to elevated MTORC1 activation. These findings reveal that, by directly repressing MTORC1 activity, CDKN1B couples the cell cycle and cell growth machineries during metabolic stress.

Main text

Every proliferating cell must ensure that it has enough mass and energy prior to division. Therefore, cells must rapidly respond to continuously changing nutrient levels, activating kinase cascades that promote either anabolic reactions, leading to cell growth, or catabolic reactions, such as autophagy, which are associated with energy production. The MTORC1 pathway adapts cell metabolism to environmental conditions by promoting cell growth and proliferation and inhibiting autophagy when nutrients are plentiful. Whereas MTORC1 regulation has been extensively studied in the context of acute increase of nutrient levels, little is known about how cells coordinate their metabolism in conditions of prolonged starvation, to which cancer cells are often exposed because of poor tumor vascularization. Previous studies showed that the cell cycle inhibitor CDKN1B/p27^{Kip1} contributes to the response of cancer cell to metabolic stress and that CDKN1B status determines whether cells survive prolonged serum and/or glucose starvation or undergo apoptosis.

CDKN1B is considered as a tumor suppressor due to its anti-proliferative activity via inhibition of cyclin-CDK complexes. However, in some cancers, CDKN1B may promote tumorigenesis via CDK-independent mechanisms, as shown in CDKN1B knockin mice in which CDKN1B cannot bind to and inhibit cyclin-CDKs (CDKN1B/p27^{CK-}). CDKN1B shuttles between the nucleus and cytoplasm, and cytoplasmic localization of CDKN1B is

associated with poor prognosis in many types of cancers, suggesting that CDKN1B exhibits its pro-oncogenic functions outside the nucleus. Importantly, previous studies showed that in response to starvation, CDKN1B relocates to the cytoplasm and promotes autophagy, but neither the subcellular compartment in which CDKN1B localizes in starved cells, nor the molecular mechanism underlying the pro-autophagic role of CDKN1B was investigated in detail.

Using mouse embryonic fibroblasts (MEFs) isolated from *Cdkn1b*^{+/+} and *cdkn1b*^{-/-} mice, we confirmed the pro-autophagic role of CDKN1B in cells deprived of amino acids for prolonged periods of time (>24 h) [1]. Surprisingly, *Cdkn1b*^{+/+} cells are more susceptible to apoptosis induced by amino acid deprivation than *cdkn1b*^{-/-} cells, despite reduced autophagy in the latter. *cdkn1b*^{-/-} cells also display increased MTORC1 signaling, consistent with the role of MTORC1 in inhibiting autophagy. Further experiments showed that impaired autophagy in *cdkn1b*^{-/-} cells is rescued by MTOR inhibition, suggesting that CDKN1B acts upstream of the MTOR pathway. To determine whether promotion of autophagy by CDKN1B requires binding to CDKs or its cytoplasmic relocation, we used knockin MEFs expressing mutant forms of CDKN1B. Whereas CDKN1B/p27^{CK-} cells, in which CDKN1B cannot bind cyclin-CDKs, display the same phenotype as *Cdkn1b*^{+/+} cells, CDKN1B^{S10A} cells, in which CDKN1B is sequestered in the nucleus, behave like *cdkn1b*^{-/-} cells, indicating that CDKN1B-mediated autophagy does not require CDK inhibition, but that cytoplasmic localization of CDKN1B is crucial. Furthermore, microscopy studies revealed that a small fraction of CDKN1B colocalizes with lysosomal proteins in fed cells, which significantly increases during prolonged amino acid starvation. Previous studies suggested the presence of CDKN1B on lysosomal and autophagy compartments via interactions with either SNX6 (sorting nexin 6) or SQSTM1/p62, but not in the context of metabolic stress. Importantly, CDKN1B was shown previously to bind to p18/p27RF-Rho, which was later renamed LAMTOR1, a lysosomal scaffold part of the Ragulator complex acting as an anchor and a GAP for RAG GTPases that recruit MTORC1 to lysosomal membrane prior to its activation in response to amino acids. Our results indicate that amino acid starvation promotes binding of CDKN1B to LAMTOR1 on lysosomes, thereby preventing the assembly of Ragulator and the translocation of RAGs and MTORC1 to lysosomes, therefore maintaining MTORC1 in an inactive state. Consequently, TFEB, a master regulator of lysosome and autophagy gene expression that is kept inactive by MTORC1, enters the nucleus to drive expression of genes required for activation of lysosomal functions and degradation of autophagic cargo. In contrast, increased MTORC1 activity in *cdkn1b*^{-/-} cells sequesters TFEB in the cytoplasm,

repressing its transcriptional activity, leading to lysosome dysfunction and accumulation of autophagy cargo.

Interestingly, lysosome number is not affected by reduced TFEB activity in *cdkn1b*^{-/-} cells. A likely explanation is that lysosomes are generated via autophagic lysosome reformation (ALR), in which lysosomes are recycled from autolysosomes during autophagy. This hypothesis is supported by the fact that i) MTORC1 signaling, which promotes ALR, is upregulated in *cdkn1b*^{-/-} cells and ii) lysosome-autophagosome fusion is not affected in the absence of CDKN1B, allowing lysosome production from autolysosomes. Recent studies highlighted the role of TFEB-driven *de novo* synthesis of lysosomes during cell cycle progression. In actively proliferating cells in the presence of nutrients, TFEB is negatively regulated by CDK4 and CDK6 in the nucleus, inducing its export to the cytoplasm. Our findings suggest that the CDK4-CDK6 inhibitor CDKN1B plays an opposite role during prolonged starvation by promoting the nuclear translocation of TFEB in a CDK-independent manner, via the regulation of MTORC1.

Importantly, the duration of starvation appears crucial for CDKN1B-mediated regulation of the autophagy-lysosomal pathway. Indeed, both MTORC1 signaling and autophagy are normal in *cdkn1b*^{-/-} cells in the first hours of starvation, and the accumulation of autophagic vesicles and elevated MTORC1 signaling become evident after 24 h of starvation. This correlates with the timing of CDKN1B recruitment to lysosomes and the induction of apoptosis in *Cdkn1b*^{+/+} cells, suggesting that the presence of CDKN1B on lysosomes switches on the apoptotic program, possibly via activation of the TFEB-BBC3/PUMA axis. Surprisingly, during prolonged amino acid starvation, increased autophagy flux in *Cdkn1b*^{+/+} cells does not protect against apoptosis and, in fact, we found that general inhibition of ATG5-dependent autophagy does not affect apoptosis rate in these conditions.

While the identification of a CDKN1B-MTORC1-TFEB axis provides an explanation for the autophagy defect in cells lacking CDKN1B, it raises questions about the ability of *cdkn1b*^{-/-} to survive long-term starvation. As lysosomal function is significantly reduced, it is unlikely that autophagy or macropinocytosis, which both require an intact lysosomal machinery, support their metabolism. Therefore, an attractive hypothesis under investigation is that the absence of CDKN1B triggers a metabolic reprogramming, similar to that described in deep quiescent cells, which are also characterized by enhanced MTORC1 signaling, impaired autophagy and accumulation of non-functional lysosomes. This metabolic reprogramming consists of decoupling metabolic pathways from nutrient availability, enabling cells to carry out anabolic reactions even when nutrients are limited. Therefore, the

recruitment of CDKN1B to lysosomal membranes may be seen as an adaptive mechanism protecting cells from uncontrolled growth and proliferation under adverse conditions. Thus, in contrast to previously described oncogenic roles of cytoplasmic CDKN1B, lysosomal CDKN1B may act as a tumor suppressor, and support the idea that the role of CDKN1B is determined by its binding partners and subcellular localization, themselves dictated by environmental conditions.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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Reference

1. Nowosad A, Jeannot P, Callot C, Creff J, Perchey RT, Joffre C, et al. p27 controls Ragulator and mTOR activity in amino acid-deprived cells to regulate the autophagy-lysosomal pathway and coordinate cell cycle and cell growth. *Nat Cell Biol* 2020; 22:1076-90.

Figure 1. CDKN1B couples the cell growth and cell cycle machineries in amino acid-deprived cells. Under full-nutrient conditions (left panel), CDKN1B shuttles between the nucleus and cytoplasm, controlling cell cycle progression. Amino acids promote Ragulator-dependent RAG activation and subsequent recruitment of MTORC1 to lysosomes, where MTORC1 is activated, leading to induction of cell growth. Activated RRAGs also recruit TFEB to lysosomes, preventing its translocation to the nucleus and induction of pro-autophagic and pro-apoptotic genes. During prolonged amino acid starvation (right panel), a fraction of CDKN1B translocates to the lysosomal surface, where it binds to the Ragulator subunit LAMTOR1, which prevents the assembly of the Ragulator-RRAG complex, precluding the recruitment of MTORC1 to lysosomes and its activation. TFEB accumulates in the nucleus and drives the expression of genes promoting autophagy and apoptosis. The figure was created using the vector image bank from Servier Medical Art by Servier, licensed under a Creative Commons Attribution 3.0 Unported License.

