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CDK4 Inhibitors Thwart Immunity by Inhibiting Phospho-RB-NF-κB Complexes

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PD-L1 plays a central role in immune recognition of cancer cells. In this issue of *Molecular Cell*, Jin et al. (2019) report that a phosphorylated retinoblastoma protein contacts the DNA-binding domain of p65 NF-κB, thereby blocking transcription of *PD-L1*.

In this issue, Jin et al. (Jin et al., 2019) unveil a molecular mechanism in which the retinoblastoma tumor suppressor protein (RB) negatively regulates the NF-κB protein p65 to block expression of *PD-L1* (Figure 1). The authors demonstrate that cyclin-dependent kinase (CDK) 4 or 6 inhibitors can suppress *PD-L1* expression through this mechanism and offer an experimental therapeutic approach to counter radiation-induced tumor immune evasion.

The RB tumor suppressor has long been recognized to be negatively regulated by CDK phosphorylation. Growth stimuli signal assembly of cyclin D and CDK4/6, creating partially phosphorylated RB that releases E2Fs, which in turn induce cyclin E expression. Cyclin E-CDK2 complexes then hyperphosphorylate RB, fully relieving repression of E2Fs and subsequent transcription of their cell cycle target genes. Not surprisingly, the CDK-RB-E2F regulatory pathway is frequently inactivated in human cancers by elevated CDK activity, and this deregulates cell proliferation (Rubin, 2013). Based on these observations, phosphorylated RB has long been considered to be inactive once a cell enters S phase, as these modifications have been shown to drastically alter its structure (Dick et al., 2018). The present publication reveals an intriguing mechanism in which CDK4 phosphorylation of RB enables an RB-NF-κB interaction to inhibit transcription, indicating that CDK phosphorylation of RB can direct protein interactions and not just disrupt them.

NF-κB p65 is one of five members of this transcription factor family, and they form homo- or heterodimers with each other to bind DNA (Perkins, 2012). Dimers are bound and negatively regulated by members of the inhibitor of κB (IκB) protein family. Upon phosphorylation of IκB, the inhibitor undergoes proteasome-dependent degradation to allow the remaining NF-κB complex to enter the nucleus and induce transcriptional expression of target genes that lead to survival, proliferation, and other cellular effects. To search for new cancer therapeutic strategies that exploit NF-κB, Jin et al. (2019) used PC-3 prostate cancer cells with shRNA gene-knockdown combinations designed to inactivate NF-κB and create synthetic lethality. The authors then searched for chemical agents that could rescue lethality. In two shRNA combinations, treatment with a CDK4/6 inhibitor, palbociclib, rescued lethality, and this was demonstrated both in cell culture and in tumor xenografts. Prompted by this drug screen, the authors then tested the effect of RB loss on synthetic lethality and found that RB knockdown similarly improved cell viability and restored expression of select NF-κB target genes, implicating RB as a negative regulator of NF-κB.

The authors elucidate a very detailed and direct molecular explanation of RB-mediated control of NF-κB transcription. They demonstrated that RB specifically binds and regulates the p65 subunit of the NF-κB family. They show that the N-terminal R-linker domain of RB interacts with the N-terminal DNA-binding domain of p65. The authors used unmodifiable mutant forms of RB to show that phosphorylation at S249 and T252 mediate its interaction with p65 and that maximal binding is dependent on cyclin D-CDK4 phosphorylation. Furthermore, Jin et al. (2019) present evidence that phosphorylation of S249 and T252 reverses electrostatic repulsion between positively charged residues in RB and p65. Replacing positively charged arginine on p65 with aspartate bypasses the need for phosphorylation of RB to induce assembly. Endogenously, RB binding to p65 inhibits DNA binding and prevents NF-κB promoter occupancy and transcriptional activation. These experiments establish the molecular determinants of RB-p65 interactions and suggest that other RB-binding proteins may be regulated by a similar mechanism in a phosphorylation-dependent manner (Gubern et al., 2016).

The discovery of this molecular interaction between RB and p65 NF-κB provides opportunities for future studies examining the relationship between these two pathways. In this report, Jin et al. (2019) focus on the implications of RB-NF-κB regulation of *PD-L1* expression because of the therapeutic utility of PD-L1/PD-1 blockade in activating anti-tumor immunity (Sun et al., 2018). Ectopic expression of an RB N-terminal fragment in PC-3 cells inhibits *PD-L1* expression, whereas a non-phosphorylatable mutant does not inhibit *PD-L1*, further providing evidence for the role of the upstream CDKs

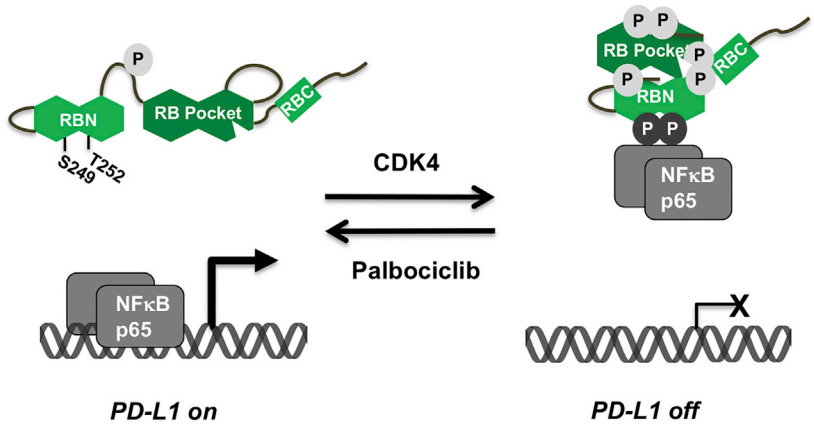

Figure 1. Phosphorylation of RB Stimulates Interaction with p65 NF-κB and Blocks Transcription

In circumstances of p65 NF-κB activation and RB underphosphorylation (pictured at left), p65 activates the *PD-L1* promoter. RB is phosphorylated on S249 and T252 by CDK4, and this induces assembly of a complex mediated by the RB N-linker and DNA-binding domain of p65. This inhibits DNA binding and blocks *PD-L1* expression (pictured at right). Treatment with CDK4/6 inhibitors such as palbociclib prevents RB phosphorylation and preserves active p65 NF-κB transcription of *PD-L1*.

mediating this effect. In addition, a short S249D/T252D phospho-mimetic peptide, but not its S249A/T252A version, inhibits *PD-L1* expression. This experiment was reproducible not only in prostate cancer cells, but also in pancreatic, lung, and liver-derived cancer cell lines, suggesting the RB-NF-κB regulatory interaction may be applicable to various cancer types. Since radiation can suppress immunity in specific circumstances, and DNA damage signaling can lead to dephosphorylation of RB, radiation may stimulate PD-L1 expression. To investigate this, syngeneic xenograft experiments were performed in which tumor cells expressing the phospho-mimetic peptide or an empty vector were irradiated and/or treated with anti-PD-L1 to stimulate immune recognition. The phospho-mimetic peptide synergized with radiation treatment to inhibit tumor growth compared to radiation or peptide alone. Moreover, it was apparent that the peptide has PD-L1-independent anti-tumor properties, as the radiation-peptide combination offered increased protection against tumor growth versus radiation and anti-PD-L1 combination treatment. As proposed by the authors, there is an intriguing yet largely unexplored role for repressed p65-regulated pro-survival genes such as *Birc2* in mediating anti-tumor effects (Yang et al., 2016). Furthermore, the RB phospho-mimetic peptide increased tumor T cell infiltration independent of PD-L1 blockade. These findings strongly suggest that an in-depth interrogation of the CDK-RB-NF-κB regulated transcriptome will reveal additional targets that mediate the anti-tumor response *in vivo*.

Interestingly, a number of recent studies using CDK4/6 inhibitors have reported upregulation of anti-tumor immunity instead of suppression (Goel et al., 2017; Zhang et al., 2018). These studies use different model systems to investigate immune recognition of tumors, and CDK4/6 inhibitors are not exclusively dependent on RB for effects on PD-L1 expression. Collectively, these studies indicate that the effect of these drugs on tumor growth and tumor immunity is complex. This highlights the challenges of predicting the outcome of various therapeutic tumor-immune system interactions that will need to be overcome in order to use these agents to improve anti-cancer immunity in the clinic.

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