MOLECULAR AND CELLULAR BIOLOGY, Feb. 2006, p. 1165–1169 Vol. 26, No. 4
0270-7306/06/$08.00+0 doi:10.1128/MCB.26.4.1165–1169.2006

MINIREVIEW

Surprising Dependency for Retinoblastoma Protein in Ras-Mediated Tumorigenesis

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Deregulation of the G_1 cyclin-dependent kinase (CDK)–retinoblastoma protein (Rb) pathway is well established in virtually all human cancers. Different cancers exhibit selective alterations of the pathway, whether by inactivation of the CDK inhibitor $p16^{Ink4a}$, by overexpression of the cyclin D1 gene, or by direct mutational inactivation of the Rb gene. The $p16^{Ink-4a}$-CDK-Rb pathway is often considered to be a linear pathway, with mutation of one component abrogating the need to mutate another. Still, it is unclear why different paths to cancer preferentially select for mutations in one component over another. A paper in this issue of *Molecular Cellular Biology* by Williams, Classon, and colleagues reveals a surprising requirement for Rb in proliferation and transformation mediated by the Ras oncogene (50). This study provides a rationale for why mutational activation of Ras and genetic disruption of Rb are rarely found together in human cancers. Thus, different cancers appear to select for different disruptions in the CDK-Rb pathway in part based on the spectrum of other mutations, such as in Ras, which also occur during tumorigenesis.

Rb was the first cloned tumor suppressor gene, isolated by virtue of its association with hereditary retinoblastoma (11). The Rb protein is a key component that regulates cell cycle entry and progression in mammalian cells, and Rb is a member of a gene family encoding structurally and functionally similar proteins, which also includes the p107 and p130 proteins (42). Like Rb, p107 and p130 are regulated during the cell cycle by CDK phosphorylation. Rb family members associate with the transcription factor E2F, negatively regulating E2F dependent transcription. E2F activity plays critical roles in cell cycle progression by regulating the transcription of genes with critical roles in cell cycle progression, including genes involved in cell cycle regulation, DNA replication, and mitosis (8, 45).

Rb plays distinct roles in transcriptional regulation relative to p107 and p130, in terms of both controlling E2F-dependent and differentiation-specific transcription (5). While Rb loss in mouse embryo fibroblasts (MEFs) is associated with the increased expression of E2F targets such as cyclin E1 and p107, the combined inactivation of p107 and p130 results in the upregulation of different E2F targets, including B-Myb and Cdc2 (13). Rb in particular has also been shown to promote the transcription of differentiation mediators (44), such as following recruitment by the CBFA1 transcription factor to osteogenic gene promoters (43).

Importantly, Rb mutation uniquely contributes to tumor suppression in mice and humans, despite the facts that tumor suppressor activity for p107 is evident in the context of Rb mutation and that the three Rb family members display functional redundancy in controlling G_1- to S-phase progression (5). On the other hand, Rb and p107/p130 have been shown to exert opposing influences on differentiation in cell cultures (6). Finally, while somatic mutations in the *RB* gene are associated with almost all sporadic retinoblastomas and small cell lung cancers (SCLCs), mutation of *RB* is much less common in other human cancers.

Ras oncoproteins, encoded by three genes (N-*ras*, K-*ras*, and H-*Ras*), are integral components of signal transduction pathways, linking extracellular signals directed by tyrosine kinase receptors to intracellular signaling cascades, and ultimately to changes in transcription (20). Signal-dependent activation of Ras results in exchange of bound GDP for GTP, and GTP-bound Ras then interacts with effectors such as the Raf serine/threonine kinase, which activates a mitogen-activated protein kinase (MAPK) signaling cascade. Once activated, Ras is quickly down-regulated by the action of a GTPase-activating protein, which promotes GTP hydrolysis. Oncogenic mutations of Ras prevent interactions with GTPase-activating proteins and thus greatly decrease GTP hydrolysis, resulting in constitutively active Ras. Oncogenic Ras mutations (henceforth denoted Ras*) are associated with almost a third of human cancers (20).

The importance of the Ras-MAPK pathway in the growth factor-dependent upregulation of cyclin D-dependent kinase activity is well established (40). In fact, growth factor-dependent Ras activation is necessary for G_1 CDK activation, Rb phosphorylation, E2F activation, and S-phase entry. Accordingly, inhibition of Ras prevents entry into S phase in wild-type but not $Rb^{-/-}$ MEFs (18, 22, 25), which seems to place Ras squarely upstream of Rb. The decreased sensitivity of Rb mutant cells to Ras inhibition could result at least in part from the substantially increased Ras activation, at the level of GTP binding, observed in $Rb^{-/-}$ cells (17). Thus, a mutual antagonism between Rb and Ras appears well established. However, as discussed below, Williams et al. now contribute another twist, a functional dependency of Ras* for Rb (50).

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In the first demonstrations of oncogene collaboration over two decades ago, Ras* and E1A or Myc were shown to synergize in transforming primary rodent cells (16, 32). The ability of E1A to cooperate with Ras* depends at least in part on its ability to disrupt complexes containing Rb family members, relieving Rb-dependent repression of transcription (23, 27, 49). Similarly, the ability of Myc and Ras* to collaborate in S-phase entry coincides with cyclin E/Cdk2 activation, Rb phosphorylation, and E2F activation (18). Ras* also cooperates with $p16^{Ink4a}$ loss to confer a transformed phenotype (21, 37, 38). In fact, Ras* overexpression results in upregulation of $p16^{Ink4a}$, which contributes to premature senescence (39). Thus, the inactivation of the CDK-Rb pathway would appear, at face value, to be important for transformation by Ras. However, in these scenarios, genetic deregulation occurs either upstream of G_1 CDKs ($p16^{Ink4a}$ loss or Myc overexpression) or via viral oncoproteins, such that inactivation of all three Rb family members occurs. Importantly, while mutations that disrupt the Rb pathway, such as in $p16^{Ink4a}$, are commonly associated with activated Ras or B-Raf in human tumors (14, 47), direct mutations of the *Rb* gene and of Ras* are rarely found together in human tumors. Why is this?

Williams and colleagues now shed light on this issue (50). They demonstrate that, quite in contrast to Rb's usual tumor suppressor role and in marked contrast to p107/p130 loss, Rb loss actually impedes the transformation of mouse NIH 3T3 fibroblasts by Ras*. In fact, cotransfection of Rb with Ras* actually increases the number and size of transformed foci. Furthermore, RNA interference-mediated knockdown of Rb inhibits the proliferation of Ras*-transformed NIH 3T3 cells, eliminating concerns that the Rb dependence of Ras* transformation is due to developmental compensation effects in $Rb^{-/-}$ embryos. The authors go on to show that the requirement for Rb in Ras*-mediated transformation extends to human cancer cells, as transfection of Ras* into Rb-deficient Saos-2 osteosarcoma cells inhibits proliferation and soft agar colony formation. In fact, while Rb-deficient cells are resistant to pharmacological inhibition of the Ras-Erk pathway (7), Williams et al. show that pharmacological inhibition of the MAPK pathway restores proliferation in Ras*-transfected Saos-2 cells. Amazingly, reexpression of Rb in these cells partially rescues Saos-2 cells from Ras*-mediated growth inhibition.

Since epithelial lineage cells are the targets for the majority of human cancers, it was important to extend the positive role for Rb in Ras*-dependent transformation to human carcinomas. Human carcinomas such as colorectal cancers with activated Ras or Raf mutations frequently exhibit high-level expression of Rb (10, 12, 51), at least in part due to a deregulated Ras-MAPK pathway. While high Rb levels could simply reflect a futile negative feedback loop resulting from deregulated E2F activation, Williams et al. rather demonstrate that RNA interference-mediated knockdown of Rb in several such Ras*-bearing carcinomas inhibits their proliferation and soft agar colony formation. Analogously, Yamamoto et al. have shown that increased Rb expression in colon cancer cells protects these cells from apoptosis (51). Thus, Rb actually stimulates proliferation in the presence of activated Ras*, and conversely, Ras* inhibits proliferation when Rb is absent.

The authors also provide a possible mechanistic explanation for why cells with Ras* need Rb: Rb, presumably in conjunction with E2F, directly limits the transcription of p107. In the absence of Rb, increased levels of p107 may be responsible for inhibiting Ras*-dependent proliferation and transformation (Fig. 1, top). Still, in the context of Ras activation, targeting Rb can be advantageous as long as p107 is also inhibited. In this regard, loss of both Rb and p107, but not either alone, overcomes Ras* inhibition of proliferation (24), and disruption of all three Rb family members in MEFs facilitates partial transformation by Ras (35). Thus, not all paths to Rb inactivation are equivalent, and prior Ras* mutation should select for events that disrupt all Rb family members, such as occurs following inactivation of $p16^{Ink4d}$ or overexpression of cyclin D1 (Fig. 1, bottom).

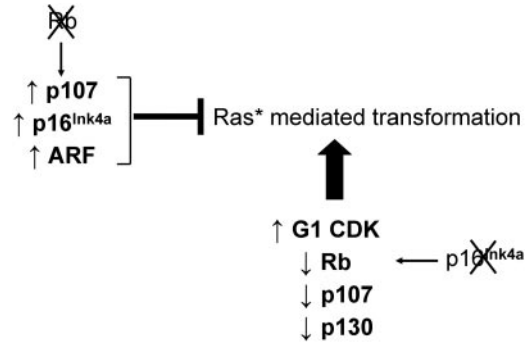

FIG. 1. Differential effects of Rb mutation versus G_1 CDK deregulation on Ras*-dependent transformation. Mutational inactivation of the *Rb* gene in a cell leads to increased expression of p107, $p16^{Ink4a}$, and ARF, which prevents Ras*-dependent transformation. In contrast, inactivation of *p16* leads to higher G_1 CDK activity, resulting in reduced function (i.e., increased phosphorylation) of all three Rb family members without ARF upregulation, which promotes Ras*-dependent transformation.

Rb loss has been shown to result in increased p107 expression in MEFs (13), and increased p107 is thought to compensate for Rb loss, restoring growth factor dependency (34). In fact, while inheritance of one mutated Rb allele results in high penetrance retinoblastoma in humans, retinoblastoma development in mice requires either mutation of both Rb and p107 or expression of viral oncoproteins that inhibit the activities of all three Rb family members (5).

Importantly, p107 probably does not act alone to inhibit Ras*-mediated transformation. Inactivation of Rb by mutation is also associated with substantial upregulation of $p16^{Ink4a}$ levels and cyclin D-dependent kinase inactivation (14), which should lead to further activation of p107 and p130. Moreover, Rb inactivation and E2F1 overexpression have been shown to promote the transcription of $p14^{ARF}$ ($p19^{ARF}$ in rodents, $p14^{ARF}$ in humans; referred to here simply as ARF), a positive regulator of p53, while normal growth stimulation or deregulated G_1 CDK activation fails to upregulate ARF (15). Finally, while perhaps not unique to Rb gene mutation, deregulated E2F activity resulting from Rb inactivation leads to increased expression of proapoptotic genes such as p73 and APAF-1 (8, 42). Therefore, a cancer with direct mutation of the *Rb* gene must be inherently resistant to or acquire the means to escape these negative feedback loops, which could in part account for cell type dependency for tumors induced by Rb loss. But in cancer cells with Ras*, the feedback loops (p107, $p16^{Ink4a}$,

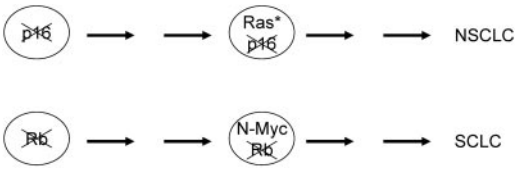

FIG. 2. Different paths to SCLC and NSCLC cancers. (Top) Should *p16* inactivation provide a competitive advantage to the appropriate progenitor cell, then subsequent Ras* mutation may provide a further selective advantage, which together with other genomic alterations leads to NSCLC. (Bottom) In contrast, according to the studies by Williams et al., early mutation of the *Rb* gene in a progenitor will disfavor subsequent Ras* mutation, but instead may be conducive to other mutations that are found in SCLC, such as those leading to increased N-*Myc* expression. Similar selections against combined mutations in Rb and Ras, and for combined mutations in Ras with those that deregulate G_1 CDK activity, probably function for other tumor types. For both NSCLC and SCLC, tumorigenesis frequently involves inactivation of the p53 pathway.

and/or ARF dependent) following Rb loss appear to more than compensate, impairing proliferation. Thus, p107, $p16^{Ink4a}$, and ARF upregulation following Rb mutation appear to represent important tumor-suppressive mechanisms that have evolved to disfavor oncogenic mutations.

It is likely that Rb dependency and these negative feedback loops are not limited to tumors with Ras*. For example, cancers with deregulated signaling pathways that lead to increased Ras activation, such as with activation of epidermal growth factor receptor (EGF-R) family members (for example, Her2/Neu in breast cancers) may also exhibit Rb dependency. In fact, *Rb* gene inactivation is rarely found to occur in breast cancers with Her2 overexpression or amplification (2, 3, 19, 46), but is instead found in breast cancers exhibiting high cyclin E and low cyclin D1 expression without Her2 overexpression. The rarity of Ras* mutations in the context of *Rb* gene loss has been proposed previously (14) to relate to low cyclin D1 expression in Rb mutant tumors, together with the demonstrated dependency of Ras*- and Neu-dependent tumorigenesis on cyclin D1 in mice (28, 52). The studies by Williams et al. now indicate that Ras*-dependent transformation actually depends on the presence of Rb itself.

Lung cancers present an interesting case of cell type dependency for specific mutations within the CDK-Rb pathway. The *Rb* gene is frequently mutated in SCLC, but *INK4A* inactivation (without *Rb* gene mutation) is implicated in the genesis of non-small cell lung cancer (NSCLC) (14, 36). Notably, Ras* mutations are not found in SCLC, but K-Ras* mutations are common in NSCLC. Furthermore, overexpression of EGF-R (which should promote Ras activation) is not detected in SCLC, but most NSCLC overexpress EGF-R (4). Inactivation of *Rb* and *INK4A* is an early event in these lung cancers, while Ras* mutations occur later (14). *Rb* inactivation may be selected for in progenitor cells for SCLC, which then alters the clonal evolutionary pathway such that Ras* mutations will not be favored (Fig. 2). In contrast, preferential inactivation of *INK4A* (frequently by gene methylation) in precursors for NSCLC may provide a positive selective pressure for Ras*. Thus, the selection for late mutations will depend on the platform of genomic alterations already present.

The order of mutations is certainly also important depending on the target cell. For example, Ras* in a target cell for NSCLC will presumably be disfavored if not preceded by $p16^{Ink4a}$ loss. What is unknown is whether *Rb* or *INK4A* inactivation helps determine the type of lung cancer, or whether the target progenitor cell type in the lung determines whether *INK4A* or *Rb* inactivation is selected for. Of note, progenitors for tumors resulting from mutation of the *Rb* gene, in both humans and mice, may share a neuroendocrine origin (14). Regardless, as highlighted in the study by Williams et al., the CDK-Rb pathway is clearly not linear. While mutations at one point in the pathway (such as in Rb) do prevent selection for alterations in other components (such as $p16^{Ink4a}$ inactivation), deregulation at distinct points of the pathways can clearly have different impacts on tumorigenesis depending on cell type and previous mutations.

Beyond an intriguing correlation in human tumors, can the relevance of the Rb dependence for Ras*-mediated transformation be demonstrated in vivo? Fortuitously, a just-published study has shown that epidermis-specific *Rb* mutation in mice actually results in fewer and smaller papillomas in a two-stage mouse skin carcinogenesis model (30). In this model, tumor initiation occurs via treatment with 7,12-dimethylbenz[*a*]anthracene (DMBA), which results in a predictable Ras* mutation. Tumor promotion is then provided by repeated phorbol ester treatment. Thus, as predicted by the Williams et al. study, Rb loss actually impairs Ras*-dependent tumorigenesis (30). Papillomas forming in $Rb^{-/-}$ skin exhibit increased E2F expression and activation, increased ARF expression and p53 activation, and decreased expression and activation of NF-κB components. In addition, increased apoptosis is observed in $Rb^{-/-}$ papillomas, which may contribute to suppressed papilloma formation.

As a further parallel to the Williams et al. study, p107 is upregulated in the $Rb^{-/-}$ epidermis (31). Still, $Rb^{-/-}$ papillomas display enhanced conversion to squamous cell carcinomas. Thus, while Rb loss may select against Ras* (or vice versa), rare or forced mutation of both Ras* and Rb could push tumor evolution towards a more malignant state, perhaps by selecting for mutations that disrupt apoptosis (such as in ARF or p53). Of note, there have been reports of combined K-Ras* and *Rb* gene mutations in human cancers (1, 9), and thus while disfavored under most contexts, activation of Ras and inactivation of Rb may contribute to tumor evolution in some cases.

Demonstrations that Ras*-mediated tumorigenesis is more efficient in the presence of Rb stand in marked contrast to studies using gene-disrupted mice, which have shown that cyclin D1 and Cdk4 are required for efficient skin carcinogenesis (28, 29), and that mice expressing a $p16^{Ink4a}$-resistant variant *Cdk4* gene display enhanced susceptibility to carcinogen-induced melanomas (41). Clearly, the requirement for cyclin D-dependent kinase activation for Ras*-mediated skin tumorigenesis is not mediated solely through Rb, but probably also involves inactivation of p107 and other possible Cdk4 targets (including p130), as well as avoiding $p16^{Ink4a}$ and ARF upregulation.

Interestingly, the dependency of Ras* on Rb may help explain studies showing the ability of transgenic expression of E2F1 to potently suppress papilloma formation in the two-stage skin cancer model or by transgenic Ras*, dependent on ARF/p53 function but apparently independent of promoting apoptosis (26, 33). Thus, in this scenario transgenic E2F1 ex-

pression may mimic Rb loss. In contrast, E2F4 expression promotes carcinogenesis in the same two-stage model (48), which could relate to the ability of E2F4 to antagonize p107 and p130 functions and the lack of ARF upregulation by E2F4 overexpression (all in contrast to E2F1).

In all, it appears that for cancers where Ras* mutations are early events, Rb mutations will be rare. Further, where Rb mutations are early events, Ras* mutations will be rare, and this may be due to upregulation of inhibitors of proliferation, including p107, $p16^{Ink4a}$, and ARF, following Rb loss. In contrast, deregulation at the level of G_1 CDK activity, such as by $p16^{Ink4a}$ inactivation or cyclin D1 overexpression, can collaborate with Ras* to promote tumorigenesis by inactivating all Rb family members without ARF activation. The paper by Williams et al., together with the other studies discussed here, provides a rationale for the concordance of distinct disruptions in the CDK-Rb pathway with Ras*.

ACKNOWLEDGMENTS

J.D. is supported by a grant from the National Institutes of Health (RO1 CA77314).

Thanks go to Bob Sclafani and David Johnson for critical review of the manuscript.

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