

# Conditional haploinsufficiency of the retinoblastoma tumor suppressor gene

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**Abbreviations:** CIN, chromosomal instability;  $\gamma$ H2AX, phosphorylated histone H2AX; KRAS, Kirsten rat sarcoma viral oncogene homolog; LOH, loss of heterozygosity; LXCXE, a peptide sequence motif containing leucine-any amino acid-cysteine-any amino acid-glutamate; PTEN, phosphatase and tensin homolog; pRB, retinoblastoma protein; *RBI*, retinoblastoma susceptibility gene; *Rb1*, murine retinoblastoma susceptibility gene homolog; *TP53*, gene encoding tumor protein p53; *Trp53*, transformation related protein p53, the murine homolog of tumor protein p53.

Recent work demonstrates that retention of a single functional retinoblastoma susceptibility (*RBI*) allele is insufficient to maintain genome stability. Haploinsufficiency of *RBI* accelerates cancer pathogenesis in concert with inactivation of tumor protein p53. Collectively, multiple lines of evidence suggest revision of the '2-hit' model to include conditional haploinsufficiency of *RBI*.

Heterozygous loss of tumor suppressor genes has emerged as functionally relevant in tumor initiation and progression. Such tumor suppressor genes are classified as 'haploinsufficient' because the remaining wild-type allele is insufficient to maintain normal cellular function. While the repertoire of haploinsufficient tumor suppressor genes increases, the paradigm of Knudson's '2-hit' hypothesis suggests that loss of both copies remains critical for eliminating retinoblastoma susceptibility (*RBI*) gene function. Our recent work challenges this model by offering a number of lines of evidence from mouse models, cancer genomic data, and analysis of non-malignant *RBI*<sup>+/-</sup> cells from hereditary retinoblastoma patients to argue that loss of one copy of *RBI* is functionally relevant.<sup>1</sup> Our study suggests conditional haploinsufficiency of *RBI*, akin to other paradigms of haploinsufficiency in cancer such as phosphatase and tensin homolog (PTEN) and tumor protein p53 (best known as p53).<sup>2</sup> In this commentary, we

analyze and interpret the advancements reported in our recent paper.

Our investigation of haploinsufficiency of *RBI* follows a number of studies that detail abnormalities in heterozygous tissues or cells from gene targeted mice.<sup>3-6</sup> Most notably, Zheng et al. published an elegant study in which embryonic stem cells heterozygous for the mouse *Rb1* gene demonstrate chromosomal instability.<sup>3</sup> In our study, we offer a number of independent approaches that implicate *RBI* heterozygosity in the maintenance of genome stability. Using fibroblast cells carrying null or partial loss of functional alleles of mouse *Rb1*, we compare wild type, heterozygous, and homozygous mutant states so that quantitative assessments can be made about the magnitude of *Rb1* function. We observe that loss of one functional *Rb1* allele recapitulates the DNA replication defects found with loss of both alleles. In particular, loss of one *Rb1* allele results in replication stress with accumulation of phosphorylated histone H2AX ( $\gamma$ H2AX) at pericentromeres and mitotic errors.

These observations regarding *Rb1* heterozygosity in mouse cells are complemented by *RBI*<sup>+/-</sup> fibroblasts from retinoblastoma patients that exhibit similar abnormalities. Lastly, using a partial loss-of-function allele of mouse *Rb1* (*Rb1* <sup>$\Delta$ L</sup>) that is viable but defective for suppressing replication stress, we compare differences in *Rb1* gene dosage in a transformation-related protein p53 (*Trp53*) knockout background. Heterozygous and homozygous *Rb1* mutants display similar degrees of cancer susceptibility and pathology, presenting tumors with similar levels of aneuploidy. Therefore, by a number of measures, loss of one copy of mouse *Rb1* is equivalent to loss of both copies in preventing replication stress and aneuploidy in cancer, establishing a paradigm where retinoblastoma protein (pRB) function is haploinsufficient.

As suggested by Berger and colleagues in their proposal of a continuum model of tumor suppressor gene function,<sup>7</sup> evidence for haploinsufficiency influencing cancer initiation and progression is highly context dependent. Our paper offers interesting

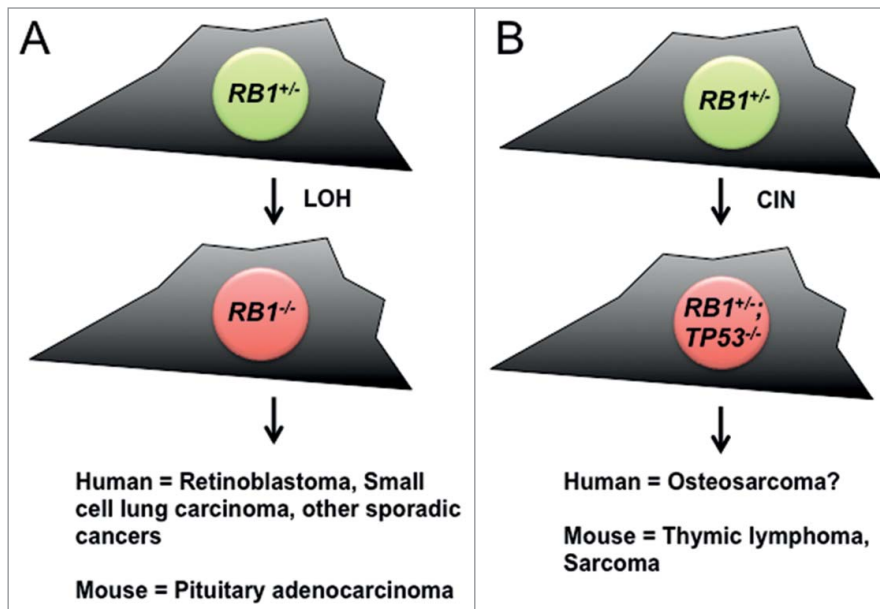
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**Figure 1.** *RB1*-related pathways in tumorigenesis. **(A)** Loss of heterozygosity (LOH) model in which the remaining wild-type retinoblastoma susceptibility gene (*RB1*) allele is eliminated and the nullizygous state contributes to malignancy. The disease sites in humans and mice where this paradigm is most relevant are depicted at the bottom. **(B)** Conditional haploinsufficiency model in which loss of one *RB1* allele contributes to chromosomal instability (CIN). Further loss of tumor protein p53 function is conducive to tumorigenesis in this paradigm and the cancer types consistent with this pathway are indicated.

parallels with their descriptions of PTEN and p53 dosage sensitivity. We report that *Rb1*<sup>ΔL</sup>, a point mutation that specifically interferes with pRB binding to chromatin regulators containing a leucine-any amino acid-cysteine-any amino acid-glutamate (LXCXE) peptide motif, accelerates cancer in a *Trp53*<sup>-/-</sup> background regardless of whether mice are heterozygous or homozygous for *Rb1*<sup>ΔL</sup>. Importantly, heterozygous tumors retain the wild-type *Rb1* allele, indicating there is no loss of heterozygosity. This is context dependent as *Rb1*<sup>ΔL/ΔL</sup> mice are not cancer prone,<sup>8</sup> indicating that p53 status is critical for this phenotype. As with studies of *PTEN* mutant mouse models of cancer,<sup>2</sup> the ability of the *Rb1*<sup>ΔL</sup> mutation to enhance cancer phenotypes is context dependent since the *Rb1*<sup>ΔL/ΔL</sup> genotype actually inhibits the ability of the Kirsten rat sarcoma viral

oncogene homolog (*KRAS*) to induce lung tumors.<sup>9</sup> From this perspective our data offer interesting similarities with other haploinsufficient tumor suppressors such that mouse *Rb1* haploinsufficiency requires key accompanying mutations and for this reason exhibits conditional effects. In addition, *Rb1*<sup>ΔL</sup>-dependent effects on cancer pathogenesis are asymmetric as they largely enhance *Trp53* inactivation-dependent characteristics rather than displaying their own. Importantly, this bears a strong similarity to cancer studies comparing *Rb1*<sup>+/-</sup>; *Trp53*<sup>-/-</sup> and *Trp53*<sup>-/-</sup> mice.<sup>3</sup> It is possible that these similarities with PTEN may form the basis of simple rules for classifying haploinsufficient tumor suppressors in the future. Why tumor suppression is conditionally haploinsufficient in these scenarios is a key question that remains to be investigated.

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In summary, a model in which *RB1* function is eliminated by loss of both copies has been widely known for some time and contributes to retinoblastoma formation among other disease sites (Fig. 1A). Our work suggests an additional pathway in which loss of one copy of *RB1* contributes to cancer pathogenesis and is most relevant when accompanied by elimination of p53 function (Fig. 1B). Examination of *RB1* loss and its correlation with genome instability in cancer cell lines suggests that mutation of *TP53* is a common event that accompanies *RB1* elimination.<sup>10</sup> It will be interesting to determine whether this is also true for *RB1*<sup>+/-</sup> cancers. We suggest that the paradigm where this new pathway of partial loss of *RB1* may be most relevant is in mesenchymal cancers. It will be interesting to see whether sequencing studies of osteosarcomas, particularly in retinoblastoma survivors, demonstrate *RB1* heterozygosity and *TP53* loss together. Since pRB status correlates with differential therapeutic responses for certain cancers,<sup>11</sup> the identification of *RB1* heterozygosity as functionally relevant suggests that gene dosage may be an important parameter to include in future diagnostic tests.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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