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AUTHOR'S VIEWS

p53 and RB1 regulate Hedgehog responsiveness via autophagy-mediated ciliogenesis

Jason E. Cain [a,b,c] and D. Neil Watkins [d,e]

[a]Centre for Cancer Research, Hudson Institute of Medical Research, Clayton, Australia; [b]Department of Translational Medicine, Clayton, Australia; [c]Department of Paediatrics, Monash University, Clayton, Australia; [d]CancerCare Manitoba, Research Institute in Oncology and Hematology, Winnipeg, Manitoba, Canada; [e]Department of Internal Medicine, Rady Faculty of Health Sciences, University of Manitoba, Winnipeg, Manitoba, Canada

ABSTRACT

Loss of tumor protein p53 (p53) and RB transcriptional corepressor 1 (RB1) in developmental and small cell lung cancer models promotes primary cilia formation and hyper-responsiveness to Hedgehog ligand. This is mediated by impaired transcription of p53 and RB1 target genes involved in autophagic degradation of primary cilia.

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The evolutionary conserved Hedgehog (Hh) signaling pathway plays fundamental roles in development and homeostasis, as well as in the initiation and progression of a wide range of cancers. In mammals, pathway activation is driven by three Hh ligands, sonic hedgehog (SHH), indian hedgehog (IHH), and desert hedgehog (DHH), that bind the receptor patched1 (PTCH1). This relieves inhibition of the G protein-coupled-like receptor smoothened (SMO), which translocates to the primary cilium, a single immotile tubulin-based structure present on most mammalian cells. Activation of SMO prevents constitutive processing of the GLI family of zinc-finger (GLI) transcription factors (GLI1, GLI2 and GLI3). Unprocessed GLI proteins in turn transcriptionally activate Hh target gene expression.[1]

Mutations in Hh pathway components leading to constitutive ligand-independent pathway activation are described is a small number of cancers, including medulloblastoma and basal cell carcinoma, and can be effectively treated with small-molecule Hh pathway inhibitors .[2] However, the vast majority of cancers in which Hh signaling is implicated do not harbor mutations in pathway components and are instead driven by ligand-dependent pathway activation. In this case, Hh ligand is secreted by tumor stroma and/or tumor cells to maintain pathway activation and self-renewal. Nevertheless, the mechanistic basis of Hh ligand-dependency is poorly understood and therefore, there are no biomarkers to predict which patients are likely to benefit from Hh inhibitor therapy.

Ligand-dependent Hh signaling is strongly implicated in the progression of small cell lung cancer (SCLC), a highly aggressive malignancy with dismal outcomes that accounts for 15% of all lung cancers .[3–5] Inactivation of tumor protein p53 (*TP53*, best known as p53) and RB transcriptional corepressor 1 (*RB1*) is a defining genetic feature of SCLC, leading us to explore the relationship between p53, RB1 and Hh-ligand dependency .[6]

Using mouse embryonic fibroblast (MEF) cell models we showed that genetic inactivation of transformation related protein 53 (*Trp53*) (*p53* KO), *Rb1* (*Rb* KO) or *Trp53* and *Rb1* (*p53Rb* KO) leads to a significant increase in canonical Hh pathway activity in response to exogenous Hh ligand treatment compared to wildtype (wt) MEFs. This response directly correlated with an increase in primary cilia frequency in *p53* KO, *Rb* KO and *p53Rb* KO MEFs. To definitively demonstrate that Hh response to ligand is dependent on primary cilia, we inhibited cilia formation in the *p53Rb* KO MEFs by knocking down kinesin family member 3A (KIF3A), a kinesin protein essential for ciliogenesis. As expected, reduced primary cilia frequency led to a proportional reduction in Hh pathway activity in response to Hh ligand. Importantly, we also showed that increased primary cilia in *p53* KO, *Rb* KO and *p53Rb* KO is independent of the cell cycle.

In order to understand the mechanism by which p53 and RB1 regulates primary cilia frequency, we considered evidence in the literature linking autophagy to primary ciliogenesis and Hh signaling, [7] and the direct transcriptional regulation of autophagy machinery genes by p53[8] and RB1 .[9] Using western blot and immunofluorescence techniques we showed that inducible autophagy was impaired in the *p53* KO, *Rb* KO and *p53Rb* KO MEFs and that this was associated with a significant reduction in the expression of critical autophagy genes and known p53 and E2 F transcription factor 1 (E2F1) targets, including autophagy related 5 (*Atg5*). Indeed, siRNA knockdown of selected dysregulated autophagy machinery genes in wt

CONTACT Jason E. Cain jason.cain@hudson.org.au Centre for Cancer Research, Hudson Institute of Medical Research, Clayton, Victoria 3178, Australia

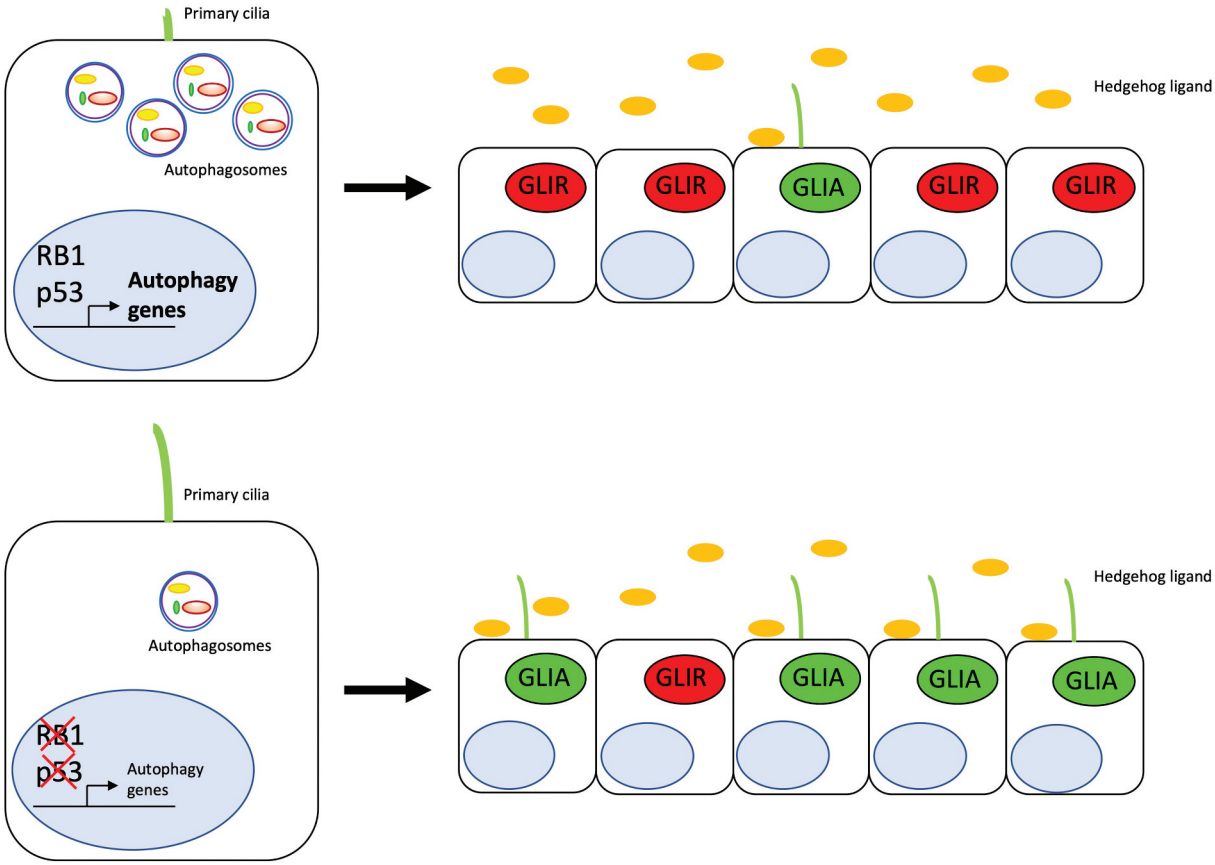

Figure 1. Potential mechanisms for p53- and RB1-dependent regulation of ligand-dependent Hh signaling. In response to cellular stress, tumor protein p53 (p53) and RB transcriptional corepressor 1 (RB1) directly regulate the expression of critical autophagy genes that in turn negatively regulates primary cilia growth. In this context, smoothned (SMO) is unable to translocate to the primary cilia and block constitutive processing of GLI family of zinc finger transcription factors (GLI) into a repressor form (GLIR). In contrast, loss of p53 and RB1 results in reduced expression of genes required for the autophagic machinery resulting in aberrant primary cilia formation. This renders cells highly sensitive to SMO activation by Hedgehog (Hh) ligand and initiation of Hh target genes by GLI transcriptional activators (GLIA).

MEFs enhanced Hh pathway activation in response to Hh ligand. Consistent with these findings, re-expression of p53 and RB1 in *p53Rb* KO MEFs resulted in restored expression of selected autophagy genes, increased autophagic flux, inhibition of primary ciliogenesis and reduced Hh pathway activation in response to Hh ligand. Collectively, these data led us to propose a model whereby loss of p53 and RB1 directly impairs the expression of important autophagy genes, leading to diminished primary cilia degradation and enhanced response to Hh ligand (Figure 1).

To test this in a clinically relevant cancer model we utilized two distinct murine lung cancer models: a model of SCLC driven by *Trp53* and *Rb1* inactivation (mSCLC); and a model of lung adenocarcinoma driven by the oncogenic KRAS proto-oncogene, GTPase (*Kras*) *G12D* mutation (mLUAD). Abundant primary cilia were detected in mSCLC tumors but were scarce in mLUAD tumors. Ablation of primary cilia in these models, via genetic inactivation of *Kif3a*, resulted in significantly reduced tumor burden in the mSCLC model but had no effect on tumor burden in the mLUAD model, highlighting the requirement for primary cilia in SCLC. Consistent with our MEF findings, p53 and RB1-deficient mSCLC cell lines were highly responsive to Hh ligand, whereas Kras-mutant mLUAD cell lines were non-responsive. This was associated with diminished inducible autophagy in mSCLC cell lines compared with mLUAD cells. We also confirmed these results in human SCLC and LUAD cell lines.

Collectively, our study resolves a long-standing controversy on the importance of ligand dependent Hh signaling in cancer, including SCLC. By establishing a direct link between loss of function mutations in *TP53* and *RB1* and dependence on Hh ligand signaling, our results provide the novel mechanistic framework for re-assessing the importance of this pathway in cancer and informs on potential genetic and molecular biomarkers of predictive response to Hh pathway inhibition.

Disclosure of potential conflicts of interest

DNW is a co-inventor on a patent relating to aspects of this work and is a financial beneficiary of a licensing agreement related to the use of SMO antagonists in small cell lung cancer. DNW and JEC are paid consultants for Mayne Pharma.

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ORCID

Jason E. Cain http://orcid.org/0000-0003-3987-5894
D. Neil Watkins http://orcid.org/0000-0001-8218-4920

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