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Editorial

Haploinsufficiency Interactions of RALBP1 and TP53 in Carcinogenesis

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Mutagenic environmental chemical or radiant stresses directly damage DNA and amplify the formation of potent endogenous mutagens from lipid peroxidation, leading to cancers that cause millions of deaths and impose enormous financial and social burdens [1,2]. The p53 protein (encoded by the *TP53* gene (*TP53*) and used here for both gene and protein), has been dubbed the 'Guardian of the Genome' because of its pleiotropic genoprotective functions. Germline mutations at 'hotspot' regions of the *TP53* gene strongly predispose cancer in persons with Li–Fraumeni syndrome and somatic mutations of *TP53* are found in the majority of cancers [3–9]. Nearly all mice carrying homozygous *TP53* deletions die of spontaneous cancer before reaching 6 months of age. In stark contrast, mice lacking the *RALBP1* ((RalA Binding Protein 1) gene, which encodes RLIP76 (also known as Rlip (RALBP1), used here for both gene and protein), a stress-responsive, anti-apoptotic protein, are highly resistant to carcinogenesis by even the most potent chemical carcinogens [10]. Targeted inhibition or depletion of RALBP1 causes regression of many types of cancer [10–16]. In studies to examine the effect of combined RALBP1 and TP53 deficiency, we discovered that hemizygous RALBP1 deficiency was sufficient to exert a strong dominant negative effect on the spontaneous carcinogenesis phenotype of homozygous TP53-null mice [10]. The studies presented here provide the first evidence for RALBP1 haploinsufficiency in the prevention of *ERBB2* (Erb-B2 Receptor Tyrosine Kinase 2) -driven breast cancer. Additionally, the molecular mechanisms through which RALBP1 may exert transcriptional regulation and DNA repair and the effects of Ω-6 fatty acid metabolites in cancer are explored here by a spectrum of researchers who have made important contributions to defining the pleiotropic molecular mechanisms that have begun to define a new paradigm in carcinogenesis.

RALBP1 is an ATPase enzyme of the mercapturic acid pathway that catalyzes the transmembrane efflux of Glutathione (GSH)-electrophile thioether conjugates (GS-E) that are formed through the glutathione S-transferase (GST)-catalyzed conjugation of GSH with electrophilic toxins [17–37]. Its ATPase activity is coupled with clathrin-dependent endocytosis (CDE), the RAL-regulated first step in the internalization and trafficking of membrane vesicles containing receptor-bound, cancer-promoting growth hormones [38,39]. CDE regulates the downstream signaling of receptors for insulin, EGF, TNFα, FGF1 and many other peptide hormones [40–43]. Concomitant deficiencies of GS-E efflux, GSH-linked antioxidant defenses and CDE-linked vesicular trafficking in RALBP1-null (RALBP1$^{-/-}$) mice show that RALBP1 functions provide an essential link between stress-induced apoptosis defenses and RAL/RAS/RHO/RAC-regulated pathways that promote cancer cell growth [44–51]. Our recent studies in lung and breast cancers as well as melanoma have confirmed that the inhibition or depletion of RALBP1 blocks CDE and inhibits signaling by cancer-promoting peptides, including EGF, WNT and FGF [10,11,13,14]. The role of RALBP1 in regulating intracellular vesicular traffic, and the signaling proteins, such as ARF6, ARNO, PI3K and RAC, that regulate vesicular traffic, is reviewed in one paper presented in this symposium issue. Furthermore, the X-ray crystallographic elucidation of

the RALBP1 structure, which sheds much-needed light on the molecular mechanisms for these pleiotropic effects, is presented.

Because GS-E conjugates are potent inhibitors of upstream xenobiotic metabolizing enzymes and GSH-linked antioxidant defenses, GS-E accumulation caused by RALBP1 deficiency profoundly impairs the ability of highly metabolically active cancer cells to defend against apoptosis caused by exogenous as well as endogenously generated toxins. Oxidative metabolism of Ω-6 polyunsaturated fatty acids triggered by radiant (X-ray, UV light and heat) or oxidative stress yields lipid hydroperoxides that degrade to toxic lipid alkenals, principally 4-hydroxynonenal (4HNE). The highly reactive alkylating agent 4HNE, with inherently greater pro-apoptotic effects against malignant as compared with non-malignant cells, is metabolized primarily to a glutathione-conjugate (GS-HNE) that is removed from cells by RALBP1 [18,26,27,36,37,52–54]. Elevated 4HNE production from the high levels of oxidative stress imposed on cancer cells by their high metabolic rates renders them very dependent on this mechanism of metabolizing and disposing of 4HNE, as is evident from the dramatic and sustained regression of multiple histological types of cancer upon depletion or inhibition of RALBP1 in mouse models without any toxicity [10,11,55]. Our findings of inhibition of cancer growth in vitro and in vivo have been confirmed by several independent groups [44,48,49,56–58]. Evidence for the role of RALBP1 and other aldehyde-metabolizing enzymes in regulating the cancer-selective apoptotic effects of Ω-6 fatty acids and 4HNE in breast cancer is presented in one of the papers in this symposium.

The inability of grafted mouse melanoma and lung cancer cells to grow in RALBP1$^{-/-}$ mice [49] and the resistance of RALBP1$^{-/-}$ mice to benzo(a)pyrene, phorbol ester or dimethylbenzanthracene-induced carcinogenesis led us test the possibility that RALBP1 has existential importance for cancer cell formation and survival [10]. Because the carcinogenic effects of TP53 loss would be opposed by the reduced survival of malignant or pre-malignant cells due to RALBP1 deficiency, we posited that RALBP1 deficiency would proportionately reduce carcinogenesis in TP53$^{-/-}$ mice. We found that weekly injections of an RALBP1-directed phosphorothioate antisense molecule (R508) to TP53$^{-/-}$ mice starting at 8 weeks of age reduced RALBP1 protein content to half of normal levels and provided complete protection from carcinogenesis. Upon crossing TP53$^{+/-}$ with RALBP1$^{+/-}$ mice, we found a profound reduction in cancer incidence in mice lacking one or two RALBP1 alleles, regardless of TP53 status, and that loss of one RALBP1 allele was sufficient for protection from spontaneous carcinogenesis. Susceptibility to benzo(a)pyrene-induced carcinogenesis was reduced to half in TP53$^{+/-}$RALBP1$^{+/-}$ mice and to one fifth in TP53$^{+/-}$RALBP1$^{-/-}$ mice [10]. The results of our subsequent studies showing distinct cancer-preventative effects of heterozygous vs. homozygous RALBP1 deficiency in ERBB2-overexpression-driven as well as PYVT (Polyoma Virus middle T antigen) -driven breast cancer in TP53$^{+/-}$ mice, presented by Singh et al. in this symposium, strongly support complex haploinsufficiency interactions underlying cancer prevention by RALBP1 deficiency. Previously reported haploinsufficiency interactions of TP53 binding partners, such as MDM2, MDM4, and HSF1, manifest as embryonic lethality in carriers of imbalanced deletions and switches in cancer histological type but do not substantially reduce cancer susceptibility. Concomitant mutations in oncogenes increase the intrinsic cancer susceptibility of TP53$^{-/-}$ mice, but genetic manipulation of single genes has not been shown to suppress carcinogenesis as effectively as RALBP1 haploinsufficiency [59–76].

The disproportionate effect on cancer protection correlated with reversion of the marked transcriptomic and promoter methylomic abnormalities in control TP53$^{-/-}$ mice to nearly wild-type in R508-treated TP53$^{-/-}$ mice, in whom RALBP1 was reduced by $\leq$50%. Whole-genome bisulfite sequencing (WGBS) showed that control TP53$^{-/-}$ mice had over 14,000 differentially methylated regions (DMRs) while R508-treated mice had less than 1000. The prevention of promoter DMRs was associated with prevention of differential expression in over 1600 genes in liver samples by RNA-Seq [10]. Detailed analyses of the transcriptomic effects of congenital RALBP1 deficiency without or with TP53 deletion with respect to age and gender are presented in this symposium issue.

Because the RALBP1-TP53 dimer cannot exist in either the $TP53^{-/-}$ or $RALBP1^{-/-}$ mouse, this complex by itself cannot explain the underlying mechanism. Instead, at least one other protein that binds both RALBP1 and TP53 would be required for an explanation. Such proteins include heat shock factor 1 (Hsf1), cdc2/cdk1, Hsp90, and PKCα. The best-studied of these is Hsf1, the master transcription factor for the chaperone response to heat shock [77] that binds RALBP1 in reciprocal inhibitory interactions [78] and binds TP53 to determine embryonic lethality as well as cancer histology [10,78]. Intriguing new results presented in the symposium on transcriptional regulation of PKCα provide an important insight into the pleotropic mechanisms through which RALBP1 could influence carcinogenesis.

Adult-onset diabetes, obesity, and metabolic syndrome are diseases characterized by insulin resistance (IR) [79] and are now strongly linked with carcinogenic risk [80]. CDE is the primary mechanism for insulin endocytosis and is activated by stressors known to exacerbate IR [38]. Despite having high levels of oxidative stress, $RALBP1^{+/-}$ mice are extraordinarily sensitive to insulin, having low blood glucose as well as lipids [81]. This topic is reviewed in the symposium. Agents that reduce RALBP1 expression or inhibit its activity could reduce cancer risk. In this issue, we present experimental evidence for cancer prevention by one such compound, 2-hydroxyflavanone, which has been shown, by us, to inhibit melanoma as well as lung, breast, and kidney cancers. This compound has served as a lead in developing the novel synthetic RALBP1 inhibitors that are discussed in one of the papers presented in this symposium. Finally, it should be noted that diabetic and obese individuals are at particularly high risk for COVID-19, a virus that enters cells through CDE. Hindle et al. present intriguing indirect evidence for a potential role of RALBP1 in the pathogenesis of this infectious disease.

In summary, we have observed a striking anticancer effect of RALBP1 that is powerful enough to overcome the universally cancer-susceptible phenotype of TP53-null mice, requiring only partial inhibition. This is in stark contrast to other targeted therapies that generally rely on abrogating the target activity, suggesting that RALBP1-targeted drugs could have lower normal tissue toxicity and fewer side effects. The complexity of the molecular mechanisms underlying the cancer-preventative effects of RALBP1 inhibition is not understood. The purpose of this symposium is to assemble a diverse array of scientific research that will collectively elucidate the molecular mechanism for this new paradigm in cancer prevention.

Conflicts of Interest: The author declares no conflict of interest.

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