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Activating p53 Function by Targeting RLIP

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Abstract

Aberrations in RLIP, p53, and PKCα represent essentially the entire spectrum of all human neoplasms. Elevated PKCα expression, failure of the cell cycle checkpoint (p53 dysfunction), and abnormal glutathione (GSH) metabolism are fundamental hallmarks of carcinogenesis and drug/radiation resistance. However, a lack of investigations into the interactions between these important regulatory nodes has fundamentally limited our understanding of carcinogenesis and the development of effective interventions for cancer prevention and therapy. Loss of p53, perhaps the most powerful tumor suppressor gene, predisposes rodents to spontaneous cancer and humans to familial, as well as acquired, cancers. Until recently, no genetic manipulation of any oncogene had been reported to abrogate spontaneous carcinogenesis in $p53^{-/-}$ rodent models. However, the overexpression of RLIP, a GSH-electrophile conjugate (GS-E) transporter, has been found to enhance cancer cell proliferation and confer drug/radiation resistance, whereas its depletion causes tumor regression, suggesting its importance in cancer and drug/radiation resistance. Indeed, RLIP is an essential effector of p53 that is necessary for broad cancer-promoting epigenetic remodeling. Interestingly, through a haploinsufficiency mechanism, the partial depletion of RLIP in $p53^{-/-}$

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mice provides complete protection from neoplasia. Furthermore, RLIP$^{-/-}$ mice exhibit altered p53 and PKCα function, marked deficiency in clathrin-dependent endocytosis (CDE), and almost total resistance to chemical carcinogenesis. Based on these findings, in this review, we present a novel and radical hypothesis that expands our understanding of the highly significant cross-talk between p53, PKCα, and GSH signaling by RLIP in multiple tumor models.

Keywords

RLIP; RalBP1; p53; PKCα; drug resistance; metastasis; glutathione-electrophile conjugate; mercapturic acid pathway; therapeutics

Overview of Mercapturic Acid Pathway

RLIP (*see Glossary*) is a 76-kDa splice variant protein encoded by the human *RALBP1* gene (chromosome 18p22.11). It is a low-affinity, high capacity transporter of glutathionylated metabolites of exogenous, as well as endogenous (lipid peroxidation-derived), and electrophilic toxins [1–6]. The importance of the **mercapturic acid pathway** (**MAP**) in the malignant phenotype was established by classical studies showing that the upregulation of **glutathione (GSH)-S-transferases (GSTs)**, which catalyze the first committed step of this pathway, is an early and very frequent event in carcinogenesis. To proceed to the next step in the pathway, the products of the GST-catalyzed metabolism of electrophilic toxins, i.e., **GSH-electrophile conjugates (GS-Es)**, must be removed from cells through active efflux. γ-Glutamyl-transpeptidase (γGT, located on the outer leaflet of cell membranes) catalyzes the deglutamylation of these conjugates prior to their re-uptake in cells and further metabolism to **mercapturic acids** [7–9].

4-hydroxynonenal (4HNE), a major downstream metabolite generated from the peroxidation of ω−6 polyunsaturated fatty acids (PUFAs), is a potent signaling lipid that can trigger cell proliferation, differentiation, apoptosis, and necrosis in a concentration-dependent manner. It is metabolized largely to mercapturic acids though glutathionylated intermediates. Conditions that lead to 4HNE accumulation in cells (i.e., oxidant or radiant stressors) result in cell proliferation and apoptosis at low levels of 4HNE or necrosis at higher levels of 4HNE [10, 11]. To prevent the accumulation of 4HNE or its glutathionylated intermediates, the latter must be removed from cells through active transport, a function that is nearly completely lost in cells and tissues of mice in which the *Ralbp1* gene is disrupted [2–6, 12, 13]. RLIP has been shown to be a dominant mechanism for the efflux of glutathionylated-4HNE (GS-HNE) from cells. Lack of functional RLIP causes the accumulation of GS-HNE, as well as its precursors, including 4HNE and the precursors of 4HNE, which include highly reactive and toxic **lipid hydroperoxides** (**LOOHs**). RLIP thus functions to minimize the accumulation of these pro-apoptotic metabolites in cells [14].

We have identified RLIP as the principal GS-E transporter that functions as a rate-determining factor in the MAP and clathrin-dependent endocytosis (CDE), a dominant anti-apoptotic effector in the stress response, and a key anti-apoptotic factor necessary for cancer cell survival and resistance to chemotherapy and radiation [2–5, 12–15]. The MAP, which is the central axis of detoxification of chemotherapy drugs and the products

of lipid peroxidation generated during **oxidative stress**, is frequently overexpressed during **carcinogenesis**, and the requirement for a functional MAP is greater in cancerous cells than in normal cells [5–7]. RLIP, which is overexpressed in many malignancies, functions as a rate-limiting factor in the cancer cell **detoxification** processes mediated by MAP, as well as in endocytosis [15–23]. Detoxification defends cells from pro-apoptotic or mutagenic electrophilic toxins, and endocytosis regulates receptor–ligand signaling, which is also frequently aberrant in cancer cells. Thus, blocking or depleting RLIP is an innately 'cancer-targeted' strategy that is expected to be highly deleterious for cancer cell survival while sparing non-malignant cells [5, 6].

Cancer-specific apoptosis upon RLIP depletion is exerted through its dual functions as a MAP transporter that defends against oxidative stress-mediated apoptosis and as a key component of the CDE mechanism that regulates the growth and survival-mediating effects of cancer-promoting peptide hormones. Studies have also demonstrated that RLIP depletion suppresses CDE with consequent inhibition of a broad spectrum of signaling pathways that promote malignancy in homozygous p53 knockout ($p53^{-/-}$) mice [24]. Specific p53 mutations can selectively impair its cell cycling, apoptotic, or transcriptional functions. Loss of its apoptotic function is mediated by mutations in regions that directly interact with or transcriptionally activate pro-apoptotic proteins (Bax, Bak) or repress anti-apoptotic proteins (Bcl2, BclXL). p53 is perhaps the most powerful tumor suppressor gene that, when missing, predisposes rodent models to spontaneous cancer. Similarly, loss of p53 predisposes humans to familial, as well as acquired cancers [25–29]. Until recently, no genetic manipulation of any oncogene had been reported to abrogate spontaneous carcinogenesis in rodent $p53^{-/-}$ knockout models. However, recent studies demonstrated, for the first time, the striking and complete suppression of carcinogenesis in $p53^{-/-}$ mice upon depletion of RLIP [24]. This review focuses on the significance of three high-impact and critical signaling nodes in cancer progression and drug resistance: RLIP, p53, and PKCα. In addition to providing significant evidence for the signaling requirements of important phases of tumor transformation, metastatic progression, and drug resistance, and we establish a novel mechanistic link between these critical proteins, which can revolutionize interventional strategies currently in various phases of pre-clinical and clinical development.

RLIP

RLIP is a stress-protective [30, 31], Ral-regulated [32–34] ATPase of the MAP that transports GS-Es [1–6, 14, 15, 35]. It is an integral component of CDE [15, 36, 37] and chaperone expression (38, 39). RLIP also plays an effector ATPase role in mechanisms that mediate cell cycling, mitochondrial fission, motility, mitosis, and exocytosis [32–34, 37, 40, 41]. It mediates resistance to oxidative stress and apoptosis caused by radiation, oxidants, alkylating agents, anthracyclines, and kinase inhibitors. Indeed, homozygous RLIP knockout ($RLIP^{-/-}$) mice have marked deficiency of GS-E transport and CDE [12–15]. Interestingly, although oxidative stress is significantly elevated in $RLIP^{-/-}$ mice [3, 13], they are paradoxically insulin-sensitive and highly resistant to **chemical carcinogenesis** [15, 42, 43]. p53 protein binds to and inhibits the transport activity of RLIP, and loss of RLIP confers constitutive p53 activation [15, 21]. Remarkably, p53 is the most differentially expressed upstream regulator in $RLIP^{-/-}$ mice [24].

RLIP$^{-/-}$ mice exhibit altered function of PKCα and p53, as well as of peptide hormone signaling pathways known to be regulated by CDE [2–6, 15]. The complete lack of CDE in the absence of RLIP and the reliance of CDE on the GS-E transport function of RLIP represent a novel paradigm. We believe that intracellular signaling in carcinogenesis and drug/radiation resistance is globally regulated by intracellular GS-E concentrations. This understanding can markedly simplify disparate models of signaling in cancer and will enable rapid the identification of inherently non-toxic cancer-targeted chemicals that can prevent and even cure cancer, as well as the development of highly reliable algorithms for individualized cancer therapy. These strategies strongly support a model in which p53 and RLIP are mutually regulatory: p53 inhibits the transport activity (and perhaps the transcription) of RLIP, and RLIP binds to and prevents the nuclear translocation of p53.

Deficient CDE and MAP activity in RLIP$^{-/-}$ mice [3, 15], as well as deficient CDE activity in RLIP mutants with deficient GS-E transport and anti-apoptotic activity [15], indicates that RLIP is a nexus linking anti-apoptotic mechanisms with the overarching role of CDE as a regulator of multiple types of membrane receptor–ligand-initiated signaling pathways that play a crucial role in the growth, survival, apoptosis resistance, metabolic derangements, and senescence of cancer cells [2, 5, 36, 37].

RLIP affects kinase signaling by regulating oxidative stress and the oxidative metabolism of PUFAs

Several studies have identified the GSH conjugate of 4HNE (HNE-SG) as a physiological substrate that is transported by RLIP [6, 14]. Blocking GS-E transport using antibodies against the 171–185-amino acid (aa) domain of RLIP, genetically knocking down RLIP in mice, or depleting RLIP by siRNA in cancer cells or mouse embryonic fibroblasts (MEFs) causes the accumulation of 4HNE and HNE-SG in cells, as well as an increase in markers of generalized oxidative stress [3, 13]. We believe that these effects occur because RLIP is an important rate-limiting catalyst for the removal of products of PUFA peroxidation; thus, inhibiting RLIP causes the accumulation of eicosanoid byproducts and the inhibition of multiple upstream GSH-linked oxidative stress defense enzymes. GS-Es are known to inhibit multiple antioxidant enzymes, including GSH reductase, GSH peroxidase, and GSTs [9, 44].

Because RLIP removes the metabolites of mutagenic compounds, its loss is expected to increase the level of mutagens in cells exposed to xenobiotics, PUFAs, or chronic oxidative stress, consequently leading to a greater incidence of cancer. Compared to wild-type mice, RLIP$^{-/-}$ mice have up to 7-fold higher levels of total lipid peroxidation, 4HNE, and GS-HNE, as well as the aldose reductase (AR)-mediated reduced metabolites dihydroxynonenol (DHN) and GS-DHN [3, 13, 45]. 4HNE is pro-apoptotic and genotoxic, whereas its metabolites are not. Higher levels of these compounds are predicted to increase cancer risk after exposure to a chemical carcinogen such as benzo[a]pyrene (BaP). BaP is known to be metabolized by cytochrome p450 to electrophilic and ultimately carcinogenic diol-epoxides, which are metabolized to GS-Es by GSTs and are also substrates for efflux by RLIP [46, 47] (Fig 1A).

PKCα

PKC is a member of the protein kinase family. Its classical isoforms (PKCα, PKCβI/II, and PKCγ) bind to and are activated by calcium and diacylglycerol (DAG), resulting in the activation of the catalytic domain in response to various stimuli. PKCα transmits signals downstream to pathways regulating cell proliferation, differentiation, survival, apoptosis, and cell cycle control in response to stressors such as the classical chemotherapy drug doxorubicin [48, 49]. The regulation of cell cycle progression by PKCα is mediated through the activation of cyclin D1 expression through enhanced AP1 binding to the cyclin D1 promoter. Loss of PKCα correlates with the induction of apoptosis. Increased PKCα-mediated augmentation of cell survival is also associated with increased levels of the anti-apoptotic protein Bcl2. PKCα activation is a key signaling event governing cell growth, stress resistance, and drug resistance. Previous studies have demonstrated that the doxorubicin resistance-mediating effects of PKCα require the presence of RLIP. Studies have also shown that RLIP is a necessary downstream effector for PKCα-mediated mitogenesis [50, 51]. The crucial role of RLIP as a regulator of cell proliferation and the central role of the RLIP–PKCα interaction in drug resistance suggest a new paradigm for understanding growth signaling in cancer (Fig 1B).

p53 (TP53)

Metastasis contributes to the vast majority of cancer-related mortality. Regulatory mechanisms of the multistep invasion-metastasis cascade are being unraveled. TP53 is the most frequently mutated gene across human cancers. Accumulating evidence has shown that mutations of TP53 not only lead to loss of function or dominant negative effects, but also promotes a gain of function. Specifically, gain of function mutant p53 promotes cancer cell motility, invasion, and metastasis. p53 (tumor protein 53, or TP53; chromosome 17p1.3) is a 53-kDa multifunctional nuclear phosphoprotein that regulates the cellular stress response, DNA repair, cell cycle arrest, apoptosis, senescence, and ubiquitination through various functional interactions with other proteins and DNA. Loss of p53 function results in unchecked cell cycling that causes genetic instability due to the propagation of unrepaired mutations. p53 loss also antagonizes the ability of cells to undergo apoptosis after acute severe genotoxic stress, such as radiation or chemotherapy, accounting for the characteristic therapy-resistant nature of neoplasms with deficient p53 function. Resistance to therapy in the setting of homozygous p53 loss is also associated with the role of p53 in the chaperone and ubiquitination responses to stress, which are directly regulated through its interactions with HSF1 and MDM2, respectively. Until recently, no genetic or pharmacological intervention had been shown to eliminate spontaneous malignancy in $p53^{-/-}$ mice, which universally die of cancer by six months of age [28, 29]. In contrast, $RLIP^{-/-}$ mice are resistant even to malignancies induced by the powerful chemical carcinogens BaP and phorbol ester/dimethylbenz anthracene (PMA/DMBA) [15]. We previously showed that the systemic depletion of RLIP using phosphorothioate antisense (R508) causes the sustained regression of multiple types of malignancies in mouse models [16–23]. Furthermore, recent studies showed that the partial depletion of RLIP using RLIP antisense completely prevented malignancies in $p53^{-/-}$ mice [24].

RLIP and p53 Interactions

RLIP$^{-/-}$ and p53$^{-/-}$ mice are polar opposites in the spectrum of cancer susceptibility [15, 24, 29]. Cancer frequently arises due to deletions or loss-of-function mutations of the p53 gene, and cancers with these genetic abnormalities are inherently resistant to therapy. 100% of p53$^{-/-}$ mice develop spontaneous malignancies by six months of age. In stark contrast, mice deficient in the protein RLIP have no spontaneous malignancies and are highly resistant to carcinogen-induced malignancies. Recent studies led to the astounding observation that p53$^{-/-}$ mice are completely protected from spontaneous malignancy for up to eight months of age by the partial depletion of RLIP via antisense therapy. This protection was associated with nearly complete reversal of the major epigenetic and gene expression changes that normally occur in p53$^{-/-}$ mice [24]. Upon RLIP depletion, the anticancer pathways that are typically inactivated by p53 loss were reactivated, and the pro-cancer pathways typically activated by p53 loss were inactivated. These observations lead us to the paradigm-shifting hypothesis that carcinogenesis in the setting of p53 loss depends on the presence of a full complement of RLIP, which cancer cells rely on to survive. Furthermore, the partial depletion of RLIP can activate p53-regulated anticancer mechanisms by preventing the epigenetic and genetic changes caused by the interactions between RLIP and p53, as well as the interactions of both with HSF1, the master transcriptional regulator of chaperone genes. These studies have major implications for preventing malignancies in genetically predisposed individuals who lack p53 or related genes and for treating the approximately 60% of cancer patients with p53-deficient malignancies. The diametrically opposed cancer susceptibility of RLIP$^{-/-}$ and p53$^{-/-}$ mice led us to hypothesize a mutually inhibitory and functionally antagonistic relationship between RLIP and p53 in carcinogenesis. We believe that ‘controlled RLIP and p53 deletion will provide efficacy and mechanistic data on the protective role of RLIP deletion against spontaneous and chemically induced carcinogenesis, which can be further tested by studying the interactions between RLIP and p53 to correlate their bidirectional regulation of cancer initiation and progression pathways’ (Figs 1C–E, 2, and 3). The abrupt change in the phenotype of p53$^{-/-}$ mice, accompanied by global changes in gene expression patterns, is reminiscent of the dramatic rescue of the embryonic lethal phenotype of Mdm2$^{-/-}$ and Mdm4$^{-/-}$ mice upon concomitant homozygous p53 deficiency and suggests a haploinsufficiency effect involving key proteins that bind to and are critical regulators of p53 [52–54]. HSF1, the master transcription factor for chaperone expression, is a p53-binding protein that co-translocates to the nucleus with p53 under stress conditions [4, 38, 39]. HSF1 inhibits the transport activity of RLIP [4], and RLIP inhibits the nuclear translocation of HSF1 [38, 39]. Analogous to Mdm2$^{-/-}$ p53$^{-/-}$ mice, HSF1$^{-/-}$ p53$^{-/-}$ mice are viable and display a remarkable change in phenotype: a switch in the lineage-specificity of spontaneous malignancy from lymphoma to carcinoma and sarcoma [55]. However, neither the lack of Mdm nor HSF1 reduces the cancer susceptibility conferred by loss of p53 [54, 55]; indeed, no previous intervention has substantially changed this phenotype. Unlike Mdm2 or HSF1 null mice, RLIP$^{-/-}$ mice survive into adulthood [3, 13], and the phenotypic change in p53$^{-/-}$ mice requires only a ‘hemizygous’ state.

The discovery that partial suppression of RLIP can restore the tumor-suppressive functions of p53 is of fundamental significance regarding our perspectives on the mechanisms of

carcinogenesis, and given the central importance of p53 in malignancy, the therapeutic implications of this discovery are broad. The characteristic resistance of a broad spectrum of p53-deficient neoplasias to curative therapy could be reversed using RLIP depletion. Because RLIP depletion does not require functional p53 to oppose carcinogenesis, its therapeutic application could extend beyond p53 malignancies. The functional activation of p53 signaling upon RLIP depletion and the effects of RLIP deficiency on cell cycling and DNA repair mechanisms suggest relevance to hereditary cancer syndromes with loss of p53-related tumor suppressors. Preventative applications can be foreseen for genetic syndromes such as Li-Fraumeni syndrome (LFS), a hereditary disease caused by haploinsufficiency of p53 that carries a lifetime cancer risk of at least 70% for male carriers and nearly 100% for female carriers, for which there is no effective prevention strategy [56–60]. Thus, the present discovery will have a major impact on cancer prevention and therapies to achieve a cancer cure [24].

Genetic alterations of RLIP and p53 in cancer

Effective cancer prevention in $p53^{-/-}$ mice by partial depletion of RLIP provides a novel means of bypassing the deleterious effects of p53 loss. Reducing the anti-apoptotic and endocytosis-promoting activities of p53, activating signaling pathways downstream of p53, and altering the ratio of heterodimers with HSF1 are plausible mechanisms by which RLIP depletions may act, based on the known interactions of these proteins (Figs 2 and 3). The remarkable anticancer effect, the breadth of effects of RLIP depletion on p53-related signaling, and the overlapping and opposite effects of p53 and RLIP loss on the expression of cancer genes indicate that the p53–RLIP interaction is an existential determinant of malignancy. Though the molecular mechanisms are yet to be established, our observations reveal a novel conceptual paradigm for how p53 loss promotes malignancy, provide a rich substrate for further mechanistic investigations, and present a new approach to develop broad-spectrum therapies to treat and prevent cancer. LFS predisposes individuals to sarcoma, melanoma, and brain, breast, adrenocortical, colon, gastric, bronchoalveolar, and hematologic malignancies. Thus, despite progress in biochemical and imaging surveillance regimens for LFS, there remains a strong need to prevent or delay the onset of cancer in these individuals. The upregulation of multiple tumor suppressor proteins upon RLIP depletion or heterozygous genetic loss of RLIP indicates that other persons afflicted with hereditary cancer syndromes due to loss-of-function mutations to one or more p53-related cancer suppressor genes affected by RLIP depletion (e.g., *BRCA1*, *BRCA2*, *CHEK2*, *ATM*, *BARD1*, *MRE11A*, *RAD50*, *MLH1*, *MSH2*, *MSH6*, *PMS2*, *NF1*, *BLM*, *RB*, *FH*, *CDKN2A*, *CDK4*, and *MAX*) could also be candidates for chemoprevention by therapies that suppress RLIP expression. Beyond these ‘orphan diseases,’ these findings apply to a broad array of malignancies that display loss of p53 function. The anti-metabolic syndrome and obesity-resistant phenotype of $RLIP^{-/-}$ mice [42, 43, 61] suggests that the side effects of RLIP depletion may actually be beneficial and provides additional rationale for developing RLIP-targeted therapeutics as well-tolerated and highly effective cancer therapies. The apparent necessity of RLIP in carcinogenesis, as indicated by our studies, is reflected by an analysis of the Cancer Genome Atlas (TCGA) database showing that loss of RLIP or HSF1 is rare in human cancer, and concomitant alterations are exceedingly rare (<0.1%). In addition, these alterations essentially only occur when p53 is altered (Fig 2). Taken together, these

findings support a model in which the three-way interactions of these proteins could serve a „driver" function that determines the existence and histology of malignancy. Hence, the role of the RLIP–PKCα–p53 complex and its regulation represents a vital signaling node in cancer progression and drug resistance.

Role of RLIP in spontaneous carcinogenesis and DNA methylation in $p53^{-/-}$ mice

The degree of lymphoma and tumor suppression, as well as the reversal of DNA hypomethylation, in $p53^{-/-}$ mice upon RLIP depletion is striking [24] and has not been observed for other interventions in these mice. A potential mechanism for this striking finding was revealed by the results of methylation-specific whole-genome sequencing studies. $RLIP^{-/-}$ mice had a wild-type phenotype, with only 45 differential methylation sites. In contrast, $p53^{-/-}$ mice had over 22,000 hypomethylated sites. The vast majority of changes in methylation were in the gene bodies, but a substantial number (~600) were in gene promoter regions. Upon RLIP depletion, hypomethylation in both the gene bodies and promoters was essentially completely reversed to wild-type levels. A heat-map of promoter methylation showed that the vast majority of genes were hypomethylated in control $p53^{-/-}$ mice, whereas RLIP depletion caused the reversal of the methylation pattern to that of wild-type or $RLIP^{-/-}$ mice. In addition, a significant number of promoters in the control $p53^{-/-}$ mice were hypermethylated. Remarkably, their methylation pattern also reverted to that of WT mice upon depletion of RLIP [24]. These findings have fundamental and paradigm-shifting implications. Identification of the gene-specific changes in promoter methylation may provide important clues to the underlying p53-related molecular mechanisms of carcinogenesis. More significantly, the dramatic reversion of both hypo and hypermethylated genes to WT methylation levels indicates a fundamental role of RLIP in governing the pattern of promoter methylation affected by p53 loss. Because DNA methylation enzymes and their signaling controls are heterogeneous, it is unlikely that all are directly affected by RLIP. Instead, these findings suggest alterations to the cellular milieu due to shifts in biochemical pathways that have global effects. Because catalysis of the ATP-dependent transmembrane transport of GS-Es and the consequent regulation of oxidative stress are the primary functions of RLIP, these findings suggest a major role of oxidative stress in regulating the patterns of DNA methylation. This model-system also represents a powerful tool for studying the effects of oxidative stress, peroxidation of essential fatty acids, and drugs on epigenetic regulation (Fig 4).

Because $p53^{-/-}$ mice with normal DNA methylation patterns did not exhibit spontaneous carcinogenesis, it is reasonable to conclude that promoter hypomethylation in $p53^{-/-}$ mouse is a powerful determinant of cancer susceptibility. Promoter-specific loss of DNA methylation in $p53^{-/-}$ mice requires the full activity of RLIP, as this defect was reversed with even partial loss of RLIP. Taken together, these observations point to a fundamental and novel hypothesis that: the presence of RLIP is an essential requirement for carcinogenesis because the balance between the methylation and demethylation of DNA is determined by the ratio of p53/RLIP activity, and this ratio is regulated by oxidative stress-derived metabolites (Fig 3). Under normal conditions, the balance between these two proteins confers a normal degree and pattern of promoter methylation. In the absence of p53, the unopposed actions of RLIP reduce promoter methylation, promoting carcinogenesis.

In the absence of RLIP, the unopposed actions of p53 augment promoter methylation, conferring resistance to carcinogenesis. We believe that: 1) increasing the expression of RLIP protein will accelerate age-dependent promoter hypomethylation, resulting in earlier onset of carcinogenesis in $p53^{-/-}$ mice; and 2) in mice with homozygous loss of both RLIP and p53, the cancer-suppressive effects of RLIP will predominate over the cancer-promoting effects of p53 loss. These changes will be associated with reductions and increases in lipid peroxidation, respectively.

Genome-wide changes in DNA methylation are considered hallmarks of cancer development [62, 63]. Many cancers, including lymphomas, exhibit gains and losses in CpG islands and repetitive elements in tumor suppressor/oncogene-related signaling genes, cell adhesion genes, and key developmental genes [63]. Although there is a lack of significant effects on oxidative or DNA repair upon RLIP deletion in $p53^{-/-}$ mice, given the profound inhibition of lymphoma observed, we considered the alternative explanation that the DNA damage prevented by RLIP depletion is epigenetic, because p53 is known to be involved in the regulation of DNA methylation [29]. Furthermore, it is possible that the interactions of TNFα with RLIP and p53 could play a role in these effects, because TNFα treatment has been shown to exert global methylation effects in cultured non-malignant cells [24].

These studies present conclusive evidence that partial RLIP depletion dramatically inhibits spontaneous neoplasia in $p53^{-/-}$ mice, which has not been previously achieved by any other intervention. This phenomenon cannot be explained by alterations in oxidative stress or DNA repair, but global alterations in peptide hormone-initiated signaling due to interruption of CDE may play a significant role. The hypoglycemic, hypolipidemic, insulin-sensitive [42, 43], obesity-resistant [61], cancer-resistant, and CDE-deficient [15, 64] phenotype of $RLIP^{-/-}$ mice strongly supports the idea of the global disruption of peptide hormone signaling.

Overall, the mechanisms underlying the protective effects of RLIP depletion against cancer in $p53^{-/-}$ mice were reviewed. These studies showed that key p53-linked pro-carcinogenic pathways were inhibited and pro-apoptotic pathways activated. The epigenomic effects of RLIP depletion were analyzed by genome-wide methylation-specific sequencing using Solexa technology, demonstrating that the genome of $p53^{-/-}$ mice is subject to extensive hypomethylation of both genes bodies and their promoters [24]. Remarkably, depletion of RLIP protein to ~50% after eight months of weekly intraperitoneal RLIP antisense injections caused a dramatic normalization of the methylation status of the $p53^{-/-}$ mouse genome. Furthermore, the overexpression of several key genes with hypomethylated promoters was reversed by depleting RLIP. These striking and novel findings indicate that altered expression of carcinogenic proteins in $p53^{-/-}$ mice depends on changes in DNA methylation regulated by RLIP [24]. Because RLIP is a primary determinant of the accumulation of cellular alkenals, such as 4HNE, and 4HNE can react with CpG islands to influence DNA methylation, we proposed the entirely novel hypothesis that global changes in DNA methylation are a major mechanism by which depletion of RLIP causes cancer suppression.

Regardless of the mechanism, which remains to be elucidated through future studies, the studies reported here create an entirely new paradigm for addressing the therapeutic

challenge of treating p53-deficient malignancies. Because the partial depletion of RLIP is sufficient to block spontaneous carcinogenesis, the chances of adverse off-target effects are relatively low compared to those if complete target inhibition was required; thus, RLIP depletion is an especially attractive potential cancer therapeutic strategy. In addition, the demonstrated beneficial side effects of RLIP blockade on metabolic syndrome characteristics could be desirable. The broad range of effects of RLIP depletion across multiple signaling pathways also suggests the possibility of synergistic therapeutic effects with other targeted therapies, especially the newly developing T cell-targeted immunotherapies. Indeed, our studies demonstrated that characteristic T cell lymphoma, which develops by six months of age in $p53^{-/-}$ mice, is completely suppressed by antisense-mediated partial depletion of RLIP. Remarkably, widespread promoter hypomethylation at baseline in $p53^{-/-}$ mice was nearly completely reversed upon prolonged suppression of RLIP by antisense. These studies demonstrate the importance of p53 and RLIP in maintaining normal DNA methylation patterns and the necessity of RLIP in cancer cell survival; however, the detailed mechanisms underlying these fundamental observations remain unknown. The discoveries outlined in review have broad implications regarding the mechanisms of carcinogenesis and apoptosis resistance and open a new avenue of investigations into the role of excess RLIP and perhaps CDE as causes of carcinogenesis in $p53^{-/-}$ mice.

RLIP, HSF1, and p53 Interactions

The dramatic change in the cancer susceptibility phenotype of $p53^{-/-}$ mice upon RLIP depletion is consistent with previous work demonstrating that $p53^{+/+}/RLIP^{+/-}$ mice are highly resistant to chemical carcinogenesis [3, 13, 15] and suggests a haploinsufficiency phenomenon, in which deficiency of a protein with multiple binding partners simultaneously alters the quantity and relative ratios of multiple heterodimers, resulting in phenotypic effects disproportionate with the level of deficiency (Fig 3). HSF1 was an obvious candidate for involvement in the effects of RLIP haploinsufficiency because HSF1 binds p53 during stress-induced nuclear translocation, RLIP inhibits its nuclear translocation to regulate chaperone expression, and its deficiency converts the histological profile of malignancies in $p53^{-/-}$ mice from lymphoma to adenocarcinoma [55, 65–67]. We believe that RLIP is an essential effector that is necessary for the broad cancer-promoting epigenetic remodeling observed in $p53^{-/-}$ mice, and thus, through a haploinsufficiency mechanism, the partial depletion of RLIP provides protection from neoplasia. We suggest a model wherein the activities of HSF1, p53, and RLIP are coordinated to regulate transcriptional responses to stress, with RLIP serving as an anti-apoptotic effector and feedback inhibitor of p53 and HSF1 (Figs 2 and 3). Specifically, only the p53–HSF1 dimer can exist in the absence of RLIP, and only the RLIP–HSF1 dimer can exist in the absence of p53; conceptually, the former would play a role in cancer prevention and the latter in promotion. These interactions between HSF1, p53, and RLIP suggest haploinsufficiency interactions between the three proteins.

Other investigators have published interesting results showing that RLIP–HSF1 binding sequesters HSF1 in the cytosol, and under stress, the HSF1–RLIP–αTubulin–HSP90 complex dissociates upon the binding of Ral–GTP to RLIP (Fig 1B) [39]. The C-terminal

region of RLIP (aa 350–415) binds HSF1 [4], and the phosphorylated S^{326} residue of p53 serves in p53–HSF1 binding. Lack of p53–RLIP binding is implicated in drug resistance, lack of the RLIP–HSF1 dimer is associated with cancer resistance, and the HSF1–p53 dimer regulates stress resistance. All three interactions are implicated in regulating oxidative stress, a condition associated with epigenetic modification. RLIP, p53, and HSF1 affect cellular oxidative stress that causes genotoxicity and apoptosis (Figs 2–4). The MAP is the chief biochemical mechanism for oxidative stress defense, and RLIP is its rate-determining step. The physiological substrates for the MAP are electrophilic end-products produced by the degradation of LOOHs derived from the oxidation of PUFAs. P450 enzymes convert LOOHs to electrophilic toxins that are converted to GS-Es by GSTs and removed from cells by RLIP. LOOHs activate normal signaling through PI3K, but high levels activate stress defenses involving MAPK, RLIP, p53, HSF1, and chaperones [2, 3, 9].

RLIP, PKCα, and p53 Interactions

RLIP is a critical regulator of endocytosis, and PKCα activates the transport function of RLIP that is required for this essential role. Previous studies have provided strong evidence demonstrating that p53 inhibits the transport function of RLIP and RLIP binds to and prevents the nuclear translocation of p53. These findings also suggest the formation of a complex containing p53, PKCα, and RLIP, all three of which were immunoprecipitated by anti-RLIP antibodies. Immunocytochemical studies also confirmed the co-localization of RLIP and p53 in cells [21]. $RLIP^{-/-}$ mice are, in a sense, the diametric opposite of $p53^{-/-}$ mice in that the latter develop malignancies spontaneously. PKCα activates RLIP, and RLIP is necessary for carcinogenesis because it directly controls the function of PKCα, p53, and cancer-promoting peptide hormones by determining the rate of CDE.

RLIP is necessary for carcinogenesis because it controls PKCα and p53 by regulating the cellular concentrations of GS-Es and the function of cancer-promoting peptide hormones via CDE. The formation of p53–RLIP complexes in the cell membrane represents a novel and critical paradigm in carcinogenesis [21]. Previous studies showing the near-complete absence of chemical carcinogenesis mediated by BaP and PMA/DMBA, along with the lack of p53 activation and PKCα function in $RLIP^{-/-}$ mice, strongly indicate that RLIP is required for neoplastic transformation [15, 64]. These findings indicate the existential reliance on RLIP in cancer cells but not in normal cells. This need for RLIP in cancer cells will help define the fundamental role of the interactions between RLIP, PKCα, and p53 in cancer and could lead to the development of novel broad-spectrum RLIP-targeted anticancer agents.

P53 inhibits the transport activity of RLIP

Because the RLIP binding motif in the RLIP-binding protein cdc2, is also found in p53, p53 could also bind to and inhibit the transport activity of RLIP [68]. The RLIP–PKCα–p53 complex distinctly regulates cancer cell proliferation, receptor endocytosis, and metastatic potential. The interaction between p53 and RLIP has unveiled a new paradigm in cancer research. We discussed the significance of p53, PKCα, and RLIP on important processes in

cancer cells, such as the initiation of apoptosis caused by toxic carcinogens, regulation of the cell cycle, and endocytosis of receptors and their downstream targets.

Signaling crosstalk between RLIP, PKCα, and p53

The PUFA metabolite 4HNE plays a vital role in regulating the interactions between RLIP, PKCα, and p53. The products of lipid peroxidation and PUFA metabolites generated in cells regulate protein cross-linking and protein interactions between lysine and histidine residues [9]. RLIP depletion effectively antagonizes chemical neoplasia in $p53^{-/-}$ mice and alters critical downstream signaling events regulated by the RLIP–PKCα–p53 complex. In summary, the RLIP–PKCα–p53 complex represents an integrated and mechanistically relevant signaling node for effectively targeting carcinogenesis and multi-drug resistance induced by the loss of p53.

Potential mechanisms of action

Glutathione (GSH), a sulfhydryl-containing tripeptide and the chief soluble nucleophile in cells, serves to protect nucleophilic sites on DNA bases by scavenging mutagenic electrophilic chemicals. The anticancer effect of blocking the MAP pathway is not entirely unexpected because GSTs, which catalyze the first committed step of the MAP, are well-known markers of carcinogenesis [9]. Indeed, GSTs are used as prognostic markers for breast cancer (e.g., the GSTM1 gene in the Oncotype-DX test) [69], as markers of the transition from *in situ* to invasive neoplasia in prostate cancer [70], and as predictors of response to therapy in lung, ovarian, and colon cancers [71–73]. These observations highlight the characteristically high MAP activity in cancers, and the protective role of this pathway in cancer cells indicates that targeting this pathway is likely to be an effective strategy for treating these malignancies. Unfortunately, the large number of GST isoenzymes with overlapping substrate specificities renders them less desirable for targeted cancer therapy. In contrast, RLIP is an ideal target because it has no close structural homologs and its inhibition causes the intracellular accumulation of GS-Es, which are potent inducers of apoptosis and excellent GST inhibitors [74, 75]. In addition, 4HNE and other eicosanoid-derived aldehydes and epoxides that are precursors of GS-Es formed during lipid peroxidation display potent cancer-selective apoptotic effects, as well as genotoxicity that contributes to genetic instability. The lipid peroxidation of eicosanoids is known to increase under nearly all oxidative stress-inducing conditions, such as oxidant chemical or xenobiotic poison exposure, mitochondrial or ER stress, and high-energy radiation [76]. One of the earliest responses to stress is the membrane localization of RLIP, and the transport activity of RLIP plays an important anti-apoptotic role under stress conditions [30, 31]. Because these oxidative stress-inducing conditions are known to exert carcinogenic effects, RLIP may play a crucial role in preventing apoptosis of stressed cells, allowing them to survive long enough to develop critical mutations that lead to the loss of tumor suppressor function and the activation of oncogenes. This theoretical construct has much indirect support in the literature but has not yet been directly proven by measuring the rate of DNA lesion accumulation under conditions of RLIP depletion and overexpression. This construct also predicts that the cancer-protective effects of RLIP loss in homozygous knockout animals could be lost if pro-apoptotic alkenals are prevented from forming due

to nutritional deficiency in their precursors, PUFAs. Furthermore, this model suggests that 4HNE levels are low in $p53^{-/-}$ mice because the inhibition of RLIP by p53 is absent (Fig 4). The present review will elucidate the structure-function relationships of the interactions between RLIP, PKCα, HSF1, and p53 to explore the implications of targeted therapies against cancer progression and drug resistance.

RLIP controls the rate of CDE, mediates GS-E transport, and controls the MAP

GSH-linked metabolism protects cancer cells through several mechanisms that prevent oxidative damage to DNA and cellular proteins, metabolize and excrete chemotherapy drugs, defend against endogenous toxins generated as a consequence of drug or radiation exposure, and regulate stress-responsive signals, enzymes, metabolites, or repair mechanisms [7–9]. The MAP functions to metabolize and excrete chemotherapy drugs, as well as endogenous PUFA-derived pro-apoptotic metabolites that are formed as a result of exposure to either chemotherapy drugs or X-irradiation [9]. Because cancer cells overexpress MAP factors and are inherently more sensitive than non-malignant cells to the apoptotic effects of PUFA-derived free-radical, electrophilic, or oxidant metabolites, the MAP seems a logical target for therapies designed to specifically and inherently kill cancer cells. Neither GSH synthesis nor GSH-recycling enzymes, which function upstream of the MAP, are good candidates for targeted cancer therapy because GSH is important for the normal physiological functions of non-malignant cells. Furthermore, cytochrome P450 enzymes that feed toxic electrophilic metabolites into the MAP are not ideal candidates either because there is a multiplicity of isoenzymes with overlapping substrate specificities [8]. The first committed step of the MAP is catalyzed by GSTs that conjugate electrophilic toxins to GSH. Unfortunately, targeted inhibition of GSTs is similarly not suitable for cancer therapy because there are multiple GST isoenzymes with overlapping substrate specificities. Because GS-E cannot be further metabolized by intracellular enzymes, and because they are anionic, their energy-dependent efflux from cells is necessary for their subsequent metabolism by γ-glutamyl transpeptidase (γGT, a cell surface enzyme), dipeptidases, and N-acetylases to mercapturic acids, primarily in the kidneys.

GS-E efflux from cells has been a controversial and poorly understood process, catalyzed by several membrane transporters [2, 5, 6]. Using affinity chromatography methods, we purified RLIP as an ATP-dependent GS-E transporter (1, 4) and showed through knockout mouse studies that RLIP is the predominant MAP transporter of GS-E, responsible for about 80% of total GS-E transport [2–6]. A series of studies subsequently established that RLIP is induced by oxidative stress, confers resistance to both chemotherapy drugs and radiation, and functions as a critical anti-apoptotic protein in cancer cells [12, 13, 30, 31]. Indeed, $RLIP^{-/-}$ mice are almost completely resistant to carcinogenesis [15] and angiogenesis (64), and recent studies show that RLIP depletion prevents spontaneous carcinogenesis in $p53^{-/-}$ mice [24]. These studies, as well as a series of studies showing regression of melanoma [16], lung [17], colon [17], kidney [18], prostate [20], breast [23], and pancreatic cancers [22] upon RLIP depletion in animal models, indicate the existential importance of RLIP in cancer cells. In these studies, we demonstrated that the antineoplastic efficacy of RLIP depletion is based on the simultaneous inhibition of multiple cancer-critical signaling mechanisms, independent of the function of p53 or other cancer-specific signaling mutations.

The mechanisms for these remarkable anticancer effects of RLIP are not completely understood, but the near absence of CDE in RLIP$^{-/-}$ MEFs and the requirement for GS-E transport to reconstitute this defect in RLIP$^{-/-}$ MEFs [12, 15] has led to us to our novel, paradigm-shifting hypothesis that GS-E transport and CDE are integrally linked because the GS-E transport-coupled ATPase activity of RLIP provides energy for CDE. The significance of these findings lies in the fact that CDE is an essential process through which hormone–receptor complexes are internalized from the plasma membrane, which is necessary for the activation or termination of signaling downstream of peptide hormones [2, 5, 15]. The present review focuses on the prediction that blocking RLIP will inhibit the MAP and simultaneously the signaling downstream of EGF, TGFβ, HGF, and WNT, which is known to be important for the survival of cancer cells. Because this mechanism is expected to be independent of aberrant signaling in individual kinase pathways, RLIP could represent an effective target for broad-spectrum cancer therapy. This review will facilitate the major signaling pathways that are dysfunctional and associated with angiogenesis and malignant characteristics, including rapid cancer cell proliferation, resistance to apoptosis, invasion, and metastasis.

Relevance to Human Health

RLIP represents a key signaling hub governing cell growth, stress resistance, and drug resistance, which are further stimulated by PKCα. Thus, the combined inhibition of RLIP and PKCα is expected to be an excellent approach for treating many cancers, even without p53-induced aggressive carcinogenesis and multi-drug resistance. The studies presented in this review expanding our understanding of the highly significant and interactive cross-talk between RLIP, p53, HSF1, PKCα, and GSH signaling in multiple tumor models, laying a strong foundation for clinical studies to evaluate RLIP-targeted anticancer therapeutics. This review provides a sound mechanistic rationale in multiple tumor models to explore the findings that RLIP is a transporter of GS-Es, which arise from xenobiotic compounds, and of endogenously generated electrophilic compounds, particularly metabolites generated by the lipid peroxidation of PUFAs (i.e., linoleic, γ-linolenic, and arachidonic). Excessive consumption of PUFAs is known to be associated with an increased risk for cancer. Overall, GS-E transport by RLIP is an integral requirement for CDE, and RLIP interacts with the tumor suppressor p53. RLIP$^{-/-}$ mice are resistant to cancer and highly sensitive to the acute toxicity of chemicals (including carcinogens) and radiation poisoning.

RLIP is necessary for carcinogenesis because it directly controls: 1) the function of PKCα and p53 by regulating cellular concentrations of GS-E, and 2) the function of cancer-promoting peptide hormones by determining the rate of CDE. RLIP represents a unique target in cancer therapy because it functions as the rate-determining step not only of the MAP but also of endocytosis. Upon confirming the dysfunction of endocytosis in RLIP$^{-/-}$ mice [15], we accumulated strong evidence supporting a novel and encompassing paradigm for carcinogenesis, apoptosis resistance, drug resistance, and radiation resistance involving the essential role of the crucial MAP transporter RLIP, the tumor suppressor p53, and the proliferative kinase PKCα. This review delineates a novel interaction between RLIP, p53, and PKCα, which has striking and practical implications for our understanding of both carcinogenesis and the emergence of drug resistance.

Clinical Impact

RLIP$^{-/-}$ mice are almost completely resistant to chemical neoplasia. BaP and PMA/DMBA are ineffective in causing neoplasia in RLIP$^{-/-}$ mice, in which PKCα, p53, JNK, and p38 signaling are blocked or functionally affected [15]. p53 is a stress-responsive, genome-protective tumor suppressor whose functions are lost or altered in nearly all neoplasia. It is considered a guardian of the genome because of its central function in cell cycle checkpoint control. Nearly 100% of p53$^{-/-}$ mice develop spontaneous lymphoma or other malignancies by six months of age (24). In stark contrast, homozygous knockout of the stress-responsive, anti-apoptotic, MAP transporter RLIP results in marked protection from chemical carcinogenesis. Intermediary metabolites of this pathway regulate p53 expression and activation, and p53 regulates the expression of key enzymes of this pathway. p53 directly binds to RLIP through a cdc2 (CDK1)-interaction domain and inhibits its transport activity, as well as endocytosis-stimulatory activity. These studies indicate that RLIP is an effector of p53 in a manner analogous to its role as an effector of the Ral pathways that regulate membrane plasticity, motility, and invasion. This translates into reduced kinase signaling downstream of membrane receptor–ligand interactions. RLIP depletion causes apoptosis in cancer cells independent of p53 status, strengthening the view that RLIP is a key downstream effector of p53 and is required for malignancy in p53$^{-/-}$ mice.

The novel finding that tumor signaling is regulated by interactions between RLIP, PKCα, HSF1, and the tumor suppressor p53 can markedly evolve our fundamental understanding of carcinogenesis and drug/radiation resistance signaling in multiple models of cancer. We believe that the studies presented here will enable the characterization of the critical RLIP–PKCα –HSF1 –p53 regulatory signaling node, which will enable the evidence-based development of highly reliable algorithms for individualized therapy and preventing tumors in patients with distinct p53 genotypes.

These findings demonstrate that RLIP is integral to endocytosis and growth factor signaling in cancer. RLIP$^{-/-}$ mice are highly sensitive to the acute toxicity of chemicals and to radiation poisoning, which indicates that the mechanisms of oncogenic transformation in the presence of toxic carcinogens are deficient in RLIP$^{-/-}$ mice. The strong resistance to the carcinogens DMBA and PMA in of RLIP$^{-/-}$ mice places RLIP among the most potent oncogenes. Furthermore, this observation highlights the existential role of RLIP in cancer; that is, inappropriately high levels of RLIP are required to trigger the subsequent steps of carcinogenesis. Hence, the interactions between RLIP, PKCα, HSF1, and p53 represent an integrated and mechanistically relevant signaling node for effectively targeting the loss of p53-induced carcinogenesis and multi-drug resistance.

Significance

The p53 protein (human *TP53* gene) is a genome protective, stress-responsive, tumor suppressor protein which loses its normal function due to mutations or other genetic alterations in a substantial proportion of nearly all types of neoplasia. The powerful tumor suppressor function of p53 is evident in mice from the universal susceptibility of p53 knockout to spontaneous neoplasia. Homozygous p53 knockout (p53$^{-/-}$) mice die of spontaneous malignancy, most commonly T-cell lymphoma, before the age of 6 months

while heterozygous p53 knockout ($p53^{+/-}$) mice develop several types of malignancy before the age of 14 months. Though the development of spontaneous neoplasia in p53 deficient mice can be accelerated and histological type altered through genetic manipulations that inactivate tumor suppressors or activate oncogenes no previous single genetic modification has completely prevented $p53^{-/-}$ mice from developing spontaneous neoplasia.

RLIP (encoded by *RALBP1* [18p11.22]) is a membrane bound *stress-responsive* nucleotidase enzyme of xenobiotic metabolism that modulates ligand-receptor signaling through its functions in CDE and binds heat-shock factor-1 (HSF1) to govern transcriptional regulation of stress-induced chaperone responses. It serves in the MAP by catalyzing transmembrane efflux of GS-Es generated from GST-catalyzed reaction between genotoxic and proapoptotic electrophilic toxins. The protective effect of RLIP in stress-mediated apoptosis is the basis of a highly effective drug for treatment of radiation and chemical poisoning, and the constitutive anti-apoptotic function of RLIP is of much greater importance in cancer than normal cells. Anti-RLIP antibodies, or RLIP-specific siRNA or antisense, cause sustained regression of xenografts of multiple histologies of human malignancy including melanoma and neuroblastoma, as well as cancers of the lung, breast, colon, kidney, pancreas, and prostate. $RLIP^{-/-}$ mice are highly resistant to chemical carcinogenesis, essentially a polar opposite of the spontaneous cancer susceptibility of $p53^{-/-}$ mouse, leading us to posit that spontaneous malignancy in $p53^{-/-}$ mice would be ameliorated by pharmacologically induced RLIP deficiency using R508, an RLIP-specific phosphorothioate antisense. We observed an astounding 100% cancer free survival of $p53^{-/-}$ mice at 32 weeks of age, unprecedented for any previous pharmacological intervention. This was accompanied by dramatic reversion of the highly aberrant methylome of $p53^{-/-}$ mice to wildtype, with over 14,000 differentially methylated regions of DNA reduced to <100 by R508 treatment. Differential expression of inflammation, immunity, cancer, and stem-cell genes in $p53^{-/-}$ mice was normalized and intracellular signaling was reverted to a wild-type pattern by RLIP depletion. R508 caused hypoglycemia and hypolipidemia, both of which are characteristic of congenital RLIP deficiency in mice. Conclusive evidence for specificity was found in studies showing that offspring of crosses between p53 and RLIP knockout mice were also highly resistant to spontaneous as well as chemically induced malignancy. This degree of efficacy in cancer prevention in $p53^{-/-}$ mice also far exceeds that observed with any prior genetic intervention. Collectively, these studies demonstrated that hemizygous RLIP deficiency exerts a striking dominant negative effect on spontaneous malignancy in $p53^{-/-}$ mice, a paradigm altering discovery that will have broad impact on cancer therapy.

The overall theme of this review is to elucidate the molecular mechanisms for fundamental discovery. Because of pleiotropic interrelated functions of p53 and RLIP will not permit full elucidation of the underlying mechanisms, we have focused this review on the general hypothesis that RLIP deficiency switches off cancer susceptible phenotype of $p53^{-/-}$ mice through a haploinsufficiency mechanism that regulates stress-induced epigenetic remodeling of DNA though binding interactions between p53, HSF, and RLIP.

Future Perspectives

We believe that the studies described in this review, which focused on the critical interactions between the major cancer-regulating proteins RLIP, p53, HSF1, and PKCα, will have a worldwide impact on the development of therapies for nearly all cancers and perhaps other oxidative stress-related disorders, including diabetes, hyperlipidemia, atherosclerosis, and neurodegeneration. The broad effects of RLIP inhibition include reductions in blood sugar, cholesterol, and triglycerides. $RLIP^{-/-}$ mice are insulin-sensitive, have low levels of cholesterol and triglycerides, and completely lack CDE [42, 43]. This last observation is highly significant because CDE is a major controlling mechanism for many of the best-known targeted cancer therapies, including those targeting RTKs (insulin, IGF1, EGF, and VEGF signaling), STKs (TGFβ signaling), death receptors (TNF, TRAIL, and Fas-L signaling), and 7-transmembrane domain receptors (WNT, Notch, and Sonic hedgehog signaling). Marked resistance of the $RLIP^{-/-}$ mice to chemically induced carcinogenesis demonstrates an existential role of RLIP in neoplasia.

No interventions have protected mice from p53 loss-induced carcinogenesis as dramatically as RLIP depletion. Thus, the findings outlined here reveal a novel means of bypassing p53 loss, the greatest barrier to treating many of the most common and deadly malignancies. Future studies to elucidate the mechanisms underlying these observations will have significant and broad implications for our basic understanding of carcinogenesis and the development of therapeutics to prevent and cure cancer. In summary, these findings represent a mechanistic milestone in our understanding of the molecular basis of cancer, as RLIP is an existential component of carcinogenesis, and the future studies will shed light on discovering novel mechanisms of mutant p53-driven cancer metastasis and developing innovative therapeutics to improve clinical outcomes in patients harboring p53 mutations.

Concluding Remarks

It is essential to investigate how RLIP and p53 together regulate DNA hypomethylation and to test the paradigm-shifting hypothesis that RLIP is necessary for the epigenetic changes that predispose individuals deficient in p53 and other p53-related tumor suppressor genes to cancer formation and growth. This work to determine the molecular mechanisms of hereditary cancer in patients with p53 deficiency will enable us to determine how to best overcome their characteristic treatment resistance and prevent their near-certain risk of death from cancer.

RLIP depletion reduced blood glucose, triglycerides, and cholesterol by ~50% each, and insulin clamp studies showed a prolonged insulin effect due to loss of CDE, a process known to function in the termination of insulin signaling [42]. In contrast, $RLIP^{-/-}$ MEFs were resistant to EGF signaling, which is activated upon ligand–receptor endocytosis by CDE. $RLIP^{-/-}$ MEFs had reduced WNT signaling and increased TGFβ signaling, consistent with literature showing that CDE functions to activate WNT and inhibit TGF signaling [13]. Notably, only the expression of GS-E transport-capable mutants of RLIP restored endocytosis to normal in $RLIP^{-/-}$ MEFs, indicating that GS-E transport is required for CDE. A strong correlation was observed between RLIP transport activity and the CDE of

fluorescence-labeled insulin or EGF in 28 different cancer cell lines. Thus, RLIP can be dissociated from CDE and continue to function as an anti-apoptotic factor by regulating the efflux of GS-E. RLIP directly interacts with Ral, Ral-GAP (Ras-GAP), cdc42, cdc2, and HSF1 [2, 4, 32–34, 36, 37, 39, 68]. Proteins included in these complexes include receptor tyrosine kinases (RTKs), serine/threonine kinases (STKs), Src, PI3K, HSP90, and p53. The global dysregulation of peptide hormone signaling (i.e., involving EGF, VEGF, IGF, TGF, WNT, Notch, and BMP) upon RLIP disruption could occur due to loss of CDE. However, we favor the alternative and somewhat more radical hypothesis that the loss or inhibition of RLIP causes the accumulation of reactive lipid alkenals, which form alkyl adducts at critical cysteine and other nucleophilic residues on key signaling proteins, thus disrupting or promoting the formation of key signaling complexes that regulate quiescence, proliferation, differentiation, apoptosis, motility, and transformation.

Consistent with the reliance of cancer cells on RLIP, we have demonstrated that depleting or inhibiting RLIP using antisense or antibodies, respectively, has striking and broad effects in a range of cellular and animal models of human neoplasia. Similarly, systemic RLIP-depleting treatments with phosphorothioate antisense (R508), antibodies, or siRNA cause tumor regression and prolonged tumor-free survival (for up to 8 months) in animal models of various cancers. Critically, the animals gain weight normally after treatment, with no adverse effects. Indeed, treatment with R508 to deplete RLIP to <1% of the level measured in control animals did not cause significant organ toxicity, suggesting that non-malignant cells do not require RLIP for survival [5, 6, 16–23]. Taken together, these findings support a critical role of RLIP in cancer but leave open important questions regarding how RLIP controls peptide hormone and intracellular kinase signaling, angiogenesis, carcinogenesis, and DNA methylation. We assume that the broad effects of RLIP depletion on these processes are a consequence of its key catalytic role in coupling GS-E efflux with CDE, simultaneously controlling peptide hormone signaling and the intracellular concentrations of oxidative metabolites of PUFA that have broad effects on intracellular signaling and genetic regulation. This review provides an essential, broad mechanistic basis for the anticancer effects of targeting interactions between RLIP, PKCα, HSF1, and p53 and activating the p53 pathway. The studies described here also provide information invaluable for the development of additional targeted anticancer interventions and will help to address the therapeutic challenges associated with p53-deficient cancers in particular.

Furthermore, many interesting aspects of mutant p53-driven metastasis remain to be elucidated (*see* Outstanding Questions). For example, how does mutant p53 affect individual steps of metastasis such as cancer cell intravasation, circulation, and extravasation? What is the role of mutant p53 in regulating metastatic cell dormancy? Does immune regulation contribute to mutant p53-driven metastasis? Future studies may provide insights into therapeutic approaches to target RLIP in primary tumors versus metastatic tumors and demonstrate the role of RLIP in metastatic tumor cell chemoresistance.

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The abbreviations used are:

CDE	clathrin-dependent endocytosis
GSH	glutathione
GS-E	glutathione-electrophile conjugate
GST	glutathione-S-transferase
4HNE	4-hydroxynonenal
LFS	Li Fraumeni syndrome
LOOH	lipid-hydroperoxide
MAP	mercapturic acid pathway
MEF	mouse embryonic fibroblast
PKC	protein kinase C
PUFA	ω−6 polyunsaturated fatty acid
RLIP	Ral-interacting protein
RTK	receptor tyrosine kinases
STK	serine/threonine kinase

Glossary

Carcinogenesis
the production of cancer.

Chemical carcinogenesis
chemical carcinogens are chemicals (such as phorbol ester, benzo pyrene, etc.) which have been demonstrated to cause tumors in mammalian species.

Epithelial-mesenchymal transition (EMT)
a cellular process where cell–cell adhesion and cell polarity is disrupted in epithelial cells and mesenchymal transcriptional programs are activated. It is an important process in development, wound healing, fibrosis, and cancer progression.

Glutathione-conjugates
a phase II detoxification reaction in the liver; glutathione combines with toxins and converts them into water-soluble mercaptates.

Glutathione S transferase (GST)

a family of enzymes involved in metabolism and in making toxic compounds less harmful to the body.

Lipid peroxidation
lipid peroxidation refers to the oxidative degradation of lipids. It is the process in which free radicals “steal” electrons from the lipids in cell membranes, resulting in cell damage. This process proceeds by a free radical chain reaction mechanism.

Mercapturic acid
a condensation product formed from the coupling of cysteine with aromatic compounds, formed as a conjugate in the liver and excreted in the urine.

Mercapturic acid pathway (MAP)
a glutathione-dependent pathway for the detoxification of a number of compounds, including arene oxides; an S-substituted glutathione is formed and ultimately converted to a mercapturic acid (N-acetylated S-cysteine), which is excreted.

Metastasis
a multistep process whereby cancer cells spread from the primary tumor and colonize in distant organs by way of blood or the lymphatic system.

Oxidative stress
oxidative stress is defined as a persistent imbalance between antioxidants and pro-oxidants in favor of the latter, resulting in irreversible cellular damages. Oxidative stress may play a part in developing chronic health conditions like cancer, heart disease and diabetes.

Reactive oxygen species
species such as superoxide ion, hydrogen peroxide, and hydroxyl radical, which have an unpaired electron. At low levels, these species may function in cell signalling processes. At higher levels, these species may damage cellular macromolecules (such as DNA and RNA) and participate in apoptosis.

RLIP
a 76 kDa ral-interacting protein; RLIP pumps out the toxic chemicals that accumulate in the cancer cell as a result of chemotherapy or radiation therapy, before they can cause cell death. Removing the RLIP, might keep the toxins in the cancer cells long enough to kill them. “RLIP works like a fan, with an exhaust sucking out the toxins from cells”.

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Outstanding Questions:

- What are the others non-ABC transporter (s) for the efflux of glutathione-electrophile conjugates (GS-Es) and other toxins from cells?
- What is the most differentially expressed upstream regulator in RLIP$^{-/-}$ mice?
- How intracellular signaling in carcinogenesis and drug/radiation resistance is regulated?
- How does RLIP and p53 together regulate DNA hypomethylation?
- How does RLIP controls peptide hormone and intracellular kinase signaling, angiogenesis, carcinogenesis, and DNA methylation?
- What is the possible relationship of the epigenome and mutant p53 in tumor metastasis?
- How does mutant p53 affect intravasation, circulating tumor cell survival, and extravasation in the metastatic cascade?
- How does mutant p53 regulate the immune system in tumor metastasis?

Highlights

The salient features of this review article with potential clinical relevance include:

- RLIP$^{-/-}$ and p53$^{-/-}$ mice are polar opposites in the spectrum of cancer susceptibility.
- RLIP is a MAP transporter protein that is integral to CDE.
- Mutual binding interactions of p53, HSF1, and RLIP
- PKCα activates RLIP, and RLIP is necessary for carcinogenesis.
- The RLIP–PKCα–p53 complex distinctly regulates cancer cell proliferation, receptor endocytosis, and metastatic potential
- p53 is the most frequently mutated gene in human cancers. Mutant p53 can exert oncogenic effects and enhance metastasis in diverse cancers.

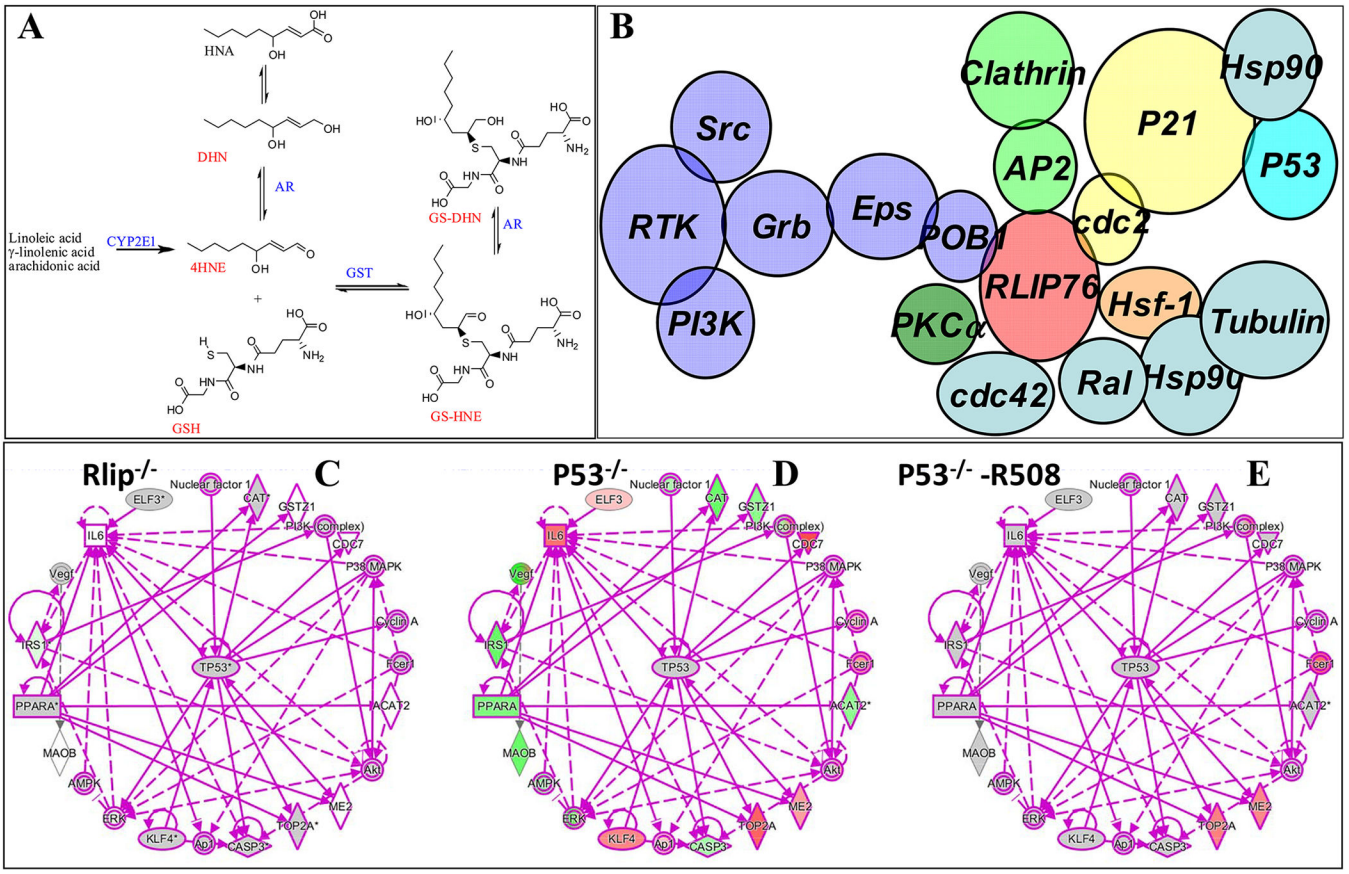

Figure 1.

(A) Oxidative metabolism of PUFAs. A model for the mechanism by which RLIP controls signaling by regulating cellular levels of HNE and its metabolites (GS-HNE and GS-DHN). Endogenous electrophilic compounds, especially those derived from essential PUFAs, are metabolized through a GSH-linked process; the rate of this metabolism controls receptor–ligand signaling. **(B)** Protein–protein complexes in which RLIP has been identified. **A proposed gene signature and network regulated by p53 and RLIP.** RNA-seq gene expression data from $p53^{-/-}$ and $RLIP^{+/-}$ mice were normalized to wild-type expression, and genes that were differentially expressed were filtered at −log (*p*-value) <7. This analysis yielded a set of 23 genes that were significantly altered in opposing directions in $p53^{-/-}$ *vs.* $RLIP^{-/-}$ mice. An overlay of data from $RLIP^{-/-}$ mice (**C**) and RLIP antisense (R508)-treated $p53^{-/-}$ mice (**E**) showed a pattern in which the deviations from wild-type expression typically observed in $p53^{-/-}$ mice (**D**) were normalized upon RLIP depletion. We predict that all mice with low RLIP expression will be cancer-resistant and exhibit the pattern seen in (**C**). Mice with normal or high RLIP levels will show a pattern that is determined by p53 or HSF. Balanced losses in RLIP and p53 should yield wild-type cancer susceptibility and a wild-type gene signature.

Mutation or Copy Number Changes in RLIP, HSF1, or p53 in Human Malignancy (TCGA Database)

A

Alterations in Rlip, p53 or Hsf1 in TCGA Provisional Database from cBioPortal

Histology	cases (#)	Genetic alterations (%)			
		Total	Rlip	p53	Hsf1
Uterine Carcinosarcoma	56	91	2	91	13
Ovarian Serous Cystadenocarcinom	311	91	3	87	33
Lung Squamous	178	81	2	79	7
Head & Neck Squamous Carcinoma	302	73	2	70	10
Pancreatic Adenocarcinoma	90	68	4	66	8
Bladder Urothelial Carcinoma	127	58	6	53	6
Colorectal Adenocarcinoma	220	58	3	54	6
Lung Adenocarcinoma	172	55	2	57	7
Glioma, Low Grade	286	52	0.3	51	9
Stomach Adenocarcinoma	220	50	3	45	6
Liver Adenocarcinoma	193	43	2	33	17
Breast Invasive Carcinoma	962	40	1	32	15
Kidney, Chromophobe Adenoma	66	36	0	33	3
Glioblastoma	273	31	0.7	30	1
Uterine Corpus Endometrial Carcin	242	31	3	28	5
Melanoma	278	25	2	17	8
Adrenocortical Carcinoma	88	22	1	19	2
Prostate Adenocarcinoma	258	21	1	15	7
Esophagus	184	19	3	2	15
Sarcoma	257	15	2	12	4
Diffuse Large B-Cell Lymphoma	48	15	4	4	6
Cervical Squamous Carcinoma	191	12	3	5	5
Acute Myelogenous Leukemia	188	9	0	8	0.5
Renal Cell Carcinoma, Clear Cell	414	3	0.2	2	1
Thyroid	400	2	0	1	1
Pheochromocytoma	158	2	0.6	1	0

B

C

Mutations in Rlip, p53 or Hsf1 Across Human Cancers*

Gene Combination	Overall Alteration Frequency	Notable Pattern Finding	Notable Pattern Frequency
Rlip	2.4%	Deletions	16.6%
p53	59.4%	Amplifications	0.9%
Hsf1	10.4%	Amplification	90.8%
p53+Rlip	0.9%	Rlip Amplified	67.9%
p53+Hsf1	4.8%	Hsf1 Amplified	89.2%
Rlip-Hsf1	0.1%	Both Mutated	40.0%

* n = 6,171 cases, TCGA provisional database, cBioportal analysis

Analysis Summary

1. **Rlip**
   1. Genetic alterations are rare in cancer (2.44% - of these 48% are Rlip amplifications and 16% deletions)
   2. Most altered cases have amplification
   3. They do not occur when p53 is normal
2. **HSF1**
   1. Mutations are very rare
   2. Alterations are more common than Rlip (10.43%, of these 91% are amplifications).
   3. They are essentially exclusive of Rlip alterations
   4. They essentially do not occur when p53 is normal
3. **P53**
   1. Loss of function alterations are frequent
   2. Hsf1 amplification frequently occurs when p53 is altered

4. p53-Rlip concomitant alterations are rare (0.85%, of these 67% have Rlip amplifications).
5. p53-Hsf1 concomitant alterations are seen in 4.78% (of these 89% have 53 loss and Hsf1 amplification).
6. Rlip-Hsf1 concomitant alterations are very rare (0.08% with both, of these 40% are dual mutations, and dual deletions are never found).
7. Rlip-Hsf1-p53 concomitant mutations are exceedingly rare in all three genes simultaneously (0.05%).

Figure 2. Genetic alterations of RLIP, HSF1, and p53 in cancer.

Genomic data from TCGA was queried using cBioportal for copy number alterations across 26 human cancers. The results (**A**) show total cases and genetic alterations in either gene. Case-wise alterations (mutation: green; **truncation: black**; deletion: blue; amplification: red; **no change: gray**) are represented for the 6,171 cases analyzed (**B**). Significant and notable findings from additional queries to determine the co-occurrence or exclusivity of these alterations are also presented (**C**), and a summary of our interpretation is given below.

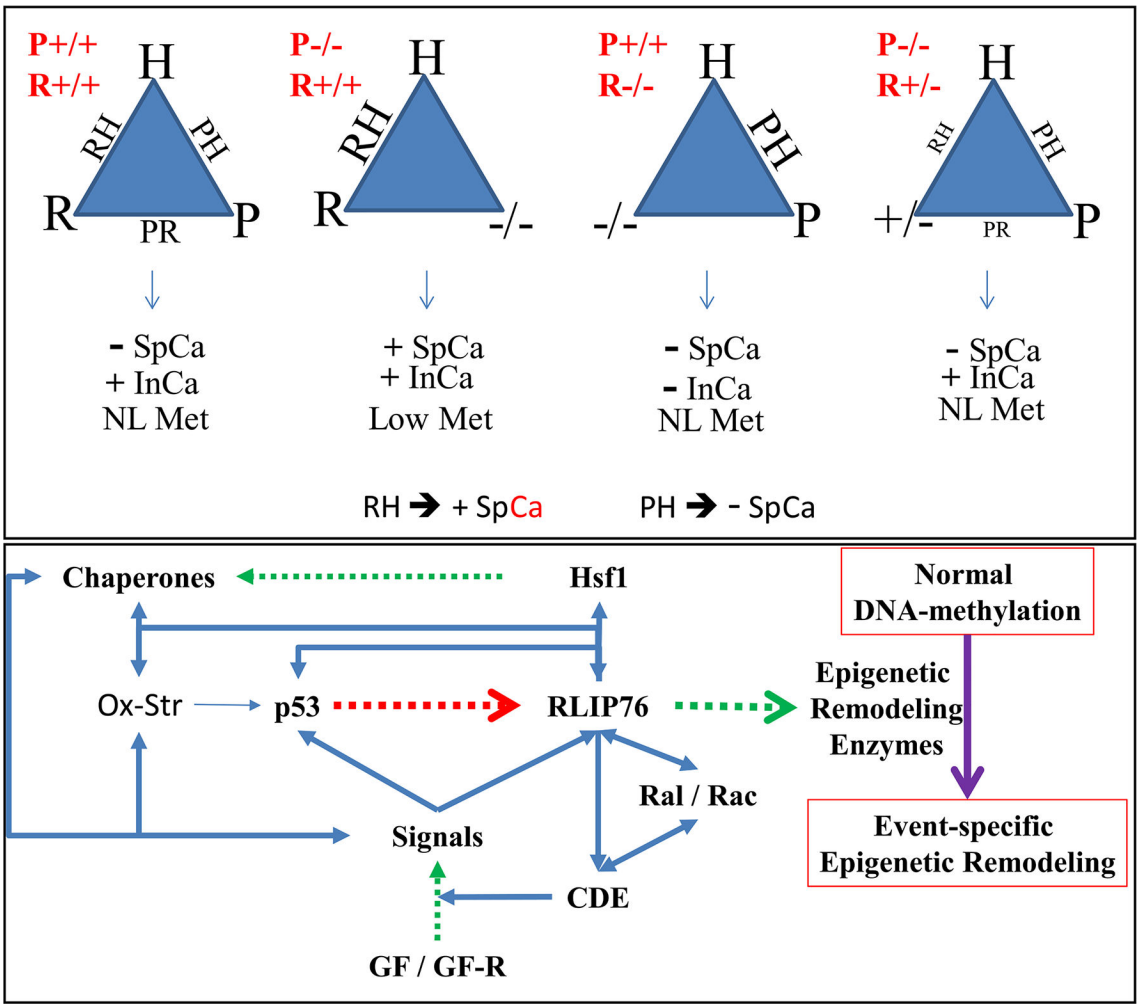

Figure 3. A hypothetical model for the role of the RLIP–p53–Hsf1 interaction on carcinogenesis and DNA methylation.

H=HSF1; P=p53; R=RLIP; −/− = homozygous knockout; +/− = heterozygous knockout; RH, PH, and PR are heterodimers; SpCa = spontaneous cancer; InCa = inducible cancer; Met = DNA methylation (**Upper Panel**). A signaling model is presented with putative inhibition (red); activation (green); mutual regulation (blue); and enzymatic signaling (purple) (**Lower Panel**).

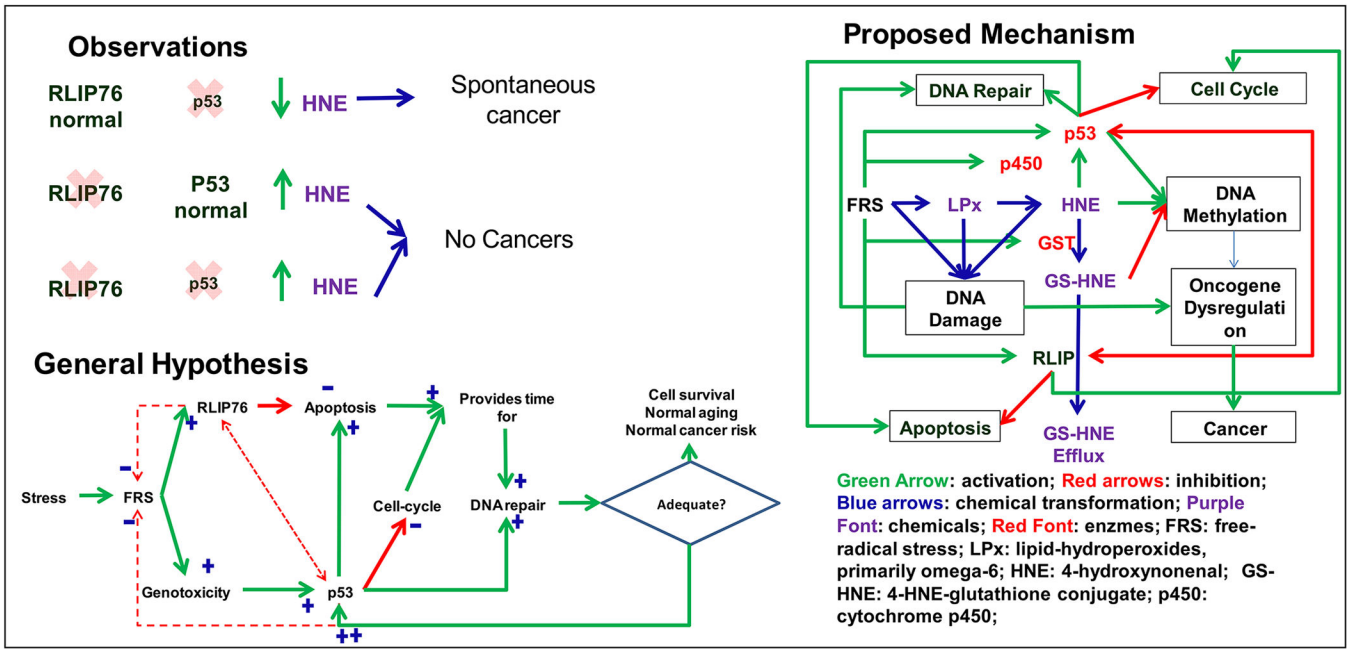

Figure 4.

Proposed mechanism of cancer prevention in $p53^{-/-}$ mice by RLIP depletion.