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Targeting Tumor Suppressor Networks for Cancer Therapeutics

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Abstract

Cancer is a consequence of mutations in genes that control cell proliferation, differentiation and cellular homeostasis. These genes are classified into two categories: oncogenes and tumor suppressor genes. Together, overexpression of oncogenes and loss of tumor suppressors are the dominant driving forces for tumorigenesis. Hence, targeting oncogenes and tumor suppressors hold tremendous therapeutic potential for cancer treatment. In the last decade, the predominant cancer drug discovery strategy has relied on a traditional reductionist approach of dissecting molecular signaling pathways and designing inhibitors for the selected oncogenic targets. Remarkable therapies have been developed using this approach; however, targeting oncogenes is only part of the picture. Our understanding of the importance of tumor suppressors in preventing tumorigenesis has also advanced significantly and provides a new therapeutic window of opportunity. Given that tumor suppressors are frequently mutated, deleted, or silenced with loss-of-function, restoring their normal functions to treat cancer holds tremendous therapeutic potential. With the rapid expansion in our knowledge on cancer over the last several decades, developing effective anticancer regimens against tumor suppressor pathways has never been more promising. In this article, we will review the concept of tumor suppression, and outline the major therapeutic strategies and challenges of targeting tumor suppressor networks for cancer therapeutics.

Keywords

tumor suppressors; RB; p53; BRCA1; BRCA2; gene therapy; small molecule inhibitors

I. An overview of tumor suppressors

a. A glimpse of history

The genetic basis for cancer was originally proposed by pioneer German pathologist, Theodor Boveri in 1914 (1). Herman J. Muller advanced the notion that cells needed to acquire multiple genetic mutations before becoming cancerous, based on observations of the

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long latent period between carcinogen exposure and cancer appearance (2). Moreover, epidemiology studies of common cancers revealed that cancer incidence increases with age, which further supported the concept that the initiation of cancer requires multiple mutations (3). Today, it is commonly understood that cancer is a consequence of multiple genetic and epigenetic alterations, which are either inherited or acquired through somatic mutations.

In general, there are two classes of genes that are frequently mutated in cancer: oncogenes and tumor suppressor genes. An oncogene is an oncogenic variant of the normal proto-oncogene that acquired a gain-of-function alteration, resulting from point mutations, chromosome rearrangements, or amplification of the proto-oncogene sequences. In 1911, Peyton Rous discovered the first oncogenic retrovirus, Rous Sarcoma Virus (RSV), and demonstrated that sarcomas could be virally induced by RSV in chickens (4). Sixty years later, in 1970, the first retroviral oncogene, *SRC* (or *v-src*), was identified in the oncogenic region of RSV (5). Shortly after the cellular proto-oncogene of *SRC* (*c-src*) was isolated, the virally transduced and mutated copy of *SRC* was demonstrated to be the causative driving force behind RSV induced tumors (6, 7). Since the first oncogene was discovered, finding for additional cancer causing genes has mushroomed.

Despite the significance of oncogenes in the genesis of tumors, many of the altered properties of cancer cells are also attributed to the inactivation or loss of normal cellular regulatory genes, known as tumor suppressor genes. In Boveri's early works, he predicted the presence of not only oncogenes but also tumor suppressor genes, "…cells of tumor with unlimited growth would arise if those 'inhibiting chromosomes' were eliminated." Tumor suppressor genes play important roles in suppressing uncontrolled proliferation, immortality, and tumorgenicity. Such tumor suppressing properties were first demonstrated in the late 1960s, when Henry Harris reverted highly malignant mouse ascities tumor cells to a nontumorigenic state by fusing the malignant cell with a normal fibroblast. The results from this study indicated that factors present in normal cells could inhibit (or suppress) the tumorigenicity of malignant cells (8). Though controversial at the time, Henry Harris' observation suggested the existence of certain intrinsic cellular factors that could suppress tumor development in a dominant manner.

b. Discovery of the first tumor suppressor: retinoblastoma susceptibility gene, RB

The very first tumor suppressor gene to be identified and characterized was the Retinoblastoma Susceptibility gene, *RB.* Retinoblastoma is a rare childhood eye tumor that can be either familial or sporadic (hereditary or non-hereditary). In approximately one-fourth of the retinoblastoma cases, tumors develop in both eyes (bilateral); whereas in the remaining cases, only one eye is affected (unilateral) (9). Using Poisson statistics, Alfred Knudson reasoned that the distribution of the observed bilateral and unilateral retinoblastoma cases could only be caused by two mutational events. This theory became known as the "two-hit" hypothesis, and suggested that tumorigenesis requires two mutational events to inactivate the two functional copies of the "tumor suppressor gene" (10). In familial retinoblastoma, the first inactivating mutation is inherited, while the second mutational event occurs spontaneously in the second allele in the same cell. In the sporadic form, both functional alleles are spontaneously mutated. For children who inherit one

mutational “hit” from their parents, the chance of getting another spontaneous mutation in the same retinal cell and developing retinoblastoma is much higher than having two spontaneous mutations on the same susceptibility locus. Consequently, familial retinoblastoma is usually bilateral while sporadic form is unilateral. Later, David Comings unified a general framework for the role of tumor suppressors for all types of cancers. He suggested that dominantly inherited tumors may result from the loss or inactivation of both alleles of suppressor genes that, when active, prevent the expression of transforming genes (possibly oncogenes) normally active only during embryogenesis (11).

Another interesting observation with retinoblastoma is that incipient cancer cells are able to invent ways to eliminate wild-type copies of tumor suppressor genes, a phenomenon now known as “Loss-of-Heterozygosity” (LOH). Using LOH as a guide, subsequent investigations on patients with both a personal and family history of retinoblastoma showed that chromosomal deletion within 13q14, or 13q deletion mosaicism, is associated with an increased risk of developing retinoblastoma (12). The retinoblastoma susceptibility gene, *RB*, was then identified, which provides a solution to the genetic puzzle of dominantly inherited neoplastic diseases (13, 14). Further analysis showed that *RB* encodes a nuclear phosphoprotein with molecular weight of about 105kD, RB or pRb, which is found to be absent or present in a defective form in retinoblastoma, osteosarcoma, breast cancer (15), and small-cell lung carcinoma (16). Homozygous knockout mice carrying non-functional *RB-1* died before the E. 14 with multiple developmental defects in the hematopoietic and nervous systems. Heterozygous knockout mice developed spontaneous pituitary tumors from 2 to 11 months of age with a nearly 100% incidence (17, 18).

Since its discovery, RB has been the subject of intense study (Figure 1). RB is now known as a universal cell cycle regulator with a central role in governing the passage of cells through the G1 phase, and particularly, the restriction point (R point), control of which is lost in most cancer cells (19). Under normal condition, RB is phosphorylated by cyclin D-CDK4/6 and cyclin E-CDK2 complexes upon mitogenic stimulus (20, 21). The activities of cyclin-CDK complexes are negatively regulated by CDK inhibitors (CKIs), including four INK4 proteins (Inhibitor of CDK4, $p16^{INK4A}$, $p15^{INK4B}$, $p18^{INK4C}$, $p19^{INK4D}$) that specifically antagonize CDK4/6, and three remaining CDK inhibitors ($p21^{Cip1}$, $p27^{Kip1}$, $p57^{Kip2}$) that have a broader inhibition spectrum (21, 22). The dephosphorylation of RB at the exit of M phase is performed by phosphatase 1α (PP1α), which has been demonstrated to compete with CDKs for a common binding site on RB (23).

When RB is unphosphorylated or hypo-phosphorylated, it binds and sequesters the transcriptional activator, E2F, to repress transcription of target genes with the help from chromatin remodeling complexes and Histone Deacetylases (HDACs). However, when hyperphosphorylated, RB dissociates from the E2Fs, allowing E2F/DP to interact with histone acetylase to activate transcription (24). This classic view of RB tumor suppressing activity is focused on this negative regulation of E2F transcriptional activation and cell cycle inhibition. It is surprising when two recent studies demonstrated that the presumably oncogenic E2F family proteins are dispensable for proliferation *in vivo* (25, 26). Such discoveries, combined with the fact that E2Fs are less frequently mutated in cancer, suggests that RB may have other functions besides E2F-dependent transcriptional regulation. So far,

RB has been demonstrated to play an important role in faithful chromosome segregation (27, 28), checkpoint control (29), apoptosis (30), senescence (31), and terminal differentiation (32). Detailed molecular studies revealed that these functions could be mediated by post-translational modifications on the C-terminal domain of RB, such as acetylation (33) and methylation (34), in response to internal or external signals (35). It is undeniable that RB suppresses tumor formation by virtue of its multiple biological activities upon receiving different signals, in addition to its role as a mediator between CDK regulatory pathways and E2F activators.

c. From oncogene to tumor suppressor: p53

Although initially discovered and inaccurately characterized as a weak oncogene, the wild-type *p53* was later confirmed to be a bona fide tumor suppressor (36–38). Sequence analysis of the oncogenic version of *p53* revealed a single base substitution mutation that led to a "gain of oncogenic function" activity. By the early 1990s, *p53* was widely recognized as the most frequently mutated tumor suppressor gene in human cancers. In ovarian cancer, esophageal cancer, colorectal cancer, and head and neck cancer, mutant alleles of *p53* or deletion of *p53* alleles are found in 40–50% of cases (39). In addition, germline *p53* mutations occur in patients with Li-Fraumeni syndrome, a hereditary predisposition to several cancers, especially soft tissue tumors (40, 41). Intriguingly, 75% of the more than 15,000 *p53* mutations identified in human tumor cell genomes are missense mutations, quite different from other tumor suppressor genes, such as *APC* (Adenomatosis Polyposis Coli), where frameshift and nonsense mutations account for more than 60% of mutations identified (42, 43). Biochemical analyses of p53 proteins showed that p53 is capable of forming tetramers, allowing the mutant protein to actively interfere with the function of wild-type protein in a dominant-negative fashion (44, 45). The presence of mutated alleles usually results in an accumulation of a faulty protein in the tumor cells, which also displays loss of heterozygosity (46).

Under normal conditions, p53 has a very short half-life of 20 minutes and a low steady-state protein level. However, upon receiving signals like UV radiation induced DNA damage, the degradation of p53 is blocked and p53 is stabilized to perform its functions (47). Many of p53's functions can be traced to its role as a transcription factor. More than 95% of *p53* mutations are found in the DNA-binding domain of the p53 protein, compared to other mutations that affect nuclear localization, oligomerization or transactivation. In response to DNA damage, p53 functions as a transcription factor that induces expression of genes such as $p21^{Cip1}$ to halt cell cycle progression at G1 phase (48). As mentioned above, $p21^{Cip1}$ inhibits RB activity by preventing the hyperphosphorylation of RB, providing another layer of regulation on cell cycle control. Delaying the cell cycle allows the cell to turn on DNA repair genes to mend the DNA damages. p53 has also been demonstrated to contribute to DNA repair directly by activating genes that facilitate nucleotide excision repair and base excision repair (49, 50). If the DNA damage is too severe to repair, wild-type p53 can redirect the cell into cell cycle arrest, senescence, or even apoptosis, by activating apoptosis-associated genes, such as *PUMA* (51). Thus, p53 plays essential roles in responding to various cellular stresses signals.

Similar to RB, p53 activities are modulated by a diverse range of post-translational modifications, which includes phosphorylation, acetylation, methylation, ubiquitination, and sumoylation (52). Intriguingly, a recent study investigating the importance of p53 acetylation on tumor suppression revealed that abolishment of p53-dependent apoptosis and/or cell-cycle arrest and senescence *in vivo* failed to induce early-onset tumorigenesis (53). This finding is consistent with previous studies that demonstrated depletion of $p21^{Cip1}$ (54) or PUMA (55), the primary mediators of p53-dependent cell-cycle arrest or apoptosis, respectively, does not lead to tumor susceptibility in the same way as loss of p53 does (56). Several other p53 mutants have also been shown to induce apoptosis or cell-cycle arrest independent tumor suppressor activities. This suggested that alternative mechanisms are involved in p53 associated tumorigenesis (57, 58). Indeed, as p53 deficient cancer cells often undergo aerobic glycolysis (also known as the Warburg effect), additional p53 functions in regulating glycolysis and Reactive Oxygen Species (ROS) have been proposed to further contribute to the tumor suppression effect of p53 (59). Understanding the role of p53 in regulating cellular metabolism and protecting cells from oxidative stress may provide new insight into p53 function and window for therapeutic intervention.

d. Breast cancer susceptibility genes: BRCA1 and BRCA2

Breast cancer is one of the most frequent malignancies with a cumulative lifetime risk of nearly 10% in women. Ovarian cancer, on the other hand, has a much lower lifetime risk of 1.8%, but is the most lethal cancers (60). In the 1990s, two Breast Cancer Susceptibility genes, *BRCA1* and *BRCA2*, were identified in patients with hereditary breast and ovarian cancer syndrome (61, 62). Female *BRCA1* or *BRCA2* mutation carriers have a lifetime breast cancer risk of 50–80% and a lifetime ovarian cancer risk of 10–20%. Studies showed that mutations in *BRCA1* account for almost all of the familial breast and ovarian cancer cases and up to 30–50% of families with hereditary breast cancer only. At one point, it was thought that *BRCA1* inactivation is not involved in sporadic breast cancers, but now inactive *BRCA1* alleles are found in about 10–15% of sporadic breast carcinomas. Mutations in *BRCA2* are linked to the other half of inherited breast cancer families and also to male breast cancer (63, 64).

Cellular and molecular studies showed that both BRCA1 and BRCA2 deficient cells tend to accumulate chromosome abnormalities, including chromosomal breaks, aberrant mitotic exchanges and aneuploidy, suggesting their normal function in DNA repair (Figure 2) (65, 66). BRCA1 is a nuclear phosphoprotein that is expressed and phosphorylated by the Ataxia Telangiectasia mutated (ATM) and Checkpoint Kinase 2 (CHK2) proteins in response to DNA damage (67, 68). BRCA1 is an essential component of the RAD50-MRE11-p95 complex, a recombination-mediated DNA double-stranded breaks (DSBs) repair complex, as well as the recombinase, RAD51, located in the nuclear foci of mitotic cells (69, 70). Similar to BRCA1, BRCA2 is also a nuclear phosphoprotein that binds to RAD51 and MRE11 to regulate DSB repair and maintain chromosome integrity (71, 72).

As aforementioned, DNA damage responses can often activate checkpoints through RB or p53 to arrest the cell cycle either at G1/S transition or G2/M transition (73), which enables the DNA repair pathway to correct and prevent the transmission of genetic errors to

daughter cells. A recent study showed that loss of p53-binding protein 1 (53BP1) could abandon the ATM-dependent checkpoint response triggered by the DBSs accumulated in BRCA1-deficient cells (74). Among different types of DNA damages, DSBs are considered to be the most harmful as the integrity of both strands of the DNA duplex are affected simultaneously. There are two major methods to repair DSBs in human, error-free homologous recombination (HR) and error-prone non-homologous end-joining (NHEJ). NHEJ is usually employed during G1 phase due to the absence of a sister chromatid; whereas HR repairs DSBs during the S and G2 phases, when an intact sister chromatid is available as a template for repair (75). Although BRCA1 and BRCA2 share many interacting proteins, their protein sequences are not conserved and they play divergent roles in regulating HR. BRCA1 not only serves as a scaffold protein that integrates different components of the HR machinery (76), but also enhances CtIP-mediated 5′-end resection of DSBs (77). In addition, cells carrying defective BRCA1 do not arrest in G2 phase after DNA damage, which indicates that BRCA1 is involved in mediating DNA-damage checkpoint control (78).

Besides its functions in DNA repair, BRCA1 also has important roles in transcriptional regulation (79, 80), differentiation (81), and ubiquitination as an E3 ligase (82–84). However, a recent study has shown that the E3 ligase activity of BRCA1 seems to be dispensable for tumor suppression (85). BRCA2, on the other side, is primarily involved in recruiting RAD51 to DNA damage sites or stalled replication forks (86). BRCA2 has also been reported to function as a RAD51 loader in facilitating telomere replication (87).

e. New classes of tumor suppressors

Classic tumor suppressors, such as RB and p53, play critical roles in governing the decisions of cells to proliferate, enter senescence or undergo apoptosis; therefore, they are termed as the “gatekeepers” of the cell. BRCA1 and BRCA2, on the other hand, function as “caretakers” to maintain the genome integrity. Loss of genome stability has now been recognized as a new cancer hallmark, which enables generation of random mutations that can potentially provide cancer cells with various growth advantages (88).

Avoiding immune destruction is another new cancer hallmark (89). The immune system can respond to cancer cells by reacting against antigens that are either unique to cancer cells (tumor-specific antigens), or differentially expressed between cancer and normal cells (tumor-associated antigens). Such antigens are often products of oncogenes and can incite strong inflammatory responses. Tumors can suppress the immune system both systemically and locally in the tumor microenvironment by expressing immuno-suppressive cytokines themselves, or activating regulatory T cells that can produce cytokines (90). Novel tumor suppressors have been identified that are capable of provoking immunity to target and destroy tumor cells.

Epigenetics is another exciting area in modern cancer biology research. Epigenetic alterations that can be somatically inherited without changing primary DNA sequences, have been proven to play a key role in tumorigenesis through activation of oncogenes or inactivation of tumor suppressors (91). A number of epigenetic modifiers like DNA methyltransferases, DNA hydroxylases, histone methyltransferases, histone

acetyltransferases and chromatin remodeling proteins, are now considered as tumor suppressors since inactivating mutations in these factors can result in decreased expression of classic tumor suppressors (92). Moreover, another group of tumor suppressors have been identified to be genes that are rarely mutated in cancer but frequently silenced by epigenetic changes like DNA methylation (93, 94).

In summary, loss of tumor suppressor genes liberates the cell from its growth-suppressing state or enhances cell survival in various manners. Tumor suppressors play an essential role in the formation of many kinds of human cancers. Therefore, targeting loss of tumor suppressors has tremendous potential as an effective therapeutic strategy against cancer.

f. Conceptual challenges of targeting tumor suppressors for cancer therapeutics

Our knowledge regarding the molecular genetics of tumor suppressors in cancer has grown tremendously over the last several decades. Studies have shown that the inactivation or deletion of certain tumor suppressor genes contribute to tumor development. Conversely, restoring normal expression of the missing tumor suppressor gene in cancer cells can re-establish normal growth suppressive properties and inhibit tumor development. Therefore, the next challenge is to translate this molecular understanding of tumor suppressor genes into viable therapeutic strategies that improve prognostic outcomes for patients.

There are two major strategies for targeting tumor suppressors: (1) reintroduce a functional copy of the tumor suppressor, i.e., gene therapy; (2) develop small molecule inhibitors that reactivate tumor suppressor function. However, both methods have been proven to be technically challenging. First, to re-express a functional tumor suppressor to comparable level as in the normal cells is easily said than done. The major limitations can be attributed to inefficiency in delivering and maintaining the tumor suppressors to all target cells within the bulk of the tumor due to host immune response, insertional mutagenesis, and transduction efficiency. Second, because cancer is a multigenic disorder, where multiple genes contribute to the development of the disease, loss of tumor suppressor function is quite often associated with gain of oncogene activity. Therefore, restoring tumor suppressor activity may not be sufficient to reverse the malignant phenotype.

Regardless the difficulties, numerous studies have shown that restoring or reactivating tumor suppressor function in tumor cells can induce cell-cycle arrest and/or apoptosis in cancer cells. Therefore, there is no doubt that targeting tumor suppressors is a therapeutically viable strategy. Although the challenges are great, there is reason to be optimistic. For example, new technological advancements in vector design have improved the efficiency and efficacy of targeted gene therapy in the last decade. Powerful computational and structural studies have also revealed new “drug-able” protein-protein interaction interfaces for small molecule inhibitors. The current endeavors in developing therapeutics that target the tumor suppressor network will be elaborated and discussed in the following sections.

II. Gene therapy

a. Concept

In the 1960s, scientists proposed to use the exogenous “good” DNA to replace the defective DNA in patients suffering from genetic diseases (95, 96). The very first trial was carried out in 1990 on a 4-year-old girl with ADA-SCID, a severe immune deficient disease (97). Since then, more than 1,900 clinical trials employing gene therapy have been conducted worldwide (For a searchable database on gene therapy clinical trials: *J. Gene Med.* Gene Therapy Clinical Trials Database. www.wiley.com).

The most common form of gene therapy utilizes DNA that encodes a functional gene to compensate for a dysfunctional gene. Alternatively, synthetic DNA that encodes a therapeutic protein drug or small interference RNA (siRNA) can also be used as treatment. Given that tumor suppressors are often deleted, mutated, or silenced, using gene therapy to restore wild-type tumor suppressor function is a logical approach for treating cancer patients. In theory, such manipulation can be performed on germline cells to correct the mutation or compensate the deletion of tumor suppressor genes; however, most of current investigations are focused on targeting somatic cells, partly due to safety concerns. Currently, there are two major methods of introducing genetic material into the body: viral and non-viral.

b. Viral delivery methods

Harnessing the innate power of viruses to deliver genetic information has been the mainstay strategy since the advent of gene therapy. Among the many different types of viruses, adenoviral vectors and adeno-associated viral vectors are most commonly used as they can be easily manipulated *in vitro* and are relatively safe *in vivo*. In the early investigations, a recombinant adenovirus carrying *RB* cDNA was injected intratumorally into spontaneous pituitary melanotroph tumors that arose in *RB* heterozygous mice (Figure 3). Such experiments demonstrated the proof of concept with promising efficacy in reducing tumor cell proliferation and prolonging the lifespan of the treated animals (98). In glioblastoma, the most malignant form of glioma, *RB* is inactivated in at least 30% of tumors and greater than 50% have disrupted RB pathway including homozygous $p16^{INK4}$ gene deletions (99). Alternative therapies are also highly desirable in glioblastoma as the existence of the blood-brain barrier renders strong resistance to most anticancer therapeutic agents due to low permeability across the barrier. However, such barrier favors viral-mediated gene therapy as the potential anti-adenoviral immune response is limited by the very blood-brain barrier (100). In addition, intratumoral injection of therapeutic adenoviruses is easily achieved using established surgical procedures for glioblastoma.

Besides RB, a large body of cancer gene therapy studies has focused on restoring p53 activity. Reactivating p53 has been of particular interest not only because loss of p53 tumor suppressor function is commonly present in most human cancers, but also because continuous p53 inactivation is required for tumor growth maintenance. Gendicine, a recombinant adenovirus carrying *p53* cDNA, was the first commercially available, viral-based gene therapy that was approved by regulatory agencies for treating head and neck

squamous cell carcinoma (China, 2003) (101). Subsequent trials have also shown Gendicine effective in other types of cancers, including hepatocellular carcinoma and gastric cancer (102, 103).

One major challenge with adenoviruses is their low infection efficiency. As a result, insufficient tumor cells are infected to show a significant therapeutic effect. To overcome this issue, tumor selective oncolytic adenoviruses were developed (104). This new generation of adenoviral vectors can selectively replicate in tumor cells but not normal cells, thereby, amplify the input viral dose and destroy the infected cells by virus-mediated cytolysis, and cause a progressive spread of virus particles in a given tumor. Oncolytic adenoviruses that can specifically replicate in tumor cells with different p53 or RB status, ONYX-015 (105) and AdΔ24 (106), respectively, are now being subjected to various phases of clinical trials in many countries. Although the initial clinical trial results suggest oncolytic adenoviruses have have limited efficacy in the clinic, combination treatment with standard chemotherapies show significantly better responses (107). In addition, great potential for targeting cancer stem cells has been reported in oncolytic adenovirus studies (108). Other types of viruses have also been tested for cancer gene therapy, including retroviruses and lentiviruses (109), as they are advantageous for their stably integration of the transferred gene into the chromosomes and capability for genetic engineering of the glycoprotein to target different cell types (110). However, oncogene activation by the retroviral and lentiviral vectors is a bigger safety concern compared to adenovirus.

Virus targeting, compounded by systemic versus local administration, is another obstacle for cancer gene therapies using viral vector. The virus with the most anticancer therapeutic potential must be capable of infecting cancer cells while sparing normal cells, although the native tropisms of viruses do not match such therapeutic needs. So far, substantial progress has been achieved with improvement in virus targeting in preclinical research (111). Alternatively, the possibility of using neural stem cells or human mesenchymal stem cells to systemically deliver the oncolytic viruses has been tested in glioblastoma. In the preclinical studies, the stem cells, which display a tropism for brain tumor, are loaded with oncolytic viruses before either intravascular or intracranial injections; hence this strategy is also referred to as “Trojan horse” strategy (112, 113). Nevertheless, the clinical efficacy for these novel viral delivery methods awaits further validation.

c. Non-viral delivery methods

Although less pursued compared to virus-mediated gene therapy methods, non-viral delivery methods possess advantages over viral delivery methods in terms of safety, low host immunogenicity, and simplicity for large scale production. Such non-viral approaches can be further divided into two categories: naked plasmids and plasmids encapsulated by liposome or polymer into nanoparticles, i.e., lipoplexes or polyplex, respectively.

Direct injection of naked plasmid DNA into muscle was first tested *in vivo* as it was simple and inexpensive. Despite a few studies showing that pressurized vascular delivery improves gene transfer in animals with a more systemic effect (114), the overall low expression levels of the transferred genes limited the potential applications of this method in the clinic. With continued improvement in technology, treatments coupling plasmid injection with

electroporation (114) or ultrasound (115), have significantly improved the efficacy of gene delivery. Electroporation, in particular, where a pair of electrode needles are inserted into the DNA injection site to deliver electric pulses, has been shown to enhance gene expression in targeted tissues by 2–3 orders of magnitude with minimal tissue damage as compared to the injection of plasmid DNA alone (116). Introducing cytokines into melanomas or head and neck cancer using direct intratumoral injection of DNA plasmids followed by electroporation is now undergoing extensive preclinical and clinical trials at various phases (117, 118). Other assisting methods such as laser and magnetic fields have also been assessed, although the efficiency of gene transfer *in vivo* needs further improvement (119, 120).

Usage of DNA encapsulating complexes is another method of huge interest. They have tremendous potential for high dosage gene encapsulation, chemical modification and genetic engineering to minimize immune response and improve targeting towards tumor cells. Various compositions have been tested for efficient gene delivery, including cationic polymers such as poly(ethylenimine) and poly(L-lysine), peptides and liposomes, where the positive charges of the capsule favor interactions with negatively charged DNA and cell membrane (121). A recent publication reported the Phase I clinical trial of *p53* cDNA administration into patients with solid tumor via a systematic liposomal nanoparticle delivery. Their results are encouraging as p53 protein specifically accumulated in metastatic tumors, but not in normal skin tissue, while the majority of patients demonstrated stable disease with minimal side effects (122).

d. Challenges for cancer gene therapy

The goal for cancer gene therapy is to safely and stably restore normal tumor suppressor functions in cancer cells to inhibit cell growth or eliminate cancer cells. Low and transient expressions of transferred gene often fail to show significant therapeutic effect, while uncontrolled transductions lead to severe side-effect, both compromising the therapeutic outcome. These problems for cancer gene therapy need to be addressed in preclinical investigations before entering human clinical trials. Nevertheless, cancer models, such as glioma and melanoma, which are malignant and lacking of effective treatment, seem to favor cancer gene therapy applications as they are more accessible for intratumoral injections to achieve high dosages of anticancer drugs at the local administration site. Advances in technology may further contribute to the development of successful cancer gene therapy.

III. Inhibitors to restore tumor suppressor functions

Currently, small molecule inhibitors are the most common anticancer therapeutic agents. Following the success of Gleevec for Chronic Myelogenous Leukemia (CML), tremendous effort has been invested in developing inhibitors for various oncogenic kinases. At present, numerous small molecule inhibitors targeting oncogenic kinases are being tested in preclinical and clinical investigations, and about a dozen have been approved by the United State Food and Drug Administration (FDA) for cancer therapy (123). The majority of these inhibitors are specifically directed toward to the ATP binding pocket to inhibit the oncogenic kinase activities. As tumor suppressor gene function is often diminished in

cancers, designing small molecule inhibitors to restore tumor suppressor gene will need to take a different approach.

a. Inhibitors targeting RB network

RB's tumor suppressor function is largely mediated through the transcriptional repression of RB/E2F, which can be alleviated by phosphorylation on RB via cyclin/CDK complexes (124). Cyclin-CDK complexes themselves are subjects of multiple signaling pathways, including Ras, β-catenin and NF-κB (125). Inhibitors for these oncogenic kinases have been shown to indirectly activate RB and restore transcriptional repressions. Such RB-reactivating agents include MEK inhibitor (Trametinib) (126), PPARα agonist (Fenofibrate) (127), and PI3K inhibitor (LY294002) (128).

Alternatively, downstream effectors of RB could also be potential targets. Hec1 (Highly Expressed in Cancer 1) was originally identified as an RB-interacting protein in a yeast two-hybrid screen. As shown in Figure 1, interaction with Hec1 has linked RB to the regulation of mitotic chromosome segregation, during which RB provides a chaperone-like activity to aid in accurate distribution of genetic material (28). Hec1 overexpression has been observed in a variety of human cancers and one of the eleven cancer gene signatures (129–132). In addition, phosphorylation of Hec1 by the mitotic kinase Nek2 is critical for the Hec1 function in cells (133). Thus, the specific interaction between Hec1 and Nek2 represents an ideal anticancer target.

Traditionally, using small molecules to interrupt protein-protein interactions have been shunned by many small molecule developers (134). Unlike the enzymatic active sites of kinases, which are often represented by well-defined pockets, protein-protein interacting interface can involve large surface areas that are shallow and lack of well-defined features (135, 136). Initially, most of researches focused on developing endogenous or engineered peptide inhibitors that mimic the target protein. However, most of the peptide inhibitors were quickly proved to be unsuccessful due to the limitation of oral bioavailability. Small molecule inhibitors provide more opportunities than peptide inhibitors, though the binding affinity, specificity and cellular permeability are still the essential criteria for evaluating small molecule inhibitors.

To find a small molecule inhibitor, we have adapted a unique reverse yeast two-hybrid system, allowing high-throughput screening for compounds that can interrupt specific protein-protein interaction (Figure 4). In this system, we genetically fused TetR with the C-terminal binding region of Hec1 (TetR-Hec1), which was then constitutively expressed in yeast. On the other hand, activation domain of GAL1 fused with Nek2 (AD-Nek2) was under the control of GAL1-inducible promoter. Without the disruption of Hec1/Nek2 interaction, the 5-FOA gene will be activated and hydrolyzed 5-FOA into toxic metabolites, inhibiting yeast growth. Conversely, if an inhibitor is able to disrupt the Hec1/Nek2 interaction, 5-FOA will not be metabolized, thus permitting yeast growth. Using this approach, we have discovered a novel compound, INH1, which can specifically disrupt the Hec1/Nek2 interaction via direct Hec1 binding. The antitumor cytotoxicity was validated both in cultured tumor cells and xenograft models (137). INH1 was further modified via organic chemistry synthesis, though the efficacies for its analogs were not significantly

improved (138). Nevertheless, this is a direct proof of concept that it is possible to target tumor suppressor gene network by targeted disruption of protein-protein interaction.

b. p53/MDM2 disruptors

Although about 50% of human tumors lack both p53 alleles or carry a mutated form of p53, in many tumors, a wild-type form of p53 is present, offering an opportunity to boost its tumor suppressor functions to eradicate the tumors. As aforementioned, p53 is a short-lived protein with a low 'steady-state' level. Activated p53 will transcriptionally activate MDM2 (also known as HDM2), an oncogene that is usually amplified in cancers, to carry out p53 degradation by the ubiquitin-proteasome pathways (139). Binding of MDM2 to p53 transactivation domain inhibits p53 mediated transcriptional activation (140), and promotes nuclear export of p53 to cytoplasm. In the cytoplasm, MDM2 functions as an E3 ligase to induce attachment of ubiquitin moieties onto p53 for degradation (141). This auto-regulatory loop can be modulated by several signaling pathways via regulating p53/MDM2 interaction (51). For example, the p53/MDM2 interaction can be disrupted by phosphorylations of p53, especially on amino acids in its N-terminal domain. These phosphorylations, which in turn stabilize p53, can be achieved by kinases activated in response to DNA damage in normal cells (142, 143). Therefore, inhibiting p53/MDM2 interaction is a promising because it not only abolishes MDM2-mediated p53 degradation, but also restores p53 transcriptional activities.

The crystal structure of the p53/MDM2 complex revealed that the interacting interface relies on a relative deep hydrophobic cleft of MDM2 that accommodates three amino acid side chains in the helical region of the p53 transactivation domain (144). This discovery led to the development of p53 mimicking peptide inhibitors as therapeutic agents to disrupt p53/MDM2 interaction in tumor cells (145–147). A number of more effective small molecule inhibitors have been identified through high-throughput screening of large chemical libraries, including Nutlins (148) and MI-219 (149), which structurally mimic the p53 peptide and bind to MDM2 pocket. On the other hand, small molecules mimicking MDM2, such as RITA (150), have also been identified to bind p53, although the cellular activities for RITA seemed to be p53-independent (151). In addition, a recent study using computational methods has revealed a novel pocket in the p53 core domain, mutation of which can abolish the reactivation of p53 by known reactivating drugs. This pocket can be a potential target for direct pharmaceutical reactivation of p53 (152).

Taken together, these inhibitors disrupt p53/MDM2 interaction and induce accumulation of wild-type p53 in cells. However, their cellular activities are quite diverse in terms p53 accumulation, and cell cycle arrest versus apoptosis. The effects on tumor cells and normal cells are not homogenous as well (153). Moreover, the presence of the other MDM2 family member, MDMX (also known as HDMX or MDM4), has been reported to reduce the efficacy of these inhibitors, probably due to redundant activities between MDM2 and MDMX (154). Some of the p53/MDM2 disruptors, such as MI-219 and Nutlin-3, which demonstrated high binding affinity and specificity to MDM2 and potent induction of cellular p53 activity, have entered advanced preclinical development or early clinical trials (155). The Phase I clinical trial for RG7112, a much more potent Nutlin analog with better

pharmacological properties, showed promising results with increased p53 activation and decreased cell proliferation in liposarcoma patients (156). Further investigations on RG7112 and other p53/MDM2 disruptors are under way.

c. PARP1 inhibitors for BRCA1/BRCA2 deficient tumors

DNA is damaged thousands of times during each cell cycle, and these genetic insults must be repaired. As aforementioned, BRCA1/2 play essential roles in DNA DSBs repair using the error-free HR mechanism. On the other hand, Poly(ADP-ribose) Polymerase 1 (PARP1) is a protein required for repairing single-strand DNA breaks (157, 158). If these “nicks” are not repaired, they can lead to double-strand breaks during replication. Since tumor cells undergo rapid cell division rate, it is obvious that tumor cells will be more susceptible for DNA breaks, making PARP1 a good therapeutic target (159). As human PARP1 catalyzes the transfer of ADP-ribose moiety from nicotinamide adenine dinucleotide (NAD^+) donor to a recipient protein, preferentially to glutamate or lysine residues, most PAPR1 inhibitors are designed to compete with NAD^+ at the enzymatic site. Currently, there are more than 40 clinical trials in development or ongoing for PARP1 inhibitors as anticancer agents, and major pharmaceutical companies are heavily investing in these PAPR1 inhibitors (160).

Treating cells with PARP1 inhibitors abolishes the cell’s ability to repair of single-strand breaks and generates multiple double strand breaks. This approach exploits a synthetic lethal strategy in cancers that harbor deficient BRCA1/2. Hence, PARP1 inhibitors have been tested in BRCA1/2-associated breast and ovarian cancers (161). A Phase I clinical trial on Olaparib (AZD2281) showed that BRCA1/2 deficient tumors are sensitive to PARP inhibitors with only mild side effects, which can be due to the selectively targeting of PARP inhibitors in BRCA1/2 defective cells, but not the normal cells with intact HR (162). This is consistent with the results that PARP knockout mice are viable and healthy in general, suggesting depletion of only PARP function is tolerable under normal condition (163, 164).

However, the clinical study by Fong *et al.* showed that not all BRCA1 or BRCA2 mutation carriers respond to the treatment (162). Similarly, some BRCA1-deficient cancers are found with null or low PARP1 expression, potentially limiting the application of PARP1 inhibitors (165). Additionally, an intragenic deletion in BRCA2 was later reported to render resistance to PARP1 inhibitors (166). These studies suggested that only in a small subset of the patient population benefitted from synthetic lethal therapies using PARP1 inhibitors. Nevertheless, in some preclinical studies, inhibiting CDK1 or PI3K can sensitize BRCA-proficient cancers to PARP inhibition (167, 168). Therefore, understanding the unique characteristics of PARP inhibition within the patient specific context is important for improving the efficacy.

d. BRCA2/RAD51 disruptor

Cancers are often under DNA replication stress and high levels of DNA damage, which require functional DNA repair pathways including HR. Elevated recombinase RAD51 expression and enhanced HR rates have been observed in many types of cancers, including CML, and often contributes to drug-resistance (169, 170). To perform HR, RAD51 needs to multimerize and form nucleo-filaments on ssDNA, critical steps which are facilitated by

many accessory factors including the breast cancer susceptibility gene product BRCA2 (71). Thus, RAD51 is an attractive target molecule for developing tumor-selective inhibitors.

Structural studies demonstrated that RAD51 directly binds to the six conserved BRC repeats of BRCA2 (171, 172). As shown in Figure 5, using the same strategy as for Hec1/Nek2, we identified a novel small molecule inhibitor, IBR2, which is capable of disrupting RAD51 multimerization, reducing ionizing radiation-induced RAD51 foci formation, impairing HR, inhibiting cancer cell growth and inducing apoptosis. In a murine CML model bearing the T315I BCR-ABL mutation that is resistant to Gleevec (173), IBR2 significantly prolonged animal survival. Consistently, IBR2 effectively inhibits the proliferation of $CD34^+$ progenitor cells derived from CML patients that are resistant to known BCR-ABL inhibitor (174). The efficacy of such small molecule inhibitor of RAD51 on other difficult-to-treat cancers warrants further investigation both preclinically and clinically.

e. Inhibitors of epigenetic modulators

Over the last decade, our understanding on how epigenetic modifiers can alter the expression of cancer-associated genes provides another opportunity for therapeutic intervention. The most extensively studied epigenetic changes that can silence tumor suppressors are DNA methylation and histone acetylation.

DNA methylation is primarily thought to play a repressive role via CpG island methylation at promoter sequences (175). It is not surprising that many have made the observation of hypermethylation phenotypes in numerous cancers (176). One of the first chemotherapeutic drugs found to modify the epigenome was a cytotoxic nucleoside analog derived in the 1960s called 5-azacytidine, or azacytidine (177). At the time, it was thought that azacytidine exerted its cytotoxic effects solely through random incorporation into the genome and RNA, leaving cells in S-phase and susceptible to cytotoxic stress. It was later discovered that this nucleoside analog could also inhibit DNA methyltransferases by covalently binding to the enzyme (178). Mechanistically, azacytidine was demonstrated to non-selectively reduce methylation levels at promoters of tumor suppressor genes and restore their function via increased gene expression. A number of DNA methyltransferase inhibitors, such as the azacytidine derivate, 5-aza-20-deoxycytidine (Decitabine), have been FDA approved and are used in chemotherapy regimens.

Mutations in pathways regulating the demethylation of DNA have also been implicated in CpG Island Hypermethylator Phenotypes (CIMP) in cancer and are currently the focus of cancer therapeutics development (179). Tet Methylcytosine Dioxygenases (TETs) and Isocitrate Dehydrogenases (IDHs), are another group of interesting epigenetic modulators that regulate DNA methylation and are found to be mutated in numerous hematological cancers and malignancies (180–182). TET family proteins are known to initiate the demethylation cascade that begins after the conversion of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC), which is eventually converted back to cytosine (183, 184). The activation of TET enzymes require α-ketoglutarate as a cofactor, while mutant IDHs have gain of function that use α-ketoglutarate to generate 2-hydroxyglutarate, which stereochemically resembles α-ketoglutarate and inhibits TETs (181). Such complicated cross-talk between TET and IDH may in part explain the observation of mutually exclusive

presence of mutant TET and IDH in glioma and leukemia (185, 186). Currently, clinical trials are underway to test the efficacy of using small molecules to inhibit 2-hydroxyglutarate production by IDH (187, 188). Such inhibition may effectively reverse the inhibition of chromatin demethylation to restore tumor suppressor expression, and demonstrate great pharmacological potential as cancer remedy.

Besides DNA modifications, histone modifications have been proven to be powerful and essential epigenetic regulation on gene expression. Acetylation of histones, for example, is required to maintain chromatin in an open state for active transcription, while HDACs remove acetyl groups to transcriptionally silence gene expression. Therefore, HDACs have become promising therapeutic targets that are under extensive study. Vorinostat and Romideosin were the first FDA approved HDAC inhibitors that came out in 2006 and 2009, respectively (189, 190). The cellular effects of these inhibitors have been shown to include the induction of apoptosis, cell cycle arrest, differentiation, inhibition of angiogenesis and alteration of gene expression patterns (191, 192). Unfortunately, these inhibitors do not spare non-transformed cells, highlighting the need for additional HDAC inhibitors that are specific to HDAC isoforms or specific to mutations found only in cancerous cells.

f. Challenges for developing inhibitors targeting tumor suppressors

Because tumor suppressors are usually mutated, deleted, or silenced in cancers, the first challenge for developing small-molecule inhibitors is to define a specific target. Therefore, possible targets may not necessarily target the tumor suppressor itself, but can target proteins that regulate the tumor suppressor activity. Hence, identification of specific structural interfaces on tumor suppressors or its regulatory proteins is crucial for the synthesis and design of novel compounds. Selecting a target with well-defined structural characteristic, such as a pocket, has proven to be beneficial as in the cases of p53/MDM2 and BRCA2/RAD50 disruptors.

Once the target is identified, multiple approaches have been developed to screen for such small molecule inhibitors, including small focused library screening, high-throughput screening of large chemical libraries, computational 3D database screening and structure-based de novo design. The advancement in computational modeling software has been demonstrated extremely useful in providing information in prediction and subsequent optimization in preclinical research. However, low efficacy and lack of specificity observed in the clinical trials have remained hurdles yet to be overcome.

IV. Concluding remarks and perspectives

A tremendous amount of financial resources and manpower have been invested to understand the malignant nature of cancer in hopes of finding a cure. For the past 40 years, our knowledge of cancer has advanced dramatically. Identifications of oncogenes and tumor suppressors have played a pivotal role in enhancing our understanding of the unique biology of cancer, as well as aiding in the development of new cancer therapies. Discovery of anticancer drug target like the oncogenic BCR-ABL has led to the development of Gleevec, one of the most successful therapeutic drugs for leukemia. On the other hand, the development of therapies that target the tumor suppressor network has failed to translate into

viable treatments options for cancer patients. Because tumor suppressor genes are often mutated, deleted or epigenetically silenced in cancer cells, effective anticancer therapies that target tumor suppressor genes must restore the normal functions of tumor suppressor genes. Therefore, gene therapies have been developed for cancers, where the loss of tumor suppressor functions is compensated via ectopic expression of wild-type genes. Various virus-mediated and non-virus-mediated delivery methods have been developed over the past 25 years. However, many experiments and clinical trials to date are hindered by efficacy and safety concerns. Alternatively, numerous inhibitors have been identified to either disrupt interactions between tumor suppressor genes and their negative regulators, or induce synthetic lethality, although the effectiveness of these inhibitors needs to be validated with solid clinical data.

One problem encountered in clinical trials for anticancer therapeutic agents is the small percentage of patients that respond to the treatment, as observed in the case of PARP1 inhibitors for BRCA mutation carriers (162). However, this is a common issue for clinical trials in general, including those for oncogene inhibitors. To overcome this obstacle, identification and incorporation of more reliable biomarkers will be beneficial or necessary to discriminate patient populations with the best responses to a particular treatment (192, 193). In addition, despite the fact that mouse models have been used as a standard system for preclinical testing, the obvious difference between mouse and human genetics cannot be ignored, which can undermine the validity of such testing. More reliable and faithful models are highly desired for testing anticancer therapeutic drugs.

Nevertheless, the ultimate judgment of any anticancer therapy targeting tumor suppressor genes will be based on the potential risk and benefit for the cancer patient. With the tremendous efforts to overcome the difficulties and discoveries of novel biotechnologies, we should expect breakthroughs in the near future towards finding the cure of cancer.

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List of Abbreviations

RSV	Rous Sarcoma Virus
SRC	Sarcoma
RB	Retinoblastoma Susceptibility gene
LOH	Loss of Heterozygosity
R Point	Restriction Point
CDK	Cyclin Dependent Kinase
CKI	Cdk Inhibitors
INK4	Inhibitor of CDK4
PP1α	Phosphatase 1α

HDAC	Histone Deacetylase
APC	Adenomatosis Polyposis Coli
ROS	Reactive Oxygen Species
PUMA	P53 Up-Regulated Modulator of Apoptosis
BRCA1/2	Breast Cancer Susceptibility gene 1/2
ATM	Ataxia Telangiectasia
CHK2	Checkpoint Kinase 2
DSB	Double-Strand Break
53BP1	p53-binding protein 1
MRE11	Meiotic Recombination 11
HR	Homologous Recombination
NHEJ	Non-Homologous End-Joining
ADA-SCID	Adenosine Deaminase Deficiency caused Severe Combined Immunodeficiency
siRNA	Small Interference RNA
CML	Chronic Myelogeneous Leukemia
FDA	The United States Food and Drug Administration
MEK	MAP Kinase Kinase
PPARα	Peroxisome Proliferator-Activated Receptor A
PI3K	Phosphoinositide 3-Kinase
Hec1	Highly Expressed in Cancer 1
Nek2	NIMA-Related Kinase 2
5-FOA	5-Fluoroorotic Acid
MDM2	Mouse Double Minute 2
PARP1	Poly(ADP-Ribose) Polymerase 1
NAD	Nicotinamide Adenine Dinucleotide
ssDNA	Single-Strand DNA
CIMP	CpG Island Hypermethylator Phenotype
TET	Tet Methylcytosin Dioxygenase
IDH	Isocitrate Dehydrogenase 1
5mc	5-methylcytosine
5hmC	5-hydroxymethylcytosine

2-HG	2-hydroxyglutarate
BCR-ABL	Breakpoint Cluster Region-Ableson

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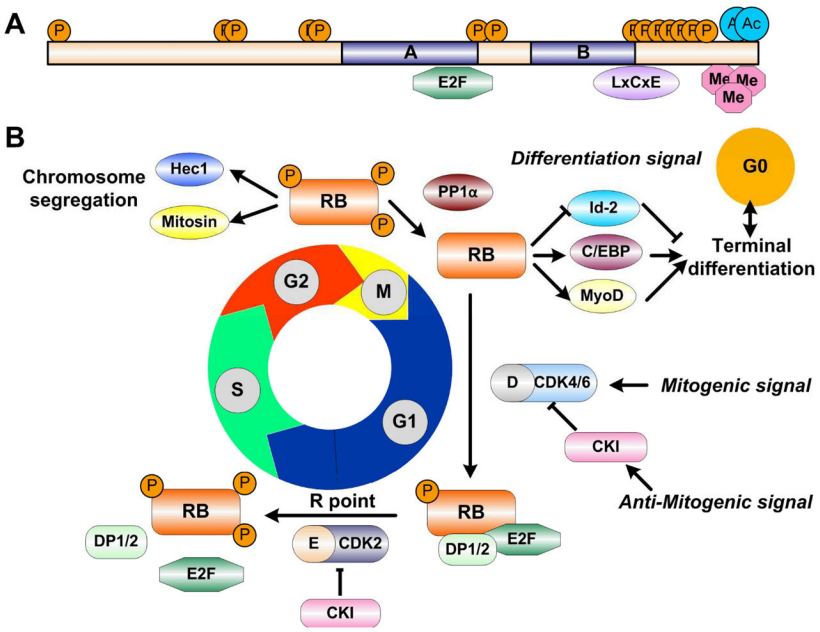

Figure 1. RB structure and functions

(A) A schematic representation of RB protein functional domains and post-translational modification sites, such as phosphorylation (P), acetylation (Ac), and methylation (Me). The central A and B domains mediate interactions with a number of proteins, such as E2Fs and LxCxE motif containing proteins. (B) The cell cycle-dependent phosphorylation status of RB determines the progression of cell cycle progression, chromosome segregation and terminal differentiation.

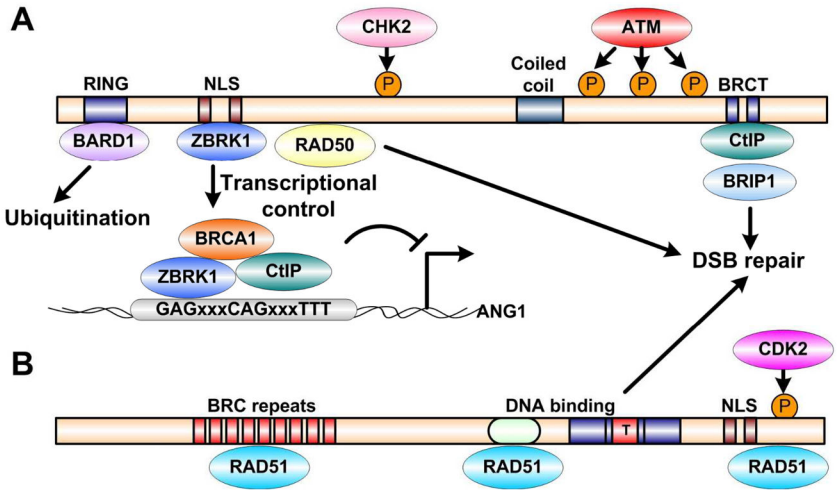

Figure 2. BRCA1 and BRCA2 structure and functions

Schematic representations of BRCA1 (A) and BRCA2 (B) structural motifs, interacting proteins, and corresponding kinase phosphorylation sites. Both BRCA1 and BRCA2 regulate DNA double-strand breaks repair; however, BRCA1 is also involved in mediating ubiquitination and transcriptional control.

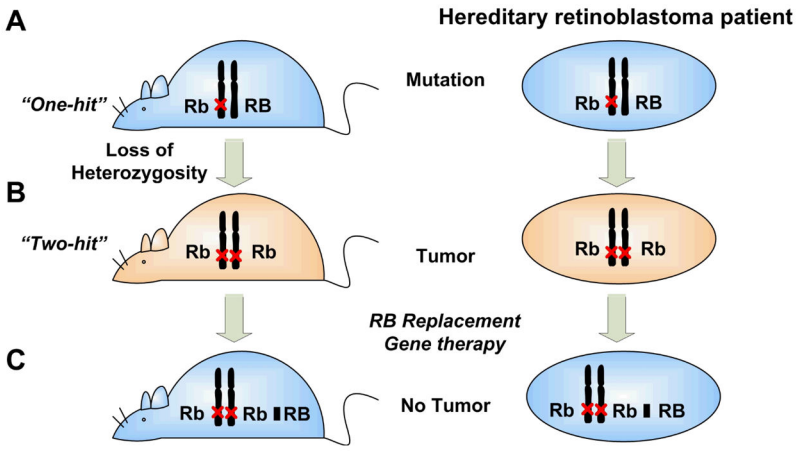

Figure 3. Representation of tumor suppressor gene therapy model

(A) For patients with hereditary retinoblastoma, one copy of the mutated or deleted *RB* gene is inherited from their parents (the first hit). (B) Acquisition of a second hit, a somatic mutation, causes "loss of heterozygosity" and retinoblastoma. (C) Replacement of the mutated or deleted *RB* with a normal *RB* cDNA inhibits tumor growth.

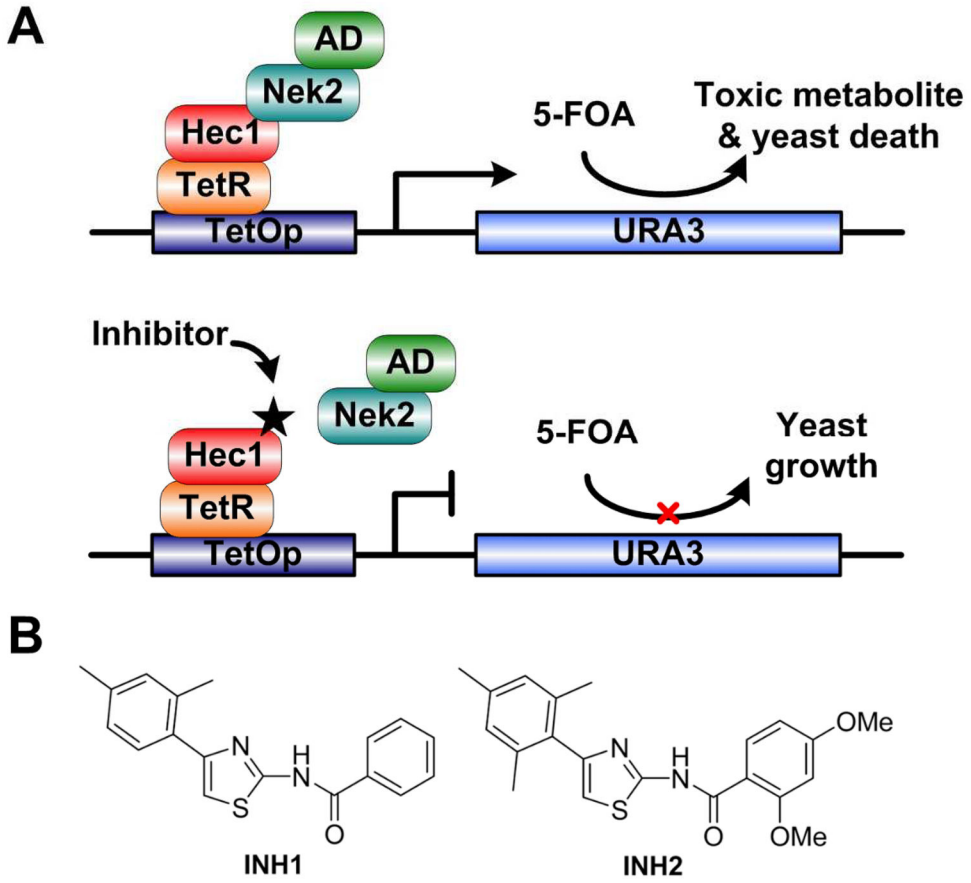

Figure 4. Small molecule inhibitors, INHs, inhibit tumor growth by disrupting interaction between RB-interacting protein, Hec1, and Nek2

(A) A schematic representation of a reverse yeast two-hybrid system for high-throughput screening for Hec1/Nek2 inhibitors. TetR fused with the C-terminal binding region of Hec1 (TetR-Hec1) was constitutively expressed; activation domain of GAL1 fused with Nek2 (AD-Nek2) was under the control of GAL1-inducible promoter. If an inhibitor abolishes the Hec1/Nek2 interaction, 5-FOA will not be metabolized, thus permitting yeast growth; otherwise, 5-FOA will be hydrolyzed into toxic metabolites and inhibit yeast growth. (B) Structures of two candidate INH compounds that promote yeast growth, i.e., disrupt Hec1/Nek2 interaction. (Figure adapted from (137))

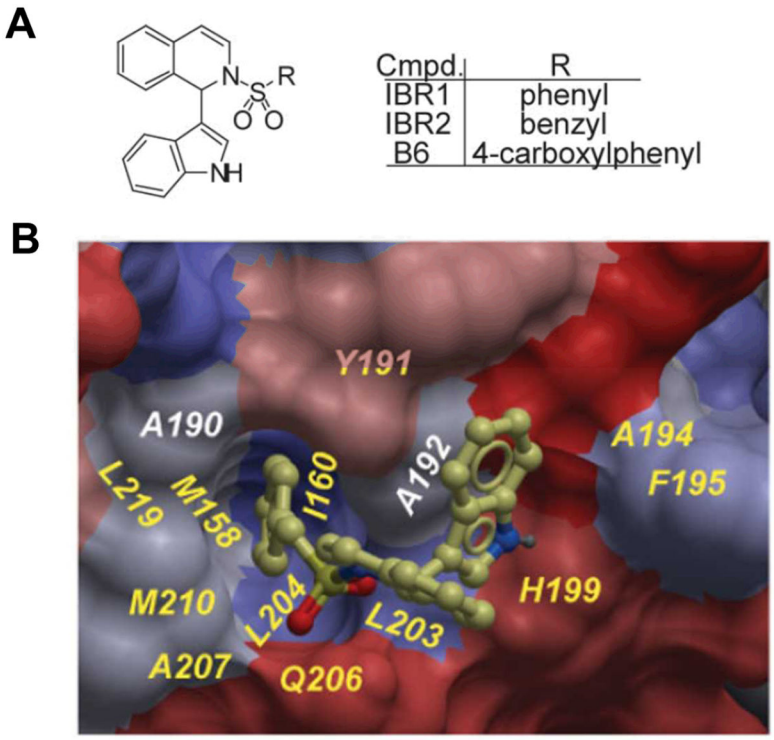

Figure 5. IBR2 inhibits tumor growth by disrupting interaction between RAD51 and BRC repeats

(A) Structures of IBR compounds (B6 is the negative compound) that disrupt RAD51/BRC repeats interaction. (B) IBR2-RAD51 docking model. IBR2 is shown in ball-and-stick model and colored by hydrophobicity. (Figure adapted from (194))