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Off and Back-On Again: A Tumor Suppressor's Tale

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Abstract

Tumor suppressor genes play critical roles orchestrating anti-cancer programs that are both context dependent and mechanistically diverse. Beyond canonical tumor suppressive programs that control cell division, cell death, and genome stability, unexpected tumor suppressor gene activities that regulate metabolism, immune surveillance, the epigenetic landscape, and others have recently emerged. This diversity underscores the important roles these genes play in maintaining cellular homeostasis to suppress cancer initiation and progression, but also highlights a tremendous challenge in discerning precise context-specific programs of tumor suppression controlled by a given tumor suppressor. Fortunately, the rapid sophistication of genetically engineered mouse models of cancer has begun to shed light on these context-dependent tumor suppressor activities. By using techniques that not only toggle *'off'* tumor suppressor genes in nascent tumors, but also facilitate the timely restoration of gene function '*back-on again'* in disease specific contexts, precise mechanisms of tumor suppression can be revealed in an unbiased manner. This review discusses the development and implementation of genetic systems designed to toggle tumor suppressor genes *off and back-on again* and their potential to uncover the *tumor suppressor's tale*.

Introduction

Identifying causal relationships between genes and the physiological programs they control is a central goal of biological research and an unmet challenge in cancer. While loss of function mutations in tumor suppressor genes are the most common genetic alterations in cancer, elucidating how these genes function within physiologically relevant *in vivo* settings

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remains difficult. For over 25 years tumor suppressor genes have been studied *in vivo* through gene knock out strategies in the mouse. Deletion of tumor suppressor genes in genetically engineered mouse models, either constitutively in the germline or conditionally in somatic cells of adult mice, typically results in spontaneous formation of cancers or progression of cancers to more advanced disease states. Though this approach has been critical for developing faithful models of human cancer and validating cancer causing mutations, deletion of tumor suppressor genes in this manner often tells us little about how these genes work to keep cancer at bay, but instead reveal only what happens when they are lost. Major advances in genome engineering have paved the way for more sophisticated methods to control gene expression *in vivo*. These methods allow not only the conditional inactivation of tumor suppressor genes, but also the acute restoration of their function in established cancers. Restoration of tumor suppressor gene function can uncover latent tumor suppressive mechanisms that are relevant in disease specific contexts. This approach has the added advantage of establishing a genetic means to assess the potential of treatment strategies that aim to reactivate tumor suppressive pathways prior to their development. Identifying the natural mechanisms that suppress and eradicate cancer upon tumor suppressor pathway activation could provide a framework on which we can develop therapeutic strategies that stimulate latent tumor suppressive programs.

Genetically engineered mouse models come in two major types. The first are those where genes are directly altered at their natural genomic locus thereby generating new alleles. These *allelic* models typically enable recombinase-dependent alterations of the locus to conditionally activate or inactivate gene function. On the other hand, *transgenic* models generally feature insertion of heterologous genetic information, or transgene, either into a random location in the genome, or precisely into a safe harbor locus that has been empirically determined to have minimal impact on natural cellular behaviors. Both of these modeling strategies, or the combination of the two, can yield powerful tools to conditionally and reversibly regulate gene function in the mouse. We have divided these approaches into their corresponding categories below and highlighted their relative strengths and weaknesses (Table 1).

ALLELIC MODELS FOR CONDITIONAL AND REVERSIBLE GENE INACTIVATION

Recombinase based alleles

Since the initial demonstration of its feasibility [1], it has become standard to generate conditional alleles using site-specific DNA recombinase-based approaches. By incorporating DNA recognition sequences that enable cognate enzymes to mediate deletion of intervening DNA segments, these alleles can be engineered to switch on or off under conditions where the recombinase is active. Several site specific recombinases exist that each act on unique DNA sequences [2]. However, the Cre-LoxP system, where Cre is the DNA recombinase and LoxP the 34 nucleotide recognition sequence, is by far the most common. Hundreds of conditional alleles have been generated that can be crossed to dozens of mouse lines that express Cre in a cell-type restrictive, or gene promoter selective manner. Alternatively, viral delivery of Cre offers the ability to conditionally recombine alleles in a temporally-

controlled and spatially-restricted manner. Additional means to temporally regulate recombinase activity can be achieved through fusion of these recombinases to fragments of hormone dependent nuclear localization domains such as the estrogen receptor (ER). Recombinase-ER fusions require addition of Tamoxifen, a synthetic estrogen, to induce site-specific recombination. Regardless of the method to introduce and control Cre, it is the specific arrangement and orientation of the LoxP sites that orchestrate the precise recombination reaction which can be tailored to either inactivate or activate expression of the engineered allele. Conditional inactivation is most commonly driven by the deletion of one or several critical exons from a gene locus. On the other hand, conditional activation of genes is typically achieved by removal of a heterologous DNA element, often called a '*STOP*' cassette, that blocks normal transcription through the locus (Figure 1A). To develop recombinase-based cancer models where tumor suppressor genes can be reactivated in established tumors in the mouse, several variations of these basic approaches have been developed.

LoxP-STOP-LoxP Alleles—When inserted into an early intron of an endogenous gene, LoxP-STOP-LoxP (LSL) cassettes functionally inactivate the host gene by terminating transcription of downstream DNA sequences (Figure 1a). Thus, LSL alleles are functionally and phenotypically equivalent to germline knock-out mice. While generating a LSL mouse model to conditionally express cancer associated mutants encoded by downstream exons of the endogenous *Trp53* (*p53*) locus, Olive and colleagues isolated embryonic stem cell clones that incorporated the LSL cassette in the first intron of the *p53* locus but not the downstream changes that would encode the mutant *p53* alleles [3, 4]. The result was a mouse line that lacked expression from the endogenous *p53* locus, but which enabled the restoration of wildtype *p53* gene expression after Cre-mediated deletion of the STOP cassette. Though this $p53^{LSL}$ allele was generated serendipitously, its potential was immediately obvious. Spontaneously arising T-cell lymphomas and soft tissue sarcomas are the dominant phenotype of p53-deficient mice [5, 6]. Therefore, by crossing the $p53^{LSL}$ mouse line to the $Rosa26^{Cre\text{-}ERT2}$ allele that is expressed ubiquitously in the mouse, it could be ascertained whether *p53* gene restoration impacted the maintenance of the established T cell lymphomas and soft tissue sarcomas (Figure 1a) [4]. Interestingly, tamoxifen-inducible restoration of *p53* had disparate effects in each tumor type. In T cell lymphomas, *p53* restoration led to rapid caspase-dependent apoptosis and robust tumor regression. Conversely, restoration of *p53* in soft tissue sarcomas instead induced features of cellular senescence that was followed by a delayed tumor regression [4]. We further explored the effects of p53 restoration in lung cancer type by crossing the $p53^{LSL}$ allele to the $Kras^{LA2}$ allele that drives oncogenic $Kras^{G12D}$ expression after a spontaneous recombination event [7]. Owing to the exquisite sensitivity of the lung epithelium to oncogenic $Kras^{G12D}$, all $Kras^{LA2}$ mice form multiple lung adenomas after birth and a small subset acquire additional changes that drive their progression to early stage carcinomas. In this setting, p53 restoration also led to tumor regression, however, the effects of p53 restoration were limited only to the subset of tumors that had progressed to the carcinomatous stage of the disease, whereas their benign adenomatous counterparts were seemingly indifferent to the effects of p53 restoration [7].

The context-specific effects of p53 reactivation highlight the power of gene restoration approaches to identify when tumor suppressor genes exert their anti-tumor effects during disease progression. These approaches also enable an unbiased analysis of relevant tumor suppressive programs that they control. Although powerful, multiple technical limitations of the LoxP-STOP-LoxP allele system have limited their application to other cancer models and tumor suppressor genes. For example, due to the mortality associated with the frequent and rapid development of sarcomas and lymphomas in p53-deficient mice, obtaining large cohorts of $Kras^{LA2};p53^{LSL/LSL}$ mice that lived long enough to restore p53 gene function within lung adenocarcinomas was difficult. Because extensive aging of the mice was not possible, we were unable to assess the effects of p53 restoration on the most clinically relevant advanced stages of lung adenocarcinomas that require extended time to develop in the model. Additionally, the LoxP-STOP-LoxP approach is not compatible with other cancer models that rely on Cre for conditional tumor initiation. Further, most tumor suppressor genes commonly inactivated in human cancers are required for normal mouse development. These genes are therefore not amenable to LoxP-STOP-LoxP approaches that block germline expression. These caveats severely limit the utility of LSL alleles to discern tumor suppressor functions and hence, this approach has not been widely employed.

Hormone dependent fusion alleles

In this allelic approach, mice harbor a knock-in allele whose gene product is the fusion of the endogenous protein of interest with a fragment of a hormone receptor (Figure 1b) [8]. Most commonly the estrogen receptor is used, but in some applications the progesterone receptor avoids unwanted physiological consequences of excessive estrogen levels in the mouse or increases functionality of the experimental system [9, 10]. Typically fusions are made with the modified estrogen receptor fragment ER^{T2} to minimize effects of endogenous mouse estrogens and better conditional regulation upon treatment with tamoxifen, a high affinity ER antagonist normally used to treat breast cancer [11, 12]. *In vivo*, tamoxifen is metabolized into 4-hydroxytamoxifen (4-OHT), its active form that displaces inhibitory chaperones such as Hsp90 and facilitates nuclear translocation of the ER-fusion protein. When applied to tumor suppressor genes, these alleles are compatible with genetically engineered, recombinase-based, carcinogen induced, and spontaneous cancer models. Tumors develop in the absence of the ER-fused tumor suppressor gene's function, and at some experimentally determined time point, allow reactivation of tumor suppressor function by tamoxifen or 4-OHT administration.

The development of an ER fusion with p53 enabled several insights into the biology of p53 action [13–16]. The $p53\text{-}ER^{TAM}$ allele enables switch-like control of p53 tumor suppressor activity whereby its functional activation is dependent on the administration of tamoxifen [13]. In this model, canonical responses to p53 stimulating stresses such as radiation induced apoptosis in the colon, thymus, and spleen, or oncogenic Ras induced senescence in cultured fibroblasts, occur only in the presence of tamoxifen [13]. In a particularly elegant experiment, it was demonstrated that p53 tumor suppressor activity is separate and temporally distinct from its ability to acutely respond to radiation induced DNA damage. Exposure to ionizing radiation induces extensive pathological responses in wildtype mice, but not in $p53^{-/-}$ mice. However, unlike wildtype mice, the p53 deficient mice are prone to

the onset of radiation induced cancer. It was widely assumed that because p53 responds acutely to noxious radiation levels and permanently eliminates damaged cells from the mouse, that this was the major tumor suppressive mechanism. Surprisingly, the tumor suppressive functions of p53 could be fully reinstated by treating the $p53ER^{TAM}$ mice with tamoxifen well after the DNA damaging insult had resolved. Thus, although untold numbers of cells in the $p53ER^{TAM}$ mice persist after exposure to levels of radiation that would have normally led to their p53-mediated demise, these cells are no more likely to initiate transformation once p53 activity is restored than cells in wildtype mice that maintained p53 all along. This result demonstrated that the critical tumor suppressive role for p53 is to suppress the outgrowth of cell clones that had suffered oncogenic mutations due to radiation, but not to respond acutely to the damage itself. This lesson was further reinforced by studies showing that tumor cell intrinsic expression of the oncogenic stress sensor $p19^{ARF}$ dictated the selective activation of p53 in $Kras^{G12D}$-induced models of lung adenocarcinoma [7, 15].

A clear advantage of the ER fusion model is the rapid response to tamoxifen and the ability to repeatedly toggle between *on* and *off* states. Toggling $p53ER^{TAM}$ on with tamoxifen leads to a rapid and potent tumor cell eradication in the transgenic Eμ-myc model of Burkitt's lymphoma and high grade-astrocytoma, thereby modeling therapeutic opportunities for p53 reactivation to treat human cancer [14, 17, 18]. Interestingly, activating and deactivating p53 in a metronomic fashion led to greater tumor suppressive potential than static p53 reactivation due to the loss of selective pressure to disable other components of the p53 pathway [18]. These observations and experimental strategies could have important implications for treatment with experimental therapeutics that aim to stabilize and activate mutant forms of p53 that are commonly found in most cancer types [19–21].

Similar to LSL alleles discussed above, a main obstacle to using ER fusion proteins is that these alleles are functionally null. Therefore, this approach is not amenable to genes that are required for either normal mouse development or other physiological programs. Of added concern is the accurate functional regulation of the protein of interest when fused to the ER fragment. ER-fusion proteins could have reduced or increased activity in the presence or absence of hormone and, though not yet documented, it is conceivable that non-physiologic, neo-morphic activities could result from these heterologous alleles. Consequently, extensive characterization of each allele and careful interpretation of experimental results are required. Finally, the ER-fusion approach is likely restricted only to nuclear-functioning proteins due to the mechanism of action driving nuclear translocation upon hormone binding. Therefore, tumor suppressors whose critical activities take place elsewhere in the cell or in both nuclear and non-nuclear compartments can not be investigated using ER fusions.

XTR (eXpressed Trapped-Restored) Alleles—To overcome many of the drawbacks associated with LSL and hormone receptor fusion alleles, we developed a synthetic gene switch coined XTR, for eXpressed-Trapped-Restored. When inserted into an early intron of a gene of interest, the XTR cassette enables the Cre-dependent inactivation and FLP-mediated reactivation of the targeted gene function (Figure 2a) [22]. At its heart, XTR is a gene trap composed of the adenoviral SV40 splice acceptor (SA), followed by the coding sequence for green fluorescent protein (GFP) and the SV40 polyadenylation transcription termination sequence. Flanking the gene trap are alternating mutant versions of loxP sites

(Lox5171 and Lox2722) that facilitate stable and permanent inversion of the gene trap due to the inverted orientation and the “double flox” configuration. In the germline, the XTR cassette is oriented oppositely to that of the targeted gene and thus *XTR* (eXpressed) alleles allow normal host gene expression. However, upon introduction of Cre, the gene trap is inverted to the *TR* (Trapped) conformation which accept transcripts from upstream donor exons, expresses a GFP reporter, and prematurely terminates transcription of host gene. Additionally, the gene trap element is flanked with FRT sites to orchestrate FLP recombinase-dependent deletion of the gene trap to form *R* (Restored) alleles that restore normal host gene expression. Thus, XTR alleles exist in three distinct conformations: *XTR* allows endogenous genes to maintain natural gene expression patterns in the germline and normal somatic cells, Cre-mediated recombination results in a *TR* allele that functionally inactivates a gene locus and expresses GFP to report endogenous gene expression, and Flp recombinase-mediated removal of the gene trap element accurately restores host gene expression and creates the *R* allele.

We validated the XTR system by targeting the cassette to the first intron of the *Rb1* and *Trp53* genes in the mouse [22]. In mouse embryo fibroblasts (MEFs) neither p53 or Rb expression is affected in XTR homozygous cells relative to wildtype MEFs (Figure 2). However, in cell lines derived from lung adenocarcinomas initiated by adenoviral delivery of Cre into the airways of $Kras^{LSL\text{-}G12D/+}; p53^{XTR/XTR}$ or $Kras^{LSL\text{-}G12D/+}; p53^{flox/flox}; Rb1^{XTR/XTR}$ mice, conversion of the *XTR* alleles to the *TR* state completely abrogated p53 and Rb expression (Figure 2). Introduction of Flp recombinase efficiently converts the *TR* allele to R conformation and restores expression of p53 and Rb (Figure 2). We demonstrated that, while $p53^{XTR/XTR}$ MEFs senesce after serial passaging, $p53^{TR/TR}$ MEFs replicate in an immortalized fashion. However, conversion to $p53^{R/R}$ by introducing Flp recombinase induced rapid cellular senescence. Consistent with the embryonic lethality of $Rb^{-/-}$ mice, we found that expressing Cre in the germline resulted in non-viable $Rb^{TR/TR}$ pups beyond embryonic day 16 [23, 24]. When crossed with the transgenic Eμ-myc model of Burkitt’s lymphoma [17], or the Cre-dependent $Kras^{LSL\text{-}G12D}$-driven model of lung adenocarcinoma [25, 26], the $p53^{TR/TR}$ tumors were phenotypically identical in tumor onset, histological progression, and limits on lifespan to those obtained from crosses with $p53^{-/-}$ or $p53^{flox/flox}$ alleles respectively [22].

Reactivation of tumor suppressor gene expression *in vivo* using the XTR approach requires temporally controlled expression or activity of Flp recombinase. We have found that the $Rosa26^{FlpO\text{-}ERT2}$ allele, allows efficient tamoxifen-dependent conversion to $p53^{R/R}$ and $Rb^{R/R}$ within *in vitro* and *in vivo* systems [22, 27]. Though somewhat difficult to assess *in vivo*, we have observed little evidence for tamoxifen-independent activity of the FlpO-ER protein and thus recommend this allele for any application. However, alternative strategies to regulate Flp expression or activity, such as doxycycline inducible systems or integration of FlpO-ER coding sequence into the XTR cassette would increase versatility of the XTR approach and therefore warrant further exploration.

The compatibility of XTR with Cre-based systems and its ability to maintain endogenous gene expression makes it an ideal tool to further dissect heterogeneity in tumor suppressor responses across different cancer types, different tumors of the same type, or even different

cells of a single tumor. Ongoing studies with XTR alleles targeted to multiple tumor suppressor genes seek to explore these possibilities in diverse tumor types. These studies will uncover new insights into the roles of important tumor suppressors in cancer relevant settings. As with most allele-based models, the development of XTR alleles also has required the use of mouse embryonic stem (ES) cells and homology-dependent recombination approaches to target specific genes of interest. The tedium, time, and cost associated with picking, screening, and injecting ES clones that have been properly targeted is significant and therefore poses a major drawback to allele-based models. However, methods that bypass the need for ES cell culturing and instead allow locus specific integration of DNA constructs in fertilized oocytes via direct injection have been validated and should facilitate a more routine utilization of allele-based models including XTR [28–31].

TRANSGENIC MODELS FOR CONDITIONAL AND REVERSIBLE GENE INACTIVATION

Regulatable RNA interference

Small interfering RNAs (siRNAs), short hairpin RNAs (shRNAs), and microRNAs (miRNAs) act post-transcriptionally by either targeting RNA transcripts for destruction, or suppressing translation of targeted mRNAs. Whereas siRNA duplexes are typically synthesized for transient transduction in cultured cells, shRNAs have the advantage that they can be expressed in cells in a predictable and stable manner downstream of heterologous Pol III promoters such as that from the U6 small nucleolar RNA gene. However, the functional dissection of mammalian miRNAs has made possible the rational design and implementation of transgenes that are processed by the endogenous RNA interference machinery to effectively repress target gene expression. An important advantage in this approach is that unlike their shRNA predecessors, miRNA-based transgenes are transcribed by RNA Pol II and are therefore easily coupled to tetracycline-regulated systems expressing tetracycline transactivators (tTA, *'tet-off'* or rtTA, *'tet-on'*) in a conditional manner, to enable temporally regulated expression of miRNA-based shRNAs [32].

The strategy of temporally regulating miRNA-based shRNA transgenes has been effectively designed and implemented for cells in culture [33–36]. Importantly, these systems have also been translated for use within *in vivo* transgenic mouse models that enable doxycycline-inducible miRNA-based shRNA expression (Figure 3a) [37, 38]. Although, multiple technically challenging steps must be followed to identify and validate sufficiently potent shRNAs that can approximate gene knock out phenotypes, this approach offers several powerful advantages. These include the ability to trace target knock down by embedding the miRNA into the 3′ untranslated region of an upstream-encoded fluorescent protein, and the ability to repeatedly toggle between states of target gene repression and expression simply by starting or stopping doxycycline treatments. Additionally, these tet-regulatable miRNAs can also be packaged into viral vectors, transferred to cells *in vitro* in a relatively high throughput or pooled manner, and then transferred back into mice to perform phenotypic screens. Perhaps most significantly for mouse model development is the establishment of ES

cell lines that enable *plug-and-play* insertion of dox-regulated miR30-based transgenes into the *Col1a1* safe harbor locus [32, 39].

These scalable dox-dependent systems have enabled the identification of surprising context-dependent anti-cancer mechanisms that are orchestrated by distinct tumor suppressors in different tumor types [40–42]. Regulated expression of *p53*-targeting miRNAs revealed that in liver cancers driven by high levels of oncogenic *HRAS*, reactivation of p53 expression in established tumors led to a robust senescence response followed by the clearance of cancer cells by multiple infiltrating innate immune cell types [40]. miRNA-dependent suppression of Pten expression in the hematopoietic compartment induces a widely disseminated T cell acute lymphoblastic leukemia. However, reactivation of Pten expression reveled that while tumor cells in circulation were eliminated, tumor cells present in hematopoietic organs were largely indifferent to re-expression of Pten [41]. In the context of the small intestine, Apc suppression with a potent miRNA can induce adenomas when expressed alone or adenocarcinoma when coupled with activation of oncogenic *Kras* and loss of *Trp53.* Reactivation of Apc led not only to tumor regressions but also reestablished completely normal tissue homeostatic capability despite the presence of the other cooperating drivers of cancer formation [42]. These examples highlight the power of tumor suppressor gene reactivation to identify unpredicted mechanisms that drive tumor regression, and discern specific contexts where reactivation of tumor suppressive pathways may be inadequate to regress cancers.

A major drawback of RNA interference approaches is the significant concern over off-target biological effects [43–46]. First, because the natural mechanism of RNAi is to knock-down cellular mRNAs even when imperfect complementarity between the mRNA target and the RNAi molecule exists, significant repression of many transcripts is expected upon shRNA expression [44]. Secondly, because imperfect complementarity of miRNAs and their cognate targets can suppress translation of mRNAs, the effects at the level of protein production could ostensibly be much more dramatic. Finally, the molar ratio of the experimental RNAi molecule (shRNA or miRNA) is typically much higher than naturally expressed endogenous miRNAs. These heterologous transgenes therefore outcompete endogenous miRNAs for access to the processing components of the RNA interference pathway and indirectly lead to the rise in transcript levels that are normally suppressed by endogenous miRNAs [47]. This phenomenon further highlights the need for screening for potent shRNAs that can then be designed to be expressed at low levels yet still lead to significant down regulation of the target mRNA [37, 48]. Unfortunately, off-target effects are difficult to experimentally control and data gleaned from RNAi-based approaches must be interpreted with caution. Regardless of these limitations, profound insights into the biology of tumor suppressor function have been made possible through these technologies.

Doxycycline inducible tumor suppressor transgenes

Though distinct in nature, allele-based and transgenic model systems could be used in concert to conditionally inactivate and restore tumor suppressor gene expression within *in vivo* cancer models. For example, combining conditional recombinase-dependent inactivation of a floxed tumor suppressor gene locus with a tetracycline inducible transgene

system, could allow inducible re-expression of the relevant tumor suppressor gene cDNA in a spatially restricted and temporally controlled manner (Figure 3b). Though this approach has been used to identify the temporal effects of transgene induction on tissue homeostasis [49], to our knowledge this approach has not yet been used to regulate tumor suppressor genes within *in vivo* cancer models. However, the incorporation of the tools described above for regulating doxycycline inducible transgenes, could position this as conceptually straightforward method. Nevertheless, dox-inducible systems typically facilitate high levels of transgene expression that may not replicate physiological programs of tumor suppression. Therefore, efforts to reduce transgene expression levels and careful interpretation of the identified phenotypes are likely required.

EMERGING TECHNOLOGIES

The CRISPR revolution has brought forth a dizzying array of technologies to not only manipulate genomes at the DNA sequence level, but also directly modulate gene expression within cell culture and *in vivo* models. Clustered regularly interspaced short palindromic repeats (CRISPR) and the CRISPR-associated endonuclease Cas9 make up the core components of an ancient adaptive immune system found in bacteria and archaea that evolved to defend against invading pathogens [50]. Cas9 is guided to precise genomic loci via RNA molecules, derived from expressed CRISPR arrays, to effectuate double strand cleavage of DNA. Although CRISPR-Cas9 systems have enabled the rational design of RNA-programmable DNA endonucleases through expression of a synthetic single guide RNA (sgRNA), Cas9 proteins can also be modified to eliminate nuclease activity and further tailored to deliver alternative biochemical activities to precise genomic locations, thereby altering gene transcription [51–53].

CRISPR interference (CRISPRi) technology is based on localizing transcriptionally repressive peptide domains to a target gene to limit transcription [54–56]. Fusing potent transcriptional repressor domains to the nuclease defective Cas9 (dCas9) from *Streptococcus pyogenes*, CRISPRi technology can repress target gene expression with similar potency to RNAi-based approaches, but with unparalleled precision (*i.e.* low to zero off target effects) [54, 55]. Localization of dCas9 fused to the transcriptionally repressive KRAB (Krüppel-associated box) domain of Kox1 to promoter regions (−50bp to +300 bp relative to the transcription start site, TSS) can robustly silence both heterologous reporter plasmids and endogenous genes up to ~100 fold [54]. The CRISPRi approach is highly amenable to genome scale library screening approaches and has been used to identify growth, differentiation, and essentiality genes [56]. While CRISPRi approaches have succeeded in vitro, it is still unclear whether CRISPRi will replicate phenotypes associated with tumor suppressor gene inactivation within *in vivo* cancer models. However, the relatively straightforward, modular, and user-friendly nature of the methodology is likely to dramatically alter future approaches to regulate gene expression and cancer modeling in the mouse.

Currently, development of CRISPRi technologies has been focused on applications that enable high-throughput functional screening and gene interaction mapping approaches. However, we believe that alternative refinements of CRISPRi techniques that allow temporal

and regulatable control over gene inhibition and activation has tremendous potential to facilitate the dissection of tumor suppressor or oncogene biology in a systematic manner (Figure 3c). To realize the potential of CRISPRi to turn tumor suppressor gene expression off and back-on again in a regulatable fashion in established tumors, several technical developments are required. Generation of knock-in mice that harbor the Lox-STOP-Lox-dCas9-KRAB fusion into a safe harbor locus in the mouse (*e.g. Rosa26*) would enable the development of a system in which gene inactivation could be conditionally regulated upon a Cre recombination event. The required sgRNA component that targets the repressive dCas9-KRAB fusion to a region of interest could be introduced via viral vectors or from a separate transgenic mouse line. However, the major unmet challenge required to create an inducible and reversible dCas9-gene repressor system is the introduction of a mechanism that is dependent on the delivery of an exogenous factor such as doxycycline or tamoxifen. Reports of tamoxifen-dependent Cas9 enzymes have emerged that rely on the integration of *in vitro*-evolved inteins that inactivate Cas9 when present, but are '*spliced*' out to activate Cas9 after exposure to tamoxifen [57, 58]. Alternative, but complex, methods to confer tamoxifen dependence split Cas9 into independent N- and C-terminal ER-fusion proteins [59], or deliver ER domains to CRISPR/Cas9 complexes through RNA aptamers appended to the sgRNA, or to soluble tandem arrays of antibody fragments fused to Cas9 [60]. On the other hand, doxycycline regulated expression of the dCas9-KRAB fusion should be relatively straightforward by inserting a dox-responsive element into the transgene as has been done for Cas9 at the *Col1a1* locus [61]. Dox-dependent expression of the sgRNA is another strategy that has shown applicability within *in vitro* cell culture systems [62, 63]. Building on the lessons learned from dox-regulatable elements, implementing a CRISPR-based system for not only conditional gene repression, but also restoration of tumor suppressor function should be possible.

An alternative RNA interference approach that relies on the RNA-guided RNA-nuclease of the Type VI CRISPR-Cas13 system has recently emerged as a novel tool to knockdown mRNA transcripts [64]. Screening the entire family of Cas13 orthologs identified PspCas13b from *Prevotella sp. P5-125* as the most effective and specific family member [65]. Although it is too early to tell whether CRISPR-Cas13b will rival other RNA interference approaches in its efficiency, its relatively high specificity is likely to aid in the characterization of knockdown phenotypes. Integrating this emerging technology into *in vivo* systems and mouse cancer models will be challenging, but its ability to control genes at the transcript level may be pivotal to discern certain biological mechanisms such as those effectuated by non-coding RNAs.

CONCLUSIONS

Fueled by an ever-expanding toolbox to regulate gene function within *in vivo* systems, genetically engineered mouse models have profoundly impacted our understanding of cancer biology. Classical gene targeting methods in ES cells laid the foundation for the development of recombinase-based conditional alleles that rapidly evolved to allow tumor suppressor genes to be toggled *'off'* in somatic cells in a cell type-restricted and temporally-controlled manner. Once switched off, studying the evolutionary adventure of tumorigenesis that ensued became possible, and these germline or conditional knock-out models provided

critical insights into the biology of cancer. However, many critical mechanisms of tumor suppression remained hidden in the shadows of these cancer models, awaiting new tools that enable inactivation of tumor suppressor gene function in a reversible manner to expose latent anti-cancer mechanisms. Thus, toggling tumor suppressor gene function *off and back-on again*, offers new and unbiased opportunities to discern context-dependent '*tales'* of tumor suppression.

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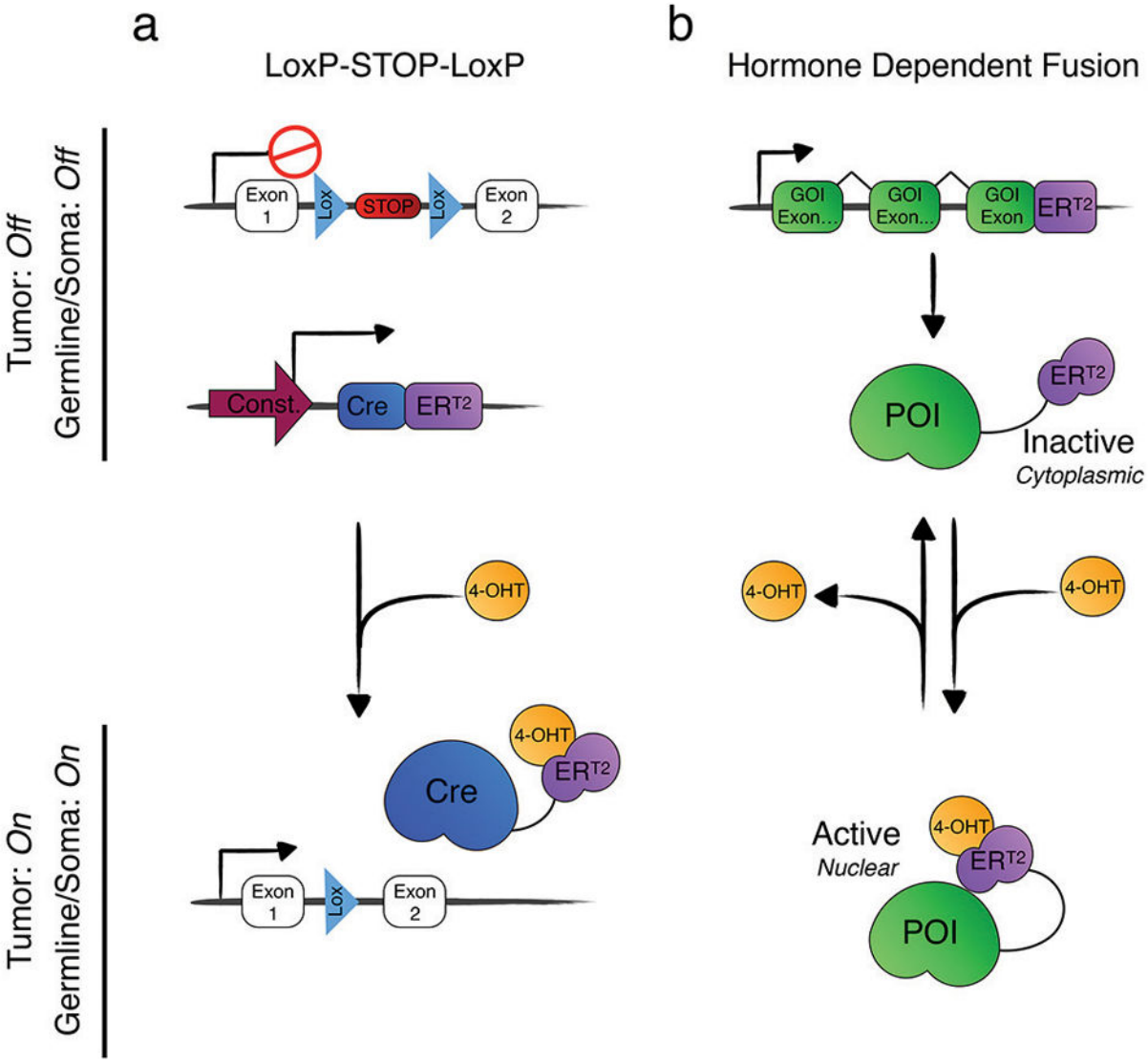

Figure 1.

Allele-based methods to regulate gene function incorporate novel regulatory elements into endogenous loci. **(a)** LoxP-STOP-LoxP (LSL): These alleles harbor transcriptional and translational inhibitory elements embedded within the STOP cassette. Some STOP cassettes also contain heterologous genes that confer antibiotic resistance (*e.g.* puromycin) to aid in the selection of properly targeted ES cells. The STOP elements are typically placed in early introns to more completely limit transcription at the locus of interest. LoxP sequences flank the STOP cassette facilitating its removal by Cre recombinase. In principle, multiple methods could be tailored for the timely introduction of Cre. However in practice, the *Rosa26*$^{Cre\text{-}ERT2}$ allele has been favored to temporally control Cre activity via the delivery of 4-hydroxytamoxifen (4-OHT). **(b)** Hormone dependent fusion alleles: Incorporation of an estrogen receptor fragment downstream of, and in frame with, the final exon of a gene of interest (GOI) generates a fusion protein of interest (POI) whose function is contingent upon the presence of 4-hydroxytamoxifen (4-OHT). Addition and removal of 4-OHT allows for

toggling between the inactive and active states. Note germline and somatic expression patterns (*On vs. Off)* for the host gene of interest.

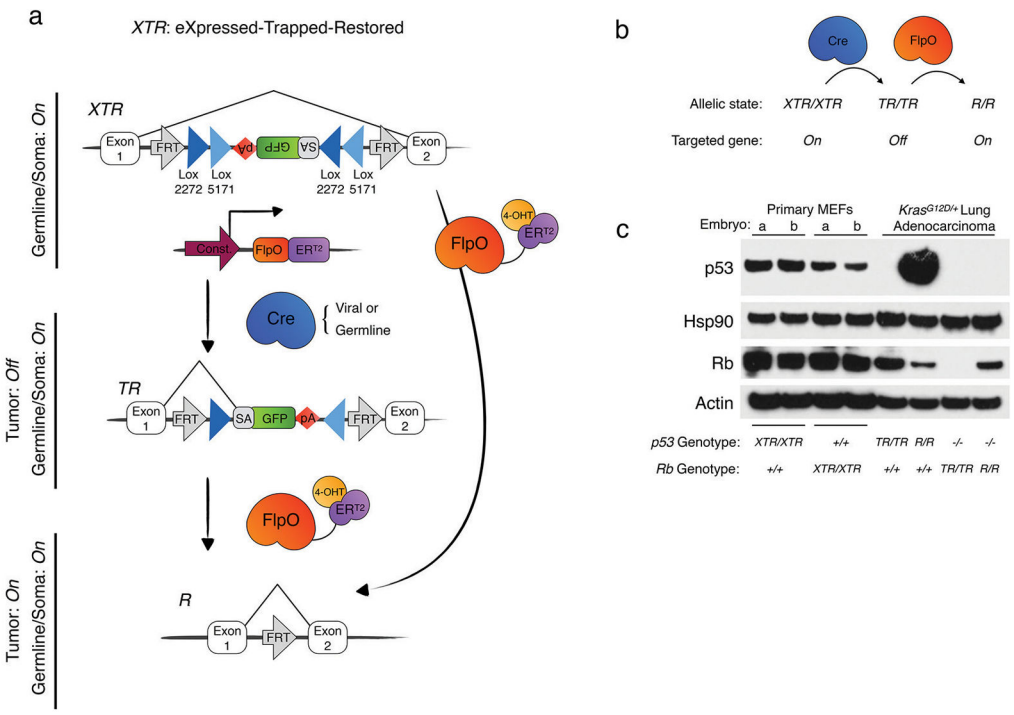

Figure 2.

XTR (e*X*pressed-*T*rapped-*R*estored): *XTR* alleles allow recombinase-based regulation of any spliced locus of interest. **(a)** Delivery of Cre via any method mediates the permanent inversion of a gene trap consisting of a splice acceptor (SA), GFP complementary DNA, and the polyadenylation transcriptional terminator sequence (pA). Inversion is facilitated by the use of two pairs of mutually-incompatible mutant LoxP sites (Lox2272 and Lox5171) arranged in the *'double-floxed'* configuration. Cre converts expressed *XTR* alleles to trapped *TR* alleles that inactive downstream gene expression. Transcripts are spliced from the upstream exon to a GFP reporter gene and downstream transcription is terminated to functionally inactivate gene function. The $Rosa26^{FlpO\text{-}ERT2}$ allele enables tamoxifen-dependent conversion of trapped *TR* to restored *R* alleles via excision of the gene trap to effectively restore tumor suppressor gene expression. *XTR* alleles can be converted directly to *R* alleles without changes in gene function. Note germline and somatic expression patterns (*On vs. Off)* for the host gene of interest. **(b)** Experimental scheme depicting conversion of *XTR/XTR* to *TR/TR* to *R/R* following the sequential delivery of Cre and FlpO recombinases. **(c)**. Immunoblot analysis of Rb and p53 expression demonstrates normal levels of p53 and Rb are expressed from *XTR/XTR* loci, *TR/TR* robustly silences p53 and Rb expression, and p53 and Rb are effectively re-expressed in *R/R* lines. Fibroblasts were derived from independent $p53^{XTR/XTR}$ and $Rb^{XTR/XTR}$ mouse embryos ('*a*' and '*b*'). Lung adenocarcinoma cell lines were established from $Kras^{LSL\text{-}G12D/+}; p53^{TR/TR}$ or $Kras^{LSL\text{-}G12D/+}; p53^{flox/flox}; Rb1^{TR/TR}$ tumors. Addition of adenoviruses expressing FlpO converted *TR/TR* to *R/R* and restoration of p53 and Rb expression. Hsp90 and Actin are loading controls for the p53 and Rb blots respectively.

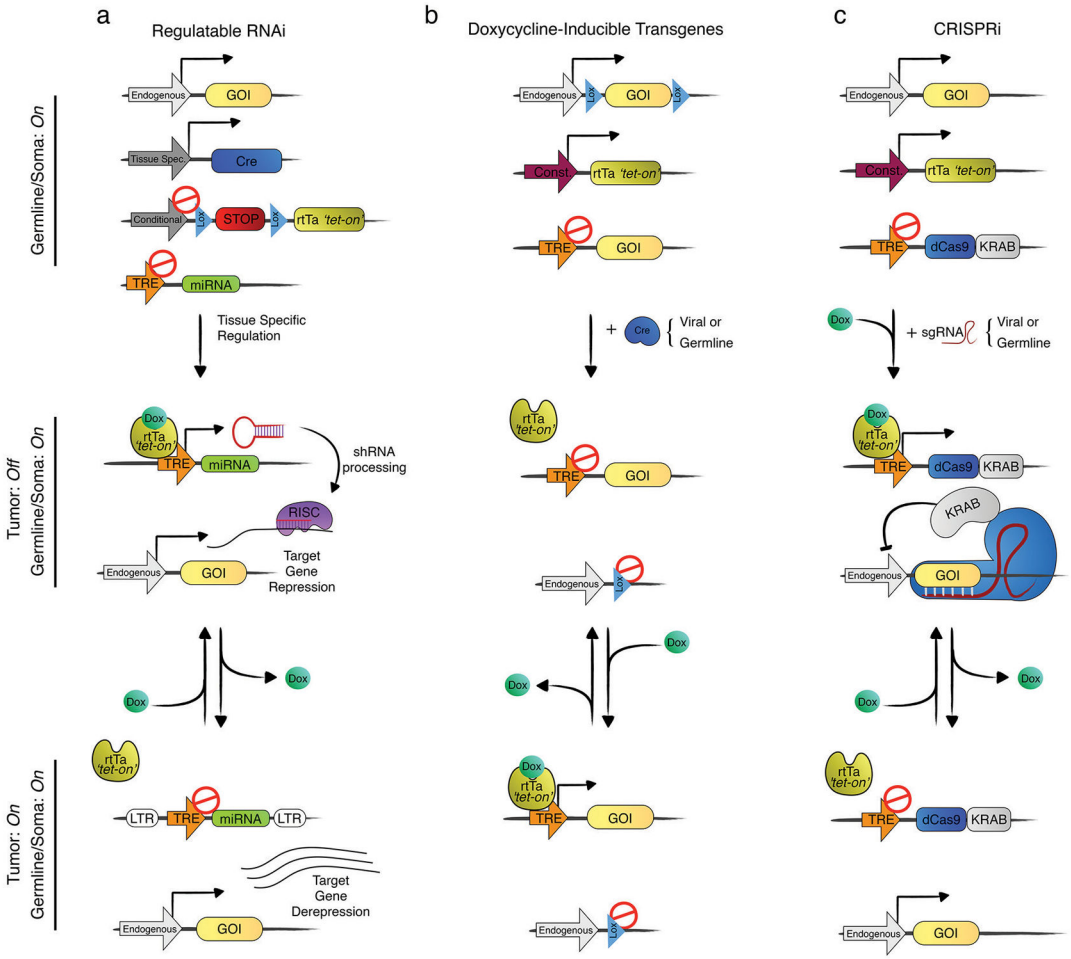

Figure 3.

Transgenic models designed for conditional and reversible tumor suppressor gene inactivation. **(a)** Regulatable RNAi: The tetracycline reverse transactivator (rtTa *'tet-on'*) protein binds to the tetracycline responsive element (TRE) present upstream of a miRNA transgene to induce shRNA expression in a doxycyclin-dependent manner. The expressed miRNA is processed by the RNA-induced silencing complex (RISC) and leads to repression of the target gene mRNA. Removal of doxycycline inhibits rtTa transactivation and the lost expression of the miRNA transgene leads to derepression of the target gene mRNA expression. Highlighted here is the example used by Dow et. al. [42] to introduce transgenes in a tissue specific manner, however several variations on this scheme are possible and include the use of tTA-based *'tet-off'* systems and retroviral delivery of transgenes (see Xue et al. [40]). **(b)** Doxycycline-inducible complementary DNAs: The rtTa *'tet-on'* requires doxycycline to bind TRE and activate expression of downstream genes of interest (GOI). Combining expression of rtTA (constitutive or conditional), a floxed endogenous GOI, and a transgene element with a TRE-controlling a cDNA of the GOI would allow for excision of the endogenous gene following delivery of Cre recombinase and Dox-dependent re-expression of the GOI. **(c)** CRISPRi: Controlled tumor suppressor gene repression with CRISPR interference relies on the constitutive (or conditional) expression of rtTa to drive the

expression of a nuclease defective Cas9 (dCas9) tethered to the transcriptionally repressive Krüppel-associated box (KRAB) domain. SgRNAs expressed from viral or germline transgenes would guide the dCas9-KRAB to any GOI to repress transcription. Doxycycline-dependent expression of dCas9-KRAB establishes control of inducible and reversible gene expression. Note germline and somatic expression patterns (*On vs. Off)* for the gene of interest.

Table 1

Summary of allelic and transgenic models to regulate tumor suppressor gene function in a conditional and reversible manner.

Method	Allelic vs. Transgenic	Germline Expression	Useful for essential genes	Physiological expression	Efficiency of knockout	Coupled to reporter	Unique hurdles to implementation	Specific Advantages	Number of alleles/ transgenes needed	Example references
Lox-STOP-Lox allele	Allelic	None	No	Yes	Similar to germline KO	No	None	None	2: LSL allele, regulatable Cre	4,7
ER Fusion knock in allele	Allelic	Tamoxifen dependent	No	Depends on post translational stability	Must be tested	No	Validating non-function and inducible function	Single allele	1: ER fusion	13,14,15
XTR allele	Allelic	Normal	Yes	Yes	Similar to germline KO	Yes (Endogenous)	Not effective in rare cases where genes are encoded by a single exon.	Plug and play potential, reports expression of disrupted gene	3: XTR allele, regulatable-FlpO, tissue specific Cre	22
Tet-regulatable RNAi transgene	Transgenic	Normal	Yes	Yes	Variable: Depends on knock down efficiency	Potentially: If shRNA has second cistron	Potential off target effects of shRNA, Identification of potent shRNA, not compatible with tet-regulated cancer models, limited availability tissue specific tTA or rtTA expression	Scalable, works as a transgene	3: TRE-shRNA allele, cre-dependent tTA or rtTA allele, tissue specific Cre	37, 40, 41, 42
Floxed allele + Tet-dependent expression of cDNA transgene	Both	Normal	Yes	Unlikely	High	Potentially: If cDNA has second cistron	Determining transgene behavior	Can induce cDNA expression to supraphysiological levels	4: floxed allele, TRE-cDNA, cre-dependent tTA or rtTA allele, tissue specific Cre	48
CRISPRi	Transgenic	Normal	Yes	Yes	Variable: Depends on repression efficiency	Potentially: If dCas9-KRAB has second cistron	Development of TRE-dCAs9-KRAB transgenic mouse line, not compatible with tet-regulated cancer models, limited availability tissue specific tTA or rtTA expression	Scalable, works as a transgene	3: TRE-dCAS9-KRAB, cre-dependent tTA or rtTA allele, tissue specific Cre	12, 13