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A Continuum Model for Tumour Suppression

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Introduction

Although hereditary predisposition to cancer was known before 1900, it was only then, after the rediscovery of Gregor Mendel's once-ignored 19th century work, that hereditary predisposition to cancer could be rationalized. By then it was also known that the pattern of chromosomes in cancer cells is abnormal. The next contribution to understanding cancer genetics was made by Theodor Boveri, who proposed that some chromosomes might stimulate cell division and others might inhibit it, but his idea was long overlooked. Now we know that there are genes of both types.

In this review, we summarize the history of the study of the latter type of genes, tumour suppressor genes (TSGs), and the evidence supporting a role for both complete and partial tumour suppressor inactivation in the pathogenesis of cancer. We integrate the classical “two-hit” hypothesis of tumour suppression with a continuum model that accounts for subtle dosage effects of tumour suppressors, and we discuss other exceptions to the two-hit hypothesis such as the emerging concept of “obligate haploinsufficiency” in which partial loss of a TSG is more tumorigenic than complete loss. The continuum model highlights the importance of subtle regulation of TSG expression or activity such as regulation by miRNAs. Finally, we discuss the implications of this model on the diagnosis and therapy of cancer.

The “two-hit” hypothesis

The first evidence for a genetic abnormality as a cause of cancer came with the discovery, in 1960, of the abnormal “Philadelphia” chromosome in chronic myelogenous leukaemia cells¹. Later, in 1973, it was discovered that this chromosome was a translocation between chromosomes 9 and 22² and in 1977, a translocation of chromosomes 15 and 17 was identified in acute promyelocytic leukaemia⁴. Eventually, the genes at the breakpoints of these translocations were cloned - BCR/ABL in 1983³ and PML/RAR α in 1991⁵. Meanwhile, seminal work demonstrated that the cancer-causing gene within the avian sarcoma virus genome, v-src, was actually a co-opted and mutated version of a normal cellular gene termed a proto-oncogene, now called c-src^{6,7}. These observations demonstrated that normal cellular genes, when mutated or altered, are able to cause cancer, and were followed by the identification of numerous other cellular oncogenes that are activated by mutation, chromosome translocation, or amplification. One of Boveri's predictions was proven to be correct.

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In the 1980s, scientists verified Boveri's other prediction with the identification of a second class of genes involved in cancer, "tumour suppressor genes," that inhibit cancer development and oppose oncogene function. In a normal cell, a physiological balance between tumour suppressors and oncogenes maintains homeostasis and allows carefully regulated cell proliferation without unrestrained malignant tumour growth. Somatic cell fusion experiments pointed to the existence of such TSGs, because fusion of a normal cell with a malignant cell could revert the malignant cell to a normal phenotype⁸. One paradox that puzzled cancer researchers was that cancer susceptibility syndromes usually display a dominant mode of inheritance whereas the hypothesized "tumour suppressor genes" appeared to function in a recessive manner in the *in vitro* cell fusion experiments.

The resolution of this problem was provided by the analysis of a tumour of children, retinoblastoma (RB), which is sometimes present even at birth⁹. Statistical modelling indicated that hereditary cases likely developed after only one somatic mutational event. It was hypothesized that that one mutant allele is inherited and the other is generated somatically during growth of the developing eye. Because of the high chance of the second "hit" occurring, almost all individuals with the inherited first hit (the mutant allele) will develop retinoblastoma, and therefore the cancer susceptibility phenotype is inherited in a dominant manner (Figure 1). In contrast, tumour initiation requires both hits, and therefore tumourigenesis is recessive. A similar mechanism could be extended to sporadic retinoblastoma, the only difference being that in the sporadic cases both the first and second hits occur in somatic tissue. At the time of the original analysis, it wasn't known if these two hits occurred in the same or distinct genes, but it was clear that the search for the secondary mutation would start with the disease gene itself.

The success of the two-hit model came with the identification of deletion of a locus on chromosome 13 in RB tumours and the subsequent cloning of the *RB1* gene as mutated in familial retinoblastoma¹⁰⁻¹⁷. This discovery was made possible by the development of restriction fragment length polymorphism (RFLP) mapping technology. Using this approach, it was shown that many families with familial retinoblastoma did indeed harbour mutations or deletions of *RB1* in the germline (the first hit), and tumours from RB patients nearly always contained mutation or loss of the other *RB1* allele¹⁰⁻¹⁷ (the second hit). This latter event most often results from somatic mitotic recombination, while in a minority of cases, the normal allele acquires a distinct mutation. The role of recessive TSGs in dominantly inherited cancer susceptibility syndromes could then be understood, and Boveri's second prediction was substantiated.

These findings and others established the two-hit hypothesis as a model that not only explained hereditary cancer susceptibility but also provided a proof-of-principle that TSGs could be identified by the study of chromosomal deletions and genetic linkage in hereditary cases of a cancer and subsequent analysis of the second allele of the locus. Such a strategy was critical to the ultimate identification of *TP53* (commonly called *p53*) as a TSG¹⁸ and was used successfully in the identification of other TSGs such as *APC*¹⁹, *BRCA1*²⁰, and *BRCA2*²¹. Now the advent of high-throughput sequencing and genome-wide copy number profiling is allowing further systematic identification of regions of mutation and deletion in tumour genomes^{22,23}.

It should be noted that it was initially hypothesized that two hits would be sufficient for the initiation of some tumours such as paediatric cancers, but additional mutations would be necessary for others. It is now thought that non-hereditary cancers require at approximately four or more distinct mutation events that result in perturbation of critical cellular signalling pathways such as PI3K, p53, or RB²⁴. Malignancy is thought to result from an iterative process of somatic mutation followed by clonal expansion²⁵. In the context of hereditary

cancer syndromes, full inactivation of the involved disease gene may be the rate-limiting step for tumour initiation, but other events may occur, particularly to promote or enhance tumour progression. In this respect, the “two-hit hypothesis” should be applied directly to the TSG, with two hits representing the number of events necessary for inactivation of the TSG, but not necessarily for tumorigenesis. Haploinsufficiency, as we discuss below, must also be interpreted in this context; in many cases, a gene might be haploinsufficient for a specific cellular function, but TSG haploinsufficiency may promote tumour formation only in the context of other genetic or environmental insults.

Haploinsufficiency and quasi-sufficiency

The two-hit hypothesis clearly explained the differences in tumour number and age of onset of RB in hereditary vs. sporadic cases of RB. Moreover, it led to the eventual cloning of *RB1* as the first bona fide human TSG. The hypothesis has been useful in cloning genes for hereditary cancers and for their non-hereditary counterparts. However, this success has led to a questionable dogma in the field that all important tumour suppressors should behave according to the “two-hit” model.

Investigators sought to apply the principles learned from the two-hit model to sporadic cancers in order to identify novel TSGs. Many recurring regions of chromosomal deletion have been identified in sporadic cancers, suggestive of the presence of a TSG. Unfortunately, it has become apparent that not all regions of consistent loss are accompanied by obvious aberrations on the other allele, raising the question of whether these deletions may represent “passenger” events not relevant to carcinogenesis. Indeed, very few of the identified regions of chromosomal losses have yielded clear TSGs in those loci that conform to the two-hit hypothesis. This finding has led many to conclude that the relevant tumour suppressors in those regions have not been identified or do not exist, when in fact many genes in those loci are known to have tumour suppressive properties *in vitro* or *in vivo*.

Alternatively, these regions could harbour bona fide TSGs but may represent regions/genes where single copy mutation or loss plays a role in tumorigenesis. These single copy events may be even be selected for during tumorigenesis instead of biallelic TSG loss. One possibility is that the initial lesion, when reduced to homozygosity, may lead to cell death or senescence. The lethality could be due to homozygosity of the disease gene itself or of other distinct genes included in the initial “hit,” since recombination is a principal “second hit” in tumorigenesis²⁶. Such a scenario would result in an “obligate haploinsufficiency” in which selection pressure during tumorigenesis favours partial, but not complete, loss of the TSG.

A second possibility is that the single copy mutation of a TSG functions as a dominant-negative towards the wild-type gene/protein. After mutation of one allele, the mutant protein product interferes with the normal wild-type protein produced from the remaining wild-type allele. Because the complete normal function of the TSG is already impaired with only one hit, there is no selection pressure by the tumour for loss or mutation of the wild-type allele.

A third possibility is that a gene or genes in the region of consistent deletion exhibits haploinsufficiency for tumour suppressor function and single-copy loss of the TSG is sufficient for aberrant TSG function and promotion of cancer (Figure 1). In contrast with classical TSGs that are insensitive to large reductions in expression or activity, the function of haploinsufficient TSGs is impaired by a 50% partial reduction in expression or activity, or sometimes by subtler changes, as in a “quasi-insufficiency” of TSG function that we discuss below.

The role of haploinsufficiency in cancer has been met with scepticism, despite the well-established role of haploinsufficiency in numerous developmental disorders such as, for

instance, aniridia (caused by haploinsufficiency for *PAX6*) and Grieg syndrome (caused by haploinsufficiency of *GLI3*)²⁷. Just as increased dosage of genes can result in developmental syndromes, such as Down syndrome, increased dosage or activity of oncogenes is an established genetic mechanism of malignancy²⁸, exemplified by the role of *MYC* amplification or *RAS* hyperactivity in cancer. However, the notion that subtle decreases in gene dosage or protein activity can be relevant to cancer has gained only limited acceptance in the scientific community.

One reason for this scepticism stems from the difficulty in definitively proving a haploinsufficient TSG is involved in tumourigenesis. Whereas the rare TSGs that fully conform to the two-hit hypothesis can be identified by their homozygous deletion or mutation in cancer, haploinsufficient TSGs cannot be identified in such a manner. Moreover, large regions of the genome, encompassing many genes, can be targeted by allelic loss and likewise, many genes may acquire somatic mutations during the course of tumour development. It is assumed that only a portion of these genes is responsible for the cancer, while the rest are passenger mutations. What then could be the gold standard by which to determine which gene or genes in the region are causative and which are non-involved bystanders? Unlike the analysis of traditional TSGs, no one assay or approach offers definitive proof that a haploinsufficient TSG is causally involved in tumourigenesis. Instead, an integrated approach involving human genetic analysis, *in vitro* and *ex vivo* functional studies, and murine cancer genetic modelling is critical to ascertain whether a gene may function as a haploinsufficient gene in cancer. Although there are caveats of each analysis on its own, a body of evidence from each of these analyses can strongly implicate a gene in dosage-sensitive tumour suppression.

Throughout this review, we use *PTEN* as a model dosage-sensitive TSG, but numerous other TSGs exhibit haploinsufficiency and dosage-sensitivity. Notably, *TP53*, commonly referred to as *p53*, exhibits haploinsufficiency. Mice with heterozygous (+/-) *p53* mutation show an intermediate survival to that of *p53* homozygous mutants and wild-type mice, and tumours that develop in the +/- animals do not always display loss of the remaining wild-type allele²⁹. Similarly, tumours in patients with Li-Fraumeni syndrome, a cancer susceptibility syndrome caused by germline mutation of *p53*, do not always exhibit loss of the wild-type *p53* allele, suggesting that haploinsufficiency of *p53* may be sufficient for tumour initiation in humans as well³⁰. A caveat with these analyses is that the effect of *p53* haploinsufficiency on cancer initiation in mouse models and Li-Fraumeni patients may be due to complex non-cell autonomous effects stemming from the partial loss of *p53* throughout the body. However, *ex vivo* analysis of murine *p53* heterozygous thymocytes showed that these cells have an impaired apoptotic response to ionizing radiation or etoposide³¹. Similarly, a *p53*-deleted HCT116 isogenic cell line expressed only 25% the normal level of *p53* mRNA and exhibited an impairment in induction of *p53*-responsive genes and apoptosis after exposure to UV radiation³². These studies demonstrate that reductions in *p53* dosage and function can impact on a cells' ability to respond to oncogenic stimuli and strengthen the notion that *p53* haploinsufficiency may drive tumourigenesis.

Similarly, other classic cancer susceptibility genes such as *BRCA1* and *BRCA2* may exhibit haploinsufficiency and/or dosage effects. Primary breast and ovarian cells from patients with heterozygous germline mutation of *BRCA1* or *BRCA2* have altered mRNA profiles compared to *BRCA* wild-type cells, suggesting that single hits in these genes can confer phenotypic differences that could in principle impact on breast and/or ovarian tumourigenesis³³. Indeed, *ex vivo* studies of human *BRCA1* mutant breast cells have found that these cells exhibit enhanced colony formation potential³⁴ and show impaired lineage commitment in differentiation assays³⁵. Although tumour formation in *BRCA1* and *BRCA2* carriers does appear to require the "second hit," these "one-hit" premalignant changes could

impact tumourigenesis by either promoting loss of the second TSG allele or of other TSGs, or by altering the cellular phenotype to predispose to certain tumour subtypes.

Another haploinsufficient TSG is the transcription factor *PAX5*. *PAX5* is found mutated, deleted, or translocated in approximately 30% of acute lymphoblastic leukaemia cases, but the aberrant mutations/deletions are almost invariably mono-allelic and appear to function as hypomorphs, not dominant negative alleles³⁶. Similarly, the E3 ligase *FBW7* is mutated in approximately 10% of human colorectal cancers³⁷. In 70% of these cases, the mutations are mono-allelic, suggestive of haploinsufficiency. Conditional mono-allelic deletion of *Fbw7* in the murine intestine cooperated with the tumorigenic *APC^{min}* allele in intestinal tumourigenesis, albeit to a lesser degree than biallelic deletion of *Fbw7*, suggesting that *Fbw7* is dosage-sensitive³⁷.

Like complete loss of TSGs, the effect of TSG haploinsufficiency can be highly tissue-specific and context dependent (Figure 2). Thus, the cellular and molecular context in which TSG function is altered will determine the outcome of such impairment of TSG function. The differing outcomes of TSG expression level in differing situations may reflect distinct thresholds of protein expression or activity needed for certain processes or in differing cell contexts. For example, in one cell type there may be other compensatory proteins that mask the potential phenotype caused by TSG haploinsufficiency whereas in other cells these proteins are not expressed and so the haploinsufficiency of the TSG manifests as tissue-specific cancer susceptibility.

Two hits for tumour suppression

The genetic background of the individual or tumour will also impact the phenotypic outcome of TSG haploinsufficiency. In some cases, combinatorial effects could arise whereby haploinsufficiency or mutation of other genetic loci is required for a phenotype caused by TSG haploinsufficiency. A powerful example of this context dependency is provided by the interaction between *Pten* deficiency or haploinsufficiency and the presence or absence of mutated *p53*³⁸ (Figure 2). In the context of wild-type *p53*, *Pten* haploinsufficiency is actually *more* tumourigenic in the prostate than complete loss of *Pten*, because the latter triggers a *p53*-dependent fail-safe senescence mechanism called *Pten*-loss induced cellular senescence (PICS). Haploinsufficiency of *Pten* enhances proliferation without activating PICS, making partial loss of *Pten* more detrimental in this context than complete loss of *Pten* (Figure 2). Similarly, complete loss of *PTEN* in the blood induces hematopoietic stem cell exhaustion or bone marrow failure, unless loss of *PTEN* is accompanied by other genetic events³⁹. After accumulation of aneuploidy or other mutations, such as loss of *p53*, complete loss of *PTEN* in the blood induces fatal leukemias^{39,40} (Figure 2).

This phenomenon defines a novel paradigm of “obligate haploinsufficiency” in which selection pressure during tumour growth may favour haploinsufficiency of a TSG over complete loss in certain scenarios (Figure 1). However, in advanced cancers with *p53* mutation or loss, PICS cannot be induced and complete loss of *Pten* enhances proliferation and tumourigenesis to a greater degree than *Pten* haploinsufficiency. This remarkable finding likely explains why complete loss of *PTEN* is typically restricted to advanced cancers and also emphasizes the importance of single-hit haploinsufficiency of TSGs in cancer initiation. Understanding the combinatorial and contextual dependencies of genetic events should not only enhance our ability to effectively treat tumours with differing combinations on genetic “hits” but will also inform chemopreventative strategies for eradicating cancer by attacking mechanisms necessary for its development.

Like *PTEN*, many other TSGs exhibit “obligate haploinsufficiency” where partial loss of the TSG is tumorigenic but complete loss is lethal to the cell. One example is the microRNA-processing enzyme *DICER1*. Mono-allelic deletion or mRNA downregulation of *DICER1* is observed in diverse cancers^{41,42}. In murine models, mono-allelic conditional deletion of *Dicer1* enhances lung tumorigenesis driven by oncogenic *Kras*⁴¹, sarcoma development induced by *Kras*^{G12D} and loss of *p53*⁴¹, and retinoblastoma formation induced by deletion of *Rb1* and *p107*⁴². In the lung model, complete inactivation of *Dicer1* improved survival compared to animals with mono-allelic *Dicer1* deletion⁴¹. Tumours that did develop in the *Dicer1*^{fllox/fllox} animals retained one wild-type allele and partial *Dicer1* expression, strongly suggesting that partial loss of *Dicer1* promotes tumorigenesis.

A recent *in vivo* shRNA-based screening approach identified lymphoma tumour suppressors, including one, the cell cycle regulator *Rad17*, which appeared to function as a TSG with obligate haploinsufficiency⁴³. The use of multiple hairpins targeting *Rad17* led to the discovery that only hairpins that partially suppressed *Rad17* promoted lymphomagenesis whereas hairpins that completely repressed *Rad17* expression were selected against in the lymphomas. This study highlights the utility of shRNA knockdown and screening technologies for the identification of haploinsufficient TSGs.

NPM1, found mutated and translocated in hematopoietic malignancies, also displays obligate haploinsufficiency. Whereas single-allelic deletion of *NPM1* induces tumour formation in mice⁴⁴, biallelic loss induces embryonic lethality and is also incompatible with cell growth after conditional deletion *ex vivo*⁴⁵. Although more data is needed, the TSGs *TAp63* and *PinX1* may also display obligate haploinsufficiency; heterozygosity for *TAp63* promoted tumorigenesis to a greater degree than complete loss of *TAp63* in genetically engineered mice, and tumours that developed in the heterozygous mice did not display loss of the wild-type allele⁴⁶. Similarly, *PinX1*, recently identified as a haploinsufficient tumour suppressor⁴⁷, may also display obligate haploinsufficiency; heterozygous loss of *PinX1* in a mouse model promoted cancer development in multiple organs, and tumours did not lose the wild-type allele or expression of *PinX1*⁴⁷.

A genome-wide screening study in yeast indicated that about 3% of the genes in the yeast genome displayed haploinsufficiency when assayed under specific growth conditions⁴⁸. These haploinsufficient genes were enriched for ribosomal genes and genes that are highly expressed. Currently, mammalian tumour suppressors that exhibit haploinsufficiency do not appear to conform to one specific functional class. Besides *p53* and *PTEN*, dozens of other TSGs exhibit haploinsufficiency for tumour suppression^{49,50}, many of which we have discussed above. These TSGs come from many functional classes, including transcription factors (e.g. *PAX5* and *TAp63*), cell cycle checkpoint genes (*RAD17*), E3 ligases (*FBW7*), ribosomal or associated proteins (*NPM1*), and other diverse genes such as *Dicer1* and *PinX1*.

A continuum model for tumour suppression

Rather than TSG function and activity following discrete, step-wise changes, evidence is emerging that supports the dosage-dependency of TSG function. Data from an allelic series of targeted *Pten* alleles that results in varying levels of *Pten* *in vivo* in genetically-engineered mice^{51,52} demonstrate the tight correlation between *Pten* expression level and function. Taking advantage of a null *Pten* allele in combination with a *Pten* “hy” allele, which is expressed at a lower level than the wild-type allele, an allelic series was created in which *Pten* expression follows a decreasing pattern in the order *Pten*^{+/+} > *Pten*^{hy/+} > *Pten*^{+/-} > *Pten*^{hy/-}. Surprisingly, *Pten*^{hy/+} animals, with cells expressing 80% of the normal level of *Pten*, did display many of the phenotypes observed in *Pten*^{+/-} animals, such as

lymphadenopathy, splenomegaly, and mammary gland tumours⁵². Moreover, survival of *Pten*^{hy/+} animals is compromised compared to *Pten*^{+/+} controls, but not to the same degree as the survival impairment in *Pten*^{+/-} animals⁵². Thus, the tumour incidence and survival of the animals correlate with the *Pten* expression level⁵². Critically, mammary tumours from *Pten*^{hy/+} animals do not show loss of *Pten* expression or loss of the wild-type *Pten* allele and exhibit a *Pten* dose-dependent increase in activation of Akt, further demonstrating that a subtle reduction in *Pten* dose can result in deregulated signalling and cancer⁵². Similarly, in the murine prostate, a progressive lowering of *Pten* levels correlates with Akt activation, prostate enlargement, proliferation of prostate cells and tumourigenesis⁵¹. These *in vivo* studies demonstrate that even a subtle 20% reduction of protein level could constitute an important “hit” involved in the development of cancer. We termed this state: TSG “quasi-insufficiency”⁵² (Figure 1).

Thus, protein function can be a continuum that is related to the level of expression or activity of the TSG rather than to discrete step-by-step changes in gene copy number. We therefore propose a shift from the classical discrete model of tumour suppression to a continuum model (Figure 3). This model takes into account the fact that subtle regulation of TSGs can have profound consequences on cancer susceptibility and/or progression, and in turn has important implications for cancer diagnosis and therapy. Note that while the function of *PTEN* appears to be strongly coupled to its expression level, for other TSGs it will be the activity of the protein and not the expression level that may be critical. This situation is readily observed in tumours with *p53* mutation where the mutant allele is highly expressed but functionally inactive or acting as a dominant negative.

Like the effect on TSGs, dosage can be extremely important for regulating the cellular effects of oncogene expression. Dramatically different consequences for the cell and for cancer development can occur depending on the level of oncogene expression. For example, high expression of oncogenic, mutated *RAS* genes drives senescence, not proliferation⁵³. Similarly, robust expression of *MYC* induces apoptosis, not proliferation⁵⁴. These examples mirror the paradigm of PICS and “obligate haploinsufficiency” and suggest that tumours may select for optimal expression of oncogenes. Data to support this notion exist already in the rampant amplification of mutated oncogenes, such as the amplification of the mutated *EGFR* gene in human lung cancer^{55,56}. Such data suggests that it is not only mutation of an oncogene that is important for cancer, but also its expression level. In this respect, the continuum model could also apply to oncogenes (Figure 3).

It appears now that some TSGs are exquisitely sensitive to dose (e.g. *PTEN*), some are intermediately sensitive (e.g. *p53*) and others are more resistant to expression level and/or activity changes (e.g. *RBI*). Those that are most resistant to changes in expression or activity, such as *RBI*, will be most likely to conform to the two-hit hypothesis whereas those that are exquisitely sensitive to dose may exhibit frequent monoallelic deletion/mutation or be misregulated in cancer via other means, such as miRNA overexpression. Moreover, tumour suppressors that exhibit “obligate haploinsufficiency” due to the lethality of homozygous inactivation of the TSG will never exhibit complete inactivation in cancer unless there are other context- or genotype-dependent events that favour their complete inactivation (as in the case of *Pten/p53* discussed above).

The dogma that every TSG must behave according to the two-hit paradigm could significantly slow the pace of cancer research by dismissing potentially critical genes as unimportant. The pace of cancer drug development will in turn be delayed by a lack of appreciation of the involvement of these potentially druggable pathways in tumour development and maintenance. A very real challenge for cancer biologists in the next decade will be first to define the TSGs affected by haploinsufficiency and then to model these subtle

dosage effects in a way that can yield quantitative models of cancer risk or outcome. Since human genetics analysis alone is often not enough to unequivocally identify critical haploinsufficient genes, mouse models will prove essential to define which genes exhibit haploinsufficiency for TSG function and in which specific cellular and genetic contexts.

Regulation of TSG activity

The continuum model of tumour suppression holds that precise regulation of TSG expression and activity is critical and thus, mechanisms of regulation of expression and function may powerfully contribute to tumour suppression. One of the most widespread networks of expression regulation is the post-transcriptional regulatory network of microRNAs (miRNAs) and competing endogenous RNAs (ceRNAs). In addition to these networks, TSG dosage/activity is regulated by transcriptional, translational, and post-translational mechanisms (Figure 4). These networks open up the realm of genes that could be contextually important for tumourigenesis, especially when considering mechanisms that tune TSG activity and not only those that function as on/off switches.

As an example, a powerful PTEN-targeting miRNA network was recently identified. MiRNAs are small non-coding RNA molecules that regulate protein expression through inhibition of protein translation or induction of direct cleavage of their mRNA targets via RISC⁵⁷. Many of the *PTEN*-targeting miRNAs are amplified or overexpressed in cancer such as miR-26 in glioma⁵⁸ and miR-22 in prostate cancer⁵⁹. The regulation of coding RNAs by miRNAs is made additionally complex through the interaction of miRNAs with ceRNAs^{60,61}. We coined the term “competing endogenous RNA” (ceRNA), to refer to an RNA – coding or noncoding – that shares miRNA binding sites with another RNA with which it competes for miRNA binding^{60,61}. Any two or more mRNAs with shared miRNA recognition elements (MRE) can act as ceRNAs towards each other by competing for miRNA binding and thereby co-regulating each other's expression. As the expression of the ceRNA increases, the miRNA binding sites on the ceRNA compete with the miRNA binding sites on the other transcript, and less miRNA is available for binding to the TSG transcript (Figure 4). CeRNA expression (e.g. expression of the *PTEN* pseudogene⁶⁰), hence results in sustained TSG expression by preventing miRNA binding to the TSG and subsequent mRNA or protein downregulation. Conversely, loss of ceRNA expression could result in low TSG expression through diversion of additional miRNA molecules to the TSG transcript^{60,61}. Even non-coding ceRNAs that are not expressed at the protein level could function as TSGs by regulating expression of protein-coding TSGs, and mutation or deletion of ceRNA genes could be important in cancer^{60,61}.

Implications for cancer susceptibility and therapy

An appreciation of the continuous nature of tumour suppressive function must in turn lead to a re-evaluation of the role of TSG dosage in cancer susceptibility, diagnosis, and treatment. Subtle variation in TSG expression or activity could underlie genetic variation in cancer susceptibility in the population. Inherent, constitutive differences in gene expression could be caused by polymorphic variation in TSG promoter regions or in miRNA binding sites or other regulatory regions. Importantly, synonymous SNPs that do not change the protein sequence of an expressed gene could still have a powerful impact by altering the level of TSG expression through alteration of miRNA binding. Moreover, SNPs in non-coding RNAs could impact tumour development through ceRNA effects. Alternatively, on the basis of a continuum model, susceptibility to cancer could be induced by environmental factors through subtle transcriptional up- or down-regulation of relevant genes. Development of drugs that prevent and counter these fluctuations could be used as chemopreventative agents

to reduce the impact of environmental carcinogens or to correct the effects of inherited predisposing factors.

Just as transcriptional or post-transcriptional regulation may determine cancer susceptibility, it could also be harnessed for cancer prevention and therapy (**Table 1**). As an example, statins, which are already in clinical use, were recently found to upregulate PTEN through PPAR γ signaling⁶². Statins or PTEN-enhancing drugs could therefore be utilized not only to treat dyslipidemic cases but also to elevate PTEN expression in patients with tumours with lowered levels of PTEN (**Figure 5**). Importantly, such an approach would require a quantitative analysis of the expression levels of TSGs and oncogenes and not solely a genetic assessment of their presence or absence.

TSG-targeting microRNAs add another dimension to genotype/phenotype considerations in targeted cancer prevention and therapy. Overexpression of one or more of TSG-targeting miRNAs in cancer could confer cancer susceptibility, and tumours not showing genetic alterations in the TSG but with aberrant TSG levels due to miRNA misregulation could behave similarly to tumours with genetic deletion or mutation of the gene. Mapping the interactions between miRNAs and TSGs could be useful for defining and predicting tumour response to therapy.

Provocatively, tumours with partial loss of TSGs like *PTEN* that display “obligate haploinsufficiency” might be treated with agents to fully suppress the expression of the TSG, thereby suppressing tumour growth via senescence or other fail-safe mechanisms (**Figure 5**). Alternatively, when a TSG is completely lost, activation of alternative tumour suppressive pathways or synthetic lethality approaches could be considered⁶³ (**Figure 5**). The success of such strategies would obviously depend on the integrity of the fail-safe response and the expression level of the TSG, once again requiring a careful annotation of the molecular characteristics of the cancer lesion subjected to treatment. The need for the same careful annotation is not restricted to targeted therapies but applies to any cancer therapy since variations of oncogene or TSG dosage may well be at the core of failure of more conventional radio- or chemo-therapies.

On this basis, the scientific challenge going forward will be to develop quantitative assays that can assess and score subtle differences in TSG gene expression to allow accurate diagnostics and prediction of risk/prognosis. Such diagnostics will also be important for analysis and prediction of response to therapeutics, since subtle variations could also determine response to therapy. A tumour with a partial downregulation of a TSG could respond differently from a tumour that has either no alteration of the TSG or complete mutation/loss of the gene (**Figure 5**). Critically, in cases where a tumour shows downregulation of a TSG via transcriptional repression, epigenetic downregulation, or aberrant miRNA and ceRNA regulation, there is an opportunity to explore therapeutic restoration of TSG expression.

The recent explosion of knowledge in the field of non-coding RNA biology has opened a new frontier in understanding gene dosage regulation and its involvement in tumorigenesis. In the months and years to come, careful experimentation is required to map out the networks of gene regulation of TSGs and oncogenes to allow drug development and creation of diagnostics from this information. Forty years after the two-hit hypothesis, we are refining and extending our understanding of TSG inactivation in cancer. Milestones made in these past forty years have contributed to the decline in cancer deaths observed in the US since 1990⁶⁴. The next decades promise further relief from the burden of cancer as we continue to tease out its molecular genetic basis.

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Preface

2011 marks the forty-year anniversary of the statistical analysis of retinoblastoma that provided the first evidence that tumorigenesis could be initiated by as few as two mutations. This work provided the foundation for the “two-hit” hypothesis that explained the role of recessive tumour suppressor genes (TSGs) in dominantly inherited cancer susceptibility syndromes. However, four decades later, it is now known that even partial inactivation of tumour suppressors can critically contribute to tumorigenesis. Here we analyse this evidence and propose a continuum model of TSG function to explain the full range of TSG mutations found in cancer.

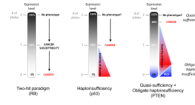


Figure 1. Paradigms of tumour suppression

A black gradient represents a continuum of expression that is also related to the number of alleles present (gray numbering). Left, the two-hit paradigm as exemplified by the tumour suppressor, RB. Loss of one allele induces cancer susceptibility; loss of two alleles induces cancer. Middle, classic haploinsufficiency. Loss of one allele is sufficient for induction of cancer. Right, Quasi-sufficiency and obligate haploinsufficiency. Quasi-sufficiency refers to the phenomenon whereby tumour suppression is impaired after subtle expression downregulation without loss of even one allele. Obligate haploinsufficiency occurs when TSG haploinsufficiency is more tumourigenic than complete loss of the TSG, usually due to the activation of fail-safe mechanisms following complete loss of TSG expression.

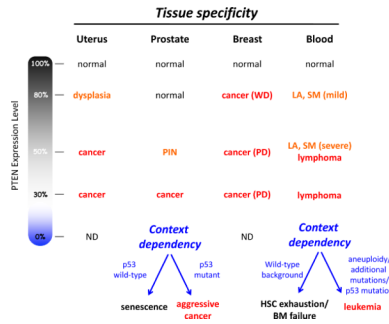


Figure 2. Tissue specificity and context dependency of tumour suppression

The phenotypic outcome of a reduction in *PTEN* expression is differentially manifested depending upon tissue type and genetic background. WD, well-differentiated. PD, poorly-differentiated. LA, lymphadenopathy. SM, splenomegaly. HSC, hematopoietic stem cell. BM, bone marrow. The effect of complete loss of *PTEN* is highly context dependent due to the obligate haploinsufficiency caused by *PTEN* loss-induced cellular senescence (PICS). Data summarized here come from multiple groups and studies of genetically-engineered mice with differing *PTEN* alleles and expression^{38–40,52,65–68}.

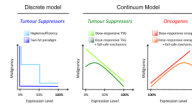


Figure 3. The continuum model of tumour suppression

The classical discrete, stepwise model of tumour suppression (left) is contrasted with a continuum model of tumour suppression and oncogenesis (centre and right, respectively). In the discrete model, tumourigenesis is induced by either complete loss of a TSG (two-hit paradigm, dark blue) or after single-copy loss of a TSG (haploinsufficiency, light blue). In contrast, we propose a continuum model (centre and right), in which tumour suppression is related to a continuum of TSG expression, rather than to discrete changes in DNA copy number. A continuum of increasing TSG expression will generally be negatively correlated with malignancy (centre, light green) whereas increasing oncogene expression will generally be positively correlated with malignancy (right, red). A linear relationship is depicted for schematic purposes, but the dose-response relationship need not be linear. In some cases, fail-safe mechanisms are induced by complete loss of TSG expression or by massive oncogene overexpression. In these cases, complete loss of TSG expression (centre, dark green) or massive overexpression of an oncogene (right, orange) will be negatively correlated with malignancy, as shown.

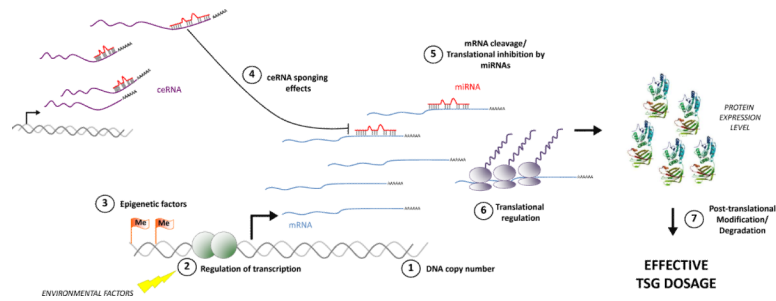


Figure 4. Mechanisms of regulation of TSG dosage

The interaction between coding and non-coding factors determines final TSG dosage. Classic mechanisms such as DNA copy number (1), transcriptional regulation (2), and epigenetic silencing can impact expression of TSG mRNA. TSG mRNA level or translation into protein is then regulated by miRNAs (4). The availability of miRNAs for TSG downregulation is further regulated by ceRNA-mediated sponging effects (3). Finally, additional translational regulation (6) or post-translational modifications contribute to the final protein expression, function and effective dosage. The protein structure shown is PTEN (PDB ID: 1D5R).

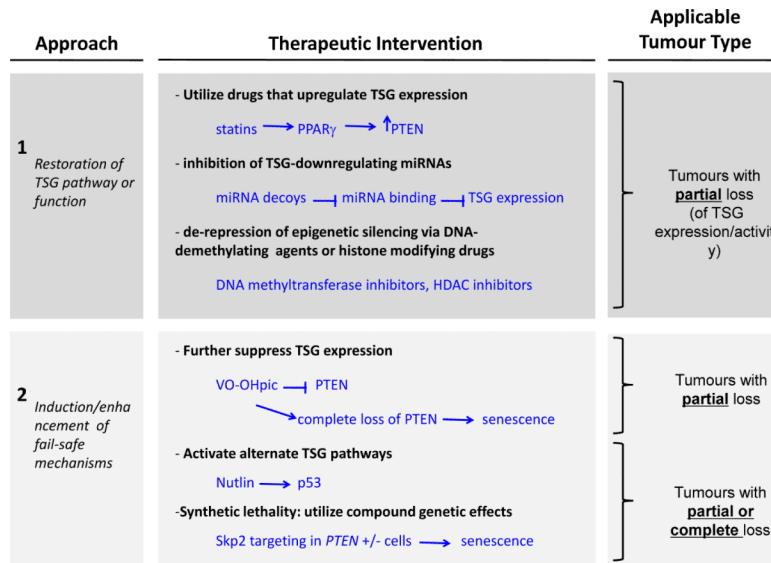


Figure 5. Opportunities for therapeutic intervention after partial or complete loss of TSG expression

Tumour genotype ultimately determines which therapies can be considered for intervention. Broadly, two general approaches can be taken: restoration of the TSG pathway or function (upper panel) or induction of fail-safe mechanisms by complete downregulation of the TSG (lower panel). In tumours with downregulation of TSG expression in the absence of complete mutation or deletion, expression of the TSG can be attempted using drugs that induce the TSG transcription, inhibit miRNA-induced TSG downregulation, or relieve epigenetic silencing. Alternatively, fail-safe mechanisms may be induced by further downregulation of the TSG, such as inhibition of PTEN by the small molecule VO-OHpic⁶⁹. In tumours with mutation or complete loss of the TSG, activation of other TSG pathways could be pursued. Alternatively, if a synthetic lethality relationship with the TSG is known, then the synthetic lethal gene can be targeted to induce cell death in the cells with complete loss of the TSG.