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Redefining tumour suppressor genes: exceptions to the two-hit hypothesis

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Abstract. Knudson's two-hit model of tumour suppressor genes supposes that two mutations are required to cause a tumour, one occurring in each of the two alleles of the gene. Many such cancer genes exhibiting biallelic disruption and truncating point mutations have been identified, revealing the success of the model. Despite changes in our concept of cancer genes, two inactivating point mutations are still considered the hallmark of tumour suppressor genes. Recently, however, more and more reports describe candidate tumour suppressors that do not conform to this standard definition, including haploinsufficient genes requiring inactivation of only one allele, and genes inactivated not by mutation but rather epigenetic hypermethylation. This review describes some of these exceptions and proposes a revised tumour suppressor gene definition to facilitate the identification of this new generation of tumour suppressor loci.

Key words. Cancer; tumour suppressor genes; Knudson two-hit hypothesis; haploinsufficiency; hypermethylation.

Introduction

Cancer development involves several discrete stages, which have been hypothesised to correlate with the accumulation of specific genetic mutations. The late 1960s saw the first paper to predict the required number of mutation events (between 3 and 7) calculated to give rise to the common cancers [1]. But it was in 1971 that Alfred Knudson Jr published his seminal statistical analysis of the childhood cancer retinoblastoma [2]. Knudson realised that bilateral cases, where cancers arose in both eyes, represented individuals inheriting a germline mutation, and who therefore required one less somatic mutation to cause a tumour. In contrast, unilateral cases primarily consisted of individuals with no hereditary mutation, in whom all the cancer-causing mutations were somatic, and who thus would be highly unlikely to exhibit multiple tumours. Using a cohort of 48 retinoblastoma patients, Knudson retrospectively plotted their age of disease onset against the proportion of cases not yet apparent at that age. He revealed a remarkable agreement between bilateral cases and a one-mutation model, and a close association between unilateral cases and a two-mutation model. This observation suggested that at least one form of cancer, retinoblastoma, was caused by only two mutation events, both sporadic events in most unilateral cases, and one sporadic and one hereditary event in bilateral cases. Later, cloning of the retinoblastoma gene confirmed the requirement for two mutations, revealing that they occurred in the two alleles of a single gene. Cancer was caused by disruption of both *RB1* alleles, and subsequent loss of RB1 protein [3]. Thus was born the 'two-hit hypothesis'. This represented a new class of cancer genes, the recessive anti-oncogenes, now known as tumour suppressor genes (TSGs) [4–6].

In 1997 Kinzler and Vogelstein proposed a further division of cancer genes into 'caretakers' and 'gatekeepers' [7]. Unlike the mutation-based definition of oncogenes and TSGs, the division of cancer loci into caretakers and gatekeepers is based on function. Many TSGs, such as

RB1, are involved in the control of cell cycle and proliferation. Loss of these gatekeeper genes results in disregulation of cell cycle and hence uncontrolled proliferation. It had recently become clear that a second type of TSG existed, however. Caretaker genes are those loci that have a role in maintaining genome integrity (e.g. *BRCA1*, *BLM* and *ATM*), preventing aberrations such as aneuploidy and microsatellite instability (see later for discussion of these). Disruption of a caretaker gene would therefore lead to increased genomic instability and cellular mutation rates, increasing the probability that a gatekeeper gene will be inactivated. Mutation of caretakers therefore indirectly leads to cancer by increasing the chance of mutating a gatekeeper gene. Such a cancer, they predicted, would result not from two mutations, but four, one in each allele of both the caretaker and gatekeeper loci. This proposal fits both the Knudson two-hit hypothesis (for each TSG involved) and the suggestion by Ashley that 3–7 mutation events are required for most adult tumours [1].

More recently, Hanahan and Weinberg have postulated a very interesting biological basis for why 3–7 separate mutation events are required for tumourigenesis [8]. They propose that all cancers must necessarily acquire 'six essential alterations in cell physiology that collectively dictate malignant growth'. These six traits are self-sufficiency in growth signals; insensitivity to growth inhibitory signals; evasion of programmed cell death; limitless replicative potential; sustained angiogenesis; and tissue invasion and metastasis. This model predicts that each individual tumour will exhibit as many mutation events, in as many genes, as are necessary to acquire these six traits. Although the model does not modify the definition of a TSG, it provides an interesting and exciting framework for understanding the interaction of TSGs and oncogenes in terms of the interconnected biological circuitry of cancer cells.

Many candidate TSGs have been identified in the last 30 years based upon evidence of mutation or altered gene expression, in vitro functional screens or mouse knockout models. Knudson's two-hit hypothesis has become the hallmark of TSGs, confirmed not only by *RB1*, but also by other tumour suppressor genes such as *TP53* and *WT1*. Even when Kinzler and Vogelstein proposed the 'caretaker-and-gatekeeper' model, they suggested that TSGs of both types required two inactivating mutation events. Thus, for example, their model did not predict any increased genetic instability (and therefore tumourigenicity) resulting from loss of a single allele of a caretaker gene. In 1997, Haber and Harlow proposed a strict definition for genes to be classified as a TSG, that they are 'genes that sustain loss of function mutations in the development of cancer' [9]. However, the authors had, in the words of Bruce Ponder, 'a practical rather than a conceptual purpose' [10]. Their intention was to provide a rigid criterion by which the validity of the myriad candidate TSGs could be judged. Like Knudson, this classification is grounded in the concept of mutation, emphasising this over gene function. Thus many today consider two inactivating hits in a cancer to be the only acceptable proof of a tumour suppressor gene. This definition excludes many potential TSGs, such as those inactivated by epigenetic mechanisms such as methylation, genes that can be disrupted by a single mutation event and genetic modifiers of tumourigenesis, which may represent polymorphic variants rather than mutated alleles. In recent years, numerous examples of candidate tumour suppressor genes have been identified that do not fit the formal definition of a TSG, as defined by *RB1* (see fig. 1). Even some of the canonical TSGs such as *TP53* and *APC* have been reported to demonstrate atypical TSG behaviour in certain situations. So are these nonstandard TSGs merely heresy, or are they indications that the accepted definition of a TSG is too limited? This review will describe some of the examples of nonstandard TSGs in the literature, and will suggest an updated definition for identifying tumour suppressor genes.

One-hit wonders

Possibly the most contested exceptions to the two-hit hypothesis are cancer-associated genes whose function can be abolished by mutation of a single allele. A number of mechanisms have been proposed to explain this phenomenon.

Haploinsufficiency

CDKN1B (p27, Kip1) is a member of the CIP/KIP class of cell cycle regulators, whose role is to inhibit the function of cyclin/cyclin-dependent kinase complexes and thus cause a cell cycle arrest. Deregulation of the cyclin-dependent signalling of cell growth is known to result in human cancer, with the best examples being the absence of growth arrest following TP53 or RB1 loss. It was therefore predicted that CDKN1B loss would also lead to tumour formation. Indeed, loss of heterozygosity (LOH) at chromosome 12p13, encompassing the *CDKN1B* locus, has been reported [11–14], and reduced CDKN1B protein levels have been identified in numerous cancer types (reviewed [15]). Furthermore, reduced CDKN1B protein is significantly associated with high-grade and high-stage disease in breast [16], colon [17] and gastric [18] tumours, and with reduced survival in breast [16], colon [19], gastric [20], ovary [21], prostate [22], bladder [23] and oesophageal [24] cancer. Despite the strong connection between reduced CDKN1B expression and cancer, only a few examples of inactivating point mutations within *CDKN1B* have been described [25, 26]. The com-

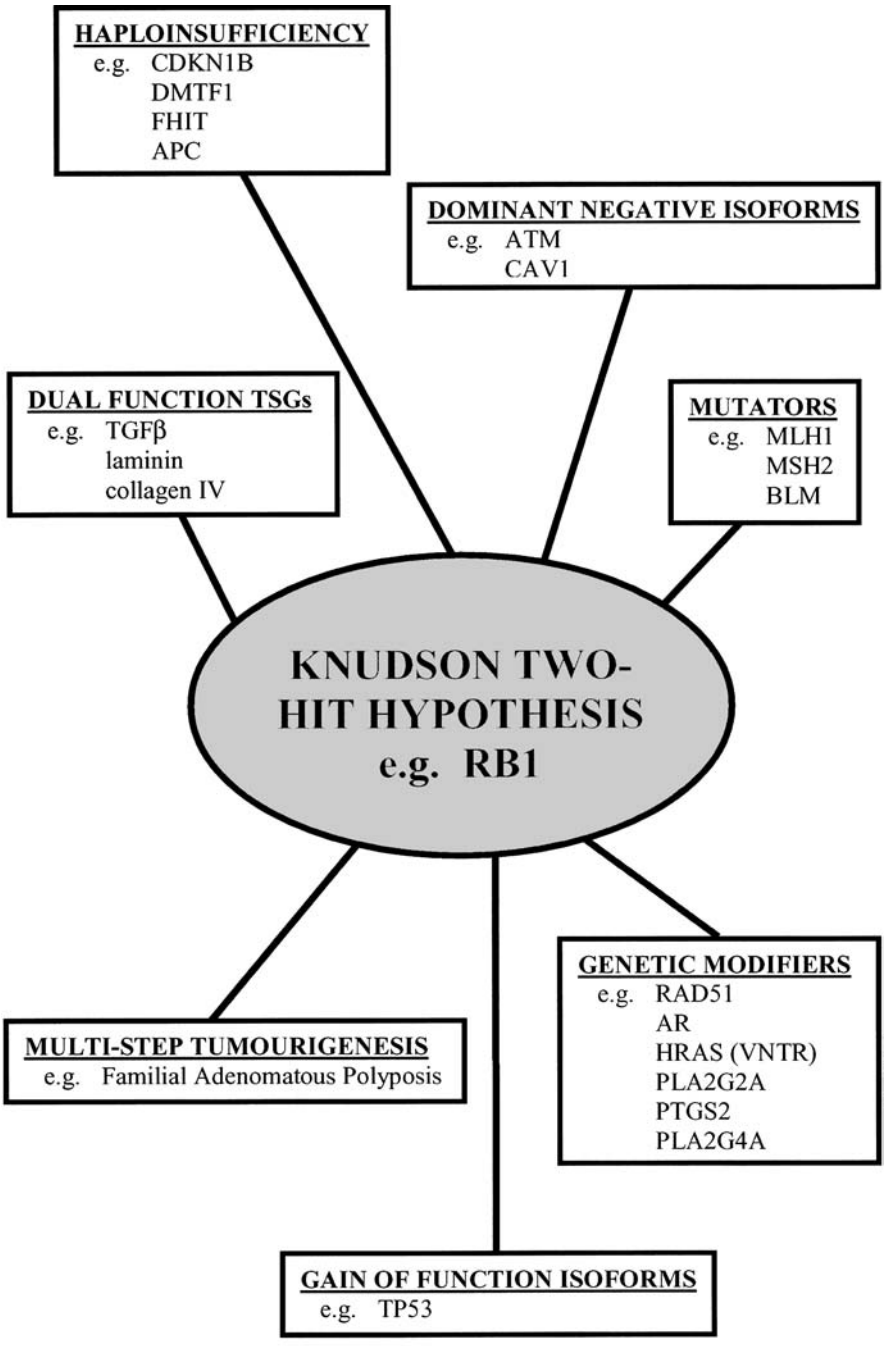

Figure 1. The Knudson two-hit hypothesis has dominated the identification of tumour suppressor genes. Surrounding these canonical TSGs are many candidate TSGs that do not conform to this model. These genes deviate from the Knudson model as tumours arise from (i) monoallelic disruption (haploinsufficiency, dominant negative isoforms or gain-of-function isoforms); (ii) multiple gene interaction (multi-step tumourigenesis, genetic modifier genes or mutator phenotypes) and (iii) competing tumour suppressive and tumour-promoting functions (dual-function TSGs). Examples of each are given and are discussed in the text.

bination of high-frequency LOH and rare point mutations has led to the suggestion that *CDKN1B* demonstrates haploinsufficiency (absent or reduced function due to the loss or inactivation of a single allele). This is in stark contrast to Knudson's two-hit model, which states that both alleles of the TSG must be inactivated. Evidence for the haploinsufficient model arises from studies of the *Cdkn1b* knockout mouse. *Cdkn1b*$^{-/-}$ mice demonstrate increased body size compared with wild-type animals due to increased cellular proliferation [27]. The *Cdkn1b*$^{+/-}$ mice show an intermediate body size, indicating a dose-dependent effect of CDKN1B on proliferation. All ho-

mozygous knockout mice developed benign pituitary adenomas, which were not found in wild-type mice [27]. *Cdkn1b*$^{+/-}$ mice also developed pituitary tumours, but with a decreased penetrance (32%) and longer median latency [28]. No other tumour types occurred in either heterozygous or homozygous knockout mice. However, following exposure to γ-radiation or the mutagen *N*-ethyl-*N*-nitrosourea (ENU), both knockout mice and heterozygotes showed a significantly elevated incidence of intestinal and lung adenomas, as compared with wild-type mice [28]. Increased frequencies of intestinal adenocarcinomas, adrenal tumours and lesions of the female reproductive tract (including granulosa cell tumours of the ovary, endometrial adenocarcinomas, endometrial polyps, angiosarcomas and fibromas) were also seen. For each tumour type, the frequency of tumours in the heterozygote was intermediate between those of the wild-type and *Cdkn1b* nullizygous animals. *Cdkn1b*$^{+/-}$ also showed an intermediate degree of tumour mortality following exposure to γ-radiation, with a median survival of ~66 weeks, compared with 40 weeks for *Cdkn1b*$^{-/-}$ mice and a 70% survival rate in wild-type animals at the end of the experiment (70 weeks). Analysis of tumours taken from the *Cdkn1b*$^{+/-}$ mice revealed that the second *Cdkn1b* allele remained intact, and that nuclear CDKN1B protein was still expressed in the tumours (at ~50% the levels in wild-type animals). Therefore, no second hit had occurred in these animals, indicating that loss of only one allele of *Cdkn1b* in mice increased their sensitivity to γ-radiation and ENU induced tumours.

Knockout mouse models of two other cancer-associated genes, *Dmtf1* and *Fhit*, have indicated further haploinsufficient TSGs. DMTF1 (cyclin D-binding, myb-like transcription factor 1, previously named DMP1) was originally identified as a cyclin D-binding protein [29, 30] and subsequently shown to transactivate genes containing an Ets consensus sequence in their promoter [31]. One important target for DMTF1 transactivation is the *Cdkn2a* (*p19*^{Arf}) gene. Induction of CDKN2A (p19^{Arf}) results in MDM2 inactivation, stabilisation of TRP53 and cell cycle arrest, and this induction is inhibited by the binding of DMTF1 protein to D-family cyclins [31]. The important role of DMTF1 in regulating the TRP53 response pathway has been demonstrated in mouse embryo fibroblasts (MEFs) lacking DMTF1, which show decreased CDKN2A (p19^{Arf}) levels, are resistant to senescence, can be transformed by *Hras1* and rarely accumulate *Trp53* mutations [32]. The *Dmtf1* knockout mouse shows a broad spectrum of spontaneous tumours, including lung adenomas and adenocarcinomas, hepatocellular tumours, B cell lymphomas and hemangiomas with a median latency of 83 weeks [33]. *Dmtf1*$^{+/-}$ mice also show a variety of spontaneous tumours with a slightly longer median latency of 110 weeks. In comparison, only about 15% of wild-type mice develop spontaneous tumours by 110 weeks. Furthermore, heterozygous and nullizygous *Dmtf1* knockout mice exhibit similar, increased tumour incidences, compared with wild-type animals, following exposure to dimethyl-benzanthracene (DMBA) or γ-radiation. As was shown for the *Cdkn1b*$^{+/-}$ mice, tumours taken from *Dmtf1*$^{+/-}$ animals had not undergone a second hit as predicted by Knudson, and still expressed DMTF1 protein or messenger RNA (mRNA) in the majority of the tumours.

Further evidence of haploinsufficiency for *Dmtf1* was found in a murine model of lymphomagenesis. Transgenic mice overexpressing *Eμ-Myc* (the *Myc* oncogene under the control of an enhancer specific to B cell progenitor cells) are known to develop Burkitt-type B-cell tumours with a median latency of 6 months [34]. Crossing the *Eμ-Myc* transgene onto a *Dmtf1*$^{+/-}$ or *Dmtf1*$^{-/-}$ background resulted in increased lymphomagenesis with a median tumour latency of 12 weeks [33]. Again, *Dmtf1*$^{+/-}$ tumours retained expression of DMTF1 protein, suggesting no second hit, and this protein was shown to be still capable of DNA binding. One explanation of the haploinsufficiency of *Dmtf1* is that the low level of protein produced by monoallelic expression is sequestered by cyclin D proteins and is thus unable to activate CDKN2A (p19^{Arf}) and suppress tumourigenesis. That the tumour suppressive function of DMTF1 is mediated through its regulation of CDKN2A (p19^{Arf}) seems certain. Whereas Eμ-Myc-induced lymphomas on a wild-type background exhibit TRP53 overexpression (indicating mutant TRP53) or *Cdkn2a* (*p19*^{Arf}) deletion in 48% of tumours, when arising on a *Dmtf1*$^{+/-}$ or *Dmtf1*$^{-/-}$ background *Trp53* or *Cdkn2a* (*p19*^{Arf}) disruption is detected in only 14 or 9% of tumours respectively. This likely indicates that the loss or reduction of DMTF1 protein abrogates the CDKN2A (p19^{Arf})/TRP53 cell cycle arrest response, thus removing any selection pressure for further mutation of this pathway.

The *FHIT* gene is a member of the histidine triad protein family and encodes a diadenosine triphosphate (AP3A) hydrolase enzyme [35]. The *FHIT* locus exhibits a high frequency of LOH in a wide variety of human cancers and has been shown to be disrupted by homozygous deletion in tumours [36]. Initial reports described aberrant transcripts of *FHIT* in primary tumours and cancer cell lines [37], although later studies identified these transcripts in normal tissues, throwing the role of *FHIT* as a TSG into doubt [38]. To assess the tumour suppressive ability of FHIT, Fong and colleagues generated a knockout mouse model [39]. Both the heterozygous and nullizygous *Fhit* mutant mice exhibited an increased frequency of tumours, both sporadic and induced by the mutagen NMBA (nitrosomethylbenzylamine), compared with wild-type mice [39, 40]. The tumours were of multiple types, including sebaceous tumours, squamous papillomas and lymphomas, and the incidence of these tumours could be

suppressed in NMBA-treated *Fhit*$^{+/-}$ mice by adenoviral-mediated *FHIT* gene therapy [41]. The frequencies of tumours in the *Fhit*$^{+/-}$ and *Fhit*$^{-/-}$ mice were very similar, suggesting that *Fhit* tumour suppressor function demonstrates haploinsufficiency [40]. In the initial study, analysis of tumours arising in the *Fhit*$^{+/-}$ mice showed no evidence of exonic deletion, genic rearrangement or point mutation in the second *Fhit* allele, although immunohistochemical staining of the tumours indicated loss of FHIT protein [39]. However, in a subsequent study of the same heterozygous mouse model, the authors reported that FHIT protein expression was retained in some of the tumours, implying that loss of expression from the second *Fhit* allele is not an absolute requirement for tumour formation in these mice [40].

Even the canonical *APC* tumour suppressor may demonstrate haploinsufficiency. Yan and colleagues examined the relative levels of *APC* mRNA expression from each allele of five affected members in a pedigree with familial adenomatous polyposis (FAP) [42]. In each case, one allele was found to express 66% of the total *APC* mRNA, with the disease-linked allele expressing only 33%. A further patient from a separate FAP pedigree displayed an allelic ratio of 71:29, whereas 24 unrelated individuals without FAP showed the expected 50:50 allele ratios. Furthermore, of 38 benign tumours from these six patients, 30 tumours showed LOH, with 28 (93%) exhibiting loss of the normal, higher-expressing allele. Thus it appears that a 25% decrease in overall *APC* transcript levels, changing to a 75% reduction in expression in tumours with LOH, may predispose to cancer.

More and more studies in recent years have suggested the existence of haploinsufficient TSGs (see [43]), strengthening the case for tumour suppressors that deviate from the accepted standard definition. A recent review proposed that the extremely high lifetime risk of cancer in humans (approximately one in three) could be explained in part by haploinsufficiency [44]. The authors suggest that many TSGs might show haploinsufficiency to a greater or lesser extent, such that heterozygous cells will undergo a clonal expansion. This would increase the population of target cells available for further mutation in a multistep tumourigenesis pathway. By this model, even TSGs giving rise to predominantly benign tumours (e.g. murine *Fhit*) may be significant to the development of malignant disease, particularly during the longer life span of humans. However, haploinsufficiency is not the only possible mechanism for a one-hit tumourigenesis model.

Dominant negative isoforms

Whilst the identification of truncating point mutations in potential TSGs is considered essential proof, the existence of a missense alteration in a candidate TSG is often believed to be less important, or even incidental. However, some studies reveal that missense mutations can be even more disruptive to normal cell function than a null mutation.

The ATM protein is a component of the cellular response to ionising radiation (IR)-induced genomic damage. ATM acts as a sensor of genomic stress, which can activate TP53 and BASC (BRCA1-associated surveillance complex) repair pathways. Mutations in *ATM* cause ataxia telangiectasia (AT), a disease characterised by radiosensitivity, genome instability and cancer predisposition. Increased risk of cancer, particularly breast cancer, has also been reported in relatives of AT patients [45–49]. Curiously, the most common type of *ATM* mutation in breast cancer patients is missense mutation, in contrast to the predominance of truncating mutations detected in the AT patients themselves [50–53]. This suggests that certain missense mutations of *ATM* have functional significance. Scott and colleagues transfected an AT cell line (AT1ABR) with 1 of 10 inducible constructs that express ATM proteins containing missense or amino acid deletion mutations that had been previously identified in AT or breast cancer patients [54]. Whereas transfection of wild-type ATM was able to restore IR-induced TP53 activation, one mutant protein from the breast cancer patients (a missense alteration) and four mutant proteins from the AT patients (three missense changes and the Atm-ΔSRI mutation, which comprises a three amino acid deletion) were unable to reconstitute ATM function, despite expressing stable proteins. This reveals that these five mutations abrogate ATM function. What is more, when these mutant proteins were transfected into C3ABR cells, which produce wild-type ATM, their expression was found to prevent IR-induced TP53 activation by the endogenous protein. Thus, not only are these missense proteins nonfunctional, they can also interfere with the function of coexpressed wild-type ATM in a dominant negative fashion. It seems likely that breast cancer is associated with missense (not null) mutation of *ATM* since, unlike truncating mutations that are likely to result in ~50% normal ATM levels, the dominant negative role of some missense ATM proteins may result in much greater reductions in ATM response. This greatly reduced response to genomic damage could thus lead to tumour formation.

Additional proof of this model is demonstrated in vivo by a transgenic mouse model of the Atm-ΔSRI mutation mentioned above. Heterozygotes carrying the mutated *Atm* allele develop increased numbers of tumours, primarily sarcomas and lymphomas, compared with wild-type littermates [55]. In contrast, heterozygotes of a null *Atm* mutation are no more susceptible to tumour formation than their wild-type littermates. This confirms that tumourigenesis is related to the dominant negative role of the Atm-ΔSRI mutation and not merely to reduced ATM expression.

A second likely example of a dominant negative TSG is the caveolin 1 (*CAV1*) locus. CAV1 is expressed in normal breast epithelia but is downregulated in several breast cancer cell lines and in primary breast tumour [56]. Whereas in vitro studies have shown tumour suppressive functions of CAV1 [57–59] and *Cav* null mice exhibit mammary epithelial cell hyperplasia, mutation screening of breast tumour samples has identified only heterozygous P132L mutations in up to 16% of cases [60]. A recent study revealed that the P132L mutant form of CAV1 is a dominant negative mutation [61]. HEK-293T cells were transfected with either MYC-tagged wild-type CAV1 or MYC-tagged P132L, and the cellular location of the proteins was determined by immunostaining and cell fractionation. While wild-type CAV1 protein was located at the plasma membrane, the P132L protein was retained intracellularly in a perinuclear Golgi compartment. Furthermore, when wild-type and P132L CAV1 proteins were coexpressed in Cos7 cells, or when P132L protein was expressed with endogenous wild-type CAV1 in HME1 cells, almost all CAV1 immunostaining was confined to the perinuclear Golgi compartment, revealing that P132L can dominant negatively interfere with wild-type CAV1 localisation. When total protein from the wild-type CAV1 and P132L transfected cells was extracted and size fractionated, the wild-type CAV1 was primarily contained within the 150-kDa and 200-kDa fractions, suggesting oligomerisation. However, the P132L protein was predominantly found either in the 29–66-kDa fractions, suggesting a monomer or dimer organisation, or in the high molecular mass (>443 kDa) fraction, indicating high molecular mass aggregates. The apparent multimerisation of CAV1 and the disruption of this process by P132L suggest that this is a likely mechanism underlying the dominant negative interference of P132L on normal CAV1 function. It seems likely that breast tumours harbouring a heterozygous P132L mutation are inactive for CAV1 function, due to improper oligomerisation or cellular localisation. Thus, tumourigenesis occurs despite the production of wild-type CAV1 protein, again in contrast to the standard definition of a TSG.

Gain-of-function isoforms

A related phenomenon to dominant negative mutation is that of gain-of-function mutants. However, whilst a dominant negative mutation in only a single allele of a TSG causes loss of gene function mimicking a null mutation, a heterozygous gain-of-function mutation in a TSG creates a mutant protein with a novel tumour-promoting function. Whereas tumour-promoting, gain-of-function mutations are by definition oncogenic, rather than tumour suppressive, it is of interest to note that certain monoallelic mutations in proven TSGs can promote cancer by gain of function rather than the expected loss of function predicted by the classical TSG definition. Gain-of-function mutations in the TP53 tumour suppressor have been identified from studies in Li Fraumeni syndrome (LFS) patients [62]. Fibroblasts from LFS patients heterozygous for any of four class II (protein conformation altering) mutations in *TP53* were noted to become polyploid when cultured in the presence of colcemid. In contrast, LFS fibroblasts containing truncating or class I (mutations within the DNA binding domain) mutations of *TP53* exhibited normal ploidy. When these class II mutant *TP53* genes, but not the truncating or class I mutant forms, were transfected into normal fibroblasts with endogenous wild-type TP53, they again caused polyploidy, showing that they can dominantly interfere with normal TP53 protein function. This differs from TP53 null cells that display normal ploidy. Furthermore, when transfected into these TP53 null cells, the class II mutants again caused polyploidy, confirming that these conformational mutations produce gain-of-function proteins that interfere with normal spindle checkpoint regulation. In other studies, some, but not all, missense mutations of *TP53* have also been shown to increase resistance to chemotherapeutic drugs in a dominant gain-of-function manner, revealing that *TP53* mutations can give rise to several novel functions [63, 64].

In summary, while some missense mutations in TSGs result simply in abrogated function, others may result in dominant negative proteins or novel gains of function. In contrast to the standard definition of TSGs, dominant negative and gain-of-function mutations and haploinsufficient genes are able to give rise to tumours following a single mutation event. Furthermore, in the case of gain-of-function mutations it is possible for a tumour suppressor to be mutated into a dominant tumour promoter.

Ménage-à-trois (quatre, cinq…)

The previous section described how for some genes in certain situations, one mutation might be sufficient to promote cancer formation or progression, in contrast to the standard two-hit model. Other cancers, however, appear to arise from a multi-hit carcinogenesis mechanism. There are several different forms of multi-hit models: cumulative mutation corresponding to multi-step cancer progression, genetic modifier effects and mutator phenotypes.

Multi-step cancer progression

It is estimated that between three and seven mutations are required for most common cancers [1, 65]. The explanation for this is that many tumours arise from the accumulated disruption of multiple genes. This is not held to contradict the Knudson hypothesis, since it is accepted doc-

trine that each TSG involved will be inactivated by a two-hit mechanism. Nonetheless, it is conceptually different from the two-hit model illustrated by RB1, in that multiple genes and multiple hits are necessary to produce a tumour. Multi-step tumourigenesis resulting from accumulation of mutations is elegantly, and most frequently, illustrated by colon cancer. Pathologists have demonstrated a clear progression from benign adenomatous polyps, through dysplastic polyps, to carcinoma and ultimately metastases for this tumour type. Furthermore, it seems clear that each step in this tumour progression correlates with additional mutations, as was first theorised by Fearon and Vogelstein [66]. In their seminal 1990 paper, Fearon and Vogelstein documented the most frequently occurring mutation events at each step of colon tumourigenesis. They discovered that several different loci were mutated frequently in colon cancer, often in the same tumour, and that the more advanced the tumour, the more mutations it had accumulated. Subsequent studies to identify the gene targets of these mutations have revealed the following tumourigenesis pathway. Loss of both copies of the *APC* TSG represents an early event that results in the benign adenomatous polyps. In fact, germline mutation of one copy of *APC* is a frequent predisposition event for colon cancer in the inherited syndrome familial adenomatous polyposis [67]. Subsequent mutation of the *RAS* oncogene causes a further increase in cell proliferation and the formation of dysplastic polyps [66]. The eventual conversion of the tumour to a carcinoma, however, is frequently associated with mutation of both copies of the *TP53* TSG [68]. It has proven more difficult to determine the specific mutation events necessary for metastasis, due to the extensive alterations arising from chromosomal instability following TP53 loss. Nevertheless, progression to colon carcinoma appears to require five mutation events, with each step providing a further growth advantage over the last. Although similar multistep progression for most other tumours is less clear, statistical analysis of the number of tumours versus the age of onset of disease suggests most adult cancers require multiple hits.

Genetic modifier genes

A related multiple-hit tumourigenesis model involves modifiers of TSGs, i.e. genes that affect the tumourigenic potential of TSGs.

The existence of cancer modifier genes is supported by the intrafamilial variation in disease observed in many hereditary cancers. A prime example of this variation is exhibited in cases of familial breast and ovarian cancers in pedigrees segregating mutations in the *BRCA1* and *BRCA2* genes. *BRCA1* mutation carriers have an 80% risk of developing breast cancer and a 40% risk of ovarian cancer by the age of 70. *BRCA2* mutation carriers show a similar risk of breast cancer, but a lower risk of developing ovarian tumours by the same age [69]. Although this effect can in part be attributed to environmental factors, some of the variation is likely to result from the influence of genetic modifiers. Possible modifiers of the *BRCA* tumour suppressor genes include the *RAD51*, androgen receptor (*AR*) and *HRAS* variable number of tandem repeat (VNTR) loci.

RAD51 is a homologue of the *Escherichia coli* recA protein and is involved in recombination and repair of DNA double-strand breaks. Both BRCA1 and BRCA2 have been shown to colocalise with RAD51 in nuclear foci in eukaryotic cells, suggesting interaction between these proteins in DNA repair. Two groups independently reported analysis of a G→C single nucleotide polymorphism (SNP) in the 5′-untranslated region of *RAD51* in groups of *BRCA1* and *BRCA2* mutation carriers [70, 71]. They compared the frequencies of the *RAD51* G-allele and C-allele in cancer patients and related healthy mutation carriers. Both studies identified a significantly increased risk of developing breast cancer in individuals carrying *BRCA2* mutations associated with the presence of at least one copy of the *RAD51* C-allele. The presence of this allele did not increase the risk of ovarian cancer in these individuals, however, and no association was found between the *RAD51* C-allele and cancer risk in *BRCA1* carriers. Thus RAD51 may be a modifier of BRCA2 related breast, but not ovarian, cancer.

In two similar studies, *BRCA1* mutation carriers (both unaffected women and those diagnosed with breast and/or ovarian cancer) were screened for polymorphisms in *AR* and *HRAS*. AR is a ligand-dependent transcriptional activator whose role in androgen response has led to the suggestion that AR may be an important factor in hormone responsive cancers, including breast and ovarian cancer. The CAG repeat in exon 1 of the *AR* gene is highly polymorphic, and its size is inversely associated with the transcriptional activity of AR. Rebbeck and colleagues analysed the size of this trinucleotide repeat in 304 *BRCA1* mutation carriers [72]. Allele sizes varied between 8 and 32 copies of the repeat, and larger alleles were noted in women with cancer compared with unaffected women. Women carrying alleles with ≥28 repeats showed increased risk of breast cancer and earlier age of onset than those with smaller alleles. Furthermore, the risk of breast cancer increased and average age of onset decreased with increasing allele size. Thus, androgen responsiveness appears to modify cancer risk in *BRCA1* families, and the role of androgens in breast cancer requires further investigation.

The variable repeat (VNTR) locus located 1 kb downstream of the *HRAS* oncogene is also highly polymorphic. When the VNTR size was measured in 307 *BRCA1* mutation carriers (173 women with breast cancer, 42 with ovarian cancer and 112 presymptomatic carriers), five

frequent alleles (with frequencies of 56, 7.2, 10.8, 8.8 and 5.2%) and several rare alleles were identified [73]. Although no association between allele sizes and breast cancer in these individuals was shown, a highly significant association between history of ovarian cancer and the presence of the rare VNTR alleles was identified. This association was still found after correction for covariates such as age, parity and age at last birth. Although a clear modifier effect on *BRCA1*-associated ovarian cancer is suggested in this study, the significance of an association with a VNTR is unknown. Whether the repeat may influence the transcription of *HRAS* or other neighbouring gene, or is merely linked to an unidentified ovarian cancer modifier locus remains to be determined.

The above studies are examples of potential modifiers of human breast and ovarian cancer. However, such studies in humans are complicated by small pedigrees, a broad mutation spectrum and extensive genetic heterogeneity. Thus many modifier studies are instead performed in mouse. The Min (multiple intestinal neoplasia) mouse possesses a nonsense mutation in the *Apc* gene (Apc^{Min}) and consequently exhibits multiple intestinal polyps. Although these polyps rarely progress to carcinomas during the short lifetime of the mouse, the Min mouse represents a useful model of human familial adenomatous polyposis.

Min mice exhibit on average 28.5 ± 7.9 intestinal tumours on the C57BL/6 background [74]. However, when these mice were intercrossed onto a different strain of laboratory mouse (AKR mice) they exhibited only 5.8 ± 4.3 tumours and a significantly longer life span, suggesting that the AKR strain expresses a genetic modifier (Mom-1, modifier of Min 1) that suppresses the intestinal tumour phenotype normally associated with *Apc* mutation [74]. Further backcrossing the *Apc* mutation onto AKR mice resulted in an even greater reduction in tumours (1.7 ± 1.7) with almost a third of the animals being tumour free, indicating that homozygosity for Mom-1 is more protective than heterozygosity. The Mom-1 locus was mapped to chromosome 4 and subsequently revealed to be a complex locus composed of more than one closely linked locus [75, 76]. This was revealed by generating congenic mice carrying the Mom-1 locus from the AKR mouse strain ($Mom1^{AKR}$) on the C57BL/6 genetic background. Whereas mice inheriting the complete $Mom1^{AKR}$ locus showed strong resistance to intestinal adenomas, as previously reported by Dietrich, mice inheriting only the proximal part of Mom-1 showed a partial resistance to tumour formation [76]. Similarly, C57BL/6 Min mice inheriting only the distal $Mom1^{AKR}$ region also showed partial resistance to developing adenomas [75], suggesting that the Mom-1 locus contains at least two modifiers of APC-null intestinal polyps. The proximal Mom-1 locus appears to provide the greater tumour resistance, and modifies tumour initiation along the length of the intestine, whereas the distal Mom-1 locus provides weaker resistance that is limited to tumour formation in the medial small intestine. The distal Mom-1 locus remains unknown; however, the proximal locus has been identified as the secretary phospholipase gene *Pla2g2a* [76–78]. The *Pla2g2a* gene was found to possess a frameshift mutation in the intestinal tumour-sensitive C57BL/6, 129 and BTBR mouse strains, but was wild type in AKR mice and five other resistant mouse strains. In agreement, transgenic mice expressing wild-type PLA2G2A on the C57BL/6 genetic background exhibit partial resistance to the Apc^{Min} adenoma phenotype [75].

Mom-1 is by no means the only modifier of the Apc^{Min} phenotype, and crossing the *Apc* mutation onto different genetic backgrounds or crossbreeding Min mice with many other mutant mice can modify the polyposis phenotype. Two further examples of genes known to modify the Apc^{Min} phenotype are *PTGS2* and *PLA2G4A*. PTGS2 (previously named COX-2) converts arachidonic acid to prostaglandin H_2, and is believed to be the therapeutic target of the non-steroidal anti-inflammatory drugs (NSAIDs) used in the treatment of colon cancer. PTGS2 is not expressed in normal intestine, but is present in colonic polyps and polyps of the small intestine greater than 2 mm in diameter [79]. When a mutated *Apc* allele ($Apc^{\Delta 716}$) was crossed onto a *Ptgs2* knockout background, a significant reduction in the number of polyps was found ($Apc^{+/-}$ $Ptgs2^{+/+}$, 652 ± 198; $Apc^{+/-}$ $Ptgs2^{+/-}$, 224 ± 123; $Apc^{+/-}$ $Ptgs2^{-/-}$, 93 ± 98) [79]. Furthermore, those polyps that did form on the $Ptgs^{-/-}$ background were considerably smaller than in *Ptgs2* normal mice. Similar decreased polyp number and size was seen when $Apc^{+/-}$ $Ptgs2^{+/+}$ mice were treated with the PTGS2 inhibitor, MF tricyclic, confirming that loss of PTGS2 was responsible for the altered phenotype. These results suggest that *Ptgs2* is a genetic modifier regulating the initiation and growth of polyps in *Apc* mutant mice.

PLA2G4A (previously named $cPLA_2$) is a member of the IIA phospholipase family, which also includes PLA2G2A, and is involved in the release of arachidonic acid. However, whilst PLA2G2A protein suppresses tumour formation, the cytosolic PLA2G4A is found to be overexpressed in polyps of the small intestine, though not of the colon [80]. Takaku and colleagues examined the role of PLA2G4A in tumourigenesis by crossing the $Apc^{\Delta 716}$ mutant mouse with the *Pla2g4*-knockout mouse. No difference in the number of intestinal polyps was found between the $Apc^{+/-}$ $Pla2g4^{+/+}$ and the $Apc^{+/-}$ $Pla2g4^{-/-}$ mice. However, the polyps arising in the small intestine of the $Apc^{+/-}$ $Pla2g4^{-/-}$ mice were significantly smaller than those exhibited by the PLA2G4A-expressing mice. A second study in which *Pla2g4a* knockout mice were crossed with $Apc^{Min+/-}$ mice confirmed the decreased polyp size in the small intestine, as reported by

Takaku, but also showed a reduced number of polyps in the *Apc*$^{Min+/-}$ *Pla2g4a*$^{-/-}$ mice as compared with *Apc*$^{Min+/-}$ *Pla2g4a*$^{+/+}$ mice [81]. Both studies showed PLA2G4A status to have no effect on the number or size of colonic polyps. The different effects in the two studies of PLA2G4A on polyp number in the small intestine may be the result of the different *Apc* mutations, or different genetic backgrounds. Nonetheless, both studies reveal PLA2G4A to be a modifier of polyp growth, with its effects limited to the small intestine.

The identification of these modifiers of murine colon cancer has led to the investigation of their role in human tumourigenesis. The human *PLA2G2A* gene has been mapped to chromosome 1p35-36. This region, which exhibits LOH in 31–64% of primary colorectal tumours [82–84], has been suggested to contain a modifier of colon cancer [85, 86]. A number of studies have looked for and failed to identify any germline or somatic mutations of *PLA2G2A* in colon cancer [82, 84, 87, 88], with one exception. One sporadic colon cancer patient was found to have inherited a frameshift mutation in *PLA2G2A* that results in premature truncation. Subsequent somatic loss of the wild-type allele resulted in a PLA2G2A-null tumour [83]. The lack of mutations cast doubt on the importance of PLA2G2A in human cancer. However, it is interesting to note in a separate study that 12/14 (86%) colon cancer cell lines showed no expression of *PLA2G2A*, despite no mutations being detected [84]. *PLA2G2A* expression was detectable in all 10 normal colonic mucosa examined. Therefore, differences in *PLA2G2A* expression, not related to mutation of the gene, may be of significance to human cancer. Support for this comes from a recent study of gastric cancer, which showed *PLA2G2A* expression to be highly variable in tumours [89]. When tumours expressing high levels of *PLA2G2A* were compared to low-expressing tumours, high *PLA2G2A* expression was found to significantly correlate with low stage ($p = 0.002$) and improved survival ($p = 0.0002$). The association with survival was confirmed in a second, smaller, independent data set ($p = 0.044$). Therefore alterations in *PLA2G2A* expression appear to have a modifier effect on gastric cancer, although the role of *PLA2G2A* in human colon cancer remains to be clarified.

The complexity of tumour modifier genes is well illustrated by the above examples. Modifier genes can affect any of the steps of tumour progression (initiation, growth, conversion, invasion or metastasis) or indeed several steps (e.g. PTGS2 modifying tumour initiation and growth). Their expression can cause suppression (e.g. PLA2G2A) or promotion (e.g. PLA2G4A) of tumourigenesis. Their affects may be global or regional (e.g. PLA2G2A expression modified adenoma formation throughout the intestine in *Apc*-mutant mice, whereas PLA2G4A expression affects polyps of the small intestine but not the colon). Furthermore, a single tumour type, such as breast cancer or intestinal polyps, and even a single tumour suppressor gene such as *BRCA1* or *Apc*, can be affected by multiple modifiers. The involvement of cancer modifier loci affects the two-hit model in several ways. First, two mutations in a particular TSG may not always produce a tumour, depending on the genetic background. Second, multiple hits may be required to mutate both the tumour-causing and tumour-modifying genes for tumourigenesis. Finally, although some genetic modifiers may themselves be considered tumour suppressors, their suppressive function may result from natural polymorphisms and thus not require somatic mutation.

Mutator phenotypes

The mutator phenotype of tumourigenesis arises from the realisation that TSGs can be divided into two general categories, gatekeeper and caretaker genes [7]. Gatekeeper genes are those that exert direct effects on cell division or survival, and thus loss of these TSGs results in dysregulated clonal expansion and tumours. In contrast, caretaker genes do not directly affect cell proliferation, but regulate genomic stability. Hence, loss of caretakers creates a cell with no growth advantage, but rather one with an increased mutation rate. This elevated incidence of mutations increases the risk of damage to gatekeeper genes and thus the risk of cancer. One form of mutator phenotype is the microsatellite instability (MSI) that is a feature of HNPCC (hereditary non-polyposis colon cancer) and is also detected in sporadic cancers of the stomach, endometrium and colon [90, 91]. MSI is caused by defects in the DNA mismatch repair (MMR) pathway, with germline mutations of the *MLH1* and *MSH2* genes common in HNPCC patients [92]. Mutations in other MMR genes, such as *MSH6*, *MSH3* and *PMS2*, have also been identified in MSI$^+$ tumours [93, 94]. Biallelic loss of any of these MMR genes causes genome-wide instability in mononucleotide and dinucleotide microsatellites, which, when these occur in coding regions of genes, results in the frameshift disruption of the locus. Analysis of MSI$^+$ tumours has revealed a number of common target loci of MSI that include *TGFBR2*, *BAX*, *IGF2R*, *MSH3*, *MSH6*, *BLM*, *ATR* and *CHEK1* [95–98]. In particular, *TGFBR2* and *BAX* are disrupted in 70–90% and 43–57%, respectively, of MSI$^+$ colon cancer cases, suggesting that these are important genes in the tumour development [95–97]. Mutation of *TGFBR2* is not only a frequent event in MSI$^+$ tumours, but also an early event in HNPCC patients, with loss of *TGFBR2* expression in 57% of adenomas [99]. *BAX* mutation, though, is infrequent in adenomas, but common in the carcinomas of HNPCC patients, indicating that *BAX* disruption represents a later event in tumour formation

[100]. Tumourigenesis in HNPCC involves loss of both alleles of a MMR tumour suppressor gene as the initial event (one germline mutation and one somatic). Tumour initiation does not occur, however, until the resulting MSI disrupts an important gatekeeper gene such as *TGFBR2*. Thus, four mutation events are required to generate a tumour. Subsequent MSI disruption of other TSGs (e.g. *BAX* and *IGF2R*) promotes cancer progression, while loss of repair proteins like *MSH3*, *MSH6* and *BLM* increases the genetic instability further.

A related mutator phenotype is chromosomal instability (CIN) whereby cells display aneuploidy, translocations and chromosomal aberrations. Again a set of caretaker genes, including *ATM*, *WRN*, *FANCA* and *BRCA2*, protect the cell from instability. Loss of these proteins results in disease syndromes exhibiting CIN, cellular sensitivity to radiation and cancer predisposition (ataxia telangiectasia, Werner syndrome, Fanconi's anaemia and Li Fraumeni syndrome, respectively) [101, 102]. As for the MSI^+ tumours, loss of the proteins controlling chromosome stability appear not to directly cause cancer, but instead enhance the mutation rate in the cell, which may subsequently lead to disruption of TSGs and tumourigenesis. Two recent *Science* papers have provided support for this model by examining the RecQ DNA helicase BLM, responsible for Bloom syndrome, a recessive disorder characterised by small stature, immunodeficiency, genetic instability and cancer predisposition. In a study of colon cancer in Ashkenazi Jews, the cancer patients were found to be twice as likely to carry the truncating BLM^{Ash} mutation as controls, suggesting that *BLM* heterozygosity is a risk factor for this cancer [103]. Murine models have revealed similar findings. Mice heterozygous for a null mutation of *Blm* were crossed with the Apc^{Min} colon cancer model mouse. The resulting $Blm^{+/-}$ $Apc^{Min/+}$ mice were found to develop twice the number of gastrointestinal tumours as the $Blm^{+/+}$ $Apc^{Min/+}$ [104]. In each tumour examined, the wild-type *Apc* allele had been lost but the wild-type *Blm* allele was retained. Furthermore, in 90% of the tumours in $Blm^{+/-}$ $Apc^{Min/+}$ mice, the wild-type *Apc* allele was lost via LOH, a form of cellular instability. It thus seems that heterozygosity for *Blm*, whilst not causing a tumour directly, results in increased CIN enhancing the rate of LOH at the *Apc* locus and increases the number of resulting $Apc^{-/-}$ tumours. Tumour formation in these animals is therefore a three-mutation event.

The common feature of the accumulated mutation, modifier gene and mutator phenotype models of tumourigenesis is that tumour formation and/or progression is dependent on disruption of several TSGs. The above examples illustrated multi-gene, multi-hit tumourigenesis, and in the cases of CIN, MSI and the resistance modifiers of *Apc*, *BRCA1* and *BRCA2*, three or four mutations must be acquired before a tumour forms, in contrast to the one-gene, two-hit Knudson model of cancer.

When is a TSG not a TSG?

There is, however, a small group of loci that deviate further from the canonical TSG definition. These are genes whose function is not always tumour suppressive, but which can also act to promote tumourigenesis. Perhaps the most extensively studied of these dual function cancer genes is the cytokine tumour growth factor beta 1 (TGFβ1, see the excellent review by Derynck and colleagues [105]). Extracellular TGFβ1 binds the transmembrane receptors TGFβR1 and TGFβR2, activating the intracellular signalling MADH (previously called SMAD) proteins and leading to altered gene expression. The TGFβ pathway is multifunctional, regulating the processes of cell growth, tissue remodelling and angiogenesis. Several lines of evidence have implicated *TGFB1* as a tumour suppressor gene. First, *Tgfb1*-knockout mice on an immunodeficient background rapidly develop adenomas and carcinomas of the cecum and colon compared with *Tgfb1*-expressing mice on the same background [106]. Both the rate and number of tumours is increased by the absence of TGFβ1. Second, mutations in the TGFβ-receptor genes *TGFB2R* and *TGFB1R*, and the *MADH2* and *MADH4* genes (previously named *SMAD2* and *SMAD4*), have been identified in a number of tumour types [107–111], suggesting that loss of TGFβ signalling is important to cancer development. Indeed, *TGFBR2* is a frequent target of the MSI phenotype occurring in HNPCC and some gastric cancers due to defects in the mismatch repair pathway (see above, [108]), and it has been suggested that *TGFBR2* mutation may be present in 20–25% of colon tumours [105]. Third, restoration of TGFβ signalling by reexpression of TGFβR2 in MCF-7 breast cancer cells resulted in reduced tumourigenicity in nude mice and decreased growth in soft agar [112]. Thus, there is a strong case for classing *TGFB1* as a TSG. The tumour suppressive function of TGFβ is likely a consequence of its role in growth arrest. TGFβ signalling can lead to the induction of CDKN2B ($p15^{INK4B}$) and CDKN1A ($p21^{CIP1}$), two inhibitors of cyclin/cyclin-dependent kinase complexes, and the subsequent blocking of cyclin-driven cell cycle progression. Thus, TGFβ is capable of inhibiting cell growth (and thus presumably tumour growth), and TGF signalling is disrupted in a number of tumour types.

However, despite this apparent tumour suppressive role, TGFβ1 often demonstrates increased production in tumour cells [113, 114]. This dichotomy may be in part explained by the existence of two opposing roles for TGFβ in tumourigenesis. These dual functions are illustrated by several transgenic mouse models with targeted expression of TGFβ1 in keratinocytes [115]. When treated with single-dose DMBA followed by multiple applications of the tumour promoter TPA, these mice developed significantly fewer skin tumours than nontransgenic controls,

confirming the suppressive role of TGFβ. However, those tumours that did develop in the transgenic mice showed a highly increased malignant conversion rate and greater numbers of carcinomas (as opposed to benign tumours) per mouse than the nontransgenic controls. This indicates that TGFβ is capable of inhibiting tumour formation, but promotes tumour progression to malignancy. Similar conclusions were reached by Oft and colleagues, who transfected HRAS expressing mammary epithelial cells (EpRas) and colon tumour cell lines (CT26 and C26) with a dominant negative TGFβR2 protein, thus inhibiting TGF signalling [116]. In all three cell types, expression of TGFβR2-dn resulted in significantly reduced tumourigenicity in nude mice and almost complete absence of lung metastases normally caused by injection of CT26 or C26 cells. The tumour-promoting role of TGFβ in this study was shown to be cell autonomous, possibly through its action of restoring epithelial morphology to the cells and preventing dedifferentiation. However, other tumour-promoting functions of TGFβ in extracellular matrix remodelling and increased angiogenesis have also been proposed (discussed in Derynck [105]).

TGFβ is not the only protein suggested to play dual, opposing roles in tumourigenesis. In an immunohistochemical analysis of extracellular matrix composition in 50 primary ovarian tumours, significantly reduced expression of laminin and collagen IV was detected, indicating disruption of the normal basement membrane beneath the carcinoma [117]. In total, 76% of ovarian tumours were laminin negative, and 94% were collagen IV negative. Absence of staining for laminin and collagen IV in the preneoplastic epithelium adjacent to each tumour suggests that loss of these basement membrane proteins precedes the malignant conversion, and is thus associated with early transformation of the surface epithelium. In contrast, when the authors examined 50 peritoneal metastases of ovarian tumours, positive staining for laminin was detected in 86% of tumours. This differs highly significantly from the laminin staining in the primary tumours ($p < 0.001$ in a two-tailed Fisher's exact test) suggesting that reexpression of laminin is important in metastatic disease. A small but nonsignificant increase in the number of collagen IV positive metastases compared with positive staining primary ovarian tumours was also noted, indicating a similar, but weaker, trend of reexpression for collagen IV in metastatic disease. The authors propose that loss of laminin and collagen IV expression in the ovarian surface epithelium is an early event in ovarian tumourigenesis and is an important change permitting the disorganisation of the epithelia (multi-layered epithelia and loss of basal-apical polarity) and stromal invasion that are hallmarks of malignancy. However, subsequent reexpression of laminin, and possibly collagen IV as well, promoted metastatic spread perhaps by providing a nurturing environment at the secondary site.

Therefore TGFβ, and possibly laminin and collagen IV, appears to be capable of both promoting and suppressing tumourigenesis. The overall effect on a tumour will probably reflect any imbalance between these two opposing influences. These proteins are likely to have different effects on different cancer types and at different times in tumour progression, such that loss of expression may be advantageous to some tumours, but not others. Since many mammalian proteins are known to be multi-functional, genes that can be both tumour suppressive and tumour promoting may be more common than we have realised. The existence of such genes, however, truly calls into question what constitutes a TSG.

A hit, a most palpable hit

A further, related question would be, What constitutes a TSG hit? I hitherto discussed situations in which more or less than the canonical two hits are required to generate a tumour. However, the question of what types of mutational hit are considered acceptable evidence of a TSG is also controversial. The identification of the retinoblastoma gene *RB1* revealed that mutational inactivation occurs by a combination of point mutations (including nonsense, missense, frameshift and splice site mutations), deletions and LOH. In some cases a point mutation or deletion in one *RB1* allele was combined with somatic LOH of the wild-type allele, whereas in other patients, biallelic point mutation or homozygous deletion was reported [118–121].

The same inactivating mechanisms, point mutations, deletions and LOH have been shown to disrupt many different TSGs, revealing the universal nature of Knudson's mutational hits. However, an additional mechanism of TSG inactivation was subsequently reported, that of gene silencing by CpG island hypermethylation. This differs from mutational disruption of genes since hypermethylation-induced gene silencing is reversible. One example of a hypermethylated TSG is the *CDKN2A* (*p16^{INK4A}*) gene. *CDKN2A* (*p16^{INK4}*) was suggested as an important TSG and shown to be disrupted by homozygous deletions, point mutations and LOH [122]. Analysis of *CDKN2A* (*p16^{INK4}*) in primary tumours by Merlo and colleagues revealed few point mutations, but identified hypermethylation of the gene's CpG island in 20% of the tumours analysed [123]. This promoter hypermethylation inhibits transcription of the gene and thus causes loss of CDKN2A (p16^{INK4A}) protein [123]. Epigenetic hypermethylation of *CDKN2A* (*p16^{INK4}*) is common in gastric cancer and results in loss of CDKN2A (p16^{INK4A}) protein, as determined by immunohistochemistry of the gastric tumours [124]. No hypermethylation was detected in normal tissue, revealing that like LOH, hypermethylation is a somatic event. *CDKN2A* (*p16^{INK4A}*) hypermethylation

was found in 42% of the primary gastric tumours analysed [125], thus making hypermethylation the predominant mechanism of CDKN2A ($p16^{INK4A}$) loss in gastric cancer.

Inactivation of more and more TSGs by epigenetic hypermethylation is being reported (reviewed by [126]), and thus hypermethylation is slowly being accepted as an additional, valid hit mechanism. Nonetheless, many researchers still consider tumour-associated truncating point mutation to be the definitive proof of a TSG. This view prevails to such an extent that when a candidate TSG exhibits hypermethylation but lacks truncating mutations, it often receives opposition, despite other supportive data. One such example is the Runt domain-containing transcription factor RUNX3. *Runx3*$^{-/-}$ mice show hyperplasia of the gastric epithelium, which results in perinatal death, and suggests a potential role of the gene in gastric cancer [127]. Analysis of human gastric tumours revealed very low (compared to surrounding normal mucosa) or absent *RUNX3* expression by reverse transcription-polymerase chain reaction (RT-PCR) in 60% of tumours. This loss of expression was stage associated, occurring in 41% of stage I tumours, but 88% of stage IV carcinomas. Gastric epithelial cells taken from *Runx3*$^{-/-}$ *Trp53*$^{-/-}$ mice were tumourigenic in nude mice, whilst equivalent cells from *Runx3*$^{+/+}$ *Trp53*$^{-/-}$ mice were not. Furthermore, RUNX3 overexpression in the MKN28 gastric cancer cell line suppressed its tumourigenicity. However, only one point mutation was detected in 119 gastric tumours, and that was not a truncating mutation, but rather an Arg>Cys missense change in the Runt domain. Overexpression of this R122C mutated RUNX3 in MKN28 cells did not suppress their tumourigenicity, confirming that the missense change causes a loss of function. Although point mutation of *RUNX3* in gastric cancer was found to be rare, 14/46 tumours and 3/15 cell lines exhibited hemizygous deletions. A subset of these tumours and cell lines were examined for hypermethylation of the *RUNX3* promoter. Three primary tumours and three cell lines shown to be lacking RUNX3 expression were all found to be hypermethylated. In contrast, three normal mucosa samples and three cell lines expressing RUNX3 showed no hypermethylation. One of these normal mucosa samples was from the same patient as one of the hypermethylated primary tumours, again confirming that hypermethylation is a somatic event in this tumour. Thus, although point mutation of *RUNX3* is rare, inactivation of this gene by hemizygous deletion and hypermethylation appears common in gastric cancer. However, despite the frequent loss of expression in gastric tumours associated with hypermethylation of *RUNX3*, the role of RUNX3 in suppressing tumourigenicity in transfected cells and the hyperplasia of the gastric mucosa in *Runx3* knockout mice, the absence of truncating point mutations has led to *RUNX3* being described as a 'new age' rather than a 'classical' TSG in a recent commentary [128].

Another possible new age TSG is the *WWOX* gene. The *WWOX* gene maps to chromosome 16q23, a region of frequent LOH in breast, prostate and hepatocellular carcinoma [129]. Homozygous deletion of *WWOX* exons has been detected in several tumour types, including ovarian, small cell lung and pancreatic carcinoma [130, 131]. Mutation screening in a number of different cancer types has failed to identify any truncating point mutations, although a high degree of polymorphism and several exon skipped isoforms were detected [130].

Once again the lack of truncating mutations has led to the classification of *WWOX* as a TSG to be questioned. Some have argued that the homozygous deletions are purely the consequence of the FRA16D common fragile site, an unstable chromosomal region located within an intron of *WWOX*, and are independent of the neighbouring *WWOX* gene. However, the clonal homogeneity of the PEO4 primary ovarian tumour for the homozygous deletion [130] suggests a selective advantage to the tumour arising from the absence of WWOX. Further evidence supports the tumour suppressive role of WWOX. A somatic mutation, combined with LOH of the other allele, was identified in an oesophageal cancer, a missense Leu>Pro alteration [132]. Although this mutated form of WWOX has not been functionally examined, the substitution of proline two residues away from the predicted catalytic site of the oxidoreductase domain is likely to disrupt WWOX function. In addition, transfection of a breast cancer cell line with WWOX resulted in suppressed tumourigenicity and reduced growth in soft agar [133], and studies with mouse fibroblasts have implicated murine WWOX as an apoptotic regulator in TNFα-induced cytotoxicity [134]. Further assessment of *WWOX* as a TSG awaits the generation of a knockout mouse model.

Considering the above examples, it is possible that the type of mutation will depend upon the biology of the TSG, the biology of the cancer type and the biology of the chromosomal region containing the gene. The *WWOX* gene shows few point mutations, but a bias towards inactivation by homozygous deletion. That the fragile site is the cause of the homozygous deletions seems likely, though the significance of the deletion probably relates to the loss of WWOX expression providing a selective advantage to tumour formation/progression. The presence of the fragile site may therefore provide the reason behind the predominance of homozygous deletion over point mutation in the *WWOX* gene. Similarly, the *RUNX3* gene is rarely mutated, but is primarily inactivated by hypermethylation. The *CDKN2A* (*p16*^{INK4A}) gene, meanwhile, exhibits point mutation and deletion frequently in melanoma, but rarely in gastric cancer where hypermethylation is predominant. To bias our definition of a TSG to those exhibiting truncating point mutation seems naïve

and limiting. In each case, irrespective of the type of hit, the consequence of mutation is loss of expression. Thus, in response to the question, What constitutes a hit? the answer should perhaps be point mutation, deletion, LOH, hypermethylation or other mechanism that results in loss of function of the gene.

Conclusion

The large number of exceptions to the standard two-hit TSG model, as illustrated in this review, suggests that the currently accepted definition may be too restrictive. The mutation-based description of tumour suppressors by Haber and Harlow does not allow for epigenetic inactivation of candidate loci, or for the influence of polymorphic variants of modifier genes on tumourigenesis. Furthermore, the underlying two-hit preconception excludes cancer-suppressing loci that exhibit haploinsufficiency or dominant negative behaviour. Whilst these definitions have been of great use in the past for picking a manageable number of TSGs from the myriad candidates reported, the rapidly increasing number of papers describing nonstandard suppressor genes suggests that we have already identified most of these simple, canonical TSGs. The future for TSG identification is therefore likely to increasingly involve exceptions to the two-hit model of inactivating mutation. To provide validation criteria for this next period of cancer genetics, I propose the following definition: proof of a tumour suppressor gene will require (i) identification of loss of function in the development of a cancer and (ii) demonstration that inactivation of the gene in vivo enhances tumour initiation, growth or progression.

The first part of this definition is based upon the broad but essential requirement that loss of tumour suppressor gene function is demonstrated in cancer development. Thus, the ability of a gene to suppress some aspect of tumourigenic behaviour in vitro is not considered sufficient proof of TSG status in the absence of evidence that loss of gene function occurs in cancer, echoing the sentiments of Haber and Harlow. Like their definition, this description also encompasses genes that suppress all steps of tumour development, not merely formation. However, by removing the word 'mutation' from the definition, this revised criterion includes epigenetic gene inactivation, and also does not bias the definition towards truncating point mutations over other inactivating mechanisms such as homozygous deletions. In addition, by not specifying the number of inactivating hits, this model includes haploinsufficient genes, dominant negative behaviour, and lost or reduced modifier function due to polymorphic variation. Since many candidate loci fulfilling this requirement will be ambiguous TSGs (for example, not all genes showing monoallelic mutation will be haploinsufficient), a second, functional proviso for validation is required. The second part of the definition therefore specifies that inactivation of a TSG in an in vivo model (avoiding the uncertainties of in vitro behaviour) should promote one or more of the steps of tumourigenesis. This tumour promotion may be spontaneous, follow chemical or viral carcinogenic induction, or may occur when the mutant mouse is crossed with other genetically modified animals or onto other strains. This therefore allows identification of TSGs that promote tumour growth or progression, as well as initiation. This functional component to the TSG definition offers the advantage that candidate genes are not assessed solely on a correlation between mutation and cancer, but in addition require evidence that loss of gene function in a living organism increases tumourigenesis. While this revised definition is indeed broader and more complicated than the previous one, it is likely that the new generation of tumour suppressors, deviating from the standard Knudson model, will necessitate a change to a wider definition of the tumour suppressor gene. Such conceptual evolution will complement the changing face of cancer genetics in the 21st century.

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