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REVIEW

Tumor suppressor genetics

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The observation that mutations in tumor suppressor genes can have haploinsufficient, as well as gain of function and dominant negative, phenotypes has caused a reevaluation of the 'two-hit' model of tumor suppressor inactivation. Here we examine the history of haploinsufficiency and tumor suppressors in order to understand the origin of the 'two-hit' dogma. The two-hit model of tumor suppressor gene inactivation was derived from mathematical modeling of cancer incidence. Subsequent interpretations implied that tumor suppressors were recessive, requiring mutations in both alleles. This model has provided a useful conceptual framework for three decades of research on the genetics and biology of tumor suppressor genes. Recently it has become clear that mutations in tumor suppressor genes are not always completely recessive. Haploinsufficiency occurs when one allele is insufficient to confer the full functionality produced from two wild-type alleles. Haploinsufficiency, however, is not an absolute property. It can be partial or complete and can vary depending on tissue type, other epistatic interactions, and environmental factors. In addition to simple quantitative differences (one allele versus two alleles), gene mutations can have qualitative differences, creating gain of function or dominant negative effects that can be difficult to distinguish from dosage-dependence. Like mutations in many other genes, tumor suppressor gene mutations can be haploinsufficient, dominant negative or gain of function in addition to recessive. Thus, under certain circumstances, one hit may be sufficient for inactivation. In addition, the phenotypic penetrance of these mutations can vary depending on the nature of the mutation itself, the genetic background, the tissue type, environmental factors and other variables. Incorporating these new findings into existing models of the clonal evolution will be a challenge for the future.

Defining tumor suppressor genes

The categorization of 'cancer genes' into dominant-acting oncogenes and recessive tumor suppressors is rooted in historical context. The first 'cancer genes' identified were primarily derived from cancer-causing viruses and were found to transform cells in a dominant fashion. These genes became known as oncogenes due to their ability to drive oncogenesis. Evidence for so-called 'antioncogenes' or tumor suppressors was less forthcoming. Early experiments with somatic cell hybridization had suggested that tumor suppressor genes existed and were recessive, that is tumor suppressor genes must be completely inactivated for malignancy to occur. In 1969, a series of somatic cell hybrid fusion experiments demonstrated that A9 cells suppressed the tumorigenicity of malignant cells (1), indicating that a factor responsible for the suppression of malignancy likely existed in the A9 cells and that this factor was lost in malignant cells. Subsequent work by Al Knudson among others both predicted the existence of human tumor suppressors and led to the 'two-hit' model of tumorigenesis (2,3).

In a seminal analysis comparing unilateral and bilateral retinoblastoma patients, Knudson first delineated his hypothesis that the dominantly inherited form of retinoblastoma and the nonhereditary form are mechanistically linked (2). In the hereditary form, he postulated that one mutation (the first 'hit') is inherited while a second mutation occurs in somatic cells significantly accelerating the onset of retinoblastoma and often leading to bilateral forms of the disease. In the nonhereditary form, two mutations must occur somatically prior to retinoblastoma inititiation. Following the cloning of the retinoblastoma gene, *RB*, in 1986, Knudson's elegant and unifying prediction appeared to be born out (4). Loss of heterozygosity (LOH) and mutation analysis of *RB* revealed biallelic mutations in retinoblastoma as well as other tumor types and demonstrated that both hereditary and nonhereditary retinoblastomas sustain mutation or loss of both alleles of *RB*. This precedent has developed into one of the most dominant paradigms in cancer research leading to the tenet that tumor suppressor genes are recessive at the cellular level, requiring complete loss of function in order to reveal a phenotype. Conversely, germline mutations of tumor suppressor genes function dominantly at the organismic level, predisposing the carrier to early onset of disease by supplying one of the required two hits at birth.

In the years that followed, it became clear that *RB* was only the first example of an ever-expanding class of cancer gene, the tumor suppressor. In 1969, Li and Fraumeni described a dominantly inherited familial syndrome of cancer including rhabdomyosarcoma, osteosarcoma and breast cancer in female carriers (5). By analogy with *RB*, Li-Fraumeni syndrome was later ascribed to mutation in the *TP53* gene and p53 was reinterpreted as a tumor suppressor rather than the oncogene

Abbreviations: FAP, familial adenomatous polyposis; VNTR, variable number of tandem repeats.

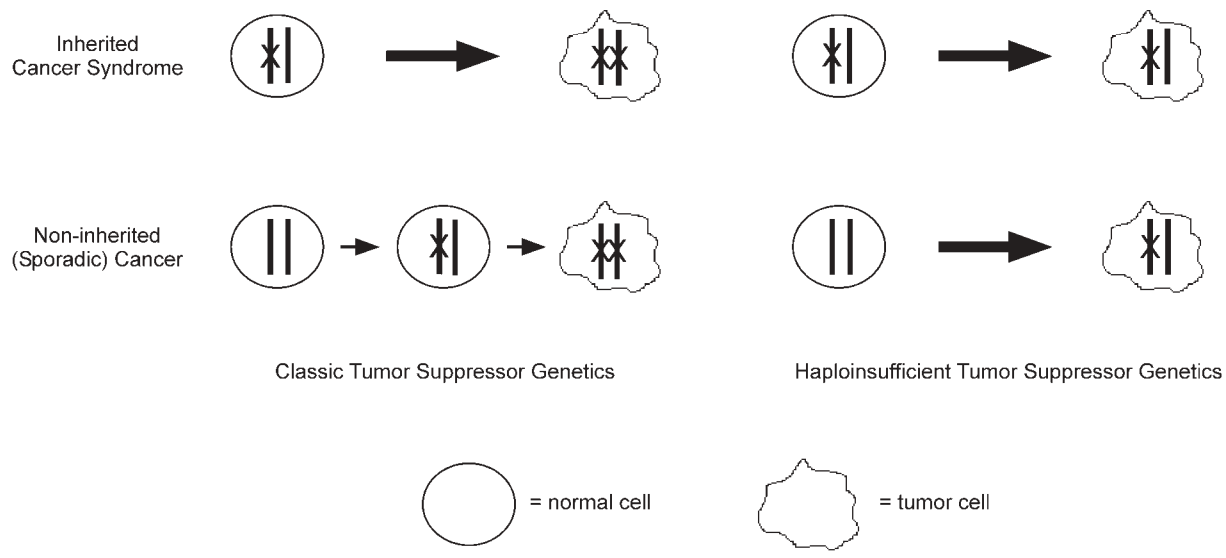

Fig. 1. Inactivation of Tumor Suppressor Genes. Classic tumor suppressor genes are inactivated via 'two hits'. In the case of inherited cancer susceptibility, one of these 'hits' is acquired in the germline with the second 'hit' being acquired somatically during tumor development. Haploinsufficient tumor suppressor genes are compromised by a single 'hit', obviating the need to sustain 'two hits' during the course of tumor development.

that it was thought to be when originally identified in its mutant form (6,7). Biallelic mutations in *TP53* were frequently (though not always) found in tumors, solidifying the belief that tumor suppressor genes require two hits for inactivation (Figure 1). The conventional wisdom that tumor suppressor genes are recessive, then, is based on historical context, not *a priori* logic.

The two-hit model, however, is difficult to reconcile with another major paradigm of cancer research, Nowell's hypothesis of clonal evolution (8). If loss of one tumor suppressor allele truly has no phenotype, then both alleles would need to be lost within a single cell before any selective advantage could occur. However, the spontaneous mutation rate is very low in cells, and accumulation of multiple mutations is required to transform normal cells to a fully malignant tumor. Given the rate of cancer, Loeb has argued that cancer cells must have a mutator phenotype to supply the requisite number of mutations within an individual's lifetime (9). If the loss of one tumor suppressor allele had no selective advantage it would be even more difficult to account for the disparity between known mutation rates and human cancer incidence. As discussed next, tumor suppressor genes are not a unique class of genes with respect to their genetics and, like many other genes involved in metabolism and development, can be haploinsufficient, showing dramatic phenotypes with loss of only a single allele.

Origins and definition of haploinsufficiency

In diploid organisms, with the exception of genes on the X and Y chromosomes and imprinted genes, two functional copies (alleles) of all genes are present. Nevertheless, for many genes, a single functional allele is sufficient to maintain normal operations. Some genes, and certain functions of other genes, however, are very sensitive to gene dosage levels. Haploinsufficiency represents that special circumstance in which one working allele of a gene is insufficient to accomplish the normal activity of that gene product in the cell. Drosophila geneticists described the concept of haploinsufficiency as early as the turn of the last century. 'Minute' mutations were first identified as dominant alleles that are lethal when homozygous. These minute mutations in many instances turned out to be chromosomal deficiencies. In the case of the haplo-IV minute in which the entire fourth chromosome is missing, it was found that a haploid complement of chromosome IV genes resulted in tardy development, reduced fertility and heavy mortality (10–12). In *Genetics*, a seminal journal of the early genetics field, the term 'haplo-insufficiency' was first applied by Curt Stern in his study of dosage effects on the *cubitus interruptus* allele of Drosophila (13) and was not used again until 1956 in describing one of Bridges' original deficiencies, *M33a* (14). Recently, however, there has been increasing interest in haploinsufficiency and the terms 'haploinsufficiency' and 'haploinsufficiency' have appeared in more than 102 journal articles published in the journal *Genetics* alone during the last decade while at least 984 references in PubMed list 'haploinsufficiency' as a keyword (http://www.ncbi.nlm.nih.gov, August 11, 2005).

Haploinsufficient tumor suppressor genes

Despite the long history of haploinsufficiency in genetics, translation of this concept to tumor suppressor genes has been slow. The delay is likely due to the lack of experimental evidence as well as a perceived conflict between haploinsufficiency (one allele is not enough) and the original definition of a tumor suppressor gene (two hits required). In traditional tumor suppressor genetics, inherited loss of one tumor suppressor allele leads to accelerated tumorigenesis due to the need to inactivate only one remaining allele. Haploinsufficient tumor suppressor genes also lead to accelerated tumorigenesis, however, without the requirement for inherited mutation of one allele (Figure 1).

In 1998, Fero *et al*. (15) reported that the cyclin-dependent kinase inhibitor $p27^{kip1}$ is haploinsufficient for tumor suppression and Venkatachalam *et al*. (16) showed that p53 could suppress tumor development in a gene dosage-dependent manner. Similarly, Tang *et al*. (17) reported that Tgfβ is

haploinsufficient for tumor suppression, although its mechanism of haploinsufficiency is fundamentally different as Tgfβ is a secreted protein and thus, functions in a non-cell-autonomous fashion.

In 1996, three labs independently created p27 knockout mice and reported similar phenotypes (18–20). $p27^{-/-}$ mice have an increased growth rate and adults are 20 to 30% larger than wild-type littermates. The increased size of the $p27^{-/-}$ animals was due to increased cellularity as opposed to increased cell size, pointing to a key role of p27 in controlling growth in all tissue compartments *in vivo* and providing the first clue to its gene dosage sensitivity. Interestingly, tissues from the $p27^{+/-}$ mice expressed roughly 50% the normal level of p27 protein and these mice showed an intermediate growth rate. The intermediate phenotype of the heterozygous animals indicated that control of proliferation and even adult animal size is extremely sensitive to the level of p27 protein. In addition, p27-deficient mice exhibited hyperplasia of the pituitary intermediate lobe and nearly 100% of $p27^{-/-}$ mice (on a 129/Sv genetic background) eventually succumbed as a result of benign pituitary adenomas (18). $p27^{-/-}$ mice have not demonstrated increased susceptibility to spontaneous tumor development in other tissues.

There is, however, an abundance of evidence from human cancers indicating that reduced p27 protein is associated with more aggressive tumors and reduced patient survival (reviewed in ref. 21). As cancer is a multistep process requiring several genetic events, a lack of spontaneous tumor predisposition in p27-deficient mice was insufficient evidence to rule out a tumor-suppressing role for p27. Other genetic events might be required to elicit latent tumor suppressing effects by p27. Indeed when p27-deficient mice were challenged with either the point mutagen ENU, or the broad spectrum mutagenic agent ionizing radiation, they showed tumor predisposition in multiple epithelial tissues (15). In a cohort challenged with a single dose of 1 Gy radiation, the median tumor-free survival was reduced from more than 70 weeks in wild-type control mice to 40 weeks in $p27^{-/-}$ mice due to increased tumor multiplicity in diverse sites including small and large intestine, lung, ovary, uterus and adrenal gland. $p27^{+/-}$ mice showed an intermediate susceptibility, both in tumor-free survival and tumor multiplicity. Genetic and biochemical analysis of tumors from the $p27^{+/-}$ mice revealed that the wild-type *p27* allele was not mutated and protein expression was not silenced in $p27^{+/-}$ tumors. Similarly, when p27-deficient mice were challenged with the carcinogen 1,2-dimethylhydrazine (DMH), an alkylating agent that induces adenomas and adenocarcinomas specifically in the colon, overall tumor-free survival was significantly reduced in $p27^{-/-}$ mice and to an intermediate extent in $p27^{+/-}$ mice relative to wild-type littermate controls (22). The incidence of colorectal adenocarcinoma as well as the ratio of adenocarcinomas to adenomas and the histological aggressiveness of tumor behavior were all significantly increased in p27-deficient mice. While $p27^{+/-}$ mice exhibited a colorectal tumor latency and histologically aggressive tumor behavior intermediate between $p27^{-/-}$ and $p27^{+/+}$ mice, the remaining wild-type allele was retained and continued to be expressed in DMH-induced colorectal tumors from $p27^{+/-}$ mice.

These results differed from those reported previously in other murine models of tumor suppressor knockouts. For example, tumors from $Rb^{+/-}$ (23,24) and $Apc^{+/-}$ (25) mice all show frequent mutation or loss of the remaining wild-type allele, consistent with Knudson's two-hit model (2). In these cases the tumor suppressor appears recessive at the cellular level: complete loss of both alleles provides a much greater selective advantage than loss of a single allele. Even in these cases, however, the possibility that the loss of one allele confers a selective advantage perhaps early in tumor development cannot be excluded. The genetic inactivation of murine *Rb* and *Apc* genes mimics that seen in human tumors where biallelic mutations in these tumor suppressor genes are observed. In contrast, biallelic mutations in *p27* are rarely seen in either human or murine tumors. The murine data unequivocally show a strong selective advantage to tumor development with loss of a single *p27* allele. The data indicate that inhibition of growth (18) and tumor development (15) is highly sensitive to the gene dosage of p27. Thus, halving the normal amount of p27 is sufficient to result in unchecked growth, suggesting that there may not be a threshold for its activity but rather a dose–response continuum.

In another well-known example, *Trp53* is inactivated via traditional 'two-hit' kinetics under certain circumstances and in certain tissues, but in other cases shows clear evidence for haploinsufficiency. Early evidence for haploinsufficient behavior of *Trp53* at the cellular level was observed by Bouffler *et al*. (26). In this study, $p53^{+/-}$ mice showed a significantly higher number of spontaneous chromosome aberrations as compared to $p53^{+/+}$ mice, with the incidence being intermediate between that of the $p53^{+/+}$ and $p53^{-/-}$ mice. Similarly, Clarke *et al*. (27) reported that apoptosis is partially impaired in $p53^{+/-}$ mice. Venkatachalam *et al*. (16) later confirmed the haploinsufficiency of *Trp53* by analysis of tumors from $p53^{+/-}$ mice. In a study of 217 $p53^{+/-}$ mice, roughly half of all tumors retained the wild-type *p53* allele. In contrast to $p53^{+/-}$ tumors that lose the remaining wild-type allele, those tumors with wild-type allele retention expressed a functional p53 protein that preserved the ability to induce apoptosis following irradiation, to induce p21 and Mdm2 expression, and to repress PCNA expression.

Since 1998, a burgeoning number of tumor suppressor genes have shown evidence of haploinsufficiency (Table I). One of the recent examples with the best understood mechanism of haploinsufficiency is Nkx3.1. *Nkx3.1* encodes a homeobox protein that is expressed specifically in the luminal epithelium of the prostate. $Nkx3.1^{+/-}$ mice develop prostatic hyperplasia without loss of the remaining wild-type allele (28). Microarray analysis identified a number of Nkx3.1 target genes that represented a range of responses to *Nkx3.1* gene dosage (29). Some genes, e.g. probasin were relatively insensitive to gene dosage with 70 to 80% of normal expression in *Nkx3.1* hemizygotes whereas expression of other genes such as intelectin was lost in *Nkx3.1* hemizygotes. In between the two extremes were a number of genes whose expression in *Nkx3.1* hemizygotes ranged from 16 to 55% of wild-type expression. The authors argued for a stochastic model of gene expression in which *Nkx3.1* gene dosage affects the probability of target gene expression in any given cell. In the case of Nkx3.1, evidence of haploinsufficiency depended largely on the specific phenotype studied (i.e. probasin expression versus intelectin expression), indicating the difficulty inherent in identifying a multifunctional tumor suppressor as haploinsufficient or not.

In most cases, demonstration of the haploinsufficiency of a tumor suppressor gene has only been possible in the more rigorously controlled genetics of mouse models. One exception is the CBFA2/AML1 tumor suppressor. Song *et al*. (30)

Table I. Haploinsufficient tumor suppressor genes

Gene	Inherited human cancer association	Sporadic human cancer association	Haploinsufficient phenotype
Anx7	?	Down-regulated in sporadic prostate, glioblastoma multiforme and hormone receptor negative breast cancers (71–73)	Multiple tissues in mouse: lymphoma, hepatocellular carcinoma, and lung adenoma among others (74)
Apc	Mutated in FAP coli syndrome (75,76)	Mutated in sporadic colorectal, gastric, pancreatic, thyroid and ovarian cancers (77)	Tumorigenicity of cell lines with activated v-Ha-ras in nude mice (78)
Arf	Mutated in familial melanoma and neural tumors (79)	Mutated in multiple sporadic cancer types (ARF-specific in T-cell acute lymphoblastic leukemia and metastatic melanoma) (80–82); Promoter hypermethylation in oral squamous cell carcinoma (83)	Human melanoma (84); murine DMBA/TPA-induced papilloma (85)
*Atm***	Mutated in Ataxia telangiectasia (86); Increased susceptibility to breast cancer in heterozygotes (reviewed in ref. 87)	Mutated in several forms of leukemia and lymphoma (reviewed in ref. 88); Rarely mutated, but frequently down-regulated in sporadic breast cancer (89–91)	Increased sensitivity to sublethal doses of ionizing radiation as determined by lifespan and premature greying (92)
Atr	Mutated in Seckel syndrome (reviewed in ref. 93)	Mutated in microsatellite unstable endometrial and gastrointestinal cancer (94,95)	Mlh1-induced murine thymic lymphoma and intestinal adenocarcinomas (96)
Beclin1	?	Down-regulated in sporadic breast cancer (97,98)	Murine lymphoma, hepatocellular carcinoma and lung adenocarcinoma (99,100)
Bim	?	Frequently down-regulated with accompanying LOH in mantle cell lymphomas (101), but otherwise few published mutation screens to date	Eμ-Myc-induced murine B-cell leukemia (102)
Blm	Mutated in Blooms syndrome (103)	Mutated in sporadic microsatellite unstable gastrointestinal cancer (104,105)	Murine leukemia virus-induced lymphoma, Apc^{min}-induced intestinal adenoma (106)
*BRCA1***	Mutated in familial breast and ovarian cancer syndrome (107,108)	Rarely mutated, but frequently down-regulated in sporadic breast (reviewed in ref. 109), ovarian (reviewed in ref. 110) and pancreatic (111) cancers	Radiosensitivy and maintenance of genome stability in human lymphoblastoid (112) and fibroblast (113) lines
*BRCA2***	Mutated in familial breast and ovarian cancer syndrome (114,115)	Rarely mutated in sporadic cancers analyzed to date (116–120)	Radiosensitivy and maintenance of genome stability in human lymphoblastoid (112) and fibroblast (113) lines
*Bub3***	?	Frequent LOH in osteosarcoma (121), but rarely mutated in sporadic cancers analyzed to date (122,123)	Maintenance of genome stability in MEFs, but no detectable difference in tumor formation *in vivo* (124)
CBFA2/AML1/RUNX1	Mutated in familial platelet disorder with predisposition to acute myelogenous leukemia (30)	Mutated in sporadic acute myelogenous leukemia (125,126)	Human acute myelogenous leukemia (30)
Cdh1 (E-cad)	Mutated in familial gastric cancer (127)	Mutated in sporadic diffuse-type gastric and lobular breast carcinoma (128); Frequently down-regulated in sporadic epithelial cancers (reviewed in ref. 129)	Apc^{1638N}-induced murine adenoma and adenocarcinoma (130)
Cdkn1a ($p21^{Waf1/Cip1}$)**	Genetic variants associated with increased risk of breast cancer and sarcoma (131), lung cancer (132,133), skin, head and neck cancer (134,135) and cervical cancer (136)	Decreased expression in hepatocellular carcinoma (137), but rarely mutated in cancers analyzed to date (137–141)	Epithelial tissues in mouse: harderian gland adenocarcinoma and granulosa cell ovarian tumor (142)
Cdkn1b ($p27^{Kip1}$)	?	Rarely mutated, but frequently down-regulated in multiple sporadic cancer types (reviewed in ref. 46)	Multiple epithelial tissues in mouse: intestinal adenoma and adenocarcinoma, lung adenoma, granulosa cell ovarian tumor, endometrial adenoma and adenocarcinoma, angiosarcoma, adrenal adenoma, pituitary adenoma, thymic lymphoma (15)
Cdkn2c ($p18^{Ink4c}$)	?	Rarely mutated, but down-regulated in some sporadic cancers (143–145)	Murine DMN-induced lung adenocarcinoma and liver hemangiosarcoma (146)
*Chk1***	?	Mutated in microsatellite unstable endometrial and gastrointestinal cancer (95,147)	Maintenance of genome stability in normal murine mammary glands (148)
Dmp1	?	Few published mutation screens to date	Eμ-Myc-induced murine lymphoma (149)
Fbxw7 (Cdc4)	?	Mutated in sporadic endometrial (150), pancreatic (151) and colorectal (152) cancers	Multiple tissues in mouse: lung adenocarcinoma, hepatocarcinoma, cholangiocarcinoma, granulose cell tumor, haemangiosarcoma, fibrosarcoma, thymic lymphoma (153)

Table I. Continued

Gene	Inherited human cancer association	Sporadic human cancer association	Haploinsufficient phenotype
Fen1	?	Rarely mutated in sporadic cancers analyzed to date (154,155)	Apc^{1638N}-induced murine adenoma and adenocarcinoma (156)
H2AX	?	Rarely mutated, but frequent LOH in B-cell lymphoma (157,158)	Murine thymic lymphoma, sarcoma and leukemia (159)
Lig4	Mutated in Lig4 syndrome (reviewed in ref. 93); Genetic variants associated with increased risk of breast cancer (160), multiple myeloma (161) and lung cancer (162)	Rarely mutated in sporadic cancers analyzed to date (163)	Murine soft-tissue sarcomas in $ink4a/arf^{-/-}$ mice (164)
Lkb1	Mutated in Peutz-Jeghers syndrome (165,166)	Mutated in sporadic lung, pancreatic and biliary cancers, but rarely mutated in other sporadic cancers associated with Peutz-Jeghers syndrome (167–171)	Murine gastric adenoma, intestinal adenoma (172)
*Mad2***	?	Frequently down-regulated in hepatocellular carcinoma (173), but rarely mutated in sporadic cancers analyzed to date (123,174,175)	Maintenance of genome stability in mouse embryonic fibroblasts (176)
*Mlhl***	Mutated in hereditary non-polyposis coli cancer (HNPCC) syndrome (177,178)	Mutated in sporadic microsatellite unstable colorectal cancer (179,180); Promoter hypermethylation in sporadic microsatellite unstable colorectal (181), gastric (182), and head and neck cancers (183) as well as melanoma (184) and retinoblastoma (185)	Mutation frequency in $Mgmt^{-/-}$ fibroblasts treated with alkylating agents (186)
Msh2	Mutated in hereditary non-polyposis coli cancer (HNPCC) syndrome (187)	Mutated in sporadic microsatellite unstable colorectal cancer (179,180,188)	Multiple tissues in mouse: lung adenoma, liver adenoma, mammary adenoma, uterine adenoma, hemangiosarcoma (189); Sister chromatid exchange in MNNG-treated MEFs (190) and oxidative damage in irradiated MEFs (191)
Mus81	?	Few published mutation screens to date	Murine thymic lymphoma, sarcoma, breast carcinoma, ovarian carcinoma (192)
Nf1	Mutated in Neurofibromatosis type 1 (193)	Mutated in sporadic colon adenocarcinoma (194), myelodisplastic syndrome (194), and astrocytoma (194,195), as well as glioblastoma, ependymoma and primitive neuroectodermal tumors (195)	Non-cell-autonomous action in mast cells surrounding murine neurofibroma (196)
Nkx3.1 (NKX3A)	?	Rarely mutated, but frequently down-regulated in sporadic testicular germ cell cancer and metastatic prostate cancer (197–199)	Murine prostatic intraepithelial neoplasia (29)
Plk4	?	Few published mutation screens to date	Murine hepatocellular carcinoma and lung adenocarcinoma (200)
Prkar1a	Mutated in Carney complex, a familial multiple neoplasia syndrome (201,202)	Frequent down-regulation and LOH in sporadic thyroid, adrenal, ovarian and colon cancers, but rarely mutated in sporadic cancers analyzed to date (reviewed in ref. 203)	Murine sarcomas and hepatocellular carcinomas (204); Human eyelid myxoma in Carney complex (205)
Ptch	Mutated in nevoid basal cell carcinoma syndrome (206,207)	Mutated in sporadic medulloblastoma (208–211)	Murine medulloblastoma (212)
Pten	Mutated in several rare autosomal dominant hamartomatous syndromes including Cowden syndrome (213)	Mutated in multiple sporadic cancers (reviewed in ref. 214)	Murine TRAMP-induced prostate adenocarcinoma (215) and murine prostatic intraepithelial neoplasia (216)
*Rb***	Mutated in familial retinoblastoma (4)	Mutated in multiple sporadic cancers including retinoblastoma (217), small cell lung (218,219), osteosarcoma (220) and ductal pancreas (221)	Marker maintenance in murine embryonic stem cells (222)
Ribosomal Protein Genes (e.g. *L35*, *L37a*, *RPS19* and *S8*)	*RPS19*, a human ribosomal protein gene, is mutated in familial Diamond-Blackfan anemia with predisposition to leukemia (evidence of haploinsufficiency in some families) (223,224)	Few published mutation screens to date	Multiple tissues in zebrafish: malignant peripheral nerve sheath tumor, lymphoma, gut adenocarcinoma, pancreatic ductal carcinoma (225)
Smad4/Dpc4	Mutated in familial juvenile polyposis (226)	Mutated in sporadic colon and pancreatic cancers (227–229)	Murine gastric adenoma (230)

Table I. Continued

Gene	Inherited human cancer association	Sporadic human cancer association	Haploinsufficient phenotype
Tsc2	Mutated in tuberous sclerosis complex (231,232)	Mutated in pulmonary lymphangioleiomyomatosis (233), but rarely mutated in other sporadic cancers analyzed to date (reviewed in ref. 234)	Murine Pten-initiated prostate adenocarcinoma (216)
Trp53	Mutated in Li-Fraumeni syndrome (reviewed in ref. 235)	Mutated in multiple sporadic cancers (reviewed in ref. 236)	Murine sarcoma, osteosarcoma and lymphoma (16); Murine urinary bladder carcinoma (237); Human Li-Fraumeni syndrome (238)
Tgfb	Mutated in Camurati-Engelmann disease, a rare bone disorder (239), (reviewed in ref. 240)	Rarely mutated in sporadic cancers analyzed to date, although many pathway components are mutated (reviewed in ref. 241)	Murine diethylnitrosamine (DEN)-induced hepatocellular adenoma, ethyl carbamate-induced lung adenoma (17)

**Haploinsufficiency for suppression of tumors *in vivo* (as determined by accelerated tumorigenesis in heterozygote organism with clear retention of a wild-type allele in the tumor) not clearly demonstrated.

were able to demonstrate heterozygous missense mutations of CBFA2 segregate with familial platelet disorder with predisposition to acute myelogenous leukemia in four different pedigrees. In the leukemic cells from these patients, no somatic mutations in the coding sequence of the wild-type allele were identified and no evidence for deletion of the wild-type allele was observed. Further, the CBFA2 protein was expressed in leukemic cells and 100% of metaphases from the leukemic bone marrow contained the karyotypic marker for the chromosome carrying CBFA2. Apart from such exceptions, evidence for the existence of haploinsufficent tumor suppressors in humans has also been suggested by cell culture experiments. In chromosome transfer studies with breast cancer cell lines it was determined that a locus on the short arm of chromosome 8, a site of frequent LOH in human breast cancers, also behaves consistently with a haploinsufficient mechanism of tumor suppression (31). The researchers transferred chromosome 8 into breast cancer cell lines in which only one allele was present for all 8p microsatellite markers analyzed. In all cases, the presence of two full copies of chromosome 8p was incompatible with cell growth and in at least one case the donor chromosome 8 was retained while the recipient chromosome 8 was lost, as opposed to the expectation for 'two-hit' tumor suppression. This is similar to a report by Islam *et al*. (32) in which introduction of a derivative chromosome 9 was accompanied by loss of the recipient chromosome 10 and hints at a potential method, albeit labor intensive, for testing the haploinsufficiency of human tumor suppressors in cases where appropriate data on inherited mutations is not available.

For some haploinsufficient genes, expression profiling has proved a useful tool for dissecting the biological pathways affected by these genes (33). Recently, an attempt to identify early molecular changes associated with dominantly inherited predisposition to renal tumors via expression profiling (34) found that heterozygosity for either the von Hippel-Lindau tumor suppressor or the tuberous sclerosis complex genes (including the haploinsufficient renal tumor suppressor gene *TSC2*) significantly altered the expression profile of phenotypically normal renal epithelial cells in a gene-specific manner. Examination of transcriptional profiles for cells bearing a single mutation in a tumor suppressor gene may yield insight into the haploinsufficient phenotype(s) of other tumor suppressor genes, provided we develop appropriate guidelines for distinguishing dominant negative mutations from haploinsufficiency.

In humans and mice, however, the known haploinsufficient tumor suppressors likely represent an underestimate of the true number due to the inherent difficulty in reliably demonstrating an absence of mutations in both alleles as well as a lack of published experiments thoroughly examining the phenotype of hemizygotes. In addition to haploinsufficiency, tumor suppressor gene mutations can also lead to dominant negative and gain of function effects as described next.

Recessive versus dominant alleles

Diploid organisms have two alleles for each autosomal gene. In simple Mendelian genetics, a dominant allele confers a phenotype in either the heterozygous or the homozygous state whereas a recessive allele confers a phenotype only in the homozygous state. Some of the earliest evidence for dominant and recessive alleles was observed in flowering pea plants. In Mendel's case, for example he found that round seed shape (R) was dominant and wrinkled seed shape (r) was recessive. The history of science is fraught with myth, however, and it is now thought that Mendel may have 'rounded the rough edges' of his data in order to simplify his conclusions. Thus, even from the beginning, the distinction between a dominant and a recessive allele was fuzzy. Words and concepts can help to clarify biological processes, but they can also limit our understanding of those same processes by imposing boundaries that do not exist in nature. The traditional view of genetics is that most recessive alleles are non-functional or have reduced functionality. However, the nature of dominant alleles is less obvious.

True nulls versus hypomorphic recessive alleles

Researchers can begin to characterize the function of a gene by the creation of an allelic series, that is a series of unique mutations within the gene of interest. In an allelic series, a true null allele has the strongest phenotype and is genetically indistinguishable from a deficiency of the gene (that is a complete absence of the chromosomal region encoding the gene). By contrast, a hypomorphic recessive allele has reduced, but not completely absent, functionality. Functionality for a

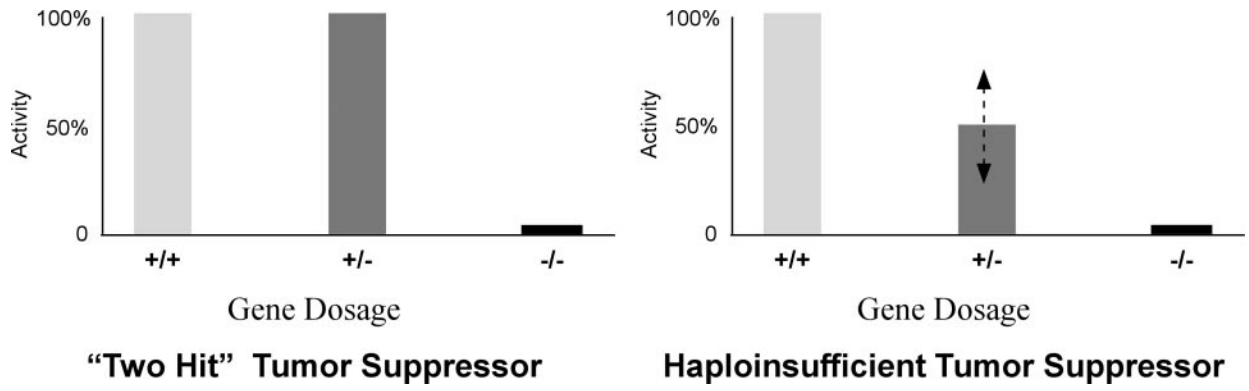

Fig. 2. Tumor suppressor activitiy versus gene dosage. Wild type (+/+) activity represents 100% of diploid gene function and true null (−/ −) represents complete loss of functionality. Haploinsufficient tumor suppressors exhibit a continuum of activity based on gene dosage with even 50% reduction sufficient for phenotypic manifestation, i.e. accelerated tumorigenesis. While some genes may be less dosage-sensitive than others (in which a true threshold of close to 0% of normal gene product is required in order to detect a phenotype), we predict that most genes will be sensitive to dosage with some threshold that varies on a continuum between 0 and 100%.

hypomorphic allele may be reduced, or in the case of genes that are responsible for multiple functions, one function may be affected while the others are preserved. Thus, even for tumor suppressors that appear to follow Knudsen's two-hit model, functionality at a given gene dosage will vary depending on whether the inactivating mutation is hypomorphic or a true null allele (Figure 2).

The distinction between a true null allele and a hypomorphic allele can sometimes be difficult to make. For example, the Apc^{min} mutation, which results in murine intestinal neoplasia via a classic two-hit mechanism, is often thought to be a loss of function allele. The Apc^{min} allele produces a truncated protein of roughly 850 amino acids. The Apc^{1638T} mutation, in which the targeted mutation results in a stable but prematurely truncated protein containing 788 amino acids more than the Apc^{min} truncated protein, is homozygously viable (unlike either Apc^{min} or Apc^{1638N}) and has no observable tumor phenotype. However, the germline Apc^{1638N}, mutation in which almost no Apc^{1638N} protein product is produced, results in reduced tumor multiplicity and increased tumor latency as compared to the Apc^{min} allele. Thus, Apc^{1638N}, a mutation in which almost no protein is produced has a less severe phenotype than Apc^{min}, in which a stable albeit truncated protein is produced. So, is the Apc^{min} allele a gain of function allele? If so, then why is the remaining wild-type allele still selectively lost in most tumors from $Apc^{min/+}$ mice? One possible explanation is that *Apc* is gene dosage-dependent. Thus impairment of *APC* function might provide gastrointestinal epithelial cells with a selective advantage, with loss of the remaining wild-type allele providing a further selective advantage. Regardless, it is clear that a more flexible paradigm involving both quantitative as well as qualitative changes in function is required to fully appreciate the spectrum of tumor suppressor gene action.

Dominant alleles of tumor suppressor genes

A haploinsufficient tumor suppressor mutation is, by nature, difficult to distinguish from a dominant negative mutation. In both cases, the wild-type allele is retained, although the reason for retention is drastically different in each case. In the case of a dominant negative mutation, the wild-type allele does not need to be inactivated because the dominant negative mutation serves that function, often by binding the wild-type protein in non-functional complexes. In the case of a haploinsufficient mutation, the wild-type allele is retained because half the normal complement of wild-type protein is insufficient for functionality. These two mutation types can often be distinguished by factoring in other known traits of the gene. For example, in the case of *p27*, it is known that mice hemizygous for *p27* show accelerated tumorigenesis without loss of the remaining wild-type allele indicating that one-half the normal complement of p27 is insufficient for tumor suppression (15) and that p27 is haploinsufficient, as opposed to inactivated by dominant negative mutation. In the case of the Wilms' tumor gene (*WT1*), however, it is known that the WT1 protein dimerizes through the N-terminal domain, indicating a potential mechanism for dominant negative action (35–38). Although milder cancer susceptibility syndromes associated with *WT1* mutation follow traditional two-hit kinetics, a severe syndrome of genitourinary malformations associated with susceptibility to Wilms' tumor and due to missense mutations in *WT1* nuclear localization signals, is thought to be caused by dominant negative mutations of *WT1* (39).

Pleiotropy

Many genes are pleiotropic, or in other words function in multiple cellular processes, and some of these functions may be more dosage-sensitive than others. For some phenotypes examined, deletion of a single allele may have little effect, while for other phenotypes deletion of a single allele of the same gene may be tantamount to a null phenotype. For example, p53 is a highly regulated gene with multiple inputs and outputs and p53 may be haploinsufficient for some functions, like inducing apoptosis (27) or maintaining genome integrity (26), but recessive for other functions like transcriptional regulation of certain genes. Quantitative phenotypic analyses of hemizygotes will improve understanding of the complex consequences conveyed by deletion of a single allele of a gene. RNAi can be useful in creating an allelic series of gene dosage as was demonstrated recently by Hemann *et al.* (40) for *Trp53* and may be useful in further differentiating between minor changes in gene dosage. The spectrum of

phenotypes between full complement of a functional tumor suppressor protein and complete deficiency for that tumor suppressor gene can be broad, especially for genes that are responsible for multiple functions within the cell. If one accepts that the definition of a gene as 'recessive' or 'haploinsufficient' is dependent on the cellular context of that mutation, then one can easily reconcile the perceived conflict between haploinsufficient tumor suppressor genes and the 'two-hit' model of tumor suppressor gene inactivation. The next sections discuss how the phenotypic penetrance of a mutation can be context-dependent.

History of modifier genes

One of the first Drosophila mutants identified by Morgan was a fly with truncated wings. The inheritance of 'truncate' was difficult to reconcile with Mendelian genetics due to the inconstant penetrance of the truncated wing phenotype. As early as 1919, Muller and Altenburg recognized that the truncated wing phenotype in Drosophila could be phenotypically modified by extragenic loci (41).

In subsequent years, genetic screens for both enhancers and suppressors of a mutant phenotype have been used to identify additional members of a signaling network. It is well known that these modifier screens function best for identifying components of pathways sensitive to gene dosage. This strategy has been put to exquisite use in Drosophila. For example, a hypomorphic allele of the receptor tyrosine kinase controlling cell-fate choice in the Drosophila eye, sevenless (*sev*), provides just enough activity for most of the photoreceptor cells to form. Further reduction of signaling, however, leads to conversion of most of the photoreceptor cells to cone cells. This sensitized background was used to identify mutations in components of the receptor tyrosine kinase signaling pathway as dominant enhancers of the cell-fate phenotype in the eye (reviewed in ref. 42).

Modifiers of tumor suppressor gene mutations

Numerous enhancers of tumor suppressor gene mutations have been identified. One of the first was the *Mom1* or 'Modifier of Min' locus on chromosome 4 in mice (43). Apc^{min} mice in a *Mom1* wild-type background, such as the AKR strain, are resistant to intestinal polyposis whereas Apc^{min} mice in a *Mom1* mutant background, such as C57Bl6/J, develop hundreds of intestinal polyps throughout the gastrointestinal tract. Mutation in the *Pla2g2a* locus, which encodes a secretory phospholipase, was eventually shown to be responsible for 50% of the enhanced intestinal polyposis of Apc^{min} mice in the C57Bl6/J background (44). Among enhancers identified by a candidate gene approach, deficiency in *Mlh1* was shown to accelerate the development of intestinal adenomas in Apc^{min} mice (45). In addition to its intrinsic tumor suppressor activity, p27 is one of the most promiscuous of these tumor suppressor enhancers. p27 functions as an enhancer of mutations in multiple genes important for human cancer, indicating that p27 may be a nodal component of diverse pathways for tumor suppression (46).

Suppressors of tumor suppressor gene mutations are known as well. *Dnmt1* is the most prominent of the three known mammalian DNA methyl transferases. Intestinal tumor multiplicity in Apc^{min} mice heterozygous for a null allele of *Dnmt1* ($Dnmt1^{S/+}$) was reduced by 60% compared to Apc^{min} mice alone (47). Using a hypomorphic allele ($Dnmt1^{N/+}$), Cormier *et al*. (48) demonstrated that the modifying effect of *Dnmt1* on Apc^{min} is independent of both *Trp53* and *Mom1* status. When two distinct hypomorphic alleles of *Dnmt1* were combined in an Apc^{min} background, intestinal polyposis was completely suppressed indicating that Dnmt1 is a very specific genetic suppressor of tumorigenesis in Apc^{min} mice (49). Similarly, deficiency for the human multidrug resistance gene, *Mdr1*, results in significantly fewer intestinal polyps in Apc^{min} mice (50). The existence of modifier loci have provided further evidence that the phenotypic expression of a primary mutation is highly dependent on multiple factors, including extragenic loci, tissue type and environmental stress.

Mechanism of genetic modifier action

Genetic modifiers serve to enhance or suppress the original mutation of interest via a number of different mechanisms. A genetic modifier can affect a parallel or redundant pathway or it can affect the localization of the mutant gene product of interest, thereby either enhancing or suppressing its activity. A genetic modifier sometimes takes the form of a stability factor affecting the gene product of interest. Finally, a genetic modifier can function as a downstream effector in a signaling cascade that amplifies the activity of the mutant gene product, as may be the case for p27.

Tissue-specific modifiers of tumor suppressor gene mutations

Gene dosage-dependence of a tumor suppressor can vary not only with the genetic background of the organism, but also between tissues. In both DMBA-TPA-induced skin carcinogenesis (51) and *Apc* mutant mice (22), *p27* tumor suppression shows a clear dosage-dependence, with *p27* hemizygotes being intermediate to $p27^{+/+}$ and $p27^{-/-}$ mice with respect to tumor-free survival. In other tissues, however, this is not the case. In the mammary gland, for example, $p27^{-/-}$ mice had longer tumor latency in *MMTV-neu* transgenics as compared to $p27^{+/+}$ mice (52). $p27^{+/-}$ mice, however, showed reduced mammary tumor latency as compared to $p27^{+/+}$ mice, as might be expected based on the established tumor suppressor function of p27. Counter intuitively, mammary tumors from $p27^{-/-}$ mice had a reduced mitotic index as compared to the mammary tumors in $p27^{+/-}$ mice. Similar results were seen in the prostate gland. In *Nkx3.1*-deficient, $Pten^{+/-}$ mice that were also hemizygous for p27, prostate tumor progression was enhanced whereas in *Nkx3.1*-deficient, $Pten^{+/-}$ mice that were $p27^{-/-}$, prostate tumor progression was inhibited (53). This result was specific to prostate epithelium as other tumor types, especially lymphoma, were enhanced in *Nkx3.1*-deficient, $Pten^{+/-}$, $p27^{-/-}$ mice. Thus, p27 haploinsufficiency is tissue-specific. Whether the unexpected effects of p27-deficiency seen in the mammary and prostate are due to extragenic tissue-specific modifiers or tissue-specific environmental exposures remains to be seen.

Modifiers of human tumor suppressor genes

Evidence of tumor suppressor modifier loci has been less forthcoming in humans than in mouse models, likely owing to the controlled genetic background available in mouse experiments (reviewed in ref. 54). Nonetheless, there is strong

evidence that such modifier loci exist in humans as well. Some of the strongest evidence comes from studying the variation in disease incidence and severity in familial adenomatous polyposis (FAP) families segregating a mutation in *APC* (55,56). Variations in the penetrance, number of polyps and the existence of extracolonic manifestations have been observed even within a given a family segregating a single mutation in *APC* (reviewed in ref. 57). The identification of *Mom1* as a modifier of the Apc^{min} phenotype in mice precipitated efforts to determine whether the human homologue of *Mom1* modifies the disease phenotype in human FAP patients. Early evidence indicated a weak linkage between the *MOM1* locus at chromosome 1p35–p36 and modification of FAP phenotype (58), however, subsequent investigation failed to support the involvement of *MOM1* (59,60). Whether *MOM1* is capable of modifying *APC* in a restricted subset of families or is merely a mouse-specific modifier of *Apc* has yet to be determined. Modelling of segregation of disease severity in FAP families has indicated that a mixed model in which a single major modifier locus acts in concert with multiple minor loci is most likely to explain the intrafamilial variation in disease phenotype (55), although these modifier loci remain to be mapped. More recently, evidence from studies of human FAP patients has indicated the possible involvement of *N*-acetyl transferase polymorphisms in modifying disease phenotype in *APC* carriers (56).

Putative modifiers of the familial breast and ovarian cancer tumor suppressors *BRCA1* and *BRCA2* have also been identified. The earliest of these was the *HRAS1* variable number of tandem repeats (VNTR) locus located 1 kb downstream of *HRAS1* (61). Rare alleles of the *HRAS1* VNTR increase the risk of ovarian cancer penetrance in *BRCA1* mutation carriers by 2.11 times. The simplest explanation of this observation is that these rare VNTR alleles somehow affect the expression of *HRAS1*, a known proto-oncogene. The occurrence of the VNTR in a non-coding region of the *HRAS1* locus does not allow a direct demonstration that *HRAS1* is the tumor suppressor modifier in question, however, and the true identity of the *BRCA1* modifier gene at this locus has not yet been demonstrated. More recently, polymorphisms of *RAD51* (62–64), the androgen receptor (reviewed in ref. 65) and *TP53* (66) have been implicated as modifiers of mutations in *BRCA1* and/or *BRCA2* and a putative *BRCA1* modifier locus has been mapped to chromosome 5q (67). In addition to *APC*, *BRCA1* and *BRCA2*, putative modifiers of tumor suppression by *CDKN2A* (68), *VHL* (69) and *NF1* (70) have been identified.

Summary

For the past 30 years, Knudsen's 'two-hit' model has provided a useful framework for interpreting the kinetics of tumor suppressor gene inactivation. However the growing list of tumor suppressor genes that exibit haploinsufficiency as well as dominant negative mutations indicates a more complex view. The Platonic view that genes function in a completely dominant or a recessive fashion is oversimplified. Past models of simple on/off switches for gene function do not account for the pleiotropy of phenotypes observed for a given gene. Much as rheostats have gradually replaced the 'toggle' light switches of old, models of tumor suppressor gene function must now encompass mutations that modulate activity without abolishing it entirely. In addition, the phenotypic expression of a primary mutation is highly dependent on multiple factors, including genetic background, tissue type, and environmental stress. Recessivity or haploinsufficiency are not absolutes but are context-dependent. Future research will need to focus more carefully on the phenotype of tumor suppressor gene mutation heterozygotes and their pleiotropic effects. A more complete understanding of tumorigenesis will only be achieved by a detailed analysis of tumor suppressor gene dosage effects on cellular phenotypes embedded within the complexity of the organism.

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