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A STRATEGY TO IDENTIFY DOMINANT POINT MUTANT MODIFIERS OF A QUANTITATIVE TRAIT

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ABSTRACT

A central goal in the analysis of complex traits is to identify genes that modify a phenotype. Modifiers of a cancer phenotype may act either intrinsically or extrinsically on the salient cell lineage. Germline point mutagenesis by ethylnitrosourea can provide alleles for a gene of interest that include loss-, gain-, or alteration-of-function. Unlike strain polymorphisms, point mutations with heterozygous quantitative phenotypes are detectable in both essential and non-essential genes, and are unlinked from other variants that might confound their identification and analysis. This report analyzes strategies seeking quantitative mutational modifiers of *Apc*^{Min} in the mouse. To identify a quantitative modifier of a phenotype of interest, a cluster of test progeny is needed. The cluster size can be increased as necessary for statistical significance if the founder is a male whose sperm is cryopreserved. A second critical element in this identification is a mapping panel free of polymorphic modifiers of the phenotype, to enable low-resolution mapping followed by targeted resequencing to identify the causative mutation. Here, we describe the development of a panel of six “isogenic mapping partner lines” for C57BL/6J, carrying single-nucleotide markers introduced by mutagenesis. One such derivative, B6.SNVg, shown to be phenotypically neutral in combination with *Apc*^{Min}, is an appropriate mapping partner to locate induced mutant modifiers of the *Apc*^{Min} phenotype. The evolved strategy can complement four current major initiatives in the genetic analysis of complex systems: the Genome-wide Association Study; the Collaborative Cross; the Knockout Mouse Project; and The Cancer Genome Atlas.

INTRODUCTION

Genetic analysis of a phenotype in a multicellular organism starts with the discovery of those genes that influence the process in the whole organism. Loss-of-function alleles provide the least ambiguous route to understand the role, direct or indirect, of the functional wildtype allele in the process of interest. Gain-of-function and neomorphic alleles can provide further functional information. The action of the gene in the whole animal is ascertained by studying animals that are mosaic or chimeric for mutant and wildtype tissue (Hotta & Benzer 1972). When a gene of interest has been identified at the molecular level, longitudinal cellular studies provide complementary information on the spatial and temporal patterns of that gene's actions.

Cancer in a metazoan is itself a multicellular process. Dramatic progress is emerging from The Cancer Genome Atlas (Vogelstein *et al.* 2013) and the Cancer Epigenome Project (Beck *et al.* 2012) to document mutations and epigenetic changes common to the neoplastic lineages of each cancer histotype. However, biologists recognize that a cancer is more than its genome. Beyond the epithelial lineage of the carcinoma, stromal elements can extrinsically influence the biology of the tumor. Such elements include leukocytes (Nosho *et al.* 2011); mast cells (Khan *et al.* 2013); neutrophils (Sinnamon *et al.* 2008); macrophages and cancer-associated fibroblasts (Edin *et al.* 2012; Herrera *et al.* 2013); and endothelial cells and the microvasculature of the tumor (Yekkala & Baudino 2007; Pitroda *et al.* 2012). Genes acting on the neoplastic lineage, intrinsically or extrinsically, to affect the cancer phenotype can be discovered using modifier genetics (Dietrich *et al.* 1993; Gould & Dove 1996; Cormier *et al.* 1997). Genome-wide discovery programs for polymorphic modifiers of cancer risk are being carried out both in human populations (Gabriel *et al.* 2002; Carvajal-Carmona *et al.* 2011; Peters *et al.* 2012; Cai *et al.* 2013) and in rodent models (Ewart-Toland *et al.* 2003; Elahi *et al.* 2009; Kwong & Dove 2009; Quan *et al.* 2011; Crist *et al.* 2011; Smits *et al.* 2011; Liu *et al.* 2011; Eversley *et al.* 2012; Nnadi *et al.* 2012). However, identifying the causative elements underlying a polymorphic quantitative risk modifier is a Herculean task (Drinkwater & Gould 2012; e.g. see Lewis & Tomlinson 2012).

An important consideration is that the inbred strains of mice or rats that provide the starting material for modifier screens must carry functional alleles in all vital genes. Thus, the informative loss-of function alleles of vital genes are absent from a modifier screen that emerges from an inbred line.

These considerations have driven us to design a strategy for the genome-wide discovery of mutagen-induced alleles that quantitatively modify the tumor-inducing phenotype of the dominant *Multiple intestinal neoplasia* (*Min*) allele of the *Adenomatous polyposis coli* (*Apc*) gene of the mouse (Moser *et al.* 1990). The mutagen N-ethyl-N-nitrosourea (ENU) predominantly causes single point mutations in the rodent germline. This simplicity has enabled facile identification of a growing set of genes, identified as ENU-induced mutant alleles (Su *et al.* 1992; King *et al.* 1997; Garcia-Garcia *et al.* 2005; van Boxtel *et al.* 2010). ENU mutagenesis produces a range of point mutations (Takahasi *et al.* 2007). The frequency of such mutations in the sperm of an efficiently mutagenized mouse or rat is in the order of 10^{-3} per locus. A "one-hit library" has a size expected to contain, on average, one mutant for each locus. Thus, a one-hit mutational library of first-generation offspring of a mutagenized male has a size in the order of 1000 founders. By contrast, transposon-mediated mutagenesis of the mouse germline is significantly less efficient and does not yield the range of alleles produced by ENU.

Mutations causing a qualitative phenotype in monogenic traits can be identified without first being mapped, using identity-by-descent in genome-wide sequencing data (Arnold *et al.* 2011; Hilton *et al.* 2011). For example, two-generation screens can be employed to identify new alleles of known genes (Shedlovsky *et al.* 1993) or new loci (Kumar *et al.* 2011). By contrast,

individual components of a highly complex trait do not show qualitative patterns of inheritance. To detect a mutant allele that quantitatively modifies a phenotype of interest, a cluster of progeny must be phenotyped. Extensive progeny testing becomes feasible if the kindred's founder is a male whose germline is cryopreserved to permit extensive retesting. Thus quantitative modifying alleles for these traits are identified by low-resolution mapping followed by targeted sequence analysis to identify the causative mutation. Such mapping requires crossing the candidate founder to a partner carrying distinct marker alleles distributed over the genome. However, outcrossing to another inbred strain may introduce polymorphic modifiers from the donor mapping strain that can obfuscate the quantitative phenotype of interest (Elahi *et al.* 2009; Crist *et al.* 2011; Liu *et al.* 2011; Eversley *et al.* 2012; Nnadi *et al.* 2012). Therefore, we have employed germline mutagenesis, followed by inbreeding, to create a set of "isogenic mapping partners" for the canonical C57BL/6J (B6) mouse strain. To begin, one of these lines, B6.SNVg, has been shown by progeny testing to be phenotypically neutral in respect to the *Apc*^{Min} phenotype. Resequencing of this line has established a set of induced SNV markers covering the genome at sufficient density to make B6.SNVg a suitable partner for mapping a new ENU-induced dominant modifier of the *Apc*^{Min} phenotype on the B6 background.

MATERIALS AND METHODS

Mice

Mice were maintained under a protocol approved by the Animal Care and Use Committee of the University of Wisconsin School of Medicine and Public Health and in a facility in the McArdle Laboratory approved by the American Association of Laboratory Animal Care. Animals were housed in standard caging with free access to mouse chow and acidified water.

BTBR/Pas (BTBR) mice, initially obtained from J-L. Guénet at the Institut Pasteur in Paris, France, were inbred in our laboratory.

C57BL/6J.*Apc*^{Min} (B6. *Apc*^{Min}) mice were obtained from the McArdle colony. The strain was maintained by backcrossing every fifth generation to C57BL6/J (B6) mice from The Jackson Laboratory (Bar Harbor, Maine). Progeny from B6.*Apc*$^{Min/+}$ mothers were fostered to ICR foster mothers to enhance their probability of survival.

Along with others, we have observed that dominant modifiers of the *Apc*^{Min} phenotype arise spontaneously in the B6 stock (Baran *et al.* 2007). Therefore we now maintain a closed colony, C57BL/6JD.*Apc*^{Min} (D for Dove) and monitor it regularly for tumor multiplicity to prevent introgression of modifying alleles that can arise through genetic drift in the B6 stock. Through monitoring, the average tumor multiplicity in the closed colony is kept at approximately 100 ± 30 for mice at 90-120 days of age..

Lines B6.SNVb, B6.SNVc, B6.SNVe, B6.SNVf, B6.SNVg and B6.SNVh were developed using ENU mutagenesis of B6 male founders, followed by at least 10 generations of inbreeding. They carry single nucleotide variant sets b, c, e, f, g and h, respectively.

ENU mutagenesis

BTBR males were mutagenized with 250 mg of ENU per kg body weight using the protocol previously described (Shedlovsky *et al.* 1986). Since B6 males are permanently sterilized at 250 mg of ENU per kg body, we mutagenized them with three weekly injections of 100 mg per kg body weight.

Tumor scoring
Each intestinal tract was removed, opened longitudinally and laid out on bibulous paper. The samples were fixed in 10% formalin overnight, then transferred to 70% ethanol for long-term storage. Tumor counts were obtained for the entire small intestine and colon using a Nikon SMZ-U dissecting microscope.

Sequencing
Paired-end 75bp reads were generated by end-sequencing DNA sheared to an average of 350bp. Sequencing was performed on the Illumina GAIIx platform to an average depth of 5.7 times genome coverage (Supporting Table S1). These data were mapped to the mm9/NCBIm37 assembly using BWA v0.5.9 (Li & Durbin 2009). Variants were called using Samtools (v0.1.17) mpileup/bcftools and then filtered selecting those variants for which the read depth was 3 or greater. Major changes in copy number or sequencing efficiency were culled by filtering out signals stronger than 2.5 times the sequencing coverage of the sample. Data are shown in Supporting File S1 .

Sequenom validation
In selecting candidates from line B6.SNVg for Sequenom validation, the goal was to find three candidates on each of the autosomes – near the centromere, middle, and telomere – fitting the following criteria:

- Phred scale genotyping quality score of 99
- No significant repeats in flanking sequence
- Appearing in both B6-SNVg and B6.SNVh (known to be related), but in no other line.
- A>T or T>A transversions were preferred, followed by A>G or T>C and G>A or C>T transitions. ENU mutagenesis enhances the proportion of AT to TA mutations, comprising 18.7% of the unique mutations in this study, in contrast to 9% of spontaneous mutations (14849300 of 164137670 B6.SNVs across 18 strains) (Keane *et al.* 2011).

Beyond the Phred scale quality rating of 99, the criteria listed above had to be relaxed in regions where mutations were sparse. Sequenom genotyping was performed as previously described (Gabriel *et al.* 2009). Chosen candidate variants in line B6.SNVg are validated in this communication; candidates in the other lines remain to be validated.

Sequencing
Paired-end 75bp reads were generated by end-sequencing DNA sheared to an average of 350bp. Sequencing was performed on the Illumina GAIIx platform to an average depth of 5.7 times genome coverage (Supporting Table S1). These data were mapped to the mm9/NCBIm37 assembly using BWA v0.5.9 (Li & Durbin 2009). Variants were called using Samtools (v0.1.17) mpileup/bcftools and then filtered selecting those variants for which the read depth was 3 or greater. Major changes in copy number or sequencing efficiency were culled by filtering out signals stronger than 2.5 times the sequencing coverage of the sample.

RESULTS

Outcross design with clustered progeny testing

Our initial strategy to discover ENU-induced alleles that quantitatively modify the *Apc*^{Min} phenotype involved outcrossing a first-generation carrier of a mutagenized BTBR paternal genome to a B6-*Apc*$^{Min/+}$ tester. BTBR carries the same sensitive allele as B6 at the known modifier *Mom1* locus (Gould *et al.* 1996), leading us to choose this partner to B6-*Apc*^{Min} in the outcross design. BTBR males, mutagenized as described in Methods, were bred to normal BTBR females to produce first generation (G1) males that were potential carriers of *Apc*^{Min} modifiers. These were first screened for such modifiers, based upon the life span of their F1(B6-*Apc*$^{Min/+}$ X G1) offspring. Figure 1A shows the breeding designs employed.

The presenting phenotype in this initial foray was that of survival. Of 172 kindreds sired by G1 males, our attention was drawn to four kindreds carrying modifiers of the *Apc*^{Min} survival phenotype: two putative dominant suppressors (258 and 201) and two putative dominant enhancers (333 and 415). Each of these kindreds was identified for further study by observing more than one animal with an extreme lifespan – long for the putative dominant suppressors and short for the putative dominant enhancers. Figure 2 displays the survival of control F1 (B6-*Apc*$^{Min/+}$ X BTBR) animals compared to F1s from all mutagenized males, as well as the survival of progeny within the above four kindreds.

Differences in the lifespan distribution between unaffected F1 animals and potentially affected G1F1 animals reflect both the rate and quantitative effect of ENU-induced modifiers. A statistical analysis of these differences considers the distribution of mutagenized G1F1 Min animals as a mixture of three components (unaffected, enhanced, suppressed), and utilizes the maximum likelihood method to estimate both the rate at which ENU induces a phenotypic modifier and the distribution of multiplicative modifier effects (Figure 3, Supporting File S2). From this calculation, we estimate that 39% of the mutagenized kindreds are free of dominant modifier alleles, 41% carry dominant suppressor alleles that lead to reduced tumor multiplicity and longer lifespan, and 20% carry dominant enhancer alleles that have the opposite phenotype. Importantly, though a high proportion (61%) of mutagenized kindreds appear to carry modifiers of *Apc*^{Min}, it is evident that most are of small effect[1] (Kwong & Dove 2009). Equation (2) in Supporing File S2 gives a formula for the enrichment in large modifier effects that can be achieved by selecting kindreds having multiple animals with extreme phenotype, as was done with kindreds 201, 258, 333, and 415 (Figures S3 and S4).

In practice, the quantitative character of this screen for dominant modifiers of the *Apc*^{Min} phenotype was first analyzed on the basis of a simplifying assumption: that the time of survival of a *Apc*$^{Min/+}$ animal was correlated with its tumor multiplicity. The four candidate kindreds, first identified by survival, were further analyzed by tumor multiplicity of test progeny. The requirement for extensive progeny testing on the basis of both survival and tumor multiplicity led to an elapsed decision time that exceeded the lifespan of the founding first-generation mice. To implement this strategy, the germlines of these founders need to be maintained through sperm cryopreservation. However, recovery of BTBR/Pas from cryopreserved sperm remains elusive, while success with B6 sperm has only recently been significantly improved (Takeo & Nakagata 2011) and confirmed in our group by KJK.

Isogenic design

Our attempts to map the presumptive dominant enhancer in kindred 333 and suppressor in kindred 258 were unsuccessful owing to modifying alleles polymorphic between BTBR/Pas and B6, such as *Mom7* (Kwong *et al.* 2007). Indeed, 18 such polymorphic modifier loci of the *Apc*^{Min} tumor multiplicity phenotype have been reported to date (Nnadi *et al.* 2012; Eversley *et al.*

2012). The requirement for quantitative precision in this genetic strategy led us to move beyond the initial, informative outcross design and to develop an "isogenic design" (Figure 1B). This method requires a set of "isogenic mapping partners" for B6 that carry markers throughout the genome. To develop these partners we mutagenized several B6 males as described in Methods and bred each to normal B6 females (Figure 1C). Progeny of these crosses were randomly selected to give seven brother by sister pairs to intercross for at least 10 generations. One line (B6.SNVd) failed to achieve 10 generations of inbreeding, presumably owing to homozygosity for a recessive lethal mutation. The other six lines achieved 10 or more generations of inbreeding without evident homozygosity for a recessive detrimental or lethal mutation. These B6.SNV lines, each with a set of single nucleotide variants, came from separate lineages with the exception of two lines (B6.SNVg and h) that had a common first-generation parent.

Progeny test of the B6.SNVg line for dominant modification of the *Apc^{Min}* phenotype

The B6.SNVg line was tested for dominant modifiers of the *Apc^{Min}* phenotype in crosses to B6-*Apc^{Min}*. Females of the B6.SNVg line were bred to B6-*Apc^{Min}* animals from the McArdle Laboratory stock. (B6.SNVg X B6-*Apc^{Min}*) and *Apc$^{Min/+}$* progeny were sacrificed at 90 to 100 days of age for tumor scoring. In parallel, tumor counts were established from (B6 X B6. *Apc$^{Min/+}$*)F1 *Apc$^{Min/+}$* control progeny, sired by the same males (Figure 4). No significant difference was observed between the two groups ($p = 0.89$ by two-sided Wilcoxon Rank Sum test).

Estimation of the size of the progeny test needed to detect a carrier of a mutant modifier of Min

The number of progeny needed to detect a Generation 1 carrier of an ENU-induced modifier at power 0.9 was estimated from the standard, overdistributed tumor multiplicity phenotype of B6.*Apc*$^{Min/+}$ (Supporting File S2). In the first iteration of this assessment, we have used tumor multiplicity alone to score the phenotype. In a second iteration, we have first pre-selected kindreds that are more likely to contain modifiers on the basis of shortened or lengthened survival, as summarized above. In each case, we have used a Negative Binomial model similar to that described more fully in the legend to Figure 5. We ask that a reported list of kindreds, controlled at 5% False Discovery Rate, be non-empty with at least 95% probability. The dimensions of this progeny test at power 0.9 for specific magnitudes of dominant effect of an ENU-induced modifier are shown in Figure S1.

In the absence of pre-selection, for example on the basis of survival times, a prohibitive number of kindreds must be screened to the following depths. As shown in Supporting Figure S1A, to be 95% confident of discovering at least one allele with a twofold enhancing effect, 350 kindreds must be screened. For such a dominant suppressor allele, 500 kindreds would be needed.

These estimates indicate that a realistic screen for dominant enhancer and suppressor alleles of the overdistributed Min phenotype must involve either pre-selection of kindreds of interest or sharpening the *Apc^{Min}* phenotype (Newton & Hastie 2006). The estimated distribution of the frequencies and fold effect of dominant enhancer and suppressor alleles in the population of Generation 1 founders (Figure 3) and the necessary sizes of test progeny clusters (Figure 5) lead in this case to the estimates summarized in Supporting Figure S1B. For example to detect at least one dominant enhancer allele with a twofold effect, approximately 13 short-lived kindreds must be tested, each with 20 *Apc$^{Min/+}$* progeny. The corresponding estimate to detect at least one dominant suppressor allele is 14 long-lived kindreds, each tested with 30 *Apc$^{Min/+}$* progeny.

Sequencing of the B6.SNV lines
Genomes of six of the B6.SNV lines were sequenced as described in Methods. We identified across all 6 B6.SNV lines a total of 22,911 high confidence candidate SNV positions from the sequencing data by comparison to the B6 (mm9/NCBIm37) reference genome (Supporting Table S1).

A B6.SNVg mapping panel of confirmed SNV sites
Because progeny tests indicated that B6.SNVg does not dominantly affect the tumor multiplicity phenotype of B6-*Apc*^{Min} (Figure 4), we have developed a mapping panel of SNV sites in this line. Thus it can serve as the isogenic mapping partner to locate new ENU-induced dominant modifiers of the *Apc*^{Min} phenotype.

A total of 180 SNV candidate marker sites were chosen for confirmation by Sequenom genotyping as described in Methods with 148 entering the final assay. Validation was carried out on DNA samples from 31 C57BL/6JD, six B6.SNVg, one B6.SNVh, and 41 (C57BL/6JD x B6.SNVg)F1 mice. The results are displayed in Supporting Data File S2. Of the 148 candidate sites tested, 8 failed to give Sequenom results, so data are shown for 140 candidates. For 5 of these 140, the results may indicate either divergence between our C57BL/6JD and the canonical B6 used in sequencing NCBIm37, or else errors in that sequence. One candidate does not vary between B6.SNVg and C57BL/6JD, probably reflecting an error in the B6.SNVg sequence. Eleven of the candidates show evidence for residual heterozygosity in the B6.SNVg line, which is consistent with the expectation of 11% for 12 generations of inbreeding (Green 1981). This heterozygosity is not obligatory; the set of progeny includes B6.SNV/B6.SNV homozygotes at each of these eleven sites. Thus, 123 sites were validated as being ENU-derived and homozygous in B6.SNVg.

The use of B6.SNVg as a mapping partner
Figure 6 presents the set of B6.SNVg sites that can serve the purpose of mapping a newly induced dominant modifier allele on B6. The meiotic maps of mammalian genomes show strong positive crossover interference (Broman *et al.* 2002). Thus, one expects that this mapping panel will permit a new mutation to be mapped to a defined region of the B6 genome. Targeted resequencing of this region in DNA from carriers of the newly induced modifier is then expected to yield one, or at most a small number, of candidate single basepair mutations; these can be confirmed by allelic substitution by the CRISP/Cas9 process (Wang *et al.* 2013).

The frequency and pattern of ENU-induced mutations
Supporting Table S1 summarizes the resequencing analysis of the six B6.SNV lines. The frequency of presumptive mutations per basepair reported from the canonical sequence of B6 (NCBIm37/mm9) was 1.3×10^4 in $6 \times 2 \times 10^9$ basepairs covered = 1.1×10^{-6} per basepair. This frequency is consistent with those previously reported (Michaud *et al.* 2005; Takahasi *et al.* 2007). Table 1 reports the numbers of line-specific mutations on each chromosome; Table 2 summarizes the spectrum of the unique mutations. Transitions from AT to GC and GC to AT ranked 1st and 2nd, while transversions between AT and TA ranked a close 3rd. This spectrum is similar to those reported in the study of Takahasi and colleagues (Takahasi *et al.* 2007) and in the review by Barbaric and colleagues (Barbaric *et al.* 2007).

The distribution across the genome of the ENU-induced alleles in the SNV lines
Are regions of the genome of a SNV line devoid of markers, owing to homozygous lethality of mutations? The complete set of line-specific SNVs for the full set of B6.SNV lines is displayed in Supporting Figure S2. Full coverage of the genome, with some heterozygosity, is observed in three of the six lines – B6.SNVb, B6.SNVe, and B6.SNVg. At least one gap of over 30Mpb is observed in each of the other lines. B6.SNVc is devoid of markers in two regions: Chr 7 at 100-

144 Mbp; and Chr 11: 28-58 Mbp. B6.SNVf (whose resequencing coverage was only 1.7-fold) is devoid of markers in seven regions: Chr 5 at 43-77 Mbp; Chr 6 at 68-99 Mbp, Chr 11 at 89-121Mbp, Chr. 12 at 26-67 Mbp; Chr 14 at 60-89 Mbp, Chr 16 at 43-77 Mbp; and Chr 1 at: 22-54 Mbp. B6.SNVh is devoid of markers on Chr 15 at 22-52 Mbp. Thus, in general the densely mutagenized genomes of the six B6.SNV lines support homozygosity, without loss of markers from particular regions.

DISCUSSION

We have developed a general genome-wide strategy to identify ENU-induced mutations that dominantly modify the *Apc*^{Min} phenotype. In important ways this strategy complements others that are being actively pursued for the analysis of complex traits in mammalian biology such as cancer. The Genome-Wide Association Studies (GWAS, http://gwas.nih.gov/) and the Collaborative Cross (CC, http://csbio.unc.edu/CCstatus/index.py) reveal germline haplotypes that contribute to disease risk in the human and mouse population, respectively. By contrast, an allelic series of ENU-induced point mutations (see Table 2 in Takahasi *et al.* 2007) enables functional analysis of each gene affecting the trait of interest. Although loss-of-function alleles are particularly informative for cause-effect analysis, these alleles cannot be present among vital genes in the inbred mouse strains that generate the CC. The cancer genome and epigenome are addressed by The Cancer Genome Atlas (TCGA, http://cancergenome.nih.gov/) and the Cancer Epigenome Project (Beck *et al.* 2012). These projects identify mutations and other somatically heritable changes that act directly within the cancer lineage but not in the stroma. By contrast, modifying alleles can act in the tumor lineage, the stroma, or the host at large.

Detection of a subtle phenotype caused by a mutation in a single gene of a complex system is enhanced by amplifying the number of progeny displaying the mutant phenotype. Here, we describe a statistical approach to detect kindreds carrying dominant alleles that modify the tumor multiplicity phenotype. Briefly, we have modeled the null and modified distribution of the average tumor count within each kindred, and calibrated this statistic, accounting for background phenotypic variation and modifier segregation. The power to detect a mutant signal is a function of the number of test progeny. Thus, the ability to recover a B6 line from cryopreserved sperm, in order to amplify a kindred under isogenic conditions, is a crucial element in implementing this quantitative modifier screen.

The advent of cost-effective sequencing of mammalian genomes led to the association (by identity-by-descent) of mutant phenotypes with particular segregating ENU-induced mutations. However, for genetically highly complex traits, it is likely to be necessary to delimit the genomic region carrying the new mutation of interest for targeted resequencing. The known point mutations preferentially caused by ENU simplify both the molecular identification of the gene of interest and the analysis of its mechanism of action. However, the distribution of distances found between adjacent candidate SNV mutations provides a measure of caution against the assumption that a single mutation is responsible for the mutant phenotype. Indeed, SNP sites in the human genome show evidence for non-random clustering (Amos 2010). We observe that the median distance (50th percentile) between in the SNV sites is 377,740 bp and the interquartile range is 48,852 – 1,115,595 (25th to 75th percentile) (see Supporting Table S2). Thus, it is likely that a new modifier allele can be traced to a single mutation, but it will ultimately be necessary to validate the candidate by methods such as genome editing (Wang *et al.* 2013).

The evolved strategy discovers modifying genes on the basis of the heterozygous phenotype of an ENU-induced mutation. In what ways does this restriction limit the genome-wide coverage to discover the full set of genes that play a causative role in the biological process of interest? Application of mutagen-induced dominant modifier genetics broadens the genetic analysis by discovering loss-of-function alleles of vital genes that must be absent from inbred populations. Although such an allele can be maintained in heterozygous form, will the heterozygote have a phenotype? Alleles at quantitative trait loci commonly show additive inheritance. Many genes that modify a tumor pathway are haploinsufficient as judged by the quantitative phenotype of heterozygotes for knockout alleles. Examples include but are not limited to *Mmp7* (Wilson *et al.* 1997); T-cell factor 4 (Angus-Hill *et al.* 2011); Kruppel-like factors 4 (Ghaleb *et al.* 2007) and 5 (McConnell *et al.* 2009); *Park2* (Poulogiannis *et al.* 2010); E3 ubiquitin ligase Fbw7 (Sancho *et al.* 2010); SGO1(Yamada *et al.* 2012); cMyc (Yekkala & Baudino 2007); and *SMAD4* (Szigeti *et al.* 2012). The quantitative nature of tumor suppression has been reviewed by Largeaspada (2001) and Payne and Kemp (2005). Berger, Knudson, and Pandolfi (2011) have generalized the dosage sensitivity hypothesis for tumor suppressor genes. Loss-of-function alleles are maximally informative in the analysis of chains of causation. As discussed above, for such alleles, the evolved strategy is biased toward cases of dominant alleles involving haploinsufficiency. This bias may favor the identification of dosage-sensitive targets of drug action.

B6.SNVg has been shown to be a phenotypically neutral isogenic partner for mapping new modifiers of B6.*Apc*^{Min}. All six B6.SNV lines are available from the McArdle Laboratory for Cancer Research at the University of Wisconsin to be developed for research purposes as candidate mapping partners for the modifier genetics of the quantitative phenotype displayed by other B6 strains carrying a dominant element such as a transgene that affects a biological pathway of interest. The sequence changes detected by Illumina resequencing of these strains are shown in Data File S1 of Supporting Information. Only the calls in strain B6.SNVg were further tested by Sequenom analysis (Data File S3 of Supporting Information).

Other derivatives of B6 that have acquired new mutations by neutral drift or by strain admixture, such C57BL/6N, may also serve as isogenic mapping partners if shown to be phenotypically neutral (Simon *et al.* 2013).

We can anticipate significant future enhancements in the modifier genetics of complex traits in experimental mammals. On the phenotypic side, we can expect advances in the molecular and cellular dissection of tumor pathways. The complexity of cancer as a genetic trait surely includes the biological complexity of the disease. Subdividing the phenotype enhances the statistical power to detect a modifying mutation, through its selective action on one component of such a complex system (see March *et al.* 2011; Green *et al.* 2011).This strategy has been demonstrated for circadian biology by Takahashi and his colleagues (Chen *et al.* 2012). On the genetic side, we can expect further improvements by a new generation of mapping partners: using the efficient one-step allelic substitution process (Wang *et al.* 2013), a geneticist can engineer an isogenic mapping partner of an inbred strain by introducing a planned set of SNV mapping sites genome-wide, designed to be functionally neutral.[2]

A genome-wide genetic screen for mutagen-induced quantitative modifiers of the complex *Apc*^{Min} tumor phenotype can expand in important directions our molecular and cellular understanding of the complexities of intestinal neoplasia. This report illustrates the obstacles that must be overcome to achieve this end, and documents the creation of isogenic mapping partners for the canonical mouse strain C57BL/6J by which such sensitive genetic screens can be developed. This strategy and its *sequelae* can complement other active programs for the analysis of complex biological processes.

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FOOTNOTES

[1] "When one tugs at a single thing in nature, he finds it attached to the rest of the world." John Muir

[2] "Isogenicity is like virtue. It is usually desired but seldom achieved." H.J. Muller

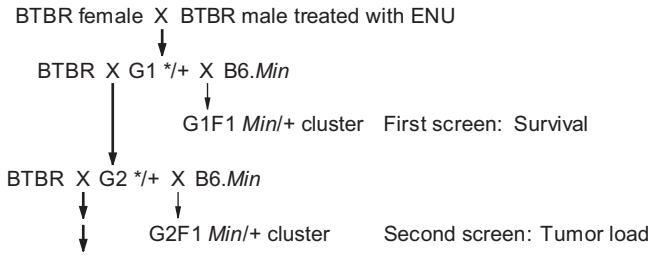
Table 1. Distribution of line-specific candidate variants by chromosome and line. In addition to the 13172 candidate variants each found only in a single line, 792 other candidate variants are each found in lines B6.SNVg and B6.SNVh, but in no other lines. B6.SNVg and B6.SNVh share founders, but the other lines are independent.

Chr	N of candidate variants						
	Only B6.SNVb	**Only B6.SNVc**	**Only B6.SNVe**	**Only B6.SNVf**	**Only B6.SNVg**	**Only B6.SNVh**	**Only B6.SNVg & B6.SNVh**
1	300	300	89	108	52	81	9
2	206	232	51	53	121	78	19
3	244	198	144	56	47	263	54
4	348	215	254	174	36	105	21
5	95	246	110	104	82	218	46
6	175	108	54	49	96	56	49
7	187	57	127	57	46	236	102
8	179	84	78	46	81	138	92
9	132	63	108	33	106	42	12
10	114	216	115	62	79	127	73
11	159	71	76	34	75	37	5
12	149	121	119	43	34	122	10
13	120	185	57	40	31	104	70
14	114	103	158	26	36	221	23
15	92	69	117	59	29	67	15
16	163	57	42	35	43	160	98
17	138	180	85	97	47	113	54
18	92	111	39	48	61	136	7
19	54	89	75	6	36	51	11
X	185	227	153	68	87	165	22
Total	**3246**	**2932**	**2051**	**1198**	**1225**	**2520**	**792**

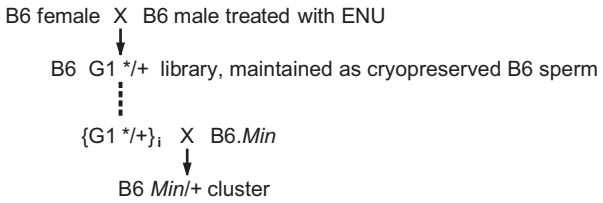
Table 2. Mutational pathway of the 13172 line-specific candidate variants.

			Line-specific candidate variants	
Mutation type	**Ref**	**Alt**	**N**	**Percent**
Transition	A	G	2142	16%
	T	C	2497	19%
	C	T	1540	12%
	G	A	1435	11%
Transversion	A	C	516	4%
	T	G	598	5%
	C	A	683	5%
	G	T	649	5%
	A	T	1114	8%
	T	A	1352	10%
	C	G	332	3%
	G	C	314	2%

A. Outcross design:



B. Isogenic design:



C. Isogenic mapping partner:

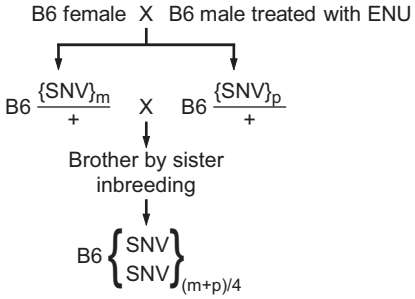


Figure 1. Breeding schemes to detect mutagen-induced dominant quantitative modifiers of the *Apc*^{Min} phenotype. Three different designs are shown. The term "cluster" indicates that multiple progeny were screened to determine whether the kindred differed significantly from the mean. Part A shows the outcross modifier screen of BTBR G1 males based first upon survival and subsequently upon tumor multiplicity. Part B shows the isogenic modifier screen of B6 mutagenized males, coupled with the ability to save sperm from candidate animals. Part C shows the design for producing inbred B6.SNV lines from mutagenized B6 males. Note that inbreeding eliminates recessive lethal or detrimental mutations. The existing B6.SNV lines have been inbred for at least 10 generations. The residual heterozygosity expected at F10 is 14% (Green 1981).

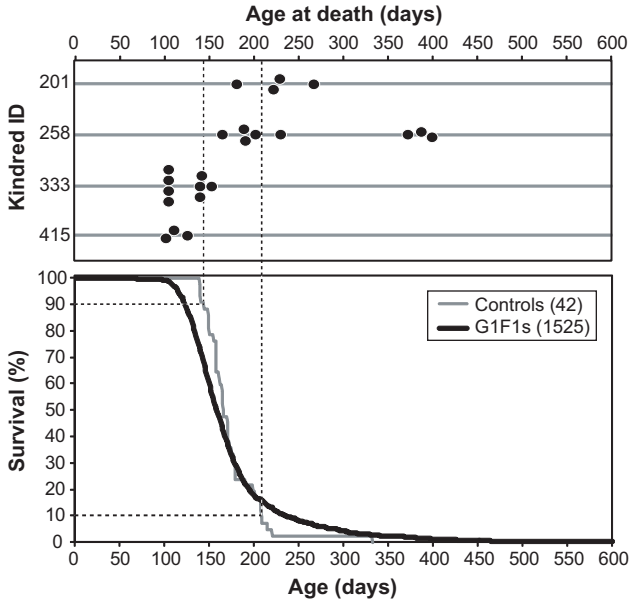


Figure 2. Candidate kindreds of dominant modifiers of *Apc*^{Min} identified by survival but not successfully mapped. The lower panel shows the survival curve of F1(B6-*Apc*^{Min} X BTBR) *Apc*$^{Min/+}$ controls (gray line) and of *Apc*$^{Min/+}$ progeny from mutagenized BTBR outcrossed to B6-*Apc*$^{Min/+}$ females (G1F1s, black line). The upper panel show the survival times of *Apc*$^{Min/+}$ progeny from four different mutagenized BTBR G1 males (each the founder of a kindred). The four kindreds shown were chosen from 172 test kindreds that generated clusters of *Apc*$^{Min/+}$ progeny with extreme short or long lifespans.

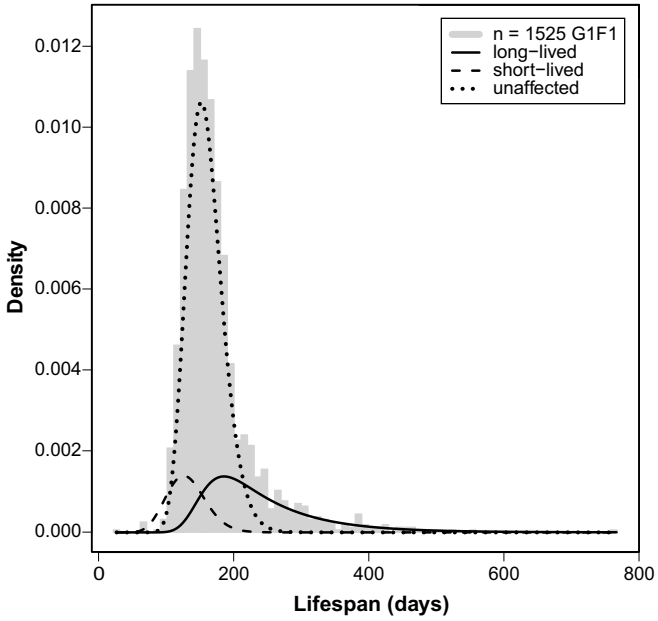


Figure 3. A statistical analysis of the differences in lifespan considers the distribution of mutagenized G1F1 Min animals as a mixture of three components (unaffected, long-lived, short-lived), and utilizes the maximum likelihood method to estimate both the rate at which ENU induces a phenotypic modifier and the distribution of multiplicative modifier effects (Figure 3, Supporting File S2). From this calculation, we estimated that 39% of the mutagenized kindreds are free of dominant modifier alleles, 41% carry dominant suppressor alleles that lead to reduced tumor multiplicity and longer lifespan, and 20% carry dominant enhancer alleles that have the opposite phenotype. A high proportion (61%) of mutagenized kindreds appear to carry modifiers of *Apc*^{Min}, but most are of small effect. Equation (2) in Supplement File S2 gives the formula to estimate the enrichment in large modifier effects that can be achieved by selecting kindreds having multiple animals with an extreme survival phenotype.

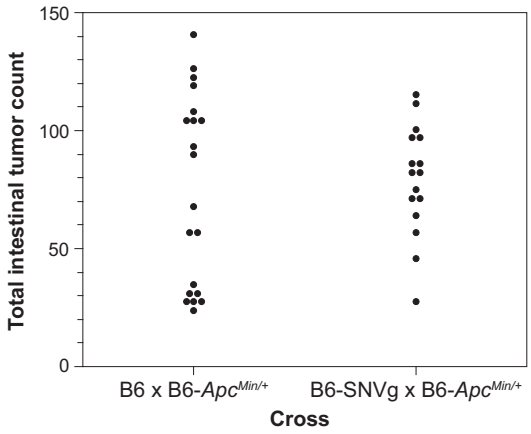


Figure 4. Progeny testing indicates that the B6.SNVg line carries no dominant modifiers of the *Apc*$^{Min/+}$ phenotype. B6-*Apc*$^{Min/+}$ males were each mated both to B6 females and B6.SNVg females. Tumors counts of *Apc*$^{Min/+}$ progeny averaged 75 ± 40 for B6 x B6-*Apc*$^{Min/+}$ and 79 ± 23 for B6.SNVg x B6-*Apc*$^{Min/+}$ ($p = 0.89$, two-sided Wilcoxon rank sum test). The analysis has been limited to comparing two sets of progeny paired by B6-*Apc*$^{Min/+}$ sires in common, because the tumor multiplicities of our B6-*Apc*$^{Min/+}$ colony varied significantly over time until the closed colony C57BL/6JD was established.

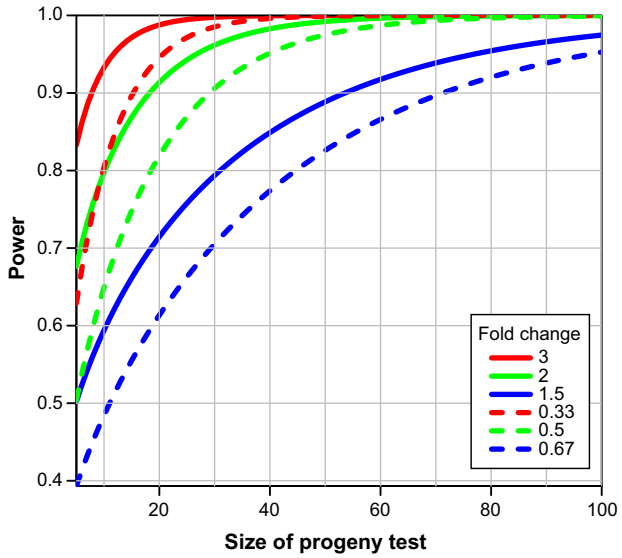


Figure 5. The number of progeny is estimated needed to test fully a mutagenized paternal gamete carried in a Generation 1 founder of a kindred. The null hypothesis tested is that the kindred carries no ENU-induced modifier. The tumor multiplicity data are assumed to follow a negative binomial distribution around a mean of 99.8 tumors and an overdispersion shape parameter of 9.8, as estimated from recent data from our closed colony, C57BL/6JD.*Apc*^{Min}. If a modifier is carried by a kindred in heterozygous form, it is assumed to segregate and to affect only the tumor multiplicity and its variance, but not the shape parameter. The number of progeny is shown on the x-axis required to achieve sufficient power (on the y-axis) to detect at a 5% significance level a fold-effect given by the lines. The power calculations are based on the approximation to a normal distribution of the average tumor multiplicities within the test kindred.

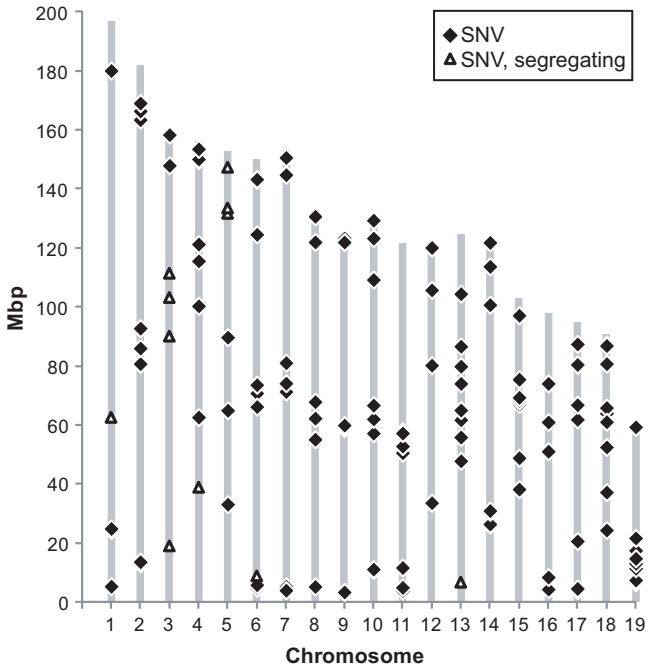


Figure 6. Location of point mutations in B6.SNVg that were validated by Sequenom testing. A total of 180 SNV candidate sites were chosen for confirmation by Sequenom genotyping, with 140 giving results in the final Sequenom assay. Shown are the 123 sites that are homozygous in B6.SNVg and the 11 sites showing residual heterozygosity. Validation was carried out on DNA samples from C57BL/6JD, B6.SNVg, B6.SNVh, and (C57BL/6JD x B6.SNVg)F1 mice (see Supporting Data File S2.) The map displays the set of validated SNV sites that can serve the purpose of mapping to low resolution a newly induced dominant modifier allele by crosses of the B6 carrier of the new modifier to B6.SNVg.

REFERENCES

Amos, W., 2010 Even small SNP clusters are non-randomly distributed: is this evidence of mutational non-independence? Proc.Biol.Sci. 277: 1443-1449.

Angus-Hill, M. L., K. M. Elbert, J. Hidalgo, and M. R. Capecchi, 2011 T-cell factor 4 functions as a tumor suppressor whose disruption modulates colon cell proliferation and tumorigenesis. Proc.Natl.Acad.Sci.U.S.A 108: 4914-4919.

Arnold, C. N., Y. Xia, P. Lin, C. Ross, M. Schwander *et al.*, 2011 Rapid identification of a disease allele in mouse through whole genome sequencing and bulk segregation analysis. Genetics 187: 633-641.

Baran, A. A., K. A. Silverman, J. Zeskand, R. Koratkar, A. Palmer *et al.*, 2007 The modifier of Min 2 (Mom2) locus: embryonic lethality of a mutation in the Atp5a1 gene suggests a novel mechanism of polyp suppression. Genome Res. 17: 566-576.

Barbaric, I., S. Wells, A. Russ, and T. N. Dear, 2007 Spectrum of ENU-induced mutations in phenotype-driven and gene-driven screens in the mouse. Environ.Mol.Mutagen. 48: 124-142.

Beck, S., B. E. Bernstein, R. M. Campbell, J. F. Costello, D. Dhanak *et al.*, 2012 A blueprint for an international cancer epigenome consortium. A report from the AACR Cancer Epigenome Task Force. Cancer Res. 72: 6319-6324.

Berger, A. H., A. G. Knudson, and P. P. Pandolfi, 2011 A continuum model for tumour suppression. Nature 476: 163-169.

Broman, K. W., L. B. Rowe, G. A. Churchill, and K. Paigen, 2002 Crossover interference in the mouse. Genetics 160: 1123-1131.

Cai, M., H. Dai, Y. Qiu, Y. Zhao, R. Zhang *et al.*, 2013 SNP set association analysis for genome-wide association studies. PLoS.ONE. 8: e62495.

Carvajal-Carmona, L. G., J. B. Cazier, A. M. Jones, K. Howarth, P. Broderick *et al.*, 2011 Fine-mapping of colorectal cancer susceptibility loci at 8q23.3, 16q22.1 and 19q13.11: refinement of association signals and use of in silico analysis to suggest functional variation and unexpected candidate target genes. Hum.Mol.Genet. 20: 2879-2888.

Chen, Z., S. H. Yoo, Y. S. Park, K. H. Kim, S. Wei *et al.*, 2012 Identification of diverse modulators of central and peripheral circadian clocks by high-throughput chemical screening. Proc.Natl.Acad.Sci.U.S A 109: 101-106.

Cormier, R. T., K. H. Hong, R. B. Halberg, T. L. Hawkins, P. Richardson *et al.*, 1997 Secretory phospholipase Pla2g2a confers resistance to intestinal tumorigenesis. Nat.Genet. 17: 88-91.

Crist, R. C., J. J. Roth, M. P. Lisanti, L. D. Siracusa, and A. M. Buchberg, 2011 Identification of Mom12 and Mom13, two novel modifier loci of Apc (Min) -mediated intestinal tumorigenesis. Cell Cycle 10: 1092-1099.

Dietrich, W. F., E. S. Lander, J. S. Smith, A. R. Moser, K. A. Gould *et al.*, 1993 Genetic identification of Mom-1, a major modifier locus affecting Min-induced intestinal neoplasia in the mouse. Cell 75: 631-639.

Drinkwater, N. R., and M. N. Gould, 2012 The Long Path from QTL to Gene. PLoS.Genet. 8: e1002975.

Edin, S., M. L. Wikberg, A. M. Dahlin, J. Rutegard, A. Oberg *et al.*, 2012 The distribution of macrophages with a M1 or M2 phenotype in relation to prognosis and the molecular characteristics of colorectal cancer. PLoS.ONE. 7: e47045.

Elahi, E., N. Suraweera, E. Volikos, J. Haines, N. Brown *et al.*, 2009 Five quantitative trait Loci control radiation-induced adenoma multiplicity in Mom 1R Apc Min/+ mice. PLoS.ONE. 4: e4388.

Eversley, C. D., X. Yuying, R. S. Pearsall, and D. W. Threadgill, 2012 Mapping six new susceptibility to colon cancer (scc) Loci using a mouse interspecific backcross. G3.(Bethesda.) 2: 1577-1584.

Ewart-Toland, A., P. Briassouli, J. P. de Koning, J. H. Mao, J. Yuan *et al.*, 2003 Identification of Stk6/STK15 as a candidate low-penetrance tumor-susceptibility gene in mouse and human. Nat.Genet. 34: 403-412.

Fraley, C., Raftery, A. E., Murphy, T. B., and Scrucca, L., 2012 mclust Version 4 for R: Normal Mixture Modeling for Model-Based Clustering, Classification, and Density Estimation. Technical Report No. 597, Department of Statistics, University of Washington.

Gabriel, S., L. Ziaugra, and D. Tabbaa, 2009 SNP genotyping using the Sequenom MassARRAY iPLEX platform. Curr.Protoc.Hum.Genet. Chapter 2.

Gabriel, S. B., S. F. Schaffner, H. Nguyen, J. M. Moore, J. Roy *et al.*, 2002 The structure of haplotype blocks in the human genome. Science 296: 2225-2229.

Garcia-Garcia, M. J., J. T. Eggenschwiler, T. Caspary, H. L. Alcorn, M. R. Wyler *et al.*, 2005 Analysis of mouse embryonic patterning and morphogenesis by forward genetics. Proc.Natl.Acad.Sci.U.S.A 102: 5913-5919.

Ghaleb, A. M., B. B. McConnell, M. O. Nandan, J. P. Katz, K. H. Kaestner *et al.*, 2007 Haploinsufficiency of Kruppel-like factor 4 promotes adenomatous polyposis coli dependent intestinal tumorigenesis. Cancer Res. 67: 7147-7154.

Gould, K. A., and W. F. Dove, 1996 Action of Min and Mom1 on neoplasia in ectopic intestinal grafts. Cell Growth Differ. 7: 1361-1368.Gould, K. A., C. Luongo, A. R. Moser, M. K. McNeley, N. Borenstein *et al.*, 1996 Genetic evaluation of candidate genes for the Mom1 modifier of intestinal neoplasia in mice. Genetics 144: 1777-1785.

Green E., 1981 *Genetics and Probability in Animal Breeding Experiments*. Oxford University Press, New York.

Green, R. A., H. L. Kao, A. Audhya, S. Arur, J. R. Mayers *et al.*, 2011 A high-resolution C. elegans essential gene network based on phenotypic profiling of a complex tissue. Cell 145: 470-482.

Herrera, M., A. Herrera, G. Dominguez, J. Silva, V. Garcia *et al.*, 2013 Cancer-associated fibroblast and M2 macrophage markers together predict outcome in colorectal cancer patients. Cancer Sci. 104: 437-444.

Hilton, J. M., M. A. Lewis, M. Grati, N. Ingham, S. Pearson *et al.*, 2011 Exome sequencing identifies a missense mutation in Isl1 associated with low penetrance otitis media in dearisch mice. Genome Biol. 12: R90.

Hotta, Y., and S. Benzer, 1972 Mapping of behaviour in Drosophila mosaics. Nature 240: 527-535.

Keane, T. M., L. Goodstadt, P. Danecek, M. A. White, K. Wong *et al.*, 2011 Mouse genomic variation and its effect on phenotypes and gene regulation. Nature 477: 289-294.

Khan, M. W., A. Keshavarzian, E. Gounaris, J. E. Melson, E. Cheon *et al.*, 2013 PI3K/AKT signaling is essential for communication between tissue infiltrating mast cells, macrophages, and epithelial cells in colitis-induced cancer. Clin.Cancer Res. 19: 2342-2354.

King, D. P., Y. Zhao, A. M. Sangoram, L. D. Wilsbacher, M. Tanaka *et al.*, 1997 Positional cloning of the mouse circadian clock gene. Cell 89: 641-653.

Kumar, V., K. Kim, C. Joseph, L. C. Thomas, H. Hong *et al.*, 2011 Second-generation high-throughput forward genetic screen in mice to isolate subtle behavioral mutants. Proceedings of the National Academy of Sciences of the United States of America 108: 15557-15564.

Kwong, L. N., and W. F. Dove, 2009 APC and its modifiers in colon cancer. Adv Exp Med Biol 656: 85-106.

Kwong, L. N., A. Shedlovsky, B. S. Biehl, L. Clipson, C. A. Pasch *et al.*, 2007 Identification of *Mom7*, a novel modifier of *Apc*$^{Min/+}$ on mouse chromosome 18. Genetics 176: 1237-1244.

Largaespada, D. A., 2001 Haploinsufficiency for tumor suppression: the hazards of being single and living a long time. J.Exp.Med. 193: F15-F18.

Lewis, A., and I. Tomlinson, 2012 Cancer. The utility of mouse models in post-GWAS research. Science 338: 1301-1302.

Li, H., and R. Durbin, 2009 Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics. 25: 1754-1760.

Liu, P., H. B. Liu, Y. Lu, W. Wen, D. Jia *et al.*, 2011 Genome-wide association and fine mapping of genetic loci predisposing to colon carcinogenesis in mice. Mol.Cancer Res. 10: 66-74.

March, H. N., A. G. Rust, N. A. Wright, J. Ten Hoeve, J. de Ridder *et al.*, 2011 Insertional mutagenesis identifies multiple networks of cooperating genes driving intestinal tumorigenesis. Nat.Genet. 43: 1202-1209.

McConnell, B. B., A. B. Bialkowska, M. O. Nandan, A. M. Ghaleb, F. J. Gordon *et al.*, 2009 Haploinsufficiency of Kruppel-like factor 5 rescues the tumor-initiating effect of the Apc(Min) mutation in the intestine. Cancer Res. 69: 4125-4133.

Michaud, E. J., C. T. Culiat, M. L. Klebig, P. E. Barker, K. T. Cain *et al.*, 2005 Efficient gene-driven germ-line point mutagenesis of C57BL/6J mice. BMC.Genomics 6: 164.

Moser, A. R., H. C. Pitot, and W. F. Dove, 1990 A dominant mutation that predisposes to multiple intestinal neoplasia in the mouse. Science 247: 322-324.

Newton, M. A., and D. I. Hastie, 2006 Assessing Poisson variation of intestinal tumor multiplicity in Min mice carrying a Robertsonian translocation. Applied Statistics 15: 123-138.

Nnadi, S. C., R. Watson, J. Innocent, G. E. Gonye, A. M. Buchberg *et al.*, 2012 Identification of Five Novel Modifier Loci of ApcMin Harbored in theBXH14 Recombinant Inbred Strain. Carcinogenesis 33: 1589-1597.

Nosho, K., G. Dranoff, C. Fuchs, and S. Ogino, 2011 Tumor-Infiltrating CD45RO+-Cell Density, but Not CD3+, CD8+, or FOXP3+-Cell Density, Has a Prognostic Role in Colorectal Cancer, Independent of Molecular Features. Laboratory Investigation 91: 160A.

Payne, S. R., and C. J. Kemp, 2005 Tumor suppressor genetics. Carcinogenesis 26: 2031-2045.

Peters, U., C. M. Hutter, L. Hsu, F. R. Schumacher, D. V. Conti *et al.*, 2012 Meta-analysis of new genome-wide association studies of colorectal cancer risk. Hum.Genet. 131: 217-234.

Pitroda, S. P., T. Zhou, R. F. Sweis, M. Filippo, E. Labay *et al.*, 2012 Tumor endothelial inflammation predicts clinical outcome in diverse human cancers. PLoS.ONE. 7: e46104.

Poulogiannis, G., R. E. McIntyre, M. Dimitriadi, J. R. Apps, C. H. Wilson *et al.*, 2010 PARK2 deletions occur frequently in sporadic colorectal cancer and accelerate adenoma development in Apc mutant mice. Proc.Natl.Acad.Sci.U.S.A 107: 15145-15150.

Quan, L., A. P. Stassen, C. A. Ruivenkamp, T. van Wezel, R. J. Fijneman *et al.*, 2011 Most lung and colon cancer susceptibility genes are pair-wise linked in mice, humans and rats. Plos One 6: e14727.

Sancho, R., A. Jandke, H. Davis, M. E. Diefenbacher, I. Tomlinson *et al.*, 2010 F-box and WD repeat domain-containing 7 regulates intestinal cell lineage commitment and is a haploinsufficient tumor suppressor. Gastroenterology 139: 929-941.

Shedlovsky, A., J. L. Guenet, L. L. Johnson, and W. F. Dove, 1986 Induction of recessive lethal mutations in the T/t-H-2 region of the mouse genome by a point mutagen. Genet.Res. 47: 135-142.

Shedlovsky, A., J. D. McDonald, D. Symula, and W. F. Dove, 1993 Mouse models of human phenylketonuria. Genetics 134: 1205-1210.

Simon, M. M., S. Greenaway, J. K. White, H. Fuchs, V. Gailus-Durner *et al.*, 2013 A comparative phenotypic and genomic analysis of C57BL/6J and C57BL/6N mouse strains. Genome Biol. 14: R82.

Sinnamon, M. J., K. J. Carter, B. Fingleton, and L. M. Matrisian, 2008 Matrix metalloproteinase-9 contributes to intestinal tumourigenesis in the adenomatous polyposis coli multiple intestinal neoplasia mouse. International Journal of Experimental Pathology 89: 466-475.

Smits, B. M., D. Sharma, D. J. Samuelson, S. Woditschka, B. Mau *et al.*, 2011 The non-protein coding breast cancer susceptibility locus Mcs5a acts in a non-mammary cell-autonomous fashion through the immune system and modulates T-cell homeostasis and functions. Breast Cancer Res. 13: R81.

Su, L. K., K. W. Kinzler, B. Vogelstein, A. C. Preisinger, A. R. Moser *et al.*, 1992 Multiple intestinal neoplasia caused by a mutation in the murine homolog of the APC gene. Science 256: 668-670.

Szigeti, R., S. A. Pangas, D. Nagy-Szakal, S. E. Dowd, R. J. Shulman *et al.*, 2012 SMAD4 haploinsufficiency associates with augmented colonic inflammation in select humans and mice. Ann.Clin.Lab Sci. 42: 401-408.

Takahasi, K. R., Y. Sakuraba, and Y. Gondo, 2007 Mutational pattern and frequency of induced nucleotide changes in mouse ENU mutagenesis. BMC.Mol.Biol. 8: 52.

Takeo, T., and N. Nakagata, 2011 Reduced glutathione enhances fertility of frozen/thawed C57BL/6 mouse sperm after exposure to methyl-beta-cyclodextrin. Biol.Reprod. 85: 1066-1072.

van Boxtel, R., M. N. Gould, E. Cuppen, and B. M. Smits, 2010 ENU mutagenesis to generate genetically modified rat models. Methods Mol.Biol. 597: 151-167.

Vogelstein, B., N. Papadopoulos, V. E. Velculescu, S. Zhou, L. A. Diaz, Jr. *et al.*, 2013 Cancer genome landscapes. Science 339: 1546-1558.

Wang, H., H. Yang, C. S. Shivalila, M. M. Dawlaty, A. W. Cheng *et al.*, 2013 One-Step Generation of Mice Carrying Mutations in Multiple Genes by CRISPR/Cas-Mediated Genome Engineering. Cell 153: 910-918.

Wilson, C. L., K. J. Heppner, P. A. Labosky, B. L. Hogan, and L. M. Matrisian, 1997 Intestinal tumorigenesis is suppressed in mice lacking the metalloproteinase matrilysin. Proc.Natl.Acad.Sci.U.S.A. 94: 1402-1407.

Yamada, H. Y., Y. Yao, X. Wang, Y. Zhang, Y. Huang *et al.*, 2012 Haploinsufficiency of SGO1 results in deregulated centrosome dynamics, enhanced chromosomal instability and colon tumorigenesis. Cell Cycle 11: 479-488.

Yekkala, K., and T. A. Baudino, 2007 Inhibition of intestinal polyposis with reduced angiogenesis in ApcMin/+ mice due to decreases in c-Myc expression. Mol.Cancer Res. 5: 1296-1303.