

# An Ancestral Haplotype Defines Susceptibility to Doxorubicin Nephropathy in the Laboratory Mouse

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Haplotype analysis was used to refine of the *DOXNPH* locus, which harbors the susceptibility gene for doxorubicin (DOX; Adriamycin) nephropathy, a Mendelian form of selective podocyte injury. Analysis of haplotype structure in three strains with contrasting susceptibility (148 single-nucleotide polymorphisms at 101-kb spacing) was complementary to analysis of recombinants in 176 F2 mice. For example, haplotype analysis but not meiotic mapping could exclude the *Abcc1* multidrug transporter, and this was confirmed further by phenotypic evaluation of *Abcc1* null mice. Next, comparison of haplotype structure (55 single-nucleotide polymorphisms at 44-kb spacing) with phenotype in 15 inbred strains revealed a risk haplotype that was shared by susceptible strains ( $P = 0.00017$ ), thereby reducing the *DOXNPH* region to a 1.3-Mb interval. These data demonstrate that susceptibility to DOX nephropathy represents a founder mutation in the laboratory mouse. Haplotype analysis can be used for identification of the *DOXNPH* gene and prediction of strain susceptibility pattern.

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Glomerular podocytes are highly specialized, terminally differentiated epithelial cells that play a critical role in maintaining permselectivity and structural integrity of the glomerular filtration barrier (1). Because of their differentiated phenotype, podocytes have limited regenerative capacity, making them vulnerable to genetic or environmental damage (1,2). For example, inherited defects in proteins that are specific or highly expressed in podocytes result in familial forms of glomerulosclerosis (3–7). Similarly, podocyte injury and depletion are key features of nephropathies that are induced by systemic disorders (*e.g.*, diabetes, immune complex disease) or environmental agents (HIV infection, pamidronate) (8–15). To demonstrate a causal effect, Wharram *et al.* (16) recently engineered a rat model of diphtheria toxin-mediated podocyte injury and observed a direct relationship between the degree of podocyte depletion and the severity of glomerulosclerosis. Progressive podocyte loss therefore has been proposed as a final common mechanism for the development of glomerulosclerosis (2,17). However, the biologic pathways that mediate podocyte damage in most secondary forms of glomerulopathy are not well understood.

To gain insight into pathways that mediate secondary forms of glomerulosclerosis, we studied the determinants of nephropathy that is induced by the anthracycline antibiotic doxorubicin (DOX; Adriamycin). Administration of this agent to susceptible

rodent strains causes selective injury to glomerular podocytes, resulting in severe proteinuria and rapidly progressive renal failure (18,19). This trait has been used extensively as an experimental model of glomerulosclerosis and may have a human counterpart in glomerulopathies that are associated with administration of chemotherapy (20–23). We recently demonstrated that susceptibility to DOX nephropathy in the laboratory mouse segregates as single gene defect with recessive inheritance (18). Using crosses between the susceptible BALB/cJ and resistant C57BL/6J strains, we mapped the trait locus to chromosome 16 A1-B1 (18). In addition, complementation tests and mapping cohorts confirmed that susceptibility/resistance in two other strains (129X1/SvJ and FVB/NJ) is attributable to variation at the *DOXNPH* locus (18).

Mendelian inheritance indicates that elucidation of DOX nephropathy is tractable to forward genetic approaches, providing an excellent opportunity to gain insight into biologic mechanisms that protect glomerular podocytes against secondary injury. Because contrasting susceptibility to DOX nephropathy among inbred strains is attributable to variation at the *DOXNPH* locus, it also strongly suggests that this trait represents a founder mutation in the laboratory mouse. This notion is supported by genealogic and sequencing data showing that the genome of the laboratory mouse can be partitioned into discrete haplotype segments, reflecting strain origin from a limited set of founders (24–26). This situation enables the utilization of haplotypes for fine mapping and evaluation of positional candidates. In this mapping strategy, haplotype blocks are compared with the strain susceptibility pattern to identify the ancestral chromosomal segment that contains the susceptibility gene(s) (25,27). This approach was validated recently for fine mapping of quantitative trait loci, as well as *in silico* localization of Mendelian and quantitative traits (25,27). Here, we investi-

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gated whether susceptibility to DOX nephropathy represents a founder mutation and whether haplotype analysis can help to refine the *DOXNPH* locus.

### Materials and Methods

#### Animal Breeding and Phenotyping

We tested eight to nine male and female mice (6 to 8 wk of age) from the various strains. The 129S1/SvImJ, AKR/J, BALB/cJ, BALB/cByJ, C57BL/6J, C57BL/10J, C3H/HeJ, CBA/J, LP/J, SJL/J, and SWR/J were obtained from Jackson Laboratories (Bar Harbor, ME). The 129S6/SvEvTac mice and *Abcc1*<sup>tm1Bor</sup> null mice on the FVB/N genetic background (FVB.129P2-*Abcc1*<sup>tm1Bor</sup> N12) were obtained from Taconic Labs (Hudson, NY). For meiotic mapping of the *DOXNPH* locus, we also produced an F2 intercross between BALB/cJ (BALB) and C57BL/6J (B6). DOX nephropathy was produced by injecting 10 mg/kg DOX by tail vein at 8 wk of age (18). Fifteen days after DOX injection, mice were killed for histologic analysis of kidneys; spontaneously voided urine was collected for urinalysis. Proteinuria was measured by spot urine dipsticks (Roche, Indianapolis, IN). Periodic acid-Schiff–stained kidneys sections were scored independently by two investigators (A.G.G. and V.D.D.), who were blinded to genetic background and genotype using a validated semiquantitative scale (18). As before, we applied dichotomous criteria to define affection status: Mice with at least 3+ proteinuria and histologic evidence of 5% or greater glomerulosclerosis at the time of death were classified as affected (18). Mice with less than 3+ proteinuria and normal histology were classified as unaffected. The protocol was approved by the Institutional Animal Care and Use Committee at Columbia University.

#### Genotyping, Haplotype Analysis, and In Silico Mapping

Genotyping was performed using informative microsatellite markers that were distributed across the *DOXNPH* interval; fluorescence primers were used to direct PCR from genomic DNA, and products were analyzed on a capillary sequencer (Spectrumedix, State College, PA). For construction of our haplotype map at the *DOXNPH* locus, we obtained marker positions and genotypes from the National Center for Biotechnology Information web site and the Mouse Phenome database (<http://aretha.jax.org/pub/cgi/phenome/mpdcgi?rtn=docs/home>). When public data were not available for relevant strains, single-nucleotide polymorphisms (SNP) were genotyped by direct sequencing.

For haplotype mapping, we used the three-SNP sliding window algorithm that was used by Pletcher *et al.* (27) (algorithm provided by Phillip McClurg and Tim Wiltshire). This algorithm applies a binomial generalized linear model to calculate the significance of association of SNP haplotypes with binary traits. In this association analysis, we used 14 strains with known phenotype and genotype data (Figure 1); BALB/cJ and BALB/cByJ were considered as a single strain because they are nearly identical (27).

### Results and Discussion

We initially constructed a haplotype map of the *DOXNPH* region for the three laboratory strains with the most public data available (C57BL/6J versus BALB/cJ and 129X1/svJ). Using public data (96 SNP) and additional resequencing (52 SNP), we generated a haplotype map with an average spacing of 101 kb (Figure 1A). Consistent with the mosaic structure that was described previously in the laboratory mouse (25), the distribution of SNP among these three strains was nonrandom, falling into blocks that range from 0.3 to 4.2 Mb in size. Several blocks are monomorphic (*i.e.*, sequence did not vary) between the

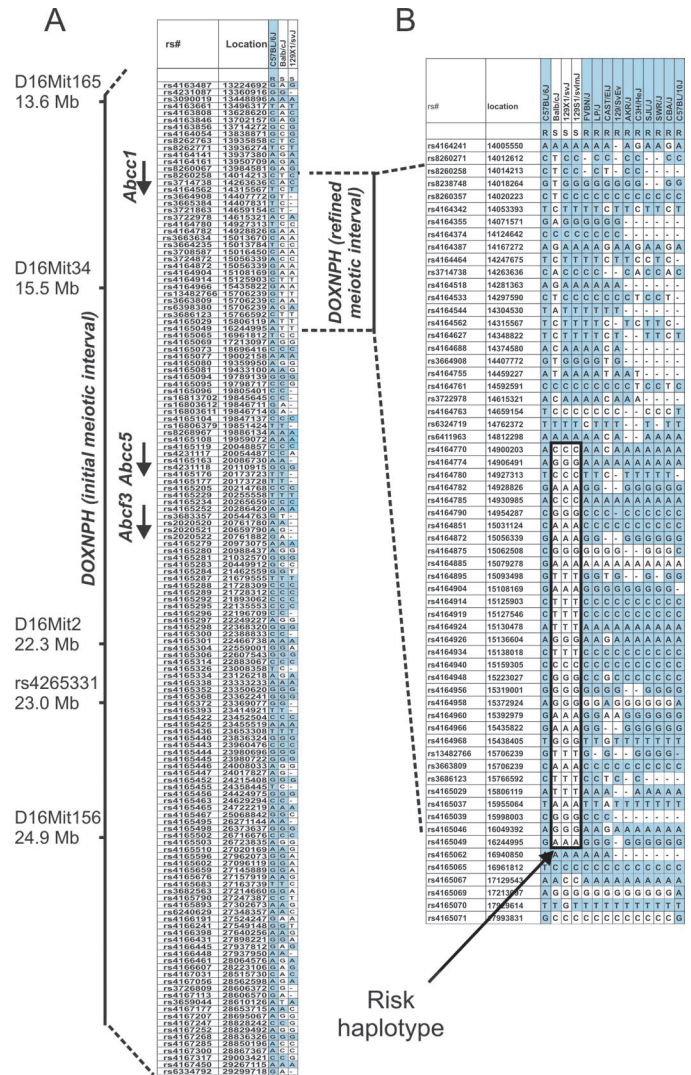


Figure 1. Map of the *DOXNPH* interval on chromosome 16 (National Center for Biotechnology Information build 35). (A) The *DOXNPH* meiotic interval as defined by our original study is indicated by the top line. The location of microsatellites is indicated by tick marks. The haplotype map of the initial *DOXNPH* interval across three strains that are susceptible (BALB/cJ and 129S1X/svJ) or resistant (C57BL/6J, B6) to doxorubicin (DOX) nephropathy. The B6 haplotype (blue) is used as reference. Single-nucleotide polymorphisms (SNP) that differ from B6 are in white. The location of the three positional candidates (*Abcc1*, *Abcc5*, and *Abcc3*) relative to the initial meiotic intervals is shown by the arrows. (B) The thick black line represents the refined meiotic interval identified in this study based on 176 F2 mice. A high-resolution haplotype map of the new recombinant interval across 14 strains is shown. Susceptible strains (S) share a common haplotype (box), enabling further refinement of the *DOXNPH* gene.

BALB/cJ and C57BL/6J strains (*e.g.*, the interval between rs14165284 and rs14165296). Such regions of identity between strains with contrasting susceptibility suggest inheritance by descent and therefore are unlikely to harbor the *DOXNPH* gene. In contrast, the centromeric portion of the interval contains a large (approx-

mately 3.4 Mb) block that is polymorphic between the resistant C57BL/6J and the susceptible BALB/cJ and 129X1/svJ strains (Figure 1A) and therefore is predicted to contain the *DOXNPH* gene.

To validate the use of haplotype analysis as a tool for fine mapping and evaluation of positional candidates, we generated 176 (BALB/cJ × C57BL/6J) F2 mice (352 recombination events) to fine-map the *DOXNPH* linkage interval. These mice were genotyped for markers at the *DOXNPH* locus, revealing six informative recombinant progenies, which subsequently were tested for susceptibility to DOX nephropathy. With additional genotyping to define the recombinant site in the informative F2 progeny, the *DOXNPH* locus was reduced to a 2.3-Mb interval between rs8260258 and rs4165049 (Figure 1B). This meiotic interval was in excellent agreement with our haplotype map (Figure 1).

The validity of haplotype mapping for gene localization was supported further by analysis of specific positional candidates. Three positional candidates in the initial 14-Mb linkage interval (*Abcc1*, *Abcc5*, and *Abcf3*) were attractive because they belong to the ATP-cassette gene family. This gene family encodes efflux pumps that mediate drug transport and are implicated in xenobiotic metabolism and multidrug resistance to chemotherapeutics (28). Haplotypes in *Abcc1*, *Abcc5*, and *Abcf3* clearly did not correlate with susceptibility to the three strains tested (Figure 1A), suggesting that mutations in these genes are not responsible for the DOX nephropathy phenotype. Consistent with these data, *Abcc5* and *Abcf3* were outside our new meiotic interval. Because *Abcc1* still remained within the meiotic interval, we studied *Abcc1* null mice on the resistant FVB/N genetic background. *Abcc1* null mice have increased susceptibility to etoposide toxicity (29), but to our knowledge, phenotypic response to anthracyclines has not been reported. If DOX nephropathy is due to a loss-of-function mutation in *Abcc1*, then we would expect that *Abcc1* knockout mice on the resistant FVB/N background would manifest the phenotype. However, these *Abcc1* null mice were completely resistant to DOX nephropathy (no proteinuria, normal histology), thereby excluding this gene (Figure 2).

We next tested 10 additional strains for susceptibility to DOX nephropathy using the same protocol used in our original mapping study. We found that AKR/J, C3H/HeJ, CBA/J, C57BL/10J, LP/J, SWR/J, SJL/J, and 129S6/SvEvTac mice are resistant to DOX nephropathy, whereas 129S1/SvImJ and BALB/cByJ mice are susceptible (Figure 1B). With the addition of strains that were characterized previously (18), this provided phenotype data for 15 strains.

We then defined the SNP haplotypes for these additional strains and generated a haplotype map of the refined meiotic interval at the *DOXNPH* locus (Figure 1B). Partitioning of strains on the basis of phenotype confirmed a risk haplotype that was common to BALB/cJ, 129S1/SvImJ, and 129X1/SvJ (Figure 1B). To detect previously unrecognized SNP blocks, we also resequenced 30 additional SNP within the new meiotic interval (44 kb average spacing between rs4164241 and rs4165049). Consistent with published reports, increased map density uncovered some additional complexity in the haplo-

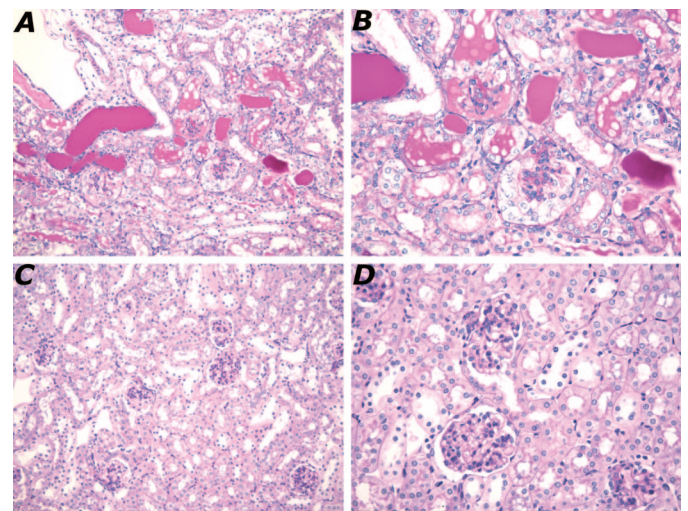


Figure 2. Renal pathology in mice with DOX nephropathy. Periodic acid-Schiff–stained kidney sections of BALB/cByJ and *Abcc1* null mice. (Left) Low-power views. (Right) Higher power magnification of glomeruli from the same mouse. (A and B) From a BALB/cByJ mouse with typical DOX nephropathy. (C and D) From an *Abcc1* null mouse. The normal histology demonstrated that *Abcc1* is not the *DOXNPH* gene.

type structure of the region (30,31), identifying some SNP that are private to the C57BL clade (e.g., rs4164875, rs4164885). However, these additional SNP did not partition the large polymorphic block further, suggesting that there are no other ancestral recombinations in the strains tested. To test the significance of the association between haplotype and strain susceptibility, we also computed an F statistic using a three-SNP haplotype window (27); this mapping statistic was highly significant across the region between rs4164770 and rs4164851 (lowest  $P = 0.00017$ ; Figure 3). Combining the results of meiotic and haplotype mapping enabled refinement of the trait locus to a 1.3-Mb region between rs4164770 and rs4165049 (Figures 1B and 3).

The positional candidates that remained in our refined interval comprise 20 genes. Of these, 13 are presently unknown/predicted and therefore will require functional annotation. The other positional candidates encode structural proteins (*Pkb2* and *Fgd4*) or proteins that are involved in mitochondrial homeostasis (*Dnm1l*), cellular stress signaling, and DNA repair (*Ube2v2*, *Prkdc*, *Mcm4*, and *Cebpd*). Defects in these pathways have been implicated either in the development of chemotherapeutic cytotoxicity or in the pathogenesis of glomerulosclerosis (3–15,32–34), making these genes plausible candidates as *DOXNPH*. We now can proceed with systematic sequence and functional analysis of these remaining positional candidates to identify the susceptibility variant. However, the common ancestral origin of laboratory mice, which facilitates fine mapping by haplotype analysis, also signifies that this population is unlikely to harbor independent susceptibility alleles for this trait. Hence, identification of the *DOXNPH* gene may require further fine mapping to achieve an interval that is sufficiently small to permit differentiation of the functional variant(s) from

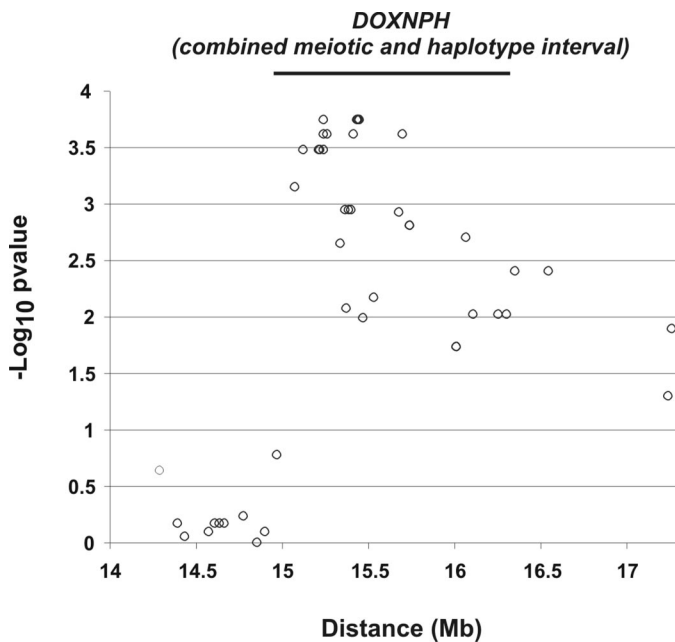


Figure 3. Results of *in silico* association study (a case-control study), using the three-SNP haplotype mapping statistic from reference (27) (62 SNP across 14 strains). The *x* axis shows the physical distance (Mb) on chromosome 16 across the minimal recombinant interval. The *y* axis is the  $-\log_{10}$  (*P* value) for the test statistic. The bar shows location of the *DOXNPH* locus based on haplotype and meiotic mapping.

linked polymorphisms and execution of bacterial artificial chromosome transgenic rescue experiments.

Identification of new meiotic recombinants offers a fail-safe but laborious route to achieve further reduction of the *DOXNPH* interval. However, our data suggest that analysis of additional laboratory strains also may identify recombinants within the ancestral haplotype. In particular, examination of wild derived strains may be very powerful for this purpose. For example, the wild derived CAST/EiJ strain shows additional recombinations within the *DOXNPH* risk haplotype (Figure 1B), but interpretation of these data is difficult because we do not know yet whether the *DOXNPH* susceptibility allele was introduced before the separation of the lineage that led to laboratory strains. Confirmation that the susceptibility allele also segregates among wild-derived strains would enable proper interpretation of the CAST/EiJ haplotype data and increase the opportunities for finding ancestral recombinants that can reduce the interval.

In rats, clipping of the renal artery during DOX infusion prevents the development of nephropathy, suggesting that this trait is independent of extrarenal drug metabolism and that direct exposure of the kidneys to anthracyclines is a requirement for the development of podocyte injury (35,36). These data predict that the *DOXNPH* gene should be expressed in the kidney and that an additional method for prioritizing positional candidates would be to determine whether they are expressed in renal tissue, particularly in glomerular podocytes.

In the meantime, our findings have practical implications for

execution and interpretation of studies that involve DOX nephropathy. Our data define the susceptibility pattern for 15 strains, enabling investigators to select the correct strain for application of this model. Moreover, we prospectively tested the predictive value of haplotypes by typing six informative loci in two additional strains (CFW and 129P2/OlaHsd); these strains have the B6 haplotype at the *DOXNPH* locus, and, as predicted by their genotype data, they were resistant to DOX nephropathy. Therefore, for strains not studied here, investigators now can determine haplotype status at the *DOXNPH* locus to predict susceptibility or resistance to DOX nephropathy.

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