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High-resolution genetic localization of a modifying locus affecting disease severity in the juvenile cystic kidneys *(jck)* mouse model of polycystic kidney disease

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Abstract

We have previously demonstrated that a locus on proximal Chr 4 modifies disease severity in the juvenile cystic kidney (*jck)* mouse, a model of polycystic kidney disease (PKD) that carries a mutation of the *Nek8* serine-threonine kinase. In this study we used QTL analysis of independently constructed B6.D2 congenic lines to confirm this and showed that this locus has a highly significant effect. We constructed sub-congenic lines to more specifically localize the modifier and have determined it resides in a 3.2 Mb interval containing 28 genes. These include *Invs* and *Anks6*, which are both excellent candidates for the modifier as mutations in these genes result in PKD and both genes are known to genetically and physically interact with *Nek8*. However, examination of strain-specific DNA sequence and kidney expression did not reveal clear differences that might implicate either gene as a modifier of PKD severity. The fact that our high-resolution analysis did not yield an unambiguous result highlights the challenge of establishing the causality of strain-specific variants as genetic modifiers, and suggests that alternative strategies be considered.

BACKGROUND

Polycystic kidney disease (PKD) represents a major cause of human morbidity and mortality. Remarkably, while PKD1 and PKD2, the genes responsible for the great majority of human autosomal dominant PKD, have both been studied for many years, the biological basis of this disorder remains elusive. This makes the development of therapies that directly target the molecular pathology that results in kidney disease difficult.

An alternative strategy for therapeutic intervention would be to identify other pathways that influence disease progression. It has been clearly shown that there can be substantial intra-familial variation in the severity of PKD, which suggests the likelihood that genetic loci

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modifying the disease are segregating in the human population (reviewed in (Rossetti and Harris 2007) Several studies have examined this specific question and concluded this is the case (Fain et al. 2005; Paterson et al. 2005). While these studies suggest that genetic loci that modify disease progression for PKD exist, identifying these in a human population would be challenging. This is because of the known influence of allelic variability on the disorder, as well as the likelihood that the modifier loci in humans are genetically heterogeneous. Thus, obtaining a large enough population in which there is sufficient power to identify multiple loci of variable effect would be challenging.

In this regard, mouse models of human disorders are potentially powerful tools for understanding heritable contributions to complex genetic traits. The availability of mutations on inbred genetic backgrounds, along with the development of extensive molecular and computational resources for genome-wide analysis, makes these systems ideal for genetic analysis. Importantly, there are numerous mouse mutants that are models of human cystic kidney disease, including those which carry defects in *Pkd1* or *Pkd2*, or other cystogenic mutations such as those associated with the nephronophthises and other ciliopathies (Happe and Peters 2014).

These include the recessive juvenile cystic kidney *(jck)* mutation (Atala et al. 1993) which we discovered and showed is caused by a missense mutation in *Nek8,* a highly conserved member of the Nek kinase family (Liu et al. 2002). Mutations in this gene are associated with the nephronophthisis disorder NPHP9 in humans (Otto et al. 2008), as well as the Lewis Polycystic Kidney rat line (McCooke et al. 2012). The course of polycystic disease in *jck* mice is very consistent in the C57BL/6J (B6) background in which it was found; this has made it very useful as a model for testing therapies (Bukanov et al. 2006; Smith et al. 2006; Natoli et al. 2010; Bukanov et al. 2012; Natoli et al. 2012; Tran et al. 2014). In our initial mapping studies to localize *jck*, we also demonstrated that interacting modifiers markedly influence the severity of the cystic disease phenotype (Iakoubova et al. 1995). Specifically, we mapped the position of the *jck* mutation to Chr 11 using an intercross of C57BL/6J (B6) carrying *jck* and DBA/2J (D2) mice. In the F2 progeny, the size of polycystic kidneys found in age-matched affected mice was markedly variable compared to that found in the parental line. This suggested that modifying loci affecting severity had been introduced from the D2 background. Initial genetic analysis revealed non-random co-segregation with disease phenotype for a B6 locus on Chr 1. Quantitative trait locus (QTL) analysis of combined kidney weight at 7 weeks of age (as a proxy for disease severity) revealed this accounted for 74% of the variance in affected F2 progeny and appeared to have its effect as a recessive locus.

A highly significant association of recessively inherited B6-related alleles on Chr 1 with severe disease was unexpected, since the PKD phenotype in the original B6 background is not severe. We proposed that the severe phenotype resulted from a genetic interaction between the B6 locus on Chr 1 and a D2 gene elsewhere in the genome. To further analyze this, *jck* was crossed into the D2 background for three generations, testing to ensure that loci spanning Chr 1 carried only D2 alleles. When these mice were intercrossed, variance in kidney size for the affected progeny was markedly less than that seen in the F2 progeny, and not significantly different than that found for the B6 parental mice (Iakoubova et al. 1999).

This observation continued to be valid after serial backcross of the *jck* mutation into D2 mice for over 10 generations, proving that the severe disease phenotype is a consequence of a genetic interaction.

However, in our QTL studies we did not identify a D2 allele that was significantly associated with disease severity. We hypothesized that the statistical effect of this locus would be obscured in an intercross, since the Chr 1 locus behaves recessively and three-fourths of the mice are therefore not informative for the interaction. To address this, we constructed a D2.B6 *jck/+* congenic strain that carries a large interval on Chr 1 as homozygous B6 and the remainder of the genome as D2. There are two predictions for this strain: firstly, the congenic strain should show a severe disease phenotype (since all mice carry the interacting modifiers). Secondly, it should be useful for a mapping analysis because when crossed with a B6 *jck/+* mouse, the congenic region on Chr 1 is fixed as B6, while the rest of the genome is segregating as in an F2 population; thus, all the affected mice should be informative for the interacting D2 locus. Both of these predictions proved true, and using this congenic line we were readily able to localize a D2 locus on proximal Chr 4 associated with severe PKD (Kuida and Beier 2000).

The determination that a locus on proximal Chr 4 contributes to severe PKD in the *jck* mutant mouse is of particular interest because this is the position of a locus which has been shown to modify the progression of PKD in two different mouse mutations, *cpk* and *pcy* (Woo et al. 1997). Modifying loci on proximal Chr 4 affecting disease severity in *cpk* mice have also been described by Guay-Woodford and colleagues (Mrug et al. 2005). This suggests that the effect of this locus may be independent of the underlying mutations, and therefore potentially relevant to human PKD progression. In this report we describe our high-resolution localization of the modifier by analysis of multiple congenic lines.

RESULTS

The localization of this modifier was facilitated by the development by Iakoubova and West of a series of congenic strains containing overlapping regions of D2 alleles of Chr 4 on a B6 background, which had been bred to the N5 generation (Iakoubova et al. 2001). Since these congenic lines were established using a genotype-assisted selection strategy, the percent of unselected D2 alleles in the genomes of these lines is very low. That is, the residual heterozygosity in these lines for the unselected regions is significantly lower than the 6.25% that would be expected by random segregation.

We therefore planned a strategy of analysis similar to that described in (Iakoubova et al. 1999), in which our aim was to characterize congenic lines corresponding to proximal, middle, and distal regions of Chr 4. However, the congenic lines utilized were somewhat different than anticipated, as the strains we obtained had not been fully inbred at the time they were sent. This actually proved fortuitous, as we were still able to test congenic lines carrying proximal and middle regions of D2 Chr 4 on an otherwise B6 background (lines P and M-C in Fig. 1). However, we also tested an additional congenic line, M-A, which had an “island” of D2 alleles from the proximal region as well as alleles from the middle region of

the chromosome (Fig. 1). As shown, this “island” encompassed the QTL peak we had previously found on Chr. 4.

Given the husbandry challenge of obtaining sufficient power to test a recessive mutation on multiple congenic lines, we elected to simply perform a QTL analysis on all of the resultant mice combined. This revealed that the D2 allele of marker *D4Mit286*, located at 43.2 MB on Chr 4, was associated with disease severity with a LOD value of 5.4 (Fig. 2). Permutation analysis was used to calculate LOD values corresponding to levels of suggestive (0.8), significant (2.5), and highly significant (5.2) association.

With this result in hand we then generated additional sub-congenic lines to further define the region carrying the modifier locus. For this high resolution analysis it was necessary to test the lines independently; we therefore employed a two-step strategy whereby we generated *jck/+* lines that were homozygous for the sub-congenic region, and intercrossed these. The data for these lines, X, Y and Z, is shown in Fig. 3. The data for males and females is shown separately, as we and others have now found there are sex-specific effects on PKD severity in the *jck* model (Smith et al. 2006). Note that lines X and Y show the more severe disease phenotype (P value for male and female cohorts combined = 1.03×10^{-8} and 6.2×10^{-11}, respectively), while Z does not (P= 0.28), revealing that the modifier lies in the more distal interval between the SNP markers *RS13477682* at 46.5 Mb and *RS6186613* at 49.7 Mb.

This 3.2 Mb interval contains 28 genes (Table 1). As discussed below, *Invs* and *Anks6* were considered as likely candidates for the modifier because they both are known to interact with *Nek8*. However, *Invs* is in a region of high haplotype similarity between B6 and D2 (Fig. 4), and has no exonic sequence variants. *Anks6* has two missense variants (H298Y and A507V); however, these are likely benign substitutions with Polyphen scores of 0.010 and 0.137, respectively (Adzhubei et al. 2010). Kidney expression of the D2 alleles of *Invs* and *Anks6* in the Y sub-congenic line was compared to that of B6 and no significant differences were found (Fig. 5).

The only other gene in this interval with a missense or nonsense variation in D2 compared to B6 is *Grin3a* (https://www.sanger.ac.uk/sanger/Mouse_SnpViewer/rel-1303; (Keane et al. 2011). This was not pursued as a candidate as the Polyphen score was 0.0 and the gene has a known role in neuronal function (Das et al. 1998).

DISCUSSION

We have previously demonstrated that a locus on Chr 4 modifies disease severity in the *jck* mutant mouse (Kuida and Beier 2000), which carries a mutation of the *Nek8* serine-threonine kinase (Liu et al. 2002). Specifically, we showed that this involved D2 alleles on Chr 4 that interacted with B6 alleles on Chr 1. In this study we used congenic lines that have regions of D2-derived alleles on Chr 4 on an otherwise B6 background to define the region that contains this locus with high resolution. Our first step was to use congenic lines developed by Iakoubova and West (Iakoubova et al. 2001) and cross these with *jck*. A combined analysis of 39 of the resultant mutant mice showed that a D2-derived locus on Chr 4 was associated with increased disease severity with high significance. This is important for

several reasons. Firstly, this was an independent confirmation of the observation we made previously, using separately derived congenic lines. Also, the fact we obtained this highly significant association with a relatively modest number of affected mice demonstrates this locus has a major effect on disease progression.

To more narrowly define the region carrying the modifier, we made several additional sub-congenic lines that were individually tested for their effect on disease severity. Two of these lines showed a highly significant increase in disease severity compared to B6 *jck/jck* mice, while a third showed no difference when compared to B6. As the boundaries of the congenic regions are defined by SNPs whose genomic position is known, this localizes the region carrying the modifier to an interval of 3.2 Mb. Again, the highly significant difference of disease severity in the lines carrying the modifier underscores the influence of this locus on PKD progression.

As shown in Table 1, this region contains 28 genes. Two of these, *Invs* and *Anks6*, were considered as excellent candidate for the modifying locus. Mutations of *Invs* in mice are well known to result in cystic kidney disease (Mochizuki et al. 1998; Morgan et al. 1998) and mutation of its human orthologue result in the nephronophthisis NPHP2, which also features cystic kidney disease (Otto et al. 2003). Further, we have shown that *Invs* and *Nek8* interact physically and genetically (Shiba et al. 2010). *Anks6* was found to be mutated in the *Pkdr1* rat (Brown et al. 2005), and more recently has been associated with cystic kidney disease in humans (Hoff et al. 2013) and mice (Czarnecki et al. 2015). Also, we and others have shown that *Nek8* and *Anks6* physically interact (Hoff et al. 2013; Czarnecki et al. 2015). Several other factors suggested that protein interactions could be key to the modifying effect. These include the observation that all of the cystogenic mutations in *Nek8* are associated with missense mutations in its presumptive regulatory protein-interacting domain. Also, we have shown that a null mutation of *Nek8* is not cystogenic, and our genetic analysis demonstrates that the *jck* missense mutation is a gain-of-function (Manning et al. 2013). In addition, a locus very close to the one we have identified has been shown to affect PKD severity in the *pcy* mutant mouse (Woo et al. 1997), which carries a mutation in *Nphp3* (Olbrich et al. 2003); we have shown that this protein interacts physically and genetically with *Invs* and *Nek8* (Shiba et al. 2010).

However, we cannot find evidence to support these loci as the PKD modifier. *Invs* is in a region of marked haplotypic similarity between B6 and D2, and has no exonic sequence variants. The haplotypes for *Anks6* are more dissimilar and the B6 and D2 alleles do have missense differences, which are predicted to be benign. Expression analysis does not reveal a significant difference of mRNA abundance for these genes when examined in adult kidneys. This by no means excludes these genes as candidates, as the predicted effect of the missense changes may be incorrect, or differences of expression may be limited to specific cell types or developmental windows.

Importantly, this analysis reveals the challenges of discovering strain-specific modifiers using a genetic approach. Despite our localization of a PKD modifier to a region comprising 0.1% of the genome, an experimentally intractable number of genes remain as candidates. Even the two excellent candidates we identify would be difficult to confirm, given the

absence of compelling sequence or expression variation. Furthermore, this underscores a hazard of the approach, as it is often biased to the consideration of those genes whose known functions potentially implicate them in the biology being studied. Finally, since this approach will usually localize a gene to region of haplotype differences, it may prove difficult to determine which of many sequence variants (most of which do not cause protein truncations) are causal.

The task of assigning causality to a specific gene within the intervals identified as significantly linked by QTL analysis has been addressed by a variety of strategies. These include creating novel genetic resources such as the Collaborative Cross (Churchill et al. 2004) and Diversity Outbred population (Svenson et al. 2012), which have highly resolved regions of linkage disequilibrium that make them amenable to association analysis. More relevant to this study is the utilization of systems genetics approaches, primarily employing mRNA expression resources, as a means to parse candidate genes within a recombinant interval (Faraji et al. 2014; Parks et al. 2015; Tian et al. 2015). The international effort to obtain phenotype data for large numbers of “knock-out” mutant mice can also inform the selection of candidate loci, with the caveat that most strain-specific protein variation is non-synonymous amino acid substitution, not null mutation (Brown and Moore 2012).

Given the difficulty of assigning causality for strain-specific modifiers, one can consider using a different strategy to ascertain genetic modifiers. ENU mutagenesis is a powerful and efficient method for creating mutations, and the merits of its utilization for the identification of quantitative phenotypes has been ably discussed (Nadeau and Frankel 2000). A challenge for this method is the identification of the causal variants; to this end, we have recently developed a protocol for positional cloning of ENU-induced mutations without employing an outcross (Gallego-Llamas et al. 2015). This allows the characterization of phenotypic variation without strain effects as a potential confounder. ENU-induced mutations are usually sufficiently distributed such that they can be resolved by a modest number of meioses, enabling causality to be readily assessed.

ENU screening strategies are well suited for the introduction of mutations for which modifiers can be assessed, and we have done this routinely using reporter genes to query specific developmental process (Dwyer et al. 2011; Stottmann et al. 2011; Ha et al. 2015). Finally, there is considerably less selection against deleteriousness for ENU-induced variants, as they are ascertained in a only a few generations, compared to those that occur in inbred strains, which must be compatible with viability and fertility. As such, the induced mutations will potentially be more severe than those fixed during the establishment of inbred strains, and their consequences for gene function may be more apparent.

METHODS

Mice and phenotype characterization

All protocols were approved by the Brigham and Women’s and the Seattle Children’s Research Institute Animal Care and Use Committees. The Brigham and Women’s and the Seattle Children’s Research Institute are fully accredited by the American Association of the Accreditation of Laboratory Animal Care. B6 *jck/+* and *jck/jck* mice are maintained in our

mouse colony. The lines we designated as “P”, “M-C” and “M-A” were obtained from Iakoubova and West, and correspond to the lines they designated as BDChr4P, BDChr4M, and BDVhr4D, respectively (Iakoubova et al. 2001). To test these congenic lines, female B6 *jck/+* mice were crossed with males of the imported strains and *jck/+* progeny were identified by genotyping for the mutation. These were intercrossed and homozygotes identified by genotype analysis. These were sacrificed at 7 weeks of age and scored for the presence of abnormal kidneys, which were removed, weighed, and fixed.

We obtained 15 homozygous mice from Line P, 13 from line M-C, and 11 from line M-A. Given these modest numbers, we elected to analyze the combined cohort. All homozygous mice were typed for 10 microsatellite markers distributed along Chr 4 (Fig. 2).

For high resolution analysis we employed a two-step strategy whereby we generated three separate *jck/+* lines that were homozygous for a sub-congenic region defined by analysis of 21 SNP markers on Chr 4 (Fig. 3), and intercrossed these. Thus all tested mice were homozygous for the sub-congenic region. Progeny homozygous for *jck* were identified by genotype analysis, and kidneys assessed as described above. The numbers of mice analyzed, separated by gender, is shown in Fig. 3.

PCR-based genotyping

Genomic DNA was prepared from tail and liver tissue according to standard techniques. Genotyping for *jck* was done by digesting a 308 bp amplicon that encompasses the mutation, generated using the primers DB 80 (CTTCCCACCTGTTGCTGTTT) and DB 81 (CAGTGGGCTTACCACCATCT), with BseY1; this restriction site is lost in the mutant allele. *Mit* microsatellite markers polymorphic between B6 and D2 were amplified by PCR and analyzed on a Metaphor agarose gels. SNPs were analyzed by amplicon sequencing. Map positions correspond to the GRCm38/mm10 assembly.

Statistical analysis

Genotype data was correlated with kidney weight trait data using Mapmanager QT (Manly and Olson 1999). Permutation tests were done in 1 cM steps for 5000 permutations. The threshold values of the permutation test, which are labeled suggestive, significant, and highly significant, are taken from the guidelines of (Lander and Kruglyak 1995) and correspond to the 37th, 95th, and 99.9th percentiles, respectively. For the comparison of the sub-congenic strains, Student’s T-tests were done, assuming a two-tailed distribution and unequal variance.

Expression analysis

Kidneys from B6 or Line Y adult mice that were wild-type at *Nek8* were obtained and RNA collected using Trizol purification and further purified using the Nucleospin RNA-Clean Up kit from Machery-Nagel. Single-stranded DNA was made using the Invitrogen Superscript III kit. qPCR was performed in 20 uL reactions (primers listed below) on a Bio-Rad CFx-96 Real-Time System and Bio-Rad C1000 Thermal Cycler. Data was collected and reviewed using the Bio-Rad CFX manager program and statistical analysis done using the ΔΔCt method (Livak and Schmittgen 2001). Primer sequences used for expression analysis: *Invs*:

Forward-GGGAGGTGGATACACAGGACA, Reverse-TGCTCGGGGTTGTTGTAGTAG. *Anks6*: Forward –TGGTGATGCTGCTGAACGAT, Reverse - GGCCGACCTCGGTCTTTATT

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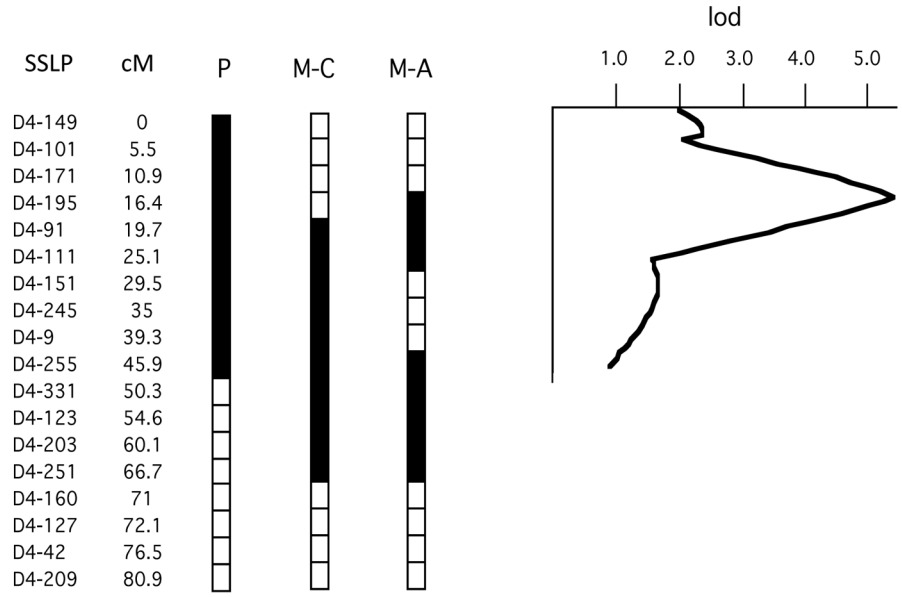


Fig. 1.

B6.D2 congenic lines that were tested for modifying effects. The SSLP markers used to characterize these lines and their position in cM are shown at left. The LOD plot of the QTL analysis for this region reported in (Kuida and Beier 2000) is aligned at right (N = 67).

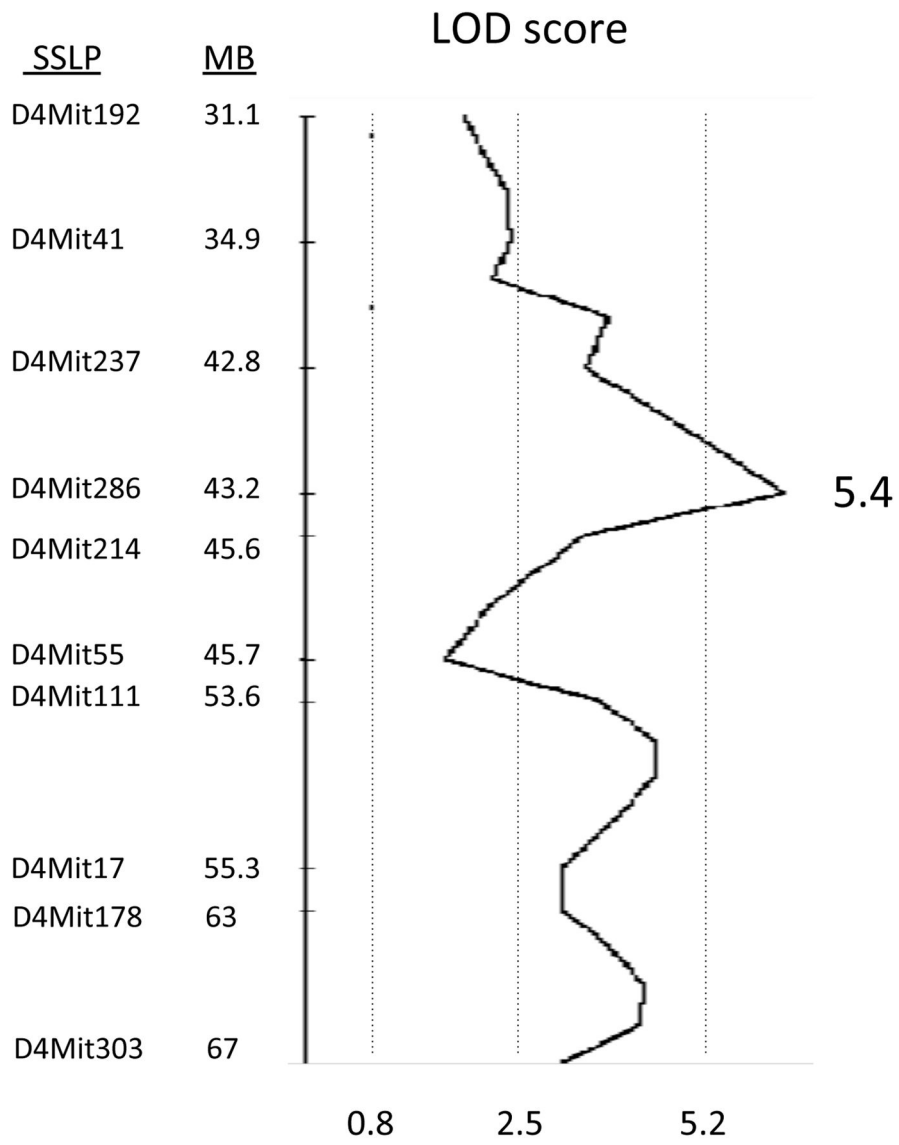
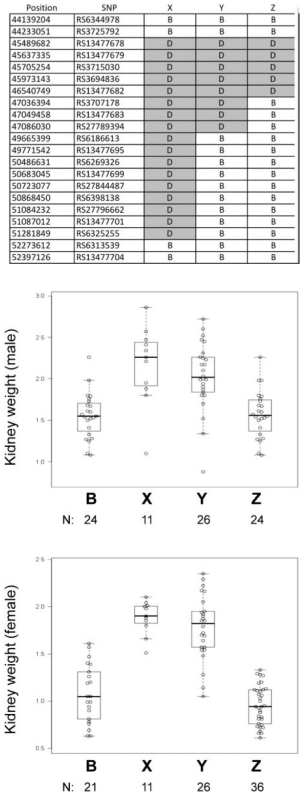


Fig. 2.

QTL analysis of all affected mice from the congenic analysis (N = 39). Permutation analysis was used to calculate LOD values corresponding to levels of suggestive (0.8), significant (2.5), and highly significant (5.2) association.



Position	SNP	X	Y	Z
44139204	RS6344978	B	B	B
44233051	RS3725792	B	B	B
45489682	RS13477678	D	D	D
45637335	RS13477679	D	D	D
45705254	RS3715030	D	D	D
45971143	RS3694836	D	D	D
46540749	RS13477682	D	D	D
47036394	RS3707178	D	D	B
47049458	RS13477683	D	D	B
47086030	RS27789394	D	D	B
49665399	RS6186613	D	B	B
49771542	RS13477695	D	B	B
50486631	RS6269326	D	B	B
50683045	RS13477699	D	B	B
50723077	RS27844487	D	B	B
50868450	RS6398138	D	B	B
51084232	RS27796662	D	B	B
51087012	RS13477701	D	B	B
51281849	RS6325255	D	B	B
52273612	RS6313539	B	B	B
52397126	RS13477704	B	B	B

Fig. 3.

Top panel: SNP genotypes of sub-congenic lines X, Y, and Z. D2-derived sequences shown in red. Combined kidney weight at 7 weeks for *jck* kidneys from these lines is shown separately for males (middle panel) and females (lower panel). The X and Y lines are significantly different from B6, while the Z line is not. (B-X - males: $p=1.4x10^{-5}$, females $p=1.8x10^{-10}$. B-Y - males: $p=1.5x10^{-3}$, females $p=3.2x10^{-9}$.)

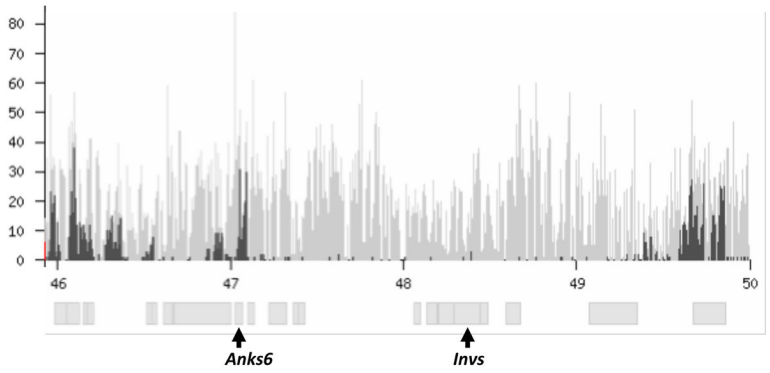


Fig. 4.

SNP variation between 46 Mb and 50 Mb on Chr 4. All variants are shown in grey. SNPs that are polymorphic between B6 and D2 are shown in black (http://phenome.jax.org/db/q?rtn=snp/distplot_staticview&chr=4). The position of the candidate genes *Anks6* and *Invs* are shown.

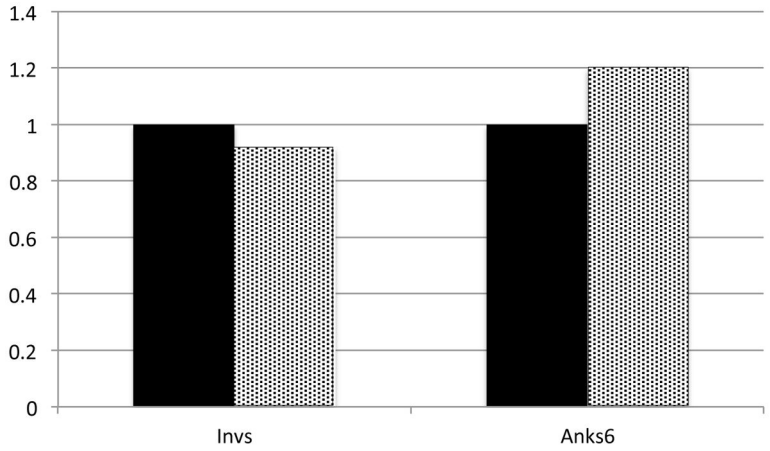


Fig. 5.

qPCR analysis of *Invs* and *Anks6* expression in adult kidneys. Black: B6 alleles (N=3). Grey: D2 alleles (N=3). Expression differences are not statistically significant.

Table 1

28 genes in the 3.2 Mb recombinant region

Genbank	Mb Ch4	Gene
NM_001164804	46549808	*Coro2a*
NM_198664	46617261	*Tbc1d2*
NM_001081141	46676769	*Gabbr2*
NM_001024136	47028560	*Anks6*
NM_172693	47104824	*Galnt12*
NM_009928	47220883	*Col15a1*
NM_009370	47366093	*Tgfbr1*
NM_019998	47482704	*Alg2*
NM_024171	47487532	*Sec61b*
NM_015743	48058176	*Nr4a3*
NM_026343	48137790	*Stx17*
NM_029572	48206202	*Erp44*
NR_035484	48225990	*Mir1958*
NM_001281977	48292631	*Invs*
NM_172304	48443827	*Tex10*
NM_001145925	48552817	*Msantd3*
NM_021436	48598064	*Tmeff1*
NM_026509	48676385	*Murc*
NM_178756	49072333	*Plppr1*
NM_145368	49392716	*Acnat2*
NM_001164565	49456403	*Acnat1*
NM_007519	49502289	*Baat*
NM_178603	49525468	*Mrpl50*
NM_145547	49534047	*Zfp189*
NM_144903	49548864	*Aldob*
NM_025944	49597377	*Tmem246*
NM_001163263	49644931	*Rnf20*
NM_001033351	49674482	*Grin3a*