

Identification of novel chromosomal regions associated with airway hyperresponsiveness in recombinant congenic strains of mice

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Abstract Airway responsiveness is the ability of the airways to respond to bronchoconstricting stimuli by reducing their diameter. Airway hyperresponsiveness has been associated with asthma susceptibility in both humans and murine models, and it has been shown to be a complex and heritable trait. In particular, the A/J mouse strain is known to have hyperresponsive airways, while the C57BL/6 strain is known to be relatively refractory to bronchoconstricting stimuli. We analyzed recombinant congenic strains (RCS) of mice generated from these hyper- and

hypo-responsive parental strains to identify genetic loci underlying the trait of airway responsiveness in response to methacholine as assessed by whole-body plethysmography. Our screen identified 16 chromosomal regions significantly associated with airway hyperresponsiveness (genome-wide $P \leq 0.05$): 8 are supported by independent and previously published reports while 8 are entirely novel. Regions that overlap with previous reports include two regions on chromosome 2, three on chromosome 6, one on chromosome 15, and two on chromosome 17. The 8 novel regions are located on chromosome 1 (92–100 cM), chromosome 5 (>73 cM), chromosome 7 (>63 cM), chromosome 8 (52–67 cM), chromosome 10 (3–7 cM and >68 cM), and chromosome 12 (25–38 cM and >52 cM). Our data identify several likely candidate genes from the 16 regions, including *Ddr2*, *Hc*, *Fbn1*, *Flt3*, *Utrn*, *Enpp2*, and *Tsc*.

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Introduction

Asthma is a common disease of the airways that is characterized by episodes of reversible airway obstruction, airway hyperresponsiveness, and inflammation (Busse and Lemanske 2001). While there is convincing evidence that genetic factors in part are responsible for the development of asthma, most genetic studies have focused on intermediate phenotypes such as total and allergen-specific IgE levels, skin-prick test responses to a number of common allergens, eosinophil counts, and airway responsiveness to methacholine (Wills-Karp and Ewart 2004). There are several advantages to using intermediate phenotypes, rather than a diagnosis of asthma, as traits in linkage and mapping studies. The intermediate phenotypes are objective and quantitative: each is likely to be affected by only a subset of the genetic factors involved in the etiology of asthma

(Palmer et al. 2000), and they can be readily modeled in animals (Zosky and Sly 2007).

A commonly studied intermediate phenotype of asthma is airway hyperresponsiveness. Airway responsiveness is the ability of airways to respond to direct or indirect bronchoconstricting stimuli through airway smooth muscle contraction that results in a reduced airway diameter. The airways of asthmatic patients, however, respond more forcefully to bronchoconstricting stimuli than the airways of healthy nonasthmatic individuals (O'Byrne and Inman 2003). Airway responsiveness can be assessed quantitatively by employing several different techniques and has been used in many studies aimed at identifying asthma susceptibility genes. This phenotype is used because it is a heritable trait and because it precedes the development of asthma, indicating that it either contributes to or is an early marker of asthma pathogenesis (Carey et al. 1996; Cockcroft et al. 1977; Hopp et al. 1990; Palmer et al. 2001). To date, several human chromosomal regions have been linked to airway responsiveness, including regions on chromosomes 2 (Xu et al. 2001), 4 (Daniels et al. 1996), 5 (Postma et al. 1995), 6 (Nicolae et al. 2005), 7 (Daniels et al. 1996), 12 (Raby et al. 2003), and 20 (Van et al. 2002). While the identification of the genes responsible for these associations in human populations has been difficult, some such as *ADAM33*, *DPP10*, *NPSR1* (GPRA), and *HLA-G* (reviewed in Vercelli 2008) have been identified. Many more genes have been identified in association studies, and large-scale multicenter whole-genome association studies that are now underway promise to lead to the identification of significantly more candidate genes in human populations (Moffatt 2008). Animal models, and in particular mice, are necessary, however, to study the molecular networks responsible for specific phenotypes. Studying the genetic regulation of asthmatic responses in animal models offers an alternative approach for the identification of genes in smaller-scale studies where genetic and environmental heterogeneity can be controlled more tightly.

The responsiveness of murine airways is similar to that of their human counterparts and is a heritable trait that varies considerably among inbred strains (Levitt and Mitzner 1989). However, in mouse models, airway responsiveness can be assessed both before and after the experimental induction of allergic airway inflammation. In both cases, the A/J strain has long been known to be one of the most hyperresponsive among the common laboratory strains, and for this reason, crosses involving A/J mice and either the C57BL/6 or the C3H hyporesponsive strain have been the most commonly used in whole-genome association studies of airway responsiveness.

While even the earliest of these studies provided strong evidence that airway responsiveness was a polygenic trait (De Sanctis et al. 1995), the ability of individual

subsequent studies to identify multiple quantitative trait loci (QTLs) was limited by (1) the use of a terminal phenotyping method with genetically unique individuals from intercrosses and/or backcrosses resulting in a single highly variable phenotype measurement for each genotype (De Sanctis et al. 1995, 1999; Ewart et al. 1996, 2000; Zhang et al. 1999), or (2) the use of a selective serial backcross study design which, while leading to the elimination of a very large fraction of the genome, thereby greatly simplifying the genetic complexity and allowing for easier identification of associated genomic regions, can also result in the elimination of loci of interest (Ackerman et al. 2005). Nevertheless, a significant QTL for airway responsiveness following the induction of allergic airway inflammation has been identified on chromosome 2 (Ewart et al. 2000), and suggestive or significant QTLs for airway responsiveness in the absence of allergic inflammation have been identified on chromosomes 6 (De Sanctis et al. 1999; Ewart et al. 1996), 7 (De Sanctis et al. 1999), and 17 (De Sanctis et al. 1999) by employing F₂ intercrosses generated from A/J and C3H mice, while QTLs or significantly associated chromosomal regions have been identified on chromosomes 2 (Ackerman et al. 2005; De Sanctis et al. 1995), 6 (Ackerman et al. 2005), 15 (De Sanctis et al. 1995), and 17 (De Sanctis et al. 1995) by employing various breeding schemes derived from A/J and C57BL/6 mice. Among the several regions identified on these chromosomes, the only candidate gene that has been identified and validated successfully is hemolytic complement (*Hc*, formerly complement factor 5 or C5), which modulates airway responsiveness and is located within the chromosome 2 region identified by Ewart and colleagues (Ewart et al. 2000; Karp et al. 2000).

The AcB/BcA recombinant congenic strains (RCS) were derived from the A/J and C57BL/6 inbred strains of mice in order to aid in the dissection of complex traits. Due to the parental strains' drastically differing airway responsiveness phenotypes, the common use of these strains in genetic studies of airway responsiveness, the ability to phenotype many genetically identical individuals, and the ability to examine the entire genome simultaneously, these mice are an ideal platform for the dissection of this phenotype. This RCS panel has been used successfully to identify QTLs for numerous traits, including amphetamine-induced locomotion (Torkamanzehi et al. 2006), emotionality and stress response (Gill and Boyle 2005b; Thifault et al. 2008), alcohol preference (Gill and Boyle 2005a), withdrawal severity (Kest et al. 2009), and susceptibility to malaria (Fortin et al. 2001a) and *Salmonella* infection (Roy et al. 2006). Each RCS is fully inbred and contains approximately 12.5% of the genome of one parental strain on a background of the other parental strain thus allowing subsets of loci that contribute to the phenotype to be

isolated in different inbred lines (Fortin et al. 2001b). Furthermore, the association analysis is simplified by negating dominance effects and allowing multiple measurements of the phenotype to be performed on several genetically identical animals, thereby reducing the phenotypic noise.

The objective of this study was to replicate the identification of loci that have been previously identified in $A/J \times C57BL/6$ crosses and, in several cases, have not yet been replicated, as well as to detect additional chromosomal regions associated with airway responsiveness. Furthermore, the identification of informative RCS, those strains that exhibit the phenotype of their minor genetic donor, immediately allows the localization of airway responsiveness loci, enabling rapid identification of causative genes.

Materials and methods

Recombinant congenic strain panel and genotyping

Recombinant congenic strains (RCS) of mice were generated at the Montreal General Hospital Research Institute from methacholine-challenge-resistant $C57BL/6$ (B) mice and methacholine-challenge-susceptible A/J (A) mice by creating an $A/J \times C57BL/6$ F_1 cross followed by two backcrosses to each of the original parental strains (Fig. 1) (Fortin et al. 2001b). The resulting progeny (both $[(F_1 \times A) \times A]$, referred to as AcB, and $[(F_1 \times B) \times B]$, referred to as BcA) were then inbred for more than 30 generations, generating 15 distinct AcB strains containing, on average, 13.25% of the B genome on an A background

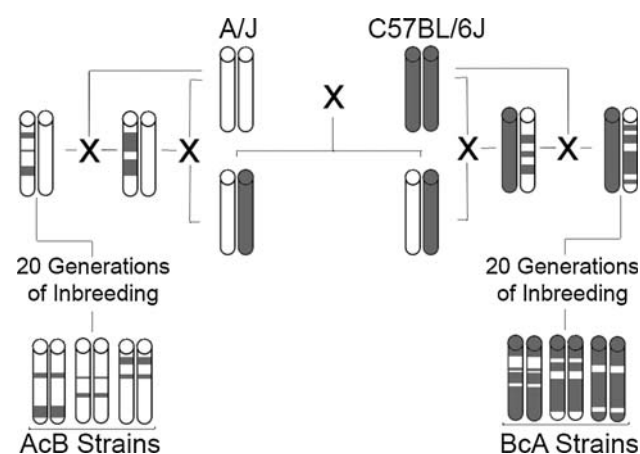


Fig. 1 Breeding scheme employed for the generation of AcB/BcA recombinant congenic strains (RCS). Methacholine-challenge hyper-responsive A/J (A) and hyporesponsive (B) mice were used as parental strains. $(F_1 \times A) \times A$ and $(F_1 \times B) \times B$ mice were inbred for at least 20 generations to produce the AcB and BcA, respectively, inbred strains that constitute the RCS panel

(on average, 84.4% of the A genome) and 22 distinct BcA strains containing, on average, 13.24% of the A genome on a B background (on average, 85.37% of the B genome). All RCS were previously genotyped using a panel of 625 informative SSLP markers (Fortin et al. 2001b). All markers from this panel that are currently mapped to a single position in the Mouse Genome Informatics (MGI) Mouse Genome Database (www.informatics.jax.org) (Bult et al. 2008) were selected for use in our analysis. This yielded 603 markers covering the entire genome with an average spacing of 2.7 cM. All procedures involving live animals were reviewed and approved by the Montreal General Hospital and McGill University animal care and use committees.

Airway responsiveness phenotyping

Airway responsiveness was assessed using an unrestrained whole-body plethysmograph (Buxco Research Systems) and is expressed as the base 2 logarithm of the enhanced pause (Penh) measurement. Ten male mice from each parental strain (A/J and $C57BL/6$) and 4–12 male mice from each of the 11 AcB and 22 BcA strains used in the study were phenotyped at 8–12 weeks of age. In all cases, mice were placed in the plethysmograph chamber and allowed to acclimate for 5 min, after which they were challenged first with a 50- μ l aerosol of phosphate-buffered saline (PBS) administered over 1 min and subsequently with similar aerosols containing 5, 10, 15, and 20 mg/ml of methacholine (in PBS), allowing the Penh parameter to return to baseline between each challenge. After each aerosol challenge the average Penh measured during the 5 min after exposure was recorded. The Penh in response to the aerosol containing 15 mg/ml of methacholine most reliably differentiated the parental strains and was selected for the phenotype, and the base 2 logarithm of the Penh values was used in all analyses in order to normalize the distribution of the phenotype values.

Comparison of the mean phenotype of the AcB strains with the mean phenotype of the BcA strains was performed using a two-tailed unpaired t test. Analysis of the phenotypes across all strains was performed with a one-way ANOVA followed by Bonferroni-corrected pairwise comparisons of every AcB strain with the A strain and every BcA strain with the B strain. In all cases, significance was set at $P \leq 0.05$.

Identification of significantly associated regions

We performed a marker-by-marker analysis using a linear model to detect associations between the observed phenotypes and the genotype, or donor strain of origin (DSO), of the marker while correcting for the variance due to the

predominant background strain. We used the log-transformed median Penh measurement from each RCS as the phenotype and performed the analysis using the following model: $P = \beta_0 dso + \beta_1 bg + \beta_2 dso \times bg + \varepsilon$, where P is the observed phenotype of the strain [median $\log_2(\text{Penh})$ at 15 mg/ml], dso is the donor strain of origin of the marker, and bg is the predominant background (A for AcB strains and B for BcA strains). A permutation analysis consisting of 45,000 permutations was then performed by shuffling the phenotypes across the genotypes (without replacement) to correct for multiple testing. Significance was taken as a P value that was smaller than the smallest P value in more than 95% of the permutations ($P \leq 0.05$ of one or more markers with a more significant P value per whole genome). Regions associated with airway responsiveness were defined as the genomic interval spanning all the sequential markers on a chromosome that were significantly associated with airway responsiveness, and that part of the genome that extends from the set of significantly associated markers to the first marker, on each side, that was not significantly associated with airway responsiveness. If two such regions were separated by a single nonsignificantly associated marker, the interval spanning both regions was considered to form a single region significantly associated with airway responsiveness. The analysis was performed using R version 2.1.0 (www.R-project.org).

Databases used and gene characterization

A gene was considered to be expressed in the lungs if it fulfilled one or more of the following criteria: (1) the MGI Gene Expression Database (GXD) (Smith et al. 2007) listed the gene as detected in the lungs at any stage of development; (2) the cDNA of the gene was listed in the MGI GXD as isolated from lung tissue; or (3) a probe set for the gene was detected in the upper 75th percentile (by signal intensity) of probe sets using Affymetrix microarrays of lung mRNA originating from either the A/J or the C57BL/6 strain (GEO accession GSE2148). The 75th percentile of signal intensity corresponds roughly to all genes whose signal was equal to or greater than the BioB control, considered to be the lower limit of detection on these arrays.

Mutations in or within 2 kb of genes contained within regions identified as significantly associated with airway responsiveness were identified by using the annotations of the Mouse Genome Database (MGD). Genes were labeled as nonsynonymous or splice site (NS/SS) mutants if they contained any variations that were polymorphic between the A/J and C57BL/6 strains and had dbSNP annotations of “Coding-Non Synonymous” or “Splice-Site.” Genes were considered to be synonymous or noncoding mutants if the variations were labeled with any other dbSNP annotation.

The frequency of single nucleotide polymorphisms (SNP) was used to identify intervals that were identical by descent (IBD) within the regions that had been identified as significantly associated with asthma. IBD intervals were inherited by both progenitor strains (A/J and C57BL/6) from a common ancestor and were defined as any continuous interval, at least 500 kb in length, that contained no more than ten SNPs across any 50 kb. IBD intervals were identified by using all SNPs, polymorphic between A/J and C57BL/6 mice, that could be extracted from the MGI MGD.

Results

Airway responsiveness

The difference in airway responsiveness between A/J and C57BL/6 mice has been well characterized by several methods (De Sanctis et al. 1995; Levitt and Mitzner 1989), including the Penh measure acquired by whole-body plethysmography (Ackerman et al. 2005). As expected, the A/J mice in our study exhibited significantly more airway responsiveness than the C57BL/6 mice (Fig. 2). Airway responsiveness was then assessed in the 11 AcB and 22 BcA strains that make up the RCS panel and the distribution of phenotypes is illustrated Fig. 2. A one-way ANOVA was performed and confirmed a strong strain effect, while Bonferroni-corrected post-tests comparing all AcB strains to their major genetic donor parental strain, the A/J mice, and all BcA mice to the C57BL/6 mice identified two AcB strains (AcB64 and AcB51) that were significantly different from the A/J strain and two BcA strains (BcA85 and BcA86) that were significantly different from the C57BL/6 strain.

While the empirical Penh parameter is commonly used as a measure of airway responsiveness in mouse studies, caution must be used when interpreting these results (Bates et al. 2004). Direct measurements of airway resistance were performed in the A/J, C57BL/6, AcB64, BcA85, and BcA86 strains and in several other RCS. We have found perfect concordance between these measurements and the Penh measurements in nonallergic mice among the strains tested, indicating that the use of the Penh parameter is appropriate in the present study (data not shown).

Two additional characteristics of the phenotype are immediately obvious from a cursory examination of Fig. 2. One is that nearly two-thirds of the BcA strains (14 of 22 strains) displayed lower airway responsiveness than the C57BL/6 parental strain, indicating that the hyperresponsive A/J strain likely contains alleles that confer resistance to the methacholine challenge, at least when they are expressed on a C57BL/6 background. Also apparent is the

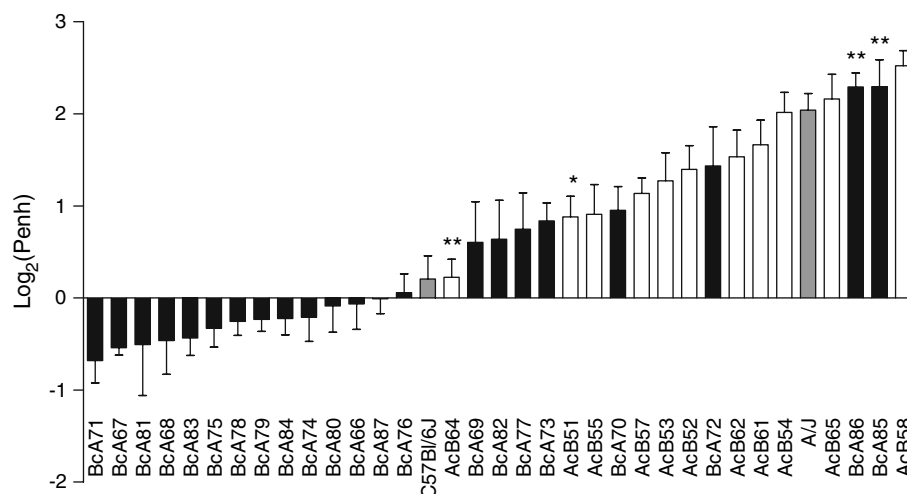


Fig. 2 Strain phenotype distribution pattern of the airway responsiveness phenotypes of the AcB and BcA recombinant congenic strains (RCS) as well as the A/J and C57BL/6 parental strains. Airway responsiveness was assessed as $\log_2(\text{Penh})$ in response to 50 μl of an aerosol containing 15 mg/ml of methacholine delivered over 1 min in

conscious and unrestrained mice using a whole-body plethysmograph. Bars represent the mean value $\pm$ SEM of each inbred strain. The AcB and BcA strains are coloured white and black, respectively, and the A/J and C57BL/6 parental strains are gray. * $P < 0.05$ and ** $P < 0.001$ by Bonferroni-corrected ANOVA

effect of the predominant background on the phenotype of the RCS, with most AcB strains showing high airway responsiveness and most BcA strains low responsiveness. In fact, the average strain mean for the 22 BcA strains was significantly lower than the average strain mean of the 11 AcB strains (Fig. 3).

Marker association

To identify chromosomal regions containing alleles that affect the airway responsiveness phenotype, we performed a test for association at each of the 603 SSLP markers for which the genotypes of the RCS panel were available. Because of the strong effect of the predominant genetic

background (Fig. 3), the test for association of marker genotypes controlled for the predominant background strain (as described in the Materials and methods section). This analysis led to the identification of 50 markers that are significantly associated with airway responsiveness and which delimited the 16 chromosomal regions listed in Table 1.

Of the 16 regions identified as significantly associated with airway responsiveness, 8 replicate QTLs or regions that have previously been implicated in the control of airway responsiveness in crosses involving the A/J strain. These eight replicated regions are distributed among four chromosomes and include the two chromosome 2 regions, the three chromosome 6 regions, the region on chromosome 15, and the two regions on chromosome 17. Furthermore, the proximal chromosome 17 region was also associated with airway responsiveness in a BP-2 $\times$ BALB/c cross. To the best of our knowledge, the remaining eight regions have never been reported to be associated with airway responsiveness. These regions are spread across six chromosomes and include one region each on chromosomes 1, 5, 7, and 8 and two regions each on chromosomes 10 and 12.

Characterization of associated regions

The size of most of the regions identified as significantly associated with airway responsiveness results in too large a number of genes for potential candidates to be identified without further analysis (Table 2). To narrow the list of potential candidates, we used several complementary in silico sequence analysis approaches that employ published

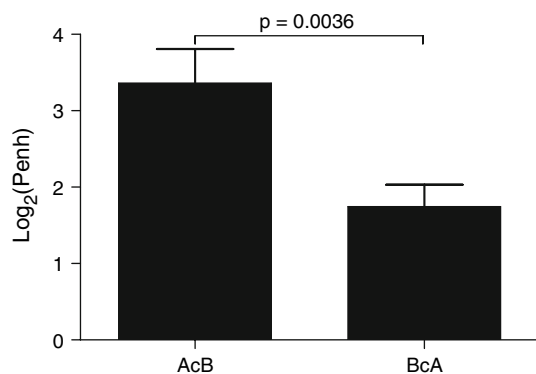


Fig. 3 Mean phenotypes of all tested AcB and BcA strains. The major background strain of each recombinant congenic strain (RCS) has a significant effect on the phenotype. Data presented as the mean $\pm$ SEM of the AcB and BcA strain means. Significance was assessed with a two-tailed t test

Table 1 Chromosomal regions associated with airway hyperresponsiveness in the AcB/BcA RCS panel

Chr.	cM range	Mb range	Significant markers	Peak <i>p</i> value	Prior evidence
1	92.3–100	171.7–179.6	<i>D1Mit113</i> , <i>D1Mit206</i>	7.62×10^{-5}	
2	5–27.3	9.3–40.6	<i>D2Mit80</i> , <i>D2Mit416</i> , <i>D2Mit81</i> , <i>D2Mit293</i> , <i>D2Mit235</i> , <i>D2Mit367</i>	3.30×10^{-5}	Ewart et al. (2000), Karp et al. (2000)
	65–68.9	117.9–148.7	<i>D2Mit133</i> , <i>D2Mit164</i>	4.12×10^{-4}	De Sanctis et al. (1995), Ackerman et al. (2005)
5	73-telomere	132.6-telomere	<i>D5Mit97</i> , <i>D5Mit31</i> , <i>D5Mit99</i> , <i>D5Mit101</i> , <i>D5Mit287</i>	5.45×10^{-5}	
6	0.6–15.6	5.3–34.7	<i>D6Mit264</i> , <i>D6Mit159</i>	4.41×10^{-5}	Ewart et al. (1996), Ackerman et al. (2005)
	38.5–43	92.6–98.5	<i>D6Mit178</i>	3.19×10^{-4}	De Sanctis et al. (1999), Ewart et al. (1996)
	60.55–65.5	125.3–136.4	<i>D6Mit290</i> , <i>D6Mit25</i>	2.48×10^{-4}	Ewart et al. (1996)
7	63.5-telomere	135.7-telomere	<i>D7Mit187</i> , <i>D7Mit259</i>	1.31×10^{-4}	
8	52–67	111.4–129.1	<i>D8Mit271</i> , <i>D8Mit200</i> , <i>D8Mit186</i>	3.04×10^{-4}	
10	3–7	8.5–18.1	<i>D10Mit80</i> , <i>D10Mit246</i>	3.15×10^{-4}	
	67.5-telomere	119.4-telomere	<i>D10Mit205</i> , <i>D10Mit103</i>	1.02×10^{-4}	
12	25–38	55.8–83.7	<i>D12Mit36</i> , <i>D12Mit52</i> , <i>D12Mit14</i>	8.86×10^{-5}	
	52-telomere	109.5-telomere	<i>D12Mit79</i> , <i>D12Mit133</i> , <i>D12Mit280</i> , <i>D12Mit196</i> , <i>D12Mit8</i> , <i>D12Mit134</i>	2.70×10^{-6}	
15	15.6–48.2	17.1–74.7	<i>D15Mit111</i> , <i>D15Mit255</i> , <i>D15Mit100</i> , <i>D15Mit115</i> , <i>D15Mit156</i> , <i>D15Mit105</i>	7.63×10^{-5}	De Sanctis et al. (1995)
17	4.1–10.4	3.9–25	<i>D17Mit113</i> , <i>D17Mit222</i>	2.99×10^{-4}	De Sanctis et al. (1995), Zhang et al. (1999) ^a
	24.5–44.5	47.9–73.9	<i>D17Mit88</i> , <i>D17Mit139</i> <i>D17Mit7</i> , <i>D17Mit159</i>	1.22×10^{-5}	De Sanctis et al. (1999)

Regions were identified as associated with airway responsiveness based on the analysis of the phenotypes of the RCS (Fig. 1) and the 603 known microsatellite polymorphisms of the parental strains (Fortin et al. 2001b). The data were analyzed using a simple generalized linear regression model that controlled for the major genetic donor strain, and significance was assessed based on randomly shuffling the phenotypes 45,000 times across the genotypes. Indicated *p* values are not adjusted for multiple testing

^a In (BP2 × BALB/c)F₂, all other previous evidence used crosses derived from A/J and C57BL/6 or A/J and C3H mice

sequence data. The genetic variation observed in inbred mice, including the RCS used in our analysis, is almost entirely ancestral in origin (it arose before the A/J and C57BL/6 strains split from each other) (Wiltshire et al. 2003). This implies that genomic intervals found in regions significantly associated with airway responsiveness, and where common haplotypes are shared in both the susceptible A/J and the resistant C57BL/6 parental strains, are unlikely to contain the polymorphism responsible for the association. Analysis of the regions identified in the present study reveals that, on average, only one-third of genes lie in intervals that we consider identical by descent (Table 2) and, therefore, by itself, this approach results in a moderate reduction in the list of potential candidate genes, as illustrated for one of the airway responsiveness-associated regions located on chromosome 2 (Table 2, Fig. 4).

Most of the genes affecting airway responsiveness are very likely to be expressed in lung and airway tissues. We used public databases and published expression data (described in the Materials and methods section) to identify which genes located within the significant regions are

expressed in mouse lungs. Analysis of the genes located within the significant regions reveals that there is evidence of pulmonary expression for approximately one-third (34%) of genes (Table 2); this allows for a significant reduction of the number of genes that are likely to be responsible for modulation of the phenotype.

Any genes that are responsible for the association of a chromosomal region with airway responsiveness must contain one or more sequence variants that are polymorphic between the A/J and C57BL/6 parental strains. Therefore, we examined all the genes within the significant regions for known sequence variations. Nearly half (46%) of the genes in these regions contained known polymorphisms that do not result in amino acid changes, while only 12% of the genes contain mutations that result in a difference at the protein level (Table 2).

Likely candidate genes

While each of the approaches used above resulted in only a moderate reduction in the number of potential candidate

Table 2 Characteristics of the genes in each of the 16 regions associated with airway responsiveness

Chr	Region (Mb)	Total genes	A/J vs. C57Bl/6 non-IBD	Synonymous and noncoding mutant genes	Genes expressed in lungs	NS or SS mutant genes	Possible candidates
1	171.8–179.6	124	123	90	56	38	18
2	9.3–40.7	495	234	218	181	32	11
	117.9–148.7	419	232	182	134	64	27
5	132.6-telomere	357	266	174	137	30	11
6	5.3–34.7	189	84	76	59	8	5
	92.6–98.5	25	20	18	8	1	1
	125.3–136.4	183	175	88	44	51	14
7	135.7-telomere	257	80	65	99	13	3
8	111.4–129.1	232	132	111	98	18	10
10	8.5–18.1	45	45	24	14	7	3
	119.4-telomere	158	130	83	80	11	4
12	55.8–83.7	224	164	135	83	18	7
	109.5-telomere	176	176	71	50	34	10
15	17.1–74.7	280	173	130	86	23	9
17	3.9–25.0	338	308	168	81	66	16
	47.9–73.9	208	135	87	74	23	8
	Total	3710	2477	1720	1284	437	157

Values are the number of genes in each region that are associated with airway responsiveness based on the RCS panel survey and which are expressed in the lungs (based on MXD database and microarray data), which are in regions that are nonidentical by descent (non-IBD) between A/J and C57Bl/6 mice (according to SNP density, data from MGI), which contain nonsynonymous (NS) or splice-site (SS) mutations (from MGI database), or which contain synonymous mutations or mutations in noncoding regions (from MGI database). Note that these numbers exclude all olfactory receptor genes which are considered extremely unlikely candidates, and include expressed sequence tags, predicted genes, and cDNA clones

genes, the simultaneous use of several approaches has the potential to produce a more manageable list of potential candidates for each region. Requiring that potential candidate genes be expressed in the lungs and that they contain a polymorphism between the A/J and C57BL/6 parental strains which leads to a change in amino acid sequence resulted in a list of 1–27 possible candidate genes for each of the chromosomal regions (Supplementary Table 1). A literature search was performed for each of the genes in order to identify those involved in processes related to asthma, lung function and development, airway function, or smooth muscle contraction. This resulted in a small number of candidate genes for some of the regions identified as significantly associated with airway responsiveness. These likely candidate genes are listed in Table 3.

Discussion

In this study we employed an RCS panel derived from the airway hyperresponsive A/J strain and the hyporesponsive C57BL/6 strain to identify chromosomal regions associated with airway responsiveness to methacholine as assessed by the whole-body plethysmography enhanced pause (Penh) method. Consistent with a complex pattern of inheritance

involving several loci, the distribution of the airway responsiveness phenotypes of the RCS panel was continuous and extended beyond both parental strains. We observed that the background strain (A/J in AcB mice and C57BL/6 in the BcA mice) had a significant effect on the airway responsiveness phenotype, as is the case with a number of other phenotypes which segregate in the AcB/BcA RCS mice (Gill and Boyle 2005b; Thifault et al. 2008).

Previous studies in crosses derived from the A/J and C57BL/6 strains have identified four QTLs, one on distal chromosome 2, and one each on chromosomes 6, 15, and 17 (Ackerman et al. 2005; De Sanctis et al. 1995), while crosses derived from the A/J and C3H strains led to the identification of distinct QTLs on proximal chromosome 2 and chromosome 7 and QTLs spanning most of chromosome 6 (De Sanctis et al. 1999; Ewart et al. 1996, 2000). Of these, the QTL on distal chromosome 2 was replicated in at least two independent studies employing crosses derived from A/J and C57BL/6 mice, and a set of partially overlapping QTLs on chromosome 6 have been identified in crosses derived from A/J and both C57BL/6 and C3H mice. The analysis performed in the present study independently identified significant association with airway responsiveness for SSLP markers overlapping all these previously identified QTLs, with the exception of the QTL on

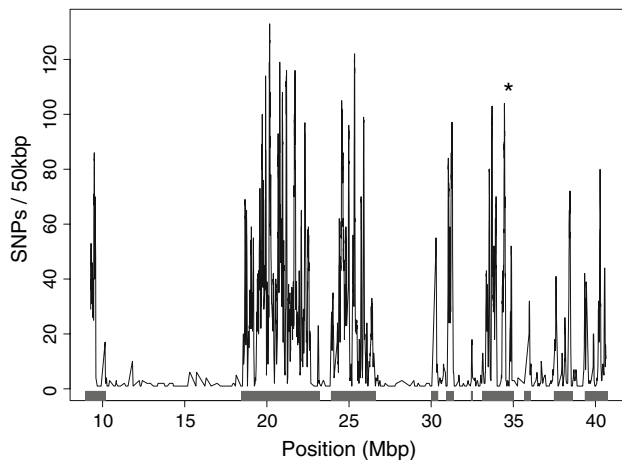


Fig. 4 The graph represents a 50-kb sliding window of the number of single nucleotide polymorphisms (SNP) between the A/J and C57BL/6 parental strains on chromosome 2. The dotted line indicates 10 SNP per 50 kb. The gray bars indicate regions that are not identical by descent based on a 10 SNP per 50 kb cutoff as detailed in the Materials and methods section. The asterisk indicates the position of *Hc* demonstrated by Karp et al. (2000) to influence airway responsiveness

chromosome 7. Given that the chromosome 7 QTL was identified in a cross involving C3H mice, it is possible that the locus responsible for the QTL is simply not polymorphic between the A/J and C57BL/6 strains. Ackerman et al. (2005) also reported the existence of significant epistatic interaction between QTLs on chromosomes 2 and 6, and while we replicated each of the QTLs, our study could not effectively test for these effects due to the large number of chromosomal regions associated with airway responsiveness and the small number of genotypes (33 RCS strains). Overall, given how well the RCS replicate QTLs derived from both A/J $\times$ C57BL/6 and A/J $\times$ C3H crosses, our results indicate that there may be significant overlap of the loci that mediate differential airway responsiveness in A/J mice when compared to both strains.

While the crosses most commonly used in the genetic analysis of airway responsiveness are derived from A/J mice, other strain combinations have also been analyzed. QTLs for airway responsiveness following the induction of allergic airway inflammation have been identified on chromosomes 9, 10, 11, and 17 in a (BALB/c $\times$ BP2)F₂

Table 3 Likely candidate genes selected from regions identified as significantly associated with airway hyperresponsiveness

Chr. (Mb)	Symbol	Name	Notes
1 (171.9)	<i>Ddr2</i>	Discoidin domain receptor family, member 2	Expressed in the lungs Contains a nonsynonymous SNP May contribute to the regulation of ECM collagen (Mihai et al. 2006)
2 (34.8)	<i>Hc</i>	Hemolytic complement	Expressed in the lungs Contains several nonsynonymous variations Has been shown to modulate airway responsiveness by Karp et al. (2000)
2 (125.3)	<i>Fbn1</i>	Fibrillin 1	Expressed in the lungs Contains a nonsynonymous SNP Involved in extracellular matrix deposition/maintenance Mutations lead to Marfan syndrome in humans Knockout mice display several lung morphology phenotypes (Neptune et al. 2003)
10 (12.6)	<i>Utrn</i>	Utrophin	Expressed in the lungs Contains a nonsynonymous SNP It is necessary for the normal clustering of acetylcholine receptors at the neuromuscular synapse (Huh and Fuhrer 2002)
15 (54.7)	<i>Enpp2</i>	Ectonucleotide pyrophosphatase/phosphodiesterase 2	Expressed in the lungs Contains a nonsynonymous SNP Previously identified as a candidate gene for the control of lung function in mice (Ganguly et al. 2007)
17 (24.7)	<i>Tsc2</i>	Tuberous sclerosis 2	Expressed in the lungs Contains a nonsynonymous SNP Implicated in the potential regulation of airway smooth muscle growth and differentiation (Krymskaya 2007)

Likely candidates identified for selected chromosomal regions associated with airway responsiveness. Candidates were identified on the basis of their genomic location, comparative analysis of sequence variants observed in the A/J and C57BL/6 parental strains, and known pulmonary expression. A literature search revealing involvement in processes known, or suspected, to modulate airway responsiveness was used to identify the most likely candidates from the list of candidate genes

cross (Zhang et al. 1999) and a locus has been identified on chromosome 11 in a cross derived from BALB/c and DBA/2 mice (McIntire et al. 2001). While one region identified in the RCS overlapped the QTL on chromosome 17, the remaining QTLs were not replicated. While these studies used an allergic model, the relative paucity of overlap between these strains and those identified in crosses derived from A/J and either C57BL/6 or C3H mice suggests that the large number of loci that modulate airway responsiveness identified in these latter strains may not represent the full spectrum of genetic factors that affect the airway responsiveness phenotype.

In addition to replicating several previously described QTLs, we have identified eight novel regions across six chromosomes. These regions are significantly associated with airway responsiveness but contain no previously reported airway responsiveness QTLs. The regions vary in size from 8 to 28 Mb and contain between 45 and 357 genes. While both these novel regions and the regions that replicate previously identified QTLs remain too broad to readily identify relevant candidate genes, we applied several concurrent *in silico* approaches to narrow the list of potential candidates. Requiring that potential candidate genes be expressed in the lungs and that they contain at least one mutation leading to an amino acid change ensures that the resulting list contains the genes that are most likely to result in significant biological effects. The potential candidate gene lists that result from applying these two criteria contained between 1 and 27 genes per region (Table 2), allowing likely candidate genes to be identified based on gene functional annotations and a review of the literature.

This approach allowed us to identify several likely candidate genes involved in smooth muscle function, including *Tsc2* (tuberous sclerosis 2), which is located within the proximal chromosome 17 region and involved in the phosphatidylinositol 3-kinase-mediated control of airway smooth muscle cell growth and proliferation (Krymskaya 2007), and *Utrn* (utrophin), which is involved in acetylcholine receptor clustering at neuromuscular junctions (Huh and Fuhrer 2002). Given that airway smooth muscle contraction is the defining feature of airway responsiveness, genes that affect smooth muscle function are likely to be capable of modulating the airway responsiveness phenotype. Other likely candidates include genes that modulate the deposition and maintenance of the extracellular matrix (ECM) which has long been associated with changes in airway function (Tran and Halayko 2007). The discoidin domain receptor family member 2 (*Ddr2*) gene is located within the region identified on chromosome 1. The extracellular domain of the *Ddr2* protein can bind collagen resulting in an inhibition of collagen fibrillogenesis (Mihai et al. 2006), thereby affecting the ECM. Fibrillin 1 (*Fbn1*) was identified as a likely candidate gene

residing in the distal chromosome 2 region and is an ECM component found in extracellular microfibrils. The severe morphological abnormalities in the lungs of *Fbn1*-deficient mice suggest that mutations in this gene could lead to significant changes in pulmonary physiology that are likely to affect airway responsiveness (Neptune et al. 2003). Two other likely candidate genes are ectonucleotide pyrophosphatase/phosphodiesterase 2 (*Enpp2*), which is located within the chromosome 15 region and has previously been associated with lung function phenotypes (Ganguly et al. 2007), and hemolytic complement (*Hc*, formerly C5), which has been demonstrated to explain, at least partially, a QTL identified in a cross derived from A/J and C3H mice that overlaps the region identified on proximal chromosome 2 (Karp et al. 2000).

The identification of informative strains from the RCS panel provides an important tool for the further dissection of the airway responsiveness trait. Of the 33 strains that made up the RCS panel, three were found to be particularly interesting in this regard. The AcB64, BcA85, and BcA86 strains exhibit a phenotype that is indistinguishable from that of their minor genetic donors while carrying only 12.5% of their genome, which includes only five or six of the regions identified as significantly associated with airway responsiveness. These three informative RCS together contain recombinant segments for 12 of the 16 regions identified in the present study; however, each strain contains a subset of only five or six regions indicating that the parental phenotypes can be reproduced with more than one set of parental alleles and implying that the effects of the loci do not act in a strictly additive manner. Because they significantly simplify the genetic model (5 or 6 loci in each strain compared with the 16 potential loci identified in the RCS panel as a whole), these informative congenic strains will provide an ideal genetic background to conduct further studies aimed at reducing the size of the regions in order to identify and confirm candidate genes.

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Supplemental Table 1: Lists of the possible candidate genes from Table 2.

Chr	Region (Mb)	Symbol	Mutation(s)	Name
1	171.8 – 179.6	Adamts4	Q30R	A disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin type 1 motif, 4
		Ai607873	K862R	Expressed sequence ai607873
		Apoa2	P28Q D43E M49V	Apolipoprotein a-II
		Atf6	V141I	Activating transcription factor 6
		Cd48	N90D	CD48 antigen
		Cd84	V27M K293E	CD84 antigen
		Copa	N984S	Coatamer protein complex subunit alpha
		Crp	P134A S191N	C-reactive protein, petaxin related
		Ddr2	D555G	Discoidin domain receptor family, member 2
		Dusp12	P80Q V42T	Dual specificity phosphatase 12
		Fcgr2b	L200Q P205T S297I	Fc receptor,IgG, low affinity IIb
		Fcgr3	W73G S74R S92A	Fc receptor,IgG, low affinity III
		Ifi203	R198Q	Interferon activated gene 203
		Klhdc9	T6P R171G	Kelch domain containing 9
		Ly9	G14S I79T I139T E377K M550I G592E	Lymphocyte antigen 9
		Pex19	P55S	Peroxisome biogenesis factor 19
		Pigm	Q206H	Phosphatidylinositol glycan, class m
		Sdhc	F4L S10G	Succinate dehydrogenase complex, subunit c, integral membrane protein
2	9.3 – 40.7	Arhgap21	F288S I1487T P1488L S1493F P1494S	Rho GTPase activating protein 21
		C8g	R168Q	Complement component 8, gamma polypeptide
		Entpd2	R149Q	Ectonucleoside triphosphate diphosphohydrolase 2
		Etl4	A71V T602A V1276M D1743G	Enhancer trap locus 4
		Hc	P699L	Hemolytic complement

117.9 – 148.7	Lhx3	N1002D D248E S249D	Lim homeobox protein 3
	Nr5a1	A172S	Fushi tarazu 1 factor homolog, (Drosophila)
	Ptgds	I115V	Prostaglandin D2 synthase (brain)
	Sec16a	M185T	SEC16 homolog A (S. Cerevisiae)
	Stxbp1	D216N	Syntaxin binding protein 1
	Wdr85	Q33R A136T N393S	WD repeat domain 85
	B2m	A105D	beta-2 microglobulin
	Blvra	R192Q	biliverdin reductase A
	Bub1	R187K	budding uninhibited by benzimidazoles 1 homolog (S. cerevisiae) [
	Catsper2	S49A K486E T515I	cation channel, sperm associated 2
	Cd93	N264H	CD93 antigen
	Dll4	Q419L G627S	delta-like 4 (Drosophila)
	Dnajc17	Q152R F273Y	DnaJ (Hsp40) homolog, subfamily C, member 17
	Epb4.2	C298S K450R S528R	erythrocyte protein band 4.2
	Fbn1	N2774S	fibrillin 1
	Hisppd2a	Y1180A	histidine acid phosphatase domain containing 2A
	Lcmt2	K56R E196K D245H V322D L630P	leucine carboxyl methyltransferase 2
	Ltk	T26A V113A V477M H563Q	leukocyte tyrosine kinase
	Mageb3	A90P	melanoma antigen, family B, 3
	Mapkbp1	H1056R Q1090R H1167R Q1212R	mitogen-activated protein kinase binding protein 1
	Ndufaf1	S32N	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, assembly factor 1
	Nusap1	T104N R201H	nucleolar and spindle associated protein 1
	Pldn	T32M	Pallidin
	Ralgapa2	K447E	Ral GTPase activating protein, alpha subunit 2 (catalytic)
	Rin2	R340S	Ras and Rab interactor 2
	Rpap1	H83R N942K R1066Q	RNA polymerase II associated protein 1
	Rrbp1	S103P	ribosome binding protein 1
	Slc30a4	H426Q	solute carrier family 30 (zinc transporter),

5	132.6 – Telomere	Stard7	Y45H	member 4
		Tmem62	I579M	START domain containing 7
		Trp53bp1	V396A	transmembrane protein 62
			A422T	transformation related protein 53 binding
			K1066N	protein 1
		Ubr1	A1496T	ubiquitin protein ligase E3 component n-
				recognin 1
		Zfyve19	S94G	zinc finger, FYVE domain containing 19u
		Clip2	V25I	CAP-GLY domain containing linker protein 2u
			N1003D	
		Cyp3a16	C28Y	cytochrome P450, family 3, subfamily a,
				polypeptide 16
		Eif2ak1	V526A	eukaryotic translation initiation factor 2 alpha
				kinase 1
		Eif4h	N155S	eukaryotic translation initiation factor 4Hu
6	5.3 – 34.7	Flt1	D85N	FMS-like tyrosine kinase 1u
			T97M	
		Flt3	L760S	FMS-like tyrosine kinase 3
		Gtf2i	V5A	general transcription factor II I
		Rnf6	P3L	ring finger protein (C3H2C3 type) 6u
			H346R	
		Zan	T2586A	Zonadhesin
			E2638D	
			A2987T	
			H3081R	
			S3092L	
			S3467T	
			Q4376E	
		Zfp68	Q180H	zinc finger protein 68
			S201P	
6	92.6 – 98.5		I275T	
		Zkscan5	V186A	zinc finger with KRAB and SCAN domains 5
	5.3 – 34.7	Akr1b3	Frame shift	aldo-keto reductase family 1, member B3
				(aldose reductase)
		Glcci1	W74STOP	glucocorticoid induced transcript 1
		Ica1	Q397R	islet cell autoantigen 1
		Smo	D54N	smoothened homolog (Drosophila)
	92.6 – 98.5		R690G	
		Tes	P122L	testis derived transcript
		Frmd4b	M581V	FERM domain containing 4B
			S932N	
	125.3 – 136.4	Dusp16	Y425C	dual specificity phosphatase 16
		Gpr19	S20G	G protein-coupled receptor 19
		Gsg1	T309A	germ cell-specific gene 1
		Hebp1	L86V	heme binding protein 1
		Klrb1a	Q110H	killer cell lectin-like receptor subfamily B
6	125.3 – 136.4		G182D	member 1A
		Klrc1	Y148H	killer cell lectin-like receptor subfamily C,
				member 1

		Klri2 Klrk1	K22E H10Y L71V	killer cell lectin-like receptor family I member 2 killer cell lectin-like receptor subfamily K, member 1
		Lrp6	T83S L317M	low density lipoprotein receptor-related protein 6
		Prh1	H116P Q130P T180P S228R	proline rich protein HaellI subfamily 1
		Prpmp5 Pzp	R274G I541V H1043R	proline-rich protein MP5 pregnancy zone protein
		Rad51ap1	I5T E114G G120D H124R T103M	RAD51 associated protein 1 [Von Willebrand factor homolog
7	135.7 - Telomere	E030019B06R ik Lrdd Nsmce4a	S231P Frame shift R23C	RIKEN cDNA E030019B06 gene leucine-rich and death domain containing non-SMC element 4 homolog A (S. cerevisiae)
		2810004N23R ik	V130G P136A	RIKEN cDNA 2810004N23 gene
8	111.4 – 129.1	Agt	I182V V203M T321A	angiotensinogen (serpin peptidase inhibitor, clade A, member 8)
		Ankrd11 Dpep1 Gm505 Gnpat Klhdc4 Sipa1l2	S77G N358S R442Q F421L S337A D3E R1649Q	ankyrin repeat domain 11 dipeptidase 1 (renal) predicted gene 505 glyceronephosphate O-acyltransferase kelch domain containing 4 signal-induced proliferation-associated 1 like 2
		Spg7 Sult5a1	R18Q R4H	spastic paraplegia 7 homolog (human) sulfotransferase family 5A, member 1
10	8.5 – 18.1	Hivep2	M2122L	human immunodeficiency virus type I enhancer binding protein 2
		Stx11 Utn	N24D S1121L	syntaxin 11 Utrophin
	119.4 - Telomere	BC048403	R169K	cDNA sequence BC048403
		Cs Pan2 Pip4k2c	H55N D822E L276M	citrate synthase pancreas protein 2 phosphatidylinositol-5-phosphate 4-kinase, type II, gamma
12	55.8 – 83.7	Clec14a	T166A	C-type lectin domain family 14, member a
		Ctage5 Dact1 Foxa1	Splice Site P504L R648P H389R	CTAGE family, member 5 dapper homolog 1, antagonist of beta-catenin (xenopus) forkhead box A1

		Gpx2 Nin	V14I S1084Y R1085Q	glutathione peroxidase 2 Ninein
		Rtn1	E76G	reticulon 1
	109.5 - Telomere	Al132487	P59S T127A	expressed sequence Al132487
		Cdca4 Cdca7l Cyp46a1	T213A N114S M271V	cell division cycle associated 4 cell division cycle associated 7-like cytochrome P450, family 46, subfamily a, polypeptide 1
		Dync1h1 Eml1	I3856V A82S	dynein cytoplasmic 1 heavy chain 1 echinoderm microtubule associated protein like 1
		Jag2	N378S R1223C	jagged 2
		Siva1 Slc25a29	S59I I24T	SIVA1, apoptosis-inducing factor solute carrier family 25 (mitochondrial carrier, palmitoylcarnitine transporter), member 29
		Traf3	R25H	TNF receptor-associated factor 3
15	17.1 – 74.7	Asap1	L879S	ArfGAP with SH# domain, ankyrin repeat and PH domain1
		Cct5	M48I	chaperonin containing Tcp1, subunit 5 (epsilon)
		Enpp2	N743D	ectonucleotide
		Laptm4b	I133V	pyrophosphatase/phosphodiesterase 2 lysosomal-associated protein transmembrane 4B
		Myo10 Rad21 Ropn1l Trio Trps1	P350S F374S K202R G78S V813L	myosin X RAD21 homolog (S. pombe) ropporin 1-like triple functional domain (PTPRF interacting) trichorhinophalangeal syndrome I (human)
17	3.9 – 25.0	4931440B09Ri k	I57V	RIKEN cDNA 4931440B09 gene
		Abca3	Q1069STO P	ATP-binding cassette, sub-family A (ABC1), member 3
		Ccnf	F1143L V508I T539A M773T	cyclin F
		Fahd1	I202V	fumarylacetoacetate hydrolase domain containing 1
		Has1	A40T H129R V461L	hyaluronan synthase1
		Mrpl18 Park2	E91K E398Q	mitochondrial ribosomal protein L18 Parkinson disease (autosomal recessive, juvenile) 2, parkin
		Pkd1	R771Q E871D I985V A1180T	polycystic kidney disease 1 homolog

		H1292R	
		A1632V	
		S1684A	
		R2085C	
		A2507V	
	Plg	G110S	Plasminogen
		L649S	
	Slc9a3r2	L4P	solute carrier family 9 (sodium/hydrogen exchanger), member 3 regulator 2
		P32S	
	Tbl3	R475Q	transducin (beta)-like 3
		A701T	
	Tmprss8	T242I	transmembrane protease, serine 8 (intestinal)
	Tsc2	Splice Site	tuberous sclerosis 2
	Zfp213	T157A	zinc finger protein 213
		E283Q	
	Zfp40	N648H	zinc finger protein 40
	Zfp598	R851K	zinc finger protein 598
47.9 – 73.9	C230073G13	C138F	RIKEN cDNA C230073G13 gene
	Rik		
	Foxp4	T776M	forkhead box P4
	Lama1	D656N	laminin, alpha 1
	Mdfi	R67G	MyoD family inhibitor
	Myom1	S1253G	myomesin 1
	Sgol1	S134P	shugoshin-like 1 (S. pombe)
		A462F	
	Tnfsf9	Q142K	tumor necrosis factor (ligand) superfamily, member 9
	Tspo2	A146T	translocator protein 2
Listed genes are located in intervals which are significantly associated with airway responsiveness in the AcB/BcA RCS panel, are expressed in the lungs of mice, and contain at least one non-synonymous or splice-site polymorphism between the A/J and C57BL/6 progenitor strains. Non-synonymous mutations are compared to the reference assembly.			