Mapping QTL associated with resistance to avian oncogenic Marek's Disease Virus (MDV) reveals major candidate genes and variants

Short title: Candidate genes for Marek's Disease resistance

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Abstract

Marek's Disease (MD) represents a significant global economic and animal welfare issue. Marek's Disease Virus (MDV) is a highly contagious oncogenic and highly immune-suppressive alpha-herpes virus, which infects chickens, causing neurological effects and tumour formation. Though partially controlled by vaccination, MD continues to have a profound impact on animal health and on the poultry industry. Genetic selection provides an alternative and complementary method to vaccination. However, even after years of study, the genetic mechanisms underlying resistance to MDV remain poorly understood. The MHC is known to play a role in disease resistance, along with a handful of other. In this study, one of the largest to date, we used a multi-facetted approach to identify QTL regions (QTLR) influencing resistance to MDV, including an F_6 population from a full-sib advanced intercross line (FSIL) between two elite commercial layer lines differing in resistance to MDV, RNA-seq information from virus challenged chicks, and genome wide association study (GWAS) from multiple commercial lines. Candidate genomic elements residing in the QTLR were further tested for association with offspring mortality in the face of MDV challenge in 8 pure lines of elite egg-layer birds. Thirty-eight QTLR were found on 19 chicken chromosomes. Candidate genes, miRNAs, lncRNAs and potentially functional mutations were identified in these regions. Association tests were carried out in 26 of the QTLR, using 8 pure lines of elite egg-layer birds. Numerous candidate genomic elements were strongly associated with MD resistance. Genomic regions significantly associated with resistance to MDV were mapped, and candidate genes identified. Various QTLR elements were shown to have strong genetic association with resistance. These results provide a large number of significant targets for mitigating the effects of MDV infection on both poultry health and the economy – whether by means of selective breeding, improved vaccine design or gene-editing technologies.

Keywords: Marek's Disease Virus, QTL, GWAS, transcriptomics, FSIL, disease resistance, candidate gene

Author summary

Marek's Disease has a huge impact on the global poultry industry in terms of both animal welfare and economic cost. For many years, researchers have sought to identify the genes underlying resistance to Marek's Disease Virus (MDV). However, this is a complex trait with each genetic locus having a small effect, so identifying causal genes and variants is no easy task. To date, it is known that the MHC confers differing susceptibility/resistance. A few other non-MHC genes have also been implicated in disease resistance, although based on experimental inbred lines and not representing real world commercial poultry. Using an F_6 intercross population of birds with differences in MDV survival, we have identified many regions of the genome involved in resistance and highlighted candidate genes, miRNAs and lncRNA. Access to DNA from phenotyped birds spanning 15 generations of 8 elite commercial lines has provided a unique opportunity for us to show genetic association of markers in these transcripts with MDV survival. This genetic study, the largest to date, provides novel targets for mitigation of Marek's Disease within the poultry industry. This could be through selective breeding strategies, improved vaccine design or future gene editing technologies.

Introduction

Marek's Disease (MD) represents a significant global economic and animal welfare issue. This immunosuppressive disease is responsible for an estimated 2 billion USD annual economic loss to the global poultry industry [1], through bird mortality, lost egg production and vaccination costs. The virus responsible, Marek's Disease Virus (MDV), is an alpha-herpes virus that initially infects B-cells, experiences a latency period and can then proceed to develop as an oncogenic disease after infection of T-cells [2]. Furthermore, if birds do not succumb to MDV itself, they are often left severely compromised with secondary infections such as *E. coli* [3]. The commercially utilized vaccines are not sterilizing vaccines. They prevent the formation of tumours, but do not prevent infection by MDV or shedding of the pathogenic virus [4]. Both vaccine and pathogenic MDVs are found in vaccinated flocks, resulting in the emergence of increasingly more virulent strains [5]. As more virulent strains emerge, vaccine treatments are becoming less and less effective [6].

In the race to keep ahead of this viral evolution, genetic selection is a possible tool to aid in breeding for viral resistance. Indeed, genetic selection following multiple generations of progeny challenge has been shown to improve survival in commercial chicken populations [7]. Selection in the poultry industry works relatively well, but knowing the causative elements for resistance would provide a route for much more precise selection. However, the underlying genetic changes, and the genes and specific variants impacting this genetic resistance are still largely unknown. For decades, researchers have sought to identify the genes responsible for MDV resistance, with limited success. It has become clear that many QTL/genes are involved in the resistance phenotype, with resistance not being a simple trait with one or a few major genes but a classic quantitative trait with many genes of small effect, thus making it difficult to identify causal variants [8].

Marek's Disease is also of interest from a clinical perspective, as it can serve as a model for human lymphoma. MDV-induced lymphomas are found to over-express the Hodgkin's disease antigen CD30, with expression correlating with the viral Meq oncogene [9]. Indeed, after infection, the chicken CD30 promoter has also been shown to be hypo-methylated [10]. Identifying the genetic mechanisms underlying MDV resistance is therefore not only of great importance to the poultry industry but will also have implications for increasing our understanding of human cancers.

Different MHC haplotypes are known to confer different susceptibilities to the virus [11-13]. Studies have also reported the influence of non-MHC regions on resistance to MDV [14]. Several studies have implicated specific genes in MDV resistance/susceptibility. These include *GH1* [15], *SCYC1* [16], *SCA2* [17], *IRG1* [18], *CD79B* [19] and *SMOC1* and *PTPN3* [20]. These studies are usually done, not with relevant commercial lines, but with experimental or inbred lines and examine whole tissues, although recent work has investigated the host response to MDV in specific cells such as macrophages, which are an early target for the virus [21,22]. Recent studies on the role of lncRNAs [23,24] and miRNAs (in both the host and virus) have also been carried out [25-27], including study of serum exosomes from lymphoma-bearing birds [28]. In addition, the role of epigenetics in resistance to MDV has been studied, with regions of differential methylation between susceptible and resistant lines of birds highlighted [29,30].

Here, the availability of large-scale, phenotyped commercial populations, genome wide analysis technologies and an F_6 advanced intercross line [31], has given us the opportunity to carry out, for the first time, a high-resolution analysis of genes underlying MDV resistance in commercially relevant populations. We use multiple genetic resources at our disposal, including the F_6 population of an advanced full-sib inter-cross line (FSIL) previously analysed in a low-resolution study for MD resistance using microsatellite markers to identify genomic

119 regions associated with survival following MD challenge [31]. Genomic DNA of the original
120 10 founder individuals and the subsequently produced F_6 was available for fine mapping
121 through genome sequencing, and/or genotyping using a genome-wide 600K SNP chip [32].
122 Furthermore, an extensive multi-generation (15) and multi-line (8) collection of DNAs from
123 progeny-challenged males was available to further examine candidate genes and related
124 variants associated with survival in the face of MDV infection.

125 In this report we reveal for the first time MD as a true complex trait, controlled by many
126 QTL. Integration of multiple lines of evidence (F_6, multi-generation/multi-line collection, host
127 gene expression responses to viral infection, genome annotations, etc.) on a large scale enabled
128 a high-resolution analysis that predicted mutations within genes, miRNAs and lncRNAs highly
129 associated with MDV response in commercial egg production lines. This analysis not only
130 provides new markers for MD resistance but also reveals more about the biology behind the
131 mechanism of MDV susceptibility, information that should lead to more precise selection
132 strategies in the future.

133

134 # Materials and methods

135 ## Experimental animals

136 *Full Sib Advanced Inter-cross Line (FSIL)*

137 F_6 birds from the FSIL were used to map QTL affecting MD resistance. The development
138 of the FSIL F_6 challenge population has been previously described [31,33,34]. It was initiated
139 from a cross of two partially inbred commercially utilized elite White Leghorn lines, known to
140 differ in their resistance to MDV. Five independent FSIL families were developed and
141 expanded over five generations. In all five families, the male parent was from the more resistant
142 line and the female parent was from the less resistant line.

143 At the F_6 generation, 1,615 chicks were challenged with vv+ MDV strain 686 following
144 the protocol of Fulton et al. [7]. The experiment was carried out in two hatches; it was
145 terminated at 152 days of age in the first hatch, and after 145 days of age in the second.
146 Resistance was measured as survival time (age at death). For birds that survived to the end of
147 the experiment, survival time was taken as age at end of the experiment. To standardise the two
148 hatches, survival for all birds that survived to end of experiment was set to 149 days. This
149 measure of resistance was used in all association tests.

150 This population was segregating for two MHC haplotypes (B^2 and B^{15}). Given the known
151 strong association of MHC-type with MD resistance, all analyses were done within MHC-type
152 within each family.

153

154 *Pure line Pedigreed sire populations with daughter progeny records for MDV mortality*

155 As part of the routine selection process within the Hy-line (HL) breeding program,
156 individual males were mated to multiple females to produce 30 half-sib female progeny per
157 sire. The dams were MD vaccinated following normal industry practices. Progeny were
158 vaccinated at 1 day of age with HVT/SB1 and at 7 days of age inoculated subcutaneously with
159 500 PFU of vv+ MDV (provided by Avian Disease and Oncology Lab, East Lansing MI).
160 Mortality was recorded from 3 to 17 weeks of age (termination of experiment), as described in
161 Fulton et al. [7]. The sire MD tolerance phenotype is the proportion of mortality among the
162 daughters upon MD challenge, with no signs of MD at the termination of the experiment.

163 Data were available from 15 generations of 8 elite lines (not the same generations in all
164 lines), with 1,081 to 1,393 males per line having MD progeny mortality data, for a total of
165 9,391 males. The lines included three different egg production breeds, namely five White
166 Leghorn lines, two White Plymouth Rock lines and one Rhode Island Red line. Pedigreed sires

and their progeny from these pure lines were used to test functional genomic elements (genes, miRNAs, lncRNAs in the mapped QTL regions (QTLR)), for association with MD progeny mortality, Also tested were a group of singleton coding SNPs predicted to have deleterious effects on protein structure and function (henceforth, potentially functional mutations, see below for details).

Genomic sequencing of founder birds of the FSIL

Genome sequence information was produced for the 10 F_0 founder individuals of the F_6 population. This was used to identify variants that differed in the genomes of the parental birds and may have functional impact. Sequencing the DNA from the 10 F_0 founder birds was carried out by the Edinburgh Genomics sequencing facility (Edinburgh, UK). Samples were prepared for sequencing using 1 ug of genomic DNA following the TruSeq PCR free kit protocol (Illumina, FC-121-3001). Resulting libraries were quality checked on an Agilent DNA 1000 bioanalyzer (Agilent Technologies, South Queensferry, UK) and then clustered onto HiSeq Rapid V2 flow cell at a concentration of 15 pM. Sequencing was carried out on an Illumina Hiseq 2500 using the HiSeq Rapid v2 SBS reagents (Illumina, Little Chesterford, UK), for 150 cycle paired end reads. Each of the 10 samples was sequenced to around 15x coverage. Quality of sequences was determined using FastQC [35], and mapping to the chicken reference genome (Galgal4) was carried out using BWA (v0.7.0) [36]. The resulting bam file was sorted with Samtools (v0.1.18) [37]. Picard tools (v1.95) was then used to add read groups and mark duplicates [http://broadinstitute.github.io/picard/]. The mpileup program within Samtools carried out SNP calling (with options: -q20 -Q20 -AB –ugf) and these variants were then filtered using the bcftools package within Samtools (v0.1.18).

Annotation of genome variants

Variants distinguishing the genomes of the F_6 parental birds were classified as exonic - synonymous or non-synonymous, intronic, 5'-upstream, 3'-downstream or intergenic, as determined by the SNPEff program (v3.6c) [38]. Non-synonymous coding variants were further designated as predicted to be highly deleterious to protein function (henceforth, "potentially functional mutations"), moderately deleterious or having a low likelihood of being deleterious to protein function. In all, a total of 5,718,725 - 6,154,628 variants (in all 5 males v all 5 females) were annotated within each sample. Some of the identified variants were used to test the QTLR, as described below.

600K high density SNP genotyping of F_6 birds

F_6 birds were genotyped genome-wide with the high density 600k Affymetrix SNP array [32] for GWAS. DNA was available from 1,615 MDV challenged birds. After quality control (QC), 1,192 F_6 birds provided high quality genotypes (178, 234, 357, 221 and 202 birds from Families 1 to 5, respectively). Genotyping was performed using 200 ng of gDNA with the standard protocol for the Axiom Affymetrix platform (Affymetrix). Samples were amplified using the Axiom 2.0 Reagent Kit (#901758, Thermo Fisher Scientific), and the resultant product checked for both quantity and quality of fragmentation. QC was performed using absorbance assay for quantity and running a sample on a 4% agarose E-Gel (#G800804, Thermo Fisher Scientific) to check for fragmentation. The samples were then hybridised to the Axiom Chicken Genotyping array (#902148, Thermo Fisher Scientific). Hybridisation, wash, stain and scanning were carried out within the GeneTitan MC Instrument. The resulting .CEL files were loaded into Axiom Analysis Suite (v2.0) for first stage analysis.

215 **GWAS of the F_6 population to identify MD resistance QTL**

216 The F_6 genotypes were used to map QTL that impacted survival following MDV
217 challenge. After applying Affymetrix standard QC the remaining markers were further filtered
218 to remove markers with minor allele frequency ≤ 0.01 and markers with significant deviation
219 from Hardy–Weinberg Equilibrium (P ≤ 0.001). An independent GWAS was then carried out
220 within each of the 5 families of this study, using JMP Genomics SNP-Trait association Trend
221 test (JMP Genomics, Version 9, SAS Institute Inc., Cary, NC, 1989-2019). Survival to the end
222 of the experiment was taken as the Censor Variable; age at death was taken as a survival trait
223 and MHC as a class variable and fixed effect. To obtain experimental significance thresholds,
224 the Proportion of False Positives (PFP) [39,40] was used to correct for multiple tests.

225

226 **Identification of QTL and their confidence intervals**

227 Although many marker x family combinations were nominally significant to highly
228 significant (comparison wise P ≤ 0.05 to P ≤ 0.001), very few remained significant after PFP
229 corrections for multiple tests. Nevertheless, visual inspection of the chromosomal Manhattan
230 plots by families showed distinct clusters of markers with high –LogP values intermixed with
231 markers with low -LogP values (**Figure 1**). Therefore, following Lipkin et al. [41], we
232 identified QTL by using a moving average of -LogP (mAvg) to smooth the Manhattan plots.
233 We used a window size of ~0.1 Mb (27 markers) with step size of 1 marker and a critical
234 threshold mAvg of -LogP = 2.0 (P = 0.01) to declare significance, and Log drop 1 [42] to define
235 QTLR boundaries.

236 Many QTLR had overlapping boundaries across two or more families, thus providing
237 replication and increased assurance of significance. Conservatively assuming such overlaps
238 represents the same underlying genetic element, we combined these QTLR within and across

239 families, taking the start and end log-drop boundaries of the QTLR as the first upstream and
240 last downstream marker across all families in the QTLR. **Table 1** shows final list of 38 QTLR
241 after consolidation within and across families. Also shown are the number of genes (Ensembl
242 database v79) and non-coding RNAs [43] found in each QTLR

243 **Table 1**. **Mapped QTLRs**.

QTLR	Chr	Start	End	Length	Genes	lncRNAs
1	1	8,854,589	9,261,317	406,729	0	1
2	1	13,268,810	14,294,877	1,026,068	12	34
3	1	52,012,381	52,517,953	505,573	2	4
4	1	71,738,977	73,085,956	1,346,980	16	28
5	1	75,288,912	77,765,499	2,476,588	86	79
6	1	91,482,501	91,803,475	320,975	10	9
7	1	101,761,945	104,477,713	2,715,769	31	37
8	1	109,395,871	110,913,451	1,517,581	20	32
9	1	169,656,958	172,228,091	2,571,134	32	44
10	1	174,394,951	175,634,386	1,239,436	23	33
11	1	194,193,118	194,788,548	595,431	18	23
12	2	15,960	883,257	867,298	45	20
13	2	46,423,161	46,868,789	445,629	11	12
14	2	105,493,833	108,988,920	3,495,088	34	47
15	2	125,168,753	127,089,304	1,920,552	25	34
16	2	138,767,830	139,701,774	933,945	4	7
17	3	108,220,206	109,260,649	1,040,444	13	9
18	4	8,308,498	11,268,107	2,959,610	54	99
19	4	84,393,173	88,579,400	4,186,228	68	73
20	5	7,568,851	8,147,837	578,987	4	3
21	5	18,806,924	19,673,354	866,431	4	3
22	6	2,077,640	2,709,412	631,773	19	13
23	6	29,536,109	29,817,337	281,229	10	16
24	6	31,006,769	31,448,342	441,574	7	6
25	7	13,062,871	16,436,053	3,373,183	55	82
26	10	22,643	1,713,384	1,690,742	63	28
27	11	7,397,790	8,440,259	1,042,470	9	28
28	12	8,996,686	9,432,693	436,008	15	16
29	13	10,363,430	12,176,727	1,813,298	27	40
30	14	8,085,563	9,335,685	1,250,123	38	50
31	14	13,138,194	15,087,518	1,949,325	82	41
32	16	1,630	490,907	489,278	47	14
33	17	3,442,598	5,634,042	2,191,445	64	84
34	18	3,196,488	4,093,129	896,642	22	32
35	24	4,489,675	5,514,833	1,025,159	42	32
36	26	4,378,168	5,036,699	658,532	22	17
37	27	1,540,112	2,270,461	730,350	23	11
38	28	1,282,726	1,571,011	288,286	15	12

244
245

246 QTLR - ordinal number of the QTLR after consolidation within and across families (see Methods); Chr -

chromosome; Start/End - Galgal4 QTLR coordinates of the first and last SNP in the QTLR; Length - Size of the QTLR (bp); Genes and lncRNA - number of genes (Ensembl database v79) and non-coding RNAs [43] found in the QTLR

RNA-Seq analysis

MDV challenge experiment

An experiment measuring host response to MDV viral strain 686 challenge (based on gene expression measured by a whole genome RNA-seq assay) was carried out with 10 male and 10 female birds from HL W36 commercial production hybrids. The parental lines used to produce the W36 commercial hybrid are closely related to the lines used to develop the F_6 experimental cross. Previously it was reported that males are more resistant to MD than females [44-46]. On this basis, we compared Differentially Expressed Genes (DEGs) of males and females as surrogate for comparison of chicks from more- and less-resistant lines. Hence, gender balanced groups were used for the RNA-seq challenge experiment, with 5 males + 5 females in each of the challenged and control groups. This allowed us to identify viral response genes in each sex. Thus, this experiment included 4 comparisons: Females - challenged v control; Males - challenged v control; Controls - males v females; Challenged - males v females. Although some of the DEGs in the cross-sex comparisons will be due to sex rather than response to MDV, others can be expected to reveal some of the genes behind the host immune responses and potential differential susceptibility.

To reflect infection in the field, ten 6-day old HL W36 commercial birds (5 males + 5 females) were infected with 500 pfu of the very virulent plus (vv+) MDV strain 686, by subcutaneous injection in the neck (virus kindly provided by the USDA/Avian Disease and Oncology Lab, East Lansing, Michigan). All birds were vaccinated at 1 day of age with HVT/SB1 vaccine. Spleen tissues from these 10 challenged and from 10 unchallenged control birds were harvested at 4 dpi, flash frozen in liquid nitrogen and stored at -80ºC for subsequent

RNA preparation and RNA-Seq analysis. All birds were housed together, with challenged and control chicks separated by 2.44 meters.

RNA preparation

RNA was prepared from the 4-dpi flash-frozen spleen tissues of each of the above 20 chicks after homogenization in Qiazol reagent (Qiagen, Manchester, UK), and subsequent preparation using RNeasy RNA isolation kit (Qiagen, Manchester, UK) as per the manufacturer's guidelines. RNA was resuspended in dH_2O.

Transcriptomic sequencing and analysis

Strand-specific 100 bp paired-end RNA-Seq was carried out by GATC (Konstanz, Germany) using an Illumina HiSeq2500 genome sequencer. 19,510,400 – 26,596,800 reads representing at least 15x coverage were produced for each sample. Sequencing quality was assessed by means of FastQC [35] and reads mapped to the reference genome (Galgal4) with Tophat2 (v2.0.14) [47] using the Bowtie2 aligner (v2.2.3) [48]. Untrimmed reads with stated insert sizes were mapped using the Ensembl 79 genome annotation. 78.1% of reads mapped to the Galgal4 reference genome. The Cuffdiff package within Cufflinks (v2.2.1) was used to quantify transcripts and determine differential expression between experimental groups [49].

QTLR tests

Selection of candidate genomic elements underlying the 38 QTLR for further testing and validation in the 8 HL elite pure lines.

In principal a random selection of markers from a QTLR region could be used for testing in the 8 elite-sire validation populations. However, we decided to take a step forward, and search the QTLR for attractive candidate elements that could tested in the validation populations. The assumption being that for a marker to show association across a number of

297 populations, it must be in high LD with the causative mutation in all of these populations. For
298 this to hold, the marker must be very close to the causative mutation, which is what we would
299 expect for markers taken from the quantitative trait element itself.

300

301 *Identification of candidate genes within QTLR*

302 The BioMart data mining tool within the Ensembl database (release 79) was used to
303 identify genes lying under the 38 QTLR identified from the F_6 data (**Table 1**). This information
304 was then compared with the gene expression data (from RNA-Seq analysis of the MDV
305 challenge experiment) in order to identify potential candidate genes for MD resistance. The
306 DAVID analysis tool (v6.8) (https://david.ncifcrf.gov/home.jsp) identified gene ontology (GO)
307 terms associated with DEGs under each QTLR. Following this, Ingenuity Pathway Analysis
308 software (IPA v2.3) (https://www.qiagenbioinformatics.com/products/ingenuity-pathway-
309 analysis/) revealed which canonical pathways were perturbed by MDV infection in the host,
310 and allowed us to analyze the gene interaction networks involved in the host response. Likely
311 upstream regulators of differentially regulated genes were identified and DEGs in male and
312 female birds were compared.

313 *Selection of genetic markers within QTLR for Genotyping*

314 Markers located in genomic elements underlying each QTLR (genes, miRNAs,
315 lncRNAs, and potentially functional mutations) were identified from genome sequences
316 aligned to the reference chicken genome for each of the 8 elite lines utilized in this study. SNPs
317 predicted to alter amino acids were preferentially chosen, but variants were also selected to
318 encompass the entire gene where possible. All SNPs selected were validated using 200 samples
319 from cohorts separated by 13-15 generations from each line to confirm that the marker was
320 polymorphic (i.e. identified both homozygote and heterozygote individuals), and to define the

minimum number of markers required to define a haplotype within each genomic element (gene, miRNA, or lncRNA) in each line. This minimum number of SNPs was then used to identify the haplotype status of each individual bird. The elite lines were selected for MD resistance, but the selection was at a low level, as selection emphasis was for production-related traits including egg numbers, shell quality and feed efficiency. If SNPs were homozygous in both generations, then the SNP composition was assumed monomorphic for the intervening and subsequent generations.

Selection of candidate coding genes

Twenty candidate coding genes (**Table 2**) were selected for further study in the 8 elite sire lines based on (i) position under a QTLR defined by this or previous genetic studies, (ii) known biology of the gene (e.g. involvement in innate immunity, cell death, or cancer), (iii) polymorphisms (presence of variants), (iv) differential expression between challenged and control, or more and less resistant birds - either in this or previous studies (e.g. Smith et al 2011), and (v) allele specific expression [50]. Specific details for the detection assays are provided in **S1 Table**.

Table 2. Candidate genes in the QTLRs, by location (Galgal4).

QTLR	Gene	Description	Chr	Start	End	Length
5	CSTA	Cystatin A (Stefin A)	1	76,253,583	76,258,549	4,967
5	LAG3	Lymphocyte-activation gene 3	1	76,676,057	76,678,087	2,031
5	C1S	Complement C1s subcomponent	1	76,859,359	76,868,459	9,101
7	ADAMTS5	ADAM metallopeptidase with thrombospondin type 1 motif, 5	1	102,403,453	102,442,746	39,294
8	SUPT20H	Spt20 homolog, saga complex component	1	171,574,831	171,612,194	37,364
10	FLT3	Fms related tyrosine kinase 3	1	175,281,894	175,309,466	27,573
11	RELT	RELT tumor necrosis factor receptor	1	194,551,973	194,555,607	3,635
13	TRANK1	Tetratricopeptide repeat and ankyrin repeat containing 1	2	46,537,531	46,576,714	39,184
19	CTNNA2	Gallus gallus catenin (cadherin-associated protein), alpha 2	4	86,948,459	87,057,215	108,757
29	HAVCR1	Hepatitis A virus cellular receptor 1 precursor	13	10,778,915	10,785,137	6,223
29	TIMD4	T-cell immunoglobulin and mucin domain-containing protein 4	13	10,789,088	10,803,683	14,596
30	SOCS1	Suppressor of cytokine signaling 1	14	8,935,374	8,936,082	709
32	TAP1	Antigen peptide transporter 1	16	67,623	76,124	8,502
32	IL4I1	Interleukin 4 induced 1	16	191,143	194,187	3,045
33	TLR4	Toll-like receptor 4 precursor	17	3,566,454	3,571,907	5,454
33	BRINP1	Bmp/retinoic acid inducible neural specific 1	17	3,985,882	4,064,290	78,409
33	CD7	T-cell antigen CD7	18	3,681,786	3,686,083	4,298
35	TAGLN	Transgelin	24	5,229,236	5,232,028	2,793
36	TREML2	Triggering receptor expressed on myeloid cells isoform 1	26	4,693,285	4,699,278	5,994
37	CD79B	CD79b molecule	27	1,622,587	1,627,022	4,436

338 QTLR - ordinal number of the QTLR (Table 1); Chr - chromosome; Start/End - Galgal4 gene coordinates (bp);
339 Length - size of the gene (bp).
340

341 *Selection of candidate non-coding RNAs*

342 With many causal variants for quantitative traits known to reside within regulatory
343 elements [51], we deemed it important to examine non-coding transcripts as well. In contrast
344 to the candidate genes, these were selected more or less on the basis of position underlying
345 QTLR alone, with no information on potential biological function.

346 *Micro RNAs (miRNAs).* A total of 46 miRNAs were found underlying the QTLR.
347 Sequence information was used to identify markers segregating in HL lines. For some
348 miRNAs, polymorphisms were not found, while for others multiple polymorphic sites were
349 observed. Since most of the miRNAs are very short, haplotype information could be derived
350 only for some of them. Ultimately, 17 miRNAs were chosen for further association analysis
351 (**Table 3**). Detection assays for 30 SNPs lying within these 17 transcripts were developed (**S1**
352 **Table**).

353 **Table 3. Candidate miRNAs in the QTLRs, by location (Galgal4).**

QTLR	Gene Name	Ensembl Gene ID	Chr	Start	End	Length
5	gga-mir-6553	ENSGALG00000026406	1	76,381,420	76,381,519	100
7	gga-mir-1738	ENSGALG00000025522	1	104,085,133	104,085,223	91
12	gga-mir-1749	ENSGALG00000025364	2	569,326	569,423	98
12	gga-mir-6710	ENSGALG00000027384	2	724,388	724,497	110
18	gga-mir-1462	ENSGALG00000025207	4	8,488,386	8,488,495	110
25	gga-mir-1659	ENSGALG00000025154	7	13,177,026	13,177,126	101
25	gga-mir-6624	ENSGALG00000026751	7	15,758,919	15,759,028	110
25	gga-mir-1570	ENSGALG00000025238	7	16,274,218	16,274,317	100
26	gga-mir-6660	ENSGALG00000028738	10	1,076,742	1,076,851	110
30	gga-mir-1644	ENSGALG00000025220	14	8,124,516	8,124,601	86
31	gga-mir-6643	ENSGALG00000027935	14	13,143,450	13,143,559	110
31	gga-mir-1588	ENSGALG00000025419	14	14,125,510	14,125,611	102
31	gga-mir-1715	ENSGALG00000025576	14	14,285,835	14,285,936	102
35	gga-mir-6612	ENSGALG00000026265	24	4,779,927	4,780,036	110
35	gga-mir-6671	ENSGALG00000027111	24	5,035,783	5,035,892	110
35	gga-mir-1745	ENSGALG00000026550	24	5,163,387	5,163,473	87
37	gga-mir-6573	ENSGALG00000028331	27	1,532,553	1,532,671	119

354

355 QTLR - ordinal number of the QTLR (Table 1); Chr - chromosome; Start/End - miRNA bp coordinates, Length -
356 size of the miRNA (bp).

Long non-coding RNAs (lncRNAs). With a view to testing some lncRNAs potentially associated with MDV resistance, five small QTLR were chosen which harboured only a few (1-6) lncRNAs. From these RNAs, up to 5 lncRNAs per QTLR were tested for association with MD. Thus, there was relatively high likelihood that one of these would be the genomic element housing the causative mutation. From among these, a further 17 transcripts were chosen for further association analysis (**Table 4**). Two lncRNAs overlapped (labelled with negative distance). Detection assays for 80 SNPs across these 17 transcripts were developed (**S1 Table**).

Table 4. Candidate lncRNA in the QTLRs, by location (Galgal4).

QTLR	gene id; transcript id; stable transcript id	Chr	Start	End	Length	Distance	Strand	Exons
1	R192;R192.1;RI_1_8950428_8950983_1_1	1	8,950,428	8,950,983	556		+	1
3	R1291;R1291.1;RI_1_52122457_52122949_1_1	1	52,122,457	52,122,949	493	43,171,475	-	1
3	R1292;R1292.1;RI_1_52183105_52322961_6_1	1	52,183,105	52,322,961	139,857	60,157	-	6
3	R1293;R1293.2;RI_1_52481252_52482976_1_1	1	52,481,252	52,482,976	1,725	158,292	-	1
3	R1293;R1293.3;RI_1_52481282_52488508_2_1	1	52,481,282	52,488,508	7,227	-1,693	-	2
20	R12773;R12773.1;RI_5_7604020_7604920_1_1	5	7,604,020	7,604,920	901		+	1
20	R12779;R12779.1;RI_5_7727117_7728010_1_1	5	7,727,117	7,728,010	894	122,198	-	1
20	R12784;R12784.1;RI_5_7960049_7961460_1_1	5	7,960,049	7,961,460	1,412	232,040	+	1
21	R13147;R13147.6;RI_5_18814123_18815319_2_1	5	18,814,123	18,815,319	1,197	10,852,664	+	2
21	R13148;R13148.1;RI_5_18861679_18862530_1_1	5	18,861,679	18,862,530	852	46,361	+	1
21	R13149;R13149.1;RI_5_19632618_19793846_5_1	5	19,632,618	19,793,846	161,229	770,089	-	5
24	R15316;R15316.1;RI_6_31138889_31157672_3_1	6	31,138,889	31,157,672	18,784		-	3
24	R15317;R15317.1;RI_6_31199730_31200561_1_1	6	31,199,730	31,200,561	832	42,059	-	1
24	R15318;R15318.1;RI_6_31204510_31210163_7_1	6	31,204,510	31,210,163	5,654	3,950	+	7
24	R15319;R15319.1;RI_6_31263768_31264516_1_1	6	31,263,768	31,264,516	749	53,606	+	1
24	R15320;R15320.2;RI_6_31277294_31279639_1_1	6	31,277,294	31,279,639	2,346	12,779	-	1
24	R15321;R15321.1;RI_6_31368941_31369400_1_1	6	31,368,941	31,369,400	460	89,303	-	1

QTLR - ordinal number of the QTLR (Table 1); gene id; transcript id; stable transcript id – unique lncRNA identifier; Chr - chromosome; Start/End - lncRNA bp coordinates; Length - size of the lncRNA (bp); Distance - bp between lncRNAs on the same chromosome; negative labelled value - overlap between this lncRNA and the previous one. Data from [43].

Selection of potentially functional variants in coding genes

Analysis of the complete sequences of the 10 F_0 birds showed multiple SNPs distinguishing the founder birds that could impact gene function. Alternative variants fixed in one parental line compared to the other and which were predicted to cause highly deleterious changes in the gene protein product, were chosen as candidates for further study. Across the genome as a whole, a total of 252 variants of this nature were identified in 217 genes, with 22

of these variants lying within genes underlying the 38 QTLR. Twelve of these variants (representing 9 genes) were segregating in one or more of the 8 elite lines and were chosen for further studies in these lines. These 12 SNPs are summarized in **Table 5**. Specific details on the detection assays are provided in **S1 Table**.

Table 5. Markers in the QTLRs with genetic variations predicted to be functionally deleterious to the gene product.

QTLR	Gene	Marker	Chr	bp	Consequence	Effect
14	ENSGALG00000015219	MD-07	2	107,385,545	S/*	Stop gained
25	gga-mir-7474	MD-16	7	14,743,048	actgat	Frame shift
26	ENSGALG00000001269	MD-20	10	48,846	P/X	Frame shift
29	ENSGALG00000004078	MD-23	13	11,619,922	gtt/gGTTTtt	Frame shift
30	IGSF6	MD-28	14	8,738,347	L/X	Frame shift
32	BG1	MD-29	16	117,493		Splice site acceptor
32	BG1	MD-30	16	118,808	Q/*	Stop gained
32	ENSGALG00000028367	MD-32	16	206,100		Splice site acceptor
32	ENSGALG00000028367	MD-33	16	206,241		Splice site acceptor
36	ENSGALG00000003188	MD-44	26	4,629,538	*C	Stop lost
37	SCN4A	MD-45	27	1,646,318	aga/agaCAGAAAT	Frame shift
37	SCN4A	MD-46	27	1,646,601	Q/*	Stop gained

QTLR - ordinal number of the QTLR (Table 1); Chr - chromosome; bp - Galgal4 location of deleterious variant; AA change - amino acid change.

Selecting QTLR markers from the candidate genomic elements, for genotyping in the 8 elite HL pure lines

Markers located in the genomic elements underlying the QTLR (genes, miRNAs, lncRNAs, and singleton variants with potentially functional effects), were identified from genome sequences aligned to the reference chicken genome for each of the lines utilized in this study. For the candidate genes underlying the QTLR, SNPs predicted to alter amino acids were preferentially chosen. For all genomic elements (not including the singleton functional mutations), variants were also selected to encompass the entire gene when possible, so as to define haplotype status of each bird for subsequent association tests. All SNPs selected were validated using the same approach as defined above

Genotyping of the eight elite lines

Genomic DNA was extracted from whole blood using salt/ethanol precipitation and stored at -20ºC until use. DNA was diluted to 25 ng/ul. Genotyping was done as single-plex assays on the SNPline system (LGC, UK). All assays used KASP chemistry, which is a fluorescent-based competitive allele-specific detection method [52]. Genotyping was done in 1,536 well plates, and results analysed with Kraken software (LGC, Hoddesdon, UK) as previously described [53]. Specific details on primers for each assay are provided in **S1 Table**.

QTLR association analysis

Association of markers and marker-haplotypes with sires progeny-tested for post-challenge survival in the 8 HL elite lines was tested using JMP Genomics SNP-Trait association Trend test (JMP Genomics, Version 9, SAS Institute Inc., Cary, NC, 1989-201). When analysing a line-marker combination, the generation and MHC background were taken as class variables and fixed effects, and when analysing a marker combining all lines together, the line was added to the fixed effects. To obtain experimental significance thresholds, the Proportion of False Positives (PFP) [39,40] was used to correct for multiple tests.

Results

High resolution MD QTL mapping in Advanced Full-Sib inter-cross F_6 families

Following genotyping of the F_6 families by the Affymetrix 600K HD SNP chip, 40-43% of SNPs (depending on family) were found to be informative and passed QC (total, 238,777 to 259,098 informative SNPs per family; average of 246,400 SNPs in a family). The distribution of the marker P-values was fairly uniform over the range of allele frequencies. A clear excess

of small P-values (an indication of presence of true QTL effects) was not seen. In accordance with this, only in families 2, 4 and 5, were markers significant at PFP ≤ 0.2. However, n_1 - the estimated number of falsified null hypotheses at PFP ≤ 0.2 [39,40], ranged from 1,718 in Family 1 (0.7% of all markers tested in the family) to 17,385 in Family 2 (6.7% of markers tested), indicating the actual presence of true QTL effects.

As described in Methods, many markers were nominally significant, and distinct clusters of nominally significant markers intermixed with non-significant markers were clearly seen. Hence, to identify QTL we used a moving average of marker -Log_{10}P values (mAvg) across a window size of 0.1 Mb (27 SNPs) to smooth the plots. Examining Manhattan plots (**Figure 1** and **S1 Figure**), showed that the smoothing did yield a good monotonic plot, allowing identification of QTL and the use of the drop method [42] to define their boundaries.

Using a threshold of mAvg ≥ 2.0 and QTLR boundaries defined by Log Drop 1, a total of 57 regions meeting our criteria for significance were identified, ranging from 4 to 16 in a family. In some families, several regions clustered within 1 Mb of one another. Nine such chromosome-by-family clusters were identified, containing 2-4 significant sub-regions per cluster. Conservatively, we counted each cluster as representing a single within-family QTLR. Similarly, aligning QTLR maps of the five families, showed that 6 significant regions overlapped or were within 1 Mb of one another across two families. Here too, counting the same QTLR across different families only once, resulted in a final total of 38 QTLR being identified (**Table 1**). These 38 QTLR were the focus of further examination of variants as defined by multiple methodologies employed below.

Annotation of QTLR

Transcripts of genes located under the 38 mapped QTLR were identified by using the

446 Biomart tool within the Ensembl database (v79) (**S2 Table**). The number of known annotated
447 genes in each QTLR ranged from 0 to 86, for a total of 1,072 genes. Using the annotation
448 developed and described by [53], which was based on characterisation of full-length transcripts
449 using long-read sequencing and a bioinformatics pipeline to define coding and non-coding
450 RNAs, we identified 1 to 99 lncRNAs within each defined QTLR, with a total 1,153 lncRNAs
451 (**Table 1**).

452

453 **Identification and Analysis of SNPs in F_0 parental birds**

454 To investigate variants residing in regions of the genome potentially associated with
455 MDV resistance, the 10 founder parents that gave rise to the F_6 population used in this study
456 were sequenced and variants determined between males and females. Combined across both
457 lines 8,273,112 SNPs were identified. Examining the 38 QTLR within the genome highlighted
458 133,394 SNPs in these regions, which were fixed for alternative SNP variants with one parental
459 line compared to the other (**S3 Table**). With a view to testing for causality, any of these variants
460 could then be tested for genetic association with the progeny-test for survival in the 8 elite HL
461 lines.

462

463 **Transcriptomic analysis of male and female commercial birds challenged with MDV**

464 Based on the observation noted above, that male and female birds tend to show
465 differential resistance, we examined the expression profiles of five male and five female W36
466 birds challenged with vv^+MDV, and 5 male and 5 female W36 non-challenged control birds
467 (see Methods). Gene expression results from this transcriptomic analysis are presented in **S4**
468 **Table**.

469 First, we compared gene expression in challenged and control chicks within each sex of

W36 birds; 185 genes (58 up- and 127 down-regulated) were differentially expressed in male chicks, while there were 114 genes (62 up- and 52 down-regulated) differentially expressed upon challenge of the female birds. When these genes are compared across sex, the individual responses are seen to be quite different, with minimal overlap of DEGs between the male and female groups (**Figure 2**).

When the different responses were compared, it is seen that biological pathways predominantly involved in the host response in female chicks include activation of cancer signalling, whereas the DEGs identified in the male birds have roles in the Th1 immune response and dendritic cell maturation amongst others (**Figure 3A**). The diseases and biofunctions associated with the genes involved in each response are depicted in **Figure 3B**. Strikingly, with MD being an oncogenic disease, functions related to cancer and tumorigenesis are seen to be up-regulated in female birds, but are repressed in the male chicks. Investigation of genes acting as upstream regulators of identified DEGs again indicates differential regulatory mechanisms in each sex. *TGFB* appears to be a key regulator of mechanisms in females, whereas *STAT1* (an interferon response gene) is suggested to have a more prominent role in the males (**Figure 3C**).

Examination of the unchallenged control birds from each group (male and female W36 birds) identified 302 genes which were expressed in an inherently different manner between the sexes. So, these DEGs, being independent of MD challenge, are due to gender differences and/or intrinsic immune differences, manifesting in differing resistance to infection. Significant DEGs include the obvious immune related genes but also various serine proteases and carboxypeptidases, which may suggest a role in resistance to MDV. Genes previously implicated in MDV resistance including MHC genes, *IRG1* [18] and *SCYC1* [16], were also highlighted.

Analysis of differentially expressed genes located in QTLR

When DEGs are located within QTLR affecting MD resistance, it is plausible that these genes may be the causative quantitative trait gene (QTG) responsible for the QTLR effect. Based on this hypothesis, the list of genes located in the 38 QTLR identified in this study was compared to the list of DEGs. Genes that were both expressed under challenge and that were also located under one of the 38 mapped QTL, were considered likely candidate QTG (**S5 Table**).

To determine if any particular types of genes were represented by the DEGs underlying significant genomic regions (identified either from this or previous studies [31,33,54-56]), the gene ontologies associated with these DEGs were studied. **S6 Table** shows that immunoglobulins, stimulus response genes and genes involved in regulating the immune response are all highly represented. Analysis of the biological pathways controlled by these genes was also examined. To look for possible common pathways, network analysis was performed. Three networks stood out significantly: cell movement, cell signalling and cancer (**Figure 4A**); cell signalling and nervous system development (**Figure 4B**) and antimicrobial resistance, inflammation and cell death (**Figure 4C**). When candidate upstream regulators of these genes were investigated, the cytokines *TNF*, *EDN1*, *IL1B* and *IFNG* were all indicated as potentially regulating many of the genes under study. Interestingly, the *TNF* gene is located in QTLR 18 on Chr 4, 387,071 bp downstream of the top window in this QTLR.

Validating QTLR for association with MDV resistance

Populations, genomic elements and markers

In a unique aspect of this study, QTLR identified in the F_6 mapping population were re-tested for association with MD resistance in 8 pure lines maintained under selection for

519 multiple commercial production traits at HL. Markers for QTLR validation were chosen from
520 among the various classes of genomic elements identified underlying the QTLR, namely genes,
521 miRNAs and lncRNAs. Also included were a group of singleton coding SNPs predicted to have
522 deleterious effects on protein structure and function (the functional mutations).

523 ***Genetic association tests***

524 When possible, a number of markers were genotyped in each candidate gene, so that
525 association could be based on marker haplotypes rather than individual markers. When this is
526 done, all of the information is in the 'Haplotypes'. The individual markers are usually in
527 complete LD and hence do not add anything to the analysis. Thus, for statistical analyses what
528 is important are the number of element x line tests, rather than number of marker x line tests.
529 We counted an element x line test as significant if the haplotype was significant, or if one or
530 more markers in the element was significant.

531 A total of 355 markers located in 66 genetic elements (genes, miRNA, lncRNA, and
532 functional mutations) underlying 26 of the 38 QTLR were tested for association with MD
533 mortality in the 8 elite lines (**S7 Table)**. In 46 of the elements, multi-marker haplotypes were
534 also tested. The association tests were carried out separately within each of the 8 elite lines,
535 and also in data combined across the 8 lines, yielding 2,032 P-values. A total of 387 element
536 by line tests were performed.

537

538 **Significance of individual candidate genes**. Twenty genes located in the QTLR were tested
539 by a total of 127 gene x line tests of markers encompassing the entire gene, A test was
540 considered significant if the haplotype x line test was significant, or if any one of the markers
541 encompassing the haplotype were significant. All but *IL4I1*, *TAGLN* and *CD79B* (in QTLR 32,
542 35 and 37), were significant in two or more marker x line tests. Thus, 17 genes may be taken

as strong candidates to be a QTG (**S8 Table**).

Association of markers located in miRNA. A total of 17 miRNAs were chosen (**Table 3**), and 28 markers tested for association (**S7 Table**) for a total of 96 element x line tests. Only 5 significant to highly significant P-values were obtained. (**S8 Table)**.

Significance of individual miRNAs. Significant results were found in only 5 out of the 17 miRNA tested. Most interesting was gg-mir-6553 with the highest proportion of significant tests among the miRNAs (**S8 Table)**. This miRNA is located in QTLR 5, along with 3 significant genes (*CSTA, LAG3, and C1S*). The results indicate two putative causative quantitative elements in this QTLR: one upstream in the region of *CSTA*, the other downstream in the region of *LAG3* and *C1S*.

Associations of markers located in lncRNAs. A total 17 lncRNAs were chosen (**Table 4**), and 80 markers were tested for association (**S7 Table)** in a total of 114 element x line tests, of which 9 were significant (**S8 Table**).

Significance of individual lncRNAs. Significant results were found with 9 of the 17 lncRNAs tested (**S8 Table**). QTLR worth mentioning are 20 and 21 on Chr5, both tested by lncRNAs only, are detailed in **S1 File**.

Associations of QTLR functional mutations in coding genes. Twelve potentially functional mutations were tested in a total of 50 element x line tests, of which 6 were significant (**Table 5**).

Significance of genes harbouring potentially functional mutations. Significant results were found in 5 of the 9 QTLR genes harbouring potentially functional mutations. In Line WPR1, the 2 markers in the gene *BG1* (markers MD-29 and MD-30 in QTLR 32 on Chr 16) had practically the same P values as the markers of the candidate gene *TAP1* (**S8 Table**), thus strengthening the results of that gene. It should be noted that *BG1* and *TAP1* lie within the

chicken MHC, as noted above, a locus known to impact MDV resistance.

Summary of element x line tests

S9 Table summarises the element x line tests by type of element. Tests of the coding genes are outstanding in the high proportion of significant element x line tests; more than double that of the other genomic elements. Since the candidate genes were chosen on the basis of much more information than the other classes of elements, the high proportion of significant tests can be taken to support the proposition that the process by which the candidate genes were selected was effective and that an appreciable fraction of the chosen candidate genes are the actual Quantitative Trait Genes. This must be qualified however, as the distribution of the different classes of genomic elements among the QTLRs was not random, and thus may be biasing the results.

Validation of QTLR

A total of 26 QTLR were tested for significance of element x line tests. Of these, eight did not have any significant element x line tests, 5 had one significant test and 14 had 2 to 6 significant element x line tests. Thus, 18 QTLR can be considered as validated. Of the unconfirmed QTLR, QTLRs 1, 3 and 24 were tested by lncRNAs only, QTLRs 12 and 18 by miRNAs only, QTLR 14 by functional mutations only, and QTLR 25 by both miRNA and functional mutations. More markers need to be tested in these QTLR to decide if the lack of significance is a result of lack of informativity (Type 2 error), or if indeed these QTLR are false positives (Type I error). More detail on the association found within each individual QTLR is presented in **S1 File.**

DISCUSSION

591 QTL mapping in an FSIL F_6 population phenotyped for survival in the face of MDV
592 challenge, identified 38 QTLR distributed over 19 autosomes. Use of such a resource allowed
593 for the identification of QTLR at a higher resolution than have been mapped previously, thus
594 allowing for easier identification of potential candidate genes. The mapped QTLR, along with
595 genomic sequences of the F_0 founder individuals, and transcriptomic information from
596 challenged and control birds, has allowed us to identify genes, miRNAs, lncRNAs and
597 potentially functional mutations located under these QTLR as candidates for association with
598 progeny mortality from Marek's Disease. Genetic association studies in multiple elite lines
599 have confirmed the significant effects of most of these candidates on MD. Here we will discuss
600 the potential role of some of the most significant candidates.

601 Many of the genes we have associated with MD response in this study have biological
602 roles clearly relevant to the pathogenesis of MDV infection. One of the primary targets of the
603 virus are B-cells and genes known to be associated with B-cells, include two of our candidates
604 - *CD7,* the T-cell leukaemia antigen which is involved in T-cell/B-cell interactions and the
605 Toll-like receptor, *TLR4* which is found on the surface of B-lymphocytes.

606 After initial infection and a period of latency, T-cells become infected. Genes related to
607 T-cell signalling pathways include our MD-associated *ADAMTS5*, *CD7*, *HAVCR1*, *LAG3*,
608 *RELT*, *TIMD4* and *TREML2*. *ADAMTS5* (ADAM Metallopeptidase With Thrombospondin
609 Type 1 Motif 5) encodes a metalloproteinase that plays an important role in inflammation and
610 cell migration. It also has a critical role in T-lymphocyte migration from draining lymph nodes
611 following viral infection. HAVCR1 - Hepatitis A Virus Cellular Receptor 1 (T-Cell
612 Immunoglobulin Mucin Receptor 1) is a receptor for TIMD4. HAVCR1 plays a critical role in
613 regulating immune cell activity, particularly regarding the host response to viral infection,
614 while TIMD4 is a T-cell immunoglobulin involved in regulating T-cell proliferation and
615 lymphotoxin signalling. *LAG3* (Lymphocyte-Activation Gene 3) belongs to the

616 immunoglobulin superfamily and acts as an inhibitory receptor on activated T-cells. It
617 negatively regulates the activation, proliferation and effector function of both $CD8^+$ and $CD4^+$
618 T-cells as well as mediating immune tolerance. RELT is a member of the TNF-receptor
619 superfamily. It can activate the NF-kappaB pathway and selectively bind TNF receptor-
620 associated factor 1 (TRAF1). This receptor acts via CD3 signalling to stimulate T-cell
621 proliferation, suggesting its regulatory role in the immune response. TREML2 (Triggering
622 Receptor Expressed On Myeloid Cells Like 2) is a cell surface receptor that may play a role in
623 both the innate and adaptive immune responses. It acts as a counter-receptor for CD276, with
624 interaction with CD276 on T-cells enhancing T-cell activation.

625 Once infection of T-cells has occurred, the disease can then proceed to become
626 oncogenic. Once again, we see that many of our MD associated genes have functions that have
627 been implicated in cancer, including *BG1* which encodes an Ig-superfamily type I
628 transmembrane receptor-like protein that contains an immuno-receptor tyrosine-based
629 inhibition motif (ITIM). BG1 has previously been documented as conferring MHC-associated
630 resistance to MDV-induced lymphoma [57]. Other candidates include *BRINP1* (silenced in
631 some bladder cancers), *CD7* (associated with leukaemia), *CSTA* (encodes a stefin that functions
632 as a cysteine protease inhibitor, suggested as a prognostic tool for cancer), *FLT3* (mutations in
633 this gene are common in acute myeloid leukaemia), and *SUPT20H* (a known tumour rejection
634 antigen).

635 One of the main pathologies of MD is its effect on the nervous system, and so it is
636 interesting to see that some of our MD associated genes are involved in the function/growth of
637 neurons (*SCN4A* and *CTNNA2*). *SCN4A* (Sodium Voltage-Gated Channel Alpha Subunit 4)
638 encodes one member of the sodium channel alpha subunit gene family involved in generation
639 and propagation of action potentials in neurons and muscle. CTNNA2 (Catenin Alpha 2) is
640 thought to be involved in regulation of cell-cell adhesion and differentiation in the nervous

641 system. It is required for proper regulation of cortical neuronal migration and neurite growth.

642 The remaining MD associated genes are seen to have general roles as immune system
643 genes: *C1S*, *TAP1* and *SOCS1*. *C1S* (Complement component 1S) encodes a serine protease
644 component of the complement system which enhances the host antibody immune response.
645 TAP1 (Transporter 1, ATP Binding Cassette Subfamily B Member) is involved in the transport
646 of antigens from the cytoplasm to the endoplasmic reticulum for association with MHC class I
647 molecules. *SOCS1* (Suppressor of Cytokine Signalling 1) encodes a protein which functions
648 downstream of cytokine receptors, and takes part in a negative feedback loop to attenuate
649 cytokine signalling. All of these candidate genes had more than one test significant at $P \leq 0.05$.

650 Examination of these genes and their significance of association with MDV resistance
651 across the elite lines indicates a few top candidates, namely: the cluster of genes in QTLR5
652 (*CSTA*, *C1S* and *LAG3*), *FLT3* in QTLR10, *CTNNA2* in QTLR19 and *TAP1* in QTLR32.

653 Genes identified in this analysis include many novel candidates for resistance as well as
654 highlighting genes proposed in previous studies. For example *CD8B* (T-cell glycoprotein),
655 *CTLA4* (immunoglobulin) and *CD72* (B-cell associated) are postulated as important lncRNA
656 target genes by You et al. [24] and are found under our QTLRs (*CD8B* - QTLR19), and
657 differentially expressed in our transcriptomic work (*CTLA4* and *CD72*). Similarly, *ATF2*
658 (involved in carcinogenesis is found in QTLR25) was proposed as an important target for the
659 miRNA gga-mir15b during MDV infection [25]. Also in QTLR25 we find gga-mir-10b,
660 previously seen to be upregulated in the spleen during MDV infection [27]. Other potentially
661 interesting miRNA targets include *PBEF1* (pre-B-cell enhancing factor) and *FCHSD2*
662 (involved in endocytosis) [26] that lie under QTLR2 and 11, respectively. Further genes
663 previously linked with MDV resistance include *GH1* (growth hormone) and *CD79B* (B-cell
664 antigen), both of which lie under our QTLR37.

One of the significant aspects of this research is that it utilized large, commercial production- relevant lines, and the challenge virus is a very virulent ++ strain, frequently encountered by production birds in the field. In contrast, most previously published MDV resistance research utilizes specialized research lines, many of which are inbred, and selected for differential response to MDV. These studies utilized laboratory strains of the virus, for which commercial production birds now appear to be resistant. Furthermore, this study investigated MD resistance genes in three distinct breeds of chickens, White Leghorn, White Plymouth Rock and Rhode Island Red, not just one laboratory line. Moreover, these MD association studies replicated the results from the FSIL study increasing our confidence in the causal nature of the QTLR, and possibly the genes and variants in MDV resistance. The response to the virus was measured as mortality in a large progeny group (approximately 30 daughters) for over 9,000 sires, using pre-existing information that had been developed within a commercially relevant production trait breeding program. This unique approach increases the relevance of the results to application into a commercial breeding program, while simultaneously provides information on the underlying mechanism of general viral resistance applicable to not only birds, but also other species. This information can provide insights into mechanisms for improving resistance or lead to the development of improved commercial vaccines.

CONCLUSIONS

Utilizing an FSIL F_6 population of birds phenotyped for response to Marek's Disease Virus infection has allowed us to map QTLR for disease resistance at high-resolution. Combining this with expression data from challenged and control birds, we have identified candidate genes, miRNAs, lncRNAs and potentially functional mutations which have been validated in genetic association tests with MD mortality in diverse, elite lines of poultry. This

690 most comprehensive genetic study to date supplies us with variants in candidate genes that can
691 now go on to be functionally tested for their utility in marker assisted selection, improved
692 vaccine development and potential future gene editing strategies.

693

694 **LIST OF ABBREVIATIONS**

695 DEG – differentially expressed gene

696 FSIL – full-sib inter-cross line

697 GWAS – genome wide association study

698 HAPS - haplotypes

699 HL - Hy-Line

700 HVT – herpesvirus of turkeys

701 mAvg - moving average of -LogP of a marker association test

702 MD – Marek's Disease

703 MDV – Marek's Disease Virus

704 MHC – major histocompatibility complex

705 PCR – polymerase chain reaction

706 PFU – plaque forming units

707 QC – quality control

708 QT - quantitative trait

709 QTG – quantitative trait gene

710 QTL – quantitative trait locus

711 QTLR - QTL region

712 SB1 – non-oncogenic MD virus

713 vv+ - very virulent plus

714 SNP – single nucleotide polymorphism

715

716 **ETHICS APPROVAL**

717 All procedures carried out on the birds involved in this study were conducted in compliance
718 with Hy-Line International Institutional Animal Care and Use Committee guidelines.

719

720 **AVAILABILITY OF DATA**

721 Data have been submitted to the European Nucleotide Archive (ENA) at EMBL-EBI under
722 study accession numbers PRJEB39142 (WGS) and PRJEB39361 (RNAseq).

723

724 **COMPETING INTERESTS**

725 The authors declare no conflicts of interest and no competing financial interests.

726

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730

731 **AUTHOR CONTRIBUTIONS**

732 JS carried out whole genome sequence, variant and RNAseq analyses, downstream biological
733 analyses and wrote the manuscript; EL and MS performed all association analyses; JF provided

734 all experimental animals, DNA samples and individual genotypes; DB conceived and managed
735 the project. All authors contributed to the interpretation of the results and edited and approved
736 the final manuscript.

737

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746

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947 **Figures**

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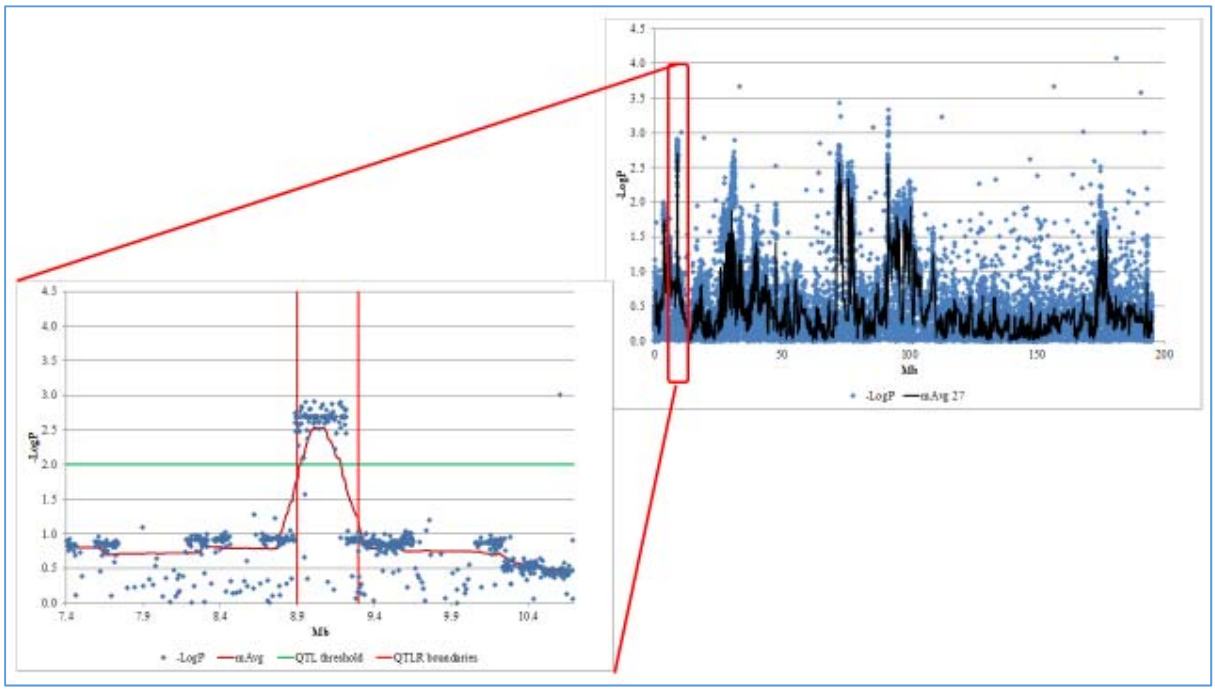

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952 **Figure 1:** Manhattan plot of a significant F_6 QTLR (Family 3: QTLR 1, Chromosome 1). LogP,
953 $-\log_{10}P$ of a single marker; mAvg, moving average of -LogP values of a window of 27 markers;
954 QTLR boundaries, boundaries of the QTL region obtained by Log Drop 1 (see Methods).

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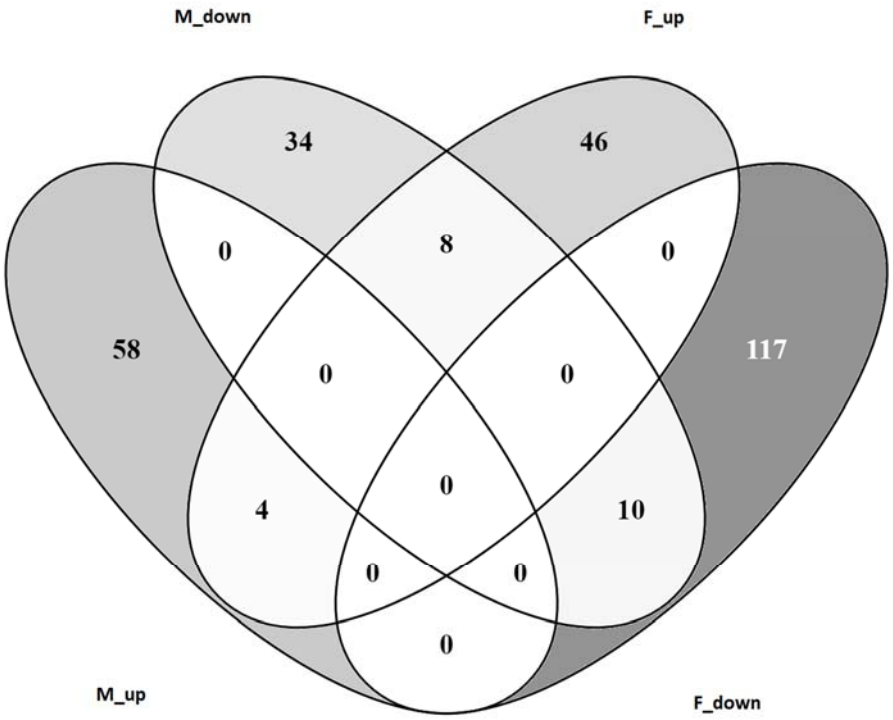

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957 **Figure 2:** Venn diagram showing the intersection of host responses in male and female W36
958 birds. M-up – number of genes up regulated in male birds; M-down - number of genes down
959 regulated in male birds; F-up – number of genes up regulated in female birds; F-down - number
960 of genes down regulated in female birds.

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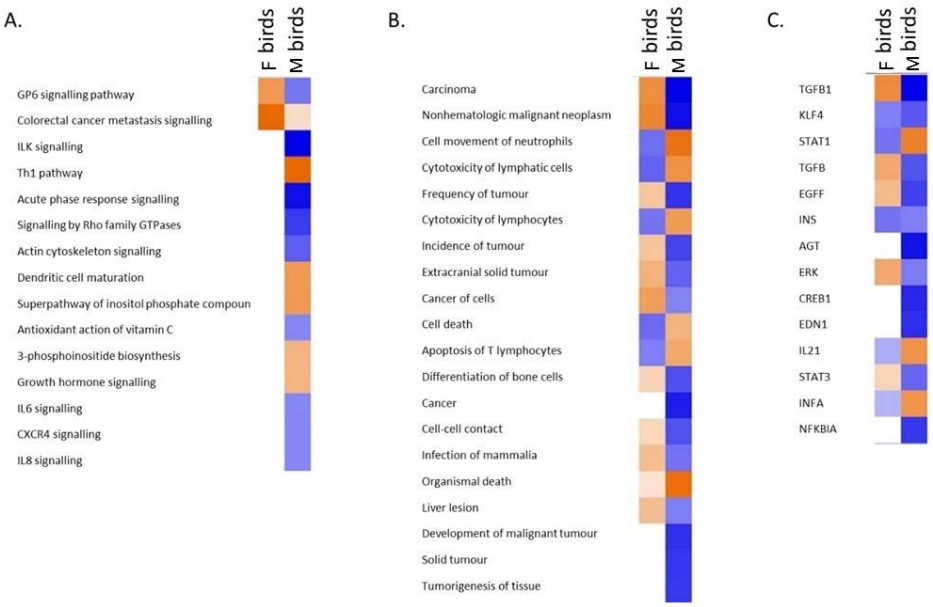

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964 **Figure 3:** Comparison of the gene expression underlying the host response in W36 birds.
965 Orange colours represent up-regulation while blue indicates down-regulation. Differential
966 activation/repression is indicated as shown: (**A**). Biological pathways (**B**). Diseases and
967 biofunctions (**C**). Upstream regulators.

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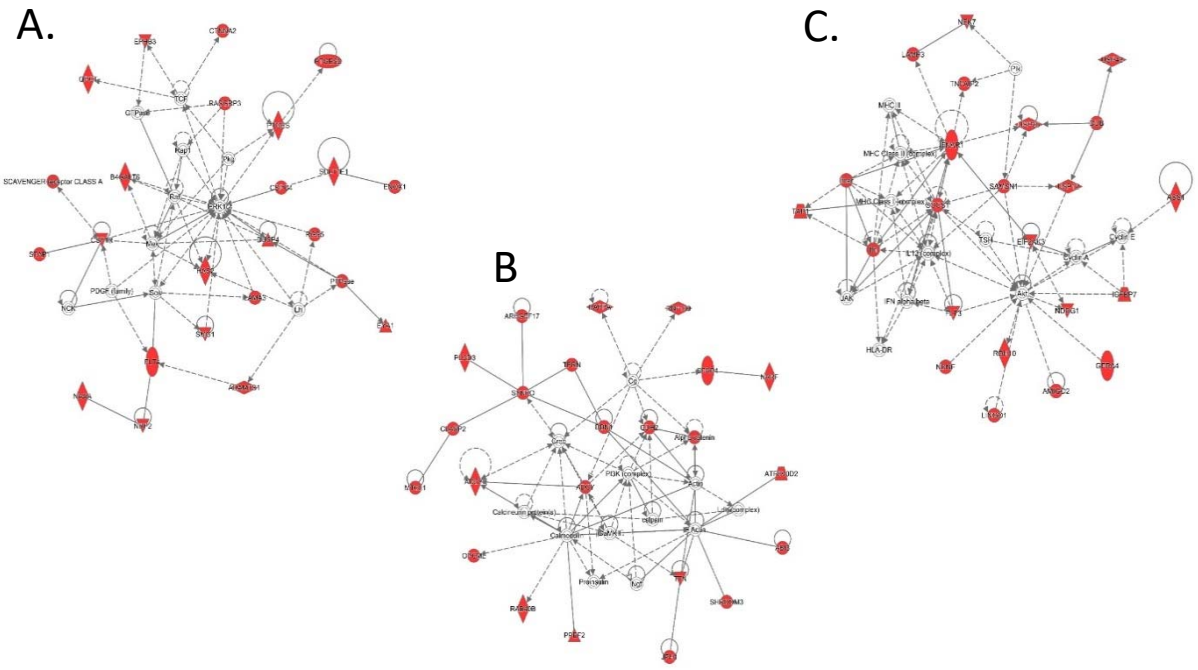


Figure 4: Gene networks represented by differentially expressed genes found under the 38 significant QTLR. Genes coloured in red are genes within the network which are in the dataset under study. (**A**). cell movement, cell signalling and cancer (**B**). Cell signalling and nervous system development and (**C**). Antimicrobial resistance, inflammation, and cell death.

Supplementary Files

S1 Table – Primers

S1 Figure – Manhattan plots of significant QTLR

S2 Table – Ensembl genes under QTLR

S3 Table – Variants under QTLR

S4 Table – Differentially expressed genes

S5 Table – QTLR genes also differentially expressed

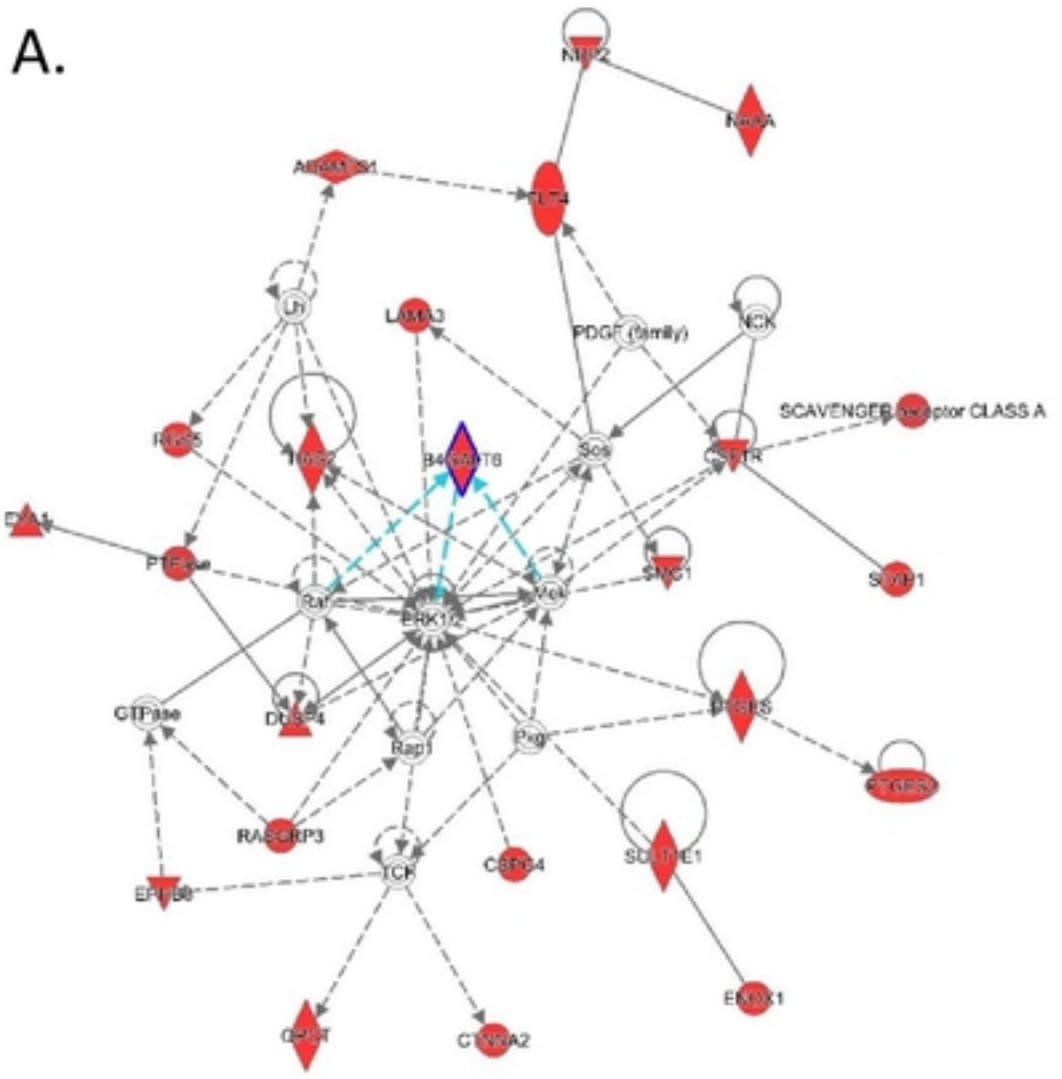
S6 Table – Gene ontology terms

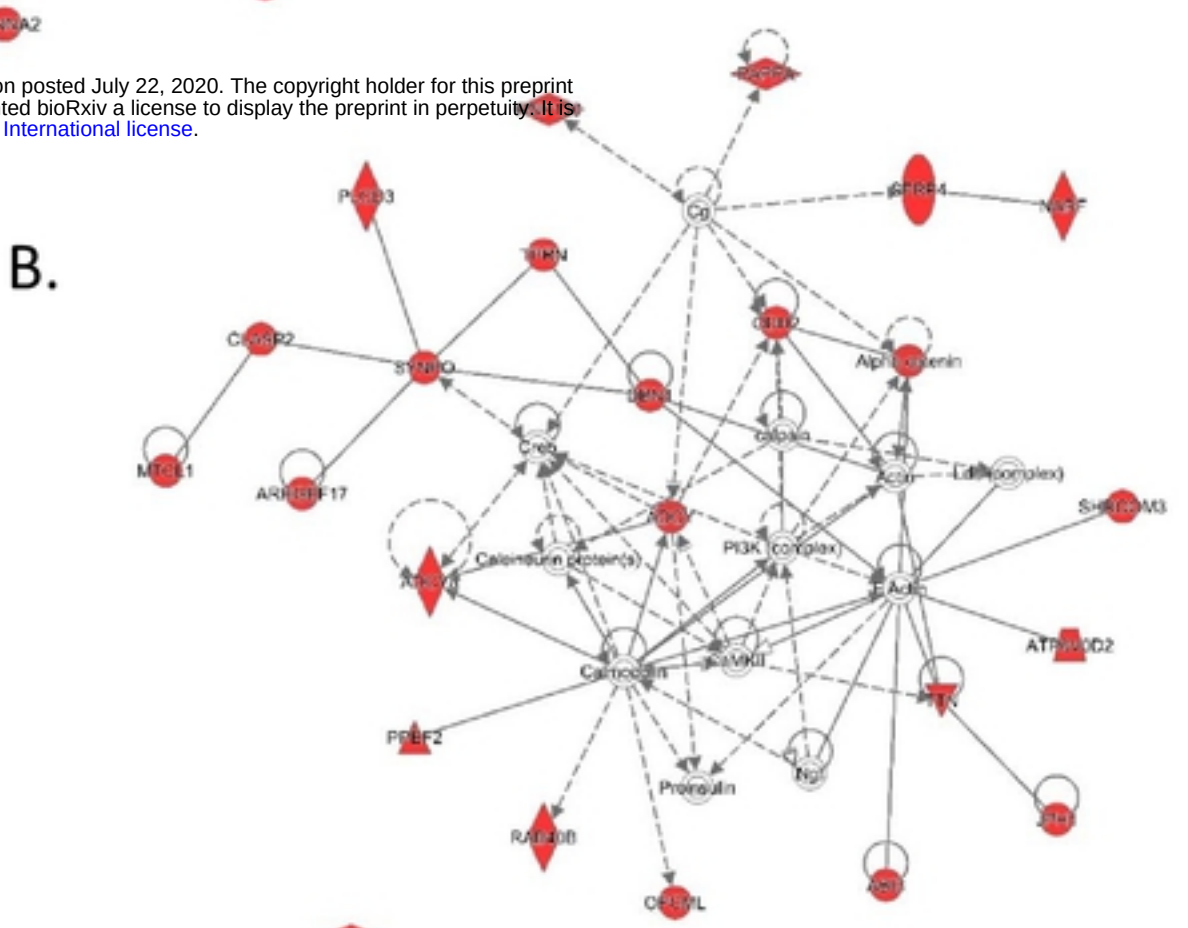
S7 Table – All markers used in association tests

989 S8 Table – Association test results (p-values)

990 S9 Table – Summary of element x line tests

991 S1 File – Details of association by individual QTLR





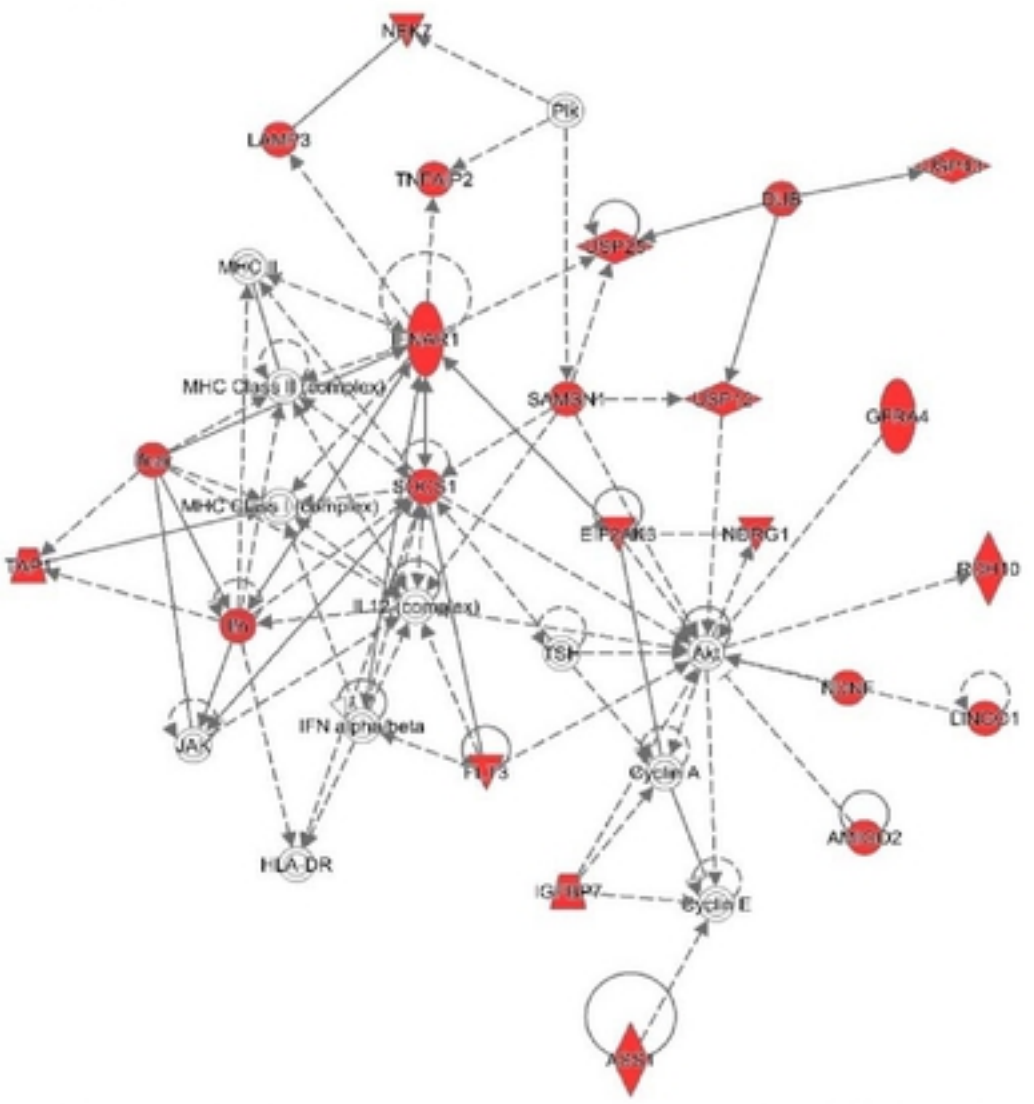


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Figure 4