HECTD2 Is Associated with Susceptibility to Mouse and Human Prion Disease

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Abstract

Prion diseases are fatal transmissible neurodegenerative disorders, which include scrapie, bovine spongiform encephalopathy (BSE), Creutzfeldt-Jakob disease (CJD), and kuru. They are characterised by a prolonged clinically silent incubation period, variation in which is determined by many factors, including genetic background. We have used a heterogeneous stock of mice to identify Hectd2, an E3 ubiquitin ligase, as a quantitative trait gene for prion disease incubation time in mice. Further, we report an association between HECTD2 haplotypes and susceptibility to the acquired human prion diseases, vCJD and kuru. We report a genotype-associated differential expression of Hectd2 mRNA in mouse brains and human lymphocytes and a significant up-regulation of transcript in mice at the terminal stage of prion disease. Although the substrate of HECTD2 is unknown, these data highlight the importance of proteosome-directed protein degradation in neurodegeneration. This is the first demonstration of a mouse quantitative trait gene that also influences susceptibility to human prion diseases. Characterisation of such genes is key to understanding human risk and the molecular basis of incubation periods.

Introduction

Prion diseases are fatal transmissible neurodegenerative disorders of animals and humans. These include the agriculturally and economically important diseases of scrapie and bovine spongiform encephalopathy (BSE) and the human diseases sporadic Creutzfeldt-Jakob disease (CJD), variant (vCJD) and kuru. Sporadic CJD has no known aetiology and vCJD is thought to have arisen following exposure to BSE prions [1]. Kuru is a prion disease that reached epidemic proportions in the 1950s in the Fore linguistic region of Papua New Guinea and is thought to have been transmitted through endocannibalism by participation in mortuary feasts [2]. Following the cessation of this practice in the late 1950’s, the incidence of disease has declined, however, it remains our only experience of a large epidemic of acquired human prion disease and provides a useful model for vCJD [3].

Although there was widespread population exposure in the UK and some other countries to BSE only around 200 have developed clinical vCJD to date, although the number infected remains unknown. This represents an on-going public health concern with a risk of iatrogenic transmission through blood and surgical instruments. vCJD has not been associated with any unusual pattern of dietary or occupational exposure to BSE prions and a significant genetic component to risk seems probable therefore the identification of susceptibility factors is key to estimating individual risk [1].

All prion diseases have prolonged clinically silent incubation periods which in humans span over 50 years [2]. Marked variation in incubation period occurs between inbred lines of mice and this is determined by multiple genetic loci in addition to the prion protein gene [4,5]. Previous studies have identified several quantitative trait loci for prion disease incubation time in mice. However, the resulting regions of interest spanned many megabases and were consequently too large for individual candidate gene analysis [6–10]. Several different strategies are available for fine mapping [11–13] and we chose to use a heterogeneous stock of mice. These are produced to model an out-bred population of mice, however they have the advantage of starting with a defined number of parental alleles. Heterogeneous stocks of mice have been shown to be a useful mapping tool because they provide a high level of recombination and the development of specific mapping software allows for convenient multipoint linkage analysis [14,15]. This approach led to the identification of Hectd2, an E3 ubiquitin ligase, as a quantitative trait gene for prion disease incubation time. Mouse models are extremely useful for studying human prion diseases as they faithfully recapitulate many key features of the disease and indeed rodents are naturally susceptible to prion diseases. It is expected that susceptibility genes and pathways identified in mice will also be relevant to human prion diseases. To test this hypothesis we carried out an association study with HECTD2 markers and samples from different human prion
**Author Summary**

Prion diseases are fatal transmissible neurodegenerative diseases of animals and humans for which there is no treatment. They include Bovine Spongiform Encephalopathy (BSE), and its human equivalent, variant Creutzfeldt-Jakob Disease (vCJD). Prion diseases are characterised by a long, silent incubation period before the disease emerges, and this time interval varies greatly between individuals. Differences in our genetic makeup are a key factor in this variability. We already know that natural variation within one key gene, the prion protein gene, has a major influence on incubation time, but it is now clear that a number of other genes are also important. Using a mouse model, we have identified one of these genes, *Hectd2*, which is thought to be involved in the process that removes unwanted proteins from the cell. We also show that *HECTD2* is associated with an increased risk of two human prion diseases—vCJD in the United Kingdom and kuru in Papua New Guinea. These data will give us a better understanding of the fundamental processes involved in these diseases and go some way to explaining why some individuals exposed to BSE have developed vCJD and others have not.

**Results**

**Identification of Mouse Quantitative Trait Gene**

To fine map regions thought to contain quantitative trait loci for prion disease incubation time we utilised the Northport heterogeneous stock (HS) of mice (gift of Robert Hitzemmann), which was produced by semi-randomly mating eight inbred lines of mice (A/J, AKR/J, BALB/cJ, C3H/HeJ, C57BL/6J, CBA/J, DBA/2J, LP/J). Approximately 1000 mice were inoculated intracerebrally with prion diseases of animals and humans for which there is no treatment. They include Bovine Spongiform Encephalopathy (BSE), and its human equivalent, variant Creutzfeldt-Jakob Disease (vCJD). Prion diseases are characterised by a long, silent incubation period before the disease emerges, and this time interval varies greatly between individuals. Differences in our genetic makeup are a key factor in this variability. We already know that natural variation within one key gene, the prion protein gene, has a major influence on incubation time, but it is now clear that a number of other genes are also important. Using a mouse model, we have identified one of these genes, *Hectd2*, which is thought to be involved in the process that removes unwanted proteins from the cell. We also show that *HECTD2* is associated with an increased risk of two human prion diseases—vCJD in the United Kingdom and kuru in Papua New Guinea. These data will give us a better understanding of the fundamental processes involved in these diseases and go some way to explaining why some individuals exposed to BSE have developed vCJD and others have not.

**Table 1. Most significant strain distribution patterns.**

| Strain distribution pattern | Genes | $-\log P$ | Comment |
|-----------------------------|-------|-----------|---------|
| (A, AKR, BALB) (C3H, C57, CBA, DBA, LP) | *Hectd2* | 6.12 | Promoter, Several intronic and 3′ UTR |
| (A, AKR, BALB) (C3H, CBA, DBA, LP) (C57) | *Hectd2* | 6.15 | Single intronic |
| (A, AKR, BALB, C57, LP) (C3H, CBA, DBA) | *Exoc6* | 6.74 | 2 intronic, 1′ UTR |
| | *Cyp26c1* | 6.15 | T18A, Q256R and other synonymous |
| | *Cyp26a1* | G202D |
| | *Plce1* | Several intronic and synonymous |
| | *Lgi1* | Single intronic |
| (A, AKR, BALB, C57) (C3H, CBA, DBA, LP) | *Hectd2* | 6.84 | Single SNP 3′ UTR |
| | *Cyp26a1* | Single intronic |

$-\log P$ values are estimated by HAPPY based on polymorphisms detected in the parental strains of the HS.

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polymorphisms occur within the predicted promoter (−226 to +25) one of which affects a potentially functional site. Sequence for the C57BL6/J allele from −216 to −210 is TGGGGCGG and the insertion of 6 Gs in the alternative allele gives TGGGGGGGGGGCG. Both variants contain the consensus sequence for a Sp1 binding site (shown in bold) however the insertion also generates an overlapping large T antigen binding site (underlined). It is unclear whether additional mouse proteins could bind to this sequence or whether Sp1 binding would be affected. The significant SDPs were spread across the whole of Hectd2 therefore we cannot exclude any of these closely linked polymorphisms either individually or collectively from a contribution to the phenotype.

Mouse Hectd2 Expression

To determine whether the polymorphisms detected in Hectd2 have an effect on expression, RNA was extracted from whole brains of 8 week old males from the parental strains of the HS mice (except LP). Samples were analysed by real time RT-PCR. To examine genotype-related differential expression, strains were grouped according to the major strain distribution pattern seen in Hectd2 (Group A = A, AKR, BALB; Group B = C3H, C57, CBA, DBA). Expression was 2.4 greater in group A than group B (P = 2.85 × 10−3, unpaired t-test) (Figure 1A). Where incubation time data are available, the increase in Hectd2 expression is associated with a shorter incubation time (R² = 0.61) [20–22] (See also Figure S2). A potential role for Hectd2 in prion disease pathogenesis was explored by comparing the mRNA expression levels between normal mice and those at the end stage of disease following infection with Chandler/RML prions. For C57BL/6, expression was ×5.0 greater in the prion infected mice (P = 2.66 × 10−5, unpaired t-test) (Figure 1B).

Human Association Study

Our data indicate that Hectd2 influences prion disease incubation time in mice. We therefore analysed HECTD2 in a hypothesis-driven association study of human prion disease. We analysed 834 samples from patients with prion disease or strong resistance to prion disease and 1162 relevant control population samples. We tested whether genetic variation at HECTD2 was associated with a phenotype of variant and sporadic CJD. In Papua New Guinea (PNG) we genotyped patients who died from the epidemic prion disease kuru, transmitted by endocannibalism, and compared these data with elderly women known to have had multiple exposures to kuru at mortuary feasts prior to the cessation of endocannibalism in the late 1950’s, but who are long-term survivors [2,23]. See methods for details of the patient data, populations, phenotype ascertainment and population stratification data.

We initially tested a single SNP, rs12249854(A/T), located in a HECTD2 intron, and showed that the minor allele (A) was significantly over-represented in vCJD (n = 117, 8.1%) compared to controls (n = 601, 3.9%), P = 0.0049, (OR 2.11, 95% CI 1.19–3.83). In addition, in a subsidiary analysis on a subset of the patient data, the minor allele of a second SNP, rs12286983(G/A), located in the 3′UTR of HECTD2, was associated with a shorter incubation time (R² = 0.61) [20–22]

Table 2. Polymorphism genotyping in HS mice.

| Gene   | Polymorphism     | Happy −logP | HS p-value (ANOVA) |
|--------|------------------|-------------|-------------------|
| Hectd2 | Promoter (G/in) | 6.12        | P = 0.0008 (n = 398) |
|        | Intron 3 A/G     | 6.12        | P = 0.0013 (n = 404) |
|        | 3′UTR A/T        | 6.84        | P = 0.0022 (n = 359) |
| Cyp26c1| Exon 1 T18A      | 6.74        | P = 0.1512 (n = 403) |
| Cyp26a1| Exon 3 G202D     | 6.74        | P = 0.2017 (n = 408) |
| Plce1  | Exon 6 T/C       | 6.74        | P = 0.1556 (n = 411) |

All polymorphisms were analysed by allele discrimination using a 7500 Fast real time PCR system (Applied Biosystems) except the Hectd2 promoter polymorphism which was typed by size using fluorescent primers on a MegaBACE1000 sequencer (GE Healthcare). For probe details see Table S5. doi:10.1371/journal.pgen.1000383.t002

Figure 1. Quantitative RT-PCR of Hectd2. cDNA was prepared from whole brains of uninfected 8 week old male mice or mice at the terminal stages of disease following intracerebral inoculation with Chandler/RML mouse-adapted scrapie prions. All samples were duplexed for Hectd2 and GAPDH fluorogenic probes and run in triplicate with n = 6 for each mouse strain/group. Mean ± s.e.m. Hectd2 mRNA expression level is expressed in arbitrary units as normalised by the quantity of GAPDH (y-axis). A, Inbred strains are grouped according to the major strain distribution pattern seen in Hectd2 (Group A = A, AKR, BALB; Group B = C3H, C57, CBA, DBA). Expression was ×2.4 greater in group A than group B (P = 2.85 × 10−3, unpaired t-test). B, Comparison of Hectd2 expression in normal and RML prion-infected C57BL6 mouse brains. Expression was ×5.0 greater in the brains of prion-infected mice, (P = 2.66 × 10−5, unpaired t-test). C, Expression of HECTD2 in cDNA prepared from lymphocytes of human blood donors (n = 140). Samples were duplexed for HECTD2 and β-actin fluorogenic probes and run four times. Mean ± s.e.m. HECTD2 mRNA expression level is expressed in arbitrary units as normalised by the quantity of β-actin (y-axis). Data are grouped according to genotypes at rs12249854 as determined from genomic DNA. Expression was ×2.3 greater in the heterozygotes (TA) than for the major allele homozygotes (TT) (P = 0.0008 Mann-Whitney test).

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3.77, trend test 1 d.f.), and between sporadic CJD (n = 452, 6.3%) and controls, P = 0.012, (OR 1.65, 95% CI 1.11–2.46, trend test 1 d.f.). Given that sample sizes are necessarily small in both sporadic and variant CJD, these data are consistent with the association of rs12249854 with risk in both prion disease categories and a large effect size. We went on to test whether the risk rs12249854 allele modified the phenotype of human prion disease. Although the age of onset of sporadic CJD with rs12249854AA was younger than other genotypes (33.5 years Vs 68.8 years for rs12249854AT and 69.0 years for rs12249854TT, P = 0.048 t-test), this genotype was rare (n = 3) and the finding therefore was not robust. There was no association between vCJD year of presentation, or age of onset with rs12249854 genotype. Insufficient data were available to look at any association with duration of illness.

We went on to analyse a further seven SNPs in HECTD2 selected to capture global genetic diversity based on Hapmap (http://hapmap.org) [24] data (Table S6). In the United Kingdom (UK), we found strong linkage disequilibrium (LD) and a single haplotype structure across the entire gene (Table 3). Three haplotypes were >1% frequency, the most common two haplotypes (1 and 2) differed at all SNPs, a third haplotype (3) was distinguished from the most common haplotype (1) by a single SNP upstream of HECTD2. Increased risk of vCJD was associated with haplotype 2, possessing rs12249854A, but the extensive LD prevented us from identifying the functional SNP. In PNG, however, we found considerably more diversity with four common haplotypes, 1, 2 and two novel haplotypes 4 and 5 (see methods for haplotype inference). Haplotype 2, most significantly associated with vCJD (haplotype association test, P = 0.006), showed no significance between kuru and the elderly female survivors of mortuary feasts. Rather, in PNG we found that a population specific haplotype (designated 4) was strongly associated with kuru (P = 0.0009). Haplotype 4 differs from haplotype 2 at a single SNP, rs12247672, which itself is significant in vCJD (P = 0.0039) but not at all in kuru (P = 0.6138). Our data suggest that there is evidence for HECTD2 association in both vCJD and kuru however the functional polymorphisms are likely to be different. This is not necessarily surprising given the distinct evolutionary history and consequent genetic differences that exist between the UK and PNG populations. It should also be noted that although vCJD and kuru are both acquired human prion diseases that share many characteristics they are also derived from different sources and caused by distinct prion strains [25,26] therefore the mechanism of HECTD2 involvement may also be different.

**HECTD2 Sequencing**

We sequenced the ORF and promoter of HECTD2 in 16 vCJD, multi kuru-exposure survivors, and both UK and PNG controls. Three polymorphisms were found, of which only one is potentially functional (Table 4). rs7081363 occurs in the promoter (−247) and the minor allele is predicted to remove an Sp1 binding site (GGCG/AGG). rs7081363 was genotyped in our samples and shown to be in complete LD with rs12249854 in the UK population (vCJD P = 0.0012; sporadic CJD P = 0.0065). We were unable to genotype the kuru samples due to poor DNA quality, however, analysis of all other samples suggest that rs7081363 is unlikely to be significant in PNG.

**Human HECTD2 Expression**

To determine whether the susceptibility alleles in the UK population are associated with differential mRNA expression, **HECTD2** expression levels in blood lymphocytes (n = 140, UK blood donors) were quantified by real-time RT-PCR. Samples were grouped according to rs12249854 genotype, however, due to the low frequency of the minor allele (A), no homozygotes (AA) were seen. The mean expression level was 2.3 greater in the heterozygotes than for the major allele homozygotes (TT) (P = 0.0008 Mann-Whitney test, Figure 1C). This suggests that a higher level of **HECTD2** mRNA expression may be linked with vCJD in the UK population.

**Discussion**

Our data show that **HECTD2** is linked to prion disease incubation time in mouse and is associated with sporadic and variant CJD and kuru in humans and an increase in expression is associated with a susceptibility genotype and disease pathogenesis. In mouse, we cannot exclude the possibility of other nearby genes or intergenic regions also being implicated as our sequencing studies were not exhaustive. However, in human, the LD block, based on HapMap [24] data, includes only **HECTD2** and does not extend into the neighbouring genes suggesting that the association observed stems from **HECTD2** and not any other gene in the area.

In mouse, the promoter, 3’UTR polymorphisms and the associated differential expression suggest a mechanism by which Hecd2 may influence the incubation time phenotype. Similarly, in the UK population a promoter polymorphism is also associated with a susceptibility phenotype and a resulting increase in expression level. This suggests that the mode of **HECTD2** action in prion disease may be independent of host and prion strain. Due to lack of available material it has not been possible to replicate these experiments in our kuru samples, however, our haplotype study suggest that a different polymorphism is likely to be functional in the PNG population. This does not rule out the possibility that differential expression is also important in PNG, through an alternative polymorphism, although this may be difficult to determine. Our expression analysis in terminally sick mice suggest that **HECTD2** is upregulated during the course of infection therefore we can speculate that a higher base line of expression reduces the time taken to reach a threshold level thereby reducing the incubation time.

The ubiquitin-proteosome system has been implicated in the pathogenesis of several neurodegenerative diseases which show an accumulation of an abnormally folded protein including prion disease, Parkinson’s disease and Alzheimer’s disease [27–29]. By homology to other family members, **HECTD2** is an E3 ubiquitin

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**Table 3. Most common HECTD2 haplotype frequencies.**

| UK | Haplotype | Name | vCJD | Control | P-value |
|----|-----------|------|------|---------|---------|
|    | 22222222  | 1    | 0.951| 0.912   | 0.02    |
|    | 11111111  | 2    | 0.026| 0.061   | 0.006   |
|    | 12222222  | 3    | 0.023| 0.027   | 0.73    |

| Papua New Guinea | Haplotype | Name | Multiple exposure | Kuru | P-value |
|------------------|-----------|------|------------------|------|---------|
|                  | 22222222  | 1    | 0.641            | 0.743| 0.014   |
|                  | 11111111  | 2    | 0.146            | 0.123| 0.45    |
|                  | 12111111  | 4    | 0.173            | 0.076| 9 × 10⁻⁴|
|                  | 22222221  | 5    | 0.040            | 0.058| 0.36    |
HECTD2 may also be a susceptibility factor for Alzheimer’s previously linked with Alzheimer’s disease [33] suggesting that Further, ataxin-1 while accelerating the Purkinje cell pathology [32]. the frequency of nuclear inclusions in mice expressing mutant in the pathogenesis of polyglutamine diseases in particular it has in patients has been shown to be associated with protein juvenile parkinsonism and loss of ubiquitin-protein ligase activity E3 ubiquitin ligase parkin are associated with autosomal recessive following BSE and secondary human prion exposure. prion pathogenesis [27,34,35] and will contribute to modelling evidence to support a role for HECTD2 in prion disease. This human prion diseases from different populations provide sufficient combined weight of data from the mouse genetic studies, mortuary feasts are of necessity small, however we believe that the disease and other neurodegenerative disorders.

Materials and Methods

Human Samples

The clinical and laboratory studies were approved by the local research ethics committee of University College London Institute of Neurology and National Hospital for Neurology and Neurosurgery and by the Medical Research Advisory Committee of the Government of PNG. Full participation of the PNG communities involved was established and maintained through discussions with village leaders, communities, families and individuals.

vCJD. 118 probable or definite vCJD patients, according to established criteria (http://www.advisorybodies.doh.gov.uk/acdp/tseguidance/tseguidance_annexb.pdf), were recruited by the National Prion Clinic (NPC), London or the National CJD Surveillance Unit (NCJDSU), Edinburgh from 1995 to 2005. Iatrogenic vCJD, acquired through blood transfusion was not included in this panel. Genomic DNA was usually extracted from peripheral blood; brain tissue was used as a source for some patients. Amplified DNA, using either multiple displacement amplification (Geneservice, Cambridge, UK) or fragmentation-PCR methods (Genomeplex, Sigma), was used for a small number <10% of samples. Samples were checked for degradation on 1% agarose gel and stored at 50 ng/μl in low concentration Tris-EDTA buffer. All patients were thought to have acquired the disease in the UK and were of white-British ethnicity; 60% were male. Mean (range) age of onset of disease onset was 29 years (13–62).

Sporadic CJD. 458 probable or definite sporadic CJD patients, according to WHO criteria, were recruited by the National Prion Clinic (NPC), London or the National CJD Surveillance Unit (NCJDSU), Edinburgh, or numerous other referrers in the UK. DNA was sourced and amplified as for vCJD. All patients were of UK or northern European origin. Although the vast majority of patients were of white-British ethnicity, and all patients of known non-white ethnicity were excluded, this information was based on name and geography for some samples. DNA preparation and storage was similar to vCJD. Over 60% had pathologically confirmed sCJD, the remainder had a diagnosis of probable sCJD according to published WHO criteria with a high specificity [36]. Mean (range) age of onset of disease was 62 years (15–87).

Kuru/elderly women resistant to kuru. Prior to 1987, kuru surveillance was conducted by many different investigators (Gajdusek, Zigas, Baker, Alpers, Hornabrook, Moir and others) and from 1987 to 1995 solely by the Kuru Surveillance Team of the Papua New Guinea Institute of Medical Research. From 1996 onwards, kuru surveillance was strengthened and a field base and basic laboratory for sample processing and storage was established in the village of Waia in the South Fore [37]. The kuru collection (n = 131) comprises young children, adolescents and adults from around the peak of the epidemic and elderly recent kuru cases with long incubation times. They resided in the South Fore [53], North Fore (40), Gimi (5), Keiaiana (10), or other linguistic groups (11) of the kuru-affected region of the Eastern Highlands Province of Papua New Guinea; in 34 cases the linguistic group within the region was not recorded.

Elderly exposed women were defined as aged over 50 years in 2000 from a kuru-exposed region (n = 115). These women were
unaffected at the time of sampling but were thought to have been exposed to kuru prions in childhood. Although these women may not be truly “resistant” to kuru prions they would have incubation times in excess of 40 years. Additional controls were obtained from the young modern day healthy population that has not been exposed to kuru but came from villages in the exposed region by matching each elderly woman (“resistant”) to at least two current residents of the same village aged less than 50 in 2000. These largely came from the South Fore, but with a significant number from the North Fore and a small number of individuals from Gimi, Keiagana and Vagaria linguistic groups. Further controls were obtained from young unexposed people from areas of PNG where no kuru has been recorded. Where identified by either genealogical data or microsatellite analysis, first degree relatives were excluded from these groups. DNA from degraded archival kuru sera, obtained from the NIH collection, was isolated by QIAGEN QIAamp Blood DNA minikit followed by whole genome amplification either through using a Phi29 protocol (Geneservice), or GenomePlex Complete Whole Genome Amplification Kit (WGA2) (Sigma).

UK controls. 116 individuals were recruited from the National Blood Service (NBS). Information was collected about gender, age, ethnicity and birthplace divided into 12 regions. Samples were very similar to vCJD for white-British ethnicity, birthplace (by 12 regions in UK) and gender. DNA was extracted from whole blood. PAXgene blood RNA samples were also collected (Preanalitix). Mean (range) of age at sampling was 34 years (18–64); 56% were male. Further UK control samples (n = 480) were purchased from the European Collection of Cell Cultures (ECACC) Human Random control (HRC) DNA panels consisting of randomly selected, non-related UK Caucasian blood donors. Total number of UK controls was n = 596.

Population structure. Population structure was considered by identity state (IBS) clustering (implemented by PLINK (http://pngu.mgh.harvard.edu/~purcell/plink/index.shtml), and principal components analysis (implemented by the EIGENSTRAT package [38]. Genome-wide data (manuscript in preparation) with high stringency filtering was used to compare vCJD and UK controls with both PLINK or EIGENSTRAT (no significant eigenvectors were detected using default procedures). For other groups of patients and PNG groups genotypes were generated for 1325 SNPs in 344 randomly selected, non-related UK Caucasian blood donors. Population structure was considered by identity state (IBS) clustering (implemented by PLINK or EIGENSTRAT (no significant eigenvectors were detected using default procedures). For other groups of patients and PNG groups genotypes were generated for 1325 SNPs in 344 randomly selected, non-related UK Caucasian blood donors provided by the European Collection of Cell Cultures (ECACC) Human Random control (HRC) DNA panels consisting of randomly selected, non-related UK Caucasian blood donors. Total number of UK controls was n = 596.

Animals

28 pairs of Northport HS mice were obtained from R. Hitzemann (Portland, Oregon, USA) at generation 35. Offspring from these pairs were randomly mated to produce a total of 49 pairs. 1000 offspring (generation 37) were used for inoculation. All other inbred lines were obtained from Harlan, UK. Mice were identified by individual transponder tags (Trovan) and tail biopsies were obtained for DNA extraction. Mice were anaesthetized with isofluorane/O2 and inoculated intra-cerebrally into the right parietal lobe with 30 µl Chandler/RML prions as previously described [6]. Incubation time was calculated retrospectively after a definite diagnosis of scrapie had been made and defined as the number of days from inoculation to the onset of clinical signs [17]. All procedures were conducted in accordance with UK regulations (Local ethics approval and Home Office regulation) and international standards on animal welfare.

Genotyping. Microsatellites were selected (Table S1) from the UCSC Mouse Genome Browser http://genome.ucsc.edu and Mouse Genome Informatics web site (www.informatics.jax.org). For the HS cross nine microsatellite markers from chromosome 19 (D19Mit86-D19Mit112 see Table S1) were genotyped in approximately 400 animals which represent the extreme 20% of both sides of the incubation time distribution. Fluorescently labelled and standard oligonucleotides were synthesized by Sigma-Genosys. PCR reactions were all carried out in 5 µl on 96-well plates using MegaMix Blue (Microzone Ltd) according to the manufacturer’s instructions using 5 pmole of each primer. PCR conditions were determined empirically but in general cycling conditions using a PTC-225 (MJ Research) thermal cycler were as follows: 94°C for 10 min; 94°C 30 s, 55°C 30 s, 72°C 30 s for 35 cycles; 72°C for 5 min. Products of appropriate size and fluorochrome were pooled before further processing. Reactions were ethanol precipitated, washed in 70% ethanol and re-suspended in a total of 10 µl including 5.8 µl MegaBACE loading solution (GE Healthcare) and 0.2 µl MegaBACE ET400-R size standard (GE Healthcare). 1/10 dilution in MegaBACE loading solution was used for analysis. Fragments were heat denatured at 94°C for 2 min before loading onto a MegaBACETM1000 capillary sequencer (GE Healthcare). Samples were injected at 3 KV for 45 s and run at 10 KV for 60 minutes. Fragment sizes were analysed using Genetic Profiler v1.1 (GE Healthcare). Multipoint linkage analysis was carried out using HAPPY. Mouse family structure was not taken into consideration for this analysis, therefore, the effect size calculated by HAPPY (http://www.well.ox.ac.uk/happy) [18] for each strain (Table S2) may be overestimated. Novel methods are under development for including family structure and more accurate estimates of effect size (personal communication Richard Mot).
for 40 cycles; 72°C for 5 min. PCR products were cleaned using Microclean (Microzone Ltd) according to the manufacturer’s instructions and re-suspended in H₂O. 100–200 ng PCR product was added to a 15 μl sequencing reaction including 5 pmol of either the forward or reverse primer, 1 μl BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems) and 5 μl Better Buffer (Microzone Ltd). Cycling conditions were: 95°C 30 s, 50°C 15 s, 60°C 120 s, for 30 cycles. Reactions were ethanol precipitated, washed in 70% ethanol and re-suspended in 10 μl MegaBACE loading solution (GE Healthcare). Products were detected on a MegaBACE 1000 capillary sequencer (GE Healthcare). Samples were injected at 3 KV for 40 s and run at 9 KV for 100 minutes.

RNA Extraction and Quantitative RT-PCR

Mouse brain. RNA was extracted from whole brains from either uninfected or RML terminally sick mice. For the HS parental lines samples were obtained for A, AKR, BALB/c C3H, C57BL/6, CBA and DBA/2 strains but not LP. Eight week old adult male mice were used for all normal brains. Tissue was homogenized using a Ribolyser according to the manufacturer’s instructions. RNA was prepared using either RNeasy Maxi (Qiagen) kit or TRIreagent (Ambion) according to the manufacturer’s instructions. Samples were treated with DNasel (Qiagen) and purified further using RNeasy Mini (Qiagen) columns according to the manufacturer’s instructions. 4 μg total RNA was reversed transcribed with AMV reverse transcriptase and random primers from the Reverse Transcription System (Promega) according to the manufacturer’s instructions. Reactions with no reverse-transcription were also carried out for each sample to ensure no genomic DNA contamination. Hectd2 real-time PCR was carried out on a 7500 Fast Real-time PCR System (Applied Biosystems) in a total volume of 15 μl using 1 μl cDNA (200–500 ng) and QuantiTect probe PCR kit (Qiagen) according to the manufacturer’s instructions. Primers (6 pmol) F- 5′-GCCGCTAGGTTACCTGAGGT-3′, R- 5′-GAGTTACTCGACCCCTTGATTTGCTG-3′, and probe (3 pmol) 5′-FamTGT-CGCTGTGCTTTCGACCCAAATGTTg-3′ were designed using PrimerExpress software (Applied Biosystems) and supplied by Sigma Genosys. Rodent GAPDH or PGK-1 (Applied Biosystems) (data not shown) was duplexed within the reaction as an endogenous control according to the manufacturer’s instructions. cDNA was diluted 1/2 in H₂O for use in downstream PCR reactions. For real-time reactions human HECTD2 primers (6 pmol) F- 5′-GCAAATGT-TACCCTTGAGGCAGTTC-3′, R- 5′-CTTCACACCTGGTCTT- CATGTGATAA -3′ and probe (3 pmol) 5′-FamCAATAT-TATGCCCTGAGGTGCGCCGATGTGATg-3′ were designed using PrimerExpress software (Applied Biosystems) and supplied by Sigma Genosys. Reactions were carried out on a 7500 Fast Real-time PCR System (Applied Biosystems) in a total volume of 15 μl using 1 μl cDNA and ROXMaxiGold Mix (Microzone Ltd) according to the manufacturer’s instructions. Human β-actin or GAPDH mRNA expression level is expressed in arbitrary units as normalised by the quantity of endogenous control. 2.5 pmole of each primer and 1 pmole of each probe was used for mouse genotyping. All reactions were carried out in 5 μl on a 7500 Fast Real-time PCR System (Applied Biosystems) using either RoxMegaMix Gold (Microzone Ltd) or QuantiTect probe PCR kit (Qiagen). Cycling conditions were the same for both enzymes (95°C 15 s, 60°C 60 s for 40 cycles) however RoxMegaMix Gold and QuantiTect probe enzyme required 5 and 15 mins initial heating at 95°C respectively. For marker rs7081363 primers were designed as above. F primer (CCCGACCGCGAGCG), R primer (CCACAGGTGCCACAGGTGTG), Probe allele C (CCTCCCGCGCCG- Vic/MGB), Probe allele T (CCCTCGTCCGCT – Fam/MGB). 10 μl reactions were carried out as above except that 1 M Betaine (Sigma) was added to the reaction and the annealing temperature was 38°C.

Supporting Information

Figure S1 HAPPY multipoint linkage analysis for Mmu19. Results are displayed on the y-axis as −log of the P value with cm or Mb distance along Mmu19 on the x-axis. A, Log probability plot (additive model) for microsatellites between D19Mit86 and D19Mit112. The peak of linkage is seen for the interval D19Mit63- D19Mit65. For details of intervals see Table S1. B, Linkage analysis for all polymorphisms detected in genes in the interval D19Mit63- D19Mit65. Details for individual SNPs are given in Table S4. Found at: doi:10.1371/journal.pgen.1000383.s001 (0.06 MB DOC)

Figure S2 Quantitative RT-PCR of Hectd2 for individual mouse strains. cDNA was prepared from whole brains of uninfected 8 week old male mice or mice at the terminal stages of disease following intracerebral inoculation with Chandler/RML mouse-adapted scrapie prions. All samples were duplexed for Hectd2 and GAPDH fluorogenic probes and run in triplicate with n = 6 for each mouse strain/group. Mean±s.e.m. Hectd2 mRNA expression levels were expressed in arbitrary units as normalised by the quantity of GAPDH (y-axis). All mouse strains carry the Pnpα allele. Published incubation times with Chandler/RML are: AKR 123±4; BALB/c 124±11; C3H 132±4; DBA/2 134±3, C57BL/6 137±0 or 145±2; CBA 140±10 [20–22]. Incubation times with RML prions are unknown for mouse strains A and LP. Found at: doi:10.1371/journal.pgen.1000383.s002 (0.02 MB DOC)

Table S1 Linkage analysis for microsatellite markers Mmu19. Found at: doi:10.1371/journal.pgen.1000383.s003 (0.03 MB DOC)

Table S2 Trait estimates for HS parental strains D19Mit63- D19Mit65. Found at: doi:10.1371/journal.pgen.1000383.s004 (0.03 MB DOC)
Table S3  Sequenced RefSeq genes from interval D19Mit63-D19Mit65. Found at: doi:10.1371/journal.pgen.1000383.s005 (0.03 MB DOC)

Table S4  Analysis of polymorphisms from D19Mit63-D19Mit65. Found at: doi:10.1371/journal.pgen.1000383.s006 (0.3 MB DOC)

Table S5  Primer and probe sequences for mouse polymorphism genotyping. Found at: doi:10.1371/journal.pgen.1000383.s007 (0.04 MB DOC)

Table S6  Genotyping results for HECTD2 tagging SNPs. Found at: doi:10.1371/journal.pgen.1000383.s008 (0.04 MB DOC)

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Author Contributions

Conceived and designed the experiments: SEL JC. Performed the experiments: SEL EGM HP JG EM JU. Analyzed the data: SEL HH SM. Contributed reagents/materials/analysis tools: JW MPA. Wrote the paper: SEL JC.