Introduction

Modifier genes have been ascribed significant influence in determining susceptibility to disease in complex traits, as well as partial penetrance and variable expressivity of monogenic conditions [1]. Moreover, modifier genes are considered largely responsible for the phenotypic variation observed when mutations are bred onto different genetic backgrounds in mice. However, identification of modifier genes and determination of their functional effects presents a considerable challenge. Understanding the genetic basis of neural tube defects (NTDs), such as spina bifida and anencephaly, typifies these difficulties. NTDs are common, severe congenital malformations resulting from failure of closure of the neural tube during embryonic development [2]. In humans, they are among the commonest birth defects, affecting around 1 per 1000 pregnancies worldwide. However, the causes are not well understood owing to their multifactorial inheritance and the potential influence of environmental factors, either predisposing or ameliorating [3,4]. The potential complexity of NTD genetics is illustrated by the fact that more than 200 different genes have been implicated as potential contributors to the overall burden of NTDs, with neural tube closure phenotypes in mouse strains carrying naturally occurring or targeted mutations [5–7]. Additionally, in many of these models penetrance is influenced by genetic background, indicating the presence of modifier genes.

The curly tail (ct) mouse mutant is among the most extensively characterised models of NTDs [8]. Approximately 5–10% of homozygous ct/ct embryos develop cranial NTDs (exencephaly), while 15–20% exhibit spinal NTDs (spina bifida), due to failure of closure of neural folds in the prospective brain and low spinal region, respectively. The major ct gene corresponds to a hypomorphic allele of the transcription factor grainyhead-like-3 (Grhl3), null mutants of which display spina bifida with 100% penetrance [9–11]. Expression of Grhl3 is diminished in the hindgut of ct mutant embryos, due to an upstream regulatory mutation, resulting in a diminished cellular proliferation rate in the hindgut endoderm [12,13]. The consequent dorso-ventral growth...
Author Summary

Failure of early development of the central nervous system leads to severe malformations termed neural tube defects (NTDs), including spina bifida and anencephaly. Inherited genetic risk factors play a major role in determining susceptibility to NTDs, but causative genes have proven difficult to identify. In this study we investigated genetic factors that could alter the risk of NTDs in an established mouse model, curly tail, in which defects result from partial loss of function of the gravyhead-like-3 (Grhl3) gene. We identified a variant of lamin B1, a key protein component of the envelope that surrounds the cell nucleus. The protein alteration reduces the structural integrity of the nuclear envelope, causes the nuclei to have altered shape, and reduces the rate of cell division. Curly tail embryos that carry the “abnormal” lamin B1 variant develop NTDs at three times the rate of those that carry the normal version. We conclude that lamin B1 function influences risk of NTDs due to effects on cell proliferation.

imbalance leads to excessive ventral curvature of the caudal region of the embryo and, hence, mechanical suppression of neural tube closure at the posterior neuropore [14]. The incidence of curly tail NTDs can be influenced by multiple environmental and genetic factors [8,15–18]. In addition, NTD frequency is also markedly affected by backcross to different strains, indicating the presence of modifier loci in the curly tail genetic background [19]. Thus, it is apparent that the genetic component of predisposition to NTDs is multifactorial in ct, as in humans.

In the current study, we identified lamin B1 as a modifier gene for NTDs in curly tail mice. Lamins are intermediate filament proteins of which the A-type, lamins A and C, are encoded by LMNA while, among the B-type, lamin B1 is encoded by LMNB1 and lamins B2 and B3, are encoded by LMNB2. The nuclear lamina is a protein complex underlying the inner nuclear membrane and composed of a meshwork of lamin polymers and lamin-binding proteins [20–22]. In addition to a key structural role in assembly and maintenance of the nuclear envelope, it has become clear that lamins have multiple functions in a diverse range of cellular properties. Thus, lamins influence nuclear shape and size as well as anchoring of protein structures, including nuclear pore complexes, in the nuclear envelope [23,24]. Additionally, lamins function in DNA synthesis and transcriptional regulation both through interaction with chromatin, to mediate sub-nuclear chromosomal positioning, and by direct interactions with transcription factors [25–29].

Highlighting the importance of lamin function, a number of clinically distinct diseases, termed laminopathies, have been found to result from mutation of LMNA [20,30]. These include muscular dystrophy disorders (e.g. Emery-Dreyfus muscular dystrophy), lipodystrophies, progeria syndromes (e.g. Hutchinson-Gilford progeria syndrome and Atypical Werner syndrome) and peripheral neuropathy (Charcot-Marie-Tooth disease type 2B1). In contrast to LMNA, coding mutations in LMNB1 have not so far been associated with human disease, although genomic duplication of LMNB1 is thought to cause a progressive dermatomyositis, adult-onset autosomal dominant leukodystrophy [31,32]. Mice homozygous for a loss of function allele of Lmnb1 die at birth with reduced growth, impaired lung development and cortical abnormalities in the brain [33,34], while Lmnb2 knockouts exhibit neuronal migration defects in the cerebral cortex and cerebellum [35]. Lmnb1/lmnb2 double knockouts exhibit a reduced thickness of the brain cortex, with altered cell cycle exit of neuronal progenitors and neuronal migration defects [29,34]. Forebrain-specific deletion of lmb1 or lmb2, allowed study of brain phenotypes at post-natal stages and showed that both genes are individually required for normal development of the cortex [34].

In the current study we identified a polymorphic variant form of lamin B1, present on the genetic background of the curly tail strain. The reduction in length of a series of glutamic acid residues, from nine to eight, was found to cause significant reduction in the stability of the lamin B1 interaction within the nuclear lamina. Genetic analysis, involving generation of curly tail sub-strains carrying combinations of the lamin B1 variant and Grhl3-/- mutation demonstrate a dramatic effect of lamin B1 on frequency of NTDs. In parallel, lamin B1 has a profound effect on nuclear morphology and proliferative capacity. Overall, our findings show that Lmnb1 can act as a modifier gene affecting risk of NTDs, an effect that appears to be mediated through impaired cell cycle regulation which summates with the effect of Grhl3 mutation.

Results

In a proteomic analysis of the curly tail mutant, two-dimensional protein gels were generated from samples at embryonic day (E) 10.5: the stage of spinal neural tube closure. Comparison of stage-matched embryos revealed differential migration of a series of three spots, which migrated to a more basic position in gels derived from ct/ct samples than the equivalent spots in congenic wildtype (+/+ +/) control gels (Figure 1A–1C). This migration change was apparent by the complete absence of the three spots that were detected in the +/+ +/+ gels from the ct/ct gels and vice versa. This difference was detected both in analysis of whole embryos and in isolated caudal regions that encompassed the posterior neuropore (PNP), the region of active neural tube closure. In both strains, these spots were identified by liquid chromatography tandem mass spectrometry as lamin B1 (Table S1). Variation in abundance of some other spots between genotypes was observed, however, no spots other than those corresponding to lamin B1 showed a difference in migration. Neither the abundance of Lmnb1 mRNA nor total lamin B1 protein abundance were found to differ between ct/ct and +/+ +/+ embryos, by real time qRT-PCR or western blot respectively (Figure 1D–1F). Moreover, the sites of Lmnb1 expression at neurulation stages were also comparable between genotypes as determined by whole mount in situ hybridisation (Figure 1G–1H). Expression was apparent throughout most of the embryo with the exception of surface ectoderm and the heart (Figure S1), where staining intensity was much lower than in other tissues.

Altered migration of lamin B1 during the isoelectric focussing step of 2-DE results from a charge difference between the protein in ct/ct and +/+ +/+ samples. Such a difference could potentially result from an alteration in primary sequence and the Lmnb1 coding region was therefore sequenced in ct/ct and +/+ +/+ genomic DNA and cDNA. A synonymous polymorphism, C612T (annotated as SNP 18: 56868078), was found in exon 1 of the ct/ct sequence. In addition, a three base-pair GAG deletion (annotated as Deletion 18: 56909394) was noted in exon 10. This deletion corresponds to one of a sequence of GAG nucleotides at position 1657–1683 of the coding sequence, encoding a stretch of nine glutamic acid (Glu) residues in the tail domain of the wild-type protein (Figure 2A, 2B). Thus, the curly tail Lmnb1 gene encodes eight Glu residues at amino acids 553–560 (here denoted Lmnb18Glu to indicate number of glutamic acids), as opposed to nine Glu residues (553–561) encoded by the +/+ +/+ sequence (denoted Lmnb19Glu). Since Glu carries a negative charge, it appeared likely that the difference in number of Glu residues is responsible for the
migration difference of lamin B1 spots on 2D gels generated from ct/ct and +/ct samples.

The Glu repeat in the lamin B1 tail domain is predicted to form an alpha-helix (PSIPRED secondary structure prediction [36]). Loss of a residue would impose a hundred degree rotation on the C-terminal region of the protein. The helix is likely to be capable of interacting with the inner nuclear phospholipid membrane [37]. Given that this region contains another strong membrane interactor, the C-terminal farnesylcysteine, we hypothesised that the interaction of lamin B1 with the nuclear membrane could be affected by variation in the number of Glu residues. We therefore used fluorescence loss in photobleaching (FLIP) to investigate possible functional effects on the stability of the lamin B1 tail domain within the nuclear envelope. Full length laminB1-YFP fusion proteins appeared to be stably integrated into the nuclear lamina without apparent difference between variants. We also tested truncated forms of the protein as these have previously been found to provide greater sensitivity to altered properties in this assay [26]. Fusion proteins comprising a nuclear localisation sequence, YFP and the forty C-terminal residues of lamin B1 were expressed in primary mouse embryonic fibroblasts (MEFs) and subjected to FLIP, as previously performed for human lamin B1 [26]. The decline in fluorescence intensity in the unbleached area of membrane was much more rapid in cells expressing Lmnb18E compared with Lmnb19E (Figure 2C). After 100 seconds, there was an approximately 43% decline in intensity in cells expressing Lmnb18E compared with only a 21% decline with Lmnb19E (p<0.001, t-test). This significant difference between variants persisted throughout the analysis, and is indicative of increased mobility, and hence decreased stability of interaction of Lmnb18E within the nuclear envelope.

Sequencing of exon 10 of lamin B1 in a series of mouse strains showed that the wild-type (Lmnb19E) variant of lamin B1 is found in the majority of strains including C57BL/6, C3H/HeJ, SWR, DBA/2J, BALB/c, LPT/Le and CAST/EiJ. However, the Lmnb18E variant occurs in CBA/Ca, a sub-strain of which (CBA/Gr) contributed to the genetic background of the curly tail strain [38]. The variant was also present in the 101 strain and hence in mice harbouring the splotch (Sp2H; Pax3) mutation, which arose in a mutagenesis experiment on a mixed CBA/101 genetic background [39]. The 18:56868078 SNP and Deletion 18:56909394 were found to be in linkage disequilibrium. Thus, the Lmnb18E variant in ct/ct is characteristic of this particular genetic background.

Embryos of the CBA/Ca strain do not exhibit developmental abnormalities under normal laboratory conditions, indicating that the Lmnb18E variant alone is insufficient to cause NTDs. Nevertheless, given the possible effect on stability of the lamina, we speculated that this variant could represent one of the modifier genes that are major determinants of penetrance of the curly tail

Figure 1. Lamin B1 shows differential protein migration by two-dimensional gel electrophoresis in curly tail and wild-type embryo samples. Protein profiles of the caudal region of stage-matched wild-type (A) and curly tail (B) embryos analysed by 2-DE (representative gels shown encompass pH 3.0–5.6 on the x-axis, basic pH to the right). A series of silver-stained protein spots exhibits a different migration pattern (C; enlarged area of gel corresponds to dashed box in A and B). On 2-DE of ct/ct samples, spots (pink arrows and pink spots on merged image) migrate to a more basic position than the corresponding spots on +/ct gels (green arrows and green spots on merged image). Spots whose migration does not differ between samples appear black on the merged image. Western blot (D–E) and qRT-PCR (F) of protein and mRNA samples from the caudal region of +/ct and ct/ct embryos at E10.5 show no significant difference between strains in relative abundance of either lamin B1 protein (normalised to beta-tubulin; arbitrary units) or mRNA (normalised to Gapdh with one wild-type sample chosen as calibrator; value set to 1.0). Number of samples, n, is shown on graphs. (G, H) The distribution of Lmnb1 mRNA at E10.5, as determined by whole mount in situ hybridisation is comparable in wild-type and ct/ct embryos (scale bar represents 1 mm).

doi:10.1371/journal.pgen.1003059.g001
Lamin B1 and Neural Tube Defects

![Graph and images related to Lamin B1 and Neural Tube Defects](image_url)
defect. To test this idea, we inter-crossed ct/ct and +/4E mice to generate sub-strains of mice carrying different combinations of the Lmb1 variant (i.e. Lmb18E and Lmb19E, abbreviated hereafter as L8E and L9E) and the Geh3 mutant allele (Geh3s or Geh3a; abbreviated as Gs and Ga). Each sub-strain was maintained in homozygous form for both Lmb1 and Geh3 alleles, that is: (i) L8E/L8E; Gs/Gs (denoted ct8E); (ii) L8E/G9E; Gs/+ (denoted +/ct8E); (iii) L9E/L9E; Ga/Ga (denoted ct9E); (iv) L9E/G9E; Ga/+ (denoted +/ct9E). In the +/ct strain the genetic background is approximately 97% curly tail [9] and in each sub-strain it is predicted to be 99.5% curly tail (see Figure S2 for breeding scheme). Embryos were collected at E11.5–15.5 and analysed for the presence or absence of NTDs.

Among embryos of the ct8E sub-strain, the range and frequency of phenotypes was closely similar to that observed in the curly tail (ct) strain which has the same genotype at the Lmb1 and Geh3 loci. Defects included spina bifida, tail flexion defects and exencephaly (Figure 3B–3D), while other embryos appeared normal (Figure 3A). Importantly, however, varying the Lmb1 genotype produced a striking difference in frequency of NTDs (Figure 3E). Thus, spina bifida occurred at significantly lower frequency in the curly tail (ct+ and +/ct) sub-strain later in development.

Although mean PNP length did not differ between curly tail and the ct8E sub-strain, embryos of the ct8E sub-strain exhibited a more rapid reduction in PNP length from the 28–29 somite stage onwards (Figure 4), indicative of an overall normalisation of spinal neural tube closure. The distribution of PNP lengths in embryos of the ct8E sub-strain was shifted towards smaller values, with a significantly lower mean PNP length. Moreover, only a few ct8E embryos showed very large PNP, whereas a greater proportion of embryos had completed PNP closure by the 30–31 somite stage (8 of 30 compared with 1 out of 20 among the ct8E sub-strain; p<0.05, t-test; Figure S3). These observations on PNP length correlate with the diminished frequency of spina bifida in the ct8E sub-strain later in development.

The genetic background of each sub-strain was predicted to be approximately 99.5% curly tail we could not exclude a possible effect of the region of DNA that is tightly linked and inherited with Lmb1. We therefore examined the possibility that a neighbouring gene to Lmb1 could vary in expression between the ct8E and ct9E sub-strains. Using a list of genes that are located within a 41 Mb interval of chromosome 18 centred on Lmb1, we interrogated microarray data generated from RNA of the caudal region of stage-matched ct/ct and +/4E embryos (E10.5; 28–29 somite stage). Among 11 differentially expressed genes (p<0.05; fold-change 1.5-fold or greater), 4 showed a similar trend of differential expression on qRT-PCR analysis of independent ct/ct and +/4E samples. However, none of these genes varied in expression when analysed by qRT-PCR in stage-matched ct8E and ct9E samples (Table S2), suggesting that the phenotypic difference between the sub-strains does not result from differential expression of genes located in proximity to Lmb1. Instead, variation in expression between ct/ct and +/4E samples seem likely to be due to downstream effects of the Geh3a mutation in ct/ct embryos.

Embryos of the wild-type congenic curly tail (ct8E) variant (4E/ct8E; Gs/+), we observed a low frequency of tail flexion defects, indicative of delayed PNP closure (Figure 3E). Exencephaly was also occasionally observed (Figure 3E). These data demonstrate that the presence of the Lmb19E variant can predispose to defects of cranial and spinal neural tube closure, even in the absence of the Geh3 mutation.

Although curly tail NTDs are partially penetrant, affected embryos can be recognised on the basis of an enlarged PNP at E10.5 [40]. In order to examine the effect of Lmb1 variants on the progress of spinal neural tube closure directly, PNP length was measured in a series of embryos at E10.5 (Figure 4). Among embryos that were wild-type-like at the Geh3 locus (+/ct and +/ct/ct), PNP length diminished rapidly between the 26 and 31 somite stages and, by the 30–31 somite stage, the PNP was very small (12 out of 37 embryos) or closed (23 of 37 embryos). There was no detectable difference between embryos with Lmb18E and Lmb19E genotypes. In contrast, mean PNP lengths were significantly larger in the Geh3ct/ct sub-strains, reflecting an overall delay in closure.
the variant lamin B1 imposes a dysmorphic phenotype on the nuclear lamina as a whole. Abnormalities were much less frequent in nuclei of the +ct;9E and C57BL/6 strains, carrying wild-type alleles of Lmnb1 and Grhl3. To provide a quantitative measure of nuclear morphology, the contour ratio (4π*area/ perimeter) of DAPI-stained nuclei was analysed (Figure 5B, 5C). The mean contour ratio was significantly lower for ct nuclei than for any of the other strains (Figure 5B). Consistent with these findings, compared with other strains examined, a significantly greater proportion of ct nuclei showed a contour ratio of less than 0.7 (Figure 5C), which is considered abnormal [42].

Figure 3. The frequency of NTDs resulting from mutation of Grhl3 is affected by Lmnb1 genotype. Embryos were scored as (A) apparently normal with a straight tail (ST), or with (B) a tail flexion defect (i.e. curly tail; CT), (C) spina bifida (SB) plus tail flexion defect, and/or (D) exencephaly (arrowhead indicates open hindbrain). Note that exencephaly can occur in association with any of the spinal phenotypes. Embryos shown are from the ct8E sub-strain. The frequency of NTD phenotypes is tabulated (E). The frequency of SB is significantly lower in the ct8E than in the ct8E and ct strains (* p<0.02, χ² test). Spina bifida and tail flexion defects were never observed among ct8E embryos but tail flexion defects did occasionally occur among ct8E embryos (** χ² versus χ²; p<0.05; Z-test), although at significantly lower frequency than among ct mutant embryos (# p<0.001; χ²). There is significant variation in the frequency of exencephaly between the sub-strains (p<0.001; χ²) with a significantly lower exencephaly rate among ct8E than ct8E embryos († significantly different from ct8E, p<0.01; Z-test). Exencephaly was not observed in the ct8E strain († indicates significant difference from ct and ct8E; p<0.001; Z-test) and ct8E (p<0.02) strains. Exencephaly was observed in the ct8E strain, albeit at a significantly lower frequency than in the ct8E strain († p<0.05; Z-test).

doi:10.1371/journal.pgen.1003059.g003

The curly tail nuclear dysmorphology phenotype was rescued by the presence of the Lmnb1 9E variant in the ct9E sub-strain (Figure 5), correlating with the apparent increased stability of the lamina when this variant is present, as observed by FLIP (Figure 2C). Interestingly, in MEFs from a transgenic ct strain, ctTgGrhl3 in which Grhl3 expression is reinstated by over-expression from a Grhl3-containing BAC [9], the nuclear morphology was intermediate between that of ct and ct8E MEFs (Figure 5). Thus, although ctTgGrhl3 mice are on an identical genetic background to ct, including the Lmnb18E variant, it appears that over-expression of Grhl3 is sufficient to partially ameliorate the nuclear dysmorphology phenotype. The mean contour ratio of nuclei was higher, and the proportion of abnormal nuclei was lower, for C57BL/6 than any of the other strains, including +ct;9E (Figure 5). Thus, in addition to lamin B1 sequence and Grhl3 expression, other factors associated with the curly tail genetic background may influence nuclear morphology. Overall, among strains with the curly tail genetic background, those that express the Lmnb19E variant (ct9E and ct8E) have a significantly higher mean contour ratio (Figure 5B) and fewer dysmorphic nuclei (Figure 5C) than those that express the Lmnb18E variant (ct and ctTgGrhl3).

The effect of the Lmnb19E variant on nuclear morphology and the known function of lamins in nuclear function, including DNA
replication [24], prompted us to investigate the effect of the Glu variant on proliferative capacity in ct cells. MEFs were plated and counted after 4 hours (t = 0) and after successive 24 hour periods up to 5 days. Growth curves showed that ctMEFs proliferate significantly more slowly than their ctE counterparts over the first four days in culture (p < 0.05; Multiple linear regression, R² = 0.948) and then undergo a ‘proliferative crisis’ where cell numbers cease to increase (Figure 6A). The experiment was performed on three separate occasions using independent cell lines, with the same result each time. Therefore, in addition to nuclear dysmorphology, the Lmnb19E variant is associated with an apparent reduction in proliferative capacity in ct cells. In contrast, ctE cells continued to proliferate at a similar rate to wild-type +ctE cells at day 5 (Figure 6A). In accordance with the growth curve data, we also noted that when MEFs were repeatedly passaged, ctE fibroblasts show a dramatic loss of proliferative capacity from passage 5 onwards, whereas ctE continue to exhibit similar doubling times up to at least passage 8.

To further investigate cell cycle properties of ctE and ctE cells, labelling with 5-ethyl-2’-deoxyuridine (EdU; to monitor S-phase progression) and immunostaining for phospho-histone H3 (pH 3; a marker of mitosis) were performed on day 0. This is well before the profound loss of proliferative capacity that occurs in ctE cells after extended culture and it was therefore predicted that differences, if present, may be subtle. However, corresponding with growth curve data, we observed significantly fewer EdU-labelled ctE cells than ctE cells (Figure 6B), together with a non-significant reduction in pH 3 labelling (cells in G2/M phase) and mitotic index. Consistent with the reduced EdU labelling in ctE cells, indicating that fewer cells had passed through S-phase, we observed a slightly lower proportion of EdU/pH 3 double-labelled nuclei. However, there was no difference between strains in the number of double-labelled cells as a proportion of the total number of EdU-labelled cells (Figure 6B), suggesting that progression from S-phase to G2 is not defective in ctE cells.

We next examined the expression of key regulators of cell cycle expression by qRT-PCR, 4 hours after plating (t = 0, as for cell cycle analysis) and after 5 days of culture (t = 5). The reduced proliferative capacity of ctE cells during the initial growth period was associated with significantly lower expression of Ccn1, encoding cyclin D1 (Figure 6C). After 5 days, the expression of Ccn2 and Cenbl (encoding cyclin A2 and cyclin B1, respectively) was also significantly reduced in ctE compared with ctE cells, consistent with diminished cell cycle progression [43,44]. Conversely, there was a dramatic increase in expression of Pldha (Figure 6C), which suppresses cell cycle progression through inhibition of cyclin D-dependent kinases [45] and is a hallmark of cells entering senescence. The expression of Pldha was also increased in ctE cells at t = 5 compared with t = 0, but to a much lesser extent. In addition, at both stages ctE cells also exhibited a significant reduction in expression of Snc2, which encodes a core component of the condensin I and II complexes that play key roles in chromosome condensation during mitosis [46,47]. We conclude that changes in expression of cell cycle-associated proteins are consistent with reduced cell cycle progression in cells expressing the Lmnb19E variant, compared with those expressing the wild-type Lmnb1E variant.

Finally, we tested whether the Lmnb1 variants were also associated with differences in cellular proliferation rate in the developing embryo. Analysis was performed on the neural folds and hindgut at the axial level of the closing PNP, at the stage at which the underlying defect in proliferation in the hindgut of affected curly tail embryos was reported [12,13]. Consistent with the findings in cultured cells, the EdU labelling index was lower in ctE than in ctE embryos, particularly in the hindgut (Figure 6D, Table S3). Mitotic index was similar in the sub-strains (Table S3). The diminished S-phase progression of cells in the hindgut of ctE embryos corresponds with the proliferation defect that is known to underlie spinal NTDs in curly tail embryos.
Figure 5. Nuclear morphology is influenced by Lamin B1 variation. (A) MEFs derived from embryos of various genotypes were stained with DAPI (blue) and antibodies to lamin B1 (green) and lamin A (red) to highlight the nuclear lamina. Abnormalities observed include lobulations (yellow arrowhead) and herniations (white arrowhead). (B, C) Analysis of MEF nuclei reveals significant differences between sub-strains in (B) mean contour ratio and (C) percentage of nuclei with contour ratio lower than 0.7, which is considered dysmorphic ($p<0.001$; ANOVA). (B) Mean contour ratio (expressed as mean ± SEM) is significantly higher in C57BL/6 and significantly lower in ct than all other strains. (C) Compared with all other strains, ct has a significantly higher frequency of dysmorphic nuclei (47.4 ±5.8%) and C57BL/6 (15.1 ±3.9%) has significantly fewer dysmorphic nuclei (** significant difference from all other strains, $p<0.01$). The proportion of dysmorphic nuclei is lower in strains with the wild-type Lmnb1 allele, but higher than in C57BL/6; * indicates significantly different from all other strains ($p<0.01$ for comparison with C57Bl/6, ct and ctTgGrhl3 and $p<0.05$ for comparison with ct or ctTgGrhl3). Over-expression of Grhl3 partially normalises nuclear phenotype in the ctTgGrhl3 strain (despite presence of Lmnb1 variant as in ct). The mean contour ratio is significantly higher than in the ct strain but lower than in ct9E or ctTgGrhl3 strains (** indicates significant difference compared with all other strains tested, $p<0.01$). Values are an average of 9–15 experiments, using 2–3 independent cell lines for each strain. Total number of cells analysed: 579 C57BL/6; 895 ct; 757 ctTgGrhl3; 882 ct9E; 837 ctTgGrhl3. doi:10.1371/journal.pgen.1003059.g005
Discussion

The multifactorial, partially penetrant genetics of the curly tail mouse provided an opportunity to investigate the Lmnb1 polymorphism as a potential modifier of susceptibility to NTDs. In the context of the genetic background of the curly tail mouse, we observed a major effect of lamin B1 on development of the neural tube, the embryonic precursor of the brain and spinal cord. Curly tail sub-strains expressing the Lmnb1 variant demonstrate failure of neural tube closure with significantly higher frequency than those that express wild-type protein. Thus, although both the curly tail sub-strains (ct9E and ct8E) are homozygous for the Grhl3 mutation, which results in diminished Grhl3 expression [9], there is a three-fold difference in the frequency of NTDs depending on the co-existing Lmnb1 genotype. Strikingly, although exencephaly occurs at much lower frequency than spina bifida, Lmnb1 also affected the penetrance of these defects to a similar extent as spinal NTDs, with approximately 65% reduction in frequency among ct9E compared with ct8E embryos. Interestingly, it appears that the Lmnb1 variant may confer susceptibility to NTDs even in the absence of a Grhl3 mutation, at least in the context of the ct genetic background. Thus, +/ct embryos that are wild-type for Grhl3 but which carry the Lmnb1 variant developed occasional tail flexion defects and/or exencephaly. In contrast, spinal NTDs can be prevented by transgenic over-expression of Grhl3 expression (ctTgGrhl3) [9], despite the presence of the Lmnb1 variant.

The possible functional effect of polymorphic variants has been explored in very few proteins, to date. We found that the loss of a single Glu, in Lmnb1, compromises the stability of lamin B1’s interaction within the nuclear lamina. Thus, +/ct embryos that are wild-type for Grhl3 but which carry the Lmnb1 variant developed occasional tail flexion defects and/or exencephaly. In contrast, spinal NTDs can be prevented by transgenic over-expression of Grhl3 expression (ctTgGrhl3) [9], despite the presence of the Lmnb1 variant.

Figure 6. Cell cycle progression is impaired in curly tail cells and embryos expressing the Lmnb1 variant. In cultured embryonic fibroblasts (A–C), analysis of growth curves (A) shows that ct8E cells proliferate significantly slower than ct9E cells (*p<0.05; multiple linear regression for days 0–4) and then undergo a ‘growth crisis’ after 4 days culture. (B) Cell cycle analysis was performed at day 0 (5 hours of culture) using EdU to label cells as they progress through S phase (Bii) and anti-phospho Histone H3 (pH 3) to label cells in G2/M (Biii–vi). Mitosis was scored visually as cells that were in prophase, metaphase (Biv), anaphase (Bv) or telophase (Bvi). Data represents the mean of three experiments, each using an independent cell line, plated in triplicate. ct9E cells show significantly reduced EdU labelling (* p<0.05; t-test). There is a trend towards reduced pH 3 labelling and mitotic index in ct8E cells, but this difference is not statistically significant. The proportion of cells double-labelled with EdU and pH 3 (Bii) does not differ between ct9E and ct8E cells. (C) Expression of cell cycle regulators determined by qRT-PCR. For each gene, significant differences in the comparison of ct9E and ct8E cells cultured for the same period are indicated (* p<0.05, ** p<0.01; ANOVA with Holm-Sidak Pairwise Comparison). Expression differences between 0 and 5 days in culture for cells of the same genotype are indicated (# p<0.05). (D) Analysis of proliferation in embryos at E10.5 showed that EdU labelling index was significantly diminished in the hindgut of ct8E compared with ct9E embryos (* p<0.02; t-test).

doi:10.1371/journal.pgen.1003059.g006
Lmnb1<sup>ct8E</sup> variant compared with Lmnb1<sup>9E</sup>. Abnormalities in lamin immunostaining and nuclear shape are reminiscent of cells with nuclear envelope abnormalities, such as from progeria models [42], lamin B1 mutant mice [33,34] and following shRNA-mediated silencing of lamin B1 [40]. Using contour ratio analysis, abnormal nuclei were observed in around 47% of primary embryonic curly tail fibroblasts (current study), compared with 68% of primary dermal fibroblasts derived from a patient with Hutchinson-Gilford progeria syndrome [42]. Only 7–15% of nuclei among control fibroblasts exhibited such abnormalities. In these previously reported examples, abnormalities of cell proliferation, chromosome position, transcription factor localisation and gene expression have all been noted [22,26,33].

We found a strong correlation between frequency of dysmorphic nuclei in MEFs derived from embryos of the ct strain, and frequency of NTDs. For example, among mice homozygous for the Ghhl<sup>3t</sup> hypomorphic allele, presence of the wild-type Lmnb1<sup>9E</sup> led to a reduced NTD frequency and an increased proportion of ‘normalised’ nuclei in MEFs. These findings suggest that nuclear lamina function plays a contributory role to the efficiency of neural tube closure during embryogenesis. Whether altered nuclear structure directly affects NTD risk or is a secondary marker of altered lamin B1 function is not known. To investigate the cellular mechanism by which lamin B1 affects embryonic development we focussed on a possible effect on cell cycle progression, in view of the known tissue-specific cell cycle defect that underlies spinal NTDs in curly tail mice [8].

Lamin B1 functions in nuclear envelope breakdown/assembly and mitotic spindle formation [23,25,49]. In addition, lamin B types are spatially associated with and required for DNA synthesis during S-phase [50]. Effects of lamin B1 dysfunction on cell cycle regulation could also be mediated through altered regulation of gene expression. For example, sequestration of the transcription factor Oct-1 at the nuclear periphery is lost in cells expressing a truncated form of lamin B1, resulting in mis-expression of target genes, including cell cycle mediators [27,51]. In ct fibroblasts expressing the Lmnb1<sup>ct9E</sup> variant, analysis of growth curves and cell cycle markers revealed diminished proliferative capacity and premature senescence, accompanied by characteristic changes in expression of cell cycle mediators. Cell labelling experiments suggest that the reduced proliferation rate of ct<sup>8E</sup> cells does not result from a defect at the S-phase/G2 transition but more likely from impairment of G1 or G1/S transition. Such an idea is consistent with the reduced expression of cyclin D1, which promotes progression through G1/S. The proliferative crisis that occurs in ct<sup>8E</sup> following extended culture is accompanied by reduced expression of cyclins A2 and B1, which function at G2/M [52], and increased expression of p16<sup>ink4a</sup>. Although our study addresses an amino acid change rather than reduced expression, these observations are consistent with recent studies showing that silencing of Lmnb1 expression reduces proliferation rate and induces premature senescence in fibroblast cell lines [53]. Altered cell cycle exit is also thought to be responsible for reduced thickness of the cortex in lmbnb1/lmbnb2 knockout embryos [29,34].

Rather than a generalised growth retardation effect of the lamin B1 variant, it appears that there is an additive effect with the Ghlh<sup>3t</sup> mutation. Cell cycle differences between ct<sup>8E</sup> and ct<sup>9E</sup> cells therefore suggest a mechanism by which Lmnb1 genotype affects the morphogenetic movements of neural tube closure in curly tail mutant embryos. It was previously found that: (i) the cellular basis of spinal NTDs in ct<sup>8E</sup> mutant embryos involves a proliferation defect in cells of the hindgut which causes excessive axial curvature [12]; and (ii) inhibition of proliferation by anti-mitotics or experimental growth retardation increases frequency of cranial NTDs [18,54]. Therefore, the reduction in cellular proliferation rate resulting from the combination of diminished Ghhl3 expression together with perturbation of lamin B1 function, would be predicted to exacerbate both spinal and cranial neurulation, as we observe. In support of this model, and correlated with prevention of NTDs in embryos, reinstatement of Ghhl3 expression in cultured cells that express the Lmnb1<sup>ct8E</sup> variant partially normalises nuclear morphology (e.g. in ct<sup>8E</sup)>Ghhl3<sup>3t</sup>) and proliferative capacity (e.g. growth curves of ct<sup>8E</sup> and ct<sup>9E</sup> cells do not differ). Moreover, in vivo analysis confirmed that proliferation is diminished in the hindgut of ct<sup>8E</sup> compared with ct<sup>9E</sup> embryos, which suggests an explanation for their greater susceptibility to spinal NTDs.

Overall, our findings show that Lmbnb1 is a modifier gene that has a significant influence on the risk of NTDs in curly tail (Ghhl3<sup>3t</sup>) embryos. We propose that the Lmbnb1<sup>9E</sup> polymorphism and Ghhl3<sup>3t</sup> mutation interact genetically to influence nuclear morphology and proliferation, and hence susceptibility to NTDs. The influence of gene–gene interactions on susceptibility to NTDs in the curly tail model parallels the apparent multigenetic etiology of the corresponding human condition. Thus, it appears possible that some individuals carry ‘risk’ alleles that are insufficient to cause NTDs when present in isolation, but confer susceptibility to NTDs when co-inherited with other predisposing alleles. We speculate that variation in human lamin B1, either in the Glu repeat or elsewhere in the protein, would be worthy of investigation in the context of human NTDs.

**Methods**

**Maintenance of mice and genotyping**

**Curly tail (ct/ct), genetically-matched (partially congenic) wild-type (ct<sup>8E</sup>/ct<sup>8E</sup>) and transgenic curly tail mice carrying a Ghhl3-expressing BAC (Ghhl3<sup>3t</sup>/Ghhl3<sup>3t</sup>;Tg(Ghhl3)1NDEG, here referred to as ct<sup>8E</sup>Ghhl3<sup>3t</sup>) were as described previously [8,9].** A two-step breeding programme (Figure S2) was used to generate mice carrying different combinations of the Ghhl3 alleles (referred to as Ghhl3<sup>3t</sup> or Ghhl3<sup>3t</sup>) and Lmbnb1 variants (referred to as Lmbnb1<sup>9E</sup> and Lmbnb1<sup>9E</sup>). Mice of genotype Ghhl3<sup>3t</sup>/Ghhl3<sup>3t</sup>; Lmbnb1<sup>9E</sup>/9E, Ghhl3<sup>3t</sup>/ct, Lmbnb1<sup>9E</sup>/9E and Ghhl3<sup>3t</sup>/ct; Lmbnb1<sup>9E</sup>/9E were selected and inter-crossed to establish independent colonies.

The Ghhl3<sup>3t</sup> allele was genotyped on the basis of the putative mutation, C-21350T, upstream of Ghhl3 by PCR amplification of genomic DNA with restriction digest of PCR products [9]. Genotyping was confirmed by PCR amplification of polymorphic microsatellite markers, D4Bwg1551e and D4Mit204, downstream of Ghhl3. The Lmbnb1 GAG repeat variant (Deletion, 18: 56909394) was genotyped by PCR amplification of genomic DNA using primers that encompass the repeat (5'-GACCCACCACATCCG-GAGGAG and 5'-TCCACAGCCTCCTGATG), with separation of products on 5% agarose gels. The C612T SNP (18: 56900708) creates a HindIII restriction site, allowing genotyping by PCR amplification of exon 1 (using primer pair 5'-GGCG CTTTGGTTTGTACCC-3' and 5'-GGCGACCCTTGTCTG-TAGTTCTA-3'), followed by restriction digest of the PCR product.

**Collection of embryos**

Experimental litters were generated by timed matings. Pregnant females were killed at embryonic day by cervical dislocation and embryos were dissected from the uterus in Dulbecco’s Modified Eagle’s Medium (Invitrogen) containing 10% fetal calf serum (Sigma). At E10.5, the caudal regions of individual embryos at the 30–31 somite stage were excised at the level of somite 15, rinsed in phosphate buffered saline (PBS) and stored at −80°C prior to analysis by 2-DE or Western blot. For in situ hybridisation embryos
were fixed in 4% paraformaldehyde (PFA) in PBS at 4°C overnight. Animal studies were carried out under regulations of the Animals (Scientific Procedures) Act 1986 of the UK Government, and in accordance with guidance issued by the Medical Research Council, UK in Responsibility in the Use of Animals for Medical Research (July 1999).

**Two-dimensional gel electrophoresis (2-DE)**

Samples, comprising whole embryos (n = 10 of each genotype) or individual caudal regions (n = 10 of each genotype), were prepared by sonication in lysis buffer as described previously [55]. Proteins were separated by isoelectric focussing on pH gradients of pH 4–7 or 3–5.6, followed by SDS-PAGE on 12% polyacrylamide gels, as described [56]. Gels were fixed and stained using PlusOne silver stain (GE Healthcare) and scanned using a GS-800 calibrated densitometer (BioRad). Gel images were analysed using Progenesis SameSpots (Non-linear Dynamics) with separate between-genotype comparisons for whole embryos (n = 5 pH 4–7 and 5 pH 3–5.6 gels for each genotype) and caudal regions (n = 5 pH 4–7 and 5 pH 3–5.6 gels for each genotype).

**Liquid chromatography electrospray tandem mass spectrometry (LC-ESI-MS/MS)**

Protein spots were excised manually from a minimum of four different gels, so that each spot was analysed at least in quadruplicate, subjected to in-gel digestion with trypsin and analyzed by LC-ESI-MS/MS (QToF-micro; Waters Corp.) as described previously [55]. Mass spectrometry data were searched against the SwissProt database using the MASCOT search algorithm (Matrix Science, London, UK). One missed cleavage per peptide was allowed.

**Sequence analysis**

Genomic DNA fragments spanning exons of Lmnb1 were amplified by PCR (see Table S4 for primer sequences). Purified PCR products were sequenced using big dye terminator chemistry (Applied Biosystems) and analysed on a MegaBACE 1000 (Amersham). Sequence reads derived from both strands were assembled, aligned and analysed for nucleotide differences using Sequencher (GeneCodes).

**Western blot**

Protein lysates (1 µg per lane) in RIPA buffer were run on 10% Bis-Tris gels (NuPage, Invitrogen) and transferred to PVDF membrane (XCell II Blot Module, Invitrogen). Immunodetection was performed by standard methodology using antibodies to lamin B1 (S-20) and β-tubulin for normalisation (primary antibodies from Santa Cruz Biotechnology and used at 1:1000). Proteins were detected using horseradish peroxidase-conjugated secondary antibodies (DAKO), followed by development with ECL plus Western blotting detection system (GE Healthcare). Films were scanned on a GS-800 Densitometer (BioRad) for quantification.

**Quantitative real–time RT–PCR**

RNA was purified (TRizol Reagent, Invitrogen) from isolated caudal regions of E10.5 embryos or from MEFs, genomic DNA removed by DNase I digestion (DNA-free, Ambion) and first strand cDNA synthesis carried out (SuperScript II, Invitrogen). qRT–PCR was performed (MESA Blue Mastermix for SYBR Assay, Eurogentec) on a 7500 Fast Real Time PCR system (Applied Biosystems), with each sample analysed in triplicate. Primers for Lmnb1 were designed to amplify a 221 bp product (nucleotides 1267–1487 of coding sequence (Ensembl NM 010721.1; ENSMUSG00000024590). Additional primer pairs were: cyclin A2 (Cen2) 5’-CATGTCACTGGCGTCTGTTCTTCTC and 5’-TGATTCTACTGCGATCTCA; cyclin B1 (Cenbl) 5’-GGAATTCTTGACACGCGT and 5’-TGCGTTTGTGACGGCTTAG; Cyclin D1 (Cenbl) 5’-GCGTACCTGACACCAATCT and 5’-CTCTCTGCCACCTGCTGCTT; Smc2 5’-AAATAGCGCCAGAAAAACT and 5’-GAGCGTTCTTGGTGTCTC. Primers for p16(ink4a) were described previously [27]. Results were normalized to Gapdh as described previously [9].

For microarray, RNA was further purified using the RNeasy Micro Kit (Qiagen), followed by cDNA synthesis, linear amplification and labelling of cRNA using GeneChip 3’ IVT Express Kit (Affymetrix). RNA and cRNA quantity and quality were determined by Nanodrop spectrophotometer and Bioanalyser 2100 (Agilent). Affymetrix Mouse 430_2 arrays were hybridised as standard (www.affymetrix.co.uk). Files were processed in GeneSpring GX (Agilent Technologies), with application of GC-RNA normalisation and Benjamimi-Hochberg multiple testing correction.

**Whole-mount in situ hybridisation**

Whole-mount in situ hybridisation, was performed as described previously [9], using a digoxigenin-labelled 561 bp cRNA probe which was complementary to nucleotides 726–1206 of the Lmnb1 transcript/coding sequence. Embryos were embedded in gelatine-albumin and sectioned at 50 µm thickness on a vibratome.

**Photobleaching experiments**

Constructs were generated in pcDNA3.1 vector by standard cloning methods, to express fusion proteins composed of a nuclear localisation signal, yellow fluorescent protein and full-length lamin B1 or C-terminal region. Plasmids were transfected into MEFs and FLIP was performed as described previously [51]. In brief, a region of interest (ROI) was photobleached at full laser power while scanning at 4% laser power elsewhere. For quantitative analysis, background intensity was subtracted, and intensities of a specific ROI outside the photobleached area were measured over time and normalized using intensities of an ROI in a transfected but non-bleached cell.

**Immunofluorescent labelling and laser-scanning confocal microscopy**

MEFs, derived from pools of 3–6 embryos at E13.5, were fixed in 4% PFA in PBS for 10 min, permeabilized with 0.4% Triton X-100 in PBS for 5 min, and blocked with 0.4% fish skin gelatine in PBS for 30 min at room temperature. Incubations with primary and secondary antibodies were for 1 h each at room temperature. Primary antibodies were mouse anti-lamin B1 (BD1; [57] and rabbit anti-lamin A (ab26300: Abcam). Secondary antibodies were donkey anti-mouse and anti-rabbit (Jackson ImmunoResearch Laboratories) conjugated to Alexa Fluor 488 and Cy5 respectively. Imaging was performed using a confocal microscope (LSM 510 META; Carl Zeiss, Inc.) or Image Browser (Carl Zeiss, Inc.) software.

**Cell cycle analysis**

MEFs were plated onto 13 mm cover slips (passage 3; 1.0×10⁵ cells per well in triplicate), cultured for 5 hours prior to addition of 10 µM EdU (Invitrogen). After 1 hour cells were fixed and processed for detection of EdU (Click-it EdU Imaging Kit). Cells were then washed in 0.1% Triton-X100 in PBS and blocked for
genotype is indicated. The genetic background of the curly tail (ct) strain was shifted towards smaller values. (TIF)

Figure S4 Migration of lamin B1 protein on 2-DE correlates with number of glutamic acid residues. Two dimensional protein gels were generated using embryo samples from wild-type (+/+), curly tail (ct), Gfh3-BAC transgenic curly tail (ct<sup>EQ<sub>Gfh3</sub></sup>) and ct<sup>8E</sup> strains. (A) Differential migration of lamin B1 (major spot arrowed) was observed in comparison of aligned gels for ct/ct and +/+/+ samples. (B) In strains expressing the 8E lamin B1 variant (ct and ct<sup>EQ<sub>Gfh3</sub></sup>), alignment of lamin B1 spots was evident (software-generated spot outline is shown), whereas the corresponding spot was absent in strains expressing the Lmnb1<sup>8E</sup> variant (+/+ and ct<sup>8E</sup>). (C) Conversely, the major lamin B1 spot (outlined) aligned in strains expressing the Lmnb1<sup>EQ</sup> variant, but was absent in strains expressing the Lmnb1<sup>8E</sup> variant. (TIF)

Table S1 Identification of lamin B1 spots by LC-MS/MS. Three spots found to migrate differentially on 2-DE of curly tail and wild-type samples were excised from gels and subjected to liquid chromatography coupled to electrospray tandem mass spectrometry (LC-MS/MS). Spots are numbered 1–3 from basic to acidic (right to left on 2-DE images in Figure 1), with spot 1 the most abundant in each case. The identified peptides are listed with the Mascot score and p-value for confidence of identification. For the most abundant spot on curly tail gels 40% coverage of the protein was achieved. (DOC)

Table S2 Expression analysis of genes located in proximity to Lmnb1 on chromosome 18. A gene-list was generated that corresponds to genes located in a 41 Mb interval surrounding Lmnb1 on chromosome 18:23:47–73:00 Mb (between markers D18Mit88 and Dev1), using UCSC Genome Browser (assembly NCBI37/mm9). This list was used to interrogate a list of genes that were found by microarray analysis to be differentially expressed (p<0.05; fold-change 1.5 or greater) in +/+/+<sup>ct</sup> and +/+/<sup>ct</sup> embryos (caudal region of embryos at the 20–29 somite stage). This analysis identified 11 genes, whose relative level of expression in +/+/+<sup>ct</sup> compared with +/+/<sup>ct</sup> microarray samples is indicated. Expression was evaluated by qRT-PCR (n = 5 of each genotype, repeated twice). Four genes (underlined) showed the same trend in expression in both microarray and qRT-PCR analysis, two of which (indicated in bold) were also found to significantly differ in expression between +/+/<sup>ct</sup> and +/+/<sup>ct</sup> (p<0.001) by qRT-PCR. (DOC)

Table S3 Analysis of cellular proliferation rate in embryos of the +/+<sup>ct</sup> and +/+<sup>ct</sup> sub-strains. The proportion of cells labelled with EdU following 90 minute treatment and the mitotic index (based visual inspection of phospho-histone H3 positive cells) was determined at the axial level of the closure point of the neural folds in +/+<sup>ct</sup> (n = 6) and +/+<sup>ct</sup> (n = 5) embryos at E10.5 (mean number of somites = 28.5 ± 0.8 and 27.6 ± 0.5, respectively). The overall distribution of PNP lengths in embryos of the +/+<sup>ct</sup> sub-strain was shifted towards smaller values. (TIF)

Figure S5 Expression of Lmb1 mRNA in curly tail and wild-type embryos. Whole mount in situ hybridisation at E10.5 shows intense expression of Lmb1 throughout most of the embryo, with the exception of the heart (shown at higher magnification in F–G) and dorsal surface (arrowheads in A and E). On sections (C, D, H, I; cut at the level of the dotted lines in A and E) the diminished or absent staining in the heart is also evident. Diminished expression at the dorsal surface in whole mounts appears to correspond to lack of staining in the dorsal neural tube and surface ectoderm, particularly evident in sections through the PNP region (D, I). We did not observe any consistent differences in staining pattern between strains. A sense control probe did not give signal (B). Scale bars represent 1 mm (A, B, E), 0.5 mm (F, G) or 0.1 mm (C, D, H, I). Abbreviations: Hg, heart; Hg, hindgut; NF, neural folds. (TIF)

Figure S2 Breeding scheme for generation of curly tail sub-strains carrying different combinations of Gfh3 and Lmnb1 alleles. The key strains of interest were ct<sup>8E</sup> (same genotype as ct/ct at Gfh3 and Lmnb1), and ct<sup>EQ</sup>, which both carry the Gfh3 mutation, but differ in Lmnb1 sequence. A third strain, +/ct<sup>8E</sup>, is wild-type for Gfh3 but carries the 8E Lmnb1 variant. The predicted frequency of each genotype is indicated. The genetic background of the +/+<sup>ct</sup> strain is approximately 97% curly tail. Therefore, following the two backcrosses to ct/ct the genetic background of the resultant ct<sup>8E</sup> and ct<sup>EQ</sup> sub-strains is predicted to be 99.5% curly tail. (TIF)

Figure S3 Posterior neuropore length of embryos from curly tail sub-strains during spinal neural tube closure. The data for individual embryos is shown, with the mean PNP length (± SEM) indicated for each strain at each stage. From the 28 somite stage, a large range of values is observed, particularly within the curly tail and +/+<sup>ct</sup> strains. Thus, at the 30–31 somite stage, PNP values ranged from closed to as much as 1 mm long, reflecting the range of possible outcomes from normal closure to spina bifida. The
EdU labelling index in the hindgut was significantly higher in α<sup>9E</sup> than in α<sup>9F</sup> embryos (p < 0.02). There was a trend towards increased EdU labelling in the neural folds of α<sup>9E</sup> embryos, but this did not reach statistical significance (p = 0.06).

**Table S4** Primers for sequencing of *Lmnb1* exons. All primers are flanking coding regions of the respective exons. Sizes of product-fragments are given in base pairs (bp), including primer sequence. T<sup>·</sup> indicates annealing temperature used for PCR amplification.

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**Acknowledgments**

The authors are grateful to Dawn Savery and Valentina Massa for technical assistance and helpful discussion.

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Conceived and designed the experiments: SCPDC. AM DJV AJC NDEG. Performed the experiments: SCPDC AM K-YL PG NDEG. Analyzed the data: SCPDC NDEG AM K-YL PG. Wrote the paper: NDEG AJC SCPDC.
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