MEIS1 Intronic Risk Haplotype Associated with Restless Legs Syndrome Affects mRNA and Protein Expression Levels

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Supplementary data

Supplementary Table S1. Primer List for All PCR Amplifications

Region	Fragment	Forward_Primer	Reverse_Primer	Amplicon length	Opt. T'
Long-range PCR	**LRP01**	GCAGGCCTACGATTGGTATTCGGAGATC	TCATATTCCTGTGCACAGAGCCAGTCTA	6861	57
	LRP02	TTGGGAGACGAAGAGGGTCAGTTCAGAG	AAGCAGGAGCCAGATCACCCCCTTCAGT	6986	59
	LRP03	ACCCAGCAGTGTAATTTCCCTCCCAGAT	ACCTCCCTCCCTTATCCCTAACTTTCAG	6858	55
	LRP04	CATGGAGCTCCCGTGCTTCCAGGAATAC	TCATTTGCAGACATTCTTTCCCCTCGTT	6919	58
	LRP05	TACATATACCTCAGTGCAGCAGTTACTG	ATCAGTCCCTTTCTACTTTGATCTTCAA	6927	55
	LRP06	ACTTAGGCAATAGAGACCTTTGAAGATC	CATTTTCCACCCACTTAGCTGCTGTTTA	6838	55
	LRP07	CCAGCTAAGGTTATAAACAGCAGCTAAG	AGCATTAAACCTGCACATGTGTATTAGG	6845	55
	LRP08	TCTCTGCAGCCAAACCAGCCTAATACA	ACTTGAGGGACATTTTCTACAGCAAACA	6853	55
	LRP09	TTGCTGTAGAAAATGTCCCTCAAGTAAC	TAATTAGCCCTGAGCCTCATCTGTCGTA	6870	55
	LRP10	GAGGGAATTTATGCATCATACGACAGAT	AATTATTTGGGGTCATGATGGACATCTA	6852	55
	LRP11	TCTGGGATAATGATAAAATACACTAAAG	ACACTGTCCACCTTATAATGATTTTGAG	6879	53
	LRP12	CCATGCAGAAGACAAGAGGCCCCCTCAA	ACGTGTTTAGAAACAAGGCCCCATCAGC	2992	57
Conserved region	**C01**	ATAAATGGCATTAAAAGCGGGGAGGTAA	TGTTTGCCATATTCAGTTTTTCTTGGTA	394	53
	C02.A	TATTGTTGGCCACACTTTAGGATTTTAT	GATTGCTTTATTCCTTTATTGAACTTCA	607	52
	C02.B	TTGGCTTTATTGACTTTCATGGGAGTTG	TATAACACCCTAAAATGGCCACCATGTC	685	55
	C03	GACATGGTGGCCATTTTAGGGTGTTATA	ATTGTGGGGGCATAGAGATGGTTCCTAT	720	56
	C04	GGAACCATCTCTATGCCCCCACAATAGG	CAGGGAAGAGGGACCCAAATCAACTGTC	640	58
	C05	GTACTGTTTTACCTTTGCACTTGAAAGT	AATTAAGAATTGTCCGTTGGAGTTTGTC	571	51
	C06	TGGCCCACTGACACAGGAAGTTCTGATC	GCACAGGCTCGGGCATTATAAGACTCTA	882	58
	C07.A	AGAGCCCAGGAAGTTGCAGGGAGAGGAT	ACCCTGCTCCTTTCCGACACAAGTTAGG	825	59
	C07.B	GGCCGGCGGTTCTCCAAGTTTGTTTAGT	CAAGCAATGCATAGTGCCTGCCTAGAAG	926	57
	C07.C	ATCTGGCTCCTGCTTGAAAGGTTTCTCC	TTCTCTGACTGGGCCAAGGTCATGTACC	826	57
	C08	CTTTGGAAGAAGTTCTTTAGACCTGACT	TTGGGGTATCTATCTAAGAAATAAGTGC	530	53
	C09	TAAGAAGAAAGTTAGACAAACCCTAGAG	CTATGTAACTTTGCTACAACCTGTCTAA	610	52

Region	Fragment	Forward_Primer	Reverse_Primer	Amplicon length	Opt. T'
	C10	TTTGGTTAAGCCTTACTACAGGGACAAA	CTACTACCTGCCCATGTGCCTACCTGTA	585	55
	C11	GTCTGGGGAGGGAAATGGAGTCGATTAT	ACCCCAGGTCAGAGGTGCATAACTACAA	433	57
	C12	GGGGTGAAGAATCTTTCCTCATGCAGAT	CCAACGGAAAGATCATTAATCAAGTTCG	826	56
	C13.A	GTTCTATTGTGGCAGCTCCATTGAGACA	GACATTCTTTCCCCTCGTTTTTGGTAAA	755	57
	C13.B	TGTAAAATTTAAGCTGGGAGGCAGAATC	TCACCTTCCAACTACAGCGTTATGAAGA	651	54
	C14	TGTATTCCCACTGCCTTGTGTACTTTAT	GTAGTCTACAATGCCATCAGAGGTCTGT	596	55
	C15	ACATACATATAGCTACCCCCTCTTAATA	AGATGGCTGTTAGATCTTTAACCTTTTC	493	52
	C16	AAATAAAGGGCTCAGTAGTCCTTCAAAC	TGAGAGCCATACCTACCAAACTCTAGTA	485	56
	C17	CTAGTTATAACCAGTTTTAGTGGGTAAA	TTTTGTTAAAGGAGAGCCAGATAAATAT	865	52
	C18	CTTTAGTTTGATTGCAAGATAAGAGTTT	CTGGGAAAATATTGTTTAGCTAGTTGAT	893	51
	C19	GTGGGCATTTTGTTTTCAAGCTGGTATC	ATTGCCTTTACAGATGCCCTGTCTAGCA	895	57
	C20	TTGGTGGAGAATTAAGAAAGCGGATGGA	TTCCCCTCCTGCATTGACTGTGAATAGC	387	57
	C21.A	CTGCAAGTTTATTTAAAGTGGGAGAAGT	AACGGCTATAACAAAGCAATAGAGGTAG	543	53
	C21.B	TCCAACATATTCAGCCAAGCAACAGAAC	ACTGGGATGGGAGGGGGATTTTAAGTTG	643	56
	C22	ATGAGTTATTCCCTTTCGCTGGGGTTCC	AAGGCCTATGTCACTCCTTGCCATCCTC	628	58
	C23	TATATATATATCCCGAACAACTGAA	TACCTGGAGCTTCAAAGGGTAGTA	826	53
	C24	TGAAATTTTGAGAGCCATATTACTACCC	CACCACTGGCACTATGTGTCATATAAAG	484	52
	C25	AGGCGTCTGCTACATCCTTTGGTCATGG	GCTTAGGGGAGGAGCTGAGGATATGGTT	640	56
	C26	GGCAATATGGTGTTTTAGAGGGAGAACG	TTTCACTTTTGAATCAACAAGCCACCAG	668	56
	C27.A	TGTGCAATACTGTTTCTAAATAAAGTGT	CTGTTAATGTAGGTGCAATAGACATCAA	499	52
	C27.B	TGGGGAAACCTTATCCTCTTTGAAGTAA	CAGCCTTGAAAATTAAGACACAGTAGCA	620	55
	C28	CAGGCGTGAGCTACTGCACCCACTTATA	GCCTTTCTCCCCTCAAATCCCATGTACT	573	55
	C29	TAGCAGCCAAGATGACACTGTTCAGAAC	TCCTAGGCAAGGAAAATCACCATACA	660	53
	C30	TCAATGGAGTAACAGGTGCGGTATATTA	GTGGGGAAAAGCTCTGAGTAAACTTATG	545	54
	C31	TAAAGTGTGCGCTCTGAAACTCTCATTC	GTCCCCTTGATTCACATAGTGAGTTTCA	697	54

Region	Fragment	Forward_Primer	Reverse_Primer	Amplicon length	Opt. T'
	C32	ATGTGAATCAAGGGGACAAATGTCTCAG	ACTGGAGCAATGCAGACCGGATCTAAGA	567	57
	C33	GTGGCTGATTGATGGACTTCTTATGATT	CCCTCAGGGAAAGAGGTCTAGTTATCTC	482	54
	C34	TTTACTTATGCCTCTTAAAATGACAGGA	TTAAAACAACATCACCTTTTCGACCTTA	478	53
	C35	AGACAGAATTCCTTCCCTCTAAGAGTCT	AACCATTAAACAGACAGGGACAGGTTT	583	52
	C36	GACTTGTCACTTTTGGAGAGGCTACAA	CTCCGGGCATTAATAAACCTGTAACCAA	848	55
	C37	AGTCCCATTTTGCCAAGATCTAGTTTTA	GAAGTATCGCCATTTTAAGCTCATAATT	854	54
Predicted gene	**PRD01.A**	TGGGATGGGGAGAGGTGACAAGAGTAGC	GCATTCCTAACAAGCTCCCAGGGACTGC	769	60
	PRD01.B	TACCCATGAGGTCACCTACTCAAAATGG	TGTCCAGGTAGATTTCTTTGCAGACAAA	765	57
	PRD01.C	TTTCAGAAACGATTGGATTTTCAGATAG	GTACAATTCTGTTCATCCGGACCACATC	628	56
	PRD02	AGCGCCTGGCACATTCTCACTTCCTGAT	TTGGTGAACCCCCTGAGTAAGGGCTGTG	497	60
	PRD03	TTTTCTGTGGTTTACCAAGTTGGCTGTG	CCTGGTCTCTTCCCCCAAAACTGAATTG	378	55
	PRD04	CAGGGACTTCATATACAAGAACTGATAT	CCTAATTCTATTTTAATGCCCACTATTA	889	52
Exon	**E01**	GACTGATTCAAGGGAAGCGAGCG	CGGCCACGTTTCAATTTAATCTCAC	578	57
	E02	GGAAGGACCCAGCTGTATTGACC	CAAGGGCACAGAGAAAGAGGGAAG	398	59
	E03	CTGGGGGAGGGGGAGGAAAAGG	GGTGAGTGGGGATGCAGATGGGTG	536	60
	E04	CCTCCCCGAGAGCCGTAGTTGC	GAGGGCGTTGGAGGTGGGAGGTAG	362	59
	E05	GGGGTGGGCTGGAGATGGTAG	GGCGTTTGTGATCCCAGTTTTAGTG	344	56
	E06	ATTGCCCTGTGTTTCCCCTATT	AAATTAAAAGCGACAAGAAACAGG	524	53
	E07	GTTGAAGGGGGATGGGAAAGGTG	TGGCCAGGTGACAGACAGTTAAGC	445	57
	E08	GCCTAGGCCTTGTTCTTTCTCTG	GTTAACAAAATCGCAATCGTGAAT	537	53
	E09	GGTTCTGCAAGTATCCTAAGTAGCT	TGAATCAACAAGCCACCAGG	420	53
	E10	CCAACTGCGATTCATCTTTTCCTC	GCCAGGCGTTCCTATACTCACTCC	261	56
	E11	AGCCTGCTATGTTCTGTCTCTTTC	GATCCTGGCTTCCCCTCTG	368	54
	E12	GTCATCCCCTCATCAACACAG	TGGGCAAGGAGAACATAAGAA	423	54
	E13.A	GGAAAATAGTGGCAAAATGTGAGTT	GGTCCAGAGTAGATGCCAAGAATG	584	56

Region	Fragment	Forward_Primer	Reverse_Primer	Amplicon length	Opt. T'
	E13.B	ATACAGGAGACCCAACAATGAGTG	CCAGGCTAGAAAGAGGGAGAGA	573	54
	E13.C	CTCTCGCCTAGGATTTTCAGCC	TGCCAACTCATACCAAACTGCTAC	602	53
	E13.D	GTCCCCATGCAACAACCAC	GGGGGGCAGTAAACAAATTTTTC	544	53
RT-PCR	**meis1_1'F**	GATTGGCCGAGCACTCCT			
	meis1_1eF	AGGTCCCGTAGACCGAAGAT			
	meis1_3R		ATGACTCTGACGAGCAGACG		
	meis1_3'F	TCCAACCTCAGATTTTCTCTCTG			
	meis1_6R		GGCATTTTCCCTTTCAAACA		
	meis1_7F	ACGGCATCTACTCGTTCAGG			
	meis1_10F	AGTGCAGCCCATGATAGACC			
	meis1_10R		GGTCTATCATGGGCTGCACT		
	meis1_12R		TCATGCCCATTCCACTCATA		
	meis1_13R		TTGATGCTGACATTGGCATT		

Supplementary Table S2. Summary of Sequence Variants Detected in Sequencing of *MEIS1* Exons and Flanking Regions

Variant	Classification	Position	Genomic region	ID	Variant	Allelewise		Genotypewise			
						MAF case/control	*P* value	Genotype frequency* case/control			*P* value
v1	intronic	66520690	intron 3	rs2271856	C/T	0.384/0.356	0.357	0.155/0.140	0.457/0.432	0.388/0.428	0.637
v2	intronic	66522040	intron 4	rs41285949	G/A	0.000/0.002	0.265	0.000/0.004	1.000/0.997	0.000/0.000	0.265
v3	intronic, ins/del	66522148	intron 5	novel ins/del	(CA)del	0.000/0.002	0.265	0.000/0.004	1.000/0.997	0.000/0.000	0.265
v4	intronic	66522155	intron 5	novel SNP	A/G	0.002/0.002	0.915	0.004/0.004	0.997/0.997	0.000/0.000	0.915
v5	intronic	66522155	intron 5	novel SNP	C/G	0.002/0.000	0.214	0.004/0.000	0.997/1.000	0.000/0.000	0.214
v6	exonic, syn	66544852	Exon 7	rs13005707	A/G	0.010/0.011	0.954	0.020/0.021	0.980/0.980	0.000/0.000	0.954
v7	intronic	66649410	intron 11	novel SNP	C/T	0.053/0.042	0.402	0.008/0.000	0.090/0.084	0.902/0.916	0.206
v8	3'UTR	66651786	Exon 13	rs2861108	T/G	0.025/0.016	0.360	0.053/0.033	0.947/0.968	0.000/0.000	0.355
v9	3'UTR	66652131	Exon 13	novel SNP	C/T	0.002/0.000	0.238	0.004/0.000	0.996/1.000	0.000/0.000	0.237
v10	3'UTR	66652293	Exon 13	novel SNP	G/C	0.000/0.004	0.114	0.000/0.007	1.000/0.993	0.000/0.000	0.114
v11	3'UTR	66652436	Exon 13	novel SNP	T/A	0.002/0.004	0.645	0.004/0.007	0.996/0.993	0.000/0.000	0.645
v12	3'UTR	66652634	Exon 13	novel SNP	A/G	0.000/0.004	0.114	0.000/0.007	1.000/0.993	0.000/0.000	0.114
v13	3'UTR	66652636	Exon 13	novel SNP	A/G	0.000/0.002	0.264	0.000/0.004	1.000/0.997	0.000/0.000	0.264
v14	3'UTR	66652708	Exon 13	novel SNP	G/T	0.002/0.000	0.219	0.004/0.000	0.996/1.000	0.000/0.000	0.219
v15	3'UTR	66652752	Exon 13	novel SNP	A/G	0.000/0.002	0.260	0.000/0.004	1.000/0.996	0.000/0.000	0.260
v16	3'UTR	66652898	Exon 13	novel SNP	G/A	0.033/0.038	0.656	0.000/0.004	0.066/0.076	0.934/0.921	0.650
v17	3'UTR	66653295	Exon 13	novel SNP	A/G	0.002/0.000	0.217	0.004/0.000	0.996/1.000	0.000/0.000	0.217
v18	3'UTR	66653332	Exon 13	novel SNP	G/T	0.000/0.002	0.262	0.000/0.004	1.000/0.996	0.000/0.000	0.262
v19	3'UTR	66653340	Exon 13	novel SNP	A/G	0.000/0.004	0.113	0.000/0.007	1.000/0.993	0.000/0.000	0.112

* genotype frequency in the order of homozygous minor allele / heterozygous /homozygous major allele; MAF: minor allele frequency

Supplementary Table S3. Marker Information and Case-Control Association Tests

dbSNP	Location	Region	MAF case	MAF control	Allelic association*	Genotypic association*
rs6546232	66,512,364	Intergenic	0.350	0.337	0.65	0.90
rs13033745	66,513,907	Intergenic	0.347	0.336	0.70	0.93
rs11883967	66,527,366	Intron6	0.376	0.362	0.62	0.70
rs6716792	66,541,347	Intron6	0.452	0.475	0.43	0.40
rs11692504	66,577,567	Intron7	0.391	0.385	0.84	0.87
rs4547518	66,580,072	Intron7	0.260	0.340	3.02×10^{-03}	8.59×10^{-03}
rs12373638	66,584,746	Intron7	0.491	0.447	0.13	0.34
rs6721499	66,597,095	Intron8	0.297	0.375	4.76×10^{-03}	**5.52×10^{-04}**
rs4300815	66,600,036	Intron8	0.250	0.331	2.47×10^{-03}	4.52×10^{-03}
rs4544423	66,603,521	Intron8	0.318	0.415	**6.64×10^{-04}**	**7.56×10^{-04}**
rs6742861	66,603,656	Intron8	0.235	0.285	0.07	0.22
rs6728018	66,603,769	Intron8	0.237	0.287	0.07	0.23
C13B_2	66,604,068	Intron8	0.190	0.081	**1.81×10^{-07}**	**2.21×10^{-6}**
rs11688599	66,610,054	Intron8	0.251	0.329	3.88×10^{-03}	8.84×10^{-03}
rs4316931	66,614,409	Intron8	0.251	0.282	0.23	0.40
rs7603236	66,615,915	Intron8	0.314	0.404	1.63×10^{-03}	2.08×10^{-03}
rs12469063	66,617,812	Intron8	0.364	0.222	**8.12×10^{-08}**	**1.19×10^{-07}**
rs11681729	66,618,204	Intron8	0.241	0.209	0.23	0.21
rs9789535	66,619,082	Intron8	0.396	0.445	0.09	0.11
rs17625724	66,622,479	Intron8	0.237	0.290	0.04	0.15
rs17625742	66,622,658	Intron8	0.232	0.294	0.02	0.08
rs11678796	66,623,539	Intron8	0.253	0.335	2.43×10^{-03}	5.68×10^{-03}
rs2300478	66,634,957	Intron9	0.373	0.243	**1.37×10^{-06}**	**9.41×10^{-06}**
rs2300483	66,641,765	Intron9	0.530	0.432	**7.73×10^{-04}**	3.39×10^{-03}
rs2300486	66,644,908	Intron9	0.292	0.287	0.84	0.72
rs1000756	66,648,338	Intron10	0.305	0.357	0.06	0.14

MAF: minor allele frequency. * Significant results are highlighted in bold.

Supplementary Table S4. Clinical Information on the Autopsy Brain Tissues from 28 Individuals with RLS

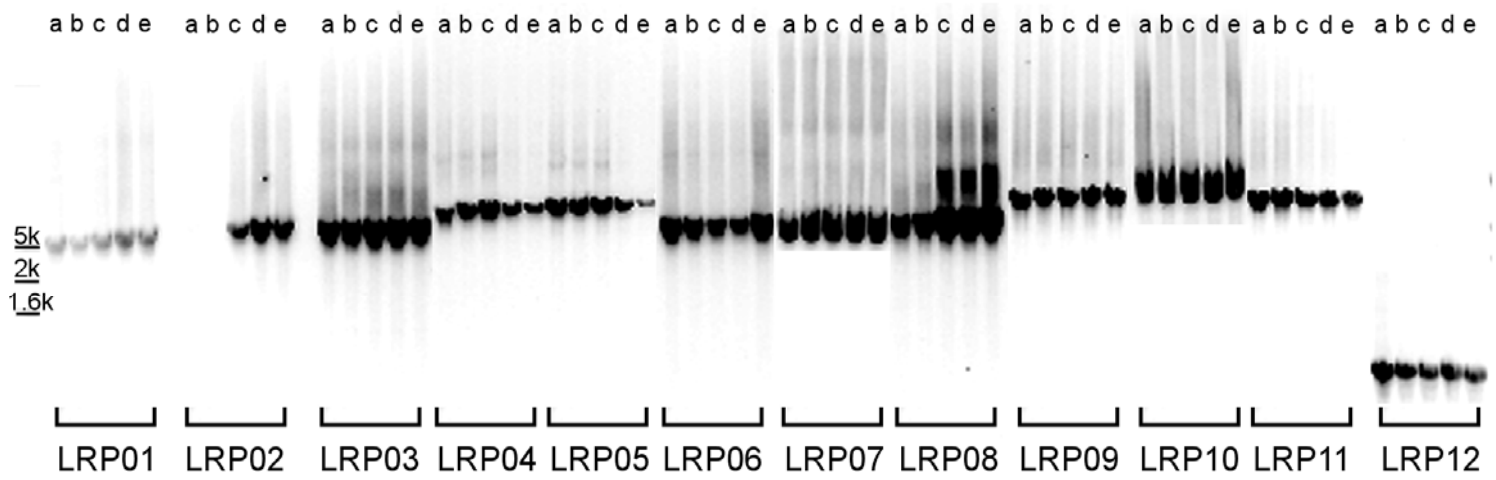
Brain #	Age	Sex	PMI	RLS diagnosis confirmation	Family history	RLS age at onset (years)	RLS progression (years symptoms progressed to daily)	RLS Treatment
B4958	77	F	11	RLS	no	6	yes (7)	vicodin
B5041	76	F	19	RLS	yes	10	yes	zanaflex
B5065	84	F	17	RLS	yes	42	yes (20-40)	bromoctriptine, propoxyphene, iron
B5164	106	F	3	RLS	yes	101	yes (1)	
B5347	53	F	4	RLS	no	<45	yes (3)	carbidopa/levodopa, neurontin
B5462	77	F	20	RLS	yes	43	yes (15)	carbidopa/levodopa, mirapex, iron, codiene
B5609	86	F	17	RLS	no	45-50	yes (20)	carbidopa/levodopa, permax, percocet, ativan
B5655	85	F	15	RLS	unknown	20	yes	codeine.
B5739	52	M	8	RLS	unknown	unknown	unknown	
B5909	71	F	19	RLS	unknown	unknown	unknown	
B6032	89	F	5	RLS	unknown	unknown	unknown	
B6050	76	F	20	RLS	unknown	unknown	unknown	
B6073	85	M	24	RLS	unknown	unknown	unknown	
B6084	82	F	19	RLS	unknown	28	yes (15)	carbidopa/levodopa, xanax, effexor, clariton D
B6106	75	F	22	RLS	unknown	unknown	unknown	
B6264	83	F	27	RLS	yes	5	yes (15+)	demerol, codeine, klonopin, durgesic patch, neurtonin
B6343	87	F	22	RLS	yes	childhood	yes	permax
B6402	87	M	22	RLS	unknown	74	unknown	mirapex
B6410	81	M	14	RLS	unknown	70	no	oxycodone, gabapentin, clonazepam, iron
B6441	83	F	17	RLS	no	35	yes (45)	carbidopa/levodopa, zanex
B6490	73	F	22	RLS	no	35	yes (23)	permax, clonazepam
B6504	90	M	16	RLS	yes	20	yes (15)	carbidopa/levodopa, permax
B6607	87	M	12	RLS	unknown	55	yes	carbidopa/levodopa, mirapex, iron
B6619	77	M	14	RLS	yes	50	no	
B6644	91	F	9	RLS	yes	40	yes	carbidopa/levodopa, vit E
B6719	78	F	13	RLS	yes	childhood	yes	ultram, ambien, vicodin, hydrocodone, neurontin
B6772	88	F	15	RLS	unknown	35	yes	iron, clonazepam
B6945	93	F	20	RLS	yes	14	yes	valium, carbidopa/levodopa

PMI: post-mortem interval.

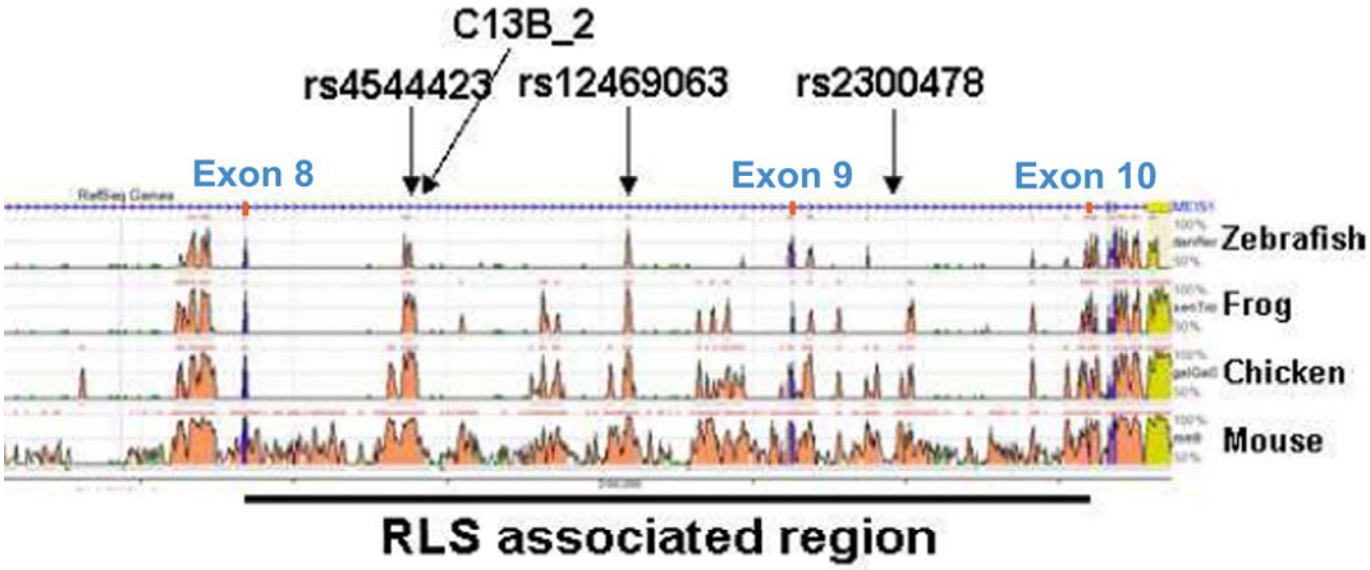
Supplementary Table S5. Clinical and Demographic Information on RLS Patients and Controls

RLS Cases	**285**
Female:male ratio	1.7 (178/107)
Age (years, mean ± SD)	54±12
Age of onset (yr, mean ± SD)	30±16
Severity score	26±11 (IRLSS[a])
Daily or nearly daily symptoms	82%
Familial cases	78%
Ferritin (μg/l)	Female: 72±51, Male:143±101
PLMS positive[b]	82%
Controls	**285**
Female:male	1.3 (162/123)
Age (yr, mean ± SD)	42 ± 13

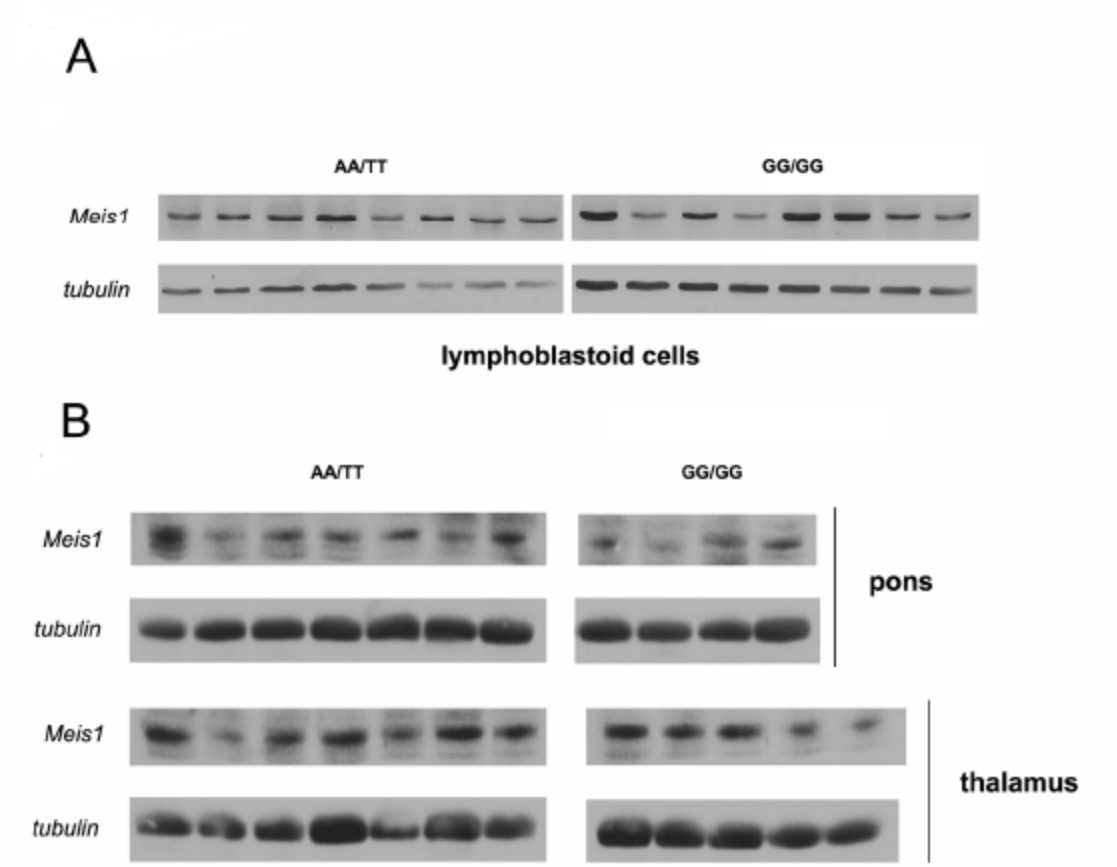
[a] The International RLS Study Group severity scale (SS) contains 10 items with a range of 0 – 4 score/each. SS: 0 – 10, mild; 11 – 20, moderate; 21 – 30, severe; 31 – 40, very severe; [b] The PLMS measurements were performed by one-night polysomnography (PSG) prior to treatment or with reduced dopaminergic medications. The PLMS index > 5.0/hr was classified as positive according to the standard.



Supplementary Figure S1: Long range PCR results. The PCR products were electrophoresed on a 0.9% agarose gel (UltraPure, Invitrogen) for 4 hours. Lambda DNA/Hind III Fragment (Invitrogen) was used as the molecular weight standard. Lane a,b,d were samples from RLS patients homozygous for the risk associated haplotype (GG/GG); lane c,e were samples from two non-RLS controls homozygous for haplotype (AA/TT). Fragments were run on three different gels based on different size range and images were assembled according to the ladders. LRP02 was repeated and two failed samples (lane a, b) were reamplified and showed no difference.



Supplemenatry Figure S2. A Schematic Showing of Comparative Genomic Analysis of the MEIS1 Gene Using the ECR Browser*. Pairwise comparisons of the human sequence with mouse, chicken, frog, and zebrafish are shown from the bottom to the top respectively. Blue peaks represent significantly evolutionary conserved exons, and peach colored peaks represent significantly evolutionary conserved intronic noncoding sequences. Black arrows point to the location of the RLS associated SNPs.
* Ref: Loots, G. and Ovcharenko, I. (2007) ECRbase: database of evolutionary conserved regions, promoters, and transcription factor binding sites in vertebrate genomes. Bioinformatics, 23, 122-124. Epub 2006 Nov 7.



Supplementary Figure S3: Western blot of MEIS1 in RLS lymphoblastoid cell lines and brain tissues. (A) Western blotting experiments illustrate significant changes in expression levels of MEIS1 in lymphoblastoid cell samples carrying the AA/TT genotype, compared with samples carrying the RLS-associated genotype GG/GG. **(B)** Western blotting experiments illustrate changes in expression levels of MEIS1 in both thalamus and pons tissue samples of RLS patients carrying the AA/TT genotype, compared with RLS patients carrying the RLS-related genotype GG/GG.

Method:

Protein extraction: Protein lysates from human thalamus, pons, and lymphoblastoid cells were homogenized in SUB lysis buffer (0.5% SDS, 8M Urea, 2% β-mercaptoethanol), centrifuged 20 min/13,000 rpm at 4 °C. The soluble fractions were quantified and diluted in loading buffer. Protein lysates were prepared three separate times.