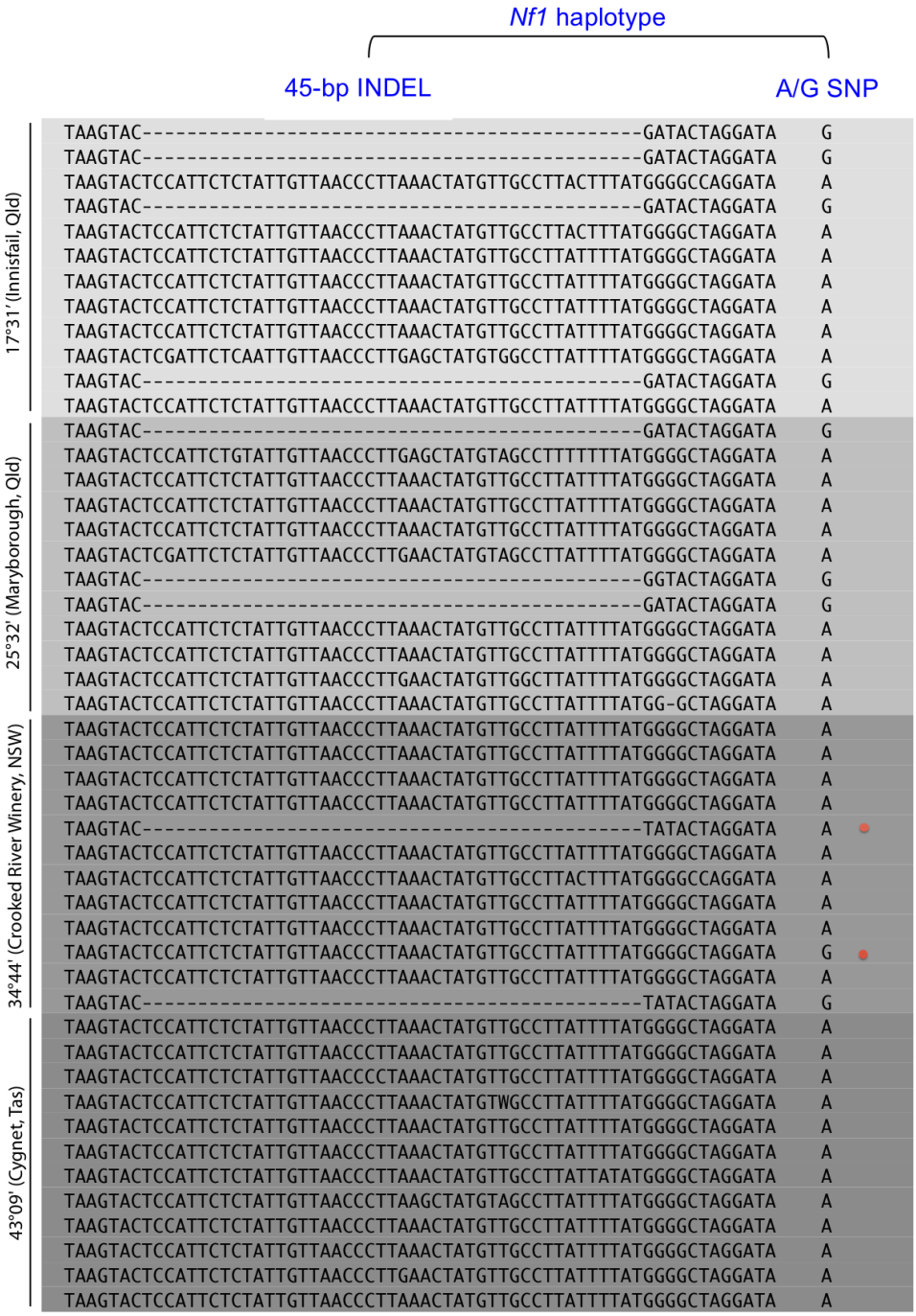
Supplemental material 1: Initial sequencing survey of the *Nf1* locus.

Table S1. Primers used in the initial sequencing survey of *Nf1*. The *Nf1* region examined spanned ~14 kb (between 3R:21806754-21820744) although we only obtained sequences that could be analysed for ~10 kb. We attempted to find clinal variations around *Nf1* locus.

Primer name	Sequence (5' to 3')
NF1upF1	CTCAGAAGGAGCCAAACCAG
NF1upR1	TGAGCTTGCGGAAAGAGTTT
NF1upF2	TCATCAAACGTCCATTGCAG
NF1upR2	GGAGTGGCAATTGGTTGAGT
NF1_F1	AAGAAACAGTGCCCGTTTTG
NF1_R1	GTGTCCTTGGTCTGGTTGGT
NF1_F2	ACCAACCAGACCAAGGACAC
NF1_R2	AGATTCGGTCACCTGTTTGG
NF1_F3	CCAAACAGGTGACCGAATCT
NF1_R3	ATCGGGCATGGTAGTTTGAT
NF1_F4	AAACTACCATGCCCGATGTG
NF1_R4	GCATTTGACGACTGCTGCTA
NF1_F5	ATGGGCCAATATGACATGGT
NF1_R5	TGGTAAGAATAGCGGCATCC
NF1_F6	CACCTCCCATCAGATTGCAC
NF1_R6	CCACCTCAAAACAGGTCTCC
NF1_F7	TCTGGATGAGGAGGAGGAGA
NF1_R7	TTCCGGCCTGGTAGAATATG
NF1_F8	GGAGGAATTCAAGACCCTCA
NF1_R8	TTGAGTAGCGCCATGTTCAG
NF1_F9	CTGAACATGGCGCTACTCAA
NF1_R9	AATTCATCCAGGGACAGTCG
NF1_F10	TACTCCGACTGTCCCTGGAT
NF1_R10	ACTTCGAACTTGTCCCGATG
NF1_F11	GAGGAAGTCCGTTCCAGATG
NF1_R11	GTGGCATTGTTCGGATCTTT
NF1_F12	ACCAAAGATCCGAACAATGC
NF1_R12	GGCAAACAGGTCTCAACCAT
NF1_F13	TCGGAGCTGTTTGTCAACTG
NF1_R13	CGCTACAAAAGGTGCTTTCA
NF1_F14	TGAAAGCACCTTTTGTAGCGTA
NF1_R14	GCCTTTAATCGCTTTCATGG

Figure S1. *Nf1* haplotype-defining polymorphisms in the Australian samples: a 45 bp INDEL in intron 2 and an A/G SNP (synonymous mutation) 2.9kb downstream. This A/G polymorphism corresponds to *L17277* in Figure S2. Shadings reflect different latitudinal origins. The results suggest potential LD between the two sites although two exceptions to linkage were detected in the Crooked River Winery population, indicated by orange dots.



Supplemental material 5: Linkage disequilibrium at the *Nf1* locus in Raleigh inbred lines

Figure S2. Level of pairwise linkage disequilibrium between the *Nf1* intronic 45-bp INDEL and nearby sites in Raleigh populations (USA). Gene content around the *Nf1* locus is shown on top. Arrows mark positions of the 45-bp INDEL polymorphism and the A/G SNP at locus L17277. Bottom dot plot shows the level of pairwise LD in R^2 between the 45-bp INDEL and all segregating sites within this 40 kb genomic region, covering the entire *Nf1* and neighbouring genes. Regions corresponding to the *Nf1* 5'UTR, structural gene and 3'UTR are shaded. Data points with LD level $0.5 < R^2 < 0.7$ are in blue, and those with $R^2 > 0.7$ are in red.

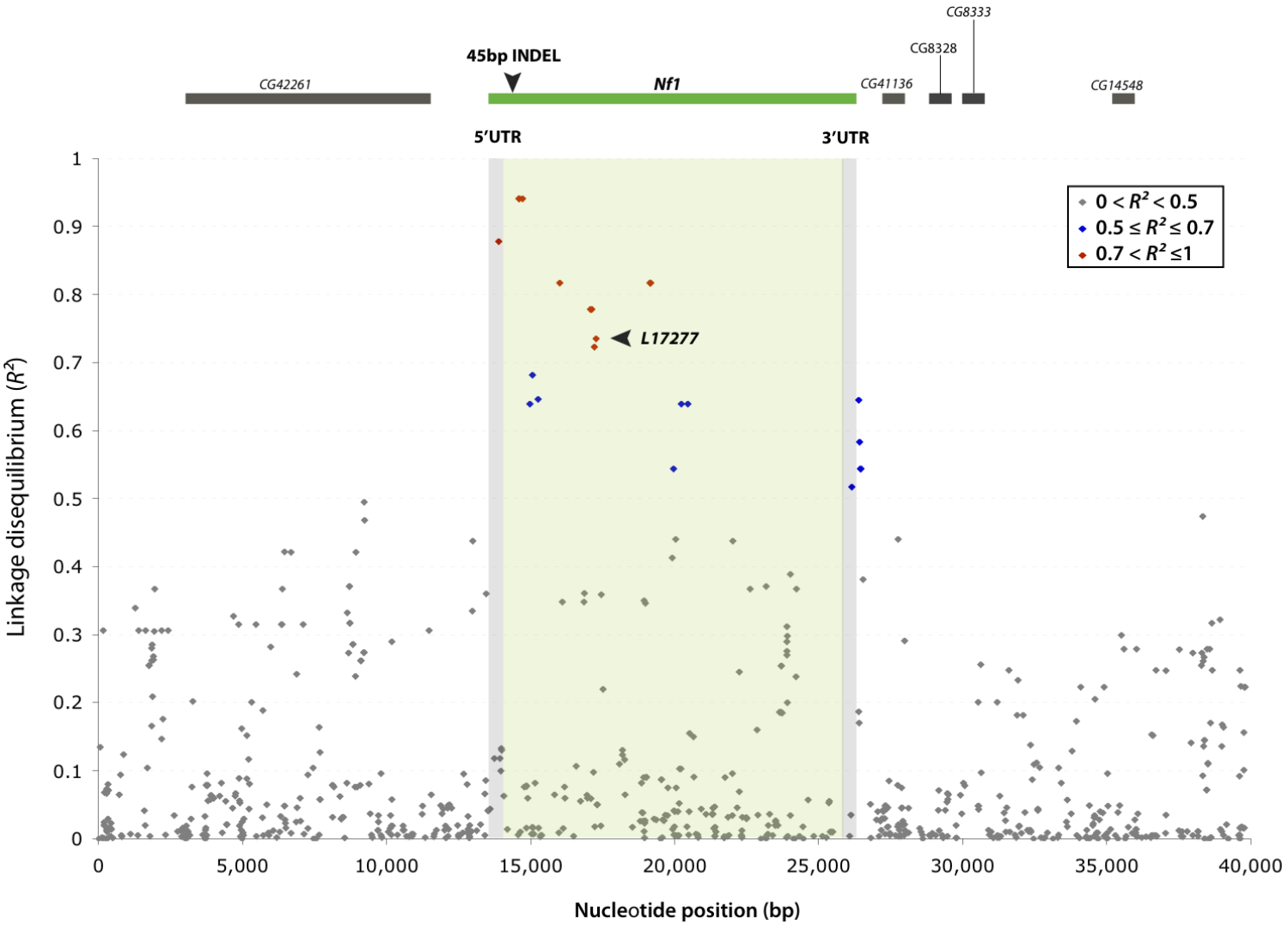

CG8333
CG8328
45bp INDEL
CG42261
Nf1
CG41136
CG14548
5'UTR
3'UTR
0 < R² < 0.5
0.5 ≤ R² ≤ 0.7
0.7 < R² ≤1
L17277
Linkage disequilibrium (R²)
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
5,000
10,000
15,000
20,000
25,000
30,000
35,000
40,000
Nucleotide position (bp)

Table S2. Summary of 19 variable sites in strong linkage disequilibrium (R^2 >0.5) with the 45-bp INDEL polymorphism in *Nf1* in Raleigh (USA) inbred lines. The preferred codons in *D. melanogaster* are in bold and underlined. Star signs (*) in the Locus ID column indicate haplotype-defining sites. For graphical representation of site locations, refer to red and blue data points in Figure S2.

Site	**Locus ID**	**Polymorphism**	**Location**	**Amino acid**	***Nf1-insertion-A* codon**	***Nf1-deletion-G* codon**
1	L13893	A/G	5'UTR	NA	NA	NA
	*L14370	Insertion/deletion	intronic	NA	45-bp insertion	45-bp deletion
2	L14595	T/A	exon3	Leucine	CTT	CTA
3	L14598	G/A	exon3	Leucine	**CTG**	CTA
4	L14718	G/A	exon3	Valine	**GTG**	GTA
5	L14983	C/T	exon4	Isoleucine	**ATC**	ATT
6	L15071	T/C	Intronic	NA	NA	NA
7	L15255	G/A	exon5	Lysine	**AAG**	AAA
8	L16015	C/T	exon6	Histidine	**CAC**	CAT
9	L17124	G/A	exon8	Glutamine	**CAG**	CAA
10	L17220	G/A	exon8	Threonine	**ACG**	ACA
11	*L17277	A/G	exon8	Valine	GTA	**GTG**
12	L19161	A/G	exon10	Arginine	CGA	**CGG**
13	L19961	C/T	exon11	Isoleucine	**ATC**	ATT
14	L20228	C/T	exon11	Glycine	**GGC**	GGT
15	L20462	A/G	exon11	Leucine	CTA	**CTG**
16	L26148	A/T	3'UTR	NA	NA	NA
17	L26397	G/T	intergenic	NA	NA	NA
18	L26420	A/G	intergenic	NA	NA	NA
19	L26471	G/A	intergenic	NA	NA	NA

Supplemental material 6: Genotyping and linkage disequilibrium between the A/G polymorphism at *L17277* and the 45-bp INDEL in Australian samples.

Figure S3. Genotyping the A/G polymorphism at *L17277* of the *Nf1* locus by high resolution melt (HRM) analysis in the Roche LightCycler®480 instrument (Genescan module). Melt curves could be grouped into the 3 distinct profiles according to their SNP genotypes (A/A, A/G and G/G). This assay was used to determine clinal frequencies of *Nf1* haplotypes (Figure 1), *Nf1*-wing size (Figure 2) as well as *Nf1*-development time association (Figure 3) analyses.

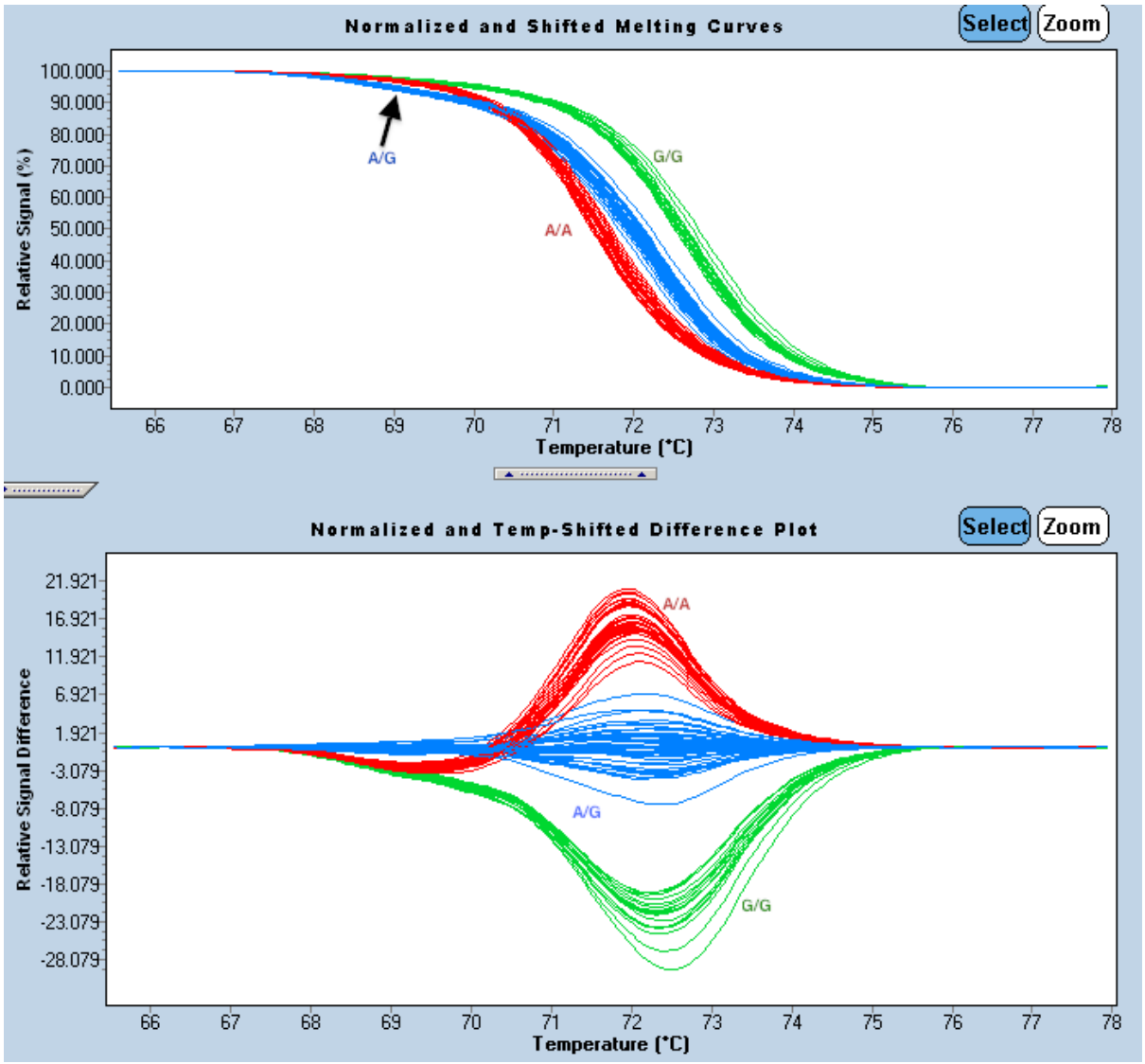


Figure S4. Genotyping the 45-bp INDEL polymorphisms of the *Nf1* locus by agarose gel electrophoresis. A typical gel image of 24 individuals is shown. The INDEL genotype indicated above each lane: M = size marker (Hyperladder V); Ins = insertion homozygote; Het = Heterozygote; Del = Deletion homozygote. The corresponding A/G SNP genotypes at *L17277* (by HRM) are shown below each lane. The extra band >200 bp present in all heterozygous individuals was a hetero-dimmer product of the Insertion and Deletion alleles.

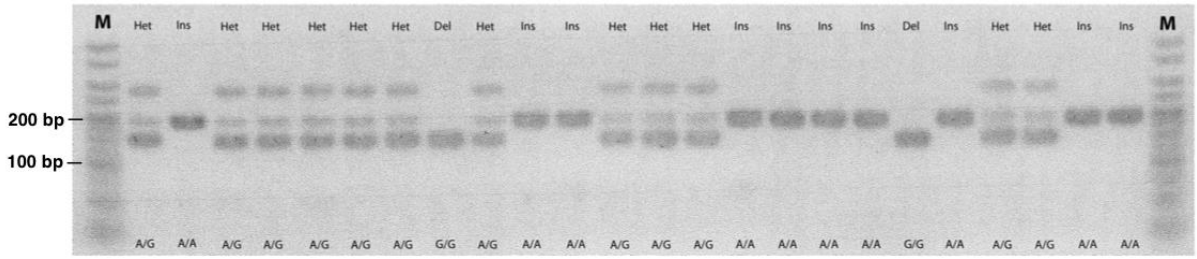


Table S3. Summary of genotyping results at the *L17277* and the 45-bp INDEL loci in the Australian samples. Based on the observed LD between the two sites, genotype of *L17277* was used as a proxy for the *Nf1* haplotype in the Australian samples. Flies used for genotyping originated from an Innisfail mass-bred population collected in 2010. Note that the two loci were successfully genotyped in 144 of the 152 individuals.

ID	**Phenotype**	**Sex**	***L17277* SNP genotype**	**45-bp INDEL genotype**
A01	Early	Male	GG	Deletion
A02	Early	Male	AA	Insertion
A03	Early	Male	AA	Insertion
A04	Early	Male	AA	Insertion
A05	Early	Male	AG	Heterozygote
A06	Early	Male	GG	Deletion
A07	Early	Male	AA	Insertion
A08	Early	Male	AA	Insertion
A09	Early	Male	AA	Insertion
A10	Early	Male	GG	Deletion
A11	Early	Male	AA	Insertion
A12	Early	Male	AG	Heterozygote
B01	Early	Male	AG	Heterozygote
B02	Early	Male	AG	Heterozygote
B03	Early	Male	AA	Insertion
B04	Early	Male	AA	Insertion
B05	Early	Male	AG	Heterozygote
B06	Early	Male	AA	Insertion
B07	Early	Male	GG	Deletion
B08	Early	Male	AA	Insertion
B09	Early	Male	GG	Deletion
B10	Early	Male	AA	Insertion
B11	Early	Male	AA	Insertion
B12	Early	Male	AA	Insertion
C01	Early	Male	AG	Heterozygote
C02	Early	Male	AG	Heterozygote
C03	Early	Male	AG	Heterozygote
C05	Early	Male	AG	Heterozygote
C06	Early	Male	AA	Insertion
C08	Early	Male	AG	Heterozygote
C09	Early	Male	AG	Heterozygote
C10	Early	Male	AG	Heterozygote
C11	Early	Male	AA	Insertion
C12	Early	Male	AG	Heterozygote
D01	Early	Male	AG	Heterozygote
D02	Early	Male	AA	Insertion
D03	Early	Male	AG	Heterozygote
E01	Late	Male	AA	Insertion
E02	Late	Male	AG	Heterozygote
E03	Late	Male	AG	Heterozygote

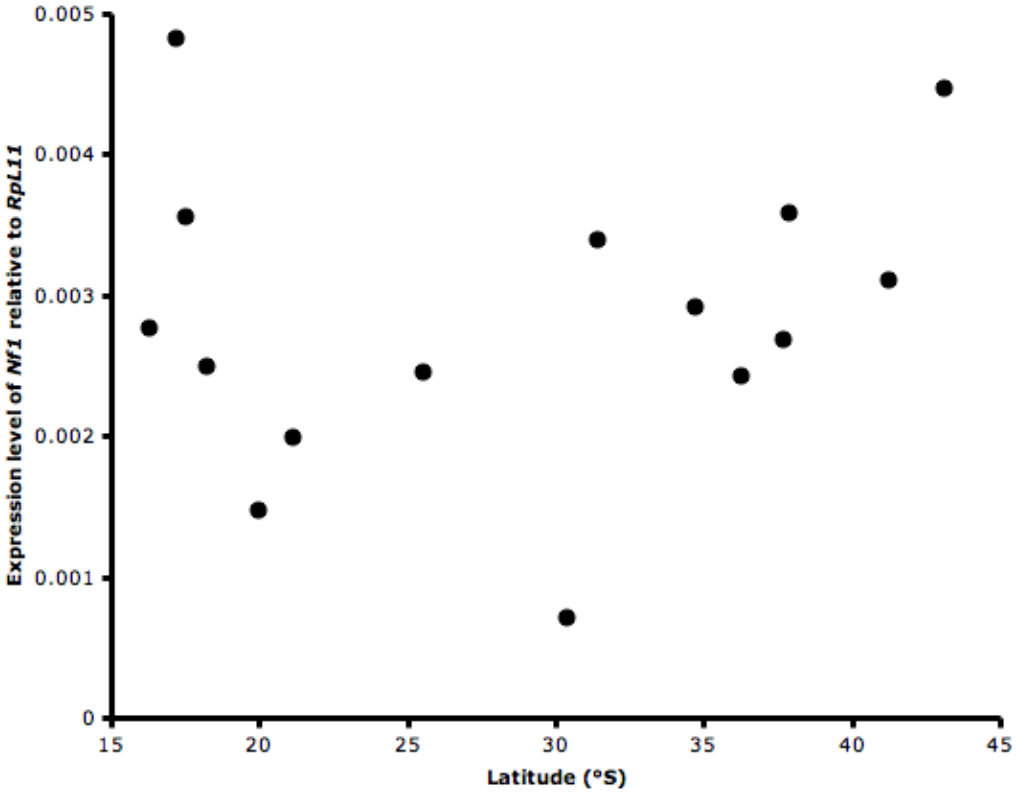
E04	Late	Male	GG	Deletion
E05	Late	Male	AA	Insertion
E06	Late	Male	AA	Insertion
E07	Late	Male	AG	Heterozygote
E08	Late	Male	AG	Heterozygote
E09	Late	Male	AA	Insertion
E10	Late	Male	AG	Heterozygote
E11	Late	Male	AA	Insertion
E12	Late	Male	GG	Deletion
F01	Late	Male	AG	Heterozygote
F02	Late	Male	AA	Insertion
F03	Late	Male	AG	Heterozygote
F04	Late	Male	AG	Heterozygote
F05	Late	Male	AG	Heterozygote
F06	Late	Male	AA	Insertion
F07	Late	Male	AA	Insertion
F08	Late	Male	AG	Heterozygote
F09	Late	Male	GG	Deletion
F10	Late	Male	AA	Insertion
F11	Late	Male	Unknown	Heterozygote
F12	Late	Male	AG	Heterozygote
G01	Late	Male	AG	Heterozygote
G02	Late	Male	AG	Heterozygote
G04	Late	Male	AA	Insertion
G05	Late	Male	AA	Insertion
G06	Late	Male	AA	Insertion
G08	Late	Male	AG	Heterozygote
G09	Late	Male	GG	Deletion
G10	Late	Male	AA	Insertion
G11	Late	Male	AG	Unknown
G12	Late	Male	AA	Unknown
H01	Late	Male	AA	Unknown
H02	Late	Male	AG	Unknown
H03	Late	Male	AG	Unknown
A01	Early	Female	AG	Heterozygote
A02	Early	Female	AA	Insertion
A03	Early	Female	AG	Heterozygote
A04	Early	Female	AG	Heterozygote
A05	Early	Female	AA	Insertion
A06	Early	Female	GG	Deletion
A07	Early	Female	AA	Insertion
A08	Early	Female	AA	Insertion
A09	Early	Female	AA	Insertion
A10	Early	Female	AG	Heterozygote
A11	Early	Female	AA	Insertion
A12	Early	Female	AA	Insertion
B01	Early	Female	GG	Deletion
B02	Early	Female	AA	Insertion
B03	Early	Female	AA	Insertion
B04	Early	Female	AG	Heterozygote
B05	Early	Female	AG	Heterozygote

B06	Early	Female	AA	Insertion
B07	Early	Female	AG	Heterozygote
B08	Early	Female	AA	Insertion
B09	Early	Female	AG	Heterozygote
B10	Early	Female	AG	Heterozygote
B11	Early	Female	AA	Insertion
B12	Early	Female	AA	Insertion
C01	Early	Female	AA	Unknown
C02	Early	Female	AG	Unknown
C03	Early	Female	AA	Insertion
C04	Early	Female	AG	Heterozygote
C05	Early	Female	AA	Insertion
C06	Early	Female	AG	Heterozygote
C07	Early	Female	AG	Heterozygote
C08	Early	Female	AA	Insertion
C09	Early	Female	AA	Insertion
C10	Early	Female	AG	Heterozygote
C11	Early	Female	AA	Insertion
C12	Early	Female	AA	Insertion
D01	Early	Female	AA	Insertion
D02	Early	Female	AA	Insertion
D03	Early	Female	AG	Heterozygote
E01	Late	Female	AG	Heterozygote
E02	Late	Female	AA	Insertion
E03	Late	Female	AG	Heterozygote
E04	Late	Female	AG	Heterozygote
E05	Late	Female	AG	Heterozygote
E06	Late	Female	AG	Heterozygote
E07	Late	Female	AG	Heterozygote
E08	Late	Female	AA	Insertion
E09	Late	Female	AG	Heterozygote
E10	Late	Female	AG	Heterozygote
E11	Late	Female	GG	Deletion
E12	Late	Female	AA	Insertion
F01	Late	Female	GG	Deletion
F02	Late	Female	GG	Deletion
F03	Late	Female	AG	Heterozygote
F04	Late	Female	AG	Heterozygote
F05	Late	Female	AG	Heterozygote
F06	Late	Female	AG	Heterozygote
F07	Late	Female	AG	Heterozygote
F08	Late	Female	GG	Deletion
F09	Late	Female	AA	Insertion
F10	Late	Female	AA	Insertion
F11	Late	Female	GG	Deletion
F12	Late	Female	AG	Heterozygote
G01	Late	Female	AG	Heterozygote
G02	Late	Female	AA	Insertion
G03	Late	Female	AG	Heterozygote
G04	Late	Female	AG	Heterozygote
G05	Late	Female	AG	Heterozygote

G06	Late	Female	AG	Heterozygote
G07	Late	Female	AG	Heterozygote
G08	Late	Female	GG	Deletion
G09	Late	Female	AG	Heterozygote
G10	Late	Female	AA	Insertion
G11	Late	Female	AA	Insertion
G12	Late	Female	AG	Heterozygote
H01	Late	Female	AG	Heterozygote
H02	Late	Female	AG	Heterozygote
H03	Late	Female	AA	Insertion

Supplemental material 7: Latitudinal variation in *Nf1* transcription

Figure S5. Latitudinal variation in *Nf1* gene expression in third instar larvae. Note: Estimation of relative expression level of *Nf1* did not take into account the potential variation in PCR efficiencies in the reference gene (*RpL11*) and the target gene (*Nf1*) in different cDNA samples.



Supplemental material 8: Transgenic over-expression of *Nf1*

We attempted to experimentally alter the *Nf1* expression with the GAL4-UAS system. A wing/haltere-specific A9-GAL4 driver line (Bloomington Drosophila Stock Center ID: 8761; FlyBase ID: FBti0009838) was crossed to the UAS-dNF1 line (a gift from Douglas Wallace) to over-express *Nf1* in the developing wings. Wing size (area in mm^2) of the F_1 progeny was measured following Gockel et al. (2002). To confirm tissue specificity, we also crossed the A9-GAL4 driver line to a UAS-GFP (Green Fluorescence Protein) line and examined the GFP staining in the third instar wing discs (Figure S6). The A9-GAL4 x w^{1118} cross was used as a control for all comparisons in wing size, gene expression, and tissue specificity.

RNA was captured from five third instar larvae of each of the four biological replicates from the A9-GAL4 x UAS-dNf1 and the A9-GAL4 x W^{1118} crosses. Total RNA was isolated using Trizol (Invitrogen) and treated with Turbo DNase (Ambion). Complementary DNA (cDNA) was prepared with the Superscript II RT system (Invitrogen). Oligo-dT was used to prime the reverse transcription. Quantitative RT-PCR (qRT-PCR) was performed with the Fast SYBR green master mix (Applied Biosystems) and run on a 7900HT Sequence Detection System (96-well Fast Block format). *Nf1* expression level in each cDNA sample was normalized to a reference gene, *actin5C*. Primers for qRT-PCR were: *dNF1*-For (5'-ACGTTTCGAGGATCAGCTG-3'), *dNF1*-Rev (5'-GCGATTTTGAAGGGCCGC-3'), *Act5C*-For (5'-CACACCAAATCTTACAAAATGTGTGA-3') and *Act5C*-Rev (5'-AATCCGGCCTTGCACATG-3'). We used the "delta-delta CP" method (Pfaffl 2001) to estimate relative expression of *Nf1*. Four biological replicates per cross type were performed and RTPCR was done in duplicates for each cDNA sample.

Over-expressing *Nf1* in the wings/halteres (A9-GAL4 x UAS-dNF1) resulted in a small but significant reduction in wing size in both sexes (-5.17% in males: $t_{16} = 14.13$; $P < 0.001$ and -2.54% in females: $t_{17} = 6.80$; $P < 0.001$). We confirmed the tissue-specificity of the A9-GAL4 driver by crossing it to a UAS-GFP line and examined the green fluorescent protein staining in third instar larval wing imaginal discs (Figure S6). Quantitative RT-PCR results using third instar larval whole bodies indicated that *Nf1* was significantly over-expressed by 22-fold in the A9-GAL4 x UAS-dNF1 compared to the control cross (A9-GAL4 x w^{1118}) ($t_3 = 4.99$; $P < 0.01$). However, we were unable to confirm *Nf1* over-expression or knockdown for the other GAL4-UAS combinations (Dah-GAL4 x UAS-dNF1; Dah-GAL4 x UAS-Nf1-RNAi; ELAV-GAL4 x UAS-dNF1; ELAV-GAL4 x UAS-Nf1-RNAi; Hsp70-GAL4 x UAS-dNF1; Hsp70-GAL4 x UAS-Nf1-RNAi; A9-GAL4 x UAS-Nf1-RNAi) (data not shown).

Figure S6. Confirmation of tissue specificity of the A9-GAL4 driver line. Green fluorescent staining is observed in the A9-GAL4 x UAS-GFP not in the control, A9-GAL4 x w^{1118}.