Dwarfism and Altered Craniofacial Development in Rabbits Is Caused by a 12.1 kb Deletion at the *HMGA2* Locus

Miguel Carneiro,*,† Dou Hu,‡,1 John Archer,* Chungang Feng,‡ Sandra Afonso,* Congying Chen,‡,§ José A. Blanco-Aguiar,*, Hervé Garreau,†† Samuel Boucher,‡‡ Paula G. Ferreira,§§ Nuno Ferrand,*,†,*** Carl-Johan Rubin,‡ and Leif Andersson‡,†††,‡‡‡**

*CIBIO/*InBIO* (Centro de Investigação em Biodiversidade e Recursos Genéticos), Campus Agrário de Vairão, Universidade do Porto, 4485-661, Vairão, Portugal, †Departamento de Biologia, Faculdade de Ciências, Universidade do Porto, 4169-007 Porto, Portugal, ‡Science for Life Laboratory Uppsala, Department of Medical Biochemistry and Microbiology, Uppsala University, SE-75123 Uppsala, Sweden, §State Key Laboratory of Pig Genetic Improvement and Production Technology, Jiangxi Agricultural University, 330045, Nanchang, China, **Instituto de Investigacion en Recursos Cinegéticos (IREC-CSIC-UCLM-JCCM), 13071, Ciudad Real, Spain, ††INRA, UMR1388 Génétique, Physiologie et Systèmes d'Elevage, University of Toulouse, F-31326 Castanet-Tolosan, France, ‡‡Fédération Française de Cuniculiculture (FFC), 75009 Paris, France, §§Departmento de Anatomia, ICBAS (Instituto de Ciências Biomédicas Abel Salazar), and UMIB (Unidade Multidisciplinar de Investigação Biomédica), Universidade do Porto, 4050-343 Porto, Portugal, ***Department of Zoology, Faculty of Sciences, University of Johannesburg, Auckland Park 2006, South Africa, †††Department of Animal Breeding and Genetics, Swedish University of Agricultural Sciences, SE-750 07 Uppsala, Sweden, ‡‡‡Department of Veterinary Integrative Biosciences, College of Veterinary Medicine and Biomedical Sciences, Texas A&M University, College Station, Texas 77843

ABSTRACT The dwarf phenotype characterizes the smallest of rabbit breeds and is governed largely by the effects of a single dwarfing allele with an incompletely dominant effect on growth. Dwarf rabbits typically weigh under 1 kg and have altered craniofacial morphology. The *dwarf* allele is recessive lethal and *dwarf* homozygotes die within a few days of birth. The dwarf phenotype is expressed in heterozygous individuals and rabbits from dwarf breeds homozygous for the wild-type allele are normal, although smaller when compared to other breeds. Here, we show that the *dwarf* allele constitutes a ~12.1 kb deletion overlapping the promoter region and first three exons of the *HMGA2* gene leading to inactivation of this gene. *HMGA2* has been frequently associated with variation in body size across species. Homozygotes for null alleles are viable in mice but not in rabbits and probably not in humans. RNA-sequencing analysis of rabbit embryos showed that very few genes (4–29 genes) were differentially expressed among the three *HMGA2/dwarf* genotypes, suggesting that dwarfism and inviability in rabbits are caused by modest changes in gene expression. Our results show that *HMGA2* is critical for normal expression of *IGF2BP2*, which encodes an RNA-binding protein. Finally, we report a catalog of regions of elevated genetic differentiation between dwarf and normal-size rabbits, including *LCORL-NCAPG*, *STC2*, *HOXD* cluster, and *IGF2BP2*. Levels and patterns of genetic diversity at the *LCORL-NCAPG* locus further suggest that small size in dwarf breeds was enhanced by crosses with wild rabbits. Overall, our results imply that small size in dwarf rabbits results from a large effect, loss-of-function (LOF) mutation in *HMGA2* combined with polygenic selection.

KEYWORDS whole-genome sequencing; RNA-seq; body size; IGF2BP2; mtDNA

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[1]Corresponding authors: Department of Medical Biochemistry and Microbiology, Uppsala University, Box 582, SE-751 23 Uppsala, Sweden. E-mail: leif.andersson@imbim.uu.se; and Campus Agrário de Vairão, Rua Padre Armando Quintas, no. 7, 4485-661 Vairão, Portugal. E-mail: miguel.carneiro@cibio.up.pt
[2]Present address: Institute of Animal Science, ETH Zürich, 8092 Zürich, Switzerland.

DOMESTICATION of rabbits was likely initiated after 500–600 AD in Southern France (Clutton-Brock 1999; Callou 2004; Whitman 2004). Rabbits have primarily been bred for meat production and most breeds of domestic rabbits are considerably larger (> 3 kg) compared with their wild ancestor (1.0–1.5 kg). However, more recently several dwarf breeds, such as Netherland dwarf, Holland lop, and long-haired dwarf rabbits, have been developed and these rabbits

are used as pets. Most, if not all, dwarf breeds are segregating for the *dwarf* (*dw*) allele (OMIA 000299-9986, http://omia.angis.org.au/OMIA000299/9986/) first described by Greene *et al.* (1934) and a comprehensive summary of the phenotypic effects of this mutation is given by Robinson (1958). The *dwarf* allele is recessive lethal. Homozygotes (*dw/dw*) are smaller than litter mates and exhibit a characteristic swollen head, tiny ears, and are usually called peanuts (Figure 1). Peanuts are viable up to the time of birth but typically die within a few days of birth. Heterozygotes (*Dw/dw*) reach ~2/3 of the size of wild-type litter mates (*Dw/Dw*) and in adulthood are typically under 1 kg in body weight, have compact and rounded bodies, a disproportionately larger head when compared to the rest of the body, small ears, and a short snout due to altered craniofacial development (Figure 1).

Using controlled crosses, dwarfism in rabbits has been shown to be caused by a single gene in genetic linkage with *Agouti* (Castle and Sawin 1941). Thus, it is expected that the *dwarf* gene is to be found on chromosome 4 where *Agouti* is located. However, it is clear that the small size of dwarf rabbits is not only caused by the *dwarf* gene. Consistent with strong selection for small size, even those animals from dwarf breeds that are homozygous wild-type at the *dwarf* locus are considerably smaller than rabbits from other breeds.

The aim of the present study was to identify the causal mutation for the *dwarf* allele and to reveal selective sweep signals at other loci in the Netherland dwarf breed using whole-genome sequencing. We demonstrate that the causal mutation is a 12.1 kb deletion that eliminates the promoter and the first three exons of the *high mobility AT-hook 2* (*HMGA2*) gene, resulting in inactivation of this gene. Taking advantage of this natural gene knockout, we carried out RNA-sequencing (RNA-seq) analysis in embryos to study how *HMGA2* inactivation affects transcriptional regulation. We show that *HMGA2* is required for the expression of *IGF2BP2* and seems to be implicated in the regulation of protein-coding mitochondrial genes. We also reveal a number of putative selective sweeps when comparing the Netherland dwarf with six other breeds of normal size.

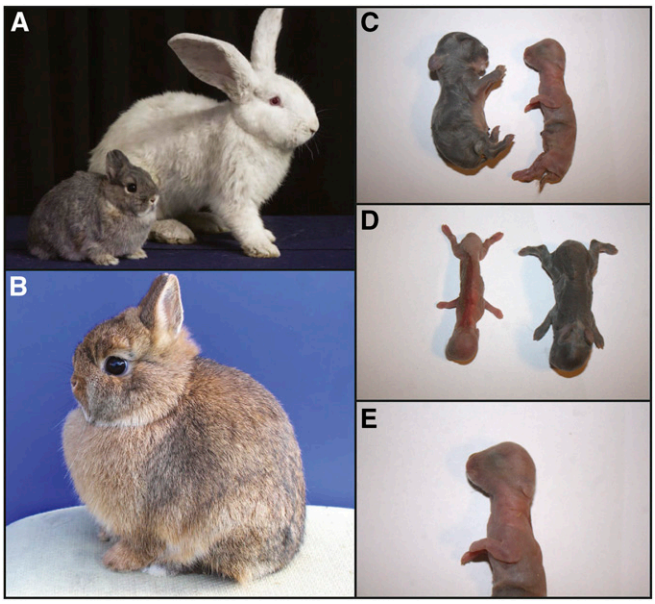

Figure 1 The dwarf and wild-type phenotypes in rabbits. (A) A dwarf individual (*Dw/dw*) side-by-side with a normal-sized New Zealand white rabbit. (B) Dwarf individual (*Dw/dw*). These animals typically weigh < 1 kg, have compact and rounded bodies, short noses, a disproportionately large head when compared to rest of the body, and small ears. (C and D) Newborn *dw/dw* individual (peanut) side by side with a *Dw/dw* (dwarf) littermate. *dw/dw* animals are born smaller than their littermates and have a disproportionate body conformation with cone-shaped heads, small ears and limbs, and prominent eyes, which are evident at the time of birth. (E) Detail of the head of a *dw/dw* individual. Photo credits: (A) Sara Gutiérrez Albarran; (B–E) Javier Lopez.

Materials and Methods

Whole-genome resequencing and SNP calling

Whole-genome resequencing data were generated for a pool of 14 unrelated *dw/dw* peanut individuals and a pool of 17 *Dw/dw* dwarf individuals collected from multiple breeders, all from the Netherland dwarf breed. Genomic DNA for *dw/dw* individuals was prepared from muscle tissue of newborn individuals donated by multiple breeders after death, and for *Dw/dw* individuals from blood extracted from live animals. Paired-end sequencing data (2 × 100 bp reads) were generated from pooled DNA using an Illumina platform. Previously published whole-genome resequencing data of pools of individuals (Carneiro *et al.* 2014) from six other breeds (Belgian hare, Champagne d'argent, Dutch, Flemish giant, French lop, and New Zealand white) were used in in the selective sweep scan. Reads were mapped to the reference genome assembly OryCun2 (Carneiro *et al.* 2014) with BWA-MEM (Li and Durbin 2009) using default parameters. Duplicate reads were flagged for subsequent analysis using Picard (http://picard.sourceforge.net).

SNP calling was performed using SAMtools (Li *et al.* 2009) and filtered by means of the mpileup2snp implemented in VarScan2 (Koboldt *et al.* 2012). We produced two sets of SNPs using different stringent criteria. For the homozygosity mapping analysis (see below) we used more stringent filtering criteria as follows: (1) a minimum depth of eight reads, (2) the allele with lower frequency was observed in at least two independent reads at a frequency ≥ 0.01, (3) a minimum Phred base quality of 15, and (4) a *P*-value threshold for calling variants of ≤ 0.01. For the SNP functional annotation (see below) we relaxed the minimum coverage to four reads to ensure that potential causative mutations could be identified within regions of lower coverage. The latter relaxed coverage requirement was also used to call indels by means of the mpileup2indel option of VarScan2 using identical filters to the SNP calling. Allele counts for each SNP/indel were extracted for subsequent analysis.

Mapping of the dwarf mutation

To search for extended regions of homozygosity and to overcome potential genotyping errors in our SNP calls, we calculated the proportion of monomorphic SNPs in the *dw/dw* pool summarized in a sliding window mode. Briefly, for

overlapping windows of 2000 SNPs iterated in steps of 20%, we divided the number of SNPs monomorphic in *dw/dw* individuals by the total number of polymorphic SNPs within domestic rabbits. We also estimated the proportion of monomorphic SNPs using only SNPs that were polymorphic in the *Dw/dw* pool. Since the total number of SNPs in this case was smaller, we performed the sliding window analysis in windows of 500 SNPs also iterated in steps of 20%, which resulted in an average physical size of ~800 kb.

We also estimated F_{ST} (*i.e.*, a measure of genetic differentiation) between the *dw/dw* and *Dw/dw* pools. To estimate F_{ST} we used the PoPoolation2 package (Kofler *et al.* 2011b), which corrects for possible allele sampling biases associated with pool sequencing as well as sequencing errors. We corrected for unequal sampling of alleles among positions using an unbiased estimator of F_{ST} (Karlsson *et al.* 2007) as implemented in the PoPoolation2 package. F_{ST} values were summarized in 1 Mb windows iterated every 200 kb. We required per position a minimum coverage of four, a maximum coverage of 30, at least two reads supporting the minor allele in polymorphic positions, and a per base Phred quality score of 20 or higher. Windows with $< 30\%$ of positions passing these quality filters were discarded.

Structural rearrangements and SNP functional annotation

The identification of structural variants was performed using Breakdancer (Chen *et al.* 2009) and restricted to the *dw/dw* pool, in which the causative mutation should be homozygous. We focused on candidates with a score ≥ 80, which were then intersected with Ensembl gene annotations (release 78). In parallel, we performed depth of coverage analysis for all breeds using nonoverlapping windows of 1000 bp. This analysis was restricted to reads with a minimum mapping quality of 10. Only windows for which at least one breed had 50% of the window covered with reads were retained.

The functional annotation of the detected variants (both SNPs and indels) was performed using the genetic variant annotation and effect prediction toolbox SNPEff (Cingolani *et al.* 2012). Using the extracted allele counts, we searched specifically for variants with an absolute allele frequency difference ≥ 0.85 between the *dw/dw* pool and breeds not carrying the *dwarf* allele. The *Dw/dw* pool was not considered in this analysis because the frequency of the causative mutation is expected to be 50% in this pool. We did not require full fixation (*i.e.*, allele frequency difference = 1) due to potential sequencing errors or mis-phenotyping.

Diagnostic PCR test for genotyping

To genotype the deletion, we designed two pairs of PCR primers; details on the PCR reactions are given in Supplemental Material, File S1. We genotyped 19 *dw/dw* individuals from three different breeds (Netherland dwarf $n = 14$, Holland lop $n = 4$, and long-haired dwarf $n = 1$), 20 *Dw/dw* individuals from a single breed (Netherland dwarf), and 40 individuals belonging to six breeds that are not expected to carry the *dwarf* allele (Belgian hare $n = 7$, Champagne d'argent $n = 6$, Dutch $n = 6$, Flemish giant $n = 7$, French lop $n = 7$, and New Zealand white $n = 7$).

Scan for selective sweeps

To identify regions of the genome that may have been subjected to selection in the Netherland dwarf breed, we estimated levels and patterns of genetic differentiation between this breed (both pools combined) and six other breeds with normal size. F_{ST} was calculated between breeds in windows of 500 kb with 250 kb steps using the PoPoolation2 package (Kofler *et al.* 2011b). We applied the same filtering requirements as before for the *dw/dw* and *Dw/dw* pools F_{ST} contrast. To summarize the magnitude of allele frequency divergence, we calculated the d_i statistic for each window (Akey *et al.* 2010). This statistic provides polarized measures of differentiation for a given breed in comparisons involving multiple breeds, by standardizing locus-specific deviations for any two breeds when compared to the genome-wide average. The final statistic was obtained by summing across all pairwise combinations including the target breed. We defined candidate regions as outlier windows falling on the top 1% of the empirical distribution. The borders of these regions were extended by aggregating windows on the top 5%. Regions separated by < 500 kb were merged into a single continuous region. Nucleotide diversity (π) within each breed was calculated using an identical sliding window approach as before for F_{ST} using the PoPoolation package (Kofler *et al.* 2011a).

Gene expression analysis

The following experimental procedures were approved by the Ethical Committee for Animal Research of the University of Castilla la Mancha, Spain (Register number CEEA: 1012.02). Female rabbits were kept under standard conditions of housing with unrestricted access to food and water; this was done according to the European Union Directive no. 86/609/CEE. The rabbits were attended by veterinary doctors and inspected at least four times daily. Females were sedated with an intramuscular injection of a mixture of xylazine (Rompun, Bayer Co., Portugal, 8 mg/kg) and ketamine (Imalgéne 1000, Merial, Portugal, 40 mg/kg), and after killed with an injection of thiopental (Tiopental 0.5 g Braun, B. Braun, Portugal, 100 mg/kg) given intravenously in the marginal vein of the ear. After the female's death, a laparotomy was performed, and the embryos were decapitated prior to snap-freezing in liquid nitrogen.

To choose an embryonic stage at which *HMGA2* is expressed at high levels, we profiled *HMGA2* expression across several developmental stages in New Zealand white rabbits (*i.e.*, *Dw/Dw*) using quantitative PCR (qPCR) (File S1). We sampled 11 embryos from the following stages: three embryos at day 9.5, two embryos at day 12, and one embryo at days 15.5, 16, 18, 21, 24, and birth. For gene expression comparisons among *HMGA2* genotypes, we chose to sample 10 embryos of the different genotypes with 15.5 days (see *Results*). Genotypes were obtained using the PCR reactions described above from DNA extracted from the tip of the left hind leg.

Before library preparation, the entire embryo was homogenized and the RNA was extracted using the Allprep DNA/RNA/miRNA Universal kit (QIAGEN, Valencia, CA). Libraries were then prepared using the TruSeq Stranded mRNA (messenger RNA) Sample Preparation Kit (Illumina), and sequenced using 125 bp paired-end reads on an Illumina instrument. We obtained an average of 39.7 million reads per individual (range 34.6–42.3) for three *dw/dw*, four *Dw/dw*, and three *Dw/Dw* individuals (Table S1), divided into two technical replicates. All the individuals derive from five different dwarf (*Dw/dw*) females crossed with a single dwarf (*Dw/dw*) male (Table S1).

To estimate differential gene expression between the three genotypes, we used the complementary (cDNA) annotation for rabbit (Ensembl release 78), which derives from a combination of empirical data across multiple tissues and gene model predictions. Mapping was performed using Bowtie2 (Langmead and Salzberg 2012) using default parameters. Following mapping, statistics for each transcript, such as number of forward and reverse reads mapped, percent covered, and read GC content, were obtained using BBMap (http://bbmap.sourceforge.net). Forward mapping read counts were extracted and used within DESeq2 (Love *et al.* 2014) to estimate changes in gene expression among the three genotypes. *P*-values were adjusted within DESeq2, using the Benjamini–Hochberg approach for multiple comparisons, to reduce the false discovery rate to $< 5\%$. Across each of the groups, the results from both technical replicates were qualitatively similar, therefore, the results described in the main text are derived from the combined counts from both technical replicates.

IGF2 is present in the rabbit reference assembly but distributed among several unmapped scaffolds. Therefore, this gene was missed by the annotation pipelines associated with the release of the rabbit reference genome (Carneiro *et al.* 2014). To profile *IGF2* expression, we remapped the entire set of reads to a composite transcriptome reference including a rabbit cDNA sequence for this gene (Thieme *et al.* 2012).

qPCR for measuring mitochondrial DNA copy number

Mitochondrial DNA (mtDNA) copy number among the three *HMGA2* genotypes was estimated using a quantitative real-time PCR performed on an ABI HT7900 real-time PCR system with a 384-well block module. We used the same individuals as used for gene expression, two independent reactions using different mtDNA primers (File S1), and three replicates per individual. The seven-point standard curve method using fourfold dilution was used to estimate the PCR efficiency. The data were normalized using *ACTA2* as an endogenous autosomal reference gene and relative copy numbers were calculated using the $2^{-\Delta\Delta Ct}$ method. Statistical significance was evaluated using the Student's *t*-test.

Data availability

Whole-genome sequencing data and RNA-seq data have been deposited in GenBank under the bioproject PRJNA354575.

Results

Mapping of the genomic region underlying dwarfism

We performed whole-genome resequencing of two pooled DNA samples of individuals homozygous (*dw/dw*, *i.e.*, peanuts) or heterozygous for the *dwarf* allele (*Dw/dw*, *i.e.*, dwarfs). All individuals belonged to the Netherland dwarf breed (Figure 1). Sequence reads were mapped to the rabbit reference genome sequence (Carneiro *et al.* 2014), resulting in an average effective coverage of 12.9× and 11.2×, respectively.

To map the specific region on chromosome 4 that was predicted to harbor the *dwarf* mutation based on previous linkage data (Castle and Sawin 1941), we started by applying a homozygosity mapping approach under the assumption that individuals homozygous for the *dwarf* allele are expected to be identical by descent for this region (Figure 2A). First, we looked for regions of high homozygosity within the *dw/dw* pool by calculating the proportion of monomorphic SNPs, of those previously found to be polymorphic among domestic rabbits (Carneiro *et al.* 2014), using a sliding window approach with a fixed number of SNPs per window ($n = 2000$). This proportion is expected to approach a value of one in the region associated with the phenotype. The average physical size of these windows was ~1.06 Mb. Given that the length scale of linkage disequilibrium (LD) within domestic breeds typically decays to r^2 values between 0.21 and 0.34 at genomic distances of 400 kb (Carneiro *et al.* 2011), this seemed an appropriate window length to avoid elevated homozygosity resulting from demography and chance events. The most extreme window in terms of the occurrence of monomorphic SNPs in *dw/dw* homozygotes was located at chromosome 4 (chr4):43,668,805–45,210,336 bp (Figure 2A).

Next, we estimated the proportion of monomorphic SNPs in *dw/dw*, but this time for SNPs that were polymorphic in the *Dw/dw* individuals (Figure 2A). The rationale behind this approach is that *Dw/dw* are expected to carry one haplotype containing the causative mutation and one containing the wild-type allele, and are thus heterozygous for mutations that differ between these haplotypes. This approach is also expected to be less influenced by fixation of long haplotype blocks in response to genetic drift or selection specific to the Netherland dwarf breed, because it uses only positions that are polymorphic within the breed. Using a similar sliding window approach as before, the top two windows are contiguous and map to the same region (chr4:43,895,910–44,998,202 bp). However, there are a few other windows in the interval chr4:40,000,000–60,000,000 that show nearly as high a degree of homozygosity as found in the top windows.

Finally, we estimated genetic differentiation between the two pools using F_{ST} (Figure 2A). Both pools consist of individuals that belong to the same breed, and although *Dw/dw* individuals carry just one copy of the causative haplotype, the region containing the gene involved in dwarfism is expected to show elevated genetic differentiation between the two groups. The top five windows with elevated F_{ST} were found to be contiguous and the top window mapped again to the

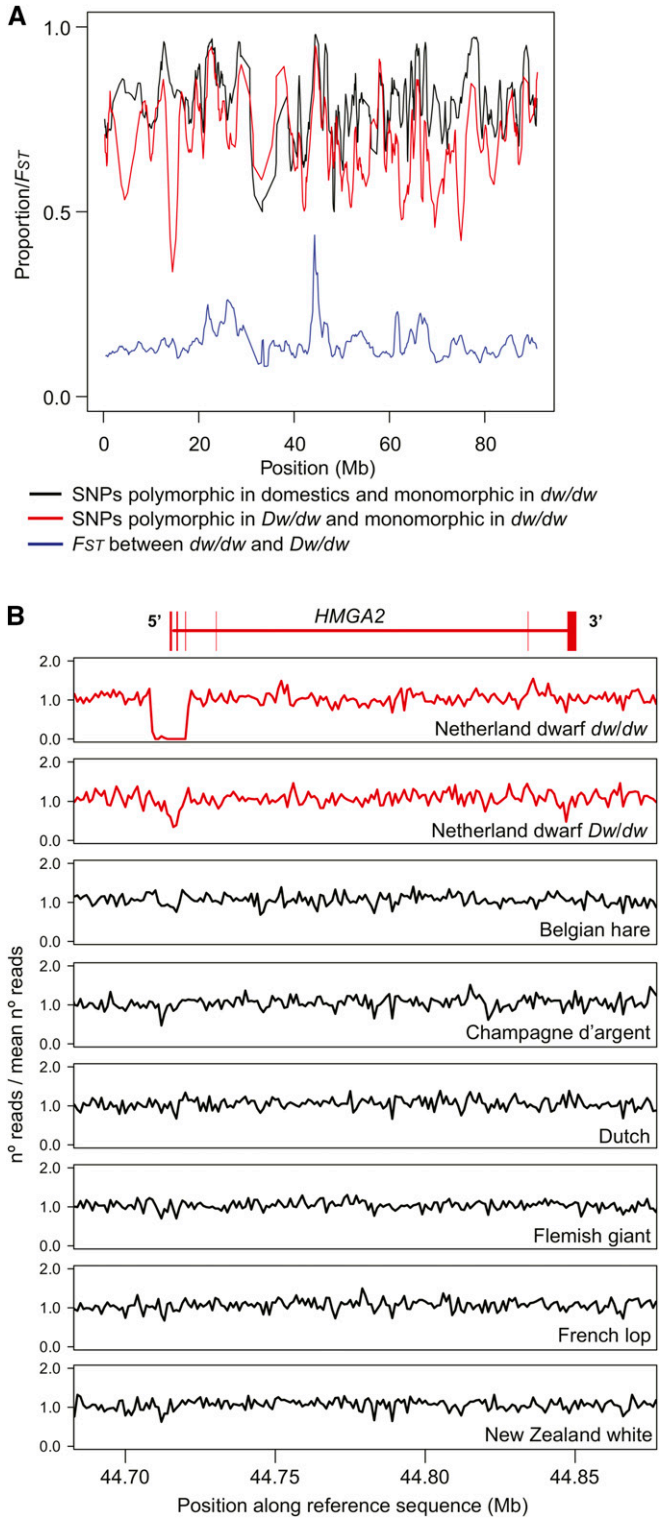

Figure 2 Mapping of the *dwarf* locus to a region on chromosome 4. (A) Homozygosity mapping and genetic differentiation. The red and black lines represent two statistics that summarize levels and patterns of homozygosity across chromosome 4. The red line represents the proportion of SNPs previously identified in domestic rabbits that were monomorphic in *dw/dw* individuals (summarized in windows of 2000 SNPs iterated every 400 SNPs); the black line represents the proportion of SNPs polymorphic in *Dw/dw* individuals that were monomorphic in *dw/dw* individuals (summarized in windows of 500 SNPs iterated every 100 SNPs). The blue line represents F_{ST} (a measure of genetic differentiation) between *dw/dw* and *Dw/dw* individuals. (B) A deletion overlapping the promoter region and the first three exons of *HMGA2* associated with the *dwarf* allele. The lines represent the normalized number of reads mapping to nonoverlapping windows of 1000 bp across the *HMGA2* region. The data for the *dw/dw* and *Dw/dw* pools are given in red at the top, while the remaining panels summarize normalized read depth for six breeds that do not carry the *dwarf* allele (Belgian hare, Champagne d'argent, Dutch, Flemish giant, French lop, and New Zealand white). The gene model for *HMGA2* is drawn in red across the top of the figure and is based on the Ensembl annotation.

region on chromosome 4 revealed by homozygosity mapping (chr4:43,800,001–44,800,000 bp). Based on the annotation of the rabbit reference genome sequence (Carneiro *et al.* 2014) and assuming the common interval among the three statistics as the most likely candidate region (chr4:43,895,910–44,800,000 bp), we found four protein-coding genes within this interval (*WIF1*, *LEMD3*, *MSRB3*, and *HMGA2*; Table 1).

A deletion encompassing the HMGA2 coding region explains dwarfism in rabbits

To reveal the gene and causative mutation underlying the dwarf phenotype, we began by performing a detailed screen for mutations of potential functional significance (nonsynonymous, stop/gain, frameshifts, and splice site mutations). We compared the pool containing *dw/dw* individuals, which are supposed to be homozygous for the causative mutation, to previously reported whole-genome sequence data from pooled DNA samples from six other breeds that are not expected to carry the *dwarf* allele (Carneiro *et al.* 2014).

We failed to find any coding changes specific to *dw/dw* when compared to the nondwarf breeds. Although a regulatory point mutation could be causative, the failure to identify a mutation altering protein structure led us to screen our candidate region for structural changes. The software Breakdancer (Chen *et al.* 2009) reported two candidate variants, both deletions, within our candidate region. Sequence depth analysis revealed a sharp decrease in read count in the *dw/dw* pool for one of the variants, confirming the existence of a deletion (Figure 2B), but not for the other. The approximate breakpoints of the candidate deletion were estimated using soft-clipped reads (*i.e.*, reads containing unaligned portions) from the whole-genome resequencing data obtained for *dw/dw* individuals. Using an individual homozygous for the *dwarf* allele, we then confirmed the exact breakpoints by PCR amplification followed by traditional Sanger sequencing. This deletion was ~12.1 kb in length (chr4:44,709,089–44,721,236) and excised the promotor region and the first three exons of *HMGA2*, expected to cause complete gene inactivation (Figure 2B). The 5′−end of this deletion overlaps a CSINE2 element, which is the largest family of short interspersed repeat elements in the rabbit genome with an estimated copy number of ~1,000,000. However, we did not detect any obvious sequence homology between the two breakpoint regions that may have promoted the excision of this sequence.

Table 1 Genes located in the region on rabbit chromosome 4 harboring the *dwarf* mutation

Gene Symbol	Name	Location (bp)
WIF1	WNT inhibitory factor 1	43,863,790–43,948,402
LEMD3	LEM domain-containing 3	44,015,308–44,091,960
MSRB3	Methionine sulfoxide reductase B3	44,126,803–44,339,285
HMGA2	High mobility group AT-hook 2	44,715,271–44,850,410

LEM, LAP2, emerin, MAN1.

HMGA2 encodes for a protein that belongs to the nonhistone chromosomal high mobility group (HMG) protein family, and functions as a transcription regulator. This gene has been previously associated with body size in humans and mice (Zhou *et al.* 1995; Alyaqoub *et al.* 2012), dogs (Webster *et al.* 2015) (OMIA 001968-9615; http://omia.angis.org.au/OMIA001968/9615/), horses (Frischknecht *et al.* 2015) (OMIA 001968-9796; http://omia.angis.org.au/OMIA001968/9796/), and with beak size among Darwin's finches (Lamichhaney *et al.* 2016) (OMIA 001992-48881; http://omia.angis.org.au/OMIA001992/48881/).

After confirmation of the exact breakpoints, we designed a four primer PCR test that amplified over the deletion, while amplifying an additional product for the wild-type allele with primers located within the deleted region. We genotyped a large cohort of samples (Table 2) including individuals belonging to the three genotypes and from breeds expected to carry and not carry the *dwarf* allele (see *Materials and Methods* for details). We found that all *dw/dw* individuals were homozygous for the deletion across three different breeds, and all individuals from nondwarf breeds were homozygous for the wild-type allele. The majority of the dwarf individuals were heterozygous (*Dw/dw*) as expected, but four individuals phenotyped as dwarf were homozygous wild-type (Table 2). This is likely explained by mis-phenotyping that can easily occur in rabbits of young age. Overall, these genotyping results support the causality of the deletion in *HMGA2*.

Differential gene expression among HMGA2 genotypes

HMGA2 functions as an architectural factor in growth regulation during embryonic development (Pfannkuche *et al.* 2009). Homozygosity for *HMGA2*-null alleles is compatible with life in mice but, as shown here, not in rabbits. To gain further insight into how *HMGA2* affects transcriptional regulation, we explored differential gene expression among *HMGA2* genotypes using RNA-seq (see Table S1 for details on the data set). We crossed *Dw/dw* heterozygotes and sampled 16-day embryos for three *dw/dw*, four *Dw/dw*, and three *Dw/Dw* individuals. We chose to study 15.5-day embryos after a preliminary screen for *HMGA2* expression across eight developmental stages (embryonic day 9.5 to birth) using progeny of New Zealand white rabbits (Figure S1). Expression analysis of different parts of wild-type embryos revealed high *HMGA2* expression during early developmental stages, from day 9.5 to day 18, and a dramatic decrease from day 21. Embryos at day 15.5 displayed the highest *HMGA2* expression.

We compared gene expression among all different contrasts (summarized in File S2 and File S3). We started by comparing differential expression between *dw/dw* and *Dw/dw* individuals and found that as few as 10 transcripts were differentially expressed (Figure 3A). The two genes receiving highest statistical support for differential expression were *HMGA2* and *IGF2BP2*, the latter encoding insulin-like growth factor 2 mRNA-binding protein 2, a member of the IGF-II mRNA-binding protein (IMP) family. The results are consistent with a complete silencing of both genes in *dw/dw* homozygotes (Figure 3B).

There were 29 differentially expressed transcripts in the contrast between *dw/dw* and *Dw/Dw* homozygotes (Figure 3A). Again, the two most significant genes were *HMGA2* and *IGF2BP2* (Figure 3B). Two interesting aspects were revealed by this contrast. First, two of the genes (*MSRB3* and *LEMD3*) showing upregulated expression in the *HMGA2* knockouts are located only ~700 kb upstream of *HMGA2*. Interestingly, *MSRB3* together with a third gene (*WIF1*) in the near vicinity (Table 1) also showed upregulated expression in the *dw/dw vs. Dw/dw* contrast (File S2). Five other genes showing differential expression between *dw/dw* and *Dw/Dw* homozygotes mapped to chromosome 4 at larger distances (> 5 Mb). Thus, 8 out of 29 genes showing differential expression are located on this chromosome (File S2). Second, and most strikingly, 11 out of the 13 protein-coding genes encoded by the mtDNA were found to be significantly upregulated in *dw/dw* homozygotes (Figure 3B).

Only four genes showed differential expression between *Dw/dw* and *Dw/Dw* rabbits (Figure 3A). The most significant transcript was *HMGA2*, which was downregulated in *Dw/dw* and the only gene in common with the *dw/dw vs. Dw/Dw* comparison. Although the levels of expression for mtDNA genes were not significantly different in this contrast, they were intermediate between *dw/dw* and *Dw/Dw* (Figure 3B).

We investigated in further detail levels and patterns of expression in other genes associated with the insulin-growth factor pathway (*IGF1*, *IGF1R*, *IGF2*, and *IGF2R*). These genes have been frequently implicated in size differences and muscle growth (Van Laere *et al.* 2003; Sutter 2007; Hoopes *et al.* 2012). However, we found no evidence for differential expression of these genes in any of the contrasts (Figure S2).

mtDNA copy number does not vary among HMGA2 genotypes

In the *dw/dw* individuals, we observed a consistent upregulation of many genes encoded in mtDNA (Figure 3C). Cell

Table 2 Genotyping results for a 12.1 kb deletion overlapping *HMGA2* at the *dwarf* locus

Group (Expected Genotype)	No. of Breeds	No. of Individuals	Genotype (Deletion)		
			del/del	*del/+*	*+/+*
Peanut (*dw/dw*)	3[a]	19	19	0	0
Dwarf (*Dw/dw*)	1[b]	20	0	16	4
Wild-type (*Dw/Dw*)	6[c]	40	0	0	40

del, deletion; +, wild-type.
[a] Netherland dwarf, Holland lop, and long-haired dwarf rabbits.
[b] Netherland dwarf.
[c] Belgian hare, Champagne d'argent, Dutch, Flemish giant, French lop, and New Zealand white.

type composition can potentially explain gene expression differences among genotypes. Thus, it is unclear whether this finding is a direct result of mtDNA misregulation caused by the *HMGA2* knockout or is explained by variation in the number of mitochondria in the sequenced sample. Therefore, we estimated mtDNA copy number for the same individuals used for the gene expression profiling using two independent qPCRs based on different sets of primers (Figure 3C). We found that copy number of mtDNA was not significantly different between most contrasts, with the exception of *Dw/dw* and *Dw/Dw* for just one of the qPCR reactions. If anything, the results were opposite to the expectation based on the gene expression pattern; *dw/dw* individuals tend to have slightly lower mtDNA copy numbers than *Dw/Dw* individuals. Another potential explanation for differential expression among genotypes is differential mapping efficiency associated with distinct mtDNA haplotypes. However, since this study is based on a within-litter comparison this explanation can be ruled out (Table S1), and gene expression is consistent among individuals within genotype class (Figure 3B). Taken together, we can conclude that mtDNA gene expression is altered as a result of the inactivation of *HMGA2*, suggesting that *HMGA2* expression might be directly associated with mitochondrial function.

Genome-wide scan for signatures of selection in Netherland dwarfs

Netherland dwarfs that do not carry the *dwarf* allele are still smaller in size when compared to most breeds, and this is true for most dwarf breeds in rabbits. To identify additional genomic regions that may have been subjected to directional selection for reduced size in Netherland dwarfs, we scanned the genome of this breed for regions of increased differentiation compared with normal-sized domestic rabbits by means of the d_i statistic (Akey *et al.* 2010).

We identified 34 genomic regions with unusual levels of population differentiation between Netherland dwarf and six other breeds (Figure 4A; outlier regions and their gene content are detailed in Table S2). These regions varied largely in size (750,000–6,250,000 bp, median 1,500,000 bp), and contained 646 genes in total. Within the most differentiated regions we noticed several genes previously associated with size or skeletal features in other species including *LCORL-NCAPG* (*e.g.*, Gudbjartsson *et al.* 2008; Rubin *et al.* 2012; Tetens *et al.* 2013; Sahana *et al.* 2015), *STC2* (Gagliardi *et al.* 2005; Rimbault *et al.* 2013), *HOXD* cluster (Zakany and Duboule 2007), *COL11A1* (Li *et al.* 1995; Annunen *et al.* 1999), and *IGF2BP2* (Dai *et al.* 2015) (Figure 4A).

In the genome-wide screen, the strongest signal overlaps the *LCORL-NCAPG* region (Figure 4A). In spite of the high F_{ST} values between Netherland dwarfs and other breeds, we failed to find a reduction of nucleotide diversity in Netherland dwarfs across this region (Figure 4B). In fact, Netherland dwarfs have the highest genetic diversity in this region, and most other breeds have nucleotide diversity values close to zero. With the exception of dwarf breeds, domestic rabbits are larger in size than wild rabbits, and our results for this region are consistent with a selective sweep for a growth-promoting allele at the *LCORL-NCAPG* locus in rabbit breeds that are large compared with wild rabbits. Given that the Netherland dwarf breed is the most recently developed breed in our data set, the increase in genetic diversity around this locus specific to Netherland dwarfs is suggestive of crosses with wild rabbits to achieve such small sizes. Historical records support such a scenario (Whitman 2004).

Discussion

The small size of Netherland dwarf rabbits and probably most, if not all, dwarf rabbits from other breeds, is due to heterozygosity for an *HMGA2* LOF allele combined with polygenic selection for small size at other loci. Although genome-wide association studies in humans have shown that body size is a highly polygenic trait, it is striking how notoriously *HMGA2* has been associated with variation in body size across species (Zhou *et al.* 1995; Alyaqoub *et al.* 2012; Frischknecht *et al.* 2015; Webster *et al.* 2015), including in humans (Buysse *et al.* 2009). We propose that *HMGA2* is a major regulator of body size in mammals and probably in other vertebrates because of an important role in regulating growth combined with minimal, if any, negative pleiotropic effects on other traits. An important difference between the *dwarf* mutation in rabbits and the *pygmy* allele in mouse (Zhou *et al.* 1995), the two *HMGA2* LOF alleles described so far, is that *pygmy* homozygotes are fully viable and show the pygmy phenotype whereas *dwarf* in rabbits is a recessive lethal (Figure 1). Human *HMGA2* LOF alleles are probably also recessive lethal, since an intragenic deletion in *HMGA2* is associated with short stature (Buysse *et al.* 2009) and no LOF homozygotes at this locus have yet been reported. Both pygmy mice and

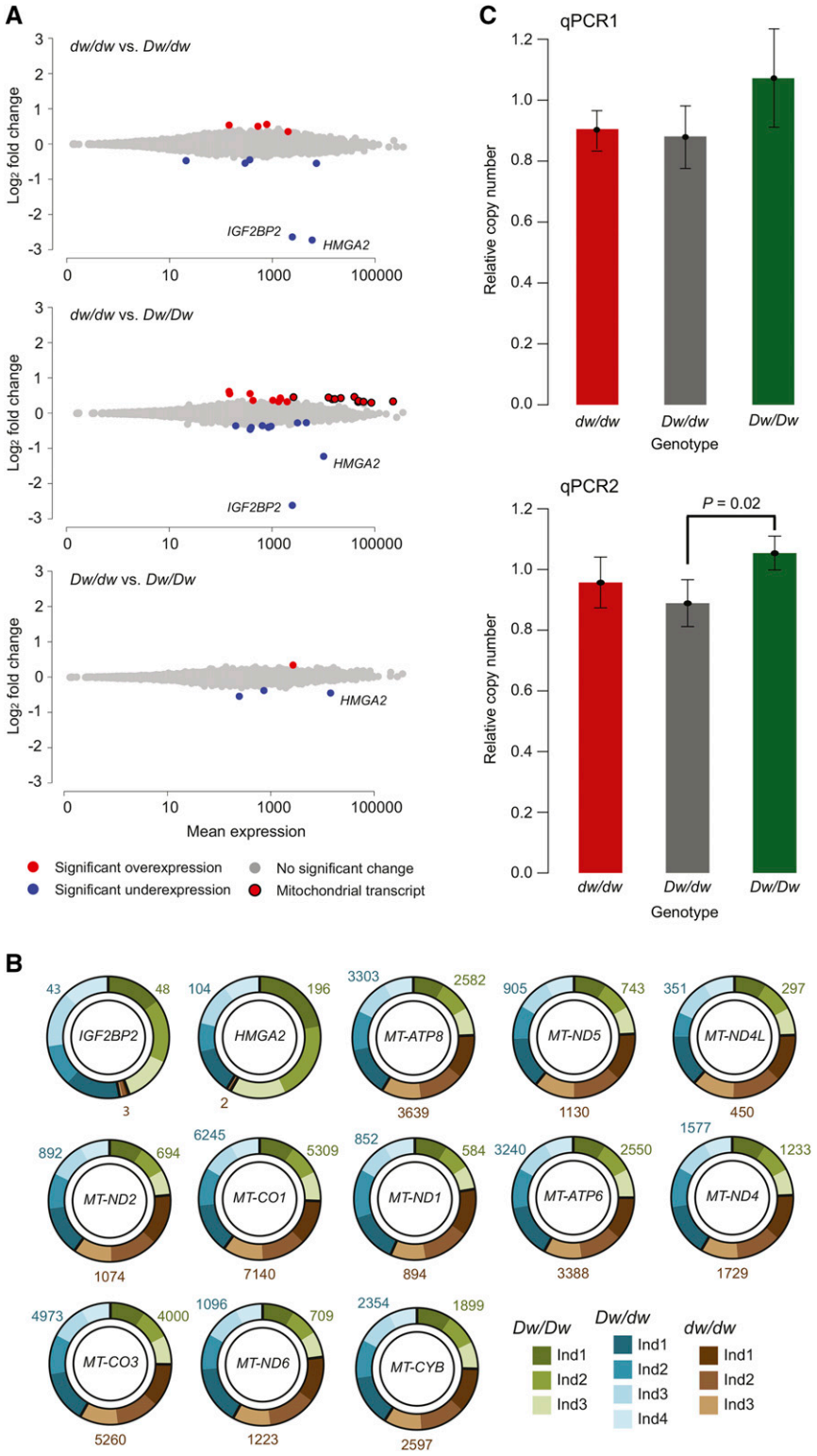

Figure 3 Differential gene expression among the three *HMGA2* genotypes and mtDNA copy number. (A) Statistically significant changes in gene expression between *HMGA2* genotypes evaluated using DESeq2 (Love *et al.* 2014). Log fold change *vs.* mean expression for the 21,588 genes included in our analysis for the different contrasts (upper panel: *dw/dw vs. Dw/dw*; middle panel: *dw/dw vs. Dw/Dw*; lower panel: *Dw/dw vs. Dw/Dw*). Red indicates significant overexpression, blue indicates significant underexpression, and gray indicates no significant change. Mitochondrial genes are circled. The data points for *HMGA2* and *IGF2BP2* are indicated. Mean FPKM values for all transcripts are given in File S3. (B) Doughnut charts summarizing levels of expression for *HMGA2*, *IGF2BP2*, and protein-coding genes encoded in mtDNA. Both technical replicates have been combined. Each chart represents one gene and the different individuals are represented by different colors. Black bars separate the genotypes (green: *Dw/Dw*; brown: *dw/dw*; and cyan: *Dw/dw*). Numbers outside of each chart indicate the mean FPKM value for each genotype. (C) mtDNA abundance in embryos of the different *HMGA2* genotypes. Barplot indicating the number of mtDNA copies relative to the autosomes for the three *HMGA2* genotypes. The bars reflect the average values and SD among three *dw/dw* (peanut), four *Dw/dw* (dwarf), and three *Dw/Dw* (wild-type) individuals. qPCR1 and qPCR2 represent two independent reactions based on different sets of primers targeting the rabbit mitochondrial genome. FPKM, fragments per kilobase million; Ind, individual; mtDNA, mitochondrial DNA; qPCR, quantitative PCR.

dwarf rabbits show altered craniofacial development, characterized by shortened heads (Zhou *et al.* 1995) (Figure 1). Interestingly, a recent study showed that *HMGA2* is a major locus affecting craniofacial development (beak size) in Darwin's finches (Lamichhaney *et al.* 2016), but in that case the phenotypic effect is likely caused by regulatory changes.

HMGA2 does not bind a specific DNA sequence but contributes to transcriptional regulation through interaction with other DNA-binding transcription factors (Reeves 2001; Pfannkuche *et al.* 2009), and it has a well-established role in cancer biology (Young and Narita 2007 and references therein). Despite the abundant genetic data demonstrating the important role of

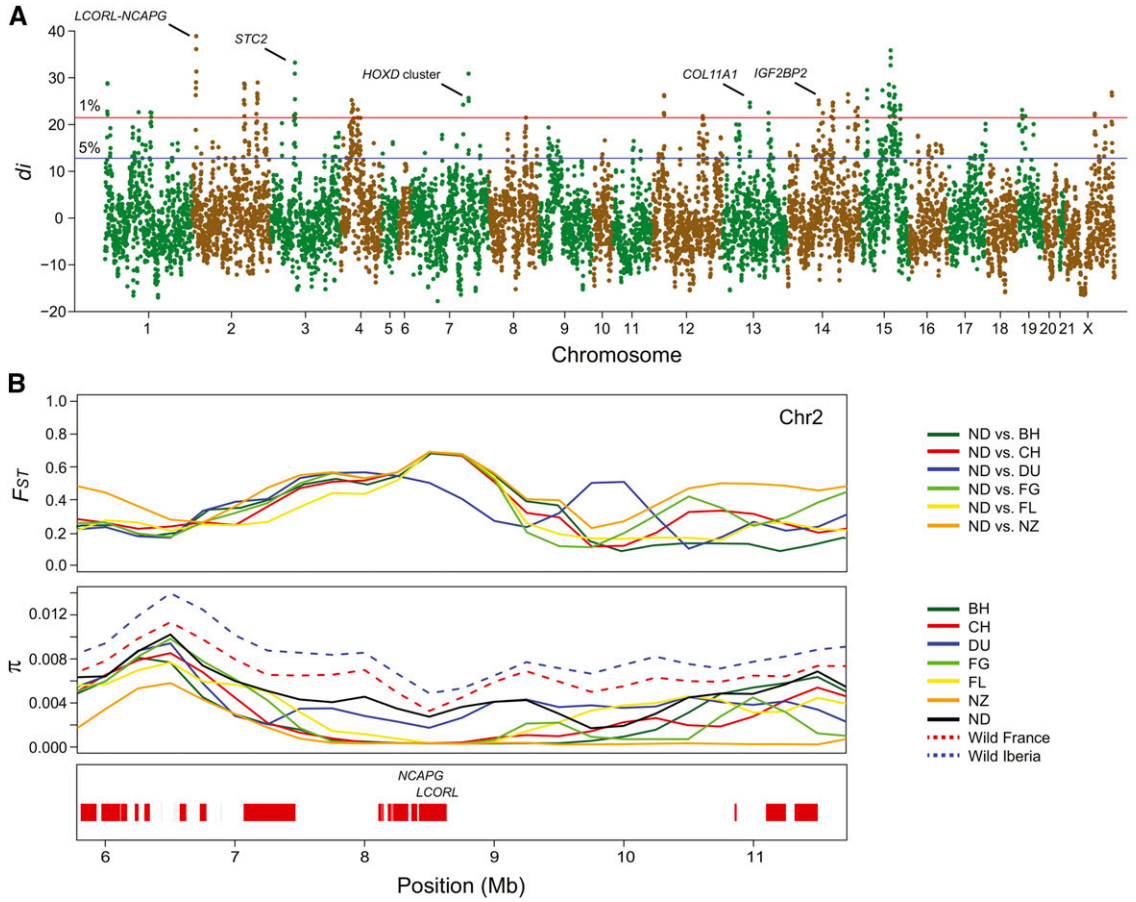

Figure 4 Selective sweep screen for additional genomic regions associated with small size in Netherland dwarf rabbits. (A) Genome-wide distribution of d_i. Values were summarized in 500 kb windows iterated every 250 kb. The red and blue lines indicate the top 1 and 5% of the empirical distribution, respectively. Unmapped contigs were included in the analysis but are not represented in the figure. (B) F_{ST} and nucleotide diversity (π) in the *LCORL-NCAPG* region. Each π and F_{ST} data point is based on a sliding window analysis using 500 kb windows with 250 kb steps. Annotated protein-coding genes are drawn across the bottom of each region, and putative candidate genes are highlighted. Nucleotide diversities of wild rabbits were averaged over three populations of wild rabbits from France (Caumont, La Roque, and Villemolaque) and two populations of wild rabbits from the Iberian Peninsula (Guadalajara and Zaragoza) belonging to the *Oryctolagus cuniculus cuniculus* subspecies; data from Carneiro *et al.* (2014). BH, Belgian hare; CH, Champagne d'argent; Chr, chromosome; DU, Dutch; FG, Flemish giant; FL, French lop; ND, Netherland dwarf; NZ, New Zealand white.

HMGA2 for controlling growth, the underlying mechanism is still poorly understood. Therefore, we carried out expression analysis of whole embryos of all three genotype classes *Dw/Dw*, *Dw/dw*, and *dw/dw*; embryos at day 15.5 were used because *HMGA2* expression was found to peak at this day. There were only three transcripts, besides *HMGA2*, that showed significant differential expression in the contrast between dwarf (*Dw/dw*) and wild-type (*Dw/Dw*) rabbits, and none of these three transcripts have an established role in regulating growth. This indicates that *HMGA2* haploinsufficiency causes dwarfism in *Dw/dw* rabbits by subtle changes in gene expression, or that the effects are very tissue-specific and therefore not revealed in our whole-embryo analysis. Only a limited number of transcripts showed significant differential expression even in the comparison between the two opposite homozygotes (*dw/dw vs. Dw/Dw*). However, there were two striking observations in this contrast: (1) silencing of *IGF2BP2* expression, and (2) mild (log2 fold change $\approx$ 0.5) upregulation of the transcripts for most mtDNA-encoded proteins.

Our result shows that the presence of HMGA2 is critical for the expression of *IGF2BP2*, consistent with a previous study showing that *Igf2bp2* undergoes a large-scale downregulation in pygmy mice (*Hmga2*-null) (Brants *et al.* 2004). Similar to our study (Figure 2A), it was essentially only the *Hmga2* and *Igf2bp2* transcripts that were significantly downregulated in pygmy 12.5-day embryos. HMGA2 also regulates expression of *Igf2bp2* in mouse myoblasts and the HMGA2-IGF2BP2 axis is an important regulator of skeletal muscle development (Li *et al.* 2012). IGF2BP2 is an RNA-binding protein and was first described as a protein that binds the *IGF2* mRNA, and it enhances *IGF2* translation (Dai *et al.* 2011). However, it also binds many other mRNAs and affects their translation (Li *et al.* 2012; Dai *et al.* 2015). Genome-wide association studies in humans have consistently shown that *IGF2BP2* is associated with susceptibility to Type 2 Diabetes (*e.g.*, Scott *et al.* 2007; Zeggini *et al.* 2007). *Igf2bp2*-deficient mice are modestly smaller, more lean, show improved glucose tolerance, higher insulin sensitivity, and have a significantly longer life span than

wild-type litter mates (Dai *et al.* 2015). The fact that *Hmga2*-null (*pygmy*) mice show a much more drastic reduction in body size than *Igf2bp2*-null mice implies that the reduced *Igf2bp2* expression in *Hmga2*-null mice and *HMGA2* heterozygous (dwarf) rabbits can only partly be mediated by reduced *IGF2BP2* expression. Interestingly, our screen for selective sweeps in Netherland dwarf rabbits indicated that genetic changes at the *IGF2BP2* locus itself might also have contributed to small size in this breed (Figure 4A).

Eleven out of the 13 protein-coding genes in mtDNA were significantly upregulated in the *HMGA2* knockout animals (Figure 3, A and B). The upregulation of mtDNA transcripts did not reflect an increased copy number of mtDNA per cell (Figure 3C), suggesting that HMGA2 has a direct or indirect role in regulating transcription of these genes. This functional role in mtDNA function is a novel finding not previously reported, but we note that the *Igf2bp2*-null mice show enhanced translation of *Ucp1* mRNA and other mRNAs encoding mitochondrial proteins, which in turn is expected to affect transcription of mtDNA genes (Dai *et al.* 2015).

Another interesting observation in this study was that the three genes *MSRB3*, *LMD3*, and *WIF1* located upstream of *HMGA2* show a mild overexpression in *HMGA2 dw/dw* and *Dw/dw* rabbits (File S2), suggesting that these four genes are coregulated to some extent. There are several possible explanations for this observation: (1) HMGA2 may be a negative regulator of these genes, (2) the 12.1 kb region deletion in *HMGA2* contains regulatory elements affecting the expression of the neighboring genes, or (3) the elimination of *HMGA2* expression may simply increase transcriptional activity from the promoters in the near vicinity. Gene targeting experiments will be required to evaluate these possible explanations.

A striking finding in our genome-wide screen for loci showing signatures of selection in Netherland dwarf rabbits was the enrichment of loci previously associated with variation in body size. The most prominent example is the two closely linked genes *LCORL* (Ligand-Dependent Nuclear Receptor Corepressor-Like), which encodes a transcription factor, and *NCAPG* (Non-SMC Condensin I Complex, Subunit G), which encodes a subunit of the condensin complex required for mitosis and meiosis progression. *LCORL-NCAPG* are, together with *HMGA2*, two of the loci that are most consistently associated with stature and body size variation across multiple studies and species. The *LCORL-NCAP* region has previously been associated with body size in human, cattle, dog, pig, and horse (Gudbjartsson *et al.* 2008; Pryce *et al.* 2011; Vaysse *et al.* 2011; Rubin *et al.* 2012; Signer-Hasler *et al.* 2012; Tetens *et al.* 2013; Sahana *et al.* 2015). Our results are consistent with a selective sweep at the *LCORL-NCAP* region related to selection for increased size in domestic rabbits, since all normal-sized rabbits included in this study are significantly larger than wild rabbits and were fixed for a single haplotype (Figure 4B). In contrast, Netherland dwarf rabbits as well as Dutch rabbits, which are well below the average size of domestic rabbits, appear to carry wild-type haplotypes at this locus. Overall, our results show that small size in dwarf rabbits results from a large effect, 12.1 kb deletion at the *HMGA2* locus, combined with polygenic selection.

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