Canine Brachycephaly is Associated with a Retrotransposon-Mediated Missplicing of SMOC2

Citation for published version:

Digital Object Identifier (DOI):
10.1016/j.cub.2017.04.057

Link:
Link to publication record in Edinburgh Research Explorer

Document Version:
Publisher's PDF, also known as Version of record

Published In:
Current Biology

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Canine Brachycephaly Is Associated with a Retrotransposon-Mediated Missplicing of SMOC2

Highlights
- A population-based genetics study of dogs that required diagnostic imaging
- Resolution of a QTL associated with face length reduction (brachycephaly)
- Association of brachycephaly with a retrotransposon that disrupts SMOC2 splicing
- The SMOC2 locus explains up to 36% of face length variation in dogs

Authors
Thomas W. Marchant,
Edward J. Johnson, Lynn McTeir, ..., Richard J. Mellanby,
Dylan N. Clements,
Jeffrey J. Schoenebeck

Correspondence
jeff.schoenebeck@roslin.ed.ac.uk

In Brief
Uncovering the molecular basis of dogs’ vastly diverse skull shapes requires individualized approaches to morphometrics and genotyping. Using data from veterinary referral patients, Marchant et al. shed light on a large effect locus that is responsible for face length reduction (brachycephaly).
Canine Brachycephaly Is Associated with a Retrotransposon-Mediated Missplicing of SMOC2


SUMMARY

In morphological terms, “form” is used to describe an object’s shape and size. In dogs, facial form is stunningly diverse. Facial retrusion, the proximodistal shortening of the snout and widening of the hard palate is common to brachycephalic dogs and is a welfare concern, as the incidence of respiratory distress and ocular trauma observed in this class of dogs is highly correlated with their skull form. Progress to identify the molecular underpinnings of facial retrusion is limited to association of a missense mutation in BMP3 among small brachycephalic dogs. Here, we used morphometrics of skull isosurfaces derived from 374 pedigree and mixed-breed dogs to dissect the genetics of skull form. Through deconvolution of facial forms, we identified quantitative trait loci that are responsible for canine facial shapes and sizes. Our novel insights include recognition that the SMOC2 gene mutation affects the facial skeleton in a dose-dependent manner. The size effects of the associated SMOC2 haplotype are profound, accounting for 36% of facial length variation in the dogs we tested. Data bring new focus to SMOC2 by highlighting its clinical implications in both human and veterinary medicine.

INTRODUCTION

The mammalian skull is an architectural wonder that illustrates the intertwined relationship of form and function. The skull facilitates ingestion and respiration, provides protection for the brain, and houses the visual, auditory, and olfactory systems. The skull also functions in communication, defense, and reproductive behaviors. The pressures of natural selection have ensured that the skull, a composite of bones, is multifunctional and is physically matched to the environmental challenges it experiences.

Human intervention through domestication and artificial selection has largely displaced the influence of natural selection on form and function across domestic species. The most profound effects of human intervention across all terrestrial species can be observed among skulls of the domestic dog, Canis familiaris [1]. Centuries of selective breeding has resulted in a broad radiation in skull form [2] whereas restraints on function have been relaxed.

Some subpopulations of dogs display morphologies that are highly reminiscent of human craniofacial anomalies, such as brachycephaly-type craniosynostosis and midface hypoplasia. In both species, brachycephaly and midface hypoplasia are risk factors for developing severe morbidities, including respiratory [3], gastrointestinal [3, 4], ear- and eye-related morbidities [3, 5], and neurological abnormalities [6]. Due to their rarity and complex clinical presentation, most human patients with brachycephaly will never receive a genetic diagnosis [7]. Conversely, dogs represent abundant examples of morphologically varied skull shapes.

Previous investigations of canine head shape using genome-wide association studies (GWASs) and selective sweep mapping highlighted an association between canine chromosome (CFA) 1 and brachycephaly [8-10]. In a binary design of brachycephalic...
versus non-brachycephalic pedigree dogs, Bannasch et al. [11] established a 296-kb haplotype that encompassed the thrombospondin 2 (THBS2) gene. This study did not identify causal genetic variants, and the effects of this locus on gene expression were not assessed [11]. Elsewhere, measurements and geometric morphometrics were used to quantify skull shape, revealing quantitative trait loci (QTL) associated with brachycephaly on CFA1, CFA5, CFA18, CFA30, and CFAX and a missense variant in the bone morphogenetic protein 3 (BMP3) gene on CFA32 [8, 9].

A limitation of the aforementioned studies is their disconnected use of phenotype and genotype data. Skulls from osteological collections were used to generate surrogate phenotypes (e.g., “breed averages”) for use in GWASs [8, 9]. Though this approach has proven successful for detecting QTL, this study design is poorly suited for identifying causal variation, which is not necessarily fixed within breeds whose complex traits are of interest. These breed average study designs cannot utilize mixed-breed dogs that represent a significant portion of extant canines and whose admixture can help separate the phenotypic effects of complex traits. Finally, mapping complex traits, such as canine brachycephaly, is confounded by the need to separate the influences that size has on shape (i.e., allometry) [12].

Our goal was to identify the causal genetic variation responsible for canine brachycephaly. Computed tomography (CT) from 374 dogs that include 84 Kennel Club (UK) recognized breeds and 83 mixed-breed dogs were analyzed using geometric morphometrics. Morphological descriptors, coupled with individuals’ genotypes, were used to conduct genome-wide association analyses of skull size and shape. Our analysis of size-controlled skull shape identified a highly significant QTL associated with canine brachycephaly on CFA1, as well as numerous other suggestive associations. Focusing on the CFA1 QTL, we defined a 187.7-kb critical interval common to 30 of 37 brachycephalic dogs. We resequenced 28 brachycephalic dogs to approximately 30-fold depth and filtered polymorphisms within the critical interval against variants called in 319 other resequenced canid genomes. Among five variants that were retained, we detected a long interspersed nuclear element (LINE-1) within the SPARC-related modulus calcium binding (SMOC2) gene. Transcript analyses revealed alternative splice isoforms that occur in the presence of the LINE-1, causing the incorporation of a premature stop codon after the eighth exon of SMOC2’s canonical 13-exon transcript. SMOC2 mRNA levels are downregulated in a dose-dependent manner with the LINE-1 element. Models of phenotypic effect indicate that the LINE-1 insertion explains up to 36% of facial retrusion observed in our study. Endogenously expressed (mouse) Smoc2 is observed in the pharyngeal arches during development, and the visceralcranial of Smoc2-null mice are dysmorphic. Our data suggest that SMOC2 dysfunction is responsible for canine brachycephaly. Understanding the developmental role of SMOC2 could have health implications in human and veterinary medicine.

RESULTS

Canine Phenotypes and GWASs

CTs of referral patients were reconstructed to produce three-dimensional isosurfaces (Figure 1A). We placed 86 landmarks across skull isosurfaces to capture subtle morphological variation within and across patients (Figures 1B–1G and S1). This study included 291 dogs that represented 84 breeds recognized by the Kennel Club (UK). Eighty-three mixed-breed dogs were also included (n = 374; Table S1). Landmarks were analyzed according to morphological substructure (neurocranium, visceralcranium, and mandible; Figure S1). Because form (size and shape considered together) differs so greatly between dogs of various breeds, we performed a Procrustes fit on the landmark data to delineate size, followed by a regression of shape on size to remove the effects of allometry (size-related shape). Principal-component (PC) analysis of distance matrices produced from the regression residuals indicated that the first component, PC1, accounted for 72.2% and 68.8% of variation in the visceralcranium and mandible data, respectively. In the positive direction of visceralcranial PC1, many of the constituent bones of the rostrum narrow mediolaterally and lengthen rostrocaudally. These are shape changes consistent with dolichocephalic dog breeds, such as the smooth collie (Figures 1B and 1F). The opposite phenomena are true for negative PC1: the rostrum broadens and shortens. This reflects the morphological changes that are consistent with brachycephalic head conformation, such as that seen in pugs (Figures 1E and 1F) [8, 13]. Individual breeds cluster together by morphological trait (e.g., visceralcranium shape and neurocranium size; Figure 1G), demonstrating the accuracy of this approach to capture phenotypes and order dogs based on their morphology.

Breeds can also be differentiated from one another by their genomic structure (Figure S2) [9, 14, 15]. Set to k = 2, STRUCTURE revealed the SNP ascertainment bias resulting from the boxer-based dog assembly; breeds closely related to the boxer including the bulldog, Dogue de Bordeaux, and Staffordshire terrier, emerge as a “molosser” subpopulation [15]. Approximately one-third of the mixed-breed dogs in our dataset also share this substructure. At k = 84, we observed that the vast majority of owner-reported breed assignments were accurate, though we note evidence of admixture among some of the pedigree dogs.

GWASs of the neurocranium size, as well as visceralcranium and mandible shapes, showed little genetic inflation (Figures 2 and S3). The analysis of neurocranium centroid size identified 32 associated SNPs, representing five genomic loci (Figure 2A; Tables 1 and S2). In a distinction from previous GWASs that investigated body size [16], our data suggest that these loci modulate skull size. This result is particularly surprising for the CFA1 locus, whose underlying FGF4 retrogene insertion is correlated with limb shortening in breeds like the Dachshund but was not known to reduce skull size, as suggested by our data [17].

Three SNPs on CFA1 at 55862036, 55983871, and 56132332 were associated with visceralcranial PC1 (Figure 2B). GWASs of mandible PC1 also highlighted the CFA1 QTL (Figure S3).

Critical Interval Determination

The CFA1 QTL of visceralcranial and mandible PC1 correspond to a broad selective sweep observed among brachycephalic pedigree dogs [8–11]. Focusing on the CFA1 QTL, we observed 16 SNPs in linkage disequilibrium (LD) (r² > 0.2) with the index SNP (BICF2P250912; visceralcranial PC1; p = 1.91 × 10⁻²⁰; Figure 3A). First, we scanned for haplotype associations...
extending 1 Mb away from the associated SNPs. This revealed a single region of highly significant haplotypes between 55,881,672 and 56,020,217 (Figure 3B). Genotypes corresponding to this interval, in addition to /C24500-kb flanking regions, were phased and ordered in rank of each subject’s viscerocranium PC1 value (Figure 3C). As the distribution of viscerocranium PC1 score is bimodal (Figure 2B, inset), with brachycephalic dogs corresponding to PC1 values less than /C240.2, we reasoned that the critical interval underlying the CFA1 QTL should be established using haplotypes from this subset of dogs, as constituents are more likely to be fixed for the underlying causal variant(s) (Figure 3C). This revealed a 187.7-kb critical interval (extending between CFA1 55,850,299 and 56,037,676) defined by a 12-SNP haplotype. The 12-SNP haplotype is highly enriched among brachycephalic dogs and was identified among 63 of 74 (85.1%) chromosomes—it is found in just 28 of 674 (4.2%) chromosomes of dogs with viscerocranium PC1 score > –0.2 (Table S3). Suggestive of an effect, the viscerocranium PC1 value of these dogs was significantly different when comparing haplotype carriers to non-carriers (Student’s t test; p = 4.86 × 10^{-49}). Curiously, we identified two Dogues de Bordeaux that did not carry the associated haplotype on CFA1. However, our STRUCTURE analysis revealed a higher degree of admixture in these two Dogues de Bordeaux compared to others of the same breed (Figure S2), suggesting that they were cryptic outbreds. Moreover, both dogs had longer viscerocrania than those Dogues de Bordeaux that were fixed for the 12-SNP haplotype (data not shown).

Eight of the twelve SNPs of this haplotype are located within the SPARC-related modular calcium-binding protein 2 (SMOC2) gene (Figure 3C). The remaining four SNPs are spread across ~43 kb of sequence downstream of the gene.
Variant Filtering Analysis

Focusing on the CFA1 critical interval, we analyzed 187,377 bp of whole-genome sequence. In total, we called 3,674 SNPs/INDELS and 162 structural variants (Table 2). After hard filtering (Table S4), four SNPs and one structural variant remained as candidates for further consideration (Table 2; see STAR Methods). All five remaining variants are located within introns of the SMOC2 gene. The structural variant is a 1,531-bp insertion, which is present in the dog reference genome (which was generated from a brachycephalic breed—a boxer). The SNPs and insertion appear in complete linkage disequilibrium (data not shown). Though we cannot formally exclude their contribution to brachycephaly, none of the SNPs fell in regions of high conservation across species (Figure S4). Thus, their potential to cause brachycephaly was poorly supported.

Conversely, the insertion is a 3’ truncated fragment of a class 1 long interspersed nuclear element (LINE-1). LINE-1 insertions are known to be mutagenic in both man and dogs [18, 19]. The LINE-1 insertion within SMOC2 is fragmented, possibly due to incomplete insertion through “abortive” retrotransposition, and includes an intact 3’ UTR and 1,302 bp of ORF2 (Figure 4A) [20]. We genotyped the LINE-1 fragment in subjects used in our GWASs. The LINE-1 fragment appears among 91.5% of chromosomes of brachycephalic dogs (viscerocranium PC1 < 0.2) compared to only 2.1% of chromosomes of non-brachycephalic dogs (Figure 4B). The LINE-1 fragment appears to have no correlation with neurocranium centroid size (Figure 4C). Grouping individuals based on the number of LINE-1 fragment alleles they carried, we observe an additive effect for all normalized linear measurements taken from skull isosurfaces, with the greatest effect observed on the length of the palatine bone (Figures 4D and 4E).

LINE-1 retrotransposons are known to alter local gene expression through a variety of mechanisms that affect transcription [21–23]. Therefore, we quantified the relative expression levels of SMOC2 mRNA at both the 3’ and 5’ ends of the transcript. A comparable additive effect on SMOC2 expression was observed across the transcript (Figure 4F). Subjects that were homozygous for the SMOC2 LINE-1 allele had an ~5-fold reduction in total SMOC2 mRNA expression compared to individuals without a copy of the allele. This observation was independently confirmed by RNA sequencing. Subjects that were homozygous carriers for the LINE-1 allele similarly had a significant reduction in total SMOC2 mRNA levels when compared to non-carriers (fold change = 3.1; Figure 4G). Three additional genes showed significantly reduced expression, including two novel genes for long non-coding RNAs (ENSCAFG0000039143 and ENSCAFG00000035778) and the protein-coding urotensin 2B (UTS2B) gene. None of these genes are located on CFA1. No changes in expression of the neighboring genes to SMOC2, THBS2, and DACT2 (dishelved-binding antagonist of beta-catenin 2) were observed (Figure 4G). Non-carriers of the SMOC2 LINE-1 exclusively transcribed the “canonical” 13-exon transcript of SMOC2 (Figure 4H). Homozygous carriers

Table 1. SNPs Showing Genome-wide Significance with Skull Datasets

<table>
<thead>
<tr>
<th>Dataset</th>
<th>Chromosome</th>
<th>Index SNP</th>
<th>Position</th>
<th>Candidate Gene</th>
<th>Allele</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neurocranium 1</td>
<td>1</td>
<td>BICF2P250912</td>
<td>55,983,871</td>
<td>SMOC2*</td>
<td>G &gt; A</td>
<td>1.91 × 10⁻²⁰</td>
</tr>
<tr>
<td>Neurocranium 1</td>
<td>1</td>
<td>BICF2P250912</td>
<td>55,983,871</td>
<td>SMOC2*</td>
<td>G &gt; A</td>
<td>8.43 × 10⁻¹⁰</td>
</tr>
<tr>
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<td>TIGRP2P56799_rs8666557</td>
<td>91,103,945</td>
<td>LCRORL/NCAPG</td>
<td>G &gt; T</td>
<td>3.64 × 10⁻⁹</td>
</tr>
<tr>
<td>Neurocranium 7</td>
<td>7</td>
<td>BICF2S23352941</td>
<td>43,719,549</td>
<td>SMAD2*</td>
<td>A &gt; G</td>
<td>5.71 × 10⁻¹³</td>
</tr>
<tr>
<td>Neurocranium 10</td>
<td>10</td>
<td>G580546S240</td>
<td>8,183,593</td>
<td>HMGA2</td>
<td>C &gt; T</td>
<td>3.06 × 10⁻¹⁵</td>
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<tr>
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<td>BICF2P355320</td>
<td>41,257,020</td>
<td>IGF1*</td>
<td>C &gt; T</td>
<td>1.73 × 10⁻¹⁹</td>
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<tr>
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<td>18</td>
<td>BICF2S23615757</td>
<td>20,272,961</td>
<td>FGF4 retrogene</td>
<td>A &gt; C</td>
<td>3.31 × 10⁻⁸</td>
</tr>
</tbody>
</table>

See also Table S2. For intragenic SNPs, genes are denoted by asterisks.

*Only index SNPs are listed. A complete list of significant SNPs is shown in Table S2.

*Derived alleles are shown after ancestral alleles.
for the SMOC2 LINE-1 similarly transcribed the canonical transcript; however, in addition to this, these individuals also transcribed multiple different isoforms of SMOC2 (Figure 4H). Using primers designed against exon 8 and the LINE, we identified three isoforms present across all individuals homozygous for the LINE-1 element and a further three rarer isoforms present in homozygous or heterozygous carriers of the LINE-1 element (Figure S5; Table S5). All isoforms incorporate the LINE-1 element and differing lengths of preceding intron into the SMOC2 mRNA following exon 8. Each of the different splice sites within intron 8 are preceded by an adenine and guanine residue (AG)—an almost invariant characteristic of mammalian splice

**Figure 3. Regional Association and Critical Interval Determination of the CFA1 Viscerocranium QTL**

(A) SNP associations with viscerocranium PC1 are shown for ~1 Mb on either side of significant SNPs on CFA1. SNPs are colored depending on the degree of LD ($r^2$) with the index SNP (BICF2P250012; 1.91 × 10^-5). (B) Ten-SNP sliding window haplotype association. (C) Genotypes between 55,881,672 and 56,020,217 (including ~500 kb of flanking sequence) were phased and ranked by their viscerocranium PC1 value. Only haplotypes from brachycephalic dogs (viscerocranium PC1 ≤ −0.2; see Figure S3) were considered. Haplotypes are paired by subject and ranked by viscerocranium PC1 value. Alleles colored light gray match the consensus haplotype; dark gray alleles are variant. A 187.7-kb critical interval is defined by at least three meiotic recombinations (indicated above by black bar). The 12 SNPs that constitute the associated haplotype (red bar) are distributed within or up to ~44 kb downstream of SMOC2. Black arrows indicate 3 of 37 dogs that have a homozygous variant haplotype. These dogs are registered as two Dogues de Bordeaux and a Chihuahua. The red arrow indicates a Japanese Chin that is homozygosed for a recombinant haplotype within the critical interval. See also Figures S2 and S6 and Table S3.
acceptors (Table S5) [24, 25]. All alternative isoforms are predicted to introduce a premature stop codon following exon 8. It is unclear whether the alternative truncated isoforms are translated; however, we predict the protein products would shear within the thyroglobulin-like domain and would have no extracellular calcium-binding domain (Figure 4) [26].

In exon 8, we observed a SNP that encodes a silent C/T substitution at position 55,939,143. Interestingly, both the C and T alleles are present across “ancestral” populations that do not carry the LINE-1 element. However, the LINE-1 element is only observed in the presence of exon 8’s T allele (Figure 4H). This suggests that the C/T variant predates the insertion of the LINE-1 variant. In heterozygous subjects, the C/T variant enables us to quantify the allele-specific transcriptional activity of SMOC2. Transcripts from a Yorkshire terrier dog that was homozygous ancestral for the SMOC2 allele (lacking the LINE-1), but heterozygous for the C/T allele, had an allele percentile ratio of 46:54, suggesting that transcripts from both alleles are equally represented (Table S6). In contrast, two Cavalier King Charles spaniels that were heterozygous for both the SMOC2 LINE-1 and the C/T allele had allele percentile ratios of ~75:25, which indicates that the DNA allele with the LINE-1 element contributes fewer of the SMOC2 reads (Table S5). A lower abundance of transcripts incorporating the LINE-1 element may suggest that they are targeted by nonsense-mediated decay, decreased transcriptional activity, or both.

**Size-Effect Modeling on Skeletal Size and Shape**

We were interested in modeling phenotypic effects of size and shape using the skull-derived QTL described by our study and elsewhere [8, 9, 16, 27]. The derived allele frequencies of associated markers of SMOC2, CFA30 QTL, BMP3, IGF1, and STC2 differ significantly according to viscerocranium PC1 (Figure 5A). These five genotypes were applied as explanatory variables in a linear stepwise model for the viscerocranium PC1. Alone, the homozygous-derived alleles of SMAD2, HMGA2, GHR(1), IGF1, FGF4, STC2, IGF1R, the LCORL/NCAPG locus, GHR(2), the CFA30 locus QTL, and BMP3 explain up to 47%, 37%, 31%, 29%, 28%, 22%, 21%, 14%, 10%, 8%, and 6% of neurocranium centroid size variation, respectively (Figure 5D).

**Species Conservation of SMOC2**

Morpholino knockdown of zebrafish smoc2 suggests it regulates head development [28, 29]. To determine whether SMOC2 function is evolutionary conserved across other species, we first assessed its regional conservation by aligning the locus to the human genome. Mouse and chick sequence conservation was strikingly reduced compared to other species, including the dog (Figure S6A). Despite this, embryonic expression in chick and mice is observed in the first pharyngeal arch (Figure S6) [30]. Notably, the cranial neural crest streams into the first arch to populate the primordia that will give rise to the maxilla, as well as other constituents of the viscerocranium and mandible [31–33]. Previous to our study, Smoc2<sup>−/−</sup> mice were generated and phenotyped for the International Mouse Phenotyping Consortium (IMPC). Although these mice are no longer maintained, adults used for phenotyping were viable and fertile. We assessed archived radiographs of Smoc2<sup>−/−</sup> (n = 8) and strain-specific controls (n = 4; Figure S7A). Principal-component analysis of the whole head revealed similar morphological variation to that which we observed in dogs. Murine PC1 variation showed mediolateral widening and rostrocaudal shortening of the skull (Figure S7B). PC1 values clustered differentially by genotype (Smoc2 knockout versus control; p < 0.001; Figure S7C); however, no such segregation was observed for sex (Figure S7D).

Total palate length was assessed from lateral radiographs. The palate was significantly shorter in transgenic mice (Student’s t test; p = 0.0011), though not when allometry was removed (Figure S7E; data not shown). Given this observation and the fact that the locus is poorly conserved might suggest species-level differences in Smoc2 function. Nonetheless, our mouse data, as well as additional bone phenotypes described by the IMPC, indicate that disruption of Smoc2 is sufficient to adversely affect craniofacial biology.

**DISCUSSION**

Studies, including ours, continue to demonstrate the effectiveness of dog breeders at propagating aesthetic traits [8, 17, 34]. This cultivation of morphologies predated the formation of breed clubs. The selective sweep and association of the CFA1 QTL with brachycephaly was recognized in the early days of dog GWASs; however, confirmation of the underlying causative genetics remained elusive. Unlike QTL mapping, fine mapping approaches based on haplotype comparisons are confounded by the occasional “outlier” within a breed that is not fixed for or does not even carry the genetic variant that drives a trait that is common to other members of its breed. Moreover, whereas dog breeds (e.g., brachycephaly) that are common across subsets of breeds are often driven by identity-by-descent genetics, this phenomenon is not absolute. To avoid these issues, as well as leverage the genetics of mixed-breed dogs, we built a study population whose phenotypes and genotypes were derived individually.
We distilled the CFA1 locus to reveal a haplotype overlapping with SMOC2 as the major contributor to brachycephaly. We strongly suspect that the insertion of a truncated transposable element into SMOC2 is most likely causal; however, we acknowledge the limitations of our study. The dog’s long-range linkage disequilibrium prevented us from disassociating four SNPs that are in linkage disequilibrium with the LINE. Whether or not these variants have functional impacts cannot be dismissed. Second, whereas our transcriptional analysis demonstrates differential expression and missplicing of SMOC2 that are associated with the LINE insertion, we cannot say whether other genes are affected by this haplotype in cis. Due to limited tissue availability, we restricted our differential expression to testis, a tissue where SMOC2 was assumed to be highly expressed based on evidence from other species [35, 36]. In the future, additional tissues will need to be tested to determine whether genes in cis...
are differentially expressed in association with the haplotype we describe.

Modeling phenotypic variance was enhanced by the inclusion of mixed-breed dogs, whose admixed genomes and lack of standardization helped separate QTL that would otherwise cosegregate. Alone, SMOC2 explains up to 38% of viscerocranium PC1 variance. Whereas clearly the locus has a large effect size, our study is currently underpowered to exhaustively detect QTL that modulate brachycephaly or, more broadly, shape of the facial skeleton. This is underscored by the fact that we have not explained canine brachycephaly as it occurs in two Dogues de Bordeaux and an Affenpinscher (the latter was used in our whole-genome sequencing); none showed evidence of a selective sweep on CFA1 nor did they carry the associated 12-SNP haplotype. Moreover, our GWASs failed to replicate the CFA32/BMP3 and CFA30 QTL associations described previously [8]. A likely explanation for this is the modest numbers of small, brachycephalic breeds in our study, as well differing

Figure 5. Size Effects of the Viscerocranium Shape and Neurocranium Centroid Size QTL

(A and C) Boxplots depicting the distribution of normalized size-corrected viscerocranium PC1 (A) and normalized neurocranium centroid size (C) for 11 loci linked with body size and skull shape. Distributions are subdivided by genotype — homozygous ancestral (AA), heterozygotes (AD), and homozygous derived (DD). *** denotes p < 0.001 in Mann-Whitney-Wilcoxon and Kolmogorov-Smirnov tests.

(B and D) A stepwise linear regression model for viscerocranium PC1 (B) and neurocranium centroid (D) determined the best explanatory model for ancestral (left) and derived (right) genotypes for each positional candidate.

Please cite this article in press as: Marchant et al., Canine Brachycephaly Is Associated with a Retrotransposon-Mediated Missplicing of SMOC2, Current Biology (2017), http://dx.doi.org/10.1016/j.cub.2017.04.057
demographics. Our study is lacking in Brussel Griffon, Pekingese, Boston terriers, and Japanese Chin—all brachycephalic breeds whose members are typically homozygous for the missense variant in BMP3.

By necessity, we cannot explain the genetics of skull shape without addressing confounding effects of allometry, which is essential in a species whose size differential can exceed 40-fold. We used subjects’ neurocranium centroid size to remove the influences of allometry from viscerocranium shape variation, as well as to explore the genetics of neurocranium size itself. A genomic association of neurocranium centroid size identified five loci. Four of these loci were previously identified in body size studies across a variety of species: SMAD family member 2 (SMAD2) [9, 16, 27], high-mobility group AT-hook 2 (HMGA2) [9, 16, 27, 37–39], insulin-like growth factor 1 (IGF1) [16, 27], and the ligand-dependent nuclear receptor corepressor-like (LCORL)/non-SMC condensin I complex, subunit G (NCAPG) locus [16, 40–42]. Our effect size data point to their relative contribution to neurocranium centroid size; the largest effect size is explained by the putative enhancer deletion at the SMAD2 locus [27]. The association of neurocranium centroid size with the fibroblast growth factor 4 (FGF4) retrogene was unexpected. Parker et al. [17] first identified an FGF4 retrogene associated with canine asymmetric chondrodysplasia, a form of dwarfism that gives breeds like the Dachshund its short legs. The same locus was associated with body weight [16], though this could be explained by reduced leg mass. Our results indicate that the bone-based structure of the neurocranium is also reduced in size by the retrogene. Similarly, Hayward et al. [16] identified an association to stature and body weight in proximity to SMOC2 [16]. Because a high proportion of the brachycephalic dog population are low-to-medium weight breeds (Figure 1A), the interpretation of their association is unclear. In our study, we see no evidence that the SMOC2 locus modulates neurocranium centroid size (Figures 2A and 5C) and, by extension, skeletal size. However, we cannot exclude the possibility that the QTL noted by the authors affect soft tissue mass or appendicular bone length.

SMOC2 belongs to the BM-40 (SPARC) family of matricellular proteins, which contain an extracellular calcium-binding module and a follistatin-like domain. SMOC2 is distinguished from the BM-40 family by the addition of two thyroglobulin domains and a novel domain unique to the SMOC subgroup [26]. The calcium-binding module facilitates the binding of multiple collagen types [43] and the interaction with several growth factors [44, 45], which permits the proteins to function in cell adhesion, cell proliferation, and matrix turnover (reviewed by [46]). The BM-40 family was first identified in bone (where SMOC2 has been shown to be differentially expressed across the growth plate) but has since been found in a wide variety of other tissues [38, 47–49]. Mounting evidence suggests the SMOC2 plays an important role in craniofacial form across species. Knockdown of zebrafish smoc2 causes severe craniofacial hypoplasia [28], a process that may act by downregulating target genes of bone morphogenetic protein (BMP) signaling [50]. In chick embryos, Smoc2 is prominently expressed in the pharyngeal arches. Murine craniofacial development undergoes dynamic growth between embryonic days 10.5 and 12.5. Throughout this window, Smoc2 is shown to have differential temporal expression in the frontalasal process and maxillary/mandibular prominences [47]—tissues that give rise to mandible and viscerocranium structures. Our geometric morphometric analysis of radiographs indicate the skulls of Smoc2-null mice cluster distinct from wild-type, though a detailed understanding of the shape changes that occur in null mice will require three-dimensional analysis (Figure S7). It is intriguing that numerous copy-number variants spanning SMOC2 are associated with human phenotypes, including brachycephaly, hydrocephalus, long face (vertical), and hypertelorism [51]. Point mutations in SMOC2 were identified independently in patients with dentin dysplasia type I syndrome, whose hallmarks include severe oligodontia and microodontia [29, 52]. Finally, deleterious mutations in SMOC2 were identified in DECODE [53] and Generation Scotland biobanks (M.L.B., unpublished data).

Leveraging the craniofacial diversity of dogs, we set out to discover candidate genes involved in human craniofacial anomalies, particularly craniosynostosis and midface hypoplasia. Our results suggest that SMOC2 should be screened as a candidate for diagnosis. Not to be ignored, the role of SMOC2 dysfunction and the associated haplotype we defined need further exploration as they concern the health of brachycephalic dogs. As our canine skull project continues to grow, we will explore the role of SMOC2 and other skeletal QTL with comparative health implications.

STAR METHODS

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SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and six tables and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2017.04.057.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments, T.W.M., E.J.J., R.J.M., D.N.C., and J.J.S.; performed the experiments, T.W.M., E.J.J., L.M., and J.J.S.; performed

ACKNOWLEDGMENTS

The authors thank the many dog owners whose beloved pets enabled this study. We would also like to acknowledge the many clinicians at the R(D)SVS Hospital for Small Animals, PTDS, Dick White Referrals, Klinetierklinik of Vetsuisse Faculty Bern, and Friendship Animal Hospital, who helped to collect biospecimens used in our DNA and RNA analysis. Gonad tissue was collected by J.J.S. with the support of E. Ostrander. Special recognition is given to D. Gaylor, S. Campbell, J. Lawrence-Rausch, R. Elders, K. Jermy, A. de Castro Marques, S. Woods, Y. Martinez-Pereira, B. Corcoran, T. Nuttall, D. Gow, K. Mellonby, and N. Bommer for their extraordinary efforts to enable patient recruitment; the Dog Biomedical Variant Database Consortium (G. Aquirre, C. Andre, D. Bannasch, D. Becker, C. Droegemueller, O. Forman, S. Friedenberg, E. Furnow, U. Giger, C. Hitte, M. Hytinen, H. Lohi, C. Mellersh, J. Mickelson, A. Oberbauer, S. Schmutz, and C. Wade) for sharing whole-genome sequencing data from control dogs; N. Russef for providing skull photography; and C. Muller, K. Mellonby, and N. Bommer for their helpful suggestions. The mouse Smocc2 riboprobe was generously provided by J. Rainger. J.J.S. is a University of Edinburgh Chancellor’s fellow and received funding from the Wellcome Trust-University of Edinburgh Institutional Strategic Support Fund (ISSF2). The Roslin Institute receives strategic funding from the Biotechnology and Biosciences Research Council; J.J.S. (BB/J004235/1 and BB/P010879/1) and M.D. (BB/J004316/1 and BB/P010327/1). J.J.S. and M.N. received funding from the Albert Heim Foundation (grant 105 09.10.2012). Funding for DECIPHER was provided by J. Rainger. J.J.S. is a University of Edinburgh Chancellor’s fellow and received funding from the Wellcome Trust-University of Edinburgh Institutional Strategic Support Fund (ISSF2). The Roslin Institute receives strategic funding from the Biotechnology and Biosciences Research Council; J.J.S. (BB/J004235/1 and BB/P010879/1) and M.D. (BB/J004316/1 and BB/P010327/1). J.J.S. and M.N. received funding from the Albert Heim Foundation (grant 101 13.03.2012). T. Leeb received funding from the Albert Heim Foundation (grant 105 09.10.2012). Funding for DECIPHER was provided by the Wellcome Trust.

Received: October 19, 2016
Revised: March 14, 2017
Accepted: April 27, 2017
Published: May 25, 2017

REFERENCES


### STAR METHODS

#### KEY RESOURCES TABLE

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Software and Algorithms

Read alignment: bwa v0.7.8

Variant caller: GATK v3.7
[56] http://gatkforums.broadinstitute.org/gatk

WGS utility: Picard
http://broadinstitute.github.io/picard
https://github.com/broadinstitute/picard/releases

Structural variant caller: Pindel v0.2.3
[57] http://gmt.genome.wustl.edu/packages/pindel/

Annotation: SNPsift v4.0

Effect prediction: HaploReg v4.1
[59] N/A

Effect prediction: CADD v3.1
[60] N/A

Utility: PLINK v1.07
[61] http://zzz.bwh.harvard.edu/plink/

Utility: PLINK v1.90 beta
[62] https://www.cog-genomics.org/plink2

Admixture assessments: STRUCTURE v2.3

Linear mixed model: GEMMA v0.94.1

Phasing: SHAPEIT v2.r837
[65] https://mathgen.stats.ox.ac.uk/genetics_software/shapeit/shapeit.html

(Continued on next page)
CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Jeffrey Schoenebeck (jeff.schoenebeck@roslin.ed.ac.uk).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Study Participants. In total, 374 canine patients (212 male, 162 female) were recruited from four veterinary practices across the United Kingdom and Switzerland: The Hospital for Small Animals, The University of Edinburgh, UK; Davies Veterinary Specialists, Hertfordshire, UK; Small Animal Medicine and Surgery Group, The Royal Veterinary College, Hertfordshire, UK; The Division of Clinical Radiology, The Vetsuisse Faculty University of Bern, Switzerland. Canine participants were admitted to referral practices for diagnostic imaging. Owners provided breed identity (when known) and consent for their dogs' participation in our study. Spiral or sequential computed tomography (CT) scans were acquired at one or two millimeter slice thickness. All scans were reviewed by a radiologist to ensure that pathologies or injuries did not compromise exterior skull integrity. All 374 individuals are represented in the viscerocranium and neurocranium dataset. Due to mandibular pathologies, a subset of 355 individuals were represented in the mandibular dataset. Participants were aged twenty-four months or above at the time of diagnostic imaging and represent eighty-four Kennel Club (UK) recognized breeds and eighty-three mixed-breed individuals (Table S1). Use of referral patient diagnostic imaging and biomaterial was reviewed and approved by the R(D)SVS’s Veterinary Ethics Review Committee.

Mouse (C57BL/6) and chick (Isa Brown) embryos used for histology were surplus biomaterial harvested prior to this study. Mouse and chick work was conducted in accordance to animal use guidelines of the Roslin Institute under UK Home Office license and with ethical review.

METHOD DETAILS

DNA Extraction and Microarray Genotyping
Genomic DNA (gDNA) was extracted from residual diagnostic whole blood stored in EDTA at 4 °C, −20 °C or at −80 °C; discarded soft tissue following surgery stored at −20 °C or oral saliva swabs (Performagene, DNA Genotek). DNA was extracted from whole blood using an adaption of Boodram salt-based protocol (http://www.protocol-online.org/prot/Protocols/Extraction-of-genomic-DNA-from-whole-blood-3171.html). For the gDNA extraction from soft tissue 750 μL extraction buffer (10 mM Tris pH 8.0, 10 mM EDTA pH 8.0, 1% SDS, 100 mM NaCl), 80 μL 0.5 M Dithiothreitol and 15 μL Protein K solution (20mg/mL, Ambion, Life Technologies) were added to approximately 4 mm³ of tissue. Following overnight digestion, 270 μL saturated NaCl solution was added and centrifuged. One mL absolute ethanol was added to 500 μL supernatant to precipitate the gDNA. gDNA was spun and following centrifugation, washed with 70% ethanol. All gDNA samples were resuspended and stored in TE buffer 4 °C. Oral mucosa swabs were processed in accordance with the Performagene protocol (http://www.dnagenotek.com/US/pdf/PD-PR-083.pdf). Genotypes
were produced using the 170,000 SNP Illumina CanineHD Whole-Genome Genotyping BeadChip by Edinburgh Genomics, UK. Genotype calls were mapped to CanFam3.1 coordinates (Broad, September 2011).

RNA Extraction and qPCR
Tests were selected for messenger RNA (mRNA) extraction due to the unattainability of appropriate embryonic-stage tissue or healthy adult tissues in the dog. SMOC2 was assumed to be expressed in the testsis based on evidence in other species [35, 36] (http://www.proteinatlas.org/). gDNA and mRNA were extracted from testes snap frozen and stored at −80°C in RNA later. gDNA was extracted from tissue following the ThermoFisher Scientific protocol (http://www.thermofisher.com/uk/en/home/references/protocols/nucleic-acid-purification-and-analysis/ma-protocol/genomic-dna-preparation-from-malater-preserved-tissues.html). gDNA samples were genotyped for the SMOC2 LINE-1 insertion to allow targeted extraction of RNA from testes. From our screening, we identified nine subjects: 3 ancestral (1 Italian greyhound, 1 whippet, 1 Yorkshire terrier), 3 heterozygous (1 Papillon, 2 Cavalier King Charles spaniels), and 3 homozygous derived (1 bulldog, 1 French bulldog, 1 pug). For RNA extractions, 1 mL chilled Trizol was added to 100 mg of testes in a matrix D lysis tube and homogenized using a FastPrep for 20 s intervals at 4 m/s. Samples were incubated at room temperature for 5 min following homogenization. Next, 200 µL 1-bromo-3-chloropropane (BCP) was added to each sample, shaken vigorously for 15 s and incubated at room temperature for 3 min. Samples were centrifuged for at 12,000 G for 15 min at 4°C and the upper aqueous phase was subsequently transferred to a fresh tube. RNA was cleaned using the QIAGEN RNeasy Minikit following and including optional steps provided in the RNeasy Mini Kit Part 1 protocol. A DNase step was not used.

Complementary DNA (cDNA) was produced from 1 µg total RNA using the SuperScript III First-Strand Synthesis SuperMix (Invitrogen) following the product protocol with oligo(dT) primers. Primers for target genes were designed to be intron-spanning using the online Roche design center. Primers for reference housekeeping genes were acquired from previously published work [54]. Relative expression profiles for SMOC2 were determined using the Roche Life Sciences probe-based real-time qPCR assay with a LightCycler 480 system (Roche). All RNA profiles were analyzed in triplicate for both technical and biological replicates. Expression of target genes were normalized with mitochondrial ribosomal protein S7 (MRPS7). Relative quantification levels were corrected for primer efficiency [72].

Sequencing Library Preparations
The integrity of genomics DNA and total RNA samples were verified by Agilent Tapestation. All RNA samples scored RIN values greater than 8.0. DNA and RNA Library preparation and sequencing services were provided by Edinburgh Genomics (UK). Briefly, DNA libraries were prepared using either SeqLab TruSeq Nano DNA library prep HT or Illumina Truseq DNA nano DNA library kits. Paired-end libraries sequences on the Illumina HiSeq 2500 had an average insert size of 550 bp and read length of 125 bp. Paired-end DNA libraries sequenced on the HiSeq X platform had an average insert size of 450 bp and 150 bp read length.

For RNA, TruSeq stranded libraries were prepared from nine preparations of total RNA according to manufacturer’s protocol. Bar-coded libraries were sequenced on three lanes of an Illumina HiSeq 4000, producing 150 bp paired-end reads (96 million + 96 million reads per library).

Histology
Whole-mount Smoc2 in situ hybridization was performed per Nieto et al. (1996) [73].

QUANTIFICATION AND STATISTICS
Morphometrics
3D reconstructions of anonymised canine skull CT scans were generated in Stratovar Checkpoint software (v2014.11.28.0324) and anatomical substructures (cranium and mandible) of resulting isosurfaces were manually landmarked by a single analyst (Figure S1). Breed designations were hidden from the analyst and CTs were analyzed in a random order. Fifty-six cranial and thirty mandibular landmarks were selected to capture morphological variation. Viscerocranium PC1 (without allometry), mandible PC1 (without allometry) and neurocranium centroid size were subsequently used as phenotypic outcomes for GWAS.

Lateral and dorsoventral radiographs of four C57BL/6J control and eight Smoc2−/− mice (Smoc2tm1.KOMP.Vlcg allele produced by The Jackson Laboratory, USA) aged thirteen weeks were landmarked in ImageJ (v1.50 g) [76] using the PointPicker plug-in (male = 5, female = 7). The raw 2D coordinates for nine lateral and fifteen dorsoventral landmarks were exported from ImageJ and analyzed in MorphoJ. Lateral and dorsoventral landmarks were analyzed as using the same approach. A generalized Procrustes fit was used to create a best fit for landmarks. A covariance matrix was calculated using the Procrutes
distance matrix of the whole head prior to PCA. A two-tailed Student’s t test assessed PC1 distribution for sex and Smoc2 background. Principal component plots were generated using a custom R script. Additional phenotypic detail regarding these mice are available form the International Mouse Phenotyping Consortium (http://www.mousephenotype.org).

Genotype Analyses
PLINK (v1.07) [61] was used to remove SNPs with a minor-allele frequency < 0.05 and individuals with > 0.1 missing markers. Genotypes were prephased using SHAPEIT (v2.r837) [63] using default parameters that includes 500 states. Imputations were done with Minimac2 (2014.9.15) using 40 rounds and 1,000 states. Post-processing by fcGene (v1.0.7) removed genotypes with R^2 < 0.3 and minor allele frequency < 0.05. In total, 139,260 SNPs remained for analysis.

Population structure was assessed using STRUCTURE (v2.3) [63], GEMMA (v0.94.1) [64], which incorporates a kinship matrix in its implementation of univariate linear mixed models, was used to perform genome-wide association tests. Sex and up to ten principal components (generated from SNP genotype data in PLINK v1.9 [62]) were used as covariates – ten covariates for neurocranium and five for viscerocranium and mandible. The number of PCs included was determined by evaluation of Q-Qplots. p values generated in the association tests were used for Q-Qplots; using the aforementioned parameters returned components (generated from SNP genotype data in PLINK v1.9) were used as covariates – ten covariates for neurocranium and five for viscerocranium and mandible. The number of PCs included was determined by evaluation of Q-Qplots. p values generated in the association tests were used for Q-Qplots; using the aforementioned parameters returned

Fine Mapping
Haplotype association testing was done using ten SNP sliding windows across ~1 megabase (Mb) flanking regions of significant SNPs in canine chromosome (CFA) 1. SHAPEIT was used to phase genotypes. Haplotypes for the region of interest were ordered by individual viscerocranium PC1 score and colored by genotypes that matched the consensus sequence. The borders of the critical interval were defined by a minimum of three meiotic recombination events across the brachycephalic individuals with a viscerocranium PC1 < −0.2.

Variant Filtering
Eight brachycephalic dogs were resequenced on an Illumina HiSeq 2000 (Edinburgh Genomics, UK) to approximately 14-33X depth. Another thirty dogs were resequenced using the Illumina HiSeq X platform to > 40X depth. Resulting paired-end reads were aligned to the reference genome (CanFam3.1, Broad September 2011) using bwa (v0.7.8) [55]. SNPs and small INDEL variants within the critical interval (CFA1:55850299-56037676) were called using GATK (v3.7) [56, 75, 76]. We compared our variant calls to those of three-hundred and four dogs and wild canids made available to the DBVCD consortium members and an additional five canids (1 Basenji, 4 wolves) from the DoGSD database [77, 78]. Variants were hard filtered using SnpSift (v4.0) [58]. Because their deep coverage and large insert sizes (> 450bp), we used our thirty-eight re-sequenced dogs to call structural variants; variants were called using Pindel (v0.2.3) [57]. Filtering criteria for both SNPs/INDELS and large structural variants were determined by the presence of the twelve SNP haplotype across selected brachycephalic and dolichocephalic individuals whose skull phenotypes were confirmed (Table S4). Our filtering criteria were based on five logical assumptions. First, genomes from brachycephalic dogs with the twelve SNP haplotype were assumed to carry, or to be fixed for, the causal variant(s) within the CFA1 critical interval. Second, haplotype sharing at the CFA1 locus suggests identity-by-descent; therefore brachycephalic dogs with the twelve SNP haplotype inherited the same causal variant(s) from a common ancestor. Third, as the dog assembly is based on the genome of a boxer (a brachycephalic dog that was fixed for the twelve SNP haplotype), the causal variant(s) could be present in the reference assembly as reference allele(s). Fourth, we expected that the causal variant(s) are derived and therefore absent from wild canid populations such as dogs’ ancestor, the gray wolf. Lastly, dolichocephalic dogs without the associated twelve SNP haplotype cannot carry the causal variant(s).

qRT-PCR
All RNA profiles were analyzed in triplicate for both technical and biological replicates. Expression of target genes were normalized with mitochondrial ribosomal protein S7 (MRPS7). Relative quantification levels were corrected for primer efficiency [72].

RNA-Seq
FASTQ files were aligned using STAR to the dog reference genome (CanFam3.1, ENSEMBL release-85). Annotated junctions were downloaded from ENSEMBL (ftp.ensembl.org/pub/release-85/gtf/canis_familiaris/Canis_familiaris.CanFam3.1.85.gtf.gz). Alignment was performed in two passes as instructed by the user manual. Using Picard tools (http://broadinstitute.github.io/picard), read groups were added, bam files were merged by sample, and reads were marked for duplicates. Using featureCounts, an analysis tool of the RSubread package (RSubread v1.22.3 installed on R v3.3.0), we quantified mapped reads to genes. Differential expression analysis was conducted at the gene level using the R package DESeq2 (v1.12.4) by comparing homozygous SMOC2 LINE-1 carriers
compared to non-carriers (three each). Detection of allelic imbalance was made possible by two of the three heterozygous dogs described above (1 Cavalier King Charles spaniel, 1 Papillon), as these dogs were also heterozygous for the C/T SNP in exon 8 (chr1:55939143) of SMOC2.

**Generation Scotland: Scottish Family Health Study**

We used whole exome sequences from the Generation Scotland: Scottish Family Health Study (GS:SFHS). Study participants had been originally recruited for population-based studies of complex traits. Details regarding the design and sequencing of human participants is described elsewhere. We extracted all sequence variants in SMOC2 which passed GATK recalibration [56]. Putative regulatory elements and functional roles of the extracted variants were assessed by the ENCODE-based prediction tool HaploReg (v4.1) [59]. To assess the predicted consequences of the variants, we examined their C-scores, which indicate the ‘deleteronousness’ of a given mutation using combined annotation dependent depletion (CADD, v3.1) [60].

**DATA AND SOFTWARE AVAILABILITY**

DNA-seq and RNA-seq data are publicly available at the European Nucleotide Archive under primary accession number ENA: PRJEB17926. Genotypes are available at Dryad Digital Repository (http://datadryad.org). The Dryad Digital Repository DOI for the genotype data reported in this paper is Dryad: 10.5061/dryad.cq612.

**ADDITIONAL RESOURCES**

**DECIPHER**

This study makes use of data generated by the DECIPHER community. A full list of centers who contributed to the generation of the data is available from https://decipher.sanger.ac.uk and via email from decipher@sanger.ac.uk. The DECIPHER database was searched for variants in human SMOC2 with reported craniofacial phenotypes.