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The correlated evolution of *Runx2* tandem repeats, transcriptional activity, and facial length in Carnivora

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SUMMARY To assess the ability of protein-coding mutations to contribute to subtle, inter-specific morphologic evolution, here, we test the hypothesis that mutations within the protein-coding region of runt-related transcription factor 2 (*Runx2*) have played a role in facial evolution in 30 species from a naturally evolving group, the mammalian order Carnivora. Consistent with this hypothesis, we find significant correlations between changes in *Runx2* glutamine–alanine tandem-repeat ratio, and both *Runx2* transcriptional activity and carnivoran facial length. Furthermore, we identify a potential evolutionary mechanism for the correlation between *Runx2* tandem repeat ratio and facial length. Specifically, our results are consistent with the *Runx2* tandem repeat system providing a flexible genetic mechanism to rapidly change the timing of ossification. These heterochronic changes, in turn, potentially act on existing allometric variation in carnivoran facial length to generate the disparity in adult facial lengths observed among carnivoran species. Our results suggest that despite potentially great pleiotropic effects, changes to the protein-coding regions of genes such as *Runx2* do occur and have the potential to affect subtle morphologic evolution across a diverse array of species in naturally evolving lineages.

INTRODUCTION

How often and under what circumstances protein-coding sequence changes play a role in the evolution of morphology remains an unresolved question in evolutionary developmental biology. Coding sequence changes have been implicated in the evolution of overall skin or coat color in mammals, birds, and fish (e.g., Majerus and Mundy 2003; Mundy and Kelly 2003; Lamason et al. 2005; Mundy 2005; Protas et al. 2006), and in major changes in body plan morphology (e.g., Galant and Carroll 2002; Ronshaugen et al. 2002; Wagner and Lynch 2005; Fidalgo et al. 2006). However, available data seem to suggest that more subtle changes in morphology (i.e., in pigment patterns of fly wings [Gompel et al. 2005]) or morphologic changes caused by genes fundamental to many aspects of development (i.e., pelvic reduction caused by *Pitx2* [Shapiro et al. 2004]) tend to be driven primarily by changes in the regulatory regions of genes. Although still under investigation, this pattern seems reasonable, as the potentially negative pleiotropic effects of protein-coding changes are much greater than those of changes to regulatory regions (Carroll 2005).

To assess the ability of protein-coding changes within a fundamental regulator of embryonic development to contribute to subtle, inter-specific morphologic evolution, we studied the runt-related transcription factor 2 (*Runx2*) gene, which encodes a transcription factor essential for osteoblast development and normal bone formation (Schroeder et al. 2005). We test here the hypothesis that mutations within the protein-coding region of *Runx2* can drive aspects of facial evolution in a naturally evolving group, the mammalian order Carnivora. Most mammalian facial bones develop via intramembrous ossification, in which osteoblasts differentiate directly from mesenchymal cells (Depew et al. 2002). During this process, *Runx2* specifies the lineage of osteoblasts from the multipotent mesenchymal cells (Kobayashi et al. 2000), enhances osteoblast differentiation at an early stage in their development, and inhibits their differentiation at later stages (Fig. 1) (Komori 2002). *Runx2* also directly induces *Ihh* expression, which directly enhances chondrocyte proliferation and indirectly inhibits chondrocyte maturation (Komori 2005). Therefore, when *Runx2* is upregulated, bone development is accelerated (via increased proliferation) and extended (via

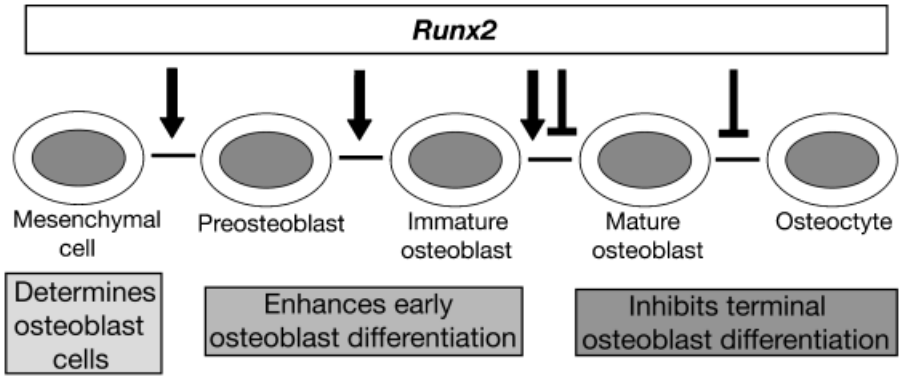

Fig. 1. *Runx2* directly regulates bone development by specifying mesenchymal cells to initially become osteoblasts, enhancing early osteoblast differentiation, and inhibiting terminal osteoblast differentiation (adapted from Komori 2002).

delayed terminal differentiation), and when it is downregulated, bone development is delayed and truncated (Komori et al. 1997; Inada et al. 1999; Ueta et al. 2001). *Runx2* possesses several functional domains, including a polyglutamine–polyalanine-rich region (QA) located in the amino-termini of the protein. Similar QA-rich regions have been found in several transcription factors (Hanna-Rose and Hansen 1996; Yeung et al. 1997; Janody et al. 2001; Galant and Carroll 2002), and are thought to mediate transcriptional activity by interacting with basal transcriptional machinery (Yeung et al. 1997; Schroeder et al. 2004). Specifically, polyglutamines drive transcription and polyalanines to repress transcription, in a length-dependent manner (Gerber et al. 1994; Galant and Carroll 2002).

Intriguingly, the ratio of the tandemly repeating polyglutamines to polyalanines in the *Runx2* QA region is positively correlated with facial length in breeds of domesticated dogs (*Canis familiaris*) (Fondon and Garner 2004). Specifically, dog breeds with higher tandem repeat ratios tend to have longer faces and taxa with lower tandem repeat ratios tend to have shorter faces. This was proposed to be due to relatively high and low levels of *Runx2* transcriptional activity, respectively, although activity was not assessed. This correlation led the original discoverers of this pattern to hypothesize that "gene-associated tandem repeat expansions and contractions are a major source of phenotypic variation in evolution," a view supported by earlier studies (see Kashi and King 2006, for examples). However, although this may be the case for tandem repeats within regulatory regions of genes, critics have stated that the protein-coding variation observed in *Runx2* "may have accompanying deleterious, pleiotropic effects that, whereas manageable under domestication, would limit its contribution under natural selection (Carroll 2005, p. 1163)." In support of this opposing viewpoint, mice mis-expressing *Runx2* exhibit a myriad of developmental problems, including dwarfism and skeletal malformations (Komori et al. 1997; Inada et al. 1999; Ueta et al. 2001). Furthermore, mutations within the *Runx2* QA region of humans are commonly associated with cleidocranial dysplasia (CCD), a rare autosomal dominant disease characterized by systemic skeletal abnormalities including a shortened face, and also shortened stature, misshapen pectoral, pelvic, thoracic and phalangial bones, abnormal permanent teeth, and premature closure of the anterior fontanel on the skull (Otto et al. 2002). Finally, many domesticated dog breeds are beset with physical disorders (e.g., hip dysplasia), prone to diseases (e.g., arthritis), and, in some cases, are only viable because of human intervention. However, it should be noted that the physical disorders of domesticated dogs have not been directly linked to pleiotropic effects of *Runx2*.

Therefore, despite the correlation between genetic mutations and morphologic change in domestic dog breeds, there are several outstanding issues that bear on the potential influence of mutations within the protein-coding region of *Runx2*, specifically within the QA domain, on subtle morphologic evolution within naturally evolving groups. In this study, we address three of these issues.

First, do changes in the ratio of *Runx2* QA tandem-repeats differentially affect transcriptional activity? If the *Runx2* tandem repeat ratio within a species does not regulate transcriptional activity, then mutations affecting the ratio (i.e., the relative amount of polyalanines to polyglutamines) are unlikely to influence facial development, and therefore most likely cannot be genetic mechanisms for evolutionary changes in facial morphology. If, on the other hand, *Runx2* tandem repeat ratio does influence transcriptional activity, then intraspecific changes that alter this ratio have the potential to drive interspecific phenotypic evolution. To address this, we functionally test the relationship between *Runx2* tandem repeat ratio and transcriptional activity using a transcriptional assay system.

Second, are *Runx2* polyglutamine to polyalanine ratios correlated with facial length in mammalian groups evolving under natural selection? We here test the correlation between *Runx2* QA tandem repeats and facial length from a wide array of species within the naturally evolving mammalian order Carnivora (Supplementary Fig. S1). The Carnivora is composed of two main suborders: the Caniformia (dogs and

their kin) and the Feliformia (cats and their kin) (Flynn et al. 2005). We selected carnivorans as the test group for this study for several reasons. First, domestic dogs, an artificially selected and evolved caniform group, were previously shown to have a correlation between *Runx2* tandem repeats and facial length among various domesticated breeds. Second, carnivorans as a group exhibit a great deal of facial length variation (Supplementary Fig. S1). Third, the phylogeny of the Carnivora is well understood (Flynn et al. 2005), which allows us to test potential evolutionary correlations among species within a phylogenetic framework.

Finally, we propose and begin to evaluate a potential evolutionary mechanism behind any observed correlations between facial length and *Runx2* tandem repeat ratio across naturally evolved carnivoran species. Despite the fact that cats have been domesticated for as long as 9500 years (Clutton-Brock 1981), cat breeds exhibit little facial length diversity (Robinson 1977). Furthermore, there are only minor differences in skull morphology among the many naturally occurring species of Felidae (Davis 1962; Ewer 1998). This is in stark contrast to the caniforms, as well as to other feliforms (although to a lesser extent). Those groups have developed a wide variety of facial morphologies, under both artificial (Wayne 1986) and, seemingly, natural selection, although the latter has never before been directly or quantitatively assessed. Among domesticated carnivorans, Wayne (1986) hypothesized that the notable difference in facial length diversity between domestic cats and dogs was driven by their contrasting modes of development, with cats exhibiting almost isometric, and dogs more allometric facial growth (Fig. 3A). In principle, allometric growth affords a greater source of variation upon which natural selection can act than does isometric growth because, under the former, changes in size are accompanied by changes in shape (Klingenberg 1998; Sears 2004). As a result, evolutionary changes in developmental timing (e.g., neoteny, progenesis, hypermorphosis, etc.) can generate novel morphologies. *Runx2*, as a regulator of bone development, has the potential to control both developmental rate and timing. Its upregulation leads to the acceleration and extension of bone development (e.g., acceleration and hypermorphosis) by increasing proliferation and early differentiation and delaying terminal differentiation of bone precursor cells, whereas its downregulation leads to the deceleration and truncation of bone development (e.g., neoteny and progenesis) (Fig. 1) (Komori et al. 1997; Inada et al. 1999; Ueta et al. 2001; Schroeder et al. 2005). We here begin to test the hypothesis that evolutionary changes to the *Runx2* tandem repeat system control *Runx2* regulation, and thus timing of skeletal development, thereby providing a mechanism for generating facial morphologic diversity among carnivoran species.

We here combine correlation and functional assays to link mutations within the protein-coding region of *Runx2* to the evolution of a specific morphology (facial length) in a naturally evolving group. Our results further suggest that *Runx2* is a potential (but almost certainly not the only) regulator of heterochrony in facial development within carnivorans, and that tandem-repeat driven variation in *Runx2* regulation is potentially at least partially responsible for the striking evolutionary differences in morphologic disparity observed across naturally evolved species of Carnivora (e.g., Holliday and Steppan 2004; Wesley-Hunt 2005) and especially between felid feliforms and caniforms.

MATERIALS AND METHODS

Data collection

Runx2 *tandem repeat data*

Tissues from 30 carnivoran species were obtained (Appendix A; see details in Flynn et al. 2005). The selected species are taxonomically diverse, incorporate a broad range of facial morphologies, and can be confidently placed within a well-supported phylogeny (Flynn et al. 2005). The goal of sampling was to get a broad phylogenetic diversity. The data set includes representatives from nine families: four feliform [Eupleridae (one species), Felidae (10 spp.), Herpestidae (four spp.), and Viverridae (four spp.)] and five caniform [Ailuridae (one sp.), Canidae (two spp.), Mustelidae (two spp.), Procyonidae (two spp.), and Ursidae (four spp.)].

DNA was extracted from tissues, and a fragment of the first exon of *Runx2* containing the tandem repeat region was amplified using PCR first with external and then internal primers. The degenerate primers designed by Fondon and Garner (2004) were used as external primers for this study, the sequences of which were generously provided by J. W. Fondon, III (U.T. Southwestern Medical Center, Dallas, TX, USA): TTGTGATGCGT ATTCCCGTA (sense) and ACAGAGCACAGGAAGTTGGG (antisense). Degenerate internal primers were designed by aligning *Runx2* sequences from human (*Homo*), mouse (*Mus*), rat (*Rattus*), dog (*Canis*), and chick (*Gallus*): ATCCGAGCACCAGCC GGCGCTTCAG (sense) and GTGGTCVGCGATGATCTCSAC (antisense). The resulting PCR product was run on a 1.5% agarose gel, and the band corresponding to the *Runx2* fragment ($\sim$ 300 bp) was extracted, purified (Qiagen Gel Purification Kit, Valencia, CA, USA), and sequenced (Molecular Biology Core Facility, Barbara Davis Center for Childhood Diabetes, University of Colorado Health Sciences Center). The resulting *Runx2* sequences were aligned and translated using MacClade 4.07 (Maddison and Maddison; Sinauer Associates Inc., Sunderland, MA, USA). Tandem repeat polyglutamine to polyalanine ratios were determined by dividing the number of consecutive glutamines by the number of consecutive alanines. To ensure that the PCR specifically amplified the correct sequence, we amplified and sequenced the *Runx2* tandem-repeat fragment of domestic dog and human and compared the resulting sequences with those available online in the NCBI database. We found our sequences to be comparable with those in the database, thereby confirming the validity of our primers and the amplification conditions.

Morphologic data

To quantify facial length and the three body size proxies used in this study, we measured distances among eight landmarks across the skull for the same species described above, using an Immersion Microscribe (San Jose, CA, USA) G2X three-dimensional digitizer. Facial length was measured as the distance between two of these landmarks (the dorsal juncture of the jugal and maxilla [at the orbital crest] and the anterior juncture of the nasal and premaxilla) (Fig. 2D).

We used three proxies for body size: (A) the juncture of the presphenoid, palatine, and pterygoid to the lateral edge of the occipital condyle, (B) the anterior juncture of the premaxilla and maxilla to the lateral edge of the occipital condyle, and (C) the juncture of the basisphenoid, presphenoid, and pterygoid to the lateral edge of the occipital condyle (Fig. 2C). We evaluated the influence of multiple potential body size proxies to ensure that our results were not merely artifactual byproducts of any given proxy. We included body size proxy A despite relatively low sampling to permit direct comparison with Fondon and Garner (2004). Data from the left and right sides of the skull were averaged for each specimen, and data from male and female specimens were averaged for each species. To correct for body size, we divided the averaged facial length for each species by one of the three body size proxies (A, B, and C) for that species to yield three relative face length metrics.

A total of 60 specimens, representing the 30 species for which tissues were sequenced for *Runx2*, were included in this analysis. Two specimens (one male and one female) were examined for each species. Only adult specimens, as determined by tooth eruption, were sampled.

Analyses

Correlation analyses

In an initial set of analyses, we performed both parametric (least-squared regression) and nonparametric (Kendall's Rank correlation) tests to assess the correlation between *Runx2* tandem repeat ratios (as the independent variable) and facial length (corrected for body size) (as the dependent variable). To assess the influence of taxonomic sampling on the results, we also performed a bootstrap analysis, in which we randomly resampled taxa with replacement one million times, and recalculated the nonparametric correlation statistics (i.e., Kendall's τ) for each pseudoreplicate data set. If tandem repeat ratio and facial length are uncorrelated, they should have nonparametric correlation statistics equal to one. Therefore, to test this null hypothesis of no correlation, we calculated the percentage of the resulting bootstrap replicates (out of one million) for which the Kendall's τ was $\leqslant 0$. We deemed the correlations significant if this percentage was $\leqslant 5\%$.

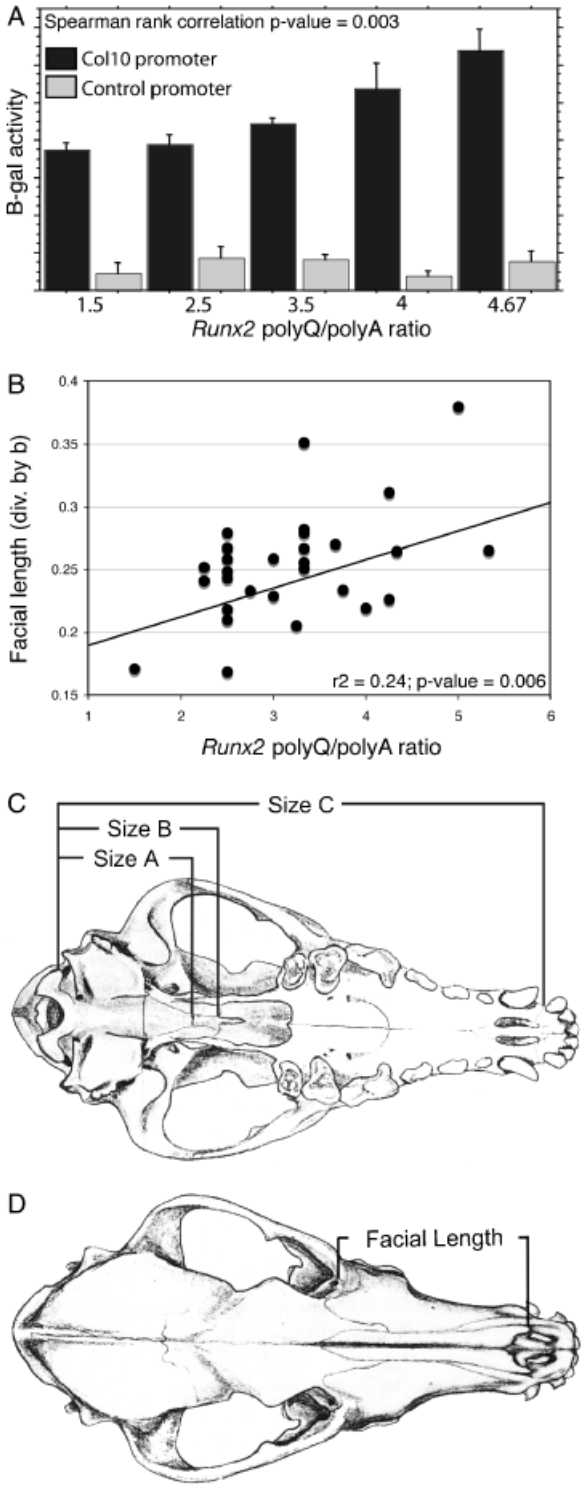

Fig. 2. (A) *Runx2* QA tandem repeat ratio is positively and significantly correlated ($P = 0.003$) with transcriptional activity, as demonstrated by assays in which *Runx2* constructs with higher tandem repeat ratios drove increased expression of a *Col10* promoter attached to a β-gal reporter ($\pm$ SEM). β-gal activity has been corrected for transfection using *Renilla* activity. (B) After correction by body size proxies (data for proxy B shown), facial length and *Runx2* tandem repeat ratio are positively and significantly correlated ($P = 0.006$). (C) Ventral view of a carnivoran skull, depicting the three body-size proxies (A, B, and C) and (D) dorsal view of the same skull, illustrating facial length.

In a second set of analyses, we sought to statistically minimize the effects of phylogenetic autocorrelation using correlations of independent contrasts (Felsenstein 1985). To do this, a composite phylogeny for the Carnivora was compiled from recent molecular studies (Veron et al. 2004; Yu et al. 2004; Bardeleben et al. 2005; Flynn et al. 2005; Johnson et al. 2006), with an emphasis on the all-Carnivora phylogeny of Flynn et al. (2005). Using MacClade 4.07, on this phylogenetic framework we reconstructed the ancestral states of the number of glutamines and alanines within the tandem repeat region of *Runx2*. We then calculated ancestral tandem repeat ratios by dividing the reconstructed number of glutamines by the number of alanines for each node. Contrasts for the resulting tandem repeat ratios were calculated according to the method outlined by Felsenstein (1985). We used the previously described phylogeny for Carnivora in the statistical program comparative analysis by independent contrasts (CAIC) (Purvis and Rambaut 1995) to calculate standardized linear contrasts for the facial length data. To assess correlation, three least-squared regression analyses that were forced through the origin were performed on the contrasts of tandem repeat ratio and facial length (divided by either body size proxy A, B, or C) (Pagel 1993).

We also assessed the strength of the correlation between tandem repeat ratio and facial length independently for caniforms and feliforms using least-squared regression. To assess the influence of taxonomic sampling on the results, we performed a bootstrap analysis on Kendall's Rank correlation data for caniforms and feliforms similar to that described above for all carnivorans.

All regression and Kendall's Rank correlation analyses were performed in StatView 5.0.1 (SAS Institute Inc.).

Analyses of tandem repeat variance and facial morphology

To measure the variation present in tandem repeat ratios within Feliformia and Caniformia, coefficient of variation (CV) was used. CV is the standard deviation of a sample divided by its mean and then multiplied by 100 (Sokal and Rohlf 1995). To determine the influence of sample size upon observed differences in CV between the two groups (19 vs. 11 species, respectively), we drew random subsamples of 11 feliform species with replacement, and calculated the CV of these subsamples. This was repeated 100,000 times to determine the frequency at which the 11-taxon caniform sample showed a higher or lower CV. This bootstrap resampling method was used to estimate the 95% confidence intervals around the means. Bootstrap percentages for the mean CV's outside the 95% confidence intervals were judged to indicate marked differences in CV between Feliformia and Caniformia (Efron and Tibshirani 1994).

Disparity (morphologic diversity) of feliform and caniform facial length (after correction for body size by division with the preferred proxy B) was measured using mean pairwise dissimilarity (MPWD), calculated as the mean squared Euclidean distance among taxa. This metric has the benefit of being proportional to total variance and relatively unbiased by sample size (Foote 1993a, b). Bootstrap resampling was used to estimate the 95% confidence intervals around the MPWD means using the method described above for CV.

Transcriptional assays

To assess the influence of altered tandem repeat ratios on *Runx2* transcription, a B-galactosidase reporter assay was performed using two vectors: one driving expression of *Runx2* constructs with experimentally determined varying tandem repeat ratios (6 glutamines to 4 alanines [ratio = 1.5], 10 to 4 [ratio = 2.5], 14 to 4 [ratio = 3.5], 16 to 4 [ratio = 4], and 14 to 3 [ratio = 4.66]) and the other carrying a 4kb *Col10a1* promoter element upstream of a β-galactosidase reporter (Zheng et al. 2003). *Col10a1* was selected for use in the *Runx2* transcriptional assays because it is a downstream target of *Runx2*, is known to be involved in bone development, and has been previously used in vitro to assess *Runx2* transcriptional activity (Zheng et al. 2003). B. Lee (Baylor College of Medicine, Houston, TX, USA) generously provided the pcDNA3.1(−) human *Runx2* expression vector and *Col10a1* promoter/β-galactosidase reporter (*Col10a1* pSAbeta-geobpA).

To develop the *Runx2* constructs, specific restriction sites flanking the tandem repeat region were identified within the *Runx2* expression vector (*Fsp*I upstream and *Not*I downstream) using Lasergene SeqBuilder (DNAStar Inc., Madison, WI, USA). Double-stranded oligonucleotides (oligos) containing these restriction sites and varying tandem repeat ratios of glutamines to alanines were designed and manufactured using PAGE purification (Integrated DNA Technologies, Coralville, IA, USA). The tandem repeat ratios of the constructed oligos varied within the experimentally determined values for carnivorans (6 glutamines to 4 alanines [ratio = 1.5], 10 to 4 [ratio = 2.5], 14 to 4 [ratio = 3.5], 16 to 4 [ratio = 4], and 14 to 3 [ratio = 4.66]). To substitute the oligos for the human *Runx2* tandem repeat sequence, the pcDNA3.1(−) *Runx2* expression vector was cut with *Fsp*I and *Not*I, and the oligos ligated into the resulting gap. Incorporation of the *Runx2* oligo was confirmed by sequencing.

Mammalian embryonic cells (3T3 fibroblast cells, National Institutes of Health) were then co-transfected (FuGENE 6 Transfection Kit, Roche Diagnostics, Boulder, CO, USA) with either the *Col10a1* promoter/β-gal vector (*Col10a1* pSAbeta-geobpA) or a promoterless control vector (pSAbeta-geobpA) and one of the designed *Runx2* expression vectors (either 6/4, 10/4, 14/4, 16/4, or 14/3). To control for transfection efficiency, cells were also co-transfected with a *Renilla* luciferase vector. Experimental treatments were replicated nine times. After transfection, cells were grown for 48 h. β-gal and luciferase activity levels were then quantified (β-gal, Applied Biosystems Dual-light Luciferase and β-galactosidase Reporter Gene Assay System, Foster City, CA, USA; *Renilla* luciferase, Promega *Renilla* Luciferase Assay System). To correct for transfection efficiency, β-gal activity was divided by *Renilla* activity for each sample. Correlation between β-gal activity (after *Renilla* correction) and *Runx2* tandem repeat ratio was assessed using Spearman's Rank correlation.

RESULTS

Runx2 tandem repeats regulate transcriptional activity

To assess whether changes in the QA ratio of the *Runx2* tandem repeat can affect the level of *Runx2* transcriptional

activity, we performed a transcriptional assay using a direct downstream target of *Runx2*, *Col10a1* (Zheng et al. 2003). In this assay, a *Runx2* expression construct was modified to contain *Runx2* tandem repeats of varying QA ratios (Q:A = 1.5, 2.5, 3.5, 4, and 4.67) matching observed interspecific sequence data. The different *Runx2* QA expression constructs were co-transfected with a *Col10a1* promoter element attached to a β-galactosidase (β-gal) reporter into NIH3T3 cells. Transcriptional activity of *Runx2*, as measured by the proxy of *Col10al*-driven β-gal output, increased linearly with *Runx2* tandem repeat ratio (Spearman's Rank correlation, *P*-value = 0.003; Fig. 2A). This result supports the hypothesis that the ratio of polyglutamines to polyalanines in the *Runx2* tandem repeat region plays a direct role in regulating *Runx2* transcriptional activity, with higher ratios resulting in higher levels of transcriptional activation of a direct target of *Runx2*.

Runx2 polyglutamine to polyalanine ratios vary between carnivoran species

To determine if the QA ratio varies in naturally evolving species, we used degenerate PCR to amplify the *Runx2* tandem repeat region from a diverse suite of 30 carnivoran species (see Appendix A for the species examined). We then sequenced these regions, and calculated the QA ratio for each by dividing the number of consecutive polyglutamines by the number of consecutive polyalanines (resulting QA ratios are shown in Appendix A). The calculated QA ratios varied from 1.5 (for the kinkajou, *Potos flavus*) to 5.33 (for the jaguar, *Panthera onca*).

Facial length is positively correlated with *Runx2* tandem repeat ratios in the Carnivora, a group that has evolved via natural selection over tens of millions of years

We next measured facial length (Fig. 2D), and three potential body size proxies based on skull dimensions (A, B, and C, Fig. 2C) for the carnivoran species listed in Appendix A. Because the correlation results for all body size proxies are generally comparable, we only discuss in detail those of body size proxy B (for complete results see Table 1). Least-squares regression ($n = 30$; $r^2 = 0.24$, *P*-value = 0.006) and Kendall's Rank correlation ($n = 30$; *P*-value = 0.030) for tandem repeat ratio and face length divided by body size proxy B were positive and significant (Fig. 2B and Table 1). This positive and significant correlation remained after bootstrapping of the included taxa (percentage of repetitions with Kendall's τ of $\leqslant 0 = 0.023$). Furthermore, this correlation was upheld ($n = 30$; $r^2 = 0.14$, *P*-value = 0.049) when phylogenetic autocorrelation was minimized using the method of independent contrasts (Felsenstein 1985). The correlation coefficients on the whole decreased after the application of independent contrasts to the data, but this was expected, as part of the uncorrected correlation was likely due to the shared phylogenetic history of the Carnivora. Thus, as the ratio of polyglutamines to polyalanines in the *Runx2* tandem repeat region increases in some carnivoran lineages, facial length also tends to increase.

Caniforms are more disparate in facial morphology than feliforms, have higher variance in *Runx2* tandem repeat ratios, and exhibit stronger correlations between facial length and *Runx2* tandem repeat ratio

The ideal way to test the hypothesis that the *Runx2* tandem repeat system generates diversity in facial morphology by controlling the timing of skeletal development would be to assess the degree to which changes between *Runx2* tandem repeats and facial length are correlated in ancestral species and their direct descendants. Unfortunately, it is not yet possible to do this because sufficient preserved genetic material has not yet been found in fossilized tissues of carnivorans. However, we can quantitatively examine two predictions of the hypothesis (note that both are predicated upon a functional relationship between tandem repeat ratio and *Runx2* transcription).

The first prediction is that caniforms should exhibit more inter-specific variation in both facial length and tandem repeat ratio than feliforms. Allometric variation in facial length has

Table 1. Correlations of *Runx2* tandem repeat ratio and face length

	LS regression		Kendall's Corr.		IC regression	
Dependent Variable	r^2	*P*-value	*P*-value	BT *P*-value	r^2	*P*-value
Face length diversity A ($n = 17$)	0.33	0.016*	0.087	0.089	0.45	0.006*
Face length diversity B ($n = 30$)	0.24	0.006*	0.030*	0.023*	0.14	0.049*
Face length diversity C ($n = 30$)	0.20	0.014*	0.043*	0.038*	0.15	0.037*

Results are shown for regressions performed without phylogenetic correction, including parametric (LS regression) and nonparametric (Kandall's Rank Correlation [Kandall's Corr]), and regressions performed after phylogenetic correction with independent contrasts (IC regression). The BT *P*-value is from the bootstrapping of the Kendall's data.

**P*-values that are significant at the 5% level. In general, correlations of tandem repeat ratio and face length are positive and significant.

only been evaluated in domesticated canids and felids, with canids exhibiting much more ontogenetic variation in facial growth than felids (Fig 3; Wayne 1986). If these patterns characterize the larger clades (Caniformia and Feliformia, respectively), then mutations that affect the *Runx2* regulatory control of developmental rate and timing would be expected to result in greater morphologic diversity in caniform face shape over evolutionary time. Therefore, caniforms should have not only more among-species variation in facial length but also more among-species variation in *Runx2* tandem repeat ratio, an underlying modulator of the facial length. To test this prediction, we used MPWD to measure disparity, or morphologic diversity. For facial length (corrected for body size proxy B), feliforms have an MPWD of 0.037 ($n = 19$), and caniforms an MPWD of 0.062 ($n = 11$). Thus, caniform facial length disparity is almost twice as great as that of feliforms, at least for the representative suite of taxa included in this study. The difference in mean MPWD for caniforms was beyond the 95% confidence intervals estimated for feliforms using bootstrap resampling, which accounts for the disparate sample sizes between the two groups. The difference between caniform and feliform MPWD was beyond the 95% confidence interval even after felids, with their isometric growth and stereotyped short faces, were removed from the feliform data set. Caniforms also have a higher CV for their tandem repeat ratios (CV = 30.84) than do feliforms (CV = 25.63). The difference in mean CV for caniforms was also beyond the 95% confidence intervals estimated for feliforms using bootstrap resampling. Again, the difference between caniform and feliform tandem repeat CV was beyond the 95% confidence interval even after felids were removed from the feliform data set. Taken together, these results support the first prediction.

The second prediction is if *Runx2* repeat mutations act upon *existing* ontogenetic variation in morphology, then facial length and tandem repeat ratio should be correlated more strongly in caniforms than in feliforms. The mutation rate of tandem repeat ratios is notably high (Fondon and Garner 2004; Kashi and King 2006; King et al. 2006), meaning that unless they are subject to stabilizing or directional selection within a group, they will quickly accrue large amounts of both intra- and inter-specific variation, which will confound their correlation with facial length. Given the nearly isometric facial growth of felid feliforms, changes in developmental rate and timing caused by *Runx2* mutations should have little effect on the range of facial lengths observed in this clade. As a result, the predominantly isometric feliforms (felids) will not be under as strong selection pressures as the highly allometric caniforms. In other words, natural selection acting upon facial length would permit more variation in repeat ratio within feliforms, resulting in a weaker correlation between the two. We found that although facial length is positively and significantly correlated with *Runx2* tandem repeat ratio in both groups when feliforms and caniforms are considered independently, the resulting correlation is stronger for caniforms ($r^2 = 0.40$; $P = 0.027$) than it is for either felids alone ($r^2 = 0.32$; $P = 0.045$) or feliforms as a whole ($r^2 = 0.20$; $P = 0.046$). However, although the feliform correlation was upheld after resampling with bootstrapping (P-value = 0.05), the caniform correlation was marginally insignificant (P-value = 0.10). Given the smaller sample size of the caniforms, this result is not unexpected, and in the future additional caniform taxa should be sampled to further test the hypothesis that differences exist between the caniform and feliform correlations. Still, taken as a whole our results also are

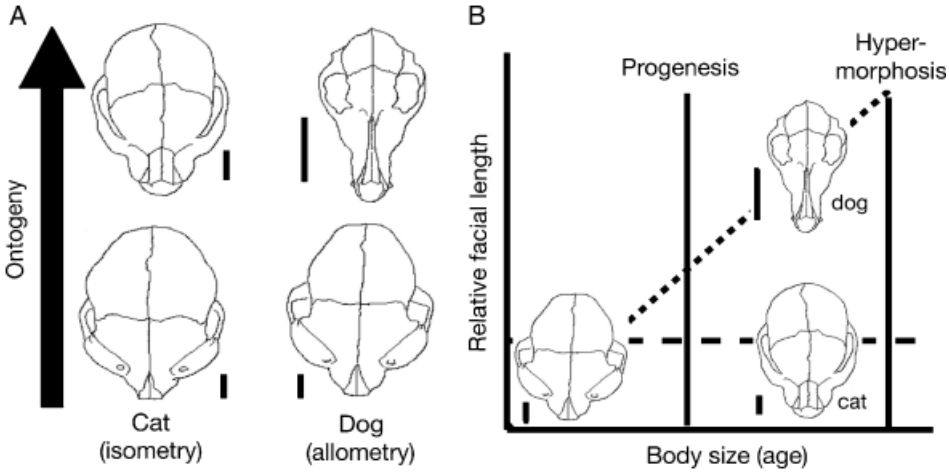

Fig. 3. *Runx2* is a candidate for regulating heterochronic changes in facial morphology. (A) Feliform (cat) face length (on the left) remains virtually constant relative to skull size during ontogeny (isometric growth), in contrast to caniform (dog) facial length (on the right) that greatly increases (allometric growth). All skulls are drawn in dorsal view and to the same length (adapted from Wayne 1986). (B) The highly allometric facial growth of dogs (line of shorter dashes) provides more raw material for natural selection to act upon via changes to developmental timing than does the nearly isometric facial growth of cats (line of longer dashes). For example, the evolutionary downregulation of *Runx2* (via a decrease in its tandem repeat ratio) would potentially result in the earlier truncation of facial bone development (progenesis) in the descendent species relative to the ancestor. In caniforms, this would result in a relatively shorter face, whereas in feliforms, relative face length would remain virtually unchanged. If *Runx2* is upregulated (via an increase in its tandem repeat ratio), facial bone development would potentially be extended (hypermorphosis), which would result in a relatively longer face in a caniform descendent, but a face of similar length in a feliform descendent.

consistent with the hypothesis that the *Runx2* tandem repeat system generates diversity in facial morphology at least in part by controlling the timing of skeletal development.

A caveat of this study is that we extrapolate from the baseline allometric data provided by domestic cats and dogs to feliforms and caniforms, respectively, because of the lack of information about facial growth in other carnivoran species. Although facial growth of domestic cats is nearly isometric, facial growth in some feliform species is undoubtedly more allometric. Furthermore, facial growth in nondomestic caniforms could also be more (or less) allometric than that observed in domestic dogs. As more information concerning facial growth in other taxa becomes available, it would be worthwhile to revisit these comparisons at a finer taxonomic scale.

DISCUSSION

Protein-coding sequence changes have been implicated in the evolution of several aspects of morphology (e.g., overall coat/skin color and major body plans). However, determining the general circumstances under which these changes can affect the evolution of morphology remains of interest to evolutionary developmental biologists. To assess the ability of protein-coding changes to affect subtle, inter-specific morphologic evolution, we tested the hypothesis that mutations within the protein-coding region of *Runx2*, specifically within the QA tandem repeat domain, play a role in the facial evolution of a group evolving under natural selection, the mammalian order Carnivora.

We found that higher ratios of polyglutamines to polyalanines within the *Runx2* tandem repeat region are significantly correlated with increased *Runx2* transcriptional activity toward one of its downstream target genes in a cell-based reporter assay. Our study represents the first time this relationship has been established for *Runx2*. Our findings also are consistent with previous studies that have found links between tandem repeats and transcriptional activity of other transcription factors (e.g., Galant and Carroll 2002).

We also demonstrate a significant and positive, albeit weak, interspecific correlation between *Runx2* tandem repeat ratio and facial length among a wide array of naturally evolving carnivoran species, a result consistent with that observed within the single species composed of domesticated dog breeds (Fondon and Garner 2004). Taken together, the correlations between *Runx2* QA tandem repeat ratio and both in vitro transcriptional activity and facial length, within the Carnivora, provide substantial support for the hypothesis (Fondon and Garner 2004) that *Runx2* protein-coding mutations can provide a genetic mechanism for morphologic evolution in groups under natural selection. Whereas we agree that such mutations will undoubtedly have pleiotropic effects (i.e., Kirschner and Gerhart 1998; Carroll 2005), our results suggest that in at least some instances such effects might not be entirely deleterious, and therefore prohibited by natural selection. Furthermore, it is possible that other changes (e.g., differential regulation) elsewhere in the *Runx2* pathway might compensate for these *Runx2* protein-coding mutations during the development of other aspects of the skeletal system, thereby modulating the resulting pleiotropic effects.

The correlation we observe between *Runx2* tandem repeat ratio and facial length, across a spectrum of species that have evolved over more than 40 million years, is all the more remarkable because of the great amount of intra-specific polymorphism and rapid evolutionary change often observed within tandem repeats (Wren et al. 2000; Fondon and Garner 2004; King et al. 2006), which would be expected to weaken such correlations. Our result suggests that *Runx2* may be under selective pressure to maintain its approximate tandem repeat ratio once established within a particular species (i.e., to maintain an optimal facial length relative to other craniodental bones). We were only able to analyze tissues from one individual per species, and thus we cannot address the potential effects of this intraspecific polymorphism. However, we predict that variation in facial length also should be directly correlated with variation in tandem repeat ratios *within* individual species. This expectation should be tested in the future by densely sampling individual species and examining the correlation between variation in tandem repeat ratio and face length.

The complexity of craniofacial development also potentially weakens the correlation between facial length and tandem repeat ratio. As an indicator of this complexity, craniofacial malformations occur in one-third of all human congenital maladies (Thorogood 1997; Gorlin et al. 2001). Consequently, there are many genes in addition to *Runx2* (e.g., *Bmp*'s, *Fgf's*, *Col10*, *Ihh*, *Pthrp*, etc.) that control growth and modeling of the facial skeleton (Depew et al. 2002). Undoubtedly, at least some of these other genes also play a role in the evolution of carnivoran facial length, which reduces the power of changes solely within *Runx2* to control evolutionary changes in facial length. To fully appreciate the relative role of mutations within the *Runx2* tandem repeat region to the evolution of carnivoran facial length, the contributions of changes within all of these genes to potential evolutionary changes in facial length should be evaluated in the future at both interspecific (naturally evolving groups) and intraspecific (populations and/or artificial breeds) levels. Furthermore, it is important to note that other mutations in *Runx2* or in genes upstream or downstream may have greater effects on *Runx2* transcription levels than does tandem repeat ratio. If any of these mutations have close associations with specific tandem repeat ratios, it is possible that they, and not the tandem repeat ratios themselves, could be driving the correlations between tandem repeat ratio and facial length that we observe.

Thus far, we have demonstrated that as *Runx2* tandem repeat ratio increases, so does the transcription of downstream targets of *Runx2*. Furthermore, *Runx2* is known to be an important regulator of the rate and timing of bone development, with its upregulation leading to the acceleration and extension of bone development, and its downregulation to the deceleration and truncation of bone development (Fig. 1) (Komori et al. 1997; Inada et al. 1999; Ueta et al. 2001; Schroeder et al. 2005). Therefore, we can predict that as *Runx2* tandem repeat ratio increases, the rate and duration of the development of bones (e.g., those comprising the face) are increased, and as tandem repeat ratio decreases, the rate and duration of bone development are decreased. Increasing and decreasing the rate and duration of growth are known evolutionary mechanisms (acceleration and neoteny for rate, and hypermorphosis and progenesis for duration, respectively) for generating heterochronic changes in adult morphology (Klingenberg 1998). Therefore, at least in theory, the *Runx2* tandem repeat system provides a reasonable genetic mechanism for driving aspects of evolution in facial morphology among carnivoran species by mediating changes in the rate and timing of skeletal development. Heterochronic changes in developmental timing have the greatest potential to drive morphologic evolution in structures exhibiting highly allometric growth (Fig. 3B) (Klingenberg 1998; Sears 2004), and domestic dog facial growth is much more highly allometric than that of domestic cats (Fig. 3A) (Wayne 1986).

Consistent with our hypothesis, we find that canids and other caniforms (vs. felids and other feliforms) exhibit greater inter-specific variation in both facial length, the product of selection, and in tandem repeat ratio, the hypothesized mechanism for selection via changes in regulation of *Runx2* activity. These results are consistent with *Runx2* tandem repeat ratio playing a role in facial evolution through heterochronic changes in developmental timing. Furthermore, facial length and tandem repeat ratio are more strongly correlated within caniforms than within feliforms. This finding is also consistent with our hypothesis, given that changes to the *Runx2* tandem repeat ratio are expected to have less of an affect on the generation of facial disparity in feliforms than caniforms as a result of at least felid feliforms exhibiting less ontogenetic variation upon which changes in developmental timing can act. Although we have examined developmental timing, changes in the transcriptional activity of *Runx2* also have the potential to influence developmental rate by mediating chondrocyte proliferation. As a result, we suggest that *Runx2* may be an important (but certainly not the only) regulator of facial heterochrony within carnivorans. In the future, this working hypothesis can be further tested as facial growth data become available for additional carnivoran species and by the creation of transgenic animals misexpresing *Runx2* with varying tandem repeat ratios. Our results suggest that *Runx2*, via changes to its tandem repeat ratio, may play a role in not only the evolution of facial length within carnivorans as a whole but also the evolutionary differences in overall facial disparity across Carnivora and between the species of the two main subgroups, the feliforms and caniforms.

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APPENDIX A

Table A1. Taxa used in study with *Runx2* tandem repeat ratios ($n = 30$)

Group	Family	Taxon/species	Common Name	Tandem. repeat ratio	Facial length (mm)	Facial length correlation by B	Voucher	Source
Caniformia								
	Ailuridae	*Ailurus fulgens*	Red (lesser) panda	3.00	23.35	0.229	NZP 83-671	National Zoological Park, Washington, DC
	Canidae	*Urocyon cinereoargenteus*	Grey fox	3.33	35.20	0.351	PL 2071	Prep lab, FMNH
		Vulpes vulpes	Red fox	5.00	39.38	0.380	PL 2023	Prep lab, FMNH

Table A1. (Contd.)

Group	Family	Taxon/species	Common Name	Tandem. repeat ratio	Facial length (mm)	Facial length correlation by B	Voucher	Source
	Mustelidae	*Martes pennanti*	Fisher	2.50	23.59	0.249	PL 4273	Prep lab, FMNH
		Taxidea taxus	American badger	4.33	28.25	0.264	PL 2542	Prep lab, FMNH
	Procyonidae	*Potos flavus*	Kinkajou	1.50	14.42	0.171	AUD CAL-1	Audubon Zoo, New Orleans
		Procyon lotor	Common raccoon	3.33	28.11	0.282	PL 2093	Prep lab, FMNH
	Ursidae	*Ailuropoda melanoleuca*	Giant panda	2.50	49.21	0.218	NZP 92-765	National Zoological Park, Washington, DC
		Melursus ursinus	Sloth bear	2.50	70.42	0.279	BZ 880109	Brookfield Zoo
		Tremarctos ornatus	Spectacled bear	3.33	49.31	0.256	PL 954	Prep lab, FMNH
		Ursus arctos	Brown bear	2.50	70.17	0.243	BZ "Doo"	Brookfield Zoo
Feliformia								
	Eupleridae	*Galidia elegans*	Ring-tailed mongoose	4.25	13.43	0.226	SMG 6499	S. Goodman, FMNH
	Felidae	*Acinonyx jubatus*	Cheetah	3.33	34.57	0.251	LSUMZ 688	Louisiana State University, Mus. of Zool.
		Felis concolor	Puma	4.00	35.00	0.219	PL 2131	Prep lab, FMNH
		Felis silvestris	Wildcat	3.25	16.99	0.205	SDZ 32735	San Diego Zoo
		Herpailurus yaguarondi	Jaguarondi	2.50	14.56	0.169	SDZ 33373	San Diego Zoo
		Lynx rufus	Bobcat	2.50	21.47	0.210	PL 2025	Prep lab, FMNH
		Neofelis nebulosa	Clouded leopard	3.75	29.85	0.234	LSUMZ 083	Louisiana State University, Mus. of Zool.
		Panthera onca	Jaguar	5.33	53.38	0.265	SZ 100266	Sacramento Zoo
		Panthera pardus	Leopard	3.00	46.80	0.259	PL 1007	Prep lab, FMNH
		Panthera tigris	Tiger	3.33	71.23	0.267	PL 942	Prep lab, FMNH
		Panthera uncia	Snow leopard	2.50	39.02	0.258	PL 2087	Prep lab, FMNH
	Herpestidae	*Cynictis pencillata*	Yellow mongoose	2.50	14.97	0.267	SDZ 30731	San Diego Zoo
		Galerella sanguinea	Slender mongoose	2.75	13.05	0.233	NMK "Karen"	National Museum of Kenya
		Helogale parvula	Dwarf mongoose	2.25	11.09	0.252	SDZ 33352	San Diego Zoo
		Herpestes auropunctatus	Java mongoose	2.25	13.35	0.241	Nellis	Prep lab, FMNH
	Viverridae	*Arctictis binturong*	Binturong	2.50	32.60	0.267	SDZ 32184	San Diego Zoo
		Civettictis civetta	African civet	4.25	40.06	0.311	SDZ 31206	San Diego Zoo
		Nandinia binotata	African palm civet	3.33	24.05	0.279	JCK 2623	J. Kerbis, FMNH
		Paradoxurus hermaphroditus	Common palm civet	3.67	23.42	0.270	LRH 3167	L. Heaney, FMNH

Family-level classification based on Wilson and Reeder (2005). FMNH, Field Museum of Natural History, Chicago (IL, USA).

SUPPLEMENTARY MATERIAL

The following supplementary material is available for this article online:

Fig. S1. Carnivoran phylogeny (adapted from Flynn et al. 2005). Groups included in this study are indicated with an asterisk.

This material is available as part of the online article from: http://www.blackwell-synergy.com/doi/abs/10.1111/j.1525-142X.2007.00196.x (This link will take you to the article abstract).

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