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Convergent Evolution of Head Crests in Two Domesticated Columbids Is Associated with Different Missense Mutations in *EphB2*

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Abstract

Head crests are important display structures in wild bird species and are also common in domesticated lineages. Many breeds of domestic rock pigeon (*Columba livia*) have crests of reversed occipital feathers, and this recessive trait is associated with a nonsynonymous coding mutation in the intracellular kinase domain of EphB2 (Ephrin receptor B2). The domestic ringneck dove (*Streptopelia risoria*) also has a recessive crested morph with reversed occipital feathers, and interspecific crosses between crested doves and pigeons produce crested offspring, suggesting a similar genetic basis for this trait in both species. We therefore investigated *EphB2* as a candidate for the head crest phenotype of ringneck doves and identified a nonsynonymous coding mutation in the intracellular kinase domain that is significantly associated with the crested morph. This mutation is over 100 amino acid positions away from the crest mutation found in rock pigeons, yet both mutations are predicted to negatively affect the function of ATP-binding pocket. Furthermore, bacterial toxicity assays suggest that "crest" mutations in both species severely impact kinase activity. We conclude that head crests are associated with different mutations in the same functional domain of the same gene in two different columbid species, thereby representing striking evolutionary convergence in morphology and molecules.

Key words: **avian, evolution, convergence, EphB2, head crest, feathers.**

Introduction

In the domestic rock pigeon, centuries of selective breeding have resulted in dramatic variation in morphological and behavioral traits. Several morphological variants are modifications of plumage traits, including the size, placement, orientation, and color of feathers (Levi 1965, 1986). For example, the head crest of domestic pigeons is a recessive phenotype characterized by reversed polarity of the neck and occipital feathers, causing the feathers to grow in a cranial rather than caudal direction (fig. 1). Previously, we identified a single amino acid substitution (Arg758Cys) in the active site of the kinase domain of Ephrin receptor B2 (EphB2) that is perfectly associated with head crests in pigeons (Shapiro et al. 2013). *EphB2* is weakly expressed in the mesenchyme of feather primordia and its precise role in feather development is not well understood (Suksaweang et al. 2012; Shapiro et al. 2013); however, other Eph family members are involved in tissue patterning, placode boundary stabilization, and morphogenesis affecting the feather cytoskeleton (Xu et al. 2000; McKinnell et al. 2004; Suksaweang et al. 2012). The same substitution was homozygous in pigeons with a wide variety of crest phenotypes, from simple peaks to elaborate hoods, demonstrating that inheritance of two copies of this variant is necessary for crest development. Furthermore, we found the same *EphB2* crest haplotype among genetically dissimilar breeds, suggesting that this trait spread by repeated introgression of the same mutant allele. Additional, unidentified modifier genes probably control the extent of crest formation by expanding the domain of reversed feather polarity and modulating feather size (Hollander 1937; Sell 1994, 2012).

Head crests have evolved in other avian species as well, including other columbids (pigeons and doves), and can be important structures in sexual selection (Price 2008; Baptista et al. 2009). In light of our finding that a variant allele of *EphB2* is associated with head crests in rock pigeons (Shapiro et al. 2013), we investigated whether similar crest structures in another species have a similar genetic basis. The ringneck dove, *Streptopelia risoria*, is a domesticated species that shared a common ancestor with the rock pigeon 23–35 Ma (Goodwin 1983; Pereira et al. 2007). Some domestic ringneck doves have head crests that are morphologically similar to the simple peak crests of rock pigeons and are also inherited as a recessive phenotype (fig. 1).

Rock pigeons and ringneck doves can be crossed to produce viable (but not fertile) offspring. This ability to generate hybrid offspring and the recessive inheritance of crests in both species makes possible intergeneric complementation tests between crested rock pigeons and crested ringneck doves. Notably, interspecies complementation crosses have offered a fruitful starting point to test for a common genetic basis for derived traits in other studies of evolutionary convergence (Sucena and Stern 2000; Parichy and Johnson 2001; Shapiro et al. 2006). Similarly, Miller and Demro (2011) crossed crested morphs of a rock pigeon and a ringneck dove and found that hybrid offsprings are also crested, suggesting that "crest" mutations inherited from each species were not complemented.

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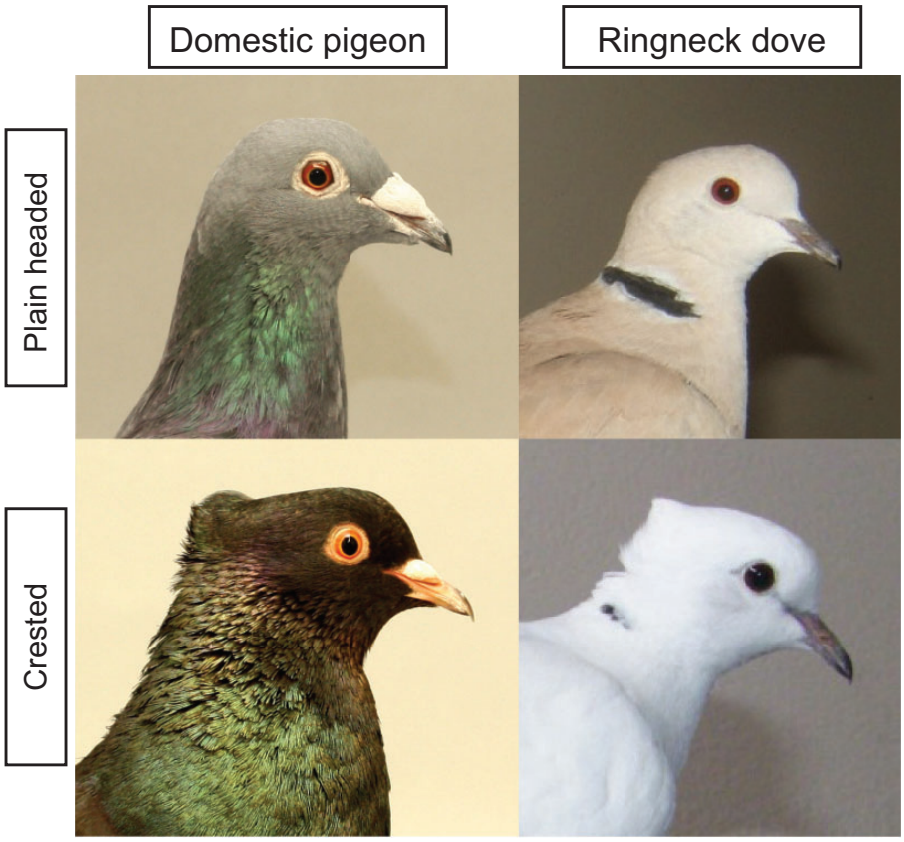

Fig. 1. Similar crested morphs occur in domestic rock pigeons (*Columba livia*) and ringneck doves (*Streptopelia risoria*). Top panels: wild-type plain-headed morphs of a domestic rock pigeon (racing homer breed, left) and a ringneck dove (right). Lower panels: peak-crested morphs of a domestic rock pigeon (archangel breed, left) and a ringneck dove (right). Peak crests in both species are inherited as recessive phenotypes. Photos courtesy of Sydney Stringham (pigeons) and Klaudia Kis (ringneck doves).

This result provided genetic evidence that the same gene might mediate crest development in both species and motivated us to directly examine variation in *EphB2* in the ringneck dove.

Results

A Gly636Arg Mutation in EphB2 Is Associated with Head Crests in Ringneck Doves

The crest mutation in *EphB2* of rock pigeons (cr^{pigeon}) occurs in the catalytic pocket of the intracellular kinase domain (Shapiro et al. 2013), and based on studies of other receptor tyrosine kinases, this amino acid substitution is predicted to abrogate kinase activity (Elder et al. 2001). To test the hypothesis that a similar mutation is associated with crests in ringneck doves, we sequenced the kinase domain-encoding regions of *EphB2* from crested and uncrested (wild-type [WT]) morphs. We identified a G to A mutation in the sixth exon of *EphB2* of crested birds (cr^{dove}); this change is predicted to result in a glycine to arginine amino acid substitution at position 636 of the protein (Gly636Arg) (fig. 2*A and* supplementary fig. S1, Supplementary Material online). The mutated amino acid residue is otherwise conserved in all Eph family members and other protein–tyrosine kinases (supplementary fig. S2, Supplementary Material online), suggesting that this could be a functionally important site (Hanks et al. 1988; Hanks and Hunter 1995). As expected, the previously reported pigeon–dove F_1 hybrids (Miller and Demro 2011) were heterozygous for the cr^{pigeon} and cr^{dove} mutations (fig. 2*A*). Their crested ringneck dove parent was homozygous for the cr^{dove} mutation, and this allele has never been detected in any rock pigeon sequenced to date (Shapiro et al. 2013).

We genotyped an additional 50 crested and 75 uncrested ringneck doves sampled from multiple breeder lofts in the United States and Hungary (supplementary table S1, Supplementary Material online) and found that the G to A mutation was nearly perfectly associated with the head crest phenotype under a recessive model (chi-square test, $P < 0.005$; fig. 2*B*). Every crested dove was homozygous for the cr^{dove} allele, and heterozygotes were plain headed. One dove was homozygous for cr^{dove} but was identified as uncrested by its owner. This may have resulted from sampling error, either in feather collection or phenotype reporting, or incomplete penetrance of the crest phenotype. Notably, hobbyists report the latter scenario in domestic pigeon breeds that are otherwise monomorphic for simple peak crests that resemble the ringneck dove crest. For example, two peak-crested Indian fantail parents will occasionally produce uncrested offspring (Kvidera T, Kral L, Skiles D, personal communication).

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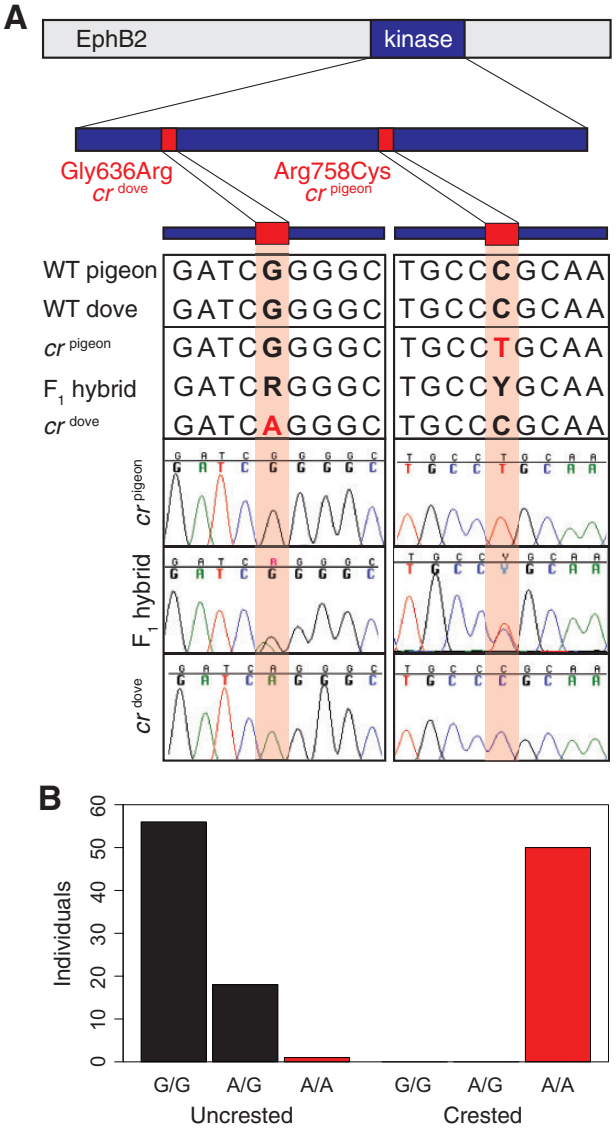

Fig. 2. A mutant allele of *EphB2* is associated with head crests in ringneck doves. (*A*) Schematic representation of the *EphB2* coding sequence. Two mutations, Arg758Cys and Gly636Arg, are associated with head crests in domestic pigeons and ringneck doves, respectively. Sequence alignments and chromatograms are shown for wild-type alleles from both species, as well as crested F_1 hybrids from a complementation cross (Miller and Demro 2011). Crested hybrids are heterozygous for both parental alleles. (*B*) Genotypes of 125 ringneck doves at the cr^{dove} locus are significantly associated with the head crest phenotype under a recessive model (chi-square test, $P < 0.005$).

EphB2 Crest Mutations Localize to the ATP-Binding Site

In an effort to understand the biochemical impact of crest mutations on EphB2 function, we located the sites of the predicted amino acid substitutions in the context of the molecular structure for the EphB2 kinase domain from mouse (Protein Data Bank [PDB] id 1jpa; Wybenga-Groot et al. 2001). Notably, both EphB2 crest mutations replace conserved residues located on the surface of the kinase domain and are positioned very close to the site of ATP binding (fig. 3*A* and *B*). Models constructed with the Arg758Cys allele found in crested rock pigeons (cr^{pigeon}) predicted that a salt bridge between Arg758 and Asp754 would be destroyed (fig. 3*C*). Because Asp754 is known to be essential for ATP catalysis (Hanks and Hunter 1995), this perturbation probably impacts protein function (discussed further below). Likewise, models constructed with the Gly636Arg allele (fig. 3*D*) found in crested ringneck doves (cr^{dove}) predicted that entry and binding of ATP and exit of adenosine diphosphate (ADP) would likely become hindered by the large arginine side chain. In this case, the ATP-binding site, normally a tight fit with glycine lining the rim of the entrance in the WT allele (Carrera et al. 1993; Hemmer et al. 1997), would be blocked. Thus, both the cr^{pigeon} and cr^{dove} alleles of *EphB2* are predicted to have deleterious effects on the function of the catalytic domains they encode.

Bacterial Toxicity Assays Suggest Crest Alleles Have Reduced Kinase Activity

Catalytically active EphB2 is toxic to bacteria (Wiesner et al. 2006), indicating that it disrupts fundamental cellular processes in bacteria. Based on our structural predictions of mutant EphB2 alleles from rock pigeons and ringneck doves (fig. 3), and the fact that dramatic amino acid substitutions in the catalytically active domains of receptor tyrosine kinases can abrogate activity (Wiest et al. 1997; Elder et al. 2001), we hypothesized that crest mutations would yield proteins with inactive (or substantially underperforming) kinase domains. A predicted consequence of this functional change is that mutant kinase domains would no longer be toxic to bacteria. Accordingly, we performed a bacterial toxicity assay to determine the functional impact of the crest alleles from both species (fig. 4). Isopropylthio-β-galactoside (IPTG)-inducible plasmid constructs were assembled containing wild-type (identical amino acid sequence for pigeon and dove), cr^{pigeon}, and cr^{dove} kinase domains. We assembled two additional constructs of the kinase domain of another Eph family member, *EphA4*, containing either a wild-type or mutagenized form with the Arg758Cys substitution found in crested rock pigeons. When kinase expression was induced, transformed bacterial cells containing wild-type *EphB2* and *EphA4* constructs did not form colonies, suggesting that the catalytically active kinase domains were cytotoxic. In contrast, all transformants containing mutant cr alleles formed colonies in the presence of IPTG, suggesting that the dove and pigeon mutations reduce or eliminate kinase activity (fig. 4*B*).

Discussion

Molecular genetic studies in a variety of organisms highlight the repeatability of phenotypic change by changes in the same genes (Gompel and Prud'homme 2009; Christin et al. 2010; Rosenblum et al. 2014). Among vertebrates, this trend is especially pronounced in studies of pigmentation diversity (despite probable discovery biases; Hoekstra and Coyne 2007; Manceau et al. 2010; Guernsey et al. 2013), but also extends to numerous other traits that result from repeated regulatory or coding mutations in the same genes (Sucena et al. 2003; Arnaud et al. 2011; Dobler et al. 2012; Liu et al.

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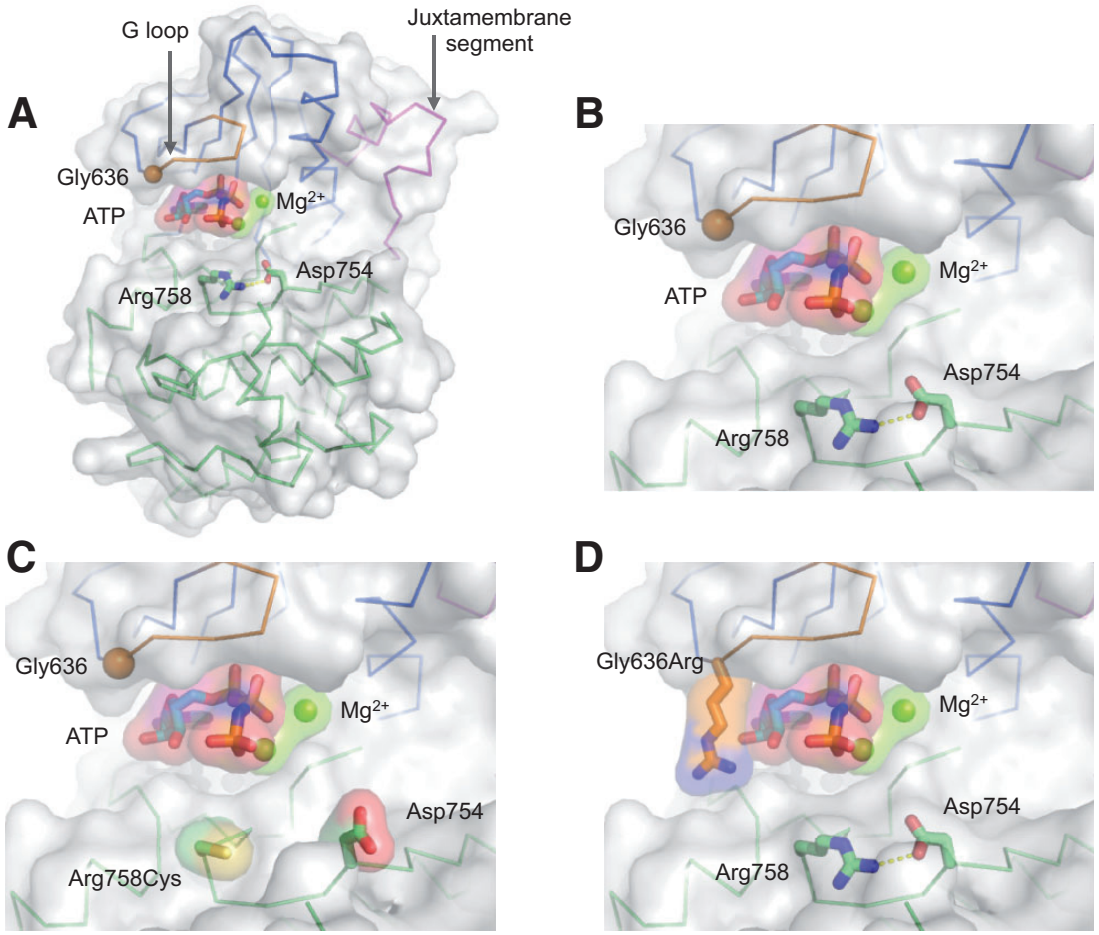

Fig. 3. Structural models predict alteration of the same catalytic domain in cr^{pigeon} and cr^{dove} alleles of EphB2. (*A*) Overview of the structure of EphB2 kinase domain (PDB id 1jpa). The small green spheres represent Mg^{2+} ions that coordinate with the phosphate groups of ATP, which have been included here by superposition of coordinates derived from a complex of the insulin receptor kinase domain and the nonhydrolyzable ATP analog ADPNP (PDB id 1ir3). Amino acid positions associated with the crest phenotype in both species are highlighted. (*B*) Detailed representation of the wild-type EphB2 kinase domain ATP-binding pocket. (*C*) Structural prediction of the cr^{pigeon} allele. Arg758 forms a salt bridge with catalytic residue Asp754 in the wild-type protein. However, Cys replaces Arg758 in cr^{pigeon}. Without the salt bridge, Asp754 would experience greater freedom and likely adopt several conformations, one of which is shown. Thus, the Arg758Cys substitution is predicted to result in severely diminished catalytic activity. (*D*) Structural consequence for the cr^{dove} allele. The Gly636Arg substitution swaps in a very large residue that would probably hinder binding of ATP and release of ADP product. Images created with PyMOL Molecular Graphics System (Schrödinger, LLC).

2012; Linnen et al. 2013; Liu, Qi, et al. 2014; Liu, Wang, et al. 2014). One dramatic example of this repeatability is the evolution of voltage-gated sodium channels (Na_v) in garter snakes, in which different amino acid substitutions confer varying resistance to toxic newt prey (Geffeney et al. 2002, 2005; Feldman et al. 2009, 2012). Remarkably, mutations in these proteins are largely limited to just a few amino acid sites, and identical substitutions have evolved in at least three paralogs in the Na_v gene family (McGlothlin et al. 2014; also see Rosenblum et al. 2014 for an extended discussion). Likewise, independent mutations at the same *Pitx1* enhancer site underlie pelvic reduction in different populations of threespine sticklebacks (Shapiro et al. 2004; Chan et al. 2010), and this same gene probably mediates the same phenotype in certain populations of the related ninespine stickleback as well (Shapiro et al. 2006; Shikano et al. 2013). Thus, similar selection pressures have had strikingly similar molecular outcomes in independent populations within and among species, as well as among paralogous genes with similar functions (McGlothlin et al. 2014; Rosenblum et al. 2014). These and other examples also highlight another emerging pattern: Not only are the same genes involved in convergent evolution among populations and species, but so are specific molecular regions. This pattern suggests constraints on the types of changes that can persist without compromising organismal viability, evolutionary paths of least resistance due to functional considerations, or perhaps properties of the genome itself, such as nucleotide sequences that are especially prone to mutation (Gompel and Prud'homme 2009; Chan et al. 2010; Rosenblum et al. 2014). A practical consequence of this pattern, predicted long before the arrival of molecular genetic tools (Haldane 1932), is that genetic studies in one population or species can nominate reasonable candidate genes for studies of similar phenotypes in another.

We previously identified an amino acid substitution in the intracellular kinase domain of EphB2 that was perfectly

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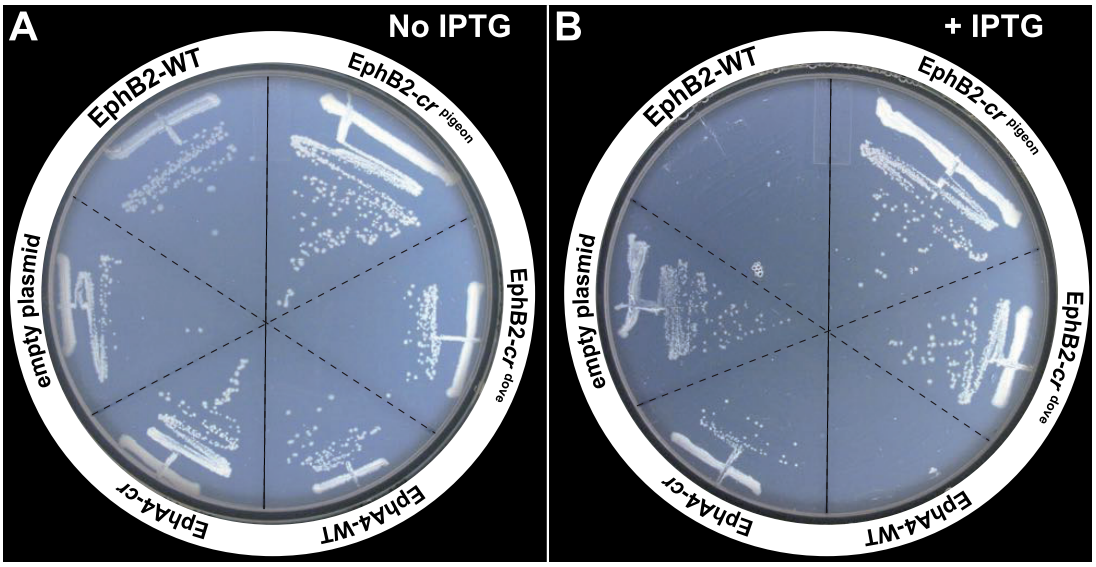

Fig. 4. Bacterial growth assay implicates reduced kinase activity of EphB2 crest alleles. DH5α cells transfected with pGex plasmids containing kinase domain inserts were grown on noninducing (LB media, *A*) or inducing media (LB-IPTG, *B*). Constructs contained (clockwise from upper left sector of each plate) the following: EphB2–WT, EphB2 wild-type kinase domain (identical amino acid sequence in domestic rock pigeons and ringneck doves); EphB2–cr^{pigeon}, EphB2 kinase domain with cr^{pigeon} mutation; EphB2–cr^{dove}, EphB2 kinase domain with cr^{dove} mutation; EphA4–WT, EphA4 wild-type kinase domain; EphA4–*cr*, pigeon EphA4 kinase domain with cr^{pigeon} mutation in orthologous position (Arg758Cys); empty pGex plasmid. EphB2 active kinase is toxic to bacteria (Wiesner et al. 2006). Absence of colonies on the inducing IPTG plate suggests toxicity and therefore an active kinase domain insert; presence of colonies suggests nontoxicity and severely reduced or absent kinase function.

associated with head crests in domestic pigeons, and we leveraged this candidate to find a different mutation in the same domain of the same gene that is associated with the same phenotype in a different species, the ringneck dove. Mutations in these two species are over a hundred amino acids apart in the primary sequence, yet in the folded protein these residues are in close proximity to each other, lining the entrance to the ATP-binding pocket of the kinase domain (fig. 3). Modeling of the structural consequences for each mutation suggests that the overall structure of the kinase domain will remain intact but that catalysis and substrate binding will be impacted. The rock pigeon allele, Arg758Cys, likely impacts catalytic efficiency because it is expected to disrupt a salt bridge found between the guanidinium group of Arg758 and the carboxylate of Asp754, a residue that is critical for catalysis (Hanks and Hunter 1995). In contrast, the ringneck dove mutation, Gly636Arg, probably occludes access to the ATP-binding pocket thereby hindering binding of ATP or release of ADP. In summary, based on modeling of EphB2 kinase domains, the kinase activities of mutant proteins in both crested rock pigeons and crested ringneck doves are predicted to be severely reduced.

Although detailed comparative anatomical studies have not been conducted on crested and uncrested pigeons or doves, birds with head crests appear to develop, behave, and fly normally. In contrast, inactivation of *EphB2* in the mouse leads to numerous developmental defects, including severe neurological and cell proliferation deficits (Henkemeyer et al. 1996; Orioli et al. 1996; Holmberg et al. 2006; Risley et al. 2009). The comparatively innocuous phenotypes observed in crested pigeons and doves could be due to functionally separable domains of the EphB2 protein. Mutations within a single modular and functionally separable domain of a protein could avoid deleterious pleiotropic effects, as the other domains remain unaffected (Lynch and Wagner 2008). For example, as noted above, both the pigeon and dove crest mutations affect only the catalytic site of the intracellular kinase domain. The cytosolic portion is otherwise predicted to be intact in both mutants and may be an essential binding member in an intracellular protein complex. As a result, other members of the complex may retain productive protein–protein interactions with EphB2. Furthermore, signaling between Eph receptors and membrane-bound ephrin ligands is bidirectional, and mutations in the intracellular kinase domain of EphB2 do not necessarily reduce or abrogate "reverse signaling" to ligand-presenting cells (Henkemeyer et al. 1996; Holland et al. 1997).

How often might the same gene be implicated in crest variation in other species, including wild birds? Mechanisms underlying crest development could be constrained by the number of genes that can potentially be altered without deleterious pleiotropic effects, or produce a magnitude of phenotypic effect that is visible to natural or artificial selection (Shapiro et al. 2006; Rosenblum et al. 2014). If mutations in the EphB2 kinase domain do not negatively impact other traits, we might find that this mechanism is involved repeatedly in the evolution of altered feather growth polarity. Ultimately, we might also observe a bias for *EphB2* mutations specifically among columbids due to their shared phylogenetic history. For example, columbids might share a developmental program that patterns the head epidermis in similar ways across different species, thereby sensitizing specific

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feather tracts to variation in *EphB2* (Mou et al. 2011; Shapiro et al. 2013). If, as this and other studies suggest, trait evolution is biased toward particular genes and pathways, a thorough understanding of the molecular basis of diversity in pigeons can yield gene candidates to investigate in other birds and vertebrates, many of which are not genetically tractable. This approach is especially relevant with the recent proliferation of vertebrate genome sequences, including many avian species (Jarvis et al. 2014), most of which are not currently amenable to experimental genetics and developmental biology.

Experimental Procedures

Sample Collection

We contacted ringneck dove breeders in the United States and abroad through e-mail or through an announcement published in the March 2013 issue of *Doveline* magazine, the quarterly newsletter of the American Dove Association. Breeders interested in contributing samples were sent feather collection packets and instructions. Feathers were mailed to the University of Utah in a return packet, along with detailed phenotypic information for each bird (determined by the breeder). Breeders were specifically instructed to submit samples from birds that were unrelated by grandparent. For samples that had a known familial relationship, breeders submitted pedigree information so that we could avoid pseudoreplication of genetic sampling within families (supplementary table S1, Supplementary Material online). Importation of international samples was approved under USDA APHIS permit 120863 to M.D.S.

DNA Extraction

DNA from 128 feather samples was extracted as previously described (Stringham et al. 2012). The concentration of each sample was measured using a Nanodrop 2000 (Thermo Scientific, Waltham) and diluted to 10 ng/μl for polymerase chain reaction (PCR) and TaqMan genotyping assays.

Exon Sequencing

Five exons that encode the *EphB2* kinase domain (exons 6–10) were amplified by PCR using primers listed in supplementary table S2, Supplementary Material online. Primer pairs were designed from the *Columba livia* reference genome sequence and annotation (Shapiro et al. 2013). PCR products were purified with either QIAquick PCR purification or gel extraction kits (Qiagen), and then Sanger sequenced at the University of Utah Sequencing Core facility.

Sequence Alignment

Sequences for each exon were edited for quality with Sequencher v.5.1 (GeneCodes, Ann Arbor, MI) and translated with SIXFRAME in SDSC Biology Workbench (http://workbench.sdsc.edu, last accessed May 5, 2015). Peptide sequences were then aligned to other EphB2 amino acid sequences downloaded from ensemble (http://www.ensembl.org, last accessed March 6, 2015) using CLUSTALW (Thompson et al. 1994) in SDSC Biology Workbench.

TaqMan Assay

Ringneck doves were genotyped for the cr^{dove} SNP using a TaqMan genotyping assay (Applied Biosystems, Foster City, CA) as described previously (Shapiro et al. 2013). Primers used to amplify the target and reporter sequences are listed in supplementary table S2, Supplementary Material online.

Protein Models

The position of each crest allele was mapped onto the structure of the mouse EphB2 kinase domain (PDB id 1jpa) by aligning amino acid sequences for mouse, human, and several bird homologs (99% sequence identity). Amino acid substitutions were modeled with the Mutate function of Coot (Emsley and Cowtan 2004; Emsley et al. 2010) and a rotamer was selected from a library of commonly encountered side chain conformations. No further energy minimization was employed because standard rotamers could be found that avoid steric clashes in each case. To obtain a structure approximating the complex with ATP, the structure of AMP-PNP•2(Mg2+) found in the structure of the kinase domain from the human insulin receptor (PDB id 1ir3) was positioned into the ATP-binding pocket of the EphB2 kinase domain by superposition of adenine moieties.

Plasmid Preparation

The nucleotide sequence encoding the kinase domain of EphB2 was PCR amplified from embryonic cDNA isolated from a plain-headed domestic pigeon using primers listed in supplementary table S2, Supplementary Material online. The fragment was cloned into pGex using Gibson assembly (Gibson et al. 2009). Gly636Arg and Arg758Cys alleles of EphB2 corresponding to cr^{dove} and cr^{pigeon}, respectively, were generated by site-directed mutagenesis in *EphB2* using primers listed in supplementary table S2, Supplementary Material online (Quick-Change Mutagenesis Kit; Stratagene, La Jolla, CA). The region of pigeon *EphA4* encoding the kinase domain was PCR amplified and cloned into pGEX by the same procedure and an R to C mutation was generated in the highly conserved DLAARN motif (analogous to Arg758Cys of the pigeon crest allele) by site-directed mutagenesis. All five plasmids were sequence verified using the standard pGex forward primer.

Bacterial Toxicity Assay

DH5α cells were transformed with empty pGex plasmid (control) or pGex containing one of the five kinase domain inserts: EphB2–WT, EphB2–cr^{pigeon}, EphB2–cr^{dove}, EphA4–WT, and EphA4–cr^{pigeon}. Transformed cells were grown overnight on LB plates containing 1 mg/ml carbenicillin (LB-carb). Transformants were then passaged through two additional LB-carb plates, and colonies from the last plate were picked and streaked on either a control LB-carb plate or an LB-carb-IPTG plate (containing 1 mg/ml IPTG) to induce expression of the kinase domain insert. Five replicates for each plate were then grown overnight at 37 °C and imaged to record colony growth.

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Supplementary Material

Supplementary figures S1–S2 and tables S1–S2 are available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org/).

Acknowledgments

Sequences for the ringneck dove ortholog of *EphB2* are deposited in GenBank (accession numbers KP903890–KP903891). We are indebted to Jim Demro, D.D.S., for providing tissue samples of birds from his hybrid cross between rock pigeons and ringneck doves. We also thank Paul Hollander, Denny Stapp, and the American ringneck dove breeder community for providing feather samples; and Klaudia Kis for photos and for her essential help with feather sample collection from Hungarian ringneck dove fanciers. We thank Kelly Hughes, Fabienne Chevance, Dan Wee, Eli Cohen, Corinne Tobler, and Markus Babst for technical assistance and advice. This work was supported by the National Science Foundation (CAREER DEB1149160 to M.D.S., IOS0955517 EDEN internship to A.I.V.), the National Institutes of Health (fellowships F32GM103077 and T32HD07491 to E.T.D.), and a Burroughs Wellcome Career Award in the Biomedical Sciences (1005281.01 to M.D.S.).

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