The Chicken Frizzle Feather Is Due to an \( \alpha \)-Keratin (\( KRT75 \)) Mutation That Causes a Defective Rachis

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\section*{Abstract}

Feathers have complex forms and are an excellent model to study the development and evolution of morphologies. Existing chicken feather mutants are especially useful for identifying genetic determinants of feather formation. This study focused on the gene \( F \), underlying the frizzle feather trait that has a characteristic curled feather rachis and barbs in domestic chickens. Our developmental biology studies identified defects in feather medulla formation, and physical studies revealed that the frizzle feather curls in a stepwise manner. The frizzle gene is transmitted in an autosomal incomplete dominant mode. A whole-genome linkage scan of five pedigrees with 2678 SNPs revealed association of the frizzle locus with a keratin gene-enriched region within the linkage group E22C19W28_E50C23. Sequence analyses of the keratin gene cluster identified a 69 bp in-frame deletion in a conserved region of \( KRT75 \), an \( \alpha \)-keratin gene. Retroviral-mediated expression of the mutated \( F \) cDNA in the wild-type rectrix qualitatively changed the bending of the rachis with some features of frizzle feathers including irregular kinks, severe bending near their distal ends, and substantially higher variations among samples in comparison to normal feathers. These results confirmed \( KRT75 \) as the \( F \) gene. This study demonstrates the potential of our approach for identifying genetic determinants of feather forms.

\section*{Introduction}

Birds have evolved many unique and interesting features, allowing them to adapt and radiate into various ecological niches. They display a great degree of diversity in feathers and other body parts. Domesticated birds exhibit an even greater diversity in phenotypes than their wild ancestors, thus providing an excellent opportunity to explore the genetic basis underlying variation in phenotypes than their wild ancestors, thus providing an excellent opportunity to explore the genetic basis underlying variation in morphology, physiology, and behavior.

As Darwin noted, the domestic chicken displays a remarkable level of phenotypic diversity \cite{1} and it is the most phenotypically variable bird, especially in terms of feather form \cite{2}. However, the genetic and developmental basis of this diversity is unclear. Understanding the genetic basis of plumage variability in the chicken would provide insight into how evolutionary diversification in morphological traits could occur rapidly during adaptive radiations or under strong sexual selection.

The development of a feather has to be coordinated by an enormous number of molecular and cellular machineries \cite{3–17}. The feather is the most complex keratinized structure of the vertebrate integument and has vital importance for physiological and functional requirements. The complex organization of feathers allows for a variety of potential morphological changes to occur. Modifications of the feather include deterioration of feather development, changes in feather structure, inhibition of feather molting, and alterations of feather growth rates \cite{18}.

The structure of feathers includes the rachis (feather backbone), ramus (branches) and barbules (branches off the ramus, which enable them to form an organ capable of moving air to provide flight) (Figure 1A). In the chicken, embryonic downy feathers are...
radially symmetric and fluffy and have a very short rachis or none at all. The branches in downy feathers only include the ramus and barbules (Figure 1A). Most adult chicken feathers are bilaterally symmetric and include a rachis, ramus, and barbules (Figure 1A). The rachis and ramus are composed of two layers: the outer, thin cortex (composed of solidly compacted squamous cells) and the inner, thick medulla (composed of empty polyhedral pith cells) [19]. Various feather types are essential characteristics of domestic chicken breeds. Although the molecular and cellular basis of feather development has been well characterized [3–6], little is known about the genes influencing feather growth, pigment pattern, length, distribution, and structure.

The existence of a reference genome sequence has placed the chicken as an important model organism for understanding genome evolution, population genetics, and the genetic basis of phenotypic traits [20–33]. Owing to the close relationships within Class Aves, the molecular and genetic understanding of phenotypic variations discovered in chickens are likely to be applicable to wild bird species. Therefore, chicken genetic and genomic studies can provide information for studying development and evolution of avian species [2,34–42].

Frizzle feathers have been described in domesticated birds and established as varietal characteristics in domestic chickens [18,43]. The contour feathers of the frizzle chicken all curl outward and upward. Due to an altered feather rachis structure and morphology, they cannot lie flat against the body. Usually, rectrices and remiges are less affected by the mutation but have an irregular appearance. Other prominent modifications, such as thickening of the barbs and barbules, alteration of the hooklets and other structural abnormalities have also been observed [44]. The frizzle mutation has been reported to occur in a single autosomal gene denoted F that shows incomplete dominant inheritance [44,45].

In order to dissect the genetic mechanism underlying frizzle feathers, we conducted a whole genome linkage scan and mapped the causative genetic mutation to the linkage group E22C19W28_E50C23. By analyzing the candidate genes in the associated interval, we found that the F mutation is caused by a deletion in a conserved region of an α-keratin. The causative effect of the KRT75-MT was confirmed by a retrovirus-mediated misexpression of the wild-type or mutated K75 protein in the feather follicle during regeneration in chickens with normal plumage.

Interestingly, mutations in KRT75 have also been identified in mammals, causing structural abnormalities in hair in humans [46,47] and mice [48]. This implies a fundamental role for K75 in building the architecture of skin appendages.

### Results

#### Physical features of the frizzle chicken

The adult frizzle chicken shows a distinct disorientation of feathers (Figure 1B). Upon hatching, the first-generation radially symmetric feathers of frizzle chicks do not show curves (Figure 1B). The frizzle phenotype starts to appear when the first-generation feathers are replaced with second-generation bilateral feathers which have a rachis. At this stage, both body and wing flight feathers twist toward a dorsal orientation.

Normally, feathers are bent along the dorsal to ventral orientation. However, in frizzle chickens, the feathers are bent along the ventral to dorsal orientation (Figure 1C). The pennaceous vanes on both dorsal and ventral sides of frizzle feathers show normal branching when compared to white leghorn controls (Figure 1C). Their rachis backbones are determined by our computer-aided analyses (Figure 1D). The definitions of “+” and “0” are shown in the schematic drawing of a feather in Figure 1E. We define the backbone of the rachis by a curve at equal distance to the two edges of the rachis, because rachis has a width. The accumulated distance from the most proximal end of the rachis along the backbone is defined as “+“. We then, arbitrarily draw a straight line as the coordinate, and define the angle between the local tangent line (the best straight-line approximation to the rachis backbone at that point) and the straight line as “0“. Thus the bending of the rachis is represented by the function $\theta(s)$, which quantitatively reflect the changing curvature of different feathers (Figure 1E). Features of $\theta(s)$ quantitatively reflect some subtle differences beyond a visual inspection of the images. For example, the heterozygous chicken feather show not only kinks along its length but also curves over the entire rachis in comparison to the homoygous chicken whose feathers have dramatically increased curvature and less kinking (Figure 1F). These features may be correlated to the growth of the feather in response to the expression of a different allele.

#### The cellular basis of the frizzle phenotype

Feather sections from the mature, top region show that the rachis of frizzle feathers has a smaller medulla compared to the normal leghorn controls (Figure 2A). The medulla is localized in the inner, ventral region of the rachis and is composed of empty polyhedral pith cells [19]. These observations suggest that the frizzle phenotype is caused by a defect in the ventral part of the rachis.

We examined rachidial morphogenesis at different regeneration time points by plucking body feathers and allowing them to regenerate for 10 days or 30 days. After regeneration, feather follicles were dissected and paraffin sections were prepared. Three different levels of cross-section of a 30-day regenerated sample, from mature (Figure 2D and S1A, level III) to immature (Figure 2D and Figure S1A, level I), were studied by H&E staining (Figure 2B). These results indicate that a clear defect in the ventral region of the rachis may be responsible for the altered medulla formation.
staining showed that the cell proliferating zone in the frizzle rachis in level I is much narrower than that of the controls (Figure 2C, upper panel). The TUNEL staining showed there is no cell death in the PCNA positive cell proliferating zone (Figure 2C, lower panel). Detailed PCNA and TUNEL staining (Figure S1B–S1E), summarized in Figure 2D and Figure S1F, show there are no differences in cell proliferation and programmed cell death between normal and frizzle feathers at level II and level III. Our cell proliferation data suggests that the cell proliferation zone at an immature level (level I) of the frizzle rachis is narrow compared to

Figure 1. The frizzle chicken phenotype. (A) Diagram of normal developing and mature embryonic and adult chicken feathers. (B) Adult, hatchling and 1-month-old frizzle chickens. Adult homozygous frizzle chicken feathers curve away from the body. This is a frizzle in White Plymouth Rock Bantam, it is not exactly the chicken we use but illustrate the phenotype. Note that the downy feathers appear normal in newborn frizzle chicks; however, by the second generation, the feathers in a one month old chick start to show a clear frizzle phenotype. (C) Comparison of body feathers from normal white leghorns and frizzle chickens. Upper panel: dorsal view, ventral view and side view. Lower panel: dorsal view (left) and ventral view (right) of branching in the pennaceous vane; D, dorsal; V, ventral. (D) Comparison of the wildtype, heterozygous and homozygous frizzle feathers. The wildtype feather image is overlaid by the computer-determined backbones of its rachis. (E) Function $\theta(s)$ describing the bending of their rachis, plotted on the length-normalized coordinate. The functions are shifted by arbitrary offsets for clarity. (F) Comparison of the feathers as shown by the qualitative change of the curves of $\theta(s)$. Red arrows highlight the kinked structures along the heterozygous feather.
that found in a normal rachis, perhaps contributing to smaller medulla formation in the frizzle rachis.

Finally, we compared the feathers of homozygous frizzle chickens with white leghorn controls at embryonic day 12. The base of the feather filament appeared normal. However, the tip of each frizzle feather filament appears to be randomly twisted in both the body and wing feathers (Figure 2E, upper two panels). To examine possible differences between frizzle and normal feather branching morphogenesis, we used whole mount in situ hybridization with a probe targeting SHH which is expressed in marginal plate cell [49]. The frizzle feathers showed the same expression of SHH as controls (Figure 2E, lower panel). This suggests that embryonic frizzle feather branching occurred normally even though the tip of frizzle feathers were randomly twisted.

Linkage analysis maps the frizzle trait to the linkage group E22C19W28_E50C23

In order to locate the gene underlying the frizzle trait, a genome scan was conducted on progeny of crosses between the same heterozygous frizzle rooster, PF1, and five different wild-type
The haplotype of SNPs segregating with the frizzle phenotype is delineated by the boxed genotypes. Haplotypes for these latter SNPs and two others from the same chromosome that were represented in the SNP panel used for genotyping are shown. The SNPs, rs16687483 and rs16687610 within the linkage group E22C19W28_E50C23 yielded LOD scores of 7.34 and 6.5, respectively. Haplotype sharing of SNPs between family members identified a shared haplotype extending from rs14689023 to rs16687610 in 21 of 22 frizzle birds. A possible recombination event in the region between rs16687483 and rs16687610 was evident in the frizzle female Y61F (Figure 3).

We isolated RNA from the feather follicles 2-weeks after plucking of normal and F/F chickens and surveyed the expression of KRT75 in the feather follicles. We confirmed that KRT75 is expressed in feather follicles of both normal and F/F chickens (Figure S5). Sequence analysis of the coding sequence of KRT75 cDNA showed that the loss of the authentic splice site at the exon5/intron 5 junction activates a ‘cryptic’ splice site in exon 5 (Figure 4B), resulting in a 69-bp in-frame deletion within the coding region (CDS positions 934–1,002). The cryptic splicing site in exon 5 contains 6-bp (5’-GTTGAAG-3’) that resembled those at the authentic splice site. The mutated K75 thus contains a deletion of 23-amino acids within a conserved region (Figure 4B and Figure S6). The deletion covers the entire part of link L2 and some parts of 23-amino acids within a conserved region (Figure S2). The deletion mutation showed complete segregation with the frizzle phenotype in all the frizzle offspring within the F1 generation of the experimental crosses (Figure S3 and Figure S4).

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Frizzle chickens sampled from different populations in Taiwan with the distinctive homoygos and heterozygous feather phenotypes demonstrated two mutant alleles and a single mutant allele, respectively (Figure 4A). The deletion was not observed in other breeds of normal chickens. Other variants discovered by sequencing genomic DNA from the frizzle chicken were also found in non-frizzle chickens except for one nonsynonymous SNP (Table S2). The effects of variants in other genes were not subjected to functional studies.

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Expression of KRT75 in embryonic and adult feathers

To locate the KRT75 transcripts in embryonic and adult feathers, we generated a KRT75 full-length antisense RNA probe. Section in situ hybridization showed that KRT75 is expressed in barb ridges but restricted to the region destined to become the rachis, at embryonic day 13 (E13) (Figure 5A). In the normal regenerating adult feather, we found that KRT75 was expressed in both the rachis and the rachis (Figure 5B). To ensure the specificity of our KRT75 probe, we made probes from the 3’untranslated region (UTR) which show the same expression pattern as our probe to the coding region (data not shown). The regenerating frizzle feathers show the same pattern of KRT75 expression as those in normal controls (Figure 5C, compared to
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A

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C

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305  Asp  306  Ser  307  Ile  308  Ile  309  Ala  310  Glu  311  Val  312  Lys
| GACAGCATCATCGCCGAGGTGAAG |
313  Ala  314  Glu  315  Tyr  316  Glu  317  Asp  318  Ile  319  Ala  320  Asn
| GCGCAGTATGAGGACATTGCCAAC |
321  Arg  322  Ser  323  Arg  324  Ala  325  Glu  326  Ala  327  Glu  328  Ser
| CGCAGTCGGGGCTGAAGCCGAGTCC |
329  Trp  330  Tyr  331  Glu  332  Thr  333  Lys
| TGGTACCAAAACCAAGgtgaagagagttgaagtctctgtgctgctgtcttcatgtcttgaataagagaagggtctgacttccaggaacaccttctcc |
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The F allele of KRT75 contains a deletion. (A) PCR products amplified from genomic DNA of phototypically normal (WT), heterozygous (+/F, lanes 2–4, 6), and homozygous (F/F, lanes 1, 5) frizzle chickens. The normal band is 851 bp and the mutant band is 767 bp. The size of the molecular marker (MW) is showed in kb. (B) Diagram of the chicken KRT75 and the cryptic splice site activated by the deletion mutation that covers positions –24 of exon 5 to +59 of intron 5. Black boxes represent exon sequences; intron 5 is designated by a line. The caret designating use of the cryptic site (position –69) is shown below, and the caret designating use of the authentic site is shown above the diagram of the pre-mRNA. (C) Partial sequence of the F allele. The 84-bp deletion in genomic DNA is shown in light gray letters. The additional deletion in exon 5 created by a cryptic splice site is shown in dark gray letters. The deletion in genomic DNA and use of the cryptic splice site together result in a deletion of 23-amino acids (position 311–333) in the K75 protein. Parts of exon 5 and intron 5 are shown in capital and small letters, respectively. The underlines show the authentic and cryptic mRNA splicing sites.

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5B), suggesting that KRT75 mRNA is expressed in the normal regions in the frizzle mutant chicken feathers and the phenotype must result from a dysfunction of the protein.

We then compared the expression of KRT75 and feather keratin at mature regions of the wildtype body feather (Figure 5D). We found that KRT75 and feather keratin were co-expressed in the rachis and ramus. KRT75 was expressed in the ventral part, whereas feather keratin was expressed in the dorsal part of the rachis and ramus.

To study the expression pattern differences between KRT75 and feather keratin, we examined the expression of these two keratins in the rachis and ramus from the immature to the mature stage of normal wing feathers (Figure 5E–5H). KRT75 is expressed in the ventral part but feather keratin is expressed in the dorsal part of the immature rachis. Ventral regions of the feather formed a medulla that expressed KRT75, whereas feather keratin was expressed in the dorsal region (Figure 5E, 5F). In the ramus, KRT75 and feather keratin were expressed in a similar pattern as in the rachis (Figure 5G, 5H). In summary, in both the rachis and ramus, KRT75 and feather keratin were expressed in a complementary pattern (Figure 5F, 5H). K75 was present in the medulla but feather keratin was not. These data confirm that although body and wing feathers have differences in symmetry and size, they show similar expression patterns of KRT75.

To further examine the alteration of K75 protein expression in the frizzle rachis, we performed double immunostaining using antibodies to both K75 and feather keratin. Figure 5I shows staining of a section at level II, adjacent to the section shown in Figure 2B. In the normal rachis, feather keratin protein (red) is expressed in the dorsal part and in regions surrounding the medulla, while K75 protein (green) is expressed in the ventral rachis as well as at lower density in the medulla. The protein expression pattern is the same as the mRNA expression pattern (Figure 5D). In the frizzle rachis, K75 protein is only expressed in the narrower ventral region but the feather keratin domain expanded to cover the medulla which is reduced in size. The perturbed keratin organization in the frizzled rachis suggests that the frizzled phenotype may be caused by the KRT75 mutation.

Effects of misexpressing KRT75-WT and KRT75-MT on embryonic feather development

To test the function of KRT75 in feather development, we constructed RCAS-KRT75-WT and RCAS-KRT75-MT viruses to misexpress the normal and mutant forms in embryonic and adult chickens. RCAS-KRT75-WT virus did not produce feather malformations in chicken embryos; however, some keratin-like depositions were found (N = 10/10) (compare middle and left panels in Figure 6A). Whole mount in situ hybridization of KRT75 confirmed the ectopic expression of KRT75 in the feather filaments (insert in Figure 6A, middle panel). In comparison, misexpression of KRT75-MT generated feathers with curved tips, mimicking the frizzle phenotype (N = 8/20) (Figure 6A, right panel).

We further characterized feather phenotypes produced as a result of KRT75 misexpression by H&E staining, PCNA staining, AMV-3C2 staining (for RCAS virus detection), KRT75 section in situ hybridization and TUNEL assay (for detection of apoptosis) in serial parasagittal sections at different levels of the filament (Figure S8). Figure 6B–6F shows sections at level III, which are close to the feather filament tip. We did not detect significant alterations based on H&E (Figure 6B) and PCNA staining (Figure 6C) among the treated samples and controls. The treated samples displayed strong AMV-3C2 staining (Figure 6D, arrows) and ectopic KRT75 expression (Figure 6E, arrows). The detailed H&E and TUNEL staining at different levels are shown in Figure S8B–S8D. In normal development, programmed cell death appeared in the peripheral epidermis at level I, II and III but eventually apoptosis occurred in all distal tip cells (level IV) (Figure S8B). Cell death was detected infrequently in the proximal to middle region of the epidermis shown in Figure S8B (red arrow). However, both treated samples induced ectopic cell apoptosis but KRT75-MT misexpression induced significantly increased TUNEL positive cells (Figure 6F, Figure S8C and S8D). We conclude that the misexpressed mutant form of KRT75 induces significant ectopic cell apoptosis, which may be responsible for the randomly curved feather morphology in the RCAS-KRT75-MT infected feathers.

Effects of misexpressing KRT75-WT and KRT75-MT on adult feather morphology

To verify that the adult frizzle phenotype is due to the identified KRT75 mutant, we misexpressed KRT75-WT or KRT75-MT by injecting the RCAS virus into adult feather follicles after plucking. Misexpressing KRT75-WT produced twisted feathers (N = 5/12) (Figure 6G). The control feathers involving only plucking or injecting RCAS-GFP did not show the twisted phenotype (N = 0/20). Cross sections of the twisted feathers showed the asymmetrical distribution of ectopically expressed KRT75 in the ramogenic zone of the feather follicle (Figure 6H). Misexpression of the mutant form of KRT75 produced the curved feathers but the curvature only existed at the tip of the feather (N = 6/10) (Figure 6I). Control feathers on the right wing did not show any unusual curvature (N = 0/10) (Figure 6I). Since misexpressing the mutant form of KRT75 in a normal feather follicle that contains numerous normal KRT75 transcripts only affects the distal feather tip, we presume that its effect may be masked by high levels of endogenous wild type transcripts and limited to the softest part of the rachis at the tip.

Images of flight feathers sampled from two wings of the same chicken in our experiments are shown in Figure 7A. Even though visual inspection of images of the control and KRT75-MT transfected feathers only reveal subtle differences, computer-aided analyses showed that ectopic expression of mutant K75 substantially changed the way the feathers bend along their rachis. Under normal circumstances, the natural bending of feathers from either side of a wildtype chicken would be expected to display reflective or mirror image symmetry to that of the opposite wing (Figure S9). Instead of the wild-type gentle inward bend, the end of the infected feather was twisted abruptly away from the body (Figure 7B), as a
Figure 5. *In situ* hybridization of KRT75 in embryonic and adult feather filaments. (A) Cross section of E13 feather filaments. KRT75 is expressed in the region that is destined to become the ramus. Note that embryonic downy feathers do not have a rachis. (B) KRT75 is expressed in the rachis and ramus in adult normal regenerating feathers. (C) KRT75 is expressed in the rachis and ramus in adult frizzle regenerating feathers. (D) A nearly mature rachis expresses KRT75 in the ventral part of the rachis. In comparison, feather keratin is expressed in the dorsal part of the rachis. (E) A KRT75 mutation causes frizzled feathers.
Sections of a rachis from adult wing feathers at different levels of maturity from the feather base (immature) to the feather tip (mature). KRT75 is expressed in the ventral part of the rachis. In comparison, feather keratin is expressed in the distal part of the rachis. (F) Schematic drawing which summarizes the expression pattern of KRT75 and feather keratin adult feather rachis. (G) Barb ridge of adult wing feathers at different levels of maturity. KRT75 is expressed in the ventral part of the ramus. In comparison, feather keratin is expressed in the distal part of the rachis. (H) Schematic drawing which summarizes the expression pattern of KRT75 and feather keratin in adult feather ramus. (I) Double immunostaining for K75 (green) and feather keratin (red) in the rachis of normal and frizzle feathers. The yellow dotted line outlines the rachis and the white dotted line indicates the medulla. D, dorsal; V, ventral; bb, barbule; rc, rachis; rm, ramus.

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Ectopic mouse K75 (Köhl) was reported to co-localize with K8, K17 and K18 in cultured PtK2 rat kangaroo kidney epithelial cells.
A KRT75 Mutation Causes Frizzled Feathers

A

Control
R7 R9 R10

KRT-75-MT
L7 L9 L10

B

Left Wing
KRT-75-MT

KRT-75-MT-L7
KRT-75-MT-L9
KRT-75-MT-L10

\[ \theta(s) \] (°)

Right wing
Control

Ctrl-R7
Ctrl-R9
Ctrl-R10

\[ s/(total \ length) \]
[56]. To explore the role of KRT75-MT in disrupting the intermediate filament structure in a dominant fashion, we transfected PtK2 cells with RCAS expressing either KRT75-WT or KRT75-MT. K18 (red) is present in the cytoplasm in a network configuration and K75 (green) is weakly positive in control PtK2 cells (Figure S8E). After transfection with wild type KRT 75, KRT 75 is expressed strongly. The keratin network is still maintained (Figure. S8F). When the mutant K75 form is expressed, both K18 and K75 accumulate around the nucleus (Figure S8G, white arrows). This is similar to what was found for mouse K75 [48]. Our data suggests that avian KRT75-MT can act in a dominant negative fashion to disrupt the keratin filament network.

Discussion

Compared to traditional model organisms, domesticated animals have a number of advantages. Selection is not based on a need for survival in nature, but rather upon human preferences due to economic or aesthetic values. This allows selection for extreme phenotypes that may not survive in the wild. Large populations and greater longevity mean that mutations of biologically important traits have a greater chance of appearing and of being maintained, giving us an excellent opportunity to identify novel functions for specific genes. In the case of chickens, the availability of breeds selected for economic value or fancy feather variants, and the progress in chicken genomics have provided a prime opportunity to study the genetic basis of feather morphogenesis [57]. We have taken advantage of the availability of genome-wide SNPs and the chicken genomic sequence to identify the molecular basis of frizzle feathers. Our work reveals the important role of α-keratin in the development and differentiation of feather structures.

Feather keratins

KRT75 is a member of the type II epithelial α-keratin gene family [58–60]. The feather mainly consists of two types of keratin proteins: α- and β-keratins. An obligate heteropolymer is formed by two types of α-keratin, an acidic type and a basic/neutral type, and culminates into the 8–10 nm-thick intermediate filament [61,62]. The polymerization partner of K75 is unclear but it may be K17 in mammals [48]. In contrast to α-keratin, a fibrous protein rich in alpha helices, β-keratin is rich in stacked β-pleated sheets. β-keratins are only found in reptiles and birds, whereas α-keratins exist in all vertebrates [50].

K75 is not a hard feather keratin per se. Although the feather mainly consists of feather-specific β-keratins, cellular and biochemical studies have shown that α-keratin plays an important role in the early formation of rachides, barbs, and barbules [51]. The molecular mechanisms for accumulating α-keratin in down feathers and regenerating feathers are still largely unknown. It has been proposed that during the development of regenerating feathers, the α-keratin in the initial tonofilaments of sheath cells is replaced by feather-specific β-keratin [51,63,64]. Ultrastructural studies indicate that bundles of keratin filaments of 8–12 nm in diameter (α-pattern) are initially formed in differentiating barb/barbule cells and later replaced by 3–4 nm-thick filaments (β-pattern) [51,65]. Our studies however, revealed that the α-keratin and β-keratin actually accumulated in different parts of the rachis and ramus. We found that KRT75 is expressed in the ventral part that is destined to become the medulla, whereas β-keratin is expressed in the dorsal part of the rachis and ramus that is destined to become the cortex.

Biochemical studies have indicated that feather α-keratins are mainly acidic, while basic α-keratins are thought to be rare in feathers [51]. Cytokeratins have been proposed to have a role in the formation of an initial and temporary scaffold for the deposition of immense quantities of compact feather keratins [51]. The identification of KRT75, which encodes a type II cytokeratin (basic), as a major determinant of normal feather structure suggests that basic α-keratins are also critical for feather formation. In the cytoplasm of rachis sheath cells, a higher quantity of α-keratin bundles is initially deposited. This observation may explain why the rachis is more severely affected than the barb and barbule in KRT75 mutant chickens.

During feather filament development, keratinocytes eventually die, either leaving space or leaving a keratinized structure. Before those events occur, localized proliferation and apoptosis of keratinocytes either add or remove cells in different places, thus shaping the feather, including the rachis [66].

Our data show that apoptosis is expanded in frizzle compared to control adult feathers in the immature feather region. In contrast, the proliferation zone is decreased in frizzle chicken feathers compared to controls. Our functional studies on embryonic chicken feathers show that apoptosis is increased within the inner epithelium of embryonic feathers expressing ectopic KRT75 compared to controls and is increased even further in embryonic feathers expressing the mutant form of KRT75. Currently, we do not know whether the mechanism is through a classic mechanical role or through an alternate pathway as was seen for KRT17 in mammalian hair follicles [67].

Our results show that KRT75 unquestionably plays a significant role in the normal development of feathers. However, the cellular mechanisms underlying the frizzle phenotype have not been specifically probed because the role of α-keratin in feather development is largely unexplored. The mutation could potentially affect feather formation in many ways such as altering the mechanical properties of the feather, weakening the initiation of keratin formation, causing abnormal scaffolding for feather keratin deposition, impairing α- and β-pattern replacement, or perturbing β-keratin polymerization. Our findings support the importance of α-keratin in feather formation. Our results also demonstrate the power of using mutants of domestic chickens as a genetic model to unravel biological functions that are difficult to reveal by traditional biochemical and cellular studies.

Effects of the chicken frizzle mutation

The action of the F gene is localized in the feather follicle and is not a consequence of a metabolic disorder [68]. However, the F gene may also have other pleiotropic effects that cause physiological abnormalities. Frizzle plumage may cause the acceleration of basal metabolism due to the loss of body heat, leading to alterations in organ size (e.g., enlargement of the heart, spleen, gizzard, and alimentary canal as well as lack of hypothermal fat deposits) and numerous physiological anomalies (e.g., higher food intake, oxygen consumption, heart rate, volume of circulating
blood as well as delayed sexual maturity or decreased fertility) [10,68–71].

An autosomal recessive modifier gene mf, which restricts the effect of F, has also been found in some chicken breeds [72,73]. Pathogen free stocks are required for RCSAS mediated gene misexpression. The available SPAFAS chickens are of White Leghorn. Landauer [72] indicated White Leghorns chickens are likely enriched for the recessive modifier of the frizzle phenotype. This may help explain the less impressive phenotypes we observed with misexpression of a virally-derived frizzle protein, although whether this is really the case remains an open question.

Mammalian KRT75 related mutations

In mammals, the α-keratin K75 (Keratin 75 or cytokeratin 75, formerly known as KOhf or hK6) is a hair follicle-specific epithelial keratin [58]. K75 plays an essential role in hair and nail formation. The KRT75 gene is specifically expressed in the companion layer [the cellular layer that lies between the outer (ORS) and inner (IRS) root sheaths], the upper germinative matrix companion layer, and the medulla of the hair shaft [74–78]. Mutations in KRT75 have been associated with the hair disorder pseudofolliculitis barbae (PFB). Pseudofolliculitis barbae is a common human hair disorder characterized by a pustular foreign body inflammatory reaction that is induced by ingrown hairs of the facial and submental (barbea) regions after regular shaving [79,80]. This abnormal hair orientation phenotype is somewhat similar to the frizzle feather we report here. Interestingly, an unusual Ala12Thr polymorphism in the 1A alpha-helical segment of K75 has been associated with PFB by examination of a three-generation Caucasian family as well as 100 individuals affected with PFB and 100 unaffected controls. Modeling and transfection studies led the investigators to conclude that the Ala12Thr substitution is disruptive at later stages of filament assembly and could represent one of the genetic factors leading to this complex phenotype. This abnormal hair orientation phenotype is somewhat similar to the frizzle feather we report here. Besides, another hair-follicle-specific epithelial keratin is also known to be associated with the autosomal dominant wooly hair syndrome [81,82].

Genomic aspects

A total of 54 functional keratin genes in the human genome can be divided into 28 type I genes and 26 type II genes [83,84]. KRT75 is located within the type II keratin gene cluster on chromosome 12 of humans and chromosome 15 of mice [85]. Twenty out of the 26 type II keratin genes are epithelial keratins and six encode hair keratins [80]. KRT75 is tightly linked with epithelial keratin genes KRT6A/B/C and hair keratin genes KRT81–86 in humans [83]. Mutations in human hair-follicle specific epithelial type II keratins are known to cause structural defects of differing severity in hair, nail, and skin [86], but the regulation of these keratins during proliferation and differentiation is yet to be elucidated. The chicken and zebra finch genomes contain only 28 and 27 α-keratin genes respectively compared to 41 genes in the anole genome [87]. β-keratin duplications occurred more frequently in birds than in reptiles [87] and they may have replaced some important roles in the formation of hard appendages in birds, thus the remaining α-keratins in the birds’ genomes should play irreplaceable roles in the formation of epithelial and epidermal appendages of birds. The frizzle feather might represent a phenotype that could also be caused by mutations in genes involved in rachis structure but other than KRT75. Thus, this group of appendage structural mutants can be considered as a sub-category of ectodermal dysplasia. Interestingly, we observed that the rachis and barbs of feathers from homozygous frizzle chickens were easily broken during handling, and they were also easier to pluck. However, no abnormalities in nails were noted (unpublished observation). Further studies will provide clues into the architectural principles controlling how various skin appendages are built. It also calls for more molecular investigation into the role of this gene cluster in the evolution and development of the feather.

Another interesting phenomenon we report is the use of the cryptic splice site in this mutant. A cryptic splice site is a suppressed splice site that is recognized but usually not utilized by the splicing machinery until a mutation activates it, either by strengthening the cryptic splice site or disrupting an authentic splice site [88,89]. Disruption of authentic splice sites crucial for identification of the 5′ or 3′ splice sites frequently result in complete exon skipping or in activating of the use of cryptic splice sites [90]. For genes with many introns, it is thought that up to 50% of mutations that cause disease actually affect splicing, either through the activation of cryptic splice sites, exon skipping, or disruption of alternative splicing [91–94].

In conclusion, we show that a single KRT75 allele is the major determinant of frizzle feathers in chickens. It is most interesting to compare the phenotypes caused by a mutation in KRT75: pseudofolliculitis barbae in human and frizzle feather in chicken. The phenotypes appear to be very different, but indeed fundamentally similar in that both exhibit appendage architecture defects. In chicken, the defect appears to be exaggerated due to the elaborate morphogenesis of feathers. Thus the ability to identify a gene that contributes to feather morphology highlights the potential contribution of chicken genetics to the understanding of feather variations. It also illustrates how the progress in chicken genomics provides a new approach to dissect basic biological questions [28], in this case, the molecular determinants of feather forms. Finally, this body of work highlights the importance of the cluster of α-keratin genes and invites further exploration into their role in normal and aberrant vertebrate developmental processes and provides an impetus for analyzing the relationship between α- and β-keratin during feather evolution.

Materials and Methods

Ethics statement

Animal care and experiments were conducted according to the guidelines established by the USC Institutional Animal Care and Use Committee.

Animals and eggs

For the linkage and association mapping, DNA from blood or feathers was opportunistically obtained as a byproduct of National Poultry Improvement Plan testing. The first pedigree was derived from a bantam White-tailed Japanese/Silver Penciled Plymouth Rock frizzle heterozygote rooster and the second from a bantam Red Cochin frizzle heterozygote rooster. These males were crossed with 9 normal feathered bantam hens of the following breeds: Araucana, Barred Plymouth Rock, and White Leghorn. Eight to eighteen offspring were evaluated from each female. The first cross produced 33 frizzles and 29 wild-type and the second 19 frizzles and 27 wild-type for a total of 108 chicks. Frizzle embryos used in embryonic studies were generated by mating a homozygous frizzle rooster. These males were crossed with 8 normal feathered bantam hens of the following breeds: Araucana, Barred Plymouth Rock, and White Leghorn. Eight to eighteen offspring were evaluated from each female. The first cross produced 33 frizzles and 29 wild-type and the second 19 frizzles and 27 wild-type for a total of 108 chicks. Frizzle embryos used in embryonic studies were generated by mating a homozygous frizzle rooster with homozygous frizzle hens. Embryos were collected at different embryonic stages. Feathers for image analysis were from sex-matched white Plymouth Rock siblings that were both heterozygous and homozygous for the F gene. Chicks were
hatched and raised in the USC animal facility. For the functional study, pathogen free fertilized eggs were purchased from SPAFAS, Preston, CT. Some of these eggs hatched and the chickens were used for functional studies on adult feather follicles. Additional frizzle chickens were opportunistically sampled from farms in Wanhua and Tamsui, Taiwan.

**Image analyses of chicken feathers**

Feathers were approximated as two-dimensional objects, which defined a primary plane for our imaging. Typical images of the feathers were taken from the dorsal side. We identified the fringes of the rachis by edge-finding algorithms we previously developed and defined the backbone of the rachis as a curve equidistant to the two fringes. The curve was smoothed by a length scale of 3 mm to suppress noises due to image errors. We parameterized this backbone by s, the accumulated distance from the proximal end that is defined as the origin (s = 0). The total length of the rachis means the accumulated distance from the origin to the distal end. We described the bending of the rachis by a function 0(s), in which 0 is an angle (with an arbitrary reference) representing the tangent of the backbone at the location s.

**Paraffin section and staining**

The generated or gene misexpressed feathers were fixed in 4% paraformaldehyde at 4°C overnight followed by procedures described by Jhang et al. (1998) for immunohistochemistry and 7 μm paraffin sections were prepared [95]. PCNA and AMV-3C2 antibodies are from Chemicon (CBL407) and Hybridoma Bank respectively. Double fluorescent immunostaining was done using K75 antibody (ab76486; Abcam, MA) and feather keratin antibody from Dr. Roger Sawyer. Section were imaged with a Zeiss 510 confocal microscope (University of Southern California Linkage analysis).

**mRNA in situ hybridization**

We performed PCR for the full length chicken KRT75 by using sense primer (5'-ATGTTCTGCGCAGTGCCACGG-3') and anti-sense primer (5'-TTAGCTTCGCTTAACCTTCC-3'). The PCR product was inserted into the p-dv plasmid (Qiagen). Antisense probe was made to detect the KRT75 mRNA by section or whole mount in situ hybridization. Non-radioactive in situ hybridization was performed according to procedures described by Chuong et al. (1996) [96]. SHH antisense probe was generated as previously described [49]. Feather keratin antisense probe was used to detect feather keratin B [97].

**Linkage analysis**

In order to locate the gene underlying the frizzle trait, a genome scan was conducted on progeny of crosses between the same heterozygous frizzle rooster, PF1, and five different wild-type feathered hens. A total of 2678 SNPs were genotyped using the Illumina GoldenGate assay. The average genotype call rate obtained for the 45 birds in the study was 99.57% (range 98.32–99.74%) providing approximately 2661 genotypes per bird. The genotype data was screened for Mendelian incompatibilities using PEDCHECK, while MERLIN was used to assess the data for occurrence of double recombination events over short genetic distances, which are most likely due to genotyping error. MLINK of the FASTLINK package was used to perform two-point linkage analysis. An autosomal dominant mode of inheritance with complete penetrance and a mutant allele frequency of 0.001 was used in the analysis.

**Positional cloning and genotyping**

Chicken genomic DNA was isolated from blood using the Blood & Cell Culture DNA Mini Kit (Qiagen, Hilden, Germany). For mutation analysis of the keratin candidate genes, we got 49 PCR amplicons of the selected candidates amplified from chicken genomic DNA (Table S1). Primers to cover some intronic and exonic regions of 14 keratin genes were designed using the CLC Bio 6.0 (Aarhus, Denmark). All amplicons were sequenced directly after treatment with exonuclease I and shrimp alkaline phosphatase by standard methods. Each amplicon was sequenced using BigDye terminator sequencing kits and standard protocols (Applied Biosystems, Santa Clara, CA). A significant mutation was detected in only one amplicon amplified by a pair of primers (5’-CCATGGGACAAACGCCGACACG-3’ and 5’-TTTGCCTTCCTGCCATCT-3’).

**RT–PCR**

Feather follicle samples were collected from a homozygous frizzle chicken 2 weeks after plucking and then were immersed in RNALater (Ambion, Austin, TX) and stored at −20°C. After thawing, total RNA was isolated by homogenization and extraction using the RNasy Tissue Mini kit (Qiagen, Hilden, Germany). Each RT-PCR reaction was carried out with 1 μg of total RNA. Primers were designed for the KRT75 gene (5’-TTTGCCTTTTCTCCCAGC-3’ and 5’-GTCTTCTCCTCCTGATTAT-3’).

**Generation of proviral constructs**

KRT75 cDNA PCR products were cloned into the pCR8/GW/TOPO Gateway entry vector (Invitrogen, Carlsbad, CA) and sequenced. An LR recombination reaction was performed to transfer the cDNAs to a Gateway compatible RCASBP-Y DV vector [98].

**Construction of RCAS-KRT75-WT and RCAS-KRT75-MT**

KRT75 wildtype (KRT75-WT) and KRT75 mutant forms (KRT75-MT) were cloned to RCAS by the Gateway system (Invitrogen, Carlsbad, CA). Virus was made according to Jhang et al., 1990 [95] and concentrated by ultra-centrifugation.

**Feather regeneration**

Around 30 contour feathers from the middle back of the body were plucked and then 10 days or 30 days were allowed to pass. At the collection points, regenerated feathers were directly plucked or dissected and the whole single follicles were prepared for sectioning.

**Functional studies of KRT75**

For embryonic studies, RCAS-KRT75-WT or RCAS-KRT75-MT virus was injected into the amniotic cavity of E3 chicken embryos. Samples were collected at E13. RCAS-GFP was injected into different embryos as a control. For adult feathers, about 100 μl of virus was injected into the empty follicles after plucking the primary flight feathers in the left wing. The feathers on the right wing were collected at same time as controls. Feather follicles from a different chicken injected with RCAS-GFP were used as an alternative control. Feather morphogenesis was observed after 1–2 months of regeneration.
Expression of KRT75 in mammalian cells

PK2 cells (American Tissue Culture Collection, MD) were transfected with plasmid (RCAS) encoding KRT75-WT or KRT75-MT by lipofection (Lipofectamine, Invitrogen, CA). After 48 hours samples were fixed in 4% paraformaldehyde and stained with antibodies to K16 (AV40206; Sigma, MO) and K75. DAPI was used to visualize the nuclei. Images were obtained using a Zeiss 510 confocal microscope.

Accession number

The sequence of F allele of KRT75 has been submitted to GenBank with the accession number JQ013796.

Supporting Information

Figure S1 Detailed PCNA and TUNEL staining in the rachis of normal and frizzle chicken regenerated body feathers at different levels. (A) Diagram of a 30 day regenerating follicle. Levels I, II and III show the planes of section from immature to mature regions of a feather follicle. (B, C) Comparison of PCNA staining between normal (B) and frizzle (C) feathers at different levels. (D, E) Comparison of TUNEL staining between normal (D) and frizzle (E) feathers at different levels. (F) Diagram to summarize the PCNA (red) and TUNEL (green) data. D, dorsal; DP, dermal papilla; V, ventral. (TIF)

Figure S2 Schematic of the F critical region. (A) The region within the chicken linkage group E22C19W28_E50C23 harbouring the F mutation in the KRT75 gene, (adapted from the Ensembl genome browser http://www.ensembl.org/). (B) The F mutation was determined to be an 84-bp deletion covering the junction of exon 5 and intron 5 in the KRT75 gene and is indicated by a yellow bar. The deletion activates a cryptic splice site in exon 5. (TIF)

Figure S3 KRT75 genotypes of parental chickens and progenies in the experimental cross. These results were obtained with DNA samples used for the genome-scan and those that were not included in the genome scan P. (A) Lab IDs, phenotypes and lane number for samples used in the genome-scan. (B) KRT75 genotypes of samples used in the genome scan. Samples 33 and 39–41 are represented by longer exposure times. (C) Lab IDs, phenotypes and lane numbers for samples not used in the genome scan. (D) KRT75 genotypes of samples not used in the genome scan. Samples 78, 81, 89 and 96 represent repeat PCR reactions. A total of 96 PCR reactions were performed and include 2 heterozygous frizzle phenotype roosters, 9 homozygous wild type hens, 38 frizzle phenotype progeny and 47 wild type phenotype progeny. In each case, the genotype showed perfect correlation with the phenotype. F, frizzle phenotype; M, DNA marker; W, wild type. (TIF)

Figure S4 The summary of KRT75 genotypes of the parents and progenies in the experimental cross. Lane 1 shows the genotype of the heterozygous father (+/F), lane 2 shows the genotype of wild-type mother, lane 3 shows the genotype of frizzle offspring, lanes 4 show the genotype of normal offspring (I do not think we need delete this). (TIF)

Figure S5 KRT75 is expressed in the frizzle feather follicles. Lanes 1–3 show the [72,73]PCR products amplified from total cDNA prepared using a poly-T oligonucleotide primer, whereas lanes 4–6 show the PCR products amplified from total cDNA prepared using random hexamer primers. Lanes 1 and 4 show the PCR products amplified using primers located in the 5’- and 3’-UTRs of KRT75 mRNA (5’-TTCCTTCTTCGCCACT-3’ and 5’- GTGTCCGCTTCCCGTGAAT-3’), whereas lanes 2, 3, 5, and 6 show the PCR products containing the complete CDS only (5’-ATGCTGCCAGTCACCG-3’ and 5’-TTAGCTTCGTAACCTTCCG-3’). (TIF)

Figure S6 Multiple sequence alignment of amniote K75 proteins. The alignment was prepared using MUSCLE implemented in the CLC Bio 6.0 (Aarhus, Denmark). The sequences were translated from AY574985.1 (red junglefowl), XM_003205996 (turkey), XM_002194971 (zebrafish KRT6A), XM_002194995 (zebrafish KRT75), XM_003216971 (lizard), XM_001362788 (opposum), XM_001504398 (horse), BC137935 (mouse), and NM_004693 (human). The sequence of F allele of KRT75 has been submitted to GenBank with the accession number JQ013796. (TIF)

Figure S7 Domains of K75. The yellow-line box highlights the deletion which removes the entire part of link L2 and some parts of the coiled-coil segments of 2A and 2B. The domain information was obtained from the Human Intermediate Filament Mutation Database (www.interfil.org). (TIF)

Figure S8 Detailed H&E and TUNEL staining at different levels among control, RCAS-KRT75-WT and RCAS-KRT75-MT misexpression samples. (A) Diagram showing levels for section from immature (level I) to mature (level IV) regions of an E13 embryonic body feather. (B-D) H&E and TUNEL staining at different levels. (B) and (B’) control; (C) and (C’) RCAS-KRT75-WT; (D) and (D’) RCAS-KRT75-MT. PK2 cells stained for KRT18 (red), K75 (green) and DAPI (blue). (E) Wildtype cells; (F) RCAS-KRT75-WT; (G) RCAS-KRT75-MT. (TIF)

Figure S9 Natural bending of feathers from both sides of a chicken. (A) Images of flight feathers from opposite sides of the same normal chicken (the R8 and L6 denote the 8th feather taken from the right and left wing, respectively). (B) The trend of curves (s) shows reflective symmetry. (TIF)

Figure S10 Contrast between feathers that have misexpressed GFP and overexpressed KRT75-WT. (A) Comparison of the feathers with misexpressed GFP (Control) and KRT75-WT. (B) Effects of the viral misexpression, as shown by the qualitative change of the curves of s(0). (TIF)

Table S1 Primers designed to screen for the causative mutation in the candidate gene regions. (PDF)

Table S2 Sequence variants within the linkage group chrE22C19W28_E50C23. (PDF)

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References


