

# Developmental expression of chicken *FOXN1* and putative target genes during feather development

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**ABSTRACT** *FOXN1* is a member of the forkhead box family of transcription factors. *FOXN1* is crucial for hair outgrowth and thymus differentiation in mammals. Unlike the thymus, which is found in all amniotes, hair is an epidermal appendage that arose after the last shared common ancestor between mammals and birds, and hair and feathers differ markedly in their differentiation and gene expression. Here, we show that *FOXN1* is expressed in embryonic chicken feathers, nails and thymus, demonstrating an evolutionary conservation that goes beyond obvious homology. At embryonic day (ED) 12, *FOXN1* is expressed in some feather buds and at ED13 expression extends along the length of the feather filament. At ED14 *FOXN1* mRNA is restricted to the proximal feather filament and is not detectable in distal feather shafts. At the base of the feather, *FOXN1* is expressed in the epithelium of the feather sheath and distal barb and marginal plate, whereas in the midsection *FOXN1* transcripts are mainly detected in the barb plates of the feather filament. *FOXN1* is also expressed in claws; however, no expression was detected in skin or scales. Despite expression of *FOXN1* in developing feathers, examination of chick homologs of five putative mammalian *FOXN1* target genes shows that, while these genes are expressed in feathers, there is little similarity to the *FOXN1* expression pattern, suggesting that some gene regulatory networks may have diverged during evolution of epidermal appendages.

**KEY WORDS:** *chick, forkhead, WHN, HFH11, thymus, nudee*

The forkhead family transcription factor *FOXN1* (formerly called Winged helix transcription factor nude, *Whn* (Meier *et al.*, 1999) and HNF-3/forkhead homolog 11, *Hfh11* (Segre *et al.*, 1995) is essential for hair and thymus development in mammals. Hair production is complex and cyclic, involving many transcription factors and their targets (Lee and Tumber, 2009; Lin *et al.*, 2009). In the murine hair follicle *FOXN1* exhibits a dynamic expression pattern that is dependent on the stage of the hair cycle. *FOXN1* appears to be strongly expressed in the anagen or growth phase and its expression decreases by the telogen or quiescent phase. In the mature hair follicle *FOXN1* is expressed in the hair shaft and the inner root sheath (Lee *et al.*, 1999). In the hair shaft, *FOXN1* is expressed in the cortex, which is consistent with absence of the cortex in *Nude* (*FOXN1<sup>nud/nud</sup>*) mice. *Nude* mice develop normal hair follicles and produce hair shafts, but the cortex fails to form most keratins so the hair does not extend. The absence of visible

hair is due to a coiling of hair shafts in the hair canal resulting in a failure to penetrate the epidermal layer of the skin or breaking off at the surface (Mecklenburg *et al.*, 2001, 2004, 2005). Similarly, humans with *FOXN1* mutation have congenital alopecia (hair loss), nail dystrophy and immunodeficiency (Frank *et al.*, 1999, Pignata *et al.*, 2009).

Although *FOXN1* function is well studied in mammals, and additional work has been done on its thymic expression in zebrafish (Ma *et al.*, 2012), frog (Lee *et al.*, 2013) and birds (Neves *et al.*, 2012), nothing is known about *FOXN1* expression in the integumentary organs in non-mammalian vertebrates. Birds, like mammals, have developed evolutionarily novel integumentary appendages, feathers, which are composed of keratins. But as hair and feathers

*Abbreviations used in this paper:* ED, embryonic day; FOX, forkhead box transcription factor.

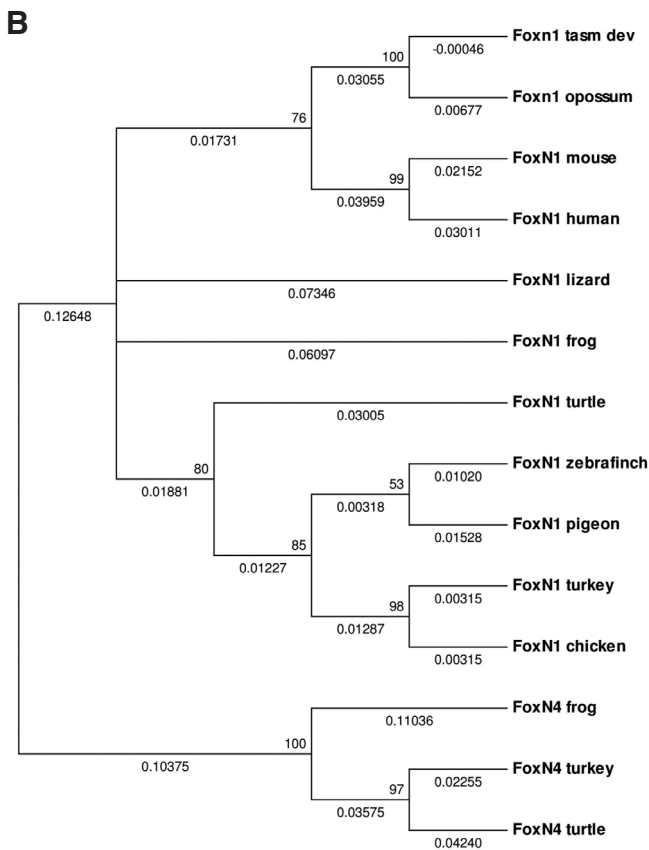
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A

FoxN1_zebrafinch	1	.....-MVSLLQDQSNIKFSSSDTLERDQQLDLMKAQGD	33
FoxN1_turkey	1	.....-MVSLLQDQSNIKFSSSDTLERDQQLDLMKAQGD	34
FoxN1_chicken	1	.....-MVSLLQDQSNIKFSSSDTLERDQQLDLMKAQGD	34
FoxN1_pigeon	1	.....-MVSLLQDQSNIKFSSSDTLERDQQLDLMKAQGD	33
FoxN1_turtle	1	.....-MVSLLQDQSNIKFSSSDTLERDQQLDLMKAQGD	34
FoxN1_lizard	1	.....-MVSLLQDQSNIKFSSSDTLERDQQLDLMKAQGD	1
FoxN1_frog	1	.....-MVSLLQDQSNIKFSSSDTLERDQQLDLMKAQGD	67
FoxN1_tasmanian devil	1	.....-MVSLLQDQSNIKFSSSDTLERDQQLDLMKAQGD	64
FoxN1_opossum	1	.....-MVSLLQDQSNIKFSSSDTLERDQQLDLMKAQGD	34
FoxN1_mouse	1	.....-MVSLLQDQSNIKFSSSDTLERDQQLDLMKAQGD	34
FoxN1_human	1	.....-MVSLLQDQSNIKFSSSDTLERDQQLDLMKAQGD	34
FoxN1_zebrafinch	34	I S P V Q Q S E N P ..... S Y S C ..... Q P Y E P D T R T E R T S E S S P S P S P A S P R Q E P T Q G R F P S S Q G I P C G T N K F R A S F S T	100
FoxN1_turkey	35	V S P I Q Q T D N P ..... S Y N C ..... Q P Y E S D S R T E R K S S E S S H S P S P A S P A Q D Q L L G R Y P P S Q G I H C S K N K F Q V S F S T	101
FoxN1_chicken	35	V S P V Q Q T D N P ..... S Y N C ..... Q P Y E S D S R T E R K S S E S S H S P S P A S P A Q D Q L L G R Y P P S Q G I H C K N K F Q V S F S T	101
FoxN1_pigeon	34	S S I A Q Q T D N L ..... G Y S C ..... Q P Y E S D S R T E R K S S E S S H S P S P A S P A Q D Q L L G R Y P P T N Q G I H C G K N K F K A S F S T	100
FoxN1_turtle	35	S S I S A Q Q T D N P ..... T F S C ..... Q S Y I D P S D R T E R K S S E S S H S P S P A S P A Q D Q T G G R F S N Q G I H T G C K F K V S P N A	101
FoxN1_frog	68	C S P I N I T D D G I L E Q A G S S S C L H M D T Y G S H T I D I Q G E P K N S D S S V S P S L S S P C Q E I Q M G F P S K ..... S S P R	134
FoxN1_tasmanian devil	65	G S P P P Q S E N P ..... G F S C ..... P P Y P G D V Q P E P E P S I S S H S P S P S P C P E Q L Q S H F A G P G ..... Q T A F R L S P T	126
FoxN1_opossum	35	G S P P P Q S E N P ..... G F S C ..... P P Y P G D V Q P E P E P S I S S H S P S P S P C P E Q L Q S H F A G P G ..... Q A A F R L S P T	96
FoxN1_mouse	35	D S P T P Q S E N P ..... N F S C ..... S S F V P D G P P E R T P S L P H S P T A S P D P E Q I G G H C T A G P G ..... P S G F R L S P T	96
FoxN1_human	35	G S P A P Q S K H A ..... G F S C ..... S S F V S D G P P E R T P S L P H S P T A S P D P E Q I G G H C T A G P G ..... P P G F R L S P S	96
FoxN1_zebrafinch	101	E K F R R C S Y E E N N A G S Y E R F L R S S R N P F H P Y K R Q L G E D V F Q E P Q Q A L P P E A S P F K S H O S I D G F E G L P G S A ..... E E P E A F P T	177
FoxN1_turkey	102	E K F R R Y S Y E E N T V G N Y D R F L K S S R N P F H P Y K R Q I S E D V F Q E A Q Q A L P P E G P S F K N T R S I E G F E G L P G S T G P D E S S F P T	180
FoxN1_chicken	102	E K F R R Y S Y E E N T V G N Y D R F L K S S R N P F H P Y K R Q I S E D V F Q E A Q Q A L P P E G P S F K N T R S I E G F E G L P G S T G P D E S S F P T	180
FoxN1_pigeon	101	E K F R R Y S Y E E N T V G N Y D R F L K S S R N P F H P Y K R Q I S E D V F Q E A H Q A L P P E A S P F K N T R S I E G F E G L P G S T G P D E S S F P T	179
FoxN1_turtle	102	E K F R R Y S Y E E N T A A N Y G R F L K S S R N P F H P Y K R F E S D V F Q E H T P A L P E A S P F Q N A R S T E G F E G L P G S T G P D E S S F P T	180
FoxN1_lizard	1	.....-E K F R R Y S Y E E N T A A N Y G R F L K S S R N P F H P Y K R F E S D V F Q E H T P A L P E A S P F Q N A R S T E G F E G L P G S T G P D E S S F P T	1
FoxN1_frog	135	V I F E R Y I S C D D T T E R G Y S N F L K D R N Q G F H P Y K R Q F S D V F P N T L D S L S A E K I F K E M N R I D T F E D V C P P P P E H I E P P V T	211
FoxN1_tasmanian devil	127	E K Y Q G F V Y E D G P A V G S P G R F L K G G R V P F H P Y K R Q F N E D V F L E A H P A L G L D G H S F K K P G C M E T F E D V A S A G ..... D T E P F	201
FoxN1_opossum	97	E K Y Q G F V Y E D G A V A G S P G R F L K G S R A P F H P Y K R Q F N E D V F L E A H P A L G L D G H S F K K P A C L E T F E D V A S A G ..... D A E P F	171
FoxN1_mouse	97	E K Y P G F V F E E G A G S P G R F L K G H M A P F H P Y K R F H E D V F L E A Q T A M A L D G H S F K T O G A L E A F E E I P V D M G ..... D A E A F	171
FoxN1_human	97	K Y P G F G F E E A A A S S P G R F L K G H M A P F H P Y K R P F H E D V F P E A E T T A L K G H S F K T P G L E A F E E I P V D V A ..... E A E A F	171
FoxN1_zebrafinch	178	H I P N T S I S E Q P W C I N L Q Y S S T G G E F G S Q L M Q D S D L K L R T S P L E G Q ..... P G L Y C Y Q P V Q Q M Y C S S H P ..... F H Q Y P S G ..... G S Y P V T	253
FoxN1_turkey	181	H I P N T S I S E Q P W C N S L Q Y S T T G Q D H T S Q V M ..... D S D L K L R T S P L E G Q ..... P G L Y C Y Q P V Q Q M Y C S S H P ..... F H Q Y P S G ..... G S Y P V T	255
FoxN1_chicken	180	H I P N T S I S E Q P W C N S L Q Y S T T G Q D H T S Q V M ..... D S D L K L R T S P L E G Q ..... P G L Y C Y Q P V Q Q M Y C S S H P ..... F H Q Y P S G ..... G S Y P V T	255
FoxN1_pigeon	181	H I P N T S I S E Q P W C N S L Q Y S T T G Q D H T S Q V M ..... D S D L K L R T S P L E G Q ..... P G L Y C Y Q P V Q Q M Y C S S H P ..... F H Q Y P S G ..... G S Y P V T	255
FoxN1_turtle	181	H I P N T S I S E Q P W C N L Q Y N P T S Q D H V S Q V M Q D S E V K L K T S P L E G Q ..... Q S L Y C Y Q P V Q Q M Y C S S H Q ..... F H Q Y S S G ..... G S Y P V T	256
FoxN1_lizard	1	.....-H I P N T S I S E Q P W C N L Q Y N P T S Q D H V S Q V M Q D S E V K L K T S P L E G Q ..... Q S L Y C Y Q P V Q Q M Y C S S H Q ..... F H Q Y S S G ..... G S Y P V T	4
FoxN1_frog	212	H I P D I S A E A A W C N S V Q Y N S ..... T D E D S Q I M M H G S E V K K R P P M M E G P ..... P G L Y C Y Q P ..... O H M Y C S S Q P ..... F H Q Y S P G N G N Y P M T	286
FoxN1_tasmanian devil	201	V I P S F S A E A A A A W C N G P H Y P S ..... Q E H S A Q I M M H G S E V K K P P A P M E G P ..... P G L Y C Y Q P ..... O H M Y C S S Q P ..... F H Q Y S P G N G N Y P M T	274
FoxN1_opossum	171	V I P S F S A E A A A A W C N G P H Y P S ..... Q E H S A Q I M M H G S E V K K P P A P M E G P ..... P G L Y C Y Q P ..... O H M Y C S S Q P ..... F H Q Y S P G N G N Y P M T	244
FoxN1_mouse	171	L P S F A E ..... A W C N G L P Y P S ..... Q E H N O I L Q G S E V K V K P P V L E S G ..... A G M F C Y Q P P L O H M Y C S S Q P P F H Q Y S P G G S Y P T P	244
FoxN1_human	171	L P S F A E ..... A W C N G L P Y P S ..... Q E H G P V L G S E V K V K P P V L E S G ..... A G M F C Y Q P P L O H M Y C S S Q P P F H Q Y S P G G S Y P T P	244
FoxN1_zebrafinch	254	Y I A S S H Y P Q R I A P Q S I Q E S Q P L F P K P I Y S Y S I L F M A L K N S K T G S L P V S E I Y N F M T E H F P Y F K T A P D G W K N S V R H N L S	333
FoxN1_turkey	256	Y I T S S H Y P Q R I A P O S T Q E S Q P L F P K P I Y S Y S I L F M A L K N S K T G S L P V S E I Y N F M T E H F P Y F K T A P D G W K N S V R H N L S	335
FoxN1_chicken	256	Y I T S S H Y P Q R I A P O S T Q E S Q P L F P K P I Y S Y S I L F M A L K N S K T G S L P V S E I Y N F M T E H F P Y F K T A P D G W K N S V R H N L S	335
FoxN1_pigeon	257	Y I A S S H Y P Q R I A P Q S I Q E S Q P L F P K P I Y S Y S I L F M A L K N S K T G S L P V S E I Y N F M T E H F P Y F K T A P D G W K N S V R H N L S	336
FoxN1_turtle	5	Y I T S S H Y P Q R I A P Q S I Q E S Q P L F P K P I Y S Y S I L F M A L K N S K T G S L P V S E I Y N F M T E H F P Y F K T A P D G W K N S V R H N L S	84
FoxN1_lizard	287	Y I S T S H Y P Q R I A P Q A S M E A Q P P L Y P K P I Y S Y S I L F M A L K N S K T G S L P V S E I Y N F M T E H F P Y F K T A P D G W K N S V R H N L S	366
FoxN1_frog	275	Y I L P S P H Y A Y Q R I A P Q A S V E S Q P L F P K P I Y S Y S I L F M A L K N S K T G S L P V S E I Y N F M T E H F P Y F K T A P D G W K N S V R H N L S	354
FoxN1_tasmanian devil	245	Y I L P S P H Y A Y Q R I A P Q A S V E S Q P L F P K P I Y S Y S I L F M A L K N S K T G S L P V S E I Y N F M T E H F P Y F K T A P D G W K N S V R H N L S	324
FoxN1_opossum	245	Y I L P S P H Y P Q R I A P Q A S T D G H Q P L F P K P I Y S Y S I L F M A L K N S K T G S L P V S E I Y N F M T E H F P Y F K T A P D G W K N S V R H N L S	324
FoxN1_mouse	245	Y I L G S S H Y P Q R I A P Q A S T D G H Q P L F P K P I Y S Y S I L F M A L K N S K T G S L P V S E I Y N F M T E H F P Y F K T A P D G W K N S V R H N L S	324
FoxN1_human	245	Y I L G S S H Y P Q R I A P Q A S T D G H Q P L F P K P I Y S Y S I L F M A L K N S K T G S L P V S E I Y N F M T E H F P Y F K T A P D G W K N S V R H N L S	324
FoxN1_zebrafinch	334	L N K C F E K V E N K S G S S R K G C L W A L N P A K I D K M Q E E L Q W K R K D P V A V R K S M A K P E E L D T L I G D K S E K L R S S A S C ..... S P N G	412
FoxN1_turkey	336	L N K C F E K V E N K S G S S R K G C L W A L N P A K I D K M Q E E L Q W K R K D P V A V R K S M A K P E E L D T L I G D K S E K L R S S A S C ..... S P N G	414
FoxN1_chicken	336	L N K C F E K V E N K S G S S R K G C L W A L N P A K I D K M Q E E L Q W K R K D P V A V R K S M A K P E E L D T L I G D K S E K L R S S A S C ..... S P N G	414
FoxN1_pigeon	336	L N K C F E K V E N K S G S S R K G C L W A L N P A K I D K M Q E E L Q W K R K D P V A V R K S M A K P E E L D T L I G D K S E K L R S S A S C ..... S P N G	414
FoxN1_turtle	337	L N K C F E K V E N K S G S S R K G C L W A L N P A K I D K M Q E E L Q W K R K D P V A V R K S M A K P E E L D T L I G D K S E K L R S S A S C ..... S P N G	415
FoxN1_lizard	85	L N K C F E K V E N K S G S S R K G C L W A L N P A K I E K M Q E E L Q W K R K D P I A V R K S M A K P E E L D T L I G D K S E K L R S S A S C ..... S P N G	164
FoxN1_frog	367	L N K C F E K V E N K S G S S R K G C L W A L N P A K I D K M Q E E L Q W K R K D P V T A V R K S M A K P E E L D T L I G D K S E K L R S S A S C ..... S A A G	445
FoxN1_tasmanian devil	357	L N K C F E K V E N K S G S S R K G C L W A L N P A K I D K M Q E E L Q W K R K D P I A V R K S M A K P E E L D T L I G D K S E K L R S S A S C ..... S P N G	435
FoxN1_opossum	325	L N K C F E K V E N K S G S S R K G C L W A L N P A K I D K M Q E E L Q W K R K D P I A V R K S M A K P E E L D T L I G D K S E K L R S S A S C ..... S P N G	403
FoxN1_mouse	325	L N K C F E K V E N K S G S S R K G C L W A L N P A K I D K M Q E E L Q W K R K D P I A V R K S M A K P E E L D T L I G D K S E K L R S S A S C ..... S P N G	403
FoxN1_human	325	L N K C F E K V E N K S G S S R K G C L W A L N P A K I D K M Q E E L Q W K R K D P I A V R K S M A K P E E L D T L I G D K S E K L R S S A S C ..... S P N G	403
FoxN1_zebrafinch	413	A A G S S L S R M A V Q P H S L G E P S L S S I P O P L P H I H T S S G A L H A K N T L P N L L G G Q O T A C Y T ..... S P G F P P I P T A L L Q H T P D ..... H	489
FoxN1_turkey	415	V A G A P L S R M A V Q P H S L G E P S L S S I P O P L P H I H T S S G A L H A K N T L P N L L G G Q O T A C Y T ..... A P G G F P P I S S A L M P O T P D ..... H	491
FoxN1_chicken	415	V A G A P L S R M A V Q P H S L G E P S L S S I P O P L P H I H T S S G A L H A K N T L P N L L G G Q O T A C Y T ..... A P G G F P P I S S A L M P O T P D ..... H	491
FoxN1_pigeon	415	V A N A S L S R M A V Q P H S L C P S L S S V P O L Q S H I H ..... A P A L H A K N T L P N L L G G Q O T A C Y T ..... S P G F P P I S S T L M Q S P D ..... I	491
FoxN1_turtle	416	V A S T V S R M V S V Q H S L C E P T L S S I P O L L H T H T ..... T P L C A K N P L P S F L G G H O T P C F T ..... S P G A F P P I S P T L M Q S P D ..... I	493
FoxN1_lizard	165	V G S ..... R V P L ..... L H ..... P S Q P P I S A O T L T T F G ..... P	190
FoxN1_frog	446	L V N S P T M L S M Q O T P L C E T T I S P V P V S I Q S M P D I S A S L H N N T H ..... L S R Q Q P P P R Y T P L P H I P I Q L S P A M I Q P P H G I S	519
FoxN1_tasmanian devil	434	L T S S G A R P M A L ..... C D P A N P T A V P O P L S L H P A P G T H A K N P M T S L L G G H P P S C Y ..... P Q S Y P H T S P A L A Q P G ..... V P	503
FoxN1_opossum	404	L P S S G A R P M A L ..... C D P A N P T A V P O P L S L H P A P G T H A K N P M T S L L G G H P P S C Y ..... P Q S Y P H T S P A L A Q P G ..... V P	473
FoxN1_mouse	404	L A G P G P I R M A L ..... S A G L S P L H P M P A P G P M P K N P L D L L G H A T S C Y ..... Q O T Y P H L S P L A P S G ..... H O	468
FoxN1_human	404	L S G S G P I R M A L ..... P A G L S P L H P A P G P I P G K N P L D L L M G H A T S C ..... Q O T Y L H L S P L A P P G ..... P	468
FoxN1_zebrafinch	490	P S L F S A G E A Q S Q L R T Q P S I P Q D S P V P A Q S P P S C G M K M L T E H S P A R T V Q D T L L Q E G D S N D I A L N P S L T D F D L Q G N L W E E	569
FoxN1_turkey	492	Q T M F P S G E A P N E L R G Q P S I T Q D S P L P A Q T P P S C G V K V M T E H C T A R T V D P L L Q E G D S N D I A L N P S L T D F D L Q G N L W E E	571
FoxN1_chicken	492	Q T M F P S G E A P N E L R G Q P S I T Q D S P L P A Q T P P S C G V K V M T E H C T A R T V D P L L Q E G D S N D I A L N P S L T D F D L Q G N L W E E	571
FoxN1_pigeon	492	Q T L F P S G E A Q S E L R T Q P S I T Q D S P V P A Q S P P S C G G K L T E P P T A R T V Q D T L M Q E G D S N D I A L N P S L T D F D L Q G N L W E E	571
FoxN1_turtle	494	H S T L P S A N A Q S E L R A Q P S T T Q D S P L P A Q T P P T G S M K M L T E H S T A R T M O D L M Q E A D M S N D I A L N P S L T D F D L Q G N L W E E	573
FoxN1_lizard	191	Q P F L P S A D G P ..... R G Q D S P V P A Q S P P V ..... L E ..... G P R S V Q D V L V P D L L Q S D L D A L N P S L T D F D L Q G N L W E E	254
FoxN1_frog	520	Q N H F S P S D N G N D L R A Q P I N P Q D S P L P A H T P P L Q T M K M L S E H S P A R T M Q E N F T E V E L S N D I D L N P S L T D F D L Q G N L W E E	598
FoxN1_tasmanian devil	504	Q N H F P P S D G H G E L R A Q P T P Q D S P L P A H T P P S H G M K M L S E P S A R T M Q D P L M P E G D L S T D I D T T I N P S L T D F D L Q G N L W E E	583
FoxN1_opossum	474	Q T L F P P S D G H G E L R A Q S G T P Q D S P L P A H T P P S H G M K M L S E P S A R T M Q D T L M P E G D L S T D I D T I N P S L T D F D L Q G N L W E E	553
FoxN1_mouse	469	Q P L F P P D G H L E L L A Q P T P Q D S P L P A H T P P S H G A K M L S E P S A R T M P D L L P D G D L G T D L D A I N P S L T D F D L Q G N L W E E	548
FoxN1_human	469	Q P L F P P D G H L E L L A Q P T P Q D S P L P A H T P P S H G A K L E P S A R T M D T L L P D G D L G T D L D A I N P S L T D F D L Q G N L W E E	548
FoxN1_zebrafinch	572	L K D D S L A V D P L V L I S S S P L ..... C Q F P S ..... H C Q M E N S N T ..... A T I P ..... N G T P H S L P D L Q L T T L Y S A F	630
FoxN1_turkey	572	L K D D S L A V D P L V L I S S S P T S S ..... C Q F P S ..... H C Q M E N S N A ..... V A I P ..... N G T P H S L P D L Q L T T L Y S A F	630
FoxN1_chicken	572	L K D D S L A V D P L V L I S S S P T S S ..... C Q F P S ..... H C Q M E N S N A ..... V A I P ..... N G T P H S L P D L Q L T T L Y S A F	630
FoxN1_pigeon	572	L K D D S L A V D P L V L I S S S P T S S ..... C Q F P S ..... H C Q M E N S S T N ..... A S M Q ..... N G T V H S L P D L Q L T T L Y S A F	632
FoxN1_turtle	574	L K D D S L A V D P L V L I S S T P T S S ..... C Q F P S ..... H C Q M D S N S S S S S S S S S S I Q ..... N S A P O N S L P D L Q L T T L Y S A F	640
FoxN1_lizard	255	L K D D S L T D P L L V S S S P C P T S S ..... G O M D N G A S T D N N N ..... N G N S H ..... C N T Q H H C L P E T L Q L T T L Y S A F	315
FoxN1_frog	599	L K D D S L V L D P L N L V S P S P T P P ..... P P ..... Q Y F L H N P T S K G F S N G T ..... G S P ..... S N N S Y G D L H L T T V Y S A F	658
FoxN1_tasmanian devil	584	L K D D S L A L D P L V L V T S S P V P S ..... H C L P S ..... H C L A G S G G S P ..... G E L G L Q ..... G S N G P S G L S D L H L T T F Y S A L	645
FoxN1_opossum	554	L K D D S L A L D P L V L V T S P L A S ..... H C P S P ..... H C L A G S G G N T ..... S E L S L P ..... N G T S G L S D L H L T T V Y S A L	615
FoxN1_mouse	549	L K D D S L A L D P L V L V T S S P T S S M L P P P P A A H C F P P G P C L E T G S A ..... G D L A A P ..... G S G S G A L G D L H L T T V Y S A F	620
FoxN1_human	549	L K D D S L A L D P L V L V T S S P T S S M P P P P P P H C F P P G P C L E T G S A ..... G D L A A P ..... G S G S G A L G D L H L T T L Y S A F	620
FoxN1_zebrafinch	631	M E L D T V S P ..... S ..... Y L S S P G S K P I A L M	652
FoxN1_turkey	631	M E L D T V S P ..... A ..... Y L S N P G S K P I A L M	652
FoxN1_chicken	631	M E L D T V S P ..... A ..... Y L G ..... T Y	644
FoxN1_pigeon	633	M E L D T V S P ..... A ..... Y L N N S G S K P I A L M	654
FoxN1_turtle	641	M E L D T V S T ..... A ..... Y L N N S G S K P I A L V	662
FoxN1_lizard	316	M E L D T G S T ..... P ..... F L N S L G A K P I A L V	337
FoxN1_frog	659	V E L D K O M P I ..... S ..... Y L S T G G N K R P M G L V	680
FoxN1_tasmanian devil	646	M D L D P V A P ..... L ..... Y L N S S S K P M G L V	666
FoxN1_opossum	616	M D L D P V A P ..... L ..... Y L N S S S K P M G L V	636
FoxN1_mouse	621	V E L E S T P P S A A A G P A V Y L S P G S K P I A L A	648
FoxN1_human	621	M E L E P T P T A P A G P S V Y L S P S K P V A L A	648



**Fig. 1 (left and above). Comparison of amino acid sequences of avian FOXN1 with orthologous proteins and phylogenetic tree of FOXN1/N4. (A)** Predicted amino acid sequences of avian FOXN1 proteins aligned with orthologous protein sequences using T-coffee algorithm. The conserved forkhead DNA-binding (Kaestner *et al.*, 2000) and transcriptional activation domains (Schüddekopf *et al.*, 1996) are underlined in blue and red, respectively. Similar amino acid residues are shaded, threshold 70%. **(B)** A phylogenetic tree of the FOXN1 proteins, with FoxN4 protein sequences used as an outgroup. A neighbor-joining method was used to construct the tree topology based on the forkhead DNA-binding domain.

arose after the last shared common ancestor between mammals and birds in the late Carboniferous period, it is not surprising that proteins in these two lineages have functionally diverged (Furlong, 2005). For example, hair is characterized by a preponderance of soft alpha keratin (Eckhart *et al.*, 2008), whereas feathers are characterized predominantly by the harder beta keratins (Glenn *et al.*, 2008). Alpha keratins are found in the feather sheath and barb ridges but are rapidly overwhelmed by deposition of beta keratins (Alibardi, 2013). Since one function of transcription factor FOXN1 in mice is the activation of alpha keratins 2-6 (Schlake *et al.*, 2000; Mecklenburg *et al.*, 2004) and birds appear primarily to use beta keratin proteins in feathers, it has not been tested whether feathers express FOXN1. We previously showed that another Forkhead transcription factor, FOXE1, is transcribed in the feather filament (Yaklichkin *et al.*, 2011), and its mammalian ortholog is implicated in hair morphogenesis (Brancaccio *et al.*, 2004). This suggests the hypothesis that FOXN1 and homologs of its mammalian regulatory targets might also be expressed in developing feathers.

In this report we show that, whereas FOXN1 and its putative

regulatory targets are indeed expressed in developing feathers, some of the gene regulatory pathways downstream of FOXN1 in epidermal appendage growth appear to have diverged since the division between mammal and bird ancestors about 310 million years ago (Kumar and Hedges, 1998).

## Results

### Phylogenetic and sequence analysis of avian FOXN1 sequences

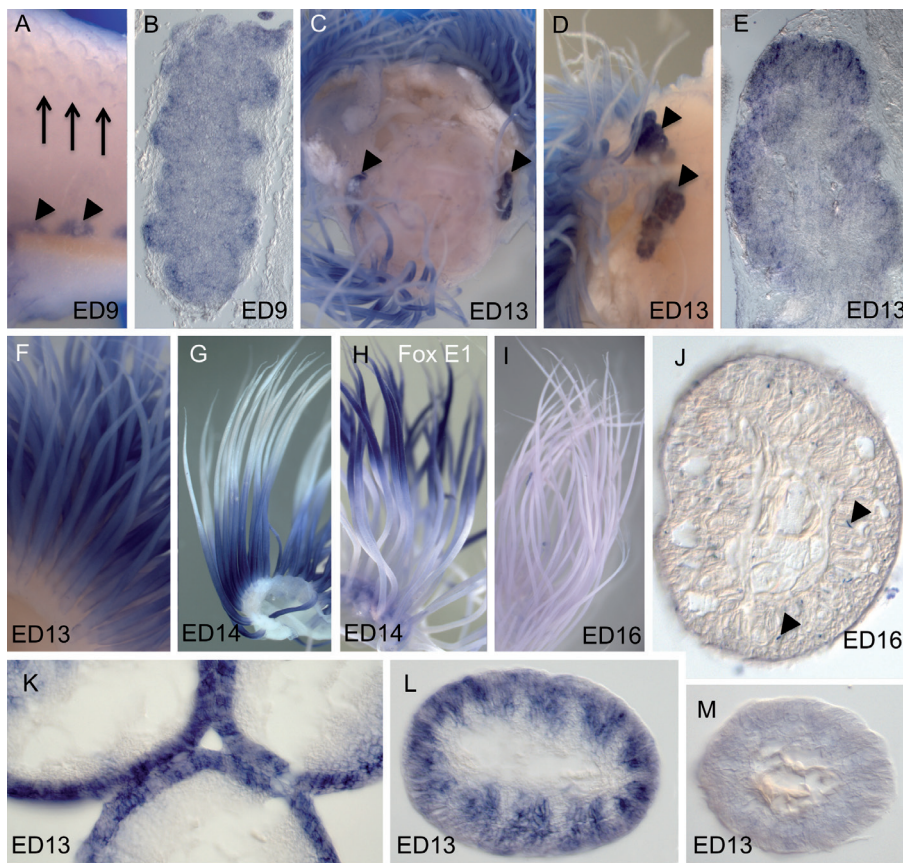
The chick FOXN1 gene encodes a 653 amino acid protein with a molecular weight of 71,957 Da. The chick FOXN1 gene shares synteny with mammalian FOXN1, thus providing unambiguous evidence for orthology. Of non-avian orthologs, chick FOXN1 protein sequence overall shares the highest sequence identity, 75%, with turtle and lizard FOXN1, although the lizard protein sequence is incomplete (Fig. 1A). For comparison, Chick FOXN1 protein shares 59% and 53-55% sequence identity with opossum and placental FOXN1 (Fig. 1A). To determine the evolutionary relatedness of avian FOXN1 gene to orthologs, a phylogenetic analysis was performed using the Neighbor-joining method (Kumar *et al.*, 2004). The phylogenetic tree shows that avian FOXN1 genes are clustered within the FOXN1 group and the closest phylogenetic relatedness is observed to the reptilian and amphibian FOXN1 genes, which is supported by the high bootstrap values (Fig. 1B).

### Expression of chick FOXN1 gene in developing thymus

We performed whole mount *in situ* hybridization analysis to examine FOXN1 expression during chick thymus development from stages ED1-16. No expression was detected from ED1-4.5 (not shown). In chicken, thymus is derived from the third and/or fourth pharyngeal pouch endoderm at stage ED5. At about ED9 thymus primordia detach from the pharynx and migrate to their mature locations in the neck, where they form bilateral multi-lobed glands near the jugular vein. We found that FOXN1 mRNA was strongly expressed bilaterally in ED9 thymus (Fig. 2A). A section through the ED9 thymus primordia shows that FOXN1 labeling is stronger in the prospective cortex with uniform lower intensity label in the medulla (Fig. 2B). This is consistent with the proposed function of mouse FOXN1 in regulation of keratin gene expression, which is present in medullary and cortical thymus epithelial cells during early thymus development (Rodewald, 2008; Nowell *et al.*, 2011). As chick embryonic development progresses (stage ED13) the thymus lobes migrate laterally and expand; expression of FOXN1 persisted (Fig. 2C-D). A section through the thymus at this stage shows increased intensity of FOXN1 mRNA labeling in the cortex (Fig. 2E). This is consistent with the observation in mouse that a FOXN1 mutant phenotype is more severe in the cortical compartment, although both cortex and medulla are affected (Nowell *et al.*, 2011); and expands on the published FOXN1 expression at older stages in chick: (ED18; Neves *et al.*, 2012).

### Expression of chick FOXN1 gene in developing feathers

Feathers are unquestionably the most complex vertebrate epidermal appendages and the specifics of their embryology are well described (e.g., Yu *et al.*, 2002, 2004; Alibardi, 2009, 2013). In short, a feather placode in the epidermis with an underlying dermal condensation sprouts into a feather filament with a dermal pulp separated from the epidermal sheath by the pulp epithelium. Barb



**Fig. 2. Expression of chick *FOXN1* in developing thymus and feathers.** (A) *FOXN1* expression (arrowheads) in ED9 chick neck showing one side of bilateral, lobular expression in the thymus glands. Early feather buds (arrows) and skin show no expression. (B) A section through the thymus lobe in panel A showing expression throughout the thymus with increased intensity in the cortex. (C) Bilateral *FOXN1* expression (arrowheads) in ED13 chick neck looking down from above after decapitation. Expression in feathers filaments can be seen peripherally. (D) Another ED13 embryonic neck from the side after removal of the skin showing two lobes of the thymus (arrowheads). (E) A section through ED13 thymus shows expression varies in intensity with stronger expression in the cortex. (F) *FOXN1* in situ hybridization of feather filaments from ED13 showing label along the entire length of the shaft, but less intense at the base and tip. (G) By ED14, *FOXN1* mRNA expression restricted to the proximal shaft, whereas in comparison with skin from a similarly-staged embryo, *FOXE1* mRNA expression (H) is limited to the distal shaft of the feather. (I) At a slightly older stage the expression of *FOXN1* mRNA in the feather was absent. Trapped precipitate (J-arrowheads) that can be seen in cross section of (I) indicates color reactants were able to penetrate. A transverse section near the base (K), midpoint (L) or tip of the shaft (M) of feathers similar to those shown in panel (F) indicates a transition in both morphology of the feather and label.

ridges begin to form in one side of the epidermis and are displaced laterally to create two vertical half-spirals or arcs between the base and their distal merger into the feather stem or rachis on the opposite side (downy feathers have no rachis). Each barb ridge appears as an arch in cross section with paired stacks of barb plates at its base and a growth zone at its apex, all surrounded by marginal cells. At maturity, a medullary core forms in each barb, the feather sheath is lost and the barbs separate.

Because *FOXN1* is important for normal hair development in mammals, we used *in situ* hybridization to examine expression of *FOXN1* in developing chicken feathers. *FOXN1* expression was at, or barely above, background levels in initial feather buds at ED9 (Fig. 2A), but strong transient expression was observed in feather filaments at stages ED13-16 (Fig. 2 F-G, I-M). Strong expression of *FOXN1* mRNA in the feather filament was detected nearly from base to apex of the feather at stage ED13 (Fig. 2F). Weaker *FOXN1* label was observed in the most proximal part of the feather filament and the most distal tip. This may indicate the beginning of the maturation of the distal region of the filament. By stage ED14 *FOXN1* transcripts become restricted to the proximal feather filament (Fig. 2G), which is a less differentiated and morphologically more active portion of the feather filament (Haake *et al.*, 1984). To verify that the failure to hybridize in the distal filament was not due to exclusion of the color reaction or another artifact, *in situ* hybridizations were performed on comparable feather filaments using a probe for another transcription factor, *FOXE1* (Fig. 2H; Yaklichkin *et al.*, 2011). *FOXE1* showed a complementary expression pattern to that of *FOXN1* in proximal/distal distribution at this stage, indicating that both proximal and distal filaments were able

to label. By stage ED16, *FOXN1* mRNA was no longer detected in the feather filament (Fig. 2I, J), which can be explained by either down-regulation of *FOXN1* expression in a differentiated feather filament or increased keratinization in the feather sheath, which may block *FOXN1* probe penetration at this stage. Small flecks of trapped color precipitate (artifact, arrowheads Fig. 2J) indicate that probe and labeling solution were able to penetrate.

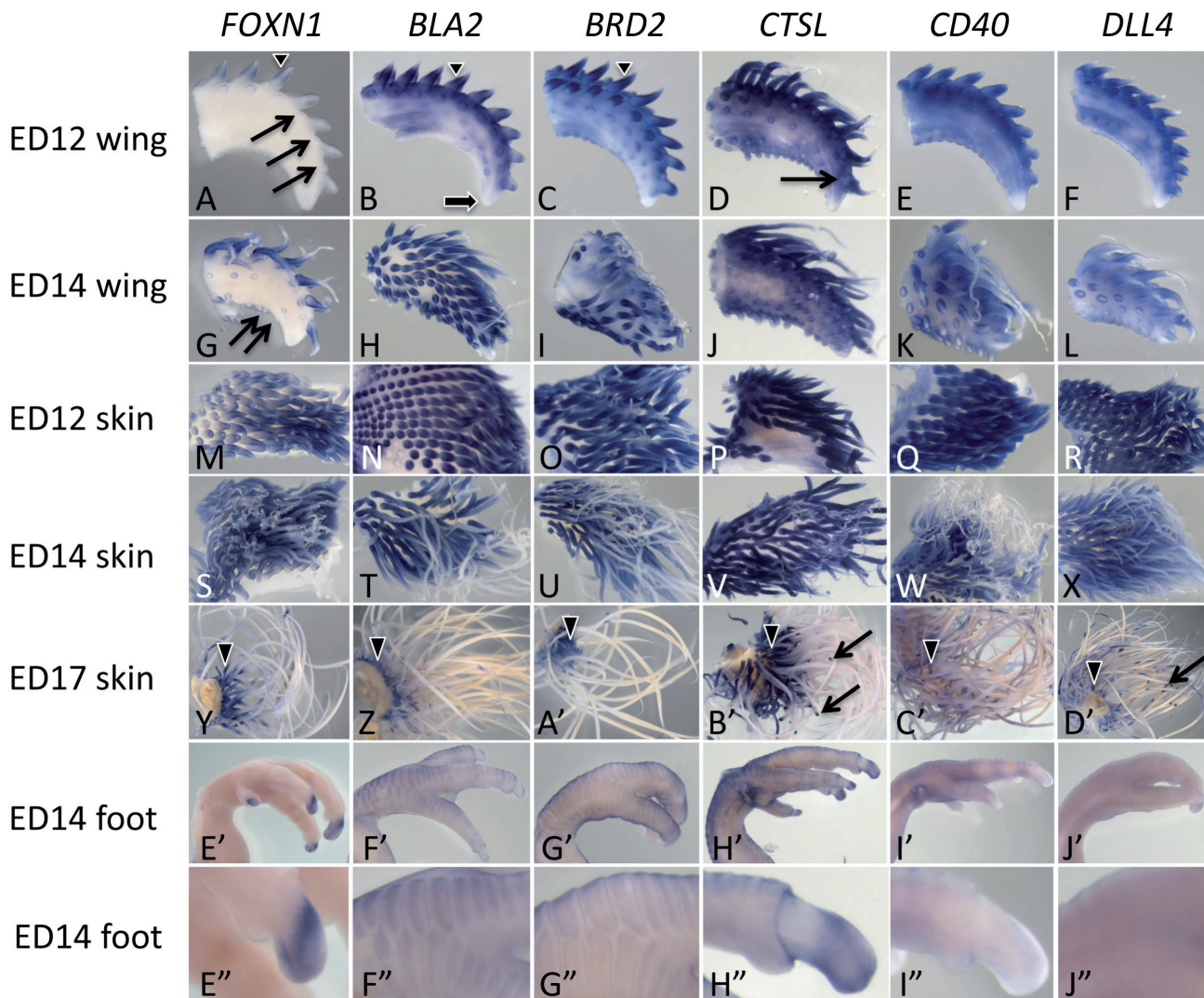
To determine specific localization of *FOXN1* mRNA within the labeled feather filament, feathers were paraffin sectioned transversely. *FOXN1* expression was evident in the proximal section of the feather filament at ED12-13 in a tight peripheral band with alternating intensity (Fig. 2K) the 'ring sheath cells' (Haake *et al.*, 1984). At the midpoint of the developing filament, *FOXN1* mRNA was expressed within the barb plates (Fig. 2L). At the feather tips, expression was much diminished (Fig. 2M). Thus, our data suggest that *FOXN1* may play a role in regulation of differentiation programs or morphogenesis of the feather filament.

#### **Expression of *FOXN1* and its putative target genes in cutaneous appendages**

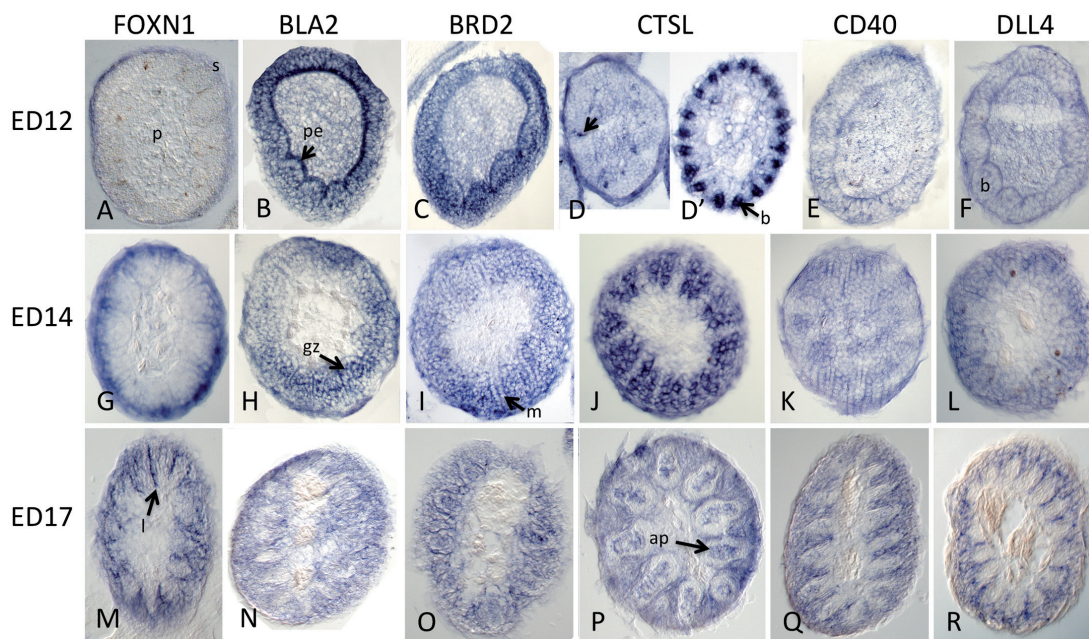
Putative downstream target genes of *FOXN1* have been identified in mouse thymocytes (Nowell *et al.*, 2011) and human embryonic stem cells (hESNet). In addition to regulating keratins (e.g., Mecklenburg *et al.*, 2004), the regulation of MHC Class II genes, *BRD2* (Bromodomain-containing gene 2), *CTSL* (Cathepsin L, a lysosomal protease), *CD40* (TNF-related receptor), and *DLL4* (delta-like gene 4) was positively correlated with *FOXN1* dosage (Nowell *et al.*, 2011; hESnet). To determine if these components represent a gene regulatory network that is conserved in other *FOXN1*-expressing

tissues, we used *in situ* hybridization to evaluate the expression of chick homologs of these genes in feathers and other epidermal appendages. Chick *BLA2* represented the MHC Class II genes, while other chick gene symbols are similar to mouse. Comparisons of expression in whole mount and feather section are shown in Figures 3 and 4. Our section photos were taken near the base of the feather filament where barb ridges are first forming on one side (Fig. 4, ED12), or at the proximodistal level corresponding to the

densest label for the majority of genes (ED14, ED17). While each of the potential targets exhibited expression in feather filaments and overlapped in expressing regions with *FOXN1*, none of the patterns were highly similar to *FOXN1* in either whole mounts (Fig. 3) or cross sections of feathers (Fig. 4). *FOXN1* expression was not detected as extensively in wing feathers or skin at ED12 (Fig. 3A), whereas the other five genes were strongly expressed (Fig. 3 B-F). At ED14, four of the putative target genes were expressed in



**Fig. 3. Expression of *FOXN1* and putative *FOXN1* regulatory targets in embryonic day 12, 14 and 17 epidermal appendages.** Photo columns are labeled with the gene name for the probe, rows indicate the days of incubation and tissue or region shown. All *in situ* probes labeled in feathers on embryonic day 12 (A-F, M-R), 14 (G-L, S-X) and 17 (Y-D'). (A-F) Embryonic day 12 wing tips. (A) *FOXN1* was expressed in some feather buds with stronger expression at the midpoint of the shaft (arrowhead) and lower expression at the base and tip. Some smaller buds (arrows) and the skin did not express. (B-F) The other five genes were also expressed more intensely in the mid-shaft (arrowhead) than at the base or tip, but also in skin and all feather buds. The distal wing tip had less expression for all genes tested (arrow in B). In wing tips at day 14, some feather buds still had no detectable *FOXN1* (G, arrows) but most feather buds showed stronger gene expression (G-L). At day 14 of incubation, wing skin had no detectable expression of *FOXN1* (G) and *BLA2* (H), but expressed other genes (I-L). Day 12 (M-R) and 14 (S-X) feathers from the back of the neck expressed all six genes similarly at this gross level. By day 17, feathers all showed probe binding proximally (all probes, arrowheads, Y-D'). Where the sheath had been bent or cut (e.g., arrows in B', D'), damaged tissue stained. No skin label was detected. ED14 foot: An evaluation of foot scales and nails, two other epidermal appendages. (E') *FOXN1* was expressed solely in the nails. Two genes were expressed in the scales (F', G'). *CTSL* (H') was expressed in both scales and nails. *CD40* (I') and *DLL4* (J') were expressed in neither scales nor nails, although it appears that *CD40* may have been expressed in some deeper tissue (I'). Final row shows close ups of (E'-J').



**Fig. 4. Expression of *FOXN1* and putative *FOXN1* regulatory targets in embryonic day 12, 14 and 17 feather cross sections.** Photo columns are labeled with the gene name for the in situ probe, rows indicate the days of incubation. Panels show the results of in situ hybridization of probes for the genes *FOXN1* (A,G,M), *BLA2* (B,H,N), *BRD2* (C,I,O), *CTSL* (D,D',J,N), *CD40* (E,K,Q) and *DLL4* (F,L,R). *FOXN1* and putative *FOXN1* target genes show dynamic and interesting expression patterns in feather compartments. However, the expression of each of the genes is distinctly different

from that of *FOXN1* at similar stages and proximodistal levels. See text for detail. Structures are labeled in various panels to help with feather terminology: ap, axial plate (P); b, barb plates (F); gz, growth zone (H); l, line (M); m, marginal cells (I); p, pulp (A); pe, pulp epithelium (B); s, sheath (A).

skin whereas *FOXN1* and *BLA2* were not. Feather expression was similar but not identical between *FOXN1* and the putative target genes for body feathers at ED12-17 (compare Fig. 3 rows ED12 skin, ED14 skin and ED17 skin). In ED14 feet (scales and nails) the difference in expression of these genes was more pronounced. *FOXN1* mRNA was expressed in nails but not in scales (Fig. 3E', E"). *BLA2* and *BRD2* were expressed in scales not nails (Fig. 3F', F", G', G"). *CTSL* was expressed in scales and nails (Fig. 3H', H"), whereas *CD40* (Fig. 3I', I") and *DLL4* (Fig. 3J', J") were expressed in neither scales nor nails. *FOXN1* and *CTSL* expression were similar in nails. We conclude that all of the tested candidate target genes for *FOXN1* are expressed in some avian epidermal appendages; however, direct *FOXN1* regulation is not sufficient to explain all of their expression patterns.

To investigate specific localization of *FOXN1* mRNA and these five candidate genes within feather filaments, hybridized feathers were paraffin sectioned transversely. At ED12 at the base of the feather where barb plates were just beginning to form, *FOXN1* transcripts were predominantly localized in the feather sheath (Fig. 4A), and more distally in the ends of the marginal plates (not shown) whereas transcripts of the five *FOXN1* candidate targets did not share this pattern (Fig. 4 B-F). For example, at ED12 *BLA2* (and somewhat less so *BRD2*) mRNA was expressed in the pulp epithelium and within proximal barb plates. *CTSL* mRNA was ubiquitously expressed with stronger intensity in the sheath and distributed cells (Fig. 4D, arrow), whereas slightly more distally strong expression was uniquely seen in the barbule plates outside the growth zone (Fig. 4D'), a pattern not shared with any of the other probes. *DLL4* and *CD40* mRNA were weakly expressed in the outside layer of the feather sheath and in the pulp epithelium (Fig. 4 E,F). At ED13-14, in a more distal part of the filament, the overlap in expression patterns was more similar but with identifiable differences (Fig. 2L, 4G-L). *FOXN1* was still expressed in the sheath, and also in the lateral barb plates and marginal cells.

Labeling of *BLA2* appeared stronger in the barb ridge (Fig. 4H), *BRD2* labeled lateral barb plates, medial barb ridges and marginal cells equally (Fig. 4I) whereas *CTSL* was strongly expressed in the barb plates (Fig. 4J). Expression of *CD40* and *DLL4* persisted fairly ubiquitously at this level (Fig. 4 K,L) with the exception of the dermal pulp for *DLL4*. By ED17 the strongest *FOXN1* label was in a thin line of unknown fate between the barbs, with barb and marginal cells labeling but no label in sheath, medullary cells of the axial plate or pulp (Fig. 4M). A similar labeling pattern was observed for the other genes with the exception of the inter-marginal line, and that *BRD2* and *CTSL* also labeled in the axial plate of the barbs (Fig. 4 N,R). Based on these data, the putative *FOXN1* target genes show dynamic and interesting expression patterns in feather compartments. However, the expression of each of the genes is distinctly different from that of *FOXN1* at similar stages and proximodistal levels.

## Discussion

In the present study, we confirm that chick *FOXN1* is expressed in the developing thymus and demonstrate its expression in some epidermal appendages, suggesting a homologous, evolutionarily conserved role for *FOXN1* across amniotes. We have identified a novel expression pattern of *FOXN1* in embryonic feathers. Chick *FOXN1* initially is expressed along the length of the feather filament, with the exception of the base and tip. As the filament elongates between ED 12 and 17, *FOXN1* expression becomes restricted to the proximal end of the feather. Although others have published differences in proximal-distal gene expression (e.g., Yu *et al.*, 2002, Chodankar *et al.*, 2003), to our knowledge *FOXN1* and *FOXE1* (Yaklichkin *et al.*, 2011), the two *FOX* genes we have characterized are the only known differentially and dynamically expressed genes along the proximal-distal axis of the elongating feather filament.

The murine ortholog of *FOXN1* regulates expression of specific

alpha keratins (Mecklenburg *et al.*, 2004). Although hair forms from soft alpha-keratins and feathers form predominantly from hard beta-keratins, it is possible *FOXN1* could be modulating expression of feather-specific keratins. Feather-specific beta-keratin expression coincides with differentiation of the barb ridges (Haake *et al.*, 1984, König and Sawyer 1985). Images of feather filaments in the Haake paper indicate the proximal to distal pattern of beta-keratin expression correlates well with the expression pattern of *FOXN1* shown here (Fig. 2). Specifically, beta-keratin expression is initiated at stage 38 (ED12) and expressed in the ring sheath cells similar to our image in Fig. 2K, whereas more distal expression is similar to Fig. 2L. Patterns of alpha-keratins also correlate with *FOXN1* expression (Alibardi, 2013). Based on these observations and the expression of both mouse and chick *FOXN1* in the nails and thymus, other keratinized epidermal structures, chick *FOXN1* may regulate expression of both alpha and beta-keratins.

Finally, expression of *FOXN1* and the other genes investigated in the feather raises more questions than it answers about the evolution of *FOXN1* function, considering that the feather is thought to have evolved from scutate scales of reptiles. Loss and gain of function studies will be required to establish a precise role for *FOXN1* transcriptional regulation in epidermal evolution and development.

## Materials and Methods

### Sequence, data and phylogenetic analysis

The protein sequences used in the analysis were obtained from the NCBI database (ncbi.nlm.nih.gov) and Ensemble database 69 (ensemble.org). The accession numbers of *FOXN1/N4* protein sequences analyzed are provided in Supplementary Table S1. Multiple sequence alignments were constructed using T-COFFEE, version 7.7.1. (tcoffee.vital-it.ch/cgi-bin/Tcoffee/tcoffee.cgi/index.cgi). Aligned *FOXN1/N4* sequences were edited using the alignment editor BioEdit 7.0.4.1. (mbio.ncsu.edu/bioedit/page2.html). A phylogenetic tree for *FOXN1/N4* proteins was generated based on the forkhead DNA-binding domain sequences (~100 residues). A neighbor-joining method was used to construct the tree topology. The phylogenetic tree was converted into a cladogram using MEGA 4 (Kumar *et al.*, 2004) (metameme.sdsc.edu).

### Incubation and isolation of chicken tissues

Fertilized white leghorn eggs (*Gallus gallus*) were incubated in a forced-draft incubator at approximately 38°C with high humidity. Slight variations in temperature between incubators or over the course of the experiment make staging approximate due to long incubation times, therefore days of incubation are given. For older embryos, neck/thorax and skin/feathers were isolated prior to fixation with paraformaldehyde.

### Molecular cloning and in situ hybridization with gene probes

To clone chicken *FOXN1* cDNA sequences for the *in situ* probe template, mRNA was isolated from day 5 chicken embryos using the Qiagen RNeasy Mini Kit (cat. number: 74104) and cDNA synthesized using standard procedures. Template for probe generation was generated by PCR with the GC-rich PCR System Kit (cat. number: 12 140 306 001), Roche Applied Science; 34 cycles of annealing at 50°C and the following primers: forward primer, 5'-TTATAAAAGCTTGCGGCCGAGAATAT**CACTACCCCTAC-CAAAGGATTGC**-3'; reverse primer: 5'-GCTCTAGAAATTAACCCTCATAAAGGGCCTCCCGCCTCCCGAAGGAAACATTG-3'. Chick *FOXN1* sequences are in bold, the T3 RNA polymerase binding site is italicized. The PCR generated template was sequenced to confirm identity (NCBI Accession number: XM\_415816.3, 904-1622 bp). A 719 nt RNA probe of *FOXN1* was produced using T3 polymerase and *in situ* hybridizations were performed using the GEISHA mRNA Detection Protocol (geisha.arizona.

edu/geisha/protocols.jsp). *FOXE1* *in situ* hybridization was performed as previously described (Yaklichkin *et al.*, 2011). Probe for MHC Class II gene, *BLA2* (NCBI Acc. #: NM\_001245061) was generated from GEISHA plasmid W34 with NotI using T7 polymerase. Probe for *BRD2* was generated from plasmid ChEST826a15 (NM\_001030674.1) with NotI and T3 polymerase. Probe for Cathepsin L, *CTSL2* (NM\_001168009) was generated from GEISHA plasmid S12 with NotI and T7 polymerase. Probe for *CD40* (*TNFRSF5*, NM\_204665) was generated by PCR: Forward primer: 5'-CTGACAAGCAGTATGAGCACAAGG-3', Reverse primer: 5'-**ATTACCCTCACTAAAGGGGAAGTCCACCTCCTCGGG**-3', where the bold sequence allows for directional amplification of the 666 nt probe using the T3 polymerase. Probe *DLL4* (XM\_421132) was generated from plasmid pgf2n.pk001.e7 (www.chickest.udel.edu) using NotI and T3 polymerase. Probe sequences and additional images are available at <http://geisha.arizona.edu/geisha>.

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