Progress Report

Molecular Cloning and Characterization of Neuronal Intermediate Filament Protein α-internexin in Chicken

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2008/07 ~ 2010/11

Introduction

- **α-internexin (INA)**
  - a ~66 kDa intermediate filament protein (Pachter and Liem, 1985)
  - The protein is present in developing neuroblasts and in the CNS of adults (Kaplan *et al*., 1990) .

- **Chicken Embryos**

http://www.springerlink.com/content/u515617p37206051/

  - an excellent model system for studying the development of vertebrates wherein growth accompanies morphogenesis

Discovery of α-internexin

1980 | 1990 | 2000

1985

Rat

Protein: Pachter and Liem, 1985.

DNA: Kaplan *et al*., 1990.

(characterization)

1994

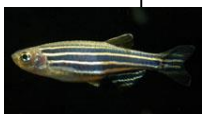
Mouse

Chien and Liem, 1994.

Zebrafish

(gefiltin)

Glasgowet *et al*., 1994.



1995

Human

Chan and Chiu, 1995.

1997

Frog *(Xenopus laevis)*

Zhao *et al*., 1997.

Chicken ***(Gallus gallus)*** **genome draft sequence**

2004

How about chicken?

Sequence and comparative analysis of the chicken genome provide unique perspectives on vertebrate evolution

International Chicken Genome Sequencing Consortium*

*Lists of participants and affiliations appear at the end of the paper

NATURE | VOL 432 | 9 DECEMBER 2004 | www.nature.com/nature

A draft genome sequence of the red jungle fowl (*Gallus gallus*)

(the 6.6X coverage draft sequence of the chicken genome)

- **38 autosomes + ZZ (male) / ZW (female)**
- **20,000-23,000 genes**
- **Lesser interspersed repeat content, pseudogenes and segmental duplications within the chicken genome.**
- **At least 70 megabases (Mb) of sequence that is highly likely to be functional in chicken and human genomes**

Aims

- **Discover the mRNA sequence encoding α-internexin from chicken embryos**
- **Study the expression of chicken α-internexin during neuronal development**
- **Confirm the physiological features of chicken α-internexin**

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- **Discover the mRNA sequence encoding α-internexin from chicken embryos**
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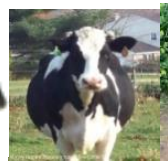
- **Confirm the physiological features of chicken α-internexin.**

Location of neural IFs in different species


Human


Rat


Mouse


Cow


Chicken


Zebra finch


Frog


Zebra fish


Chimpanzee


Platypus


Dog

Species	*Homo sapiens*	*Rattus norvegicus*	*Mus musculus*	*Bos Taurus*	*Gallus gallus*	*Taeniopygia guttata*	*Xenopus laevis*	*Danio rerio*	*Pan troglodytes*	*Ornithorhynchus anatinus*	*Canis familiaris*
Chromosome No.	46, XX / XY	42, XY / XX	40, XX / XY	60, XX / XY	78, ZW / ZZ	82, ZZ / ZW	unknown	50	46, XX / XY	52	78, XX / XY
Autosome	22 pairs	20 pairs	19 pairs	29 pairs	38 pairs	40 pairs	unknown	25 pairs	22 pairs	21 pairs	38 pairs
Sex chrosome	XX (female) / XY (male)	XX (female) / XY (male)	XX (female) / XY (male)	XX (female) / XY (male)	ZZ (male) / ZW (female)	ZZ (male) / ZW (female)	unknown	n	XX (female) / XY (male)	10 unpaired (X1-X5, Y1-Y5)	XX (female) / XY (male)
Linkage group (LG)	n	n	n	n	2 (LGE64, LGE22C19W28_E50C23)	3 (LGE22, LG2, and LG5)	unknown	n	n	n	n
Neural IF	**Chromosome (Location)**										
NF-L	8 (8p21)	15 (15p12)	14 (14 D3)	8	22	22	v	8	8	contig: NW_001794412	25
NF-M	8 (8p21)	15 (15p12)	14 (14 D1)	8	22			8	8	contig: NW_001794412	25
NF-H	22 (22q12.2)	14 (14q21)	11 (A1-A5)	17	15	15			22	contig: NW_001697718	26
α-internexin	10 (10q24.33)	1 (1q54)	19 (19 C3)	26		6	v	5	10		28
Nestin	1 (1q23.1)	2 (2q34)	3 (3 42.5 cM)	3	25			16	1		7
Vimentin	10 (10p13)	17 (17q12.3)	2 (2 7.0 cM)	13 (13q16-q17)	2	2	v	24	10	contig:NW_001656448	2 or 8 or 25
GFAP	17 (17q21)	10 (10q32.1)	11 (11 62.0 cM)	19	27			3	17		9

To find out the candidate sequence of chicken α-internexin

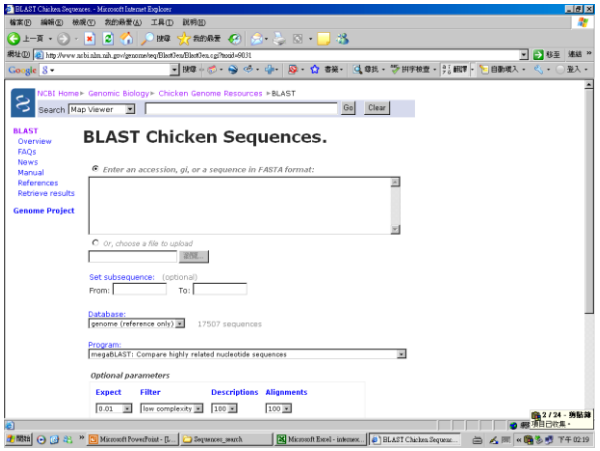
Alpha-internexin mRNA sequences

Organism	Official Symbol	Accession No.
Homo sapiens	INA	NM_032727.3
Rattus norvegicus	Ina	NM_019128.4
Mus musculus	Ina	NM_146100.4
Xenopus laevis	xefiltin	NM_001085809.1
Danio rerio	gefiltin	NM_131032.1

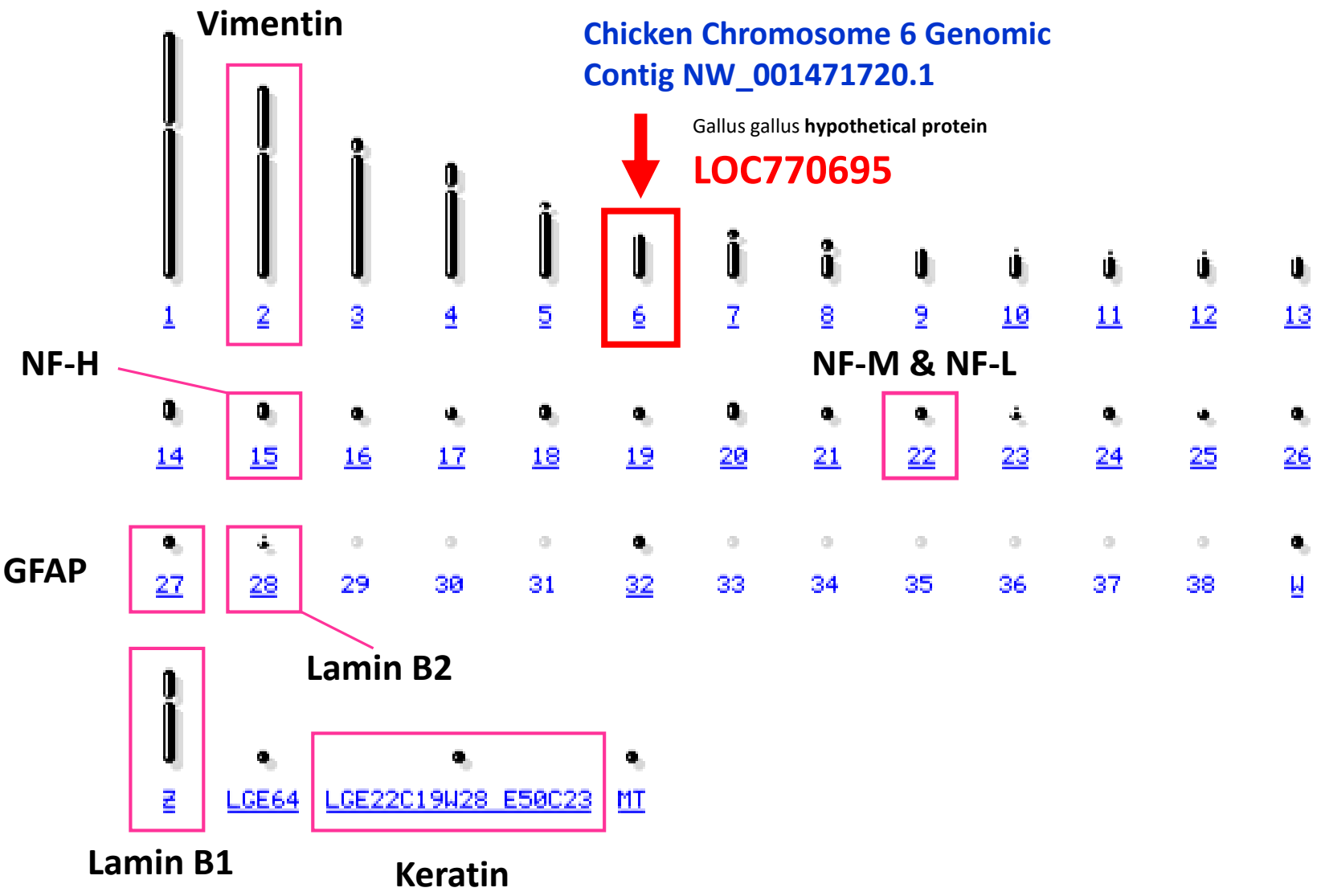
BLAST

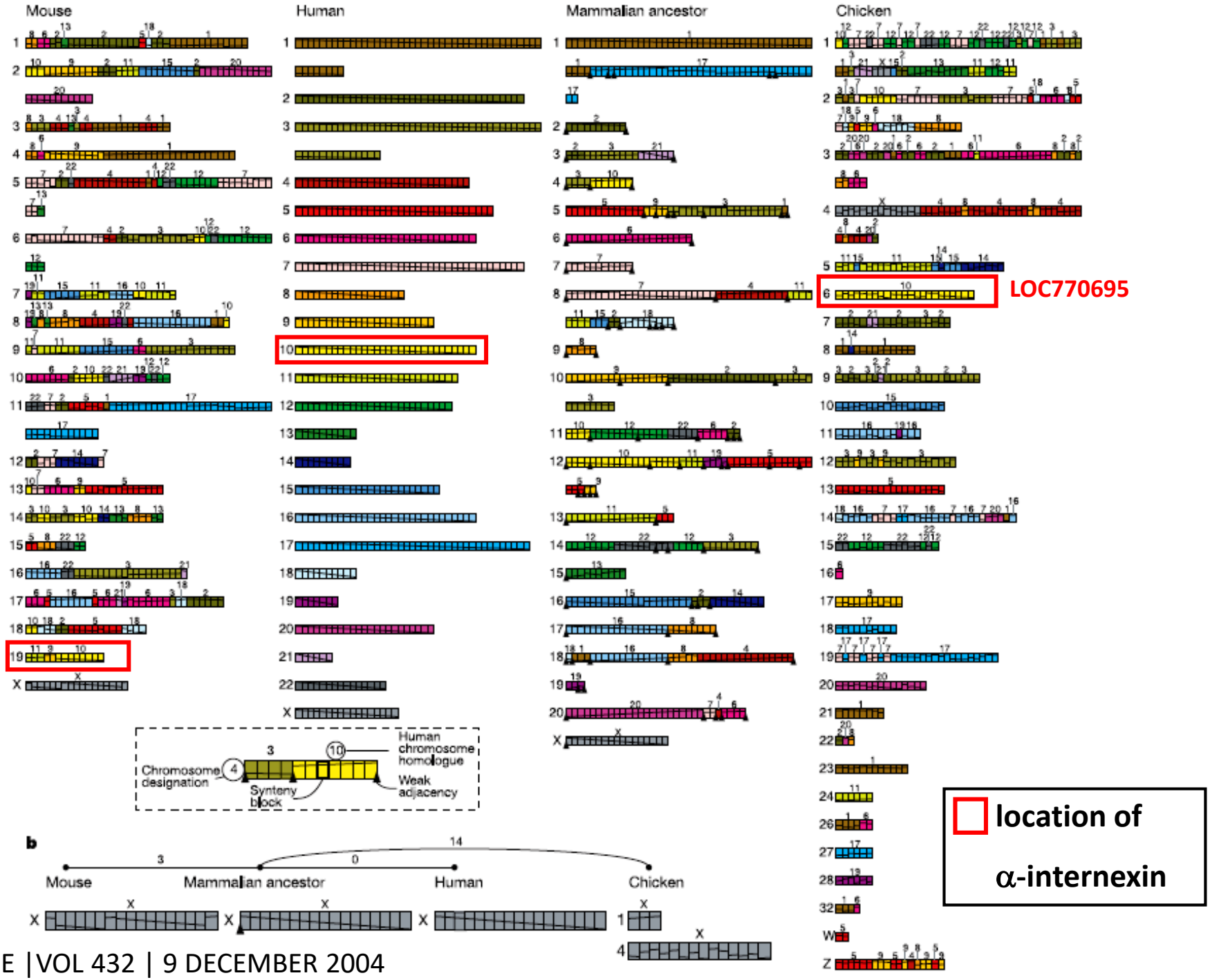
Chicken Genome Resources

http://www.ncbi.nlm.nih.gov/genome/seq/BlastGen/BlastGen.cgi?taxid=9031



Blast results

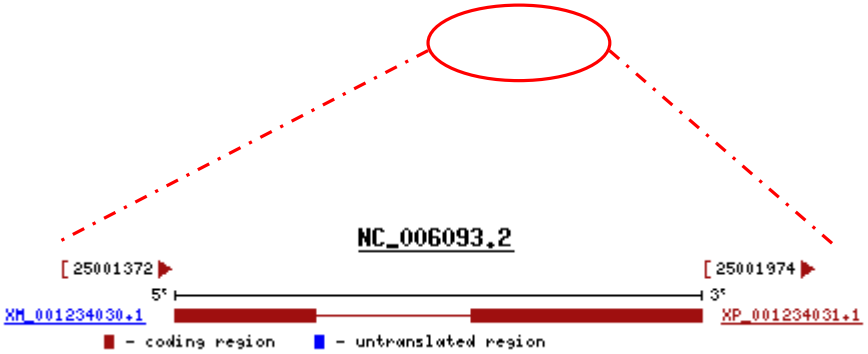



Mouse
Human
Mammalian ancestor
Chicken
LOC770695
Chromosome designation
Syntenty block
Human chromosome homologue
Weak adjacency
location of α-internexin
b
Mouse
Mammalian ancestor
Human
Chicken

Description of LOC770695 on GenBank

→ **XP_001234031.1**
PREDICTED: hypothetical protein [Gallus gallus] (**141 aa**)
→ **XM_001234030.1**
PREDICTED: Gallus gallus **hypothetical protein LOC770695**, mRNA (**426 bp**)
→ Chromosome: **6**

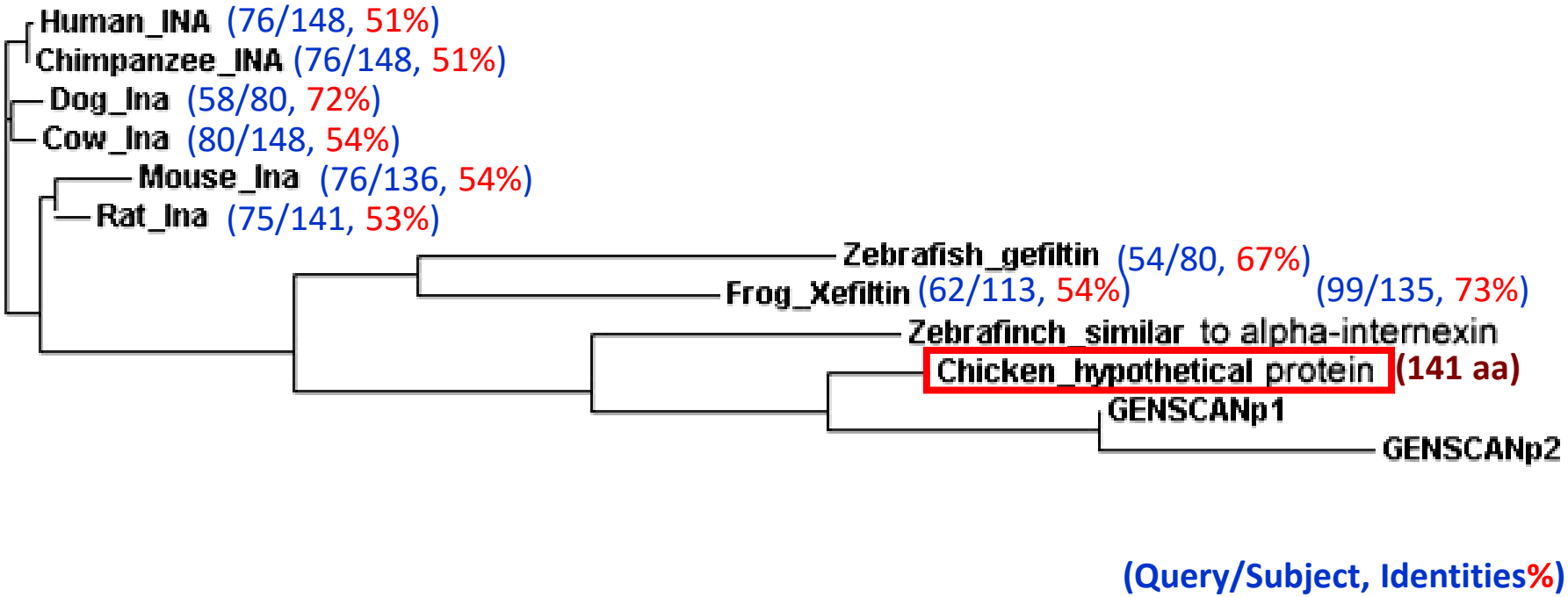
→Genomic: **NW_001471720.1**
Range: **564337..564939**



Reference Protein		Species	Id (%)	Length (aa)
XP_001234031.1	PREDICTED: hypothetical protein	*G. gallus*	100.0	140
XP_001479252.1	PREDICTED: similar to Ina protein	*M. musculus*	54.4	136
NP_116116.1	internexin neuronal intermediate filament protein, alpha	*H. sapiens*	51.7	148
NP_571107.1	gefiltin	*D. rerio*	50.4	119
NP_001079278.1	internexin neuronal intermediate filament protein, alpha	*X. laevis*	47.8	134

Cladogram of LOC770695 and INA homologous

INA, α-internexin

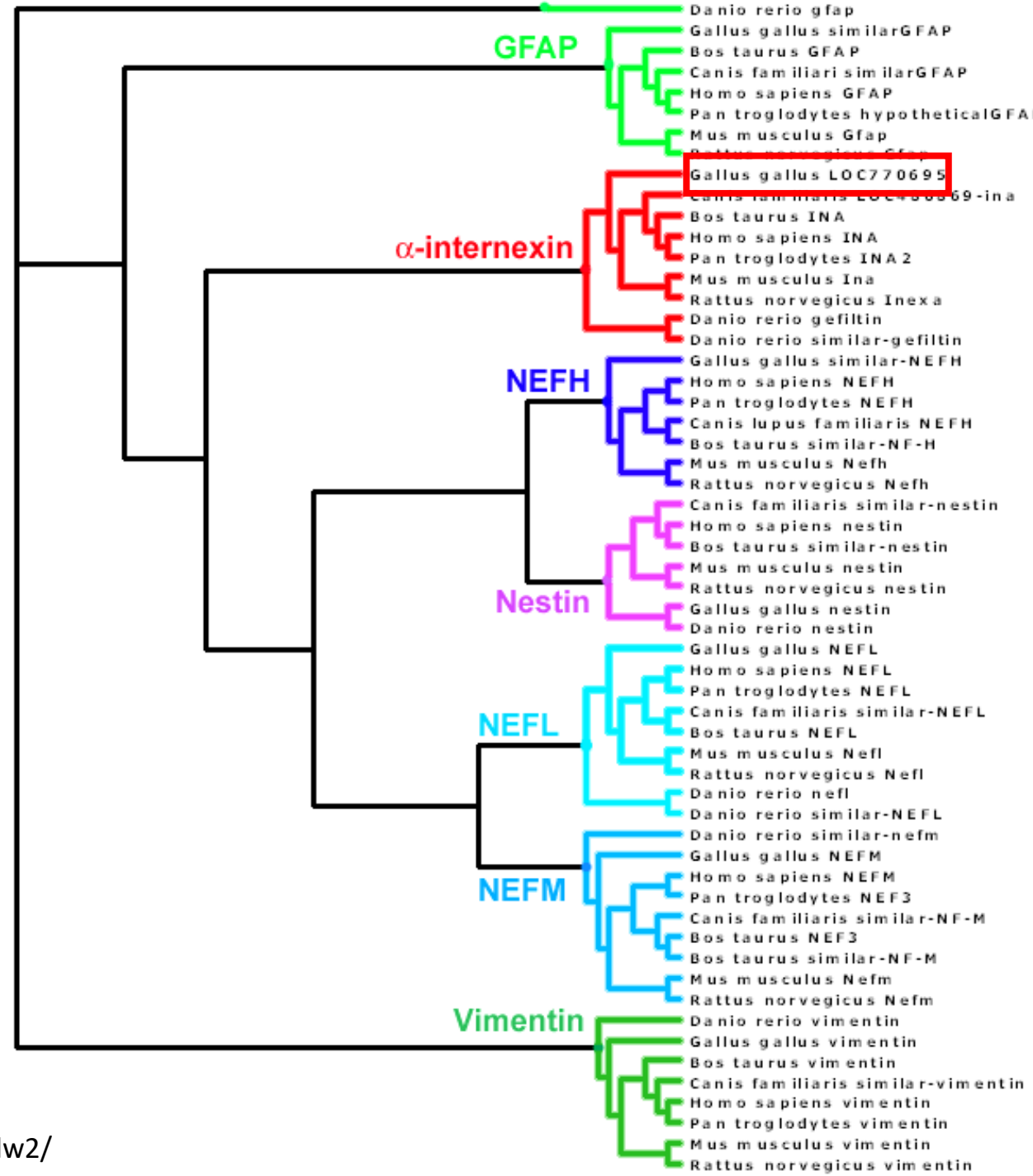


(Query/Subject, Identities%)

ClustualW2

http://www.ebi.ac.uk/Tools/msa/clustalw2/

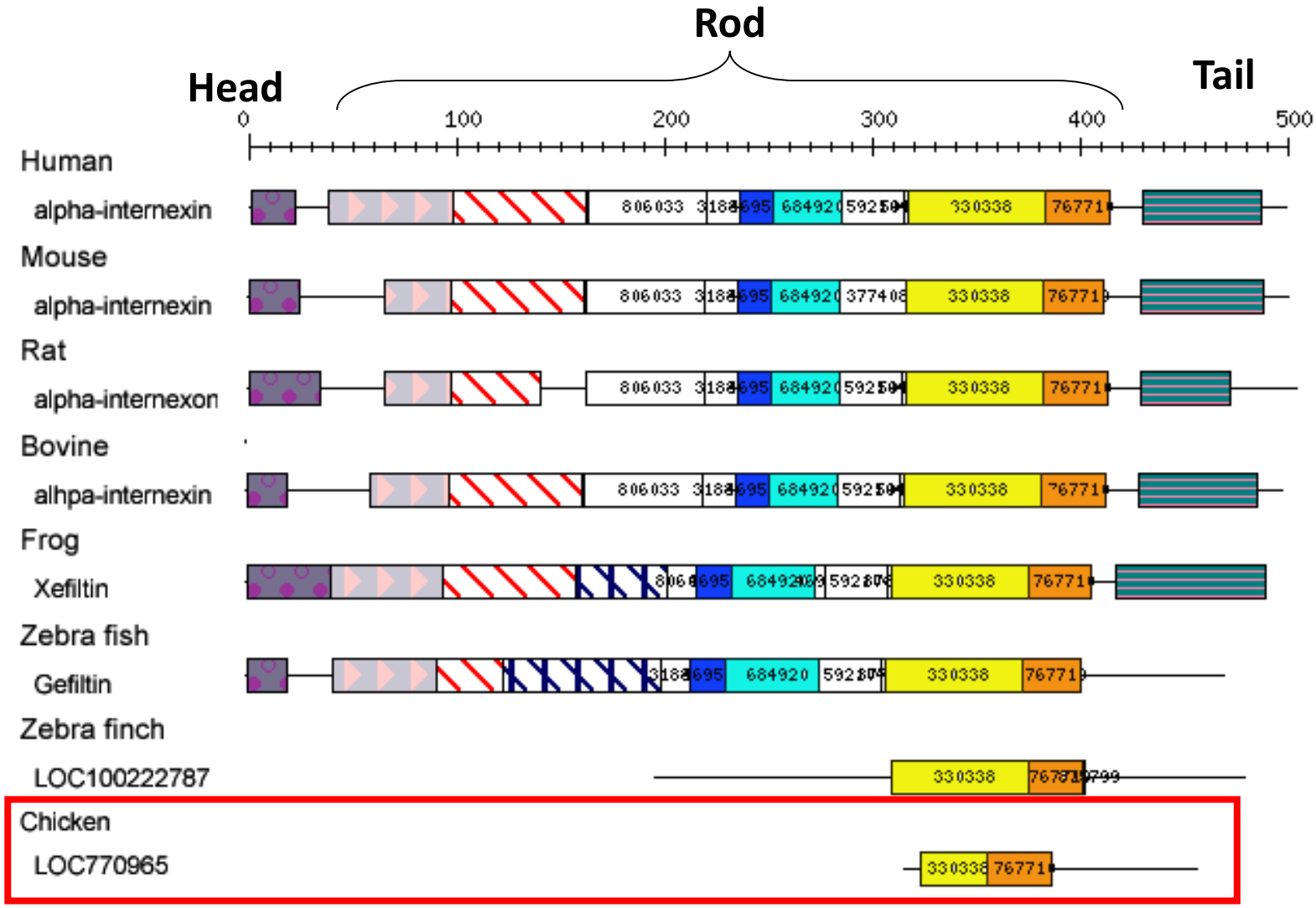
Cladogram of LOC770695 and other neural IFs



ClustualW2

http://www.ebi.ac.uk/Tools/msa/clustalw2/

Amino sequence alignments of LOC770695 and INA homologous



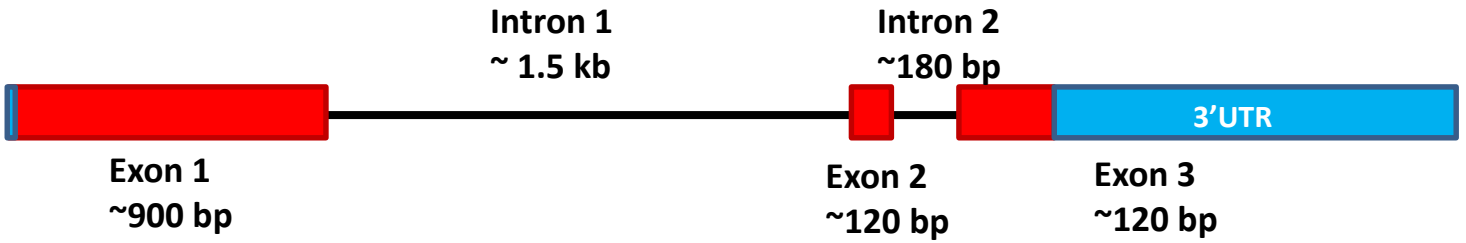
Sequence alignments were done by online tool, **ProDom.**

http://prodom.prabi.fr/prodom/current/html/form.php

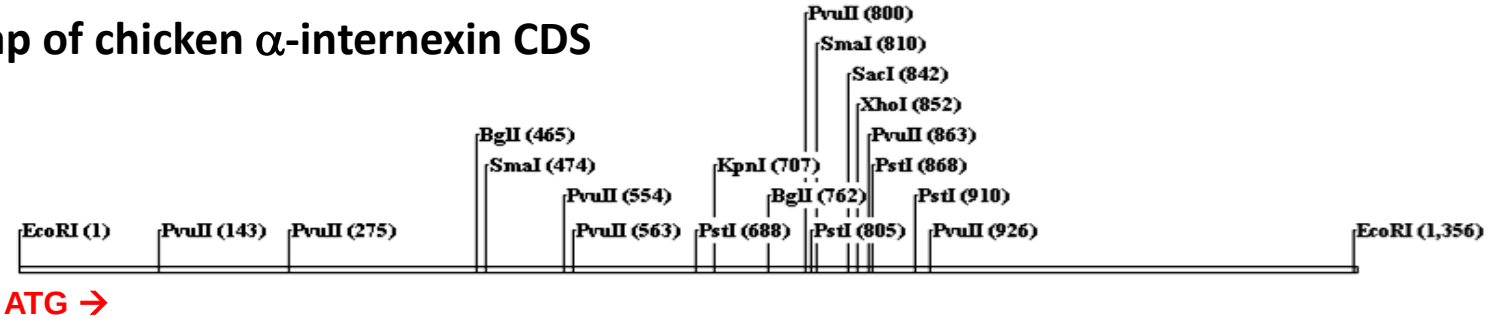
Chasing the chicken α-internexin by RACE (Rapid Amplification of cDNA Ends)

XM_001234030.1
PREDICTED: Gallus gallus hypothetical protein **LOC770695**, mRNA (**426 bp**)

Putative sequence of chicken α-internexin CDS：1302 bp

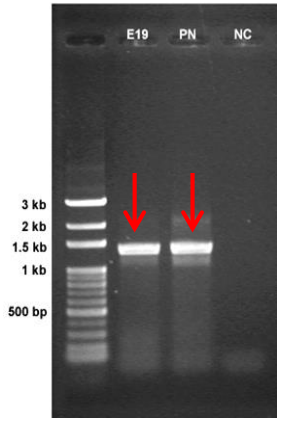


RE map of chicken α-internexin CDS



The putative cDNA sequence of chicken α-internexin (chkINT)

CDS: 1302 bp

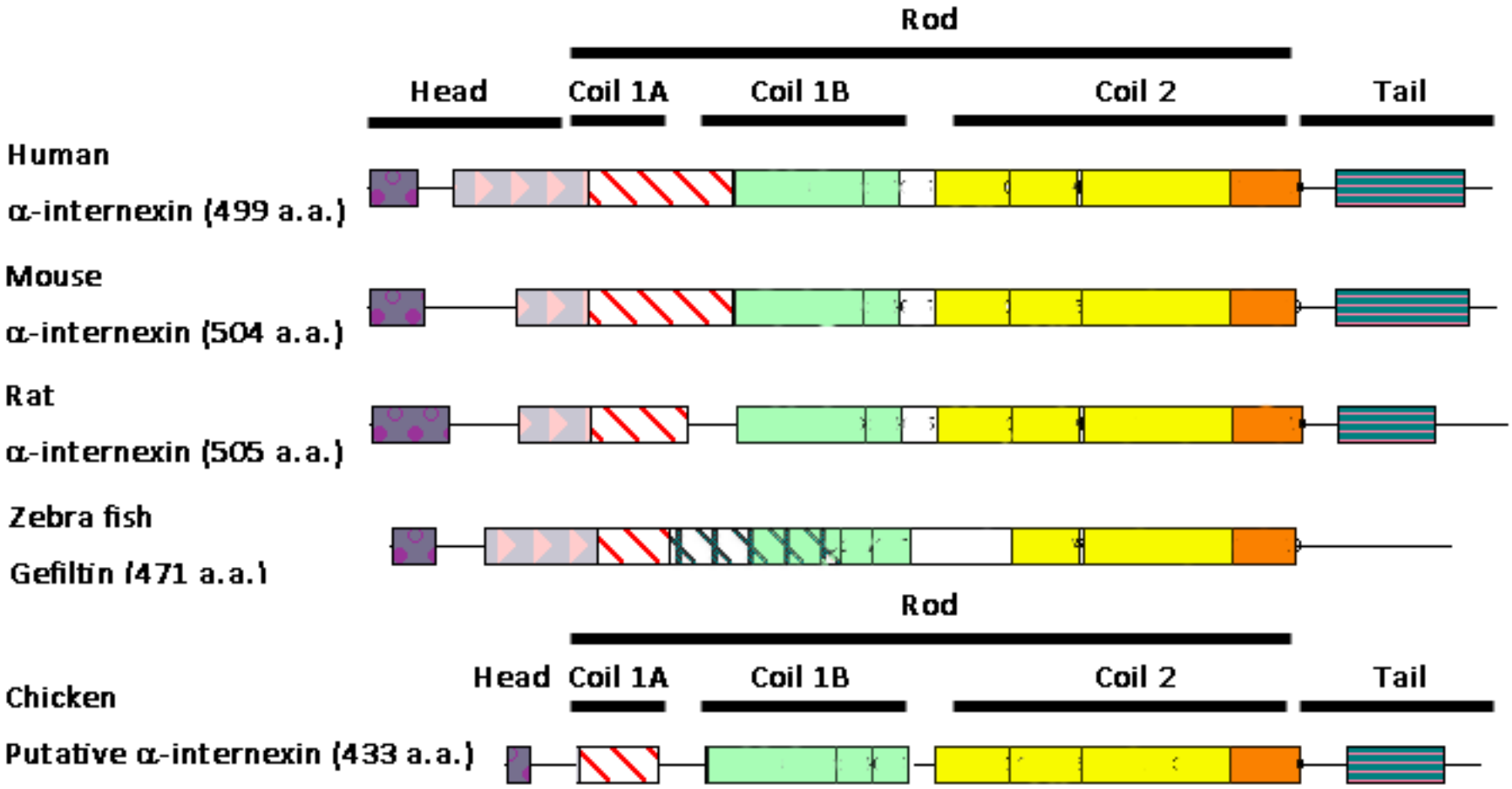


Predicted chicken α-internexin (chkpINT)

- 433 a.a.
- Theoretical pI/Mw: 9.37 / 48435.59 (**48.4 kDa**)

```
ATGAGCTACAGCGTGGAACCGCCGGCGCTAGCCGCCTCCTCCCGCCGCCTGCTCGCCCAG
 M  S  Y  S  V  E  P  P  A  L  A  A  S  S  R  R  L  L  A  Q
TCCCCGCGGCGCACGGAGGGCGCTGAGCCGCGCCGTGCTAGCGAGAAGGAGCAGCTGCGG
 S  P  R  R  T  E  G  A  E  P  R  R  A  S  E  K  E  Q  L  R
GGCCTCAACGAGCGCTTCGCCGGTTACATCGAGCGTGTCCGGGCGCTGGAAGAGCGCAAC
 G  L  N  E  R  F  A  G  Y  I  E  R  V  R  A  L  E  E  R  N
CGGGCTCTGGCCGGCGAGCTGGCGGAGCTAAGGCGGCTGCCTCCCGAACCGCGCCGTCTG
 R  A  L  A  G  E  L  A  E  L  R  R  L  P  P  E  P  R  R  L
GGGCAGCTGCTGGGCGGGGAGCTGCGGGCCCTGCGCGCCCGTTTGGAGGAGGCACACGGC
 G  Q  L  L  G  G  E  L  R  A  L  R  A  R  L  E  E  A  H  G
GAGCGGGCGCAGGCCGCGCTGGAGCGGGCGCGGCTGGCCGAGGAGACGCAGCGGCTGCGG
 E  R  A  Q  A  A  L  E  R  A  R  L  A  E  E  T  Q  R  L  R
GCGCGCTGCGAGGAGGAGGCGCGGGGCCGCGCCGAGGCGGAGCAGGCGTTGCGCGCCCGG
 A  R  C  E  E  E  A  R  G  R  A  E  A  E  Q  A  L  R  A  R
CAGCAGGCGGCCGACGGGGCGGCCCGGGCCCGCGCCGACCTGGAGCGGCGGGCGGAGGCG
 Q  Q  A  A  D  G  A  A  R  A  R  A  D  L  E  R  R  A  E  A
CTGCGGGAGGAGCTGGCGGAGCTGCGGCGCGCCCACGCCGAGCAGCTGGCCCAGCTGGGA
 L  R  E  E  L  A  E  L  R  R  A  H  A  E  Q  L  A  Q  L  G
GCCGCGCTCCGCGCCGCCGCTCCCCCGGCCTCCGGGCCCCCGACGGCGCGGCCCGACCTG
 A  A  L  R  A  A  A  P  P  A  S  G  P  P  T  A  R  P  D  L
GCGGCTGCGCTGCGGGAGCTGCGCGCTCAGTACGAGGCGCTGCCGGCCCGCAACCTGCAG
 A  A  A  L  R  E  L  R  A  Q  Y  E  A  L  P  A  R  N  L  Q
GCGGCCGAGGACTGGTACCGCGCCCGCTGCGCCAGCCTCCACGAGCGGGCGGCCCGCAGC
 A  A  E  D  W  Y  R  A  R  C  A  S  L  H  E  R  A  A  R  S
CAGGAGGCCGTCCGGGCCAGCCGCCGCGAGGCGGGCGAGTGCCGCCGGCAGCTGCAGGCC
 Q  E  A  V  R  A  S  R  R  E  A  G  E  C  R  R  Q  L  Q  A
CGGGTGGTGGAGATGGAGAGCCTGCGCGGAGCTCACGAGTCCCTCGAGAGGCAGCTGCAG
 R  V  V  E  M  E  S  L  R  G  A  H  E  S  L  E  R  Q  L  Q
GAGCTGGAGGAAAGGCACAGCGCCGAGGCCGCCGGGCTGCAGGACACCATTGGGCAGCTG
 E  L  E  E  R  H  S  A  E  A  A  G  L  Q  D  T  I  G  Q  L
GAGGCTGACCTGCGTAGCACTAAAACCGAGATGGCTCGGCACCTGAGGGAGTACCAGGAC
 E  A  D  L  R  S  T  K  T  E  M  A  R  H  L  R  E  Y  Q  D
CTGCTGAATGTCAAGATGGCCCTGGATATTGAGATTGCTGCCTACAGGAAGCTGCTGGAG
 L  L  N  V  K  M  A  L  D  I  E  I  A  A  Y  R  K  L  L  E
GGAGAGGAAAACCTGTTCAGCATGGGGAGTGTTGGCCTTCCAGCCATGAACCCCCTCCCC
 G  E  E  N  L  F  S  M  G  S  V  G  L  P  A  M  N  P  L  P
AACCCCACCTACTCTTTCCGGCCACGCTCCTCCACTCCATCCTTCAAGAAAGAGGAGCAA
 N  P  T  Y  S  F  R  P  R  S  S  T  P  S  F  K  K  E  E  Q
AGAGAGGCAGTTAGAGCGACCTCCAAGATACCATCTGGTCAGGCTGGAGTGCTTGACGGG
 R  E  A  V  R  A  T  S  K  I  P  S  G  Q  A  G  V  L  D  G
ACCATAACCACTGCTAAGAGAACGGAGAGATTCAACGTGCATGGAGGAATCATTGCAAAT
 T  I  T  T  A  K  R  T  E  R  F  N  V  H  G  G  I  I  A  N
GCTAAAGTGCAATGGTGGGAACCCCATCCCTTTTGCATTTGA
 A  K  V  Q  W  W  E  P  H  P  F  C  I  *
```

Amino sequence alignments of putative chicken α-internexin and INA homologous



Sequence alignments were done by online tool, **ProDom**, and modified by Photoshop software**.**

http://prodom.prabi.fr/prodom/current/html/form.php

The Kozak sequence of putative chicken α-internexin

Table 1. Frequency of nucleotides surrounding the initiator codon of transcripts encoding cytoplasmic proteins[a]

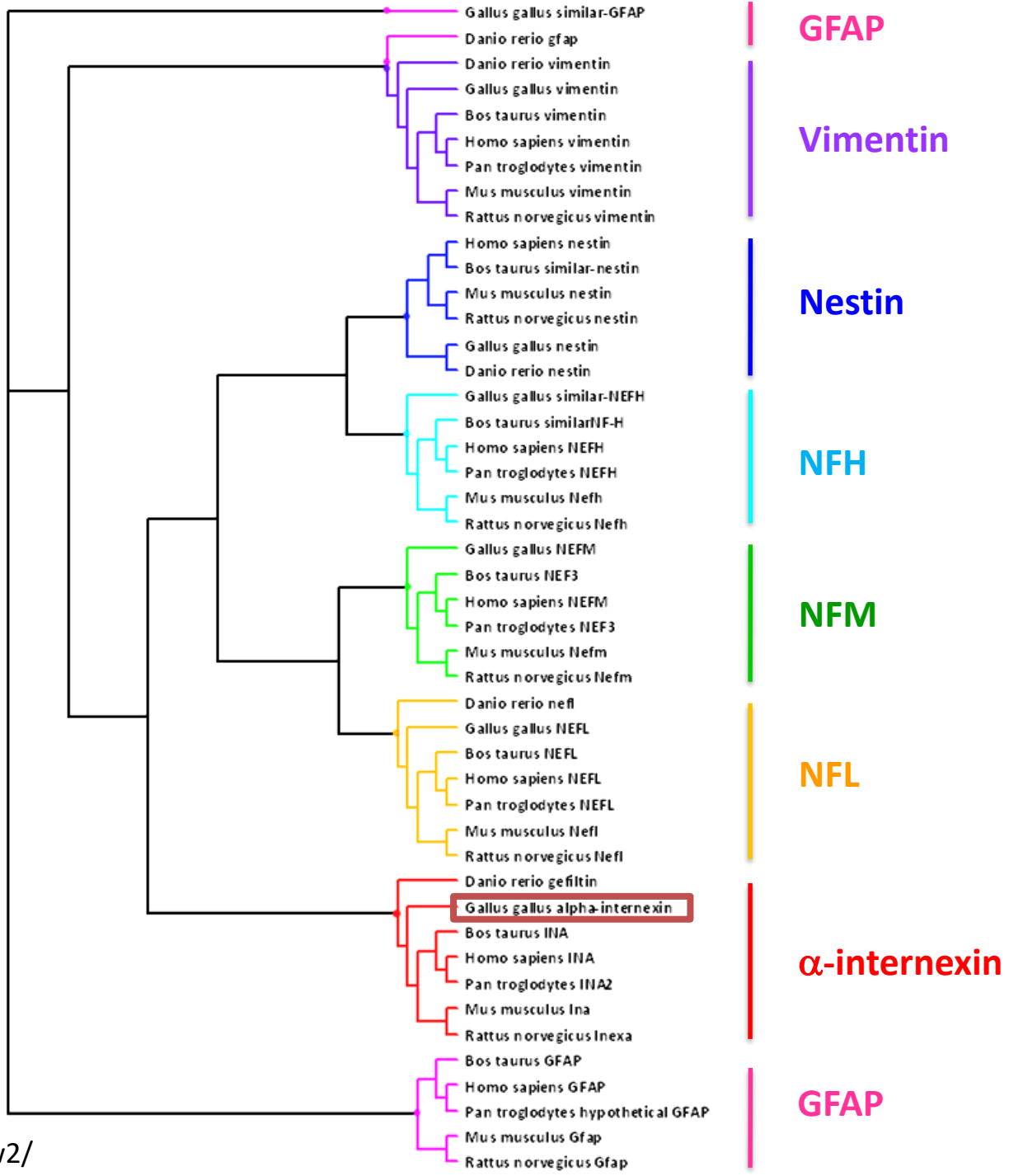
	–5	–4	–3	–2	–1	+1(A)	+2(T)	+3(G)	+4
A	17.07	21.77	47.00	30.37	20.99	100	0	0	19.36
T	19.29	10.16	5.60	10.56	6.12	0	100	0	13.36
G	31.42	28.61	37.15	18.57	27.64	0	0	100	53.38
C	32.20	39.43	10.23	40.48	45.24	0	0	0	13.88

[a]Sequences surrounding the initiator codon of 1534 manually reviewed RefSeq transcripts encoding cytoplasmic proteins. The frequency of occurrence of indicated nucleotides surrounding the initiator codon at positions –5 to +4 with respect to ATG is shown.

TRENDS in Genetics Vol.17 No.12 December 2001

Chicken (*Gallus gallus*) Neural Intermediate Filaments	Nucleotides Surrounding the Initiator Codon									
	-6	-5	-4	-3	-2	-1	+1	+2	+3	+4
NEFL (isoform 1)	A	C	C	G	C	C	A	T	G	A
NEFL (isoform 2)	A	C	A	G	C	C	A	T	G	A
NEFM	C	C	C	G	C	C	A	T	G	A
NEFH							A	T	G	C
Putative α-internexin	G	C	C	G	C	G	A	T	G	A

Cladogram of neural IFs from different species



The putative chicken α-internexin was classified into the group of α-internexin homologous.

ClustualW2
http://www.ebi.ac.uk/Tools/msa/clustalw2/

Percent identity and similarity between predicted amino acid sequence of chicken α-internexin and other species

Species	*Homo sapiens*		*Mus musculus*		*Rattus norvegicus*		*Bos taurus*		*Danio rerio*	
	Human		Mouse		Rat		Bovine		Zebra fiah	
Accession No.	NP_116116.1		NP_666212.2		NP_062001.1		NP_001069426.1		NP_571107.1	
Length (a.a.)	499		501		505		499		471	
Idenitities	55%	(242/434)	55%	(235/421)	56%	(237/418)	56%	(240/423)	52%	(179/343)
Similarity	72%	(313/434)	71%	(299/421)	72%	(302/418)	73%	(309/423)	71%	(246/343)
Region	69-499		69-489		77-494		77-499		85-423	

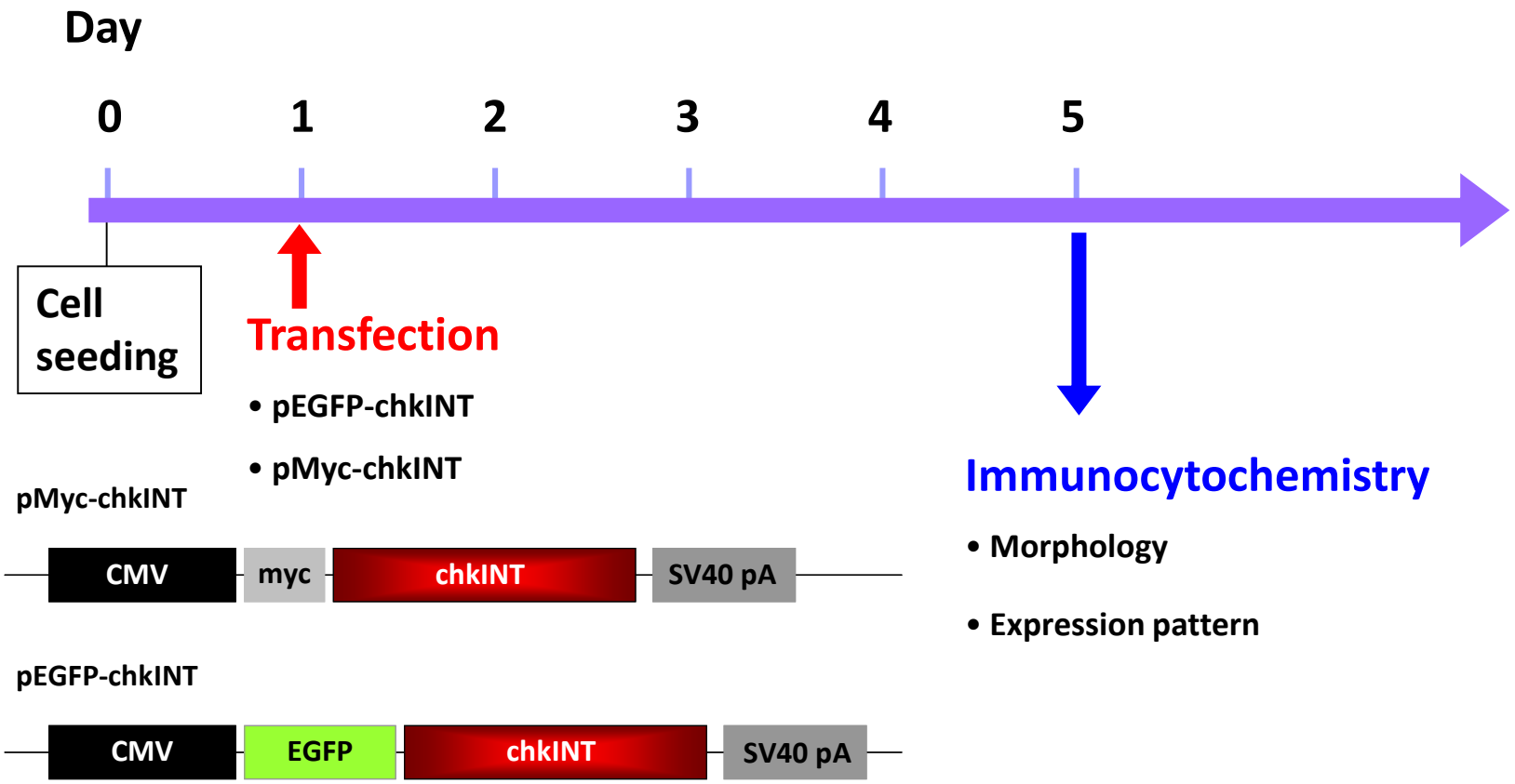
Percent identity and similarity between predicted amino acid sequence of chicken α-internexin and NF triplets

Species	*Gallus gallus*							
Homologs	similar-NEFH		NEFM		NEFL (Var.1)		NEFL (Var.2)	
Accession No.	XP_415310.2		NP_001095200.1		XP_001232615.1		XP_417679.1	
Length (a.a.)	890		858		483		556	
Idenitities	47%	(179/379)	49%	(162/328)	47%	(179/379)	58%	(71/121)
Similarity	64%	(244/379)	67%	(223/328)	64%	(244/379)	71%	(87/121)

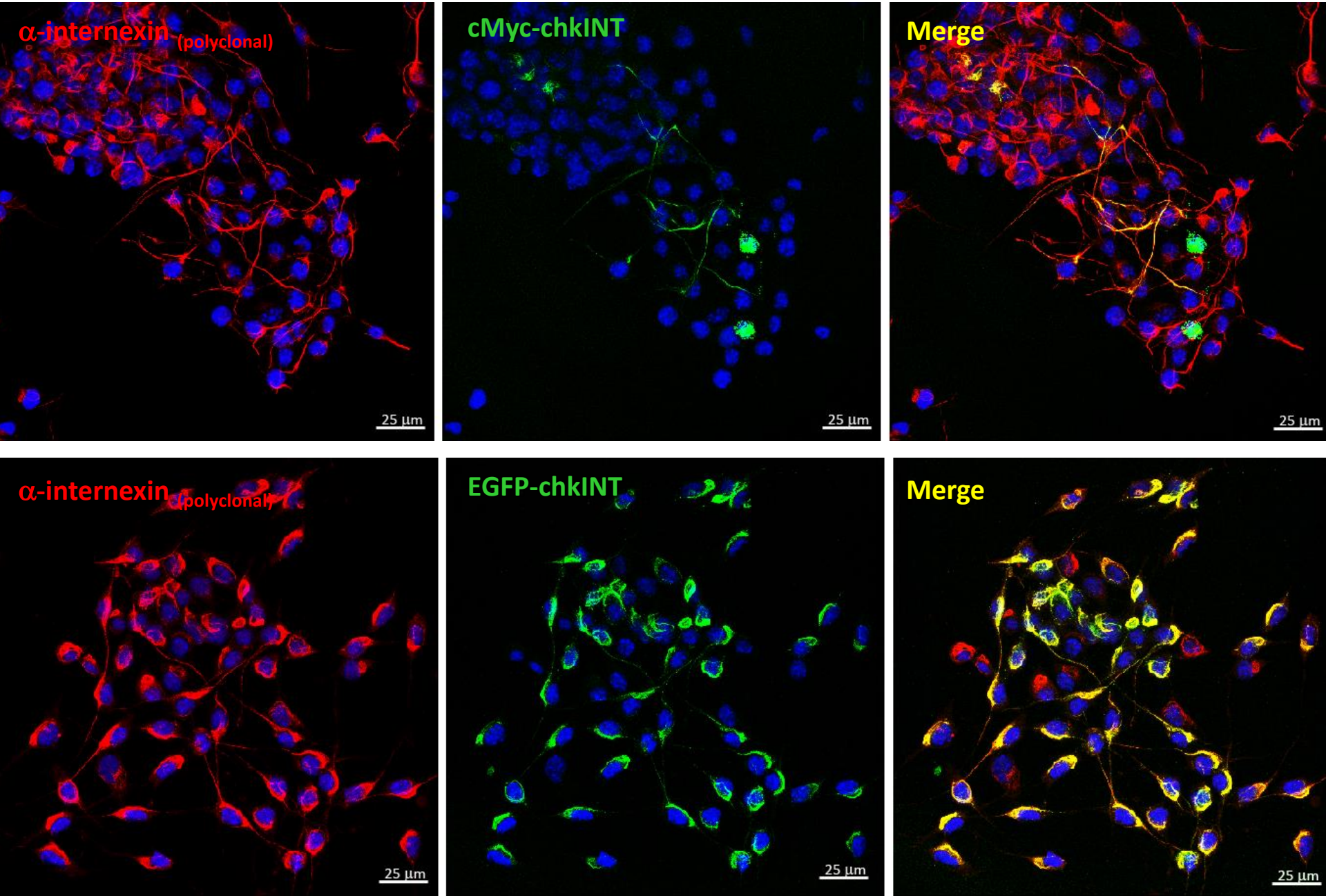
Study of the filament-assembly ability

Cells were transfected w/ pMyc-chkINT and pEGFP-chkINT constructs independently

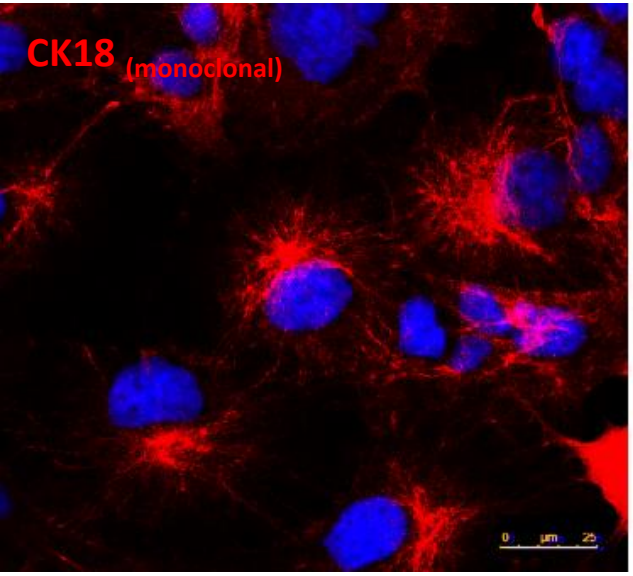
- Neuro2A (mouse, neuroblastoma)
- COS 7 (African green monkey, kidney fibroblasts)

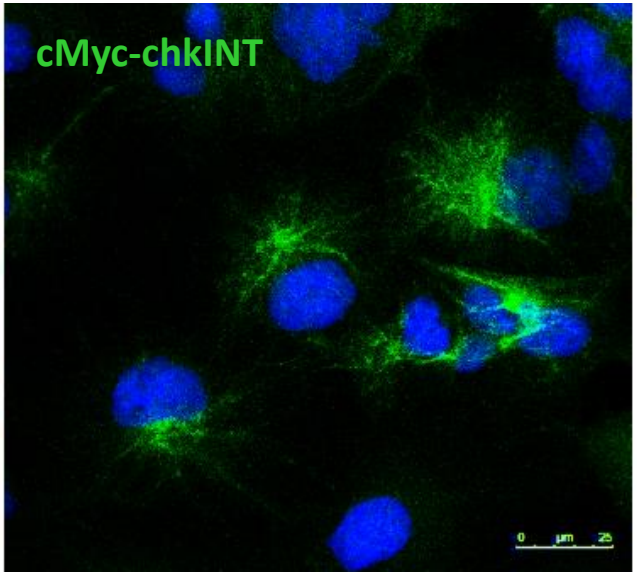


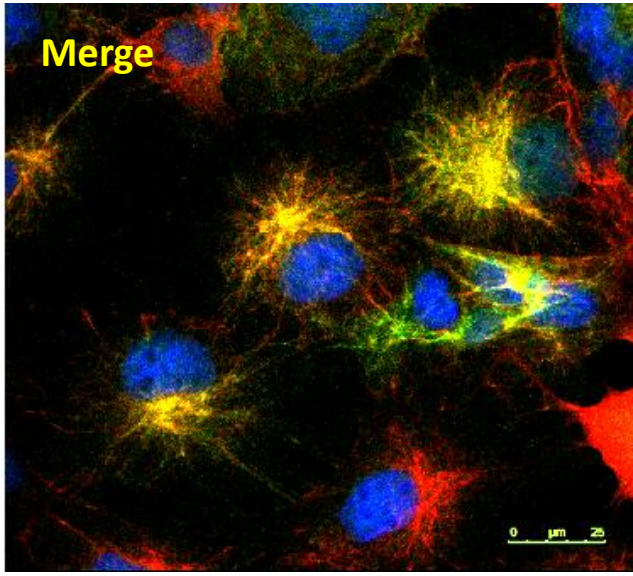
Neuro2A (pCMV-cmyc-chkINT & pEGFP-chkINT transfection)

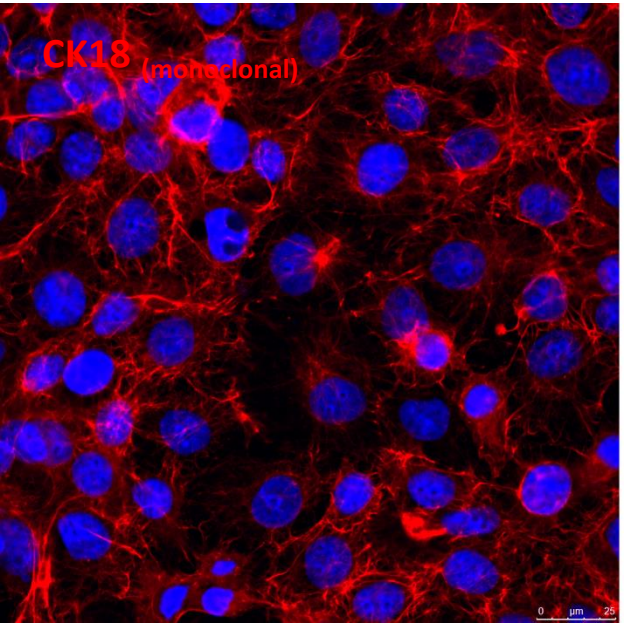


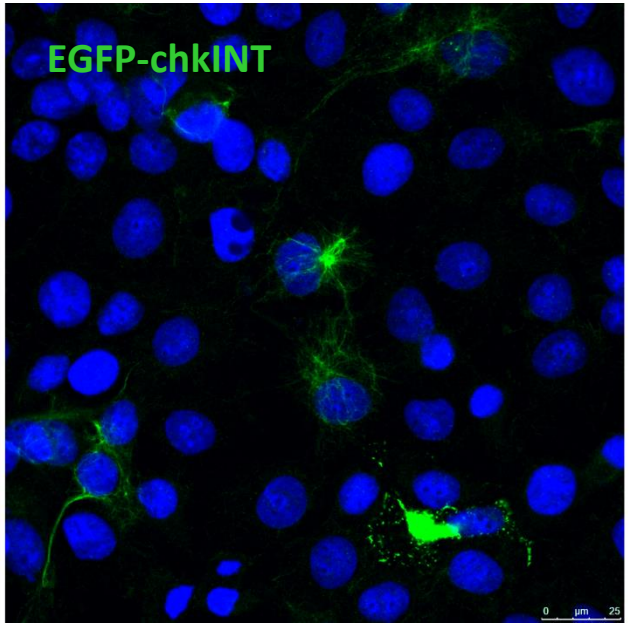
COS7 (pCMV-cmyc-chkINT & pEGFP-chkINT transfection)

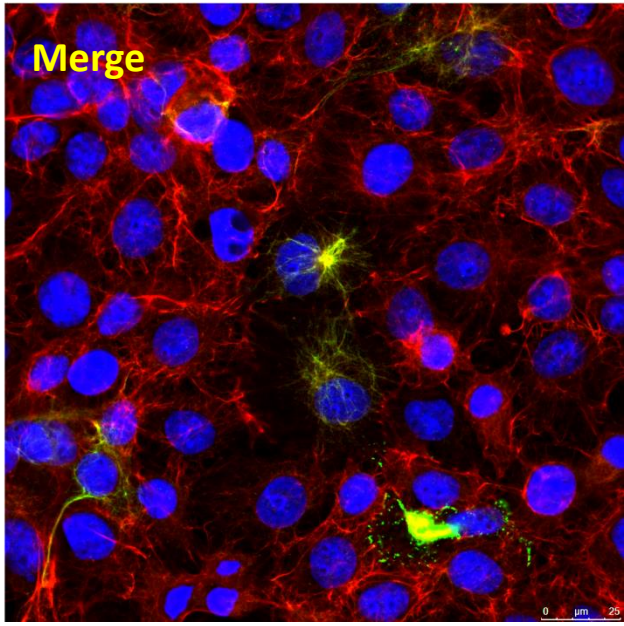








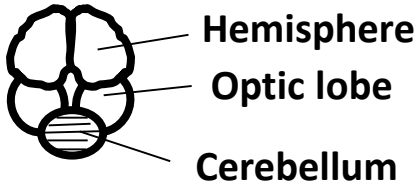




Study of the filament-assembly ability (II)

Transfection of Primary Cultured Cells

Hemisphere of E14 chicken brain



Day post-transfection (DPT)

Day *in vitro* (DIV)

0 1 2 3 4 5 6 7 8

0 1 2 3 4

Cell seeding

Transfection
- pEGFP-chkINT
- pMyc-chkINT

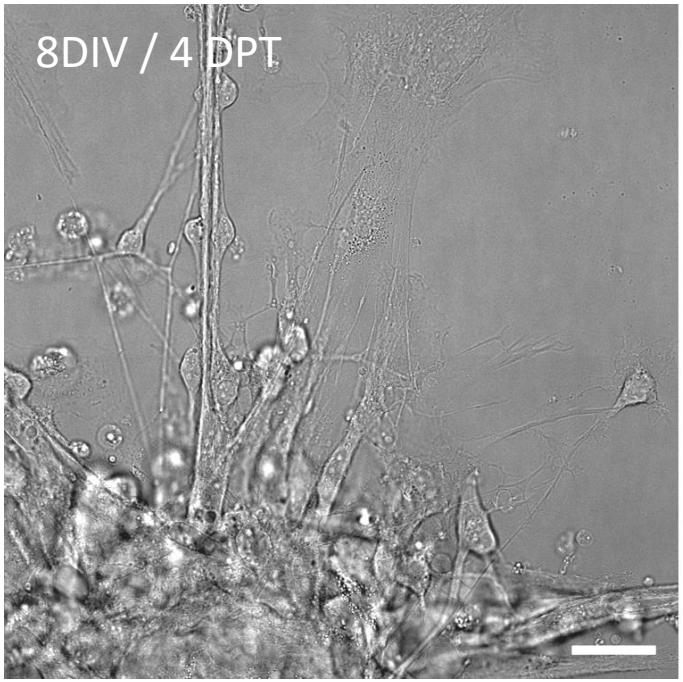
Immunocytochemistry
- Morphology
- Expression pattern

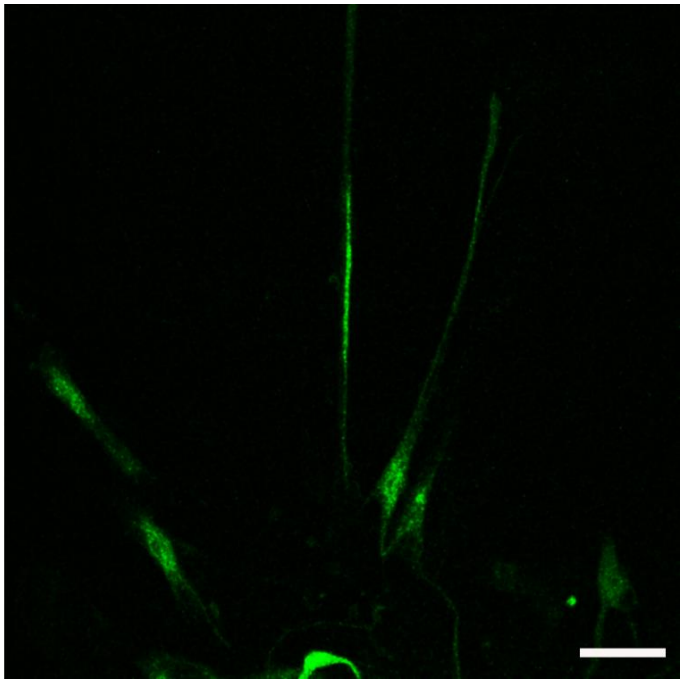
pMyc-chkINT

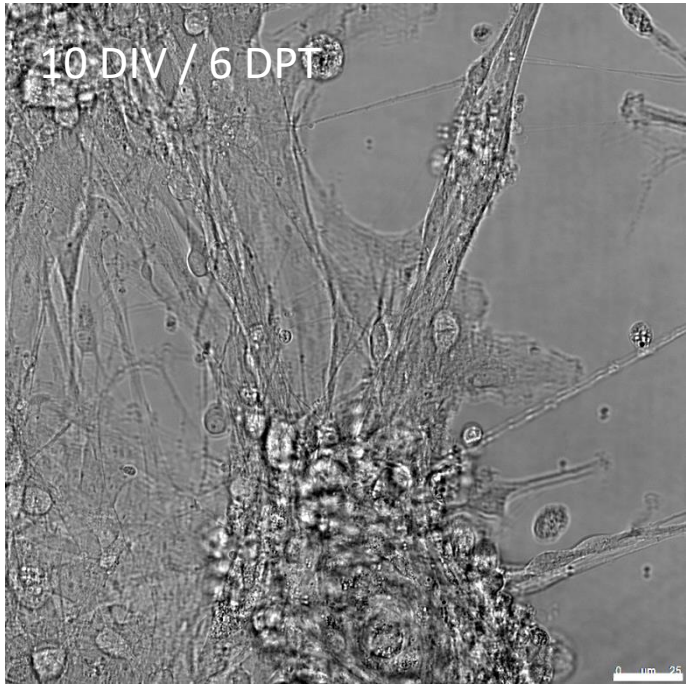
pEGFP-chkINT

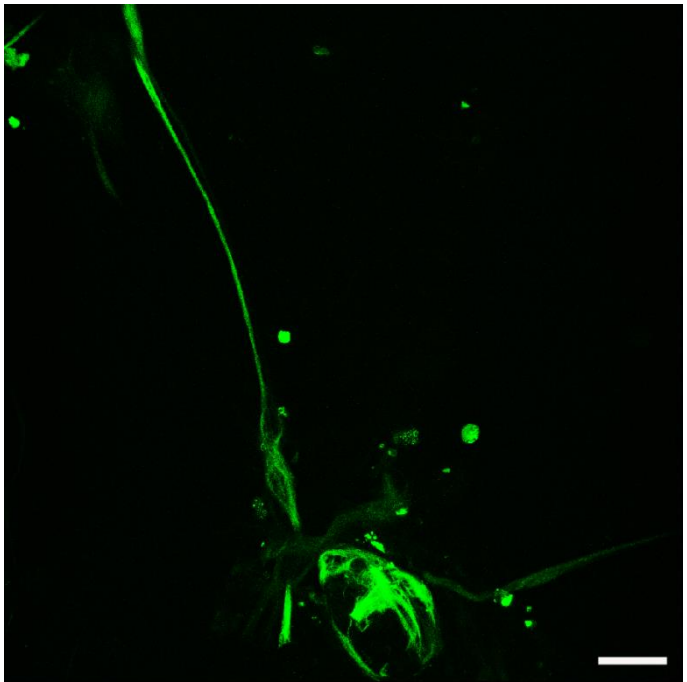
Live Cell Images

Primary chicken hemisphere cultured cells were transfected with EGFP-chkINT.









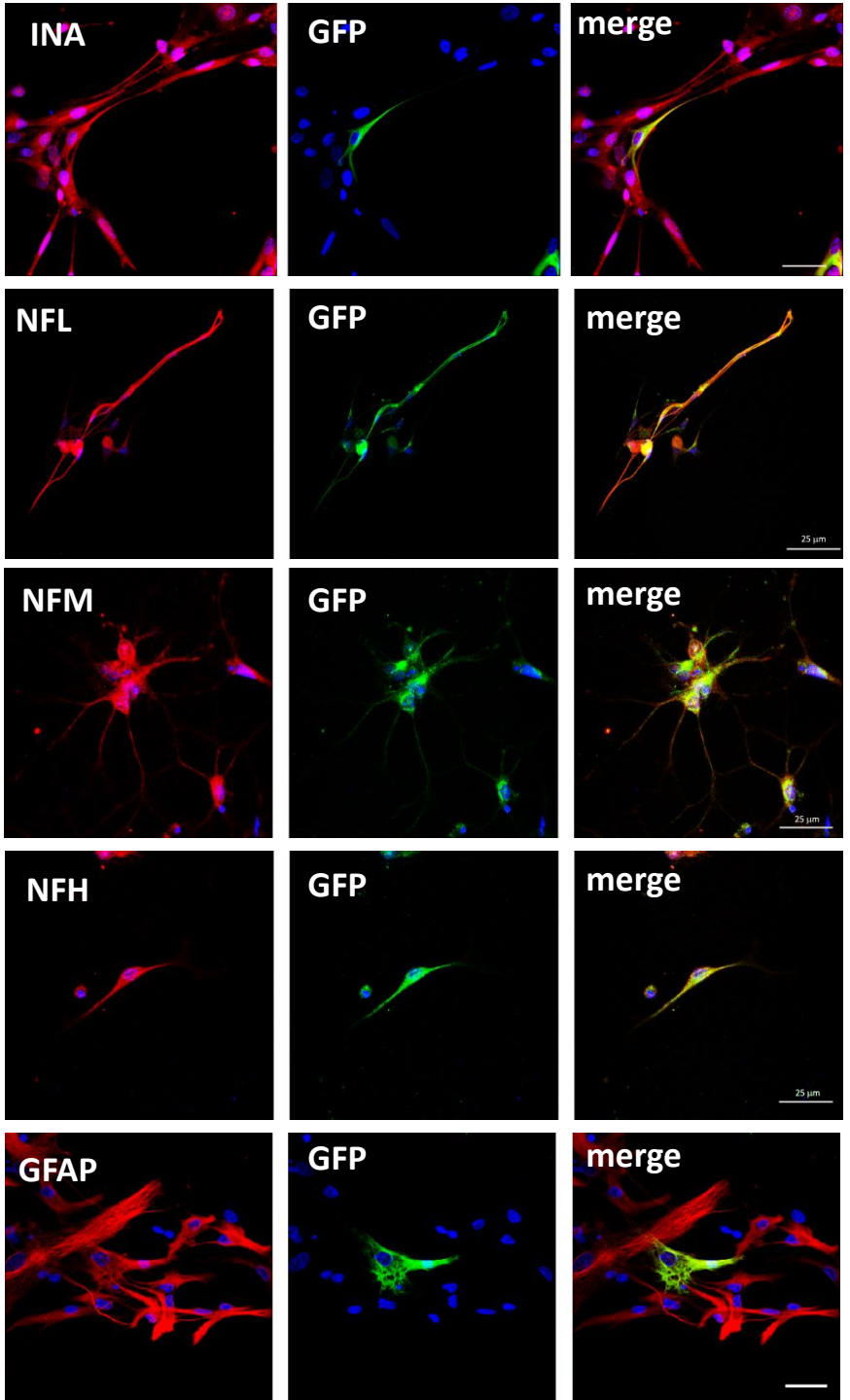
Scale bar = 25 µm

DIV, day *in vitro*
DPT, day post-transfection

ICC

Confocal microscopy images

Primary chicken hemisphere cultured cells were transfected with **EGFP-chkINT**.



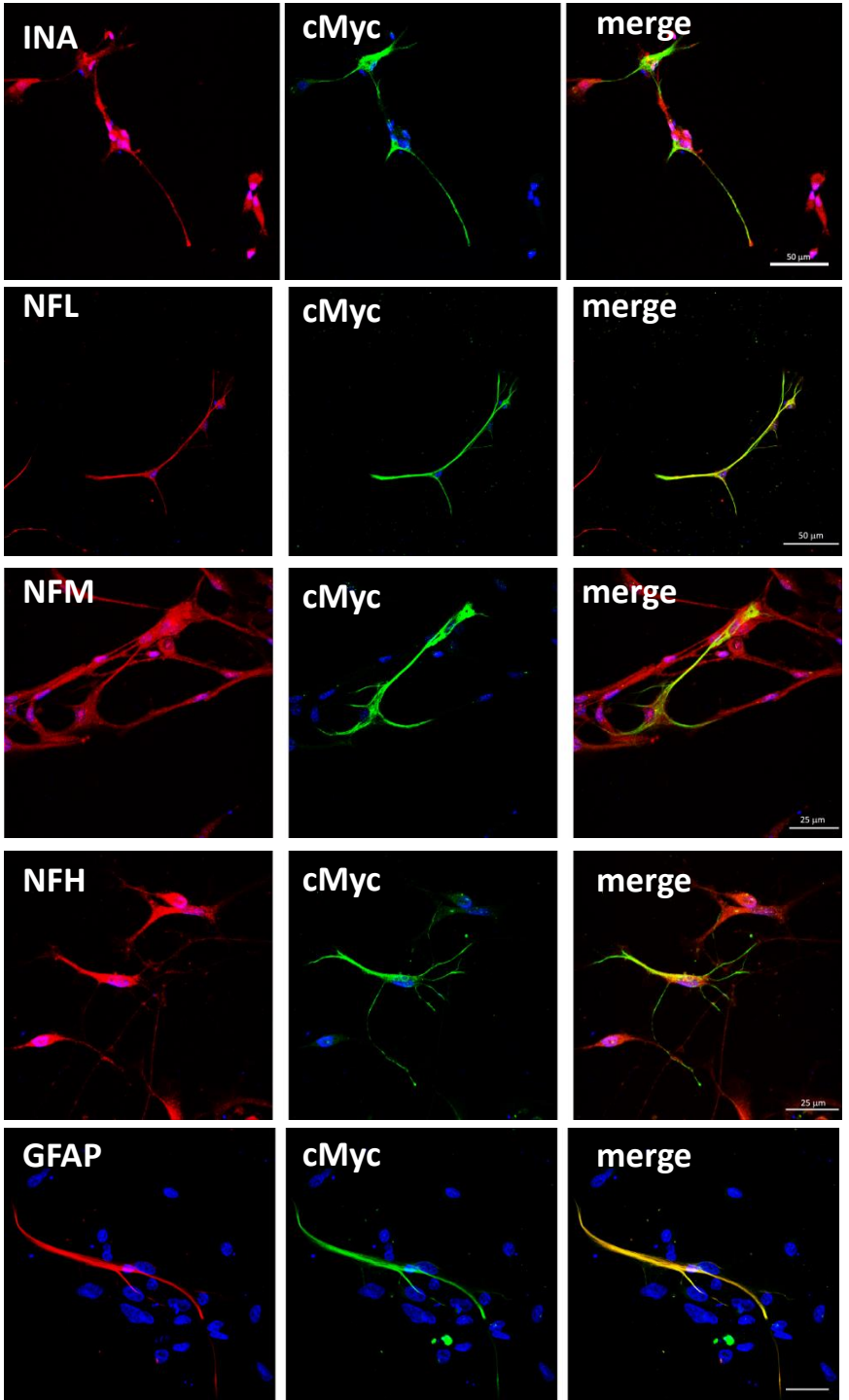
Scale bar = 25 µm

ICC

confocal microscopy images

Primary chicken hemisphere cultured cells were transfected with **cMyc-chkINT**.

The putative chicken α-internexin has the ability to form filamentous structure in the transfected cells.



Scale bar = 25 µm

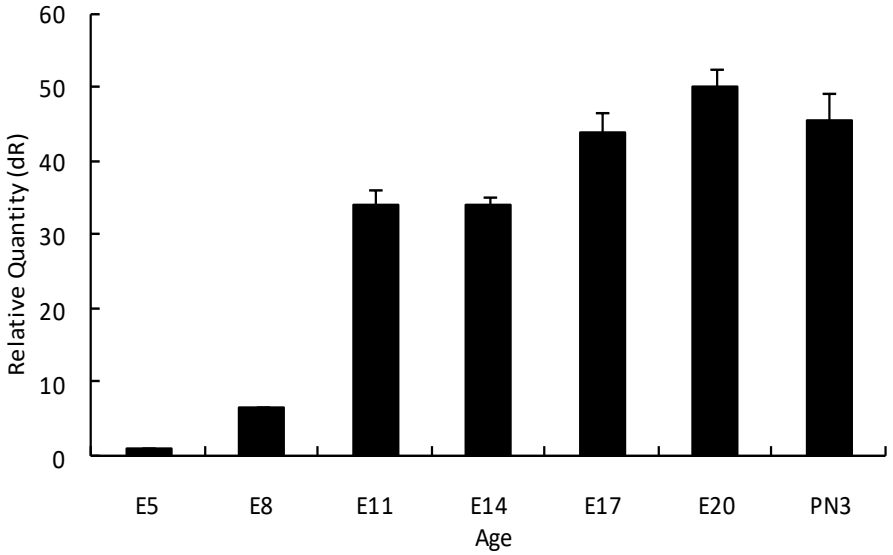
Aims

- Discover the mRNA sequence encoding α-internexin from chicken embryos.
- **Study the expression of chicken α-internexin during neuronal development**
- Confirm the physiological features of chicken α-internexin.

Q-PCR

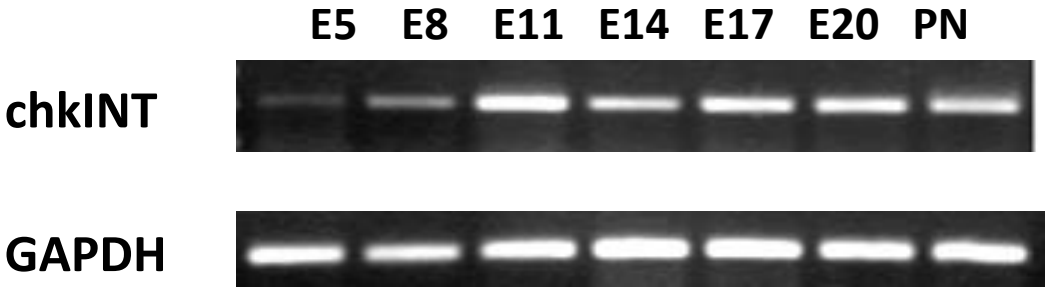
(chicken brain)

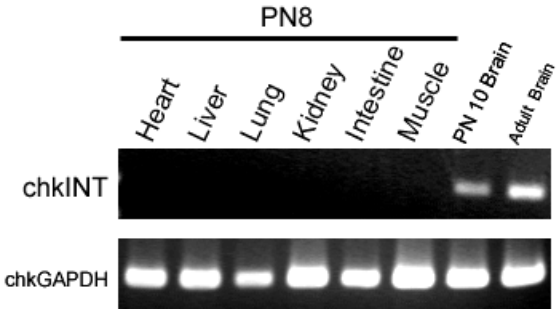
chkINT



E, embryonic day;
PN, postnatal

RT-PCR





Chicken α-internexin increased gradually during embryonic development.

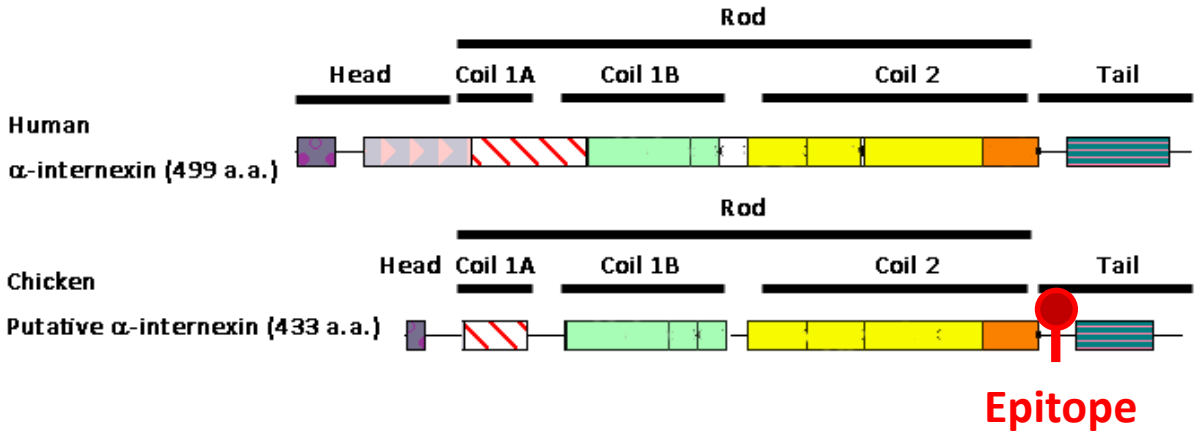
Custom Antibody – CHK 366-384

Predicted chicken α-internexin (chkpINT)

- 433 a.a.
- Theoretical pI/Mw: 9.37 / 48435.59 (**48.4 kDa**)

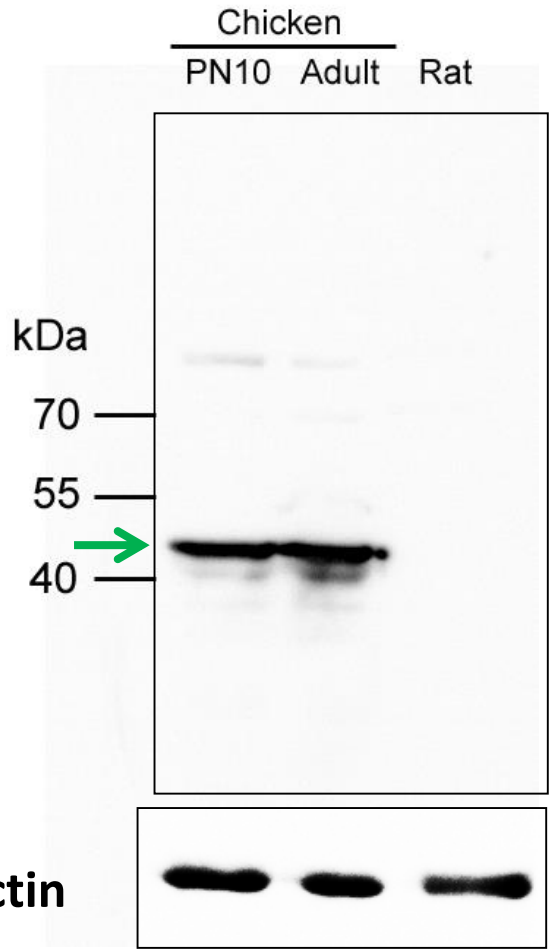
MSYSVEPPALAASSRRLLAQSPRRTEGAEPRRASEKEQLRGLNERFAGYIERVRALEERN
RALAGELAELRRLPPEPRRLGQLLGGELRALRARLEEAHGERAQAALERARLAEETQRLR
ARCEEEARGRAEAEQALRARQQAADGAARARADLERRAEALREELAELRRAHAEQLAQLG
AALRAAAPPASGPPTARPDLAAALRELRAQYEALPARNLQAAEDWYRARCASLHERAARS
QEAVRASRREAGECRRQLQARVVEMESLRGAHESLERQLQELEERHSAEAAGLQDTIGQL
EADLRSTKTEMARHLREYQDLLNVKMALDIEIAAYRKLLEGEENLFSMGSVGLPAMNPLP
NPTYSFRPRSSTPSFKKEEQREAVRATSKIPSGQAGVLDGTITTAKRTERFNVHGGIIAN
AKVQWWEPHPFCI*GGAVLITSEF

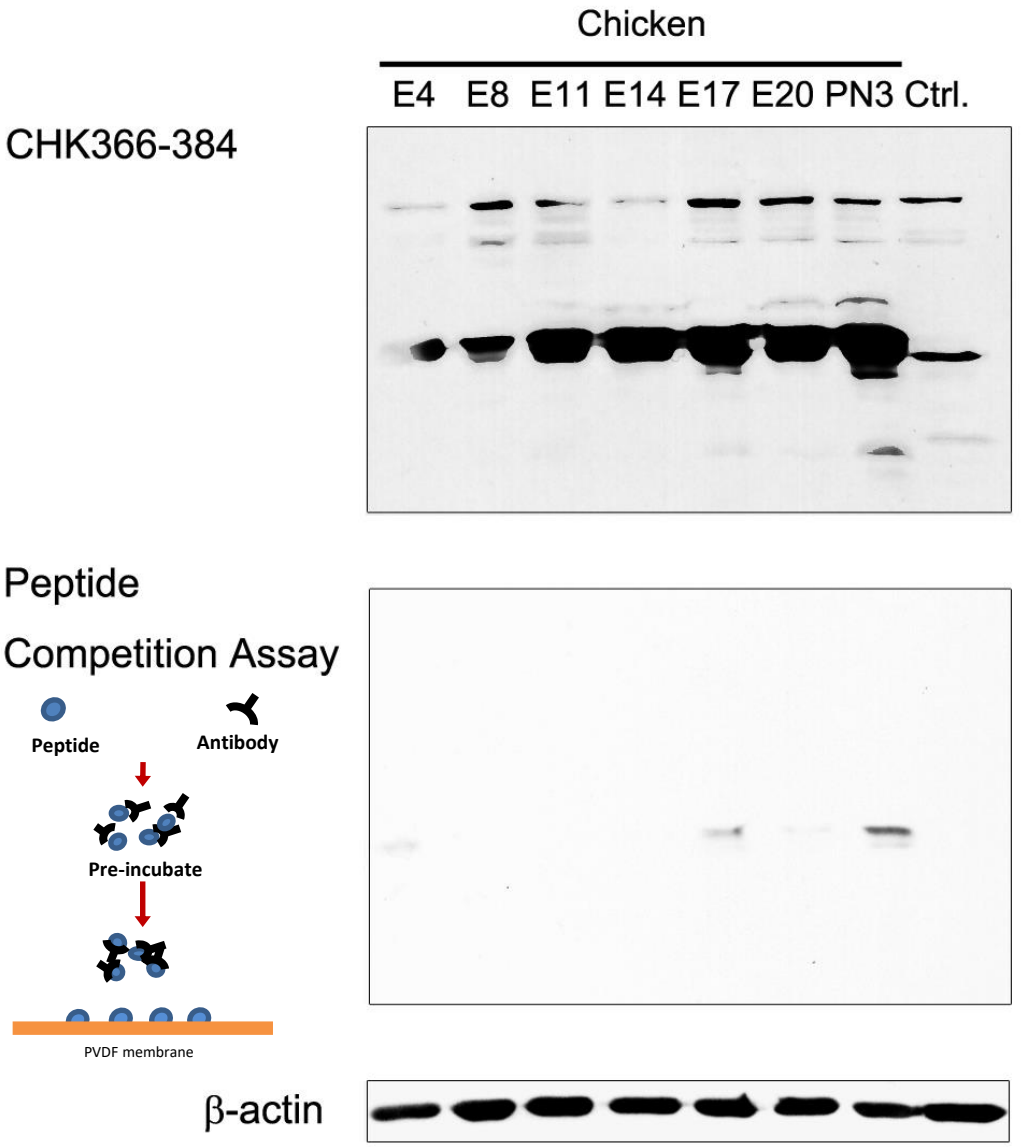
Epitope



Custom Antibody Test → Western blot

Chicken α-internexin polyclonal antibody



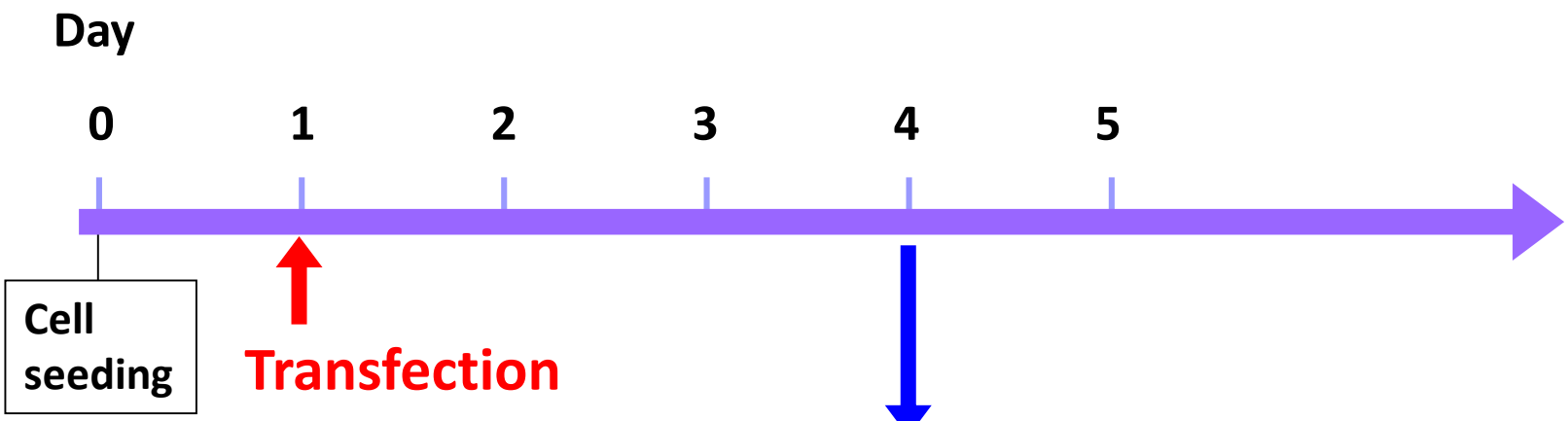


Control : mouse brain

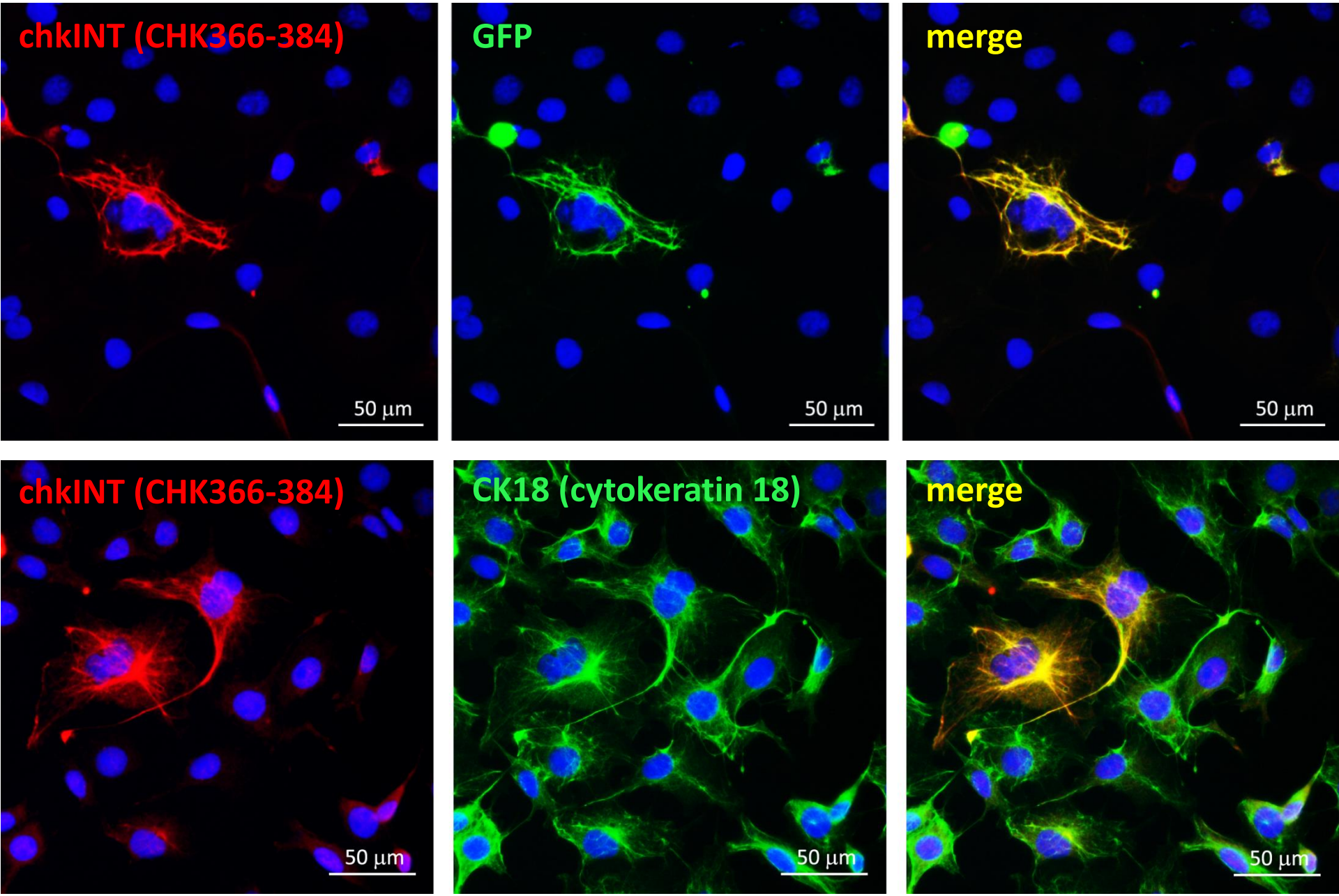
Custom Antibody Test → ICC

Cell Transfection Process

- COS 7 (African green monkey, kidney fibroblasts)

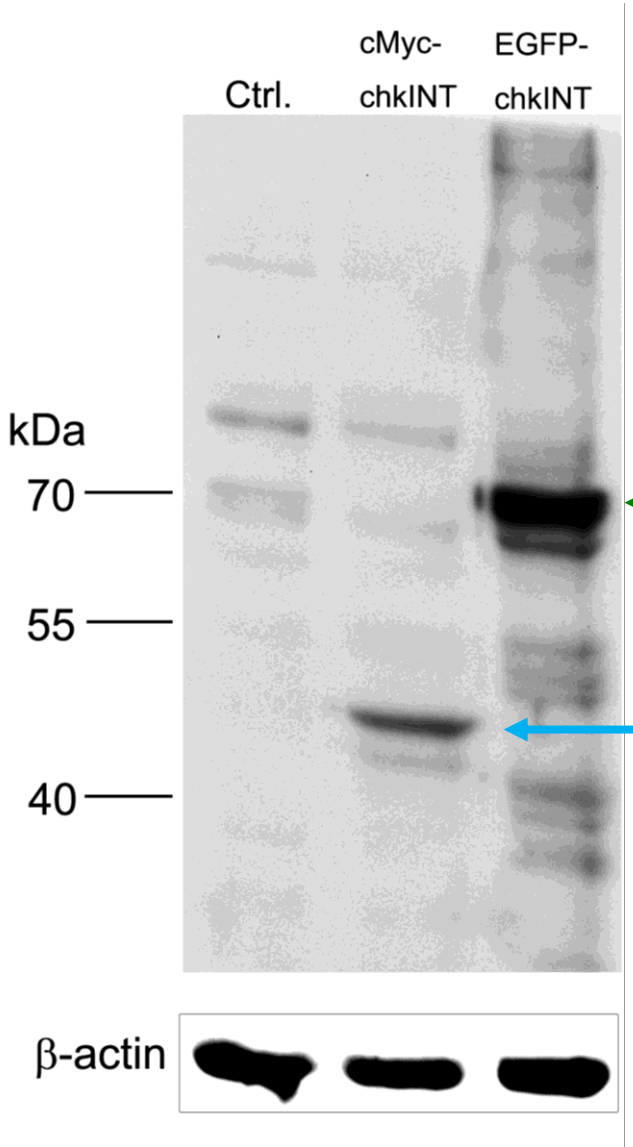


COS7 cells were transfected with **pEGFP-chkINT**.



Custom Antibody Test → Western blot

COS7 cells were transfected with cMyc-chkINT or EGFP-chkINT.



~27 kDa EGFP + ~49 kDa chkINT

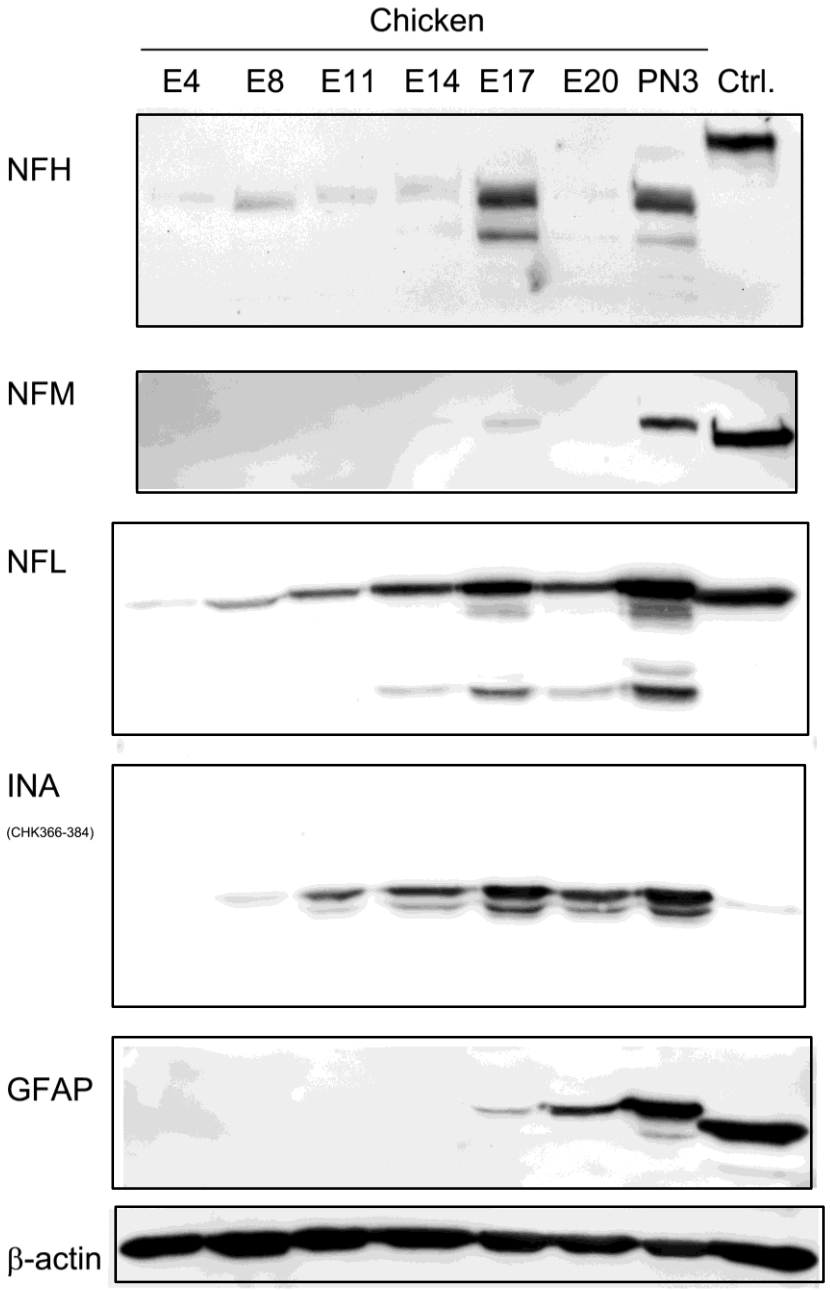
cMyc tag + ~49 kDa chkINT

Our antibody (CHK 366-384) is specific to the putative chicken α-internexin protein.

Western Bolt

- Indicate the protein level of neural IFs in different stages of chicken embryos.

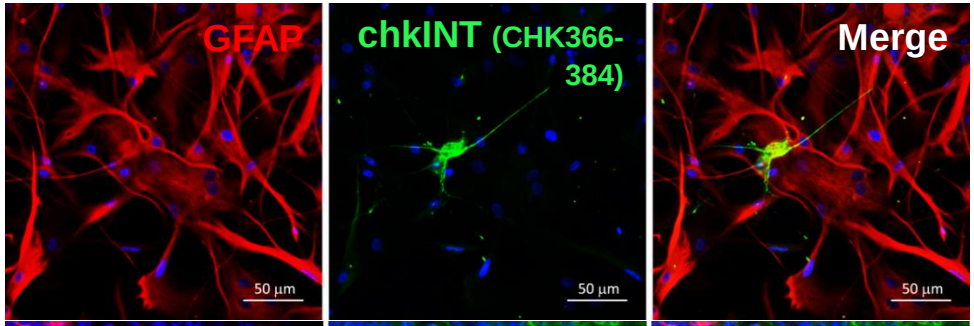
The protein level of chicken α-internexin was similar to the RNA expression level: the putative gene increased gradually during development.



Custom Antibody Test

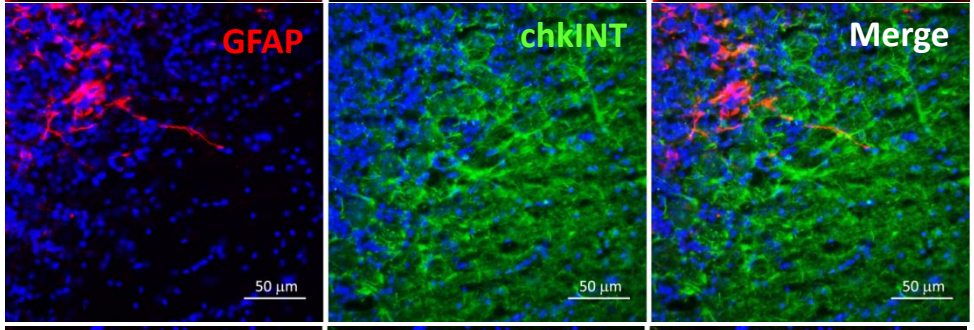
ICC

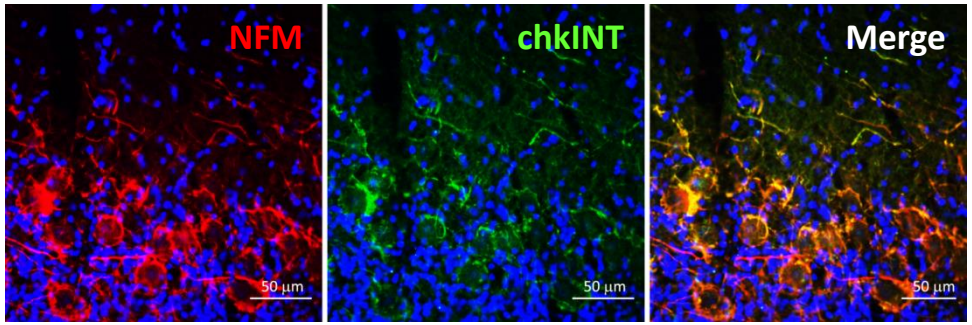
Primary cultured E17 chicken brain



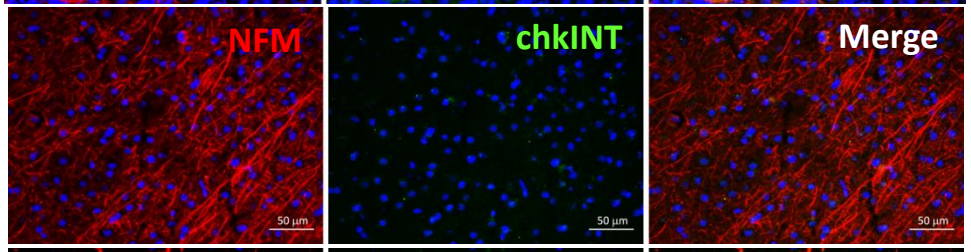
IHC

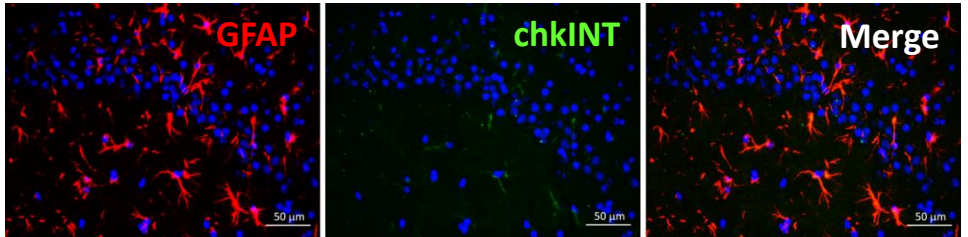
PN8 chicken brain





Mouse brain

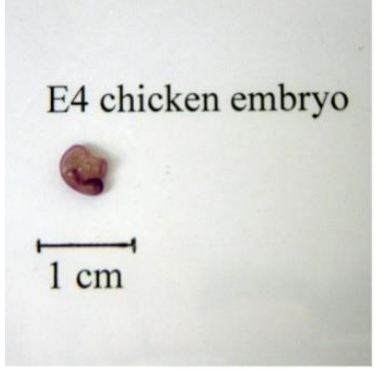


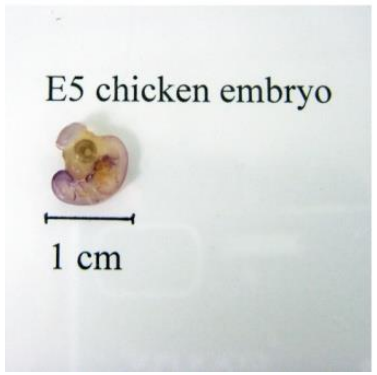


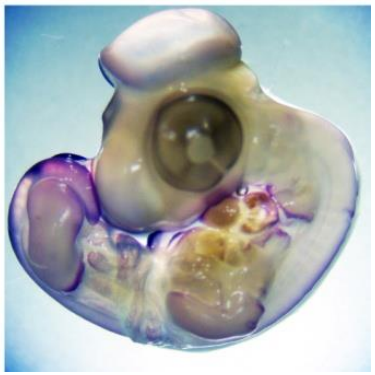
In Situ Hybridization

Whole-Mount Embryos

DIG-labeled probe to chicken α-internexin

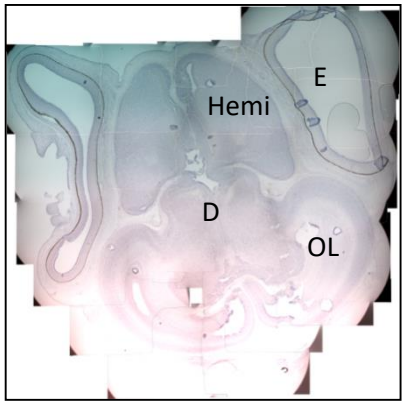




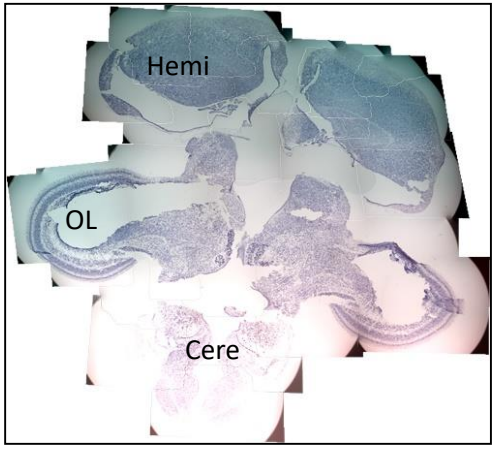


Cryostat Sections (Horizontal section)

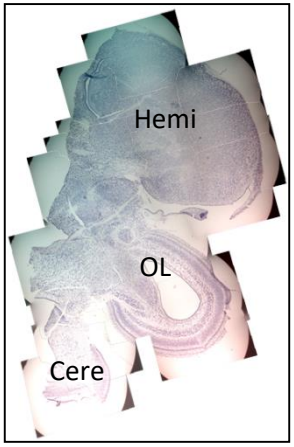
E8



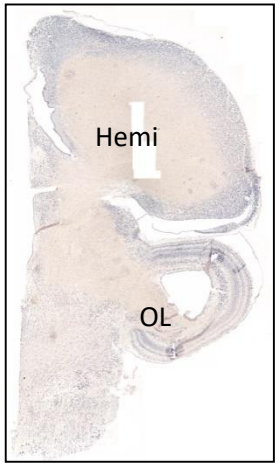
E11



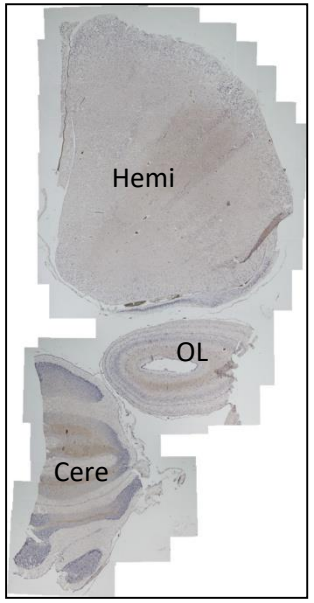
E14



E17

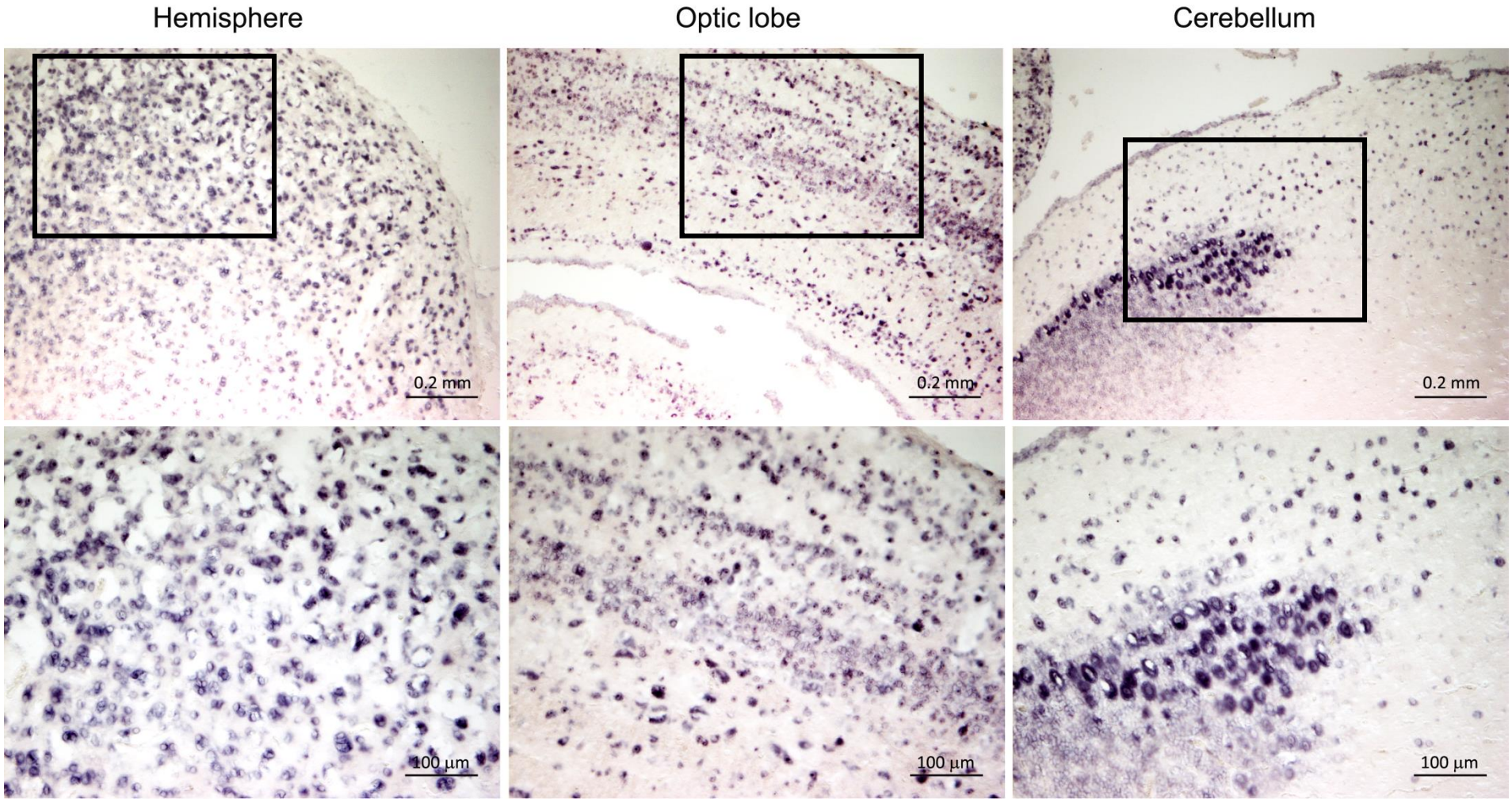


PN10



Cere, cerebellum; D, dienceophalon; E, eye; Hemi, hemisphere; OL, optic lobe

In Situ Hybridization of PN10 Chick Brain



Aims

- Discover the mRNA sequence encoding α-internexin from chicken embryos
- Study the expression of chicken α-internexin during neuronal development
- **Confirm the physiological features of chicken α-internexin**
  - **By Gene knockdown -RNAi**

Gene knockdown in chicken embryos

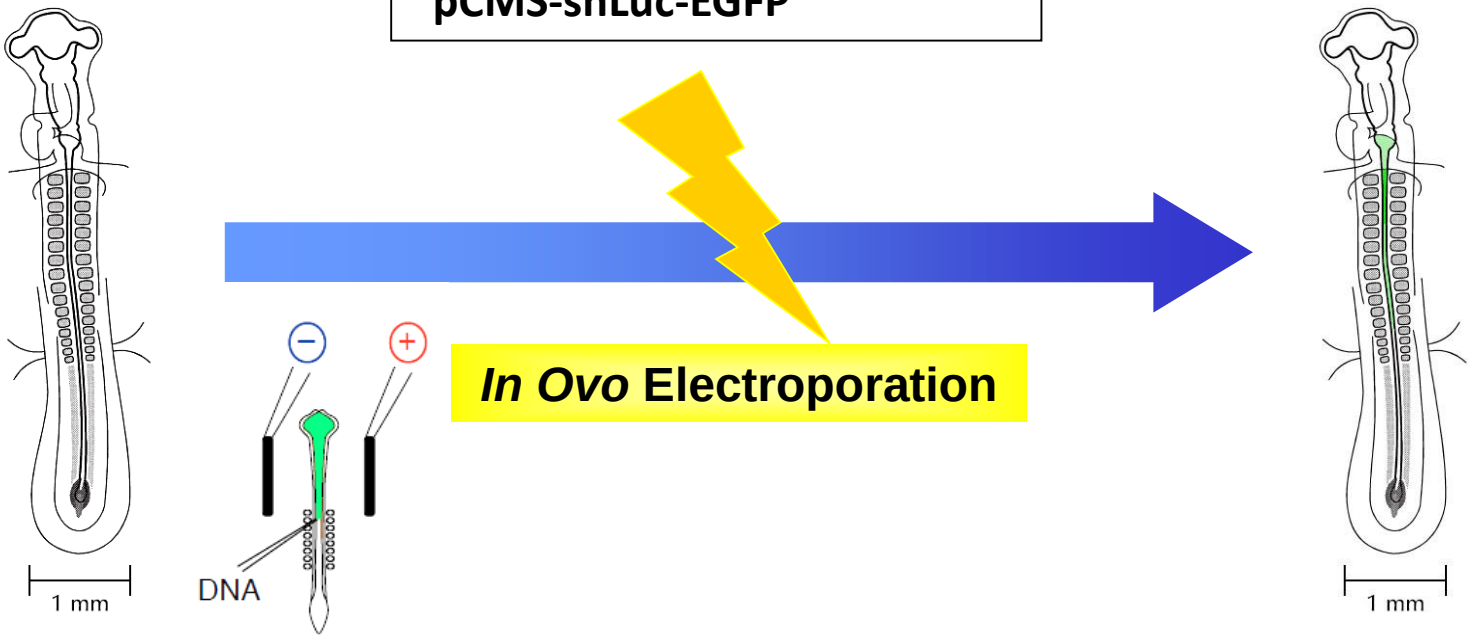
- Trancefect the shRNA constructs to the embryos by *in ovo* electroporation

Embryonic day 2 (E2) chicken embryo

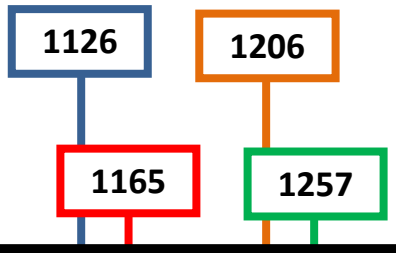
shRNA expression plasmids:

pCMS-shINT####-EGFP

pCMS-shLuc-EGFP



RNAi: shRNA constructs

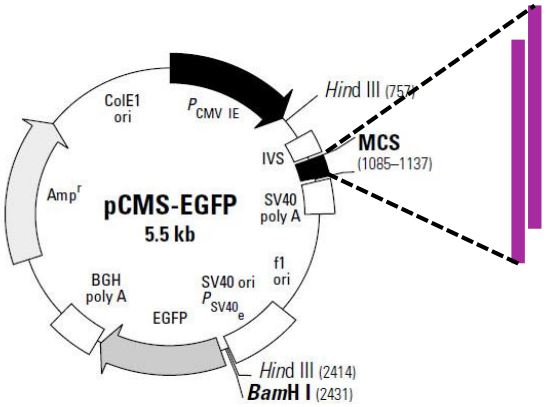


Chicken predicted α-internexin (chkpINT) -ORF 1302 bp

5’ 3’

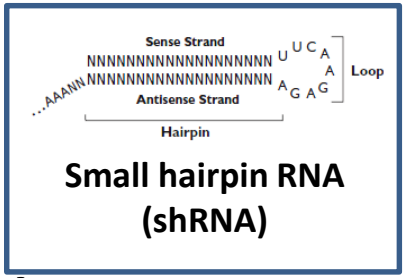
Targets of siRNA were selected by Ambion *siRNA Target Finder* .
Hairpin siRNA template oligonucleotides were designed by web-based insert design tool at the following address: www.ambion.com/techlib/misc/psilencer_converter.html

5'	Sense sequence (19 nt)	Loop	Antisense sequence (21 nt)	3'
CTCGAG	GATACCATCTGGTCAGGCT	TTCAAGAGA	AGCCTGACCAGATGGTATCTT	C
C	CTATGGTAGACCCGTCCGA	AAGTTCTCT	TCGGACTGGTCTACCATAGAA	GCCGG
Xho I				*Not* I

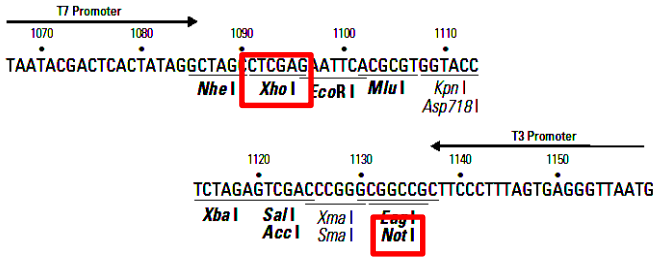


Hairpin siRNA Template Oligonucleotide

Ligation



Small hairpin RNA (shRNA)



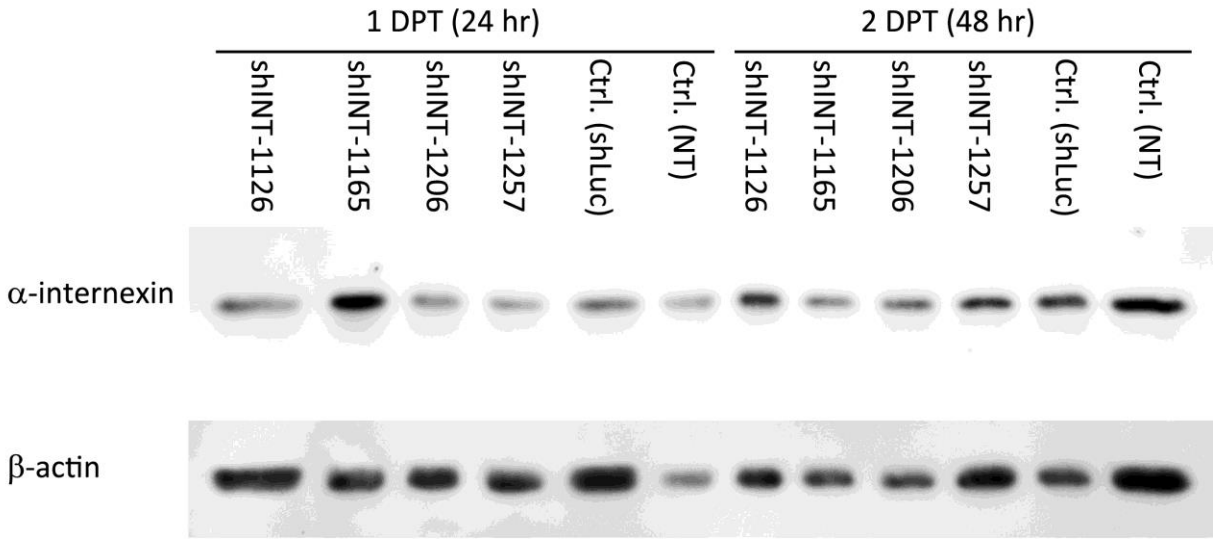
shRNA expression plasmids:

- pCMS-shINT^{1126}-EGFP
- pCMS-shINT^{1165}-EGFP
- pCMS-shINT^{1206}-EGFP
- pCMS-shINT^{1257}-EGFP
- pCMS-shLuc-EGFP

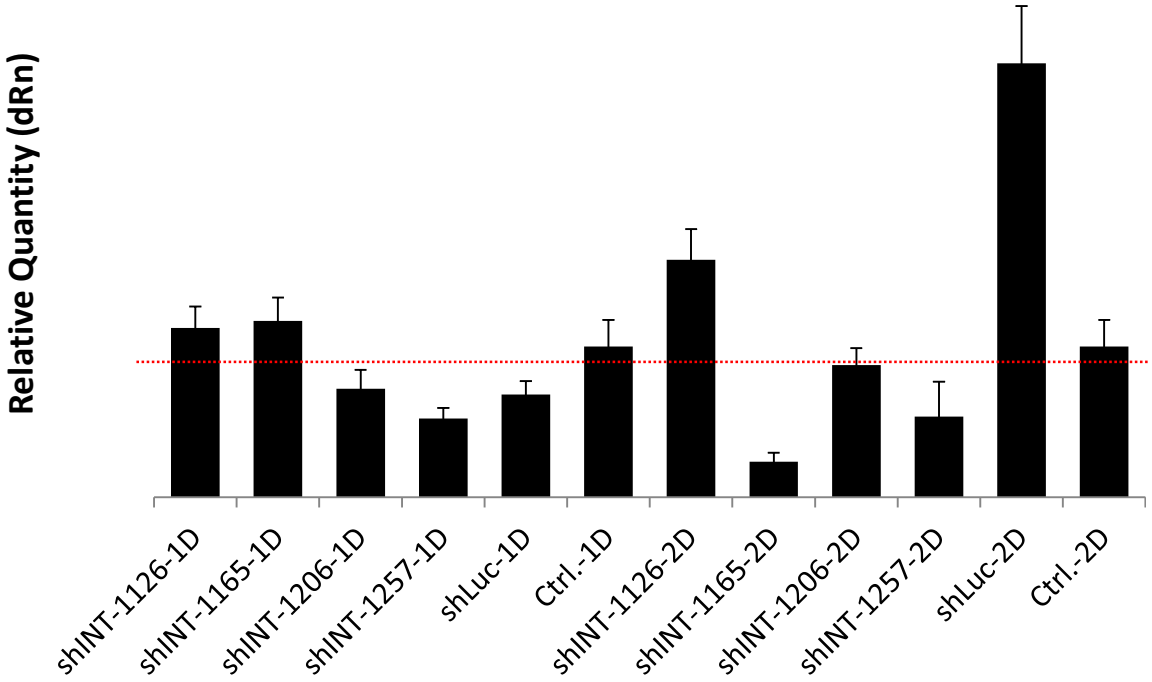
shRNA Constructs

Test

Western Blot

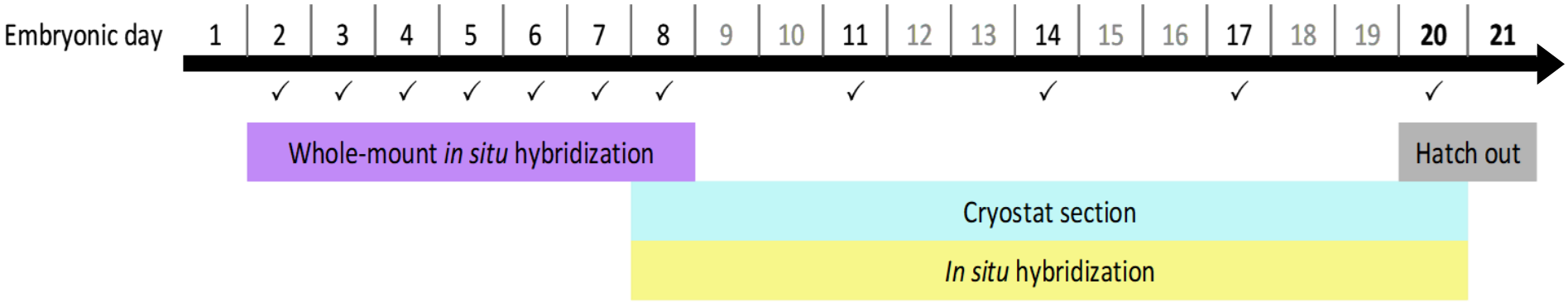


Q-PCR



Ongoing Work

- ***In situ* hybridization & immunohistochemistry**
  - To make the data more complete.



- **Generate stable chkINT expression cell lines for shRNA construct tests**
- Northern blot analysis

Future Work

- **Functional analysis by gene knockdown**

- **Investigation of the gene regulation**

  e.g. Cis-/trans-regulation of gene transcription

  Post-transcriptional control

Thank you for your attention!