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## **Recurrent *DCC* gene losses during bird evolution**

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## **Abstract**

During development, midline crossing by axons brings into play highly conserved families of receptors and ligands. The interaction between the secreted ligand Netrin-1 and its receptor Deleted in Colorectal Carcinoma (DCC) is thought to control midline attraction of crossing axons. Here, we studied the evolution of this ligand/receptor couple in birds taking advantage of a wealth of newly sequenced genomes. From phylogeny and synteny analyses we can infer that the *DCC* gene has been conserved in most extant bird species, while two independent events have led to its loss in two avian groups, passeriformes and galliformes. These convergent accidental gene loss events are likely related to chromosome Z rearrangement. We show, using whole-mount immunostaining and 3Disco clearing, that in the nervous system of all birds that have a *DCC* gene, DCC protein expression pattern is similar to other vertebrates. Surprisingly, we show that the early developmental pattern of commissural tracts is comparable in all birds, whether or not they have a DCC receptor. Interestingly, only 4 of the 5 genes encoding secreted netrins, the DCC ligands in vertebrates, were found in birds, but Netrin-5 was absent. Together, these results support a remarkable plasticity of commissural axon guidance mechanisms in birds.

## Introduction

Despite more than 600 million years of evolution, the basic components of the bilaterian brain wiring diagram are highly conserved <sup>1</sup>. One of its signature trait is the presence of two categories of projection neurons: some that connect to target cells located on the same, or ipsilateral, side of the nervous system and others that connect on the opposite, or contralateral, side. The latter are called commissural neurons and they are distributed all along the rostro-caudal axis <sup>2</sup>. The position and spatio-temporal developmental sequence of a common set of commissural tracts (anterior commissure, posterior commissure, fasciculus retroflexus, optic nerve among others) are highly similar among vertebrates <sup>3,4</sup>. However, some commissural tracts only exist in some taxa, such as the corpus callosum in placental mammals <sup>5</sup>, or the Mauthner cells in lampreys, teleosts and amphibians <sup>6</sup>. It is assumed that the appearance of novel commissural circuits has allowed the acquisition of novel brain functions and behaviours <sup>2,7</sup>.

Understanding the mechanisms controlling the development and patterning of commissural circuits has represented a daunting challenge to developmental neurobiologists since the end of the nineteenth century <sup>8,9</sup>. Significant progress has only been made during the past thirty years or so through genetic and biochemical screening. The current model favors a rather simple push-pull mechanism whereby cells at the CNS midline, such as the floor plate in vertebrates, secrete proteins that attract commissural axons and facilitate midline crossing and also repellents that force commissural axons to leave the midline <sup>8,10</sup>. Netrin-1, the first midline chemoattractant, was simultaneously identified in *C. elegans* and in chick embryo <sup>11</sup>. The vertebrate *DCC* gene (Deleted in Colorectal Carcinoma <sup>12</sup>), and its homologues



in *C.elegans*<sup>13</sup> and *Drosophila*<sup>14</sup>, encode a transmembrane receptor, mediating Netrin-1 attraction. In these species, *DCC* loss-of-function prevents many commissural axons from crossing the midline, thereby supporting *DCC* pivotal role in midline guidance<sup>14,15</sup>. In mice and human, mutations in *DCC* leads to lethality<sup>15</sup>, movement disorders<sup>16</sup> and cancers<sup>17</sup>. It was proposed that the *DCC* gene is absent from the chicken genome and that its paralogue, NEOGENIN, mediates NETRIN-1 attraction in this species<sup>18</sup>. However, multiple *in vivo* and *in vitro* studies have shown that in chick embryos, the chemotropic activity of NETRIN-1 on spinal cord commissural axons, enteric neural crest cells and oligodendrocyte precursors is blocked by anti-*DCC* antibodies<sup>19,20</sup>. Moreover, the *in ovo* electroporation of dominant negative constructs of *DCC* or *DCC* signaling partners in the chick spinal cord significantly perturbs commissural and motor axon guidance<sup>21–23</sup>. This suggests that a *DCC* gene might exist in the chick genome, which is known to be fragmented and to contain at least 30 microchromosomes<sup>24</sup>. Recently, the annotated genomes of 48 bird species were released<sup>25</sup> which led us to revisit the evolution history of *DCC* and *NETRIN* genes in birds. We have also performed a comparative analysis of the organization and development of commissural circuits in early bird embryos.

## Results

### ***DCC* gene is present in most sauropsid genomes**

We first investigated whether a *DCC* gene is present in all available sauropsid genomes. Using NCBI and *Ensembl* genome databases, we found genes annotated as “*DCC*” in several avian, crocodilian and chelonian genomes (see Supplementary Table S1). We performed a phylogenetic analysis to rule out the possibility that the

sauropsid *DCC* genes would be in fact Neogenin genes. We reconstructed vertebrate phylogeny of DCC and NEOGENIN proteins, using 47 sequences from 27 amniotes genomes, with drosophila Frazzled receptor (the DCC orthologue in flies) as outgroup (Fig. 1a). In this tree, DCC and NEOGENIN sequences cluster in two different well-supported clades, confirming that the two receptors are encoded by two distinct genes. Sauropsid DCC sequences are encompassed in vertebrate DCC group and recapitulate known phylogeny. Moreover, short branch lengths demonstrate that this gene is highly conserved among all vertebrates. Importantly, Neogenin genes could be found in all bird species investigated. The longer branch for neogenin sequences from passeriformes (*Pseudopodoces humilis*, *Geospiza fortis*, *Fiducela albicollis*) reveals a specific sequence divergence in this group. Together, these results confirm that a *DCC* gene is present in sauropsid including many bird species.

### **DCC gene was lost twice in aves evolution**

In birds, *DCC* gene is present in most birds major groups<sup>26</sup> including paleognathes, anseriformes, strisores, columbaves, gruiformes, aequorlitorithes, accipitriformes, coraciimorphes, falconiformes and psittaciformes (Supplementary Table S1). However, in agreement with a previous report<sup>18</sup>, we could not find any *DCC* gene in the presently available chicken *Gallus gallus* genome. This was also the case for two other galliformes: the turkey *Meleagris gallopovo* and the quail *Coturnix japonicus*, suggesting that *DCC* gene is absent from this group. Another striking result was the absence of *DCC* gene in a second major avian group, the passeriformes. Indeed we did not detect *DCC* sequences in any of the eleven passeriformes genomes available on NCBI including the zebra finch *Taenopygia guttata*. For both galliformes and

passeriformes we identified *DCC* genes in their respective sister group, namely anseriformes and psittaciformes, which supports independent gene losses (Supplementary Table S1).

To understand the evolutionary history of the *DCC* gene in birds, we investigated the *DCC* genomic region. We performed a physical co-localization of genetic loci on the same chromosome within an individual or species analysis (or synteny) of this particular region in amniotes genomes, i.e. mammals (human, mouse, platypus), testudinian (painted turtle), crocodilian (alligator) and various birds including paleognathes (ostrich), galloanseres (chicken, turkey, duck, goose) and 15 neoaves (Fig. 1b, Supplementary Fig. S1 and Supplementary Table S3-4-5). When present, *DCC* is always located on the sexual chromosome Z in birds, in contrast with other sauropsids and mammals in which *DCC* is on autosomes<sup>27,28</sup>.

Synteny analysis showed that this region was highly conserved in amniotes and only few gene rearrangements could be observed between sauropsids and mammals, except from two major gene block losses in galliformes and passeriformes (Fig. 1b and Supplementary Fig. S1).

In galliformes, a block of 12 genes, including *DCC*, was absent from the syntenic region: *MAPK4*, *ME2*, *ELAC1*, *SMAD4*, *MEX3C*, *DCC*, *MBD2*, *POLI*, *STARD6*, *DYNAP*, *RAB27B*, and *CCDC68* (Supplementary Fig. S1). In addition, a 13th gene, *TCF4* appeared to be missing in turkey (Supplementary Fig. S1). Only two of these genes could be detected elsewhere in these galliformes genomes using tblastn algorithm from NCBI: *MBD2* gene in chicken and *CCDC68* in turkey (Supplementary Fig. S1), but their position in the genome is still undetermined. Moreover, many chromosomal rearrangements could be observed downstream of this region in galliformes, in contrast with the stability of this genomic region in the sister group,

anseriformes. In particular, the block of genes nearby this deletion (from *TCF4* to *CPLX4*) is reversed compared to the ancestral sequence. Furthermore, *TCF4* gene, that is located at the border of the deletion block, is annotated at 875 bp from the beginning of Z chromosome (Gallus\_gallus-4.0; Ch.Z NC\_006127.3). Together this suggests that the *DCC* block was lost in galliformes during a Z chromosome extremity flip as illustrated in Figure 1c.

In passeriformes, a block of 7 genes, including *DCC*, is also absent from the locus: *DCC*, *MBD2*, *POLI*, *STARD6*, *DYNAP*, *RAB27B*, and *CCDC68* (and the quail *Coturnix japonicus*). In addition, an 8th gene, *TCF4*, is missing in the White-throated sparrow and Zebra finch available genomes (Supplementary Fig. S1). As for galliformes, none of these genes could be detected using tblastn algorithm on passeriformes genome sequences. This portion of passeriforme genomes is poorly assembled, and it is impossible to determine how these scaffolds are positioned on Z chromosome. Previous studies demonstrated that the Z chromosome has undergone major gene loss and shortening during bird evolution <sup>29</sup>. Our observations are compatible with a scenario of two independent Z chromosome rearrangements in galliformes and passeriformes leading to large chromosomal region losses, including the *DCC* gene block (Fig. 1c).

### **Netrin genes, except *NETRIN-5*, are present in all birds**

We next investigated whether genes encoding DCC main ligands, the secreted NETRINS <sup>30–32</sup>, were present in known bird genomes. In vertebrates, 5 secreted NETRIN proteins have been described (*NETRIN-1* to *5*) and are all thought to bind to DCC <sup>12,33–35</sup>. Previous study indicates that *NETRIN* genes originated prior to vertebrate radiation <sup>36</sup>. To investigate the evolutionary history of *NETRIN* genes in

birds, we reconstructed their phylogeny using amphioxus NETRIN-1 and NETRIN-4 as outgroups (Supplementary Fig. S2). This phylogenetic tree revealed that NETRIN-2 and NETRIN-3 sequences cluster in a single well-supported clade (see Supplementary Fig. S2), demonstrating that these are actually two annotations of the same gene. This observation was confirmed by synteny analysis (data not shown). No particular divergence could be observed for birds NETRIN-1, NETRIN-2/3, or *NETRIN-4* sequences, as compared to the other vertebrates. In particular, these genes were also present and conserved in both galliformes and passeriformes (Supplementary Fig. S2). In contrast, long branches suggested that NETRIN-5 sequences are highly divergent among all vertebrates. Furthermore, it was impossible to find any *NETRIN-5* gene in bird genomes (Supplementary Fig. S2). Taken together, these data show that there is no ligand modification counterpart of *DCC* gene loss in galliformes and passeriformes.

### **DCC mRNA and protein are not detectable in galliformes and passeriformes**

To confirm the loss of *DCC* in galliformes and passeriformes compared to other bird species, we assessed its mRNA and protein product by performing *in situ* hybridization and immunostaining. We used two riboprobes, cloned respectively from duck and pigeon cDNA, and two different antibodies, specific for DCC extracellular and intracellular domain (see Methods). We performed experiments on different bird embryos from species with available genomic data: chicken (*Gallus gallus*), duck (*Anas platyrhynchos*), pigeon (*Columba livia*), and zebra finch (*Taeniopygia guttata*). In addition we used three other galliformes (pheasant, partridge, and quail) assuming that they do not have a *DCC* gene. Pigeon and duck DCC antisense riboprobes could detect *DCC* mRNA in their respective species, while sense riboprobes could not

(Fig. S3). In addition, the two probes could detect DCC mRNA in both species (Fig. 2e-f). In contrast, no signal was detectable with either probe in galliformes and passeriformes (Fig. 2a-d, 2g), strongly suggesting an absence of DCC mRNA in these embryos. Similarly, the two anti-DCC antibodies labeled spinal cord commissures in duck and pigeon embryos but not in embryos from galliformes and passeriformes (Figs. 2l and 2n). Commissural axons were also immunoreactive for DCC in the hindbrain of ostrich embryos (data not shown). This result confirms genomic data of specific *DCC* gene losses in passeriformes and galliformes. Immunostaining with an antibody against the pan-neuronal marker  $\beta$ III-Tubulin showed that the overall organization of axonal tracts was highly similar in spinal cord section from the seven species tested (Fig. 2). Importantly, the ventral spinal cord commissure was present in all birds. We also performed immunostaining with antibodies against the Roundabout 3 (ROBO3) receptor, known to be expressed by growing commissural axons in the spinal cord and hindbrain in developing mammals<sup>37,38</sup>, zebrafish<sup>39</sup> and chicken<sup>40</sup>. A single *ROBO3* gene was detected in all birds (data not shown) and accordingly, ROBO3-immunopositive commissural axons were observed on spinal cord sections from all bird embryos tested (Fig. 2). Thus absence of DCC receptor does not appear to impact bird spinal cord commissural formation. To further study DCC expression pattern in birds, we performed whole-mount anti-DCC immunostaining, 3DISCO clearing and 3D imaging with light sheet microscopy on chick, pheasant, duck, pigeon and zebra finch embryos at HH21-22. In the chick, early axonal tracts<sup>41</sup> and peripheral nerves could be labeled with anti- $\beta$ III Tubulin immunostaining but none expressed DCC (Fig. 3a-b and Supplementary Movie S1). We also failed to detect any DCC expression in pheasant and zebra finch embryos (Supplementary Fig. S4), confirming what was found in the spinal cord. In contrast,

many axons were immunoreactive for DCC in duck and pigeon embryos (Fig. 3c-d). As previously shown in rodents and xenopus, DCC was broadly expressed by commissural axons in the mesencephalon, diencephalon and rhombencephalon. DCC was also detected in retinal ganglion cells, in the olfactory nerves, motor axons and fasciculus retroflexus (Fig. 3e-j and Supplementary Movie S1). Together, these data confirm that DCC was selectively lost in the galliforme and passeriforme lineages despite its highly conserved expression pattern in other tetrapods.

### **Homogenous organization of early commissural tracts in bird embryos**

To determine if the lack of DCC in passeriformes and galliformes might have resulted in a different organization of their commissural projections, we compared the early development of commissural tracts in the chick with that of pigeon and duck embryos. We used anti-ROBO3 immunostaining to specifically label all posterior commissural tracts and reconstruct their 3D organization. In addition, we used anti- $\beta$ III TUBULIN to investigate rostral commissural tracts development. In all embryos, the position, developmental sequence and density of commissural axons were comparable (Fig. 4). As expected from previous work in the mouse, when present, DCC homogeneously stained all spinal cord and hindbrain commissural axons, as does ROBO3 (Fig. 4, Supplementary Fig. S2 and Supplementary Movies S2-S3). The fasciculus retroflexus was also labeled, and no major differences were observed (data not shown). At more rostral levels where ROBO3 was not expressed, commissural tracts such as the posterior commissure or the post optic commissure tract (Supplementary Fig. S4) could be observed with anti- $\beta$ III Tubulin, and no noticeable difference was detected between chick and duck or pigeon embryos.

## Discussion

We found *DCC* genes in representative species of saurians, chelonians, crocodilians, as well as in many bird species, including paleognathes, anseriformes and numerous neoaves. In contrast, *DCC* gene is missing in chicken, in agreement with previous observations<sup>18</sup>, as well as in another galliforme, the turkey. Furthermore, *DCC* is also absent in passeriformes, such as crow, fly catcher and zebra finch. *DCC* gene was also not found in some bird genomes outside from passeriformes and galliformes (Table S1), however these bird genomes belong to low-coverage genome sequencing groups<sup>42</sup>. Moreover, In these cases, other birds from the same groups have a *DCC* gene, suggesting that its absence is more likely due to incomplete genome sequences, than to a real absence of *DCC* in these species. Phylogeny analysis clearly clustered bird *DCC* sequences with the *DCC* of the other sauropsids, and together with mammalian and actinopterygian sequences in a single *DCC* clade. By contrast, *NEOGENIN*, the *DCC* paralogue, appears to exist in all birds.

This supports that a *DCC* gene was present in bird ancestor, conserved in various avian groups, but lost in two distinct groups, galliformes and passeriformes. According to current bird phylogeny, these two groups are not closely related<sup>26,43</sup>, suggesting that two independent events of *DCC* loss occurred during bird radiation. One is aware that the absence of some genes in current bird genomes could be related to incomplete sequencing of some genomic regions<sup>44</sup>. We show here that the presence or absence of *DCC* mRNA and *DCC* protein in the brain matched with the presence or absence of *DCC* gene in the corresponding bird genomes. In the phylogeny tree, the short lengths of bird *DCC* branches indicated that bird *DCC* sequences did not diverge as compared to the other amniote sequences. In addition



DCC expression pattern when present is similar to what has been described in the mouse <sup>14,45</sup>. This indicates that the loss of *DCC* in some bird groups was not preceded by any major change of DCC structure and function in the bird lineage. Moreover, synteny analyses showed that both in galliformes and passeriformes, many genes are lost together with *DCC*. This shows that the loss of *DCC* in these birds is not targeted specifically on *DCC* gene, but concerns a whole genomic region. This differs from other recently reported gene loss in birds, such as the loss of *KISS* gene <sup>46</sup>. In this case, a degenerated sequence of *KISS* could be observed in some bird species, such as duck, zebra finch, and rock pigeon, and a specific loss of this gene in many other birds, including falcon and chicken, suggesting that accumulation of mutations and alteration of function preceded the loss of the gene <sup>46</sup>. Here, the lack of *DCC* would result from independent accidental loss events in both galliformes and passeriformes during Z chromosome recombination.

In vertebrates, DCC binds to *NETRIN-1* <sup>12,47</sup>, *NETRIN2/3* <sup>34</sup>, *NETRIN-4* <sup>33</sup> and possibly *NETRIN-5* <sup>35</sup>. Our phylogeny analyses showed that all birds possess *NETRIN-1*, *2/3*, and *4*, as the other osteichthyans with no special divergence. Interestingly, we could not retrieve any *NETRIN-5* sequence in birds, while this gene is present in other amniotes, as well as in teleost fish. This suggests an early loss of *NETRIN-5* in the bird lineage. In mammals, *NETRIN-5* is expressed in the developing brain, notably in neurogenic regions <sup>32</sup> but its function is still largely unknown. Besides the loss of *DCC* in galliformes and passeriformes, the lack of *NETRIN-5* in extant avian species represents another striking specificity of the DCC/*NETRIN* system in birds.

In the mouse, DCC has been reported to be essential for NETRIN-1 mediated attraction in many different systems<sup>15</sup> and *DCC* knockouts are not viable<sup>14</sup>. Previous *in vitro* studies have shown that in the chick as well, NETRIN-1 attracts sensory and motor neurons, spinal cord and hindbrain interneurons, GnRH neurons, enteric neural crest cells and oligodendrocyte precursors<sup>20,21,23,48–52</sup>. However, the conclusion of several previously published studies deserve to be reconsidered in the light of our results. For instance, the specificity of anti-DCC antibodies used to block NETRIN-1 activity on chick cells<sup>19,20</sup> is highly questionable as there is no DCC in chick genome. Likewise, the axon guidance defects observed after electroporation of truncated or mutated human DCC receptors, or of a ROBO1 ectodomain in chick spinal cord cannot be attributed to a dominant negative effect such as dimerization with endogenous DCC<sup>22,23</sup>. Other models, not requiring DCC, should be proposed to explain these results. For instance, the exogenous DCC receptors might bind to and perturb the function of Robo1/Robo2/Robo3 receptors, which are all expressed by chick commissural axons<sup>53,54</sup> and are known DCC partners in mouse neurons<sup>55,56</sup>. They might also trap NETRIN-1 thereby titrating it from its other receptors.

Our analysis of early bird embryos failed to reveal any major differences in the development of commissural tracts, regardless of the presence of DCC. Although such differences in commissural systems might exist at later developmental stages, the puzzling question remains on how some bird species coped with the accidental losses of *DCC* and in particular on the identity of the receptor(s) mediating NETRIN-1 chemoattractive activity in these species.

A first obvious candidate is the *DCC* paralogue NEOGENIN, as previously proposed<sup>18</sup>. In the mouse, DCC and NEOGENIN cooperate to attract spinal cord commissural axons to the floor plate, and NEOGENIN can partially compensate for DCC absence

in rodents <sup>57</sup>. Phylogeny analysis reveals a divergence in NEOGENIN sequences in passeriformes, which suggests a neofunctionalization of NEOGENIN in this group. As this bird group has lost DCC, we may raise the hypothesis that this neofunctionalization could possibly be related to NEOGENIN taking on DCC function. However, no such NEOGENIN sequence divergence was observed in galliformes, which have also lost DCC. Therefore such a scenario of NEOGENIN neofunctionalization could not apply in this later group. Although NEOGENIN is present in at least some chick spinal cord commissural neurons <sup>18</sup>, it has not yet been shown to be expressed by all NETRIN-1 responsive neurons in this species, and unlike DCC, NEOGENIN has other ligands <sup>58</sup>.

In chick embryo, NETRIN-1 was also shown to bind Down syndrome cell-adhesion molecule (DSCAM) and silencing DSCAM expression in chick spinal cord neurons impairs NETRIN-1 attraction <sup>59</sup>. In rodents, recent in vivo studies using knockout mice have challenged this model and suggest that DSCAM <sup>60</sup> is dispensable for NETRIN-1 attraction of commissural axons. It remains to confirm that this is also the case in passeriformes and galliformes.

Interestingly, DCC was also shown to bind dorsal repulsive axon guidance protein (DRAXIN) a secreted molecule which repels various classes of commissural axons <sup>61</sup>. DRAXIN and NETRIN-1 bind each other and compete for DCC binding <sup>62</sup>. How DRAXIN, which was first isolated in chick embryo <sup>61</sup>, might function in birds that have no DCC is also a mystery, but NEOGENIN could also be involved. Importantly, preliminary analysis of bird genomes indicates that, DSCAM, UNC5A-D and DRAXIN genes exist in all birds (data not shown).

In mammals, DCC plays a role in commissural axon guidance but also in the migration of neural crest cells and of GnRH neurons from the olfactory epithelium<sup>20,63</sup>. DCC influences the development of the autonomic innervation of arteries<sup>64</sup>. Moreover, DCC is a dependence receptor that can induce apoptosis in absence of NETRIN-1<sup>65</sup>, a mechanism that might explain its anti-tumorigenic properties<sup>17</sup>. Therefore, one would expect DCC loss to have important consequences on the development or function of many organs in the corresponding bird species, if not fully compensated by other receptors.

Possible loss of SMAD4, also located in the *DCC* genomic region, is particularly interesting as SMAD4 knockout mice are embryonic lethal<sup>66</sup>. However, we could find a SMAD4-like gene on chicken chromosome 25. This gene is conserved and its locus is unchanged in all birds and many vertebrates except mammals, and has been already characterized in xenopus<sup>67</sup>. It will be important to understand how some birds can cope with the absence of SMAD4, or if SMAD4-like could replace SMAD4.

Importantly, cancer has been described in all vertebrates including birds and most of the missing genes such as SMAD4<sup>66</sup>, SKA1<sup>68</sup>, MEX3C<sup>69</sup> and DCC have been linked to tumorigenesis. Interestingly, there is in chick a high incidence of spontaneous ovarian cancer with a prevalence reaching up to 35% after 3.5 years of age<sup>70,71</sup>. This correlates well with the downregulation of DCC expression described in human ovarian tumors<sup>72,73</sup>.

In conclusion, our results suggest that commissural axon guidance mechanisms are not conserved between bird species but that overall this does not seem to have a major impact on brain patterning. This illustrates the great plasticity of axon guidance mechanism, and how diverse this system can be among vertebrates. Another example of this diversity was recently reported in mammals, where mutations of few amino acids in mammalian ROBO3 receptor have completely modified its mechanism of action in commissural neurons<sup>55</sup>. To fully appreciate this diversity, it will be essential to reconstruct the phylogenic history of commissural guidance receptors and ligands in vertebrates.

## **Methods**

### **Genomic databases analysis**

Protein sequences from annotated genes were extracted from Ensembl or NCBI genome browsers (<http://www.ensembl.org/index.html> and <http://www.ncbi.nlm.nih.gov/nuccore/>). The TBLASTN algorithm of the NCBI website (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) was used on the genomic databases available when genes were not previously annotated. See supplementary Table S1 and S2 for complete genome and sequences information.

### **Phylogenetic analysis**

#### *DCC-NEOGENIN Analysis*

47 sequences composed of predicted mature netrin-receptor (DCC-NEOGENIN) with N-terminal signal peptide were first aligned using ClustalW<sup>74</sup>, then manually adjusted. The JTT (Jones, Taylor and Thornton) protein substitution matrix of the

resulting alignment was determined using ProTest software<sup>75</sup>. Phylogenetic analysis of the NEOGENIN-DCC receptors alignment was performed using the Maximum Likelihood method with 1,000 bootstrap replicates (RaxML software, <https://www.phylo.org/portal2>). DCC-NEOGENIN homologous *Drosophila melanogaster* FRAZZLED receptor was used as outgroup.

#### *NETRIN-1/2/3/5 and NETRIN-4 Analysis*

52 sequences for NETRIN-1/2/3/5 and 19 sequences for NETRIN-4, each one composed of a predicted mature NETRIN protein with N-terminal signal peptide, were aligned using ClustalW. The JTT protein substitution matrix of the resulting alignment was determined using ProTest software. Phylogenetic analysis of the NETRIN sequences alignment was performed using the Maximum Likelihood method with 1,000 bootstrap replicates (RaxML software) using *Branchiostoma floridae* NETRIN-1 and NETRIN-4 as outgroups, respectively.

#### **DCC synteny analysis**

Synteny maps of the DCC conserved genomic region were reconstructed for mammals (human, mouse, platypus), chelonian (painted turtle), crocodilians (alligator) and birds: paleognathae (ostrich), galloansers (duck, goose, chicken, turkey), and neoaves (falcon, bald eagle, royal eagle, adeli penguin, emperor penguin, pigeon, ibis, egret, cuckoo, chimney swift, hoatzin, zebra finch, sparrow, flycatcher and crow). Analyses of DCC neighbouring genes were performed manually using complete or preliminary annotated genome sequences from NCBI genome browser (<http://www.ncbi.nlm.nih.gov/gene/>), including numerous unplaced genomic scaffolds (see supplementary Table S3 for references and locations of the genes used in the synteny analysis, Table S4 for a complete list of the genes used in this

analysis, and Table S5 for a complete list of species used in the analysis). To complete this analysis we used TBLASTN algorithm on NCBI database to identify non-annotated DCC neighbouring genes and confirm gene absence (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

## **Animal sampling**

Eggs from chicken *Gallus gallus*, duck *Anas platyrhynchos*, zebra finch *Taenopygia guttata*, pigeon *Columba livia*, quail *Coturnix japonica*, pheasant *Phasianus colchicus* and partridge *Perdix perdix* were incubated at 37°C in humid conditions. Embryos were collected at different time points depending on their embryological stage. All procedures were performed in accordance to the guidelines approved by French Ministry of Agriculture and UPMC University ethic committee. Stage determination was done according to literature<sup>76-78</sup>. Exact number of embryos collected per stage is presented in supplementary Table S6. Embryos were first transferred to ice-cold PBS 1X; from E8, the nervous system were dissected and all embryos were then fixed by immersion in 4% paraformaldehyde overnight at 4°C. Samples were transferred to PBS 1X and kept at 4°C until use.

For whole-mount immunostaining, samples were dehydrated in methanol (MeOH 50%in PBS 1X - MeOH 80% in PBS 1X - MeOH 100%) and incubated overnight in MeOH with 5% $H_2O_2$  to suppress blood auto-fluorescence. Samples were then rehydrated (MeOH 100%- MeOH 80% in PBS 1X - MeOH 50%in PBS 1X - PBS 1X) and kept in PBS 1X at 4°C until use.

## **Histochemistry**

### *In situ hybridization*

Tissue sectioning and *in situ* hybridization were performed as previously described<sup>37</sup>. Pigeon *DCC* probe was designed in highly conserved domain of *DCC* gene coding from Fibronectin 5 to P1 domain. The sequence was amplified from pigeon embryos cDNA using following primers (Forward: 5'- CAGTAGGTGTCCAGGCTGTTG - 3'; Reverse: 5'- CCCGTTGGCTTCTCCATGTTC - 3') and cloned into pCRII-TOPO plasmid (ThermoFisher). The Duck *DCC* cDNA was kindly provided by Dr Sara Wilson.

#### *Sections immunostaining*

Immunostaining were performed as previously described<sup>37</sup>. The following primary antibodies were used: mouse anti- $\beta$ III Tubulin (1:1000, MMS435P-Covance), goat anti-ROBO3 (1:500, AF3076-R&D), goat anti-*DCC* (1:400, Sc-6535-Santacruz, raised against intracellular C-terminal domain of human *DCC*), mouse anti-*DCC* (1:300, AF-OP45-Calbiochem, raised against extracellular domain of human *DCC*). Corresponding secondary antibodies were used: donkey anti-rabbit Alexa488 (1:500, 711-545-152-Jackson), bovine anti-goat Cy3 (1:500, 805-165-180, Jackson), donkey anti-mouse cy3 (1:500, 715-165-150-Jackson), goat anti-mouse DL649 (1:500, 115-495-205-Jackson). Sections were counterstained with Hoechst and examined with a fluorescent microscope (DM6000, Leica) coupled to a CoolSnapHQ camera (Roper Scientific).

#### *Whole-mount Immunostaining*

Samples were incubated at room temperature (RT) in a solution (PBSGT) of PBS 1X containing 0.2% gelatin (Prolabo), 0.5% Triton X-100 (Sigma-Aldrich) and 0.01% thimerosal (Sigma-Aldrich) for 3h (E4-E6) or 8h. Samples were next transferred to



PBSGT containing the primary antibodies and placed at 37°C, with rotation at 70 rpm, for 4 days (E4-E6) or 7 days. Primary antibodies used were the following: mouse anti- $\beta$ III Tubulin (1:1000, MMS435P-Covance), goat anti-ROBO3 (1:400, AF3076 R&D), goat anti-DCC (1:400, Sc-6535 Santacruz) and mouse anti-DCC (1:300, AF-OP45-Calbiochem). Samples were then washed 3 times in PBSGT for 2h at RT and incubated for 24h at 37°C in secondary antibody diluted in PBSGT. Secondary antibodies used were the following: donkey anti-rabbit Alexa647 (1:500, 711-605-152-Jackson), bovine anti-goat Cy3 (1:500, 805-165-180, Jackson) and donkey anti-mouse Alexa488 (1:500, A21202-Lifetechnologie). After 4 washes of 2h in PBSGT at RT, samples were stored at 4°C in PBS until clearing.

Small samples (E4) were included in agarose 1.5% prior tissue clearing for better positioning in the ultramicroscope chamber.

Tissue clearing was performed using 3DISCO-clearing procedure as previously described<sup>79</sup>. Samples are stored in dibenzylether (DBE) in light protected glass vials at RT.

### *Ultramicroscopy*

3D imaging was performed with an ultramicroscope (LaVision BioTec) using InspectorPro software (LaVision BioTec). The light sheet was generated by a laser (wavelength 488 and 561nm, Coherent Sapphire Laser and 640nm, Coherent OBIS 640-100LX laser, LaVision BioTec) and two cylindrical lenses. A binocular stereomicroscope (MXV10, Olympus) with a 2X objective (MVPLAPO, Olympus) was used at different magnifications (1.25x, 1.6x, 2x, 2.5x, 3.2x and 4x). Samples were placed in an imaging reservoir made of 100% quartz (LaVision BioTec) filled with

DBE and illuminated from the side by the laser light. Images were acquired with a PCO Edge SCMOS CCD camera (LaVision BioTec).

Image processing was performed using Imaris software (Bitmap), as described previously <sup>79</sup>.

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### **Author Contributions**

F.F, performed the experiments and did the figures. A.G.L., F.F and S.D did phylogenic analyses. B.P, C.K, F.F and M.M collected bird embryos. A.C, A.G.L., F.F and S.D wrote the main manuscript. A.C. supervised the work. All authors reviewed the manuscript.

### **Competing financial interests:**

The authors declare on competing financial interests.

### **Figure Legends**

#### **Figure 1.**

**DCC gene has been lost independently twice during bird evolution. a.** Consensus phylogenetic tree of vertebrate *DCC* and *NEOGENIN*. Analysis was performed on 47 vertebrate DCC and NEOGENIN amino acid sequences using the

Maximum likelihood method, with 1000 bootstrap replicates (for sequence references, see supplementary Table S1). The tree was rooted using *Drosophila* FRAZZLED sequence as outgroup and branches displaying bootstrap values below 50 were collapsed. For better visualization, we cut out primary branches of a length equivalent to 1.2. **b.** Conserved genomic synteny of amniotes *DCC* chromosomal region. The Figure displays simplified genomic synteny map comparing positions of *DCC* and its neighboring genes in different amniotes species (For full analysis, see Supplementary Figure S1). Orthologues of each gene are represented in the same color and displayed in the same column. **c.** Proposed scenario for *DCC* gene loss in the avian lineage. *DCC* gene loss has occurred twice, independently, in galliformes and in passeriformes.

## Figure 2.

**Both *DCC* mRNA and protein are expressed in bird spinal cord except in galliformes and passeriformes.** Spinal cord sections from different birds are stained: partridge (a,h), pheasant (b,i), quail (c,j), chick (d,k), duck (e,l), pigeon (f,m) and zebra finch (g,n). a-g. *In situ* hybridization using *DCC* antisense riboprobes cloned from duck and pigeon detect strong expression of *DCC* mRNA expression in the spinal cord (e,f). Duck and pigeon riboprobes cross-react between the two species, but fail to detect *DCC* mRNA in galliformes (a-d) and passeriformes (g). h-n. Expression of  $\beta$ III-Tubulin and ROBO3 was detected in ventral commissures of all spinal cords. Anti-*DCC* antibodies against the intracellular and extracellular domains detect strong expression of *DCC* protein in ventral commissural neurons in duck (l) and pigeon (m) but fail to detect any *DCC* expression in galliformes (h-k) and

passeriformes (n). Arrows indicate the ventral midline. Abbreviations, Drg, dorsal root ganglia; Mr, motor nerve root. Scale bars: 50µm.

### **Figure 3.**

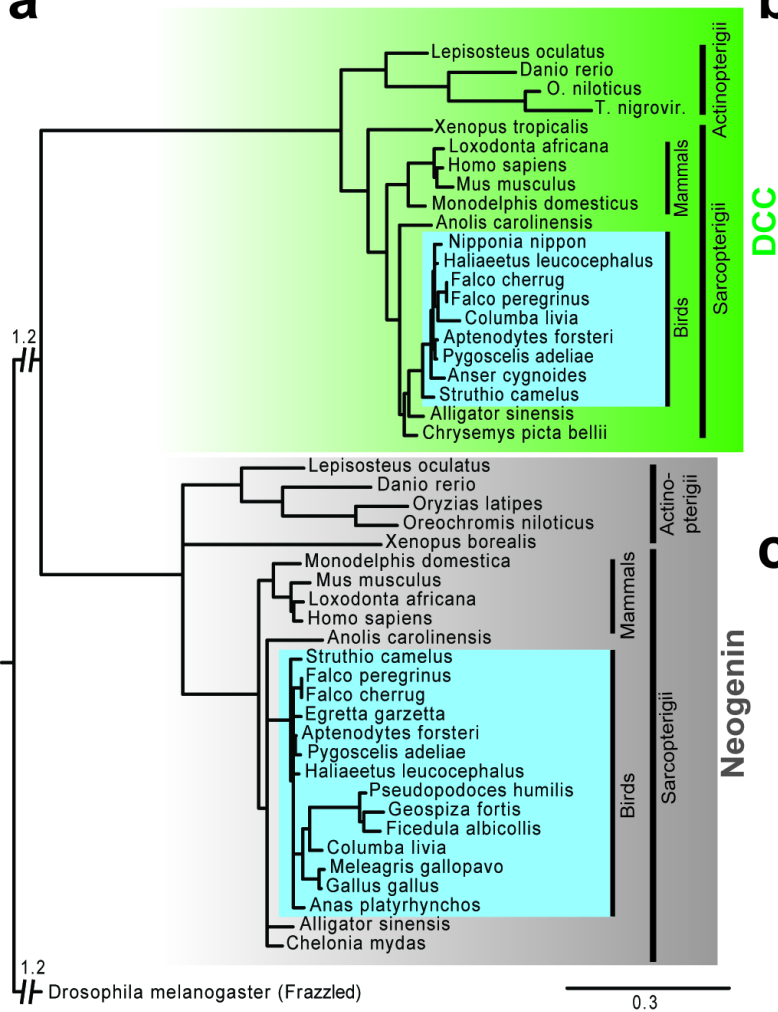
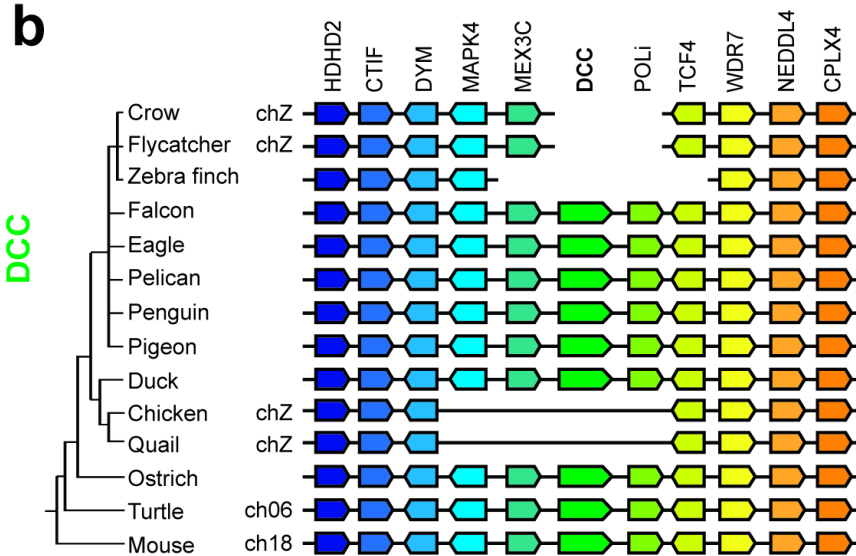
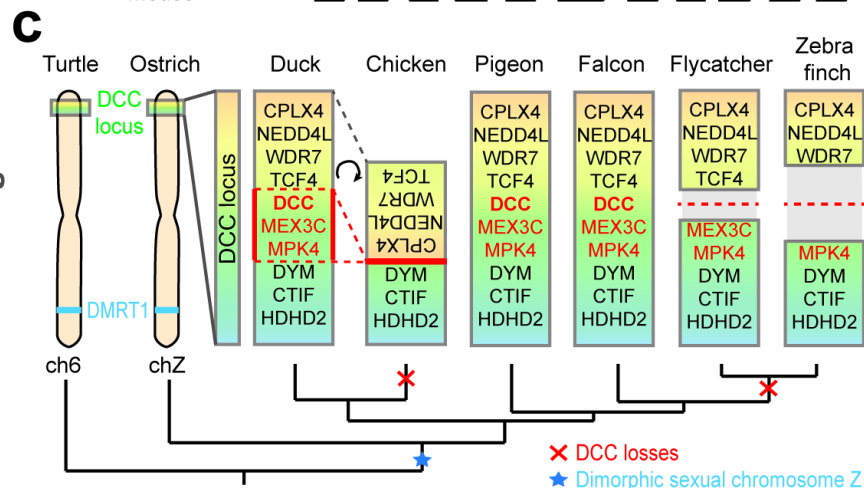
**3DISCO analysis of DCC receptor expression in chicken, pigeon and duck embryos.** DCC (a) and  $\beta$ III-Tubulin (b) whole-mount immunostaining on HH22 chicken embryos after 3DISCO clearing. No DCC expression is detected in chicken embryos whereas many axonal tracts are strongly labeled with anti- $\beta$ III-Tubulin. c-j, By contrast, DCC is strongly expressed in pigeon (c, e-g) and duck (d, h-j) embryos at stages equivalent to HH22. (h-j) in pigeon embryo, DCC is found in commissural axons crossing the floor plate (arrowhead in e), in retinal ganglion cells (Rgc) of the retina and the optic nerve (On; f), in the habenula nucleus (Ha) and fasciculus retroflexus (Fr; g). (h-j) in duck embryo, DCC is found in spinal cord (Sc) commissural axons, motor nerve roots (Mr), Rgc in the eye (i), optic nerve (On) diencephalon (Di) and olfactory nerve (Olf). Scale bars are 200µm except e, j, 100µm and f, 50µm. Abbreviations, Di: diencephalon; Hb: hindbrain; Tec, tectum; Olf: olfactory nerve; Tel, telencephalon; Tg, trigeminal ganglion.

### **Figure 4.**

**Commissural projections labeled with ROBO3 appear similar in birds with or without DCC.**

(a-o) 3D light sheet images of whole-mount bird embryos labeled with anti-Robo3 antibodies. (a-c) in H21-22 chick embryos, Robo3 is expressed by commissural axons in the tectum (Tec), ventral midbrain (Mb) and hindbrain (Hb) but not in the telencephalon (Te). The floor plate is indicated by an arrowhead in b. Commissural

axon growth cones (arrow) are seen approaching the midline in **c**. At equivalent developmental stages, the spatial pattern of Robo3<sup>+</sup> commissural projections is similar in pheasant (**d-f**), duck (**g-i**), pigeon (**j-l**) and zebra finch (**m-o**) embryos. Scale bars, 300µm in **a, d, g, j** and **m**; 150µm, in **b, e, h, k** and **n**; 100µm in **c, f, i, l** and **o**.

**a****b****c**

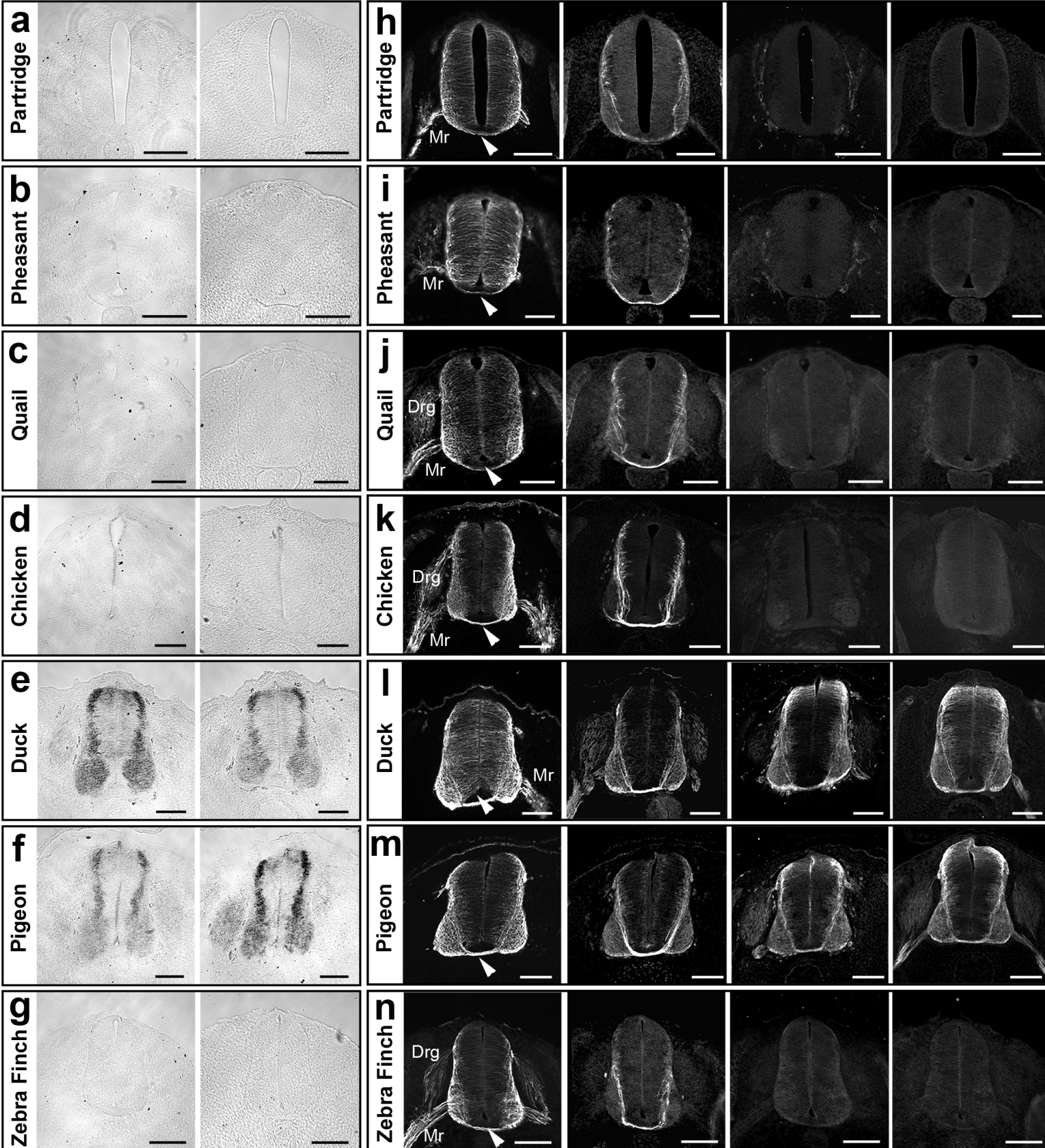


*DCC<sup>Duck</sup>**DCC<sup>Pigeon</sup>* $\beta$ III-tubulin

Robo3

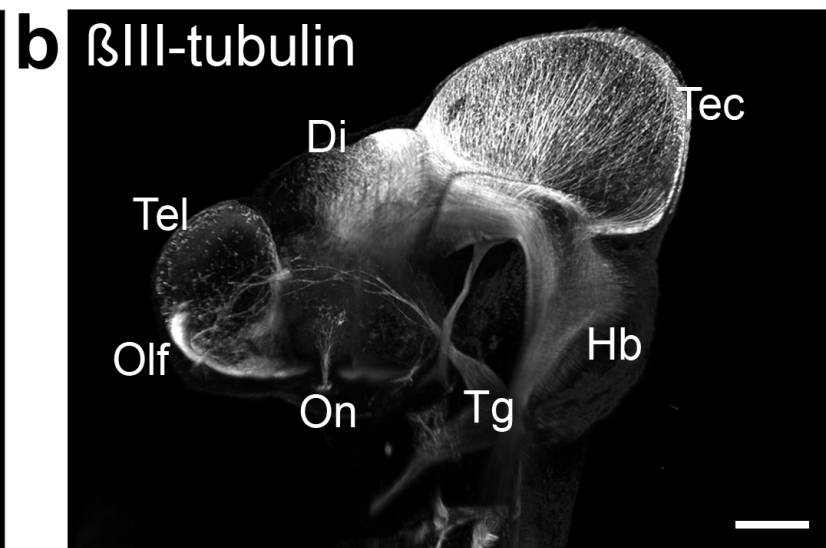
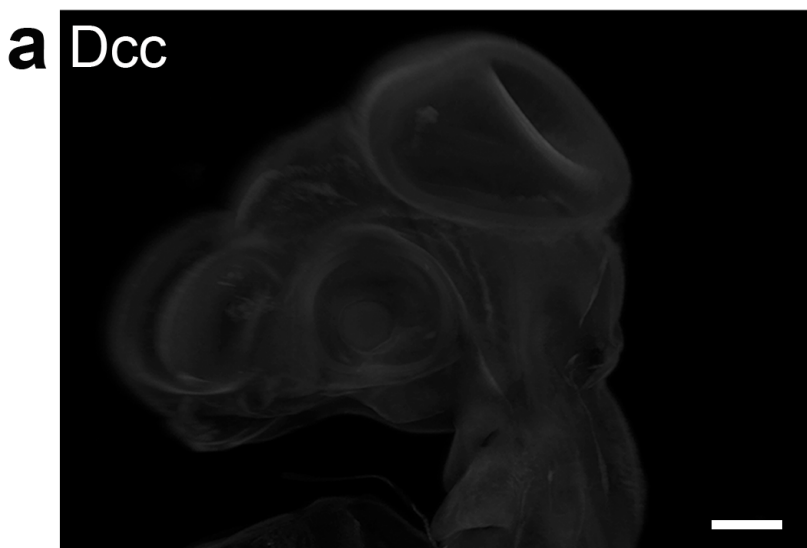
DCC intra

DCC extra

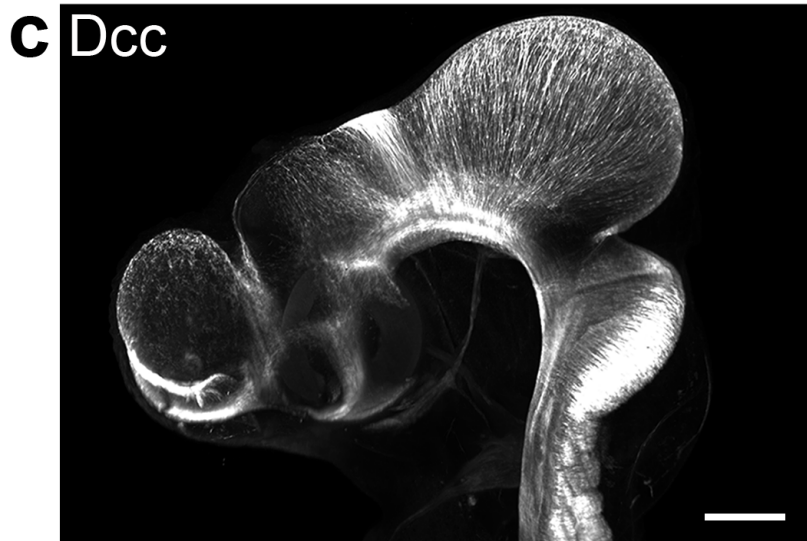




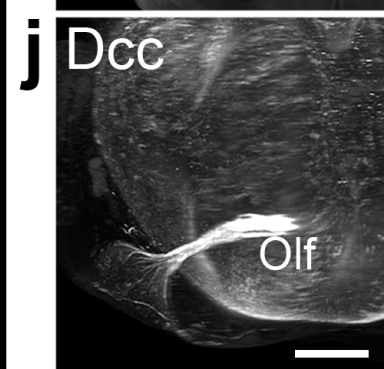
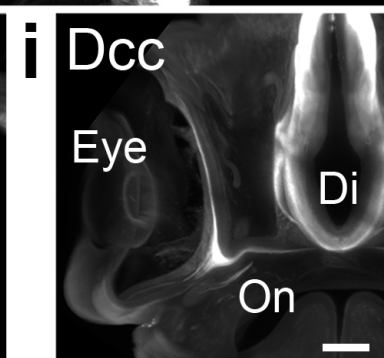
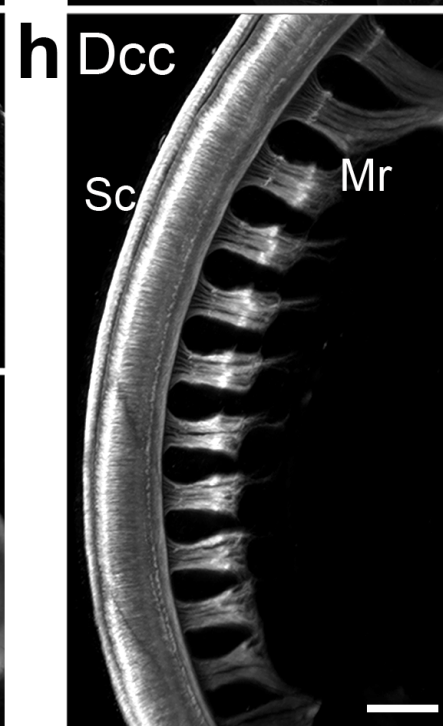
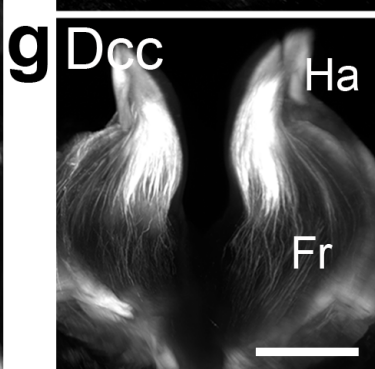
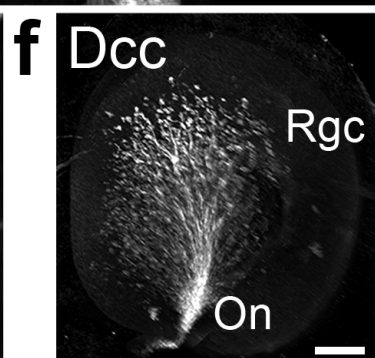
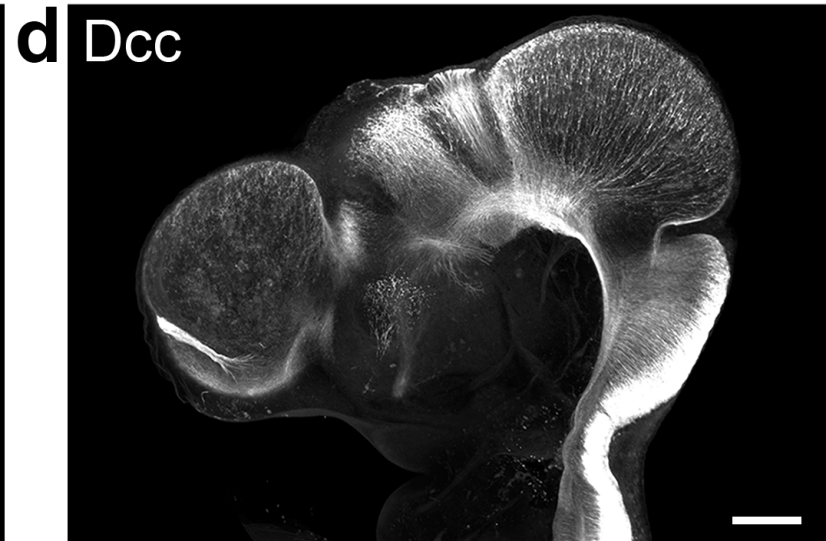
# Chicken



# Pigeon



# Duck





**Chicken**  
**Pheasant**  
**Duck**  
**Pigeon**  
**Zebra finch**

