Divergent low-density lipoprotein receptor (LDLR) linked to low VSV G-dependent viral infectivity and unique serum lipid profile in zebra finches

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The low-density lipoprotein receptor (LDLR) is key to cellular cholesterol uptake and is also the main receptor for the vesicular stomatitis virus glycoprotein (VSV G). Here we show that in songbirds LDLR is highly divergent and lacks domains critical for ligand binding and cellular trafficking, inconsistent with universal structure conservation and function across vertebrates. Linked to the LDLR functional domain loss, zebra finches show inefficient infectivity by lentiviruses (LVs) pseudotyped with VSV G, which can be rescued by the expression of human LDLR. Finches also show an atypical plasma lipid distribution that relies largely on high-density lipoprotein (HDL). These findings provide insights into the genetics and evolution of viral infectivity and cholesterol transport mechanisms in vertebrates.

cholesterol | LDLR | VSG G | lentivirus | viral transduction

The low-density lipoprotein receptor (LDLR) gene encodes the main low-density lipoprotein (LDL) receptor, a protein of highly conserved structure that plays a critical role in cholesterol uptake, contributing to the regulation of cholesterol levels in cells and blood (1, 2). Loss-of-function mutations in LDLR are the main cause of familial hypercholesterolemia, a highly prevalent human autosomal dominant disorder (1:250 to 1:500) (3), and LDLR disruptions cause hypercholesterolemia in vertebrates ranging from fish (4) to mice (5). LDLR is also the main receptor for the glycoprotein of vesicular stomatitis viruses (VSVs) (6), which accounts for the high VSV infectivity across a range of mammalian tissues and species (7). Notably, the VSV glycoprotein (VSV G) is broadly used for viral pseudotyping for gene manipulation (8) and gene therapy (9), thus a better understanding of LDLR's role in G-mediated viral uptake would have a major impact in a range of applications.

Results and Discussion

All vertebrate groups examined to date have an LDLR gene (Fig. 1*A*), and we confirm here the presence of LDLR orthologs in several bird groups (Fig. 1*B*). The human LDLR gene contains 18 exons that encode specific protein domains (Fig. 1*C*, human). This structure is considered broadly conserved across vertebrates (Fig. 1*D*, zebrafish to kakapo), consistent with a conserved role. However, we have discovered that songbirds, one of the largest and most diverse vertebrate groups are a major exception. The predicted LDLR protein in zebra finches lacks cysteine-rich repeats 1 and 2 (CR1 and CR2) and the EGF-like repeat immediately upstream of the beta-propeller blade domain (Fig. 1*C*, zebra finch). These losses are explained by the zebra finch LDLR gene lacking the equivalent of exons 2, 3, and 8 of the human gene (Fig. 1*C*, zebra finch). We confirmed that these exons are lacking in all five songbird species examined, belonging to different oscine radiations (e.g., Fig. 1*D*, thrush and crow; details in Dataset S1). This contrasts sharply with nonsongbird avian groups like parrots, eagles, and Galliformes (chicken, quail), where these exons and predicted coding domains are present (Fig. 1*D*), suggesting this domain loss is specific to passerines. CR2 is one of the two LDLR type-A repeats (the other being CR3) that are critical for VSV G protein binding and mediated viral transduction in mammals (10), whereas the EGF-like repeats together with the propeller blade region are important for receptor folding and trafficking (3). These domain losses thus provide a plausible explanation for the unusually low transduction rates seen in finch cells by VSV G-pseudotyped lentivirus (LV), which has limited the use of LV-based gene manipulation and transgenesis in this important model organism (11).

To further study the consequences of LDLR divergence in songbirds, we compared side by side the ability of VSV G LVs to infect embryonic cells in freshly laid fertilized eggs of zebra finches and chicken, using established protocols for avian transgenesis (11, 12). We observed markedly higher viral transduction (GFP expression) in somatic cells in chicken compared to finch embryos (Fig. 2 *A*, *Left*). To better assess this species difference, we compared VSV G LVs transduction in cultured embryonic fibroblasts from finch and chicken. While virtually all chicken cells were infected, very few finch cells expressed GFP (Fig. 2 *A*, *Right*). To investigate a causal link between LDLR and viral transduction, we next tested whether the expression of intact human LDLR (hLDLR) could enhance transduction in finch cells. Cultured finch embryonic fibroblasts expressing hLDLR showed a dramatic increase (nearly 10-fold) in VSV G LV transduction compared to control wild-type (WT) cells (Fig. 2 *B* and *C*, *Left* columns), an enhancement not seen in chicken cells (Fig. 2 *C*, *Right* columns). Because expression of an intact LDLR was sufficient to confer high VSV G LV transduction, these data suggest that lack of a functional high-affinity G protein receptor is a major transduction limiting factor in finch cells. These findings are also consistent with the low infectivity in neuronal cells when VSV G LVs are directly injected into the finch brain (e.g., ref. 13), and the low efficacy of

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The authors declare no competing interest.

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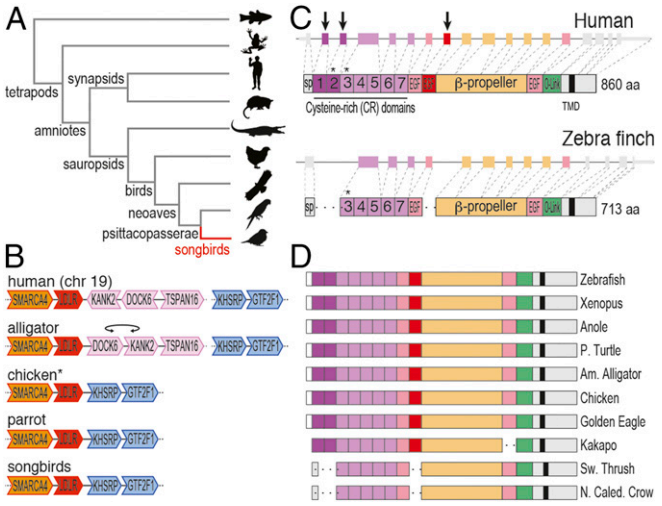

Fig. 1. LDLR gene divergence in songbirds. (*A*) Simplified cladogram of vertebrates; LDLR is present in all groups but highly divergent in songbirds (red). (*B*) LDLR gene synteny depicts broad conservation across vertebrates, with rearrangements or apparent gene losses (KANK2, DOCK6, and TSPAN16) downstream of LDLR in sauropsids. Chromosomal placement is undefined for most species, and synteny in chicken (*) derives from Pacbio p-reads (*SI Appendix*). (*C*) Diagram of LDLR gene and predicted protein in humans (*Top*) and zebra finch (*Bottom*). Dashed lines show exon correspondence to specific protein domains; arrows indicate human exons missing in songbirds; dark-colored regions indicate cysteine-rich (CR) (violet) and EGF-like (red) human protein domains missing in zebra finches; and asterisks indicate protein domains critical for VSV G binding. Relative sizes of exons (*Upper* lines) and protein domains (*Lower* lines), but not of introns, were preserved. (*D*) Predicted LDLR protein domains show conservation across vertebrates except in songbirds (e.g., Swainson's thrush and New Caledonian crow; zebra finches in *C*) where CRs 1 and 2 and one EGF-like domain are missing and the protein is shorter. We note the unique lack of the downstream EGF-like domain in kakapo. Color codes and relative scales for protein domains are as in *C*. sp, signal peptide; EGF, epidermal growth factor-like repeat; O-Link, O-linked glycosylation domain; TMD, transmembrane domain.

VSV G-based transgenesis in finch compared to chicken and quail (12, 14), two species with an intact LDLR. We also note that even in studies reporting behavioral effects from brain gene manipulations using VSV G LVs in finches, the number of infected cells is typically quite low (15–17).

To explore whether the divergent songbird LDLR might also be associated with adaptations in lipid transport, we next analyzed serum from adult male zebra finches for the content of cholesterol and triglycerides in lipoproteins. In striking contrast with humans, finches lacked an LDL-like fraction, and practically all serum cholesterol was associated with a high-density lipoprotein (HDL) fraction (Fig. 2*D*). Similar profiles were seen in both sated and fasting finches (Fig. 2 *E*, black), indicating that they are consistent across feeding states. Interestingly, triglycerides were also almost exclusively associated with the HDL fraction (Fig. 2 *E*, green). This would be expected from the loss of a functional LDLR, since triglycerides in mammals are carried by ApoB-containing lipoproteins and cleared by LDLR (18). Importantly, sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and mass spectrometry analyses revealed the presence of ApoA1 in the finch HDL-like fraction (Table 1), supporting its equivalence to mammalian HDL, and the apparent lack of ApoB-containing fractions. These findings in finches contrasted markedly with those in chicken, where an LDL fraction was detectable and triglycerides were primarily associated with the VLDL-like fraction (Fig. 2*E*), thus more reminiscent of profiles in mammals. The molar ratio of triglyceride over cholesterol for the HDL fraction was also significantly higher in finches than in chicken (Fig. 2 *F*, *Inset*).

Taken together, our findings demonstrate substantial divergence of LDLR structure and function in the songbird lineage compared to mammals, most birds, and nonavian vertebrates, contradicting a widely held belief of conserved structure and biological function of LDLR in vertebrates. Importantly, while loss of LDLR function in mammals results in severe hypercholesterolemia and large increases in LDL, this is not the case for the evolutionary loss in songbirds, or at least in finches, where most if not all cholesterol is in the HDL fraction. This finding is even more intriguing, given the apparent lack of a functional ApoE in birds (19). In mice, a double knockout of LDLR/ApoE leads to aortic atherosclerosis and high cholesterol and triglycerides (5). Loss of critical LDLR domains does not appear to be detrimental to songbirds, and this lineage seems to have evolved a unique lipid transport system that does not rely on ApoB-containing lipoproteins. Further studies in these birds could thus yield valuable new insights into lipid production, transport, and clearance mechanisms. Moreover, the divergent structure of LDLR might contribute to the low VSV infectivity of some bird species (7). Besides the practical implications for improving VSV G LV-based transgenesis and gene manipulation in songbirds, our observations open a door to study the evolution and relationship between viral infectivity and a key physiological receptor system.

Methods

For comparative gene analysis, we used BLAST searches and curation of the National Center for Biotechnology Information (NCBI) genomic databases, coupled with reciprocal alignments and synteny verification to identify avian LDLR orthologs. Determination of exon/intron boundaries was supported by transcriptome data, noting that high-quality Pacbio assemblies (as in ref. 20) were key. To compare the predicted protein sequences and structural domains of LDLR orthologs, we used Clustal with default settings in JalView1.0 and InterProScan. To study in vivo viral infectivity, freshly laid finch and chicken embryos were injected with high titer VSV G LV preps, sealed, incubated, and examined

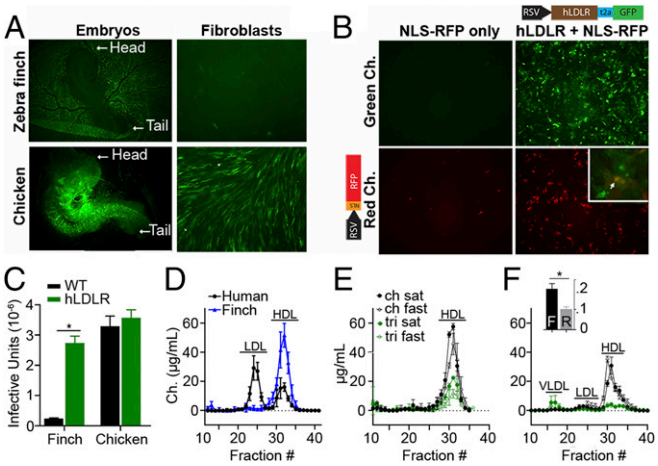

Fig. 2. LDLR-related functions in birds. (*A*) VSV G LV infectivity is higher in chicken embryos and cultured embryonic fibroblasts (*Bottom*) compared to zebra finches (*Top*). (*B*) Cultured zebra finch cells expressing hLDLR (*Right*) show higher infectivity by VSV G LV-expressing NLS-RFP (red signal) compared to wild-type cells (*Left*). Same field for green and red channels, *Inset* at *Bottom Right* shows hLDLR$^+$ cells expressing NLS-RFP (arrow), hLDLR, and NLS-RFP constructs shown at *Top Right* and *Bottom Left*, respectively. (*C*) Quantification of the experiment in *B* shows increased cell infectivity in zebra finches but not in chicken (means ± SEM, $*P = 0.0017$). (*D*) Comparison of serum cholesterol profiles for zebra finch (blue) and human (black) show absence of an LDL-like fraction and an almost exclusive association of cholesterol with an HDL-like fraction in finch. (*E* and *F*) Serum cholesterol and triglyceride profiles for sated and fasting adult male zebra finches (*E*) and roosters (*F*). (*Inset* in *F*) Comparison of molar ratio of serum triglycerides to cholesterol in HDL shows higher ratio in zebra finches (F) than roosters (R) (means ± SEM; *t* test, $P = 0.0012$). NLS, nuclear localization signal; Chol., cholesterol; Trig., triglycerides.

Table 1. Zebra finch serum cholesterol is in ApoA1-containing fractions

Gel band	Uniprot accession	Gene name	Protein name	No. unique peptides	No. PSMs	MW (kDa)
01	H0ZS92	A2M	Alpha-2-macroglobulin	25	53	164.4
01	H0ZS79	A2M	Alpha-2-macroglobulin	8	30	158.6
01	H0Z0Q3	ACTG1	Actin, cytoplasmic	9	10	41.8
02	H0Z885	TF	Ovotransferrin	39	144	77.4
02	H0YTL4	ALB	Albumin	23	47	68.1
02	H0Z0Q3	ACTG1	Actin, cytoplasmic	3	14	41.8
03	H0YTL4	ALB	Albumin	53	329	68.1
03	H0Z885	TF	Ovotransferrin	10	12	77.4
03	H0ZP14	c4	Complement C4	7	8	188.4
04	H0YTL4	ALB	Albumin	13	28	68.1
04	**H0YPF4**	**APOA1**	**Apolipoprotein A1**	**3**	**22**	**6**
04	H0YRF2	GPX1	Glutathione peroxidase 1	4	11	13.8

Protein content of SDS-PAGE-excised bands determined by mass spectrometry demonstrates the presence of ApoA1 in HDL-like and lack of ApoB or other detectable apolipoproteins in other fractions. Gel bands 01 and 02 to 04 derive respectively from finch serum fractions equivalent to 21 to 25 and 29 to 35 in Fig. 2*D*. Only the three main protein components per gel band are listed. Boldface indicates the HDL-fraction defining component.

for GFP expression 72 h later, as in ref. 11. To quantify viral infectivity and assess viral entry factors, primary cultures of zebra finch and chicken fibroblasts were prepared using a procedure modified from ref. 21, transduced with LVs containing human LDLR, and exposed after 4 to 5 d to VSV G LV expressing a red fluorescence protein. For lipid analyses, blood was collected from male birds, examined for serum cholesterol and triglyceride content, and analyzed for serum lipoprotein distribution by fast protein liquid chromatography (FPLC); protein content of lipoprotein fractions was determined by gel electrophoresis and mass spectrometry, using standard protocols. For details, see *SI Appendix*.

Data Availability. All study data are included in the article and/or supporting information.

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