# Application of zebrafish and murine models in lipoprotein metabolism and atherosclerosis research

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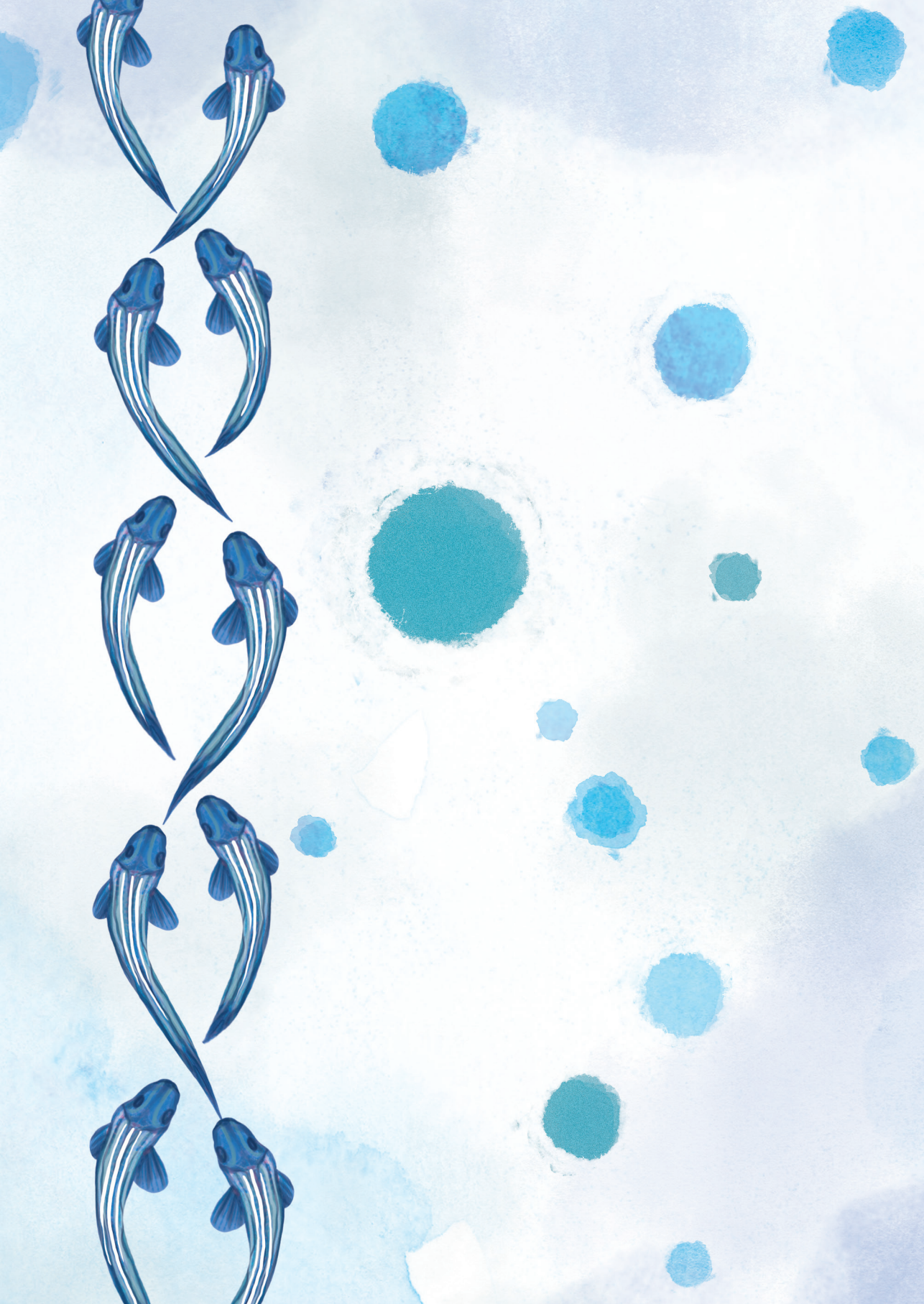

# CHAPTER 4

## ZEBRAFISH AS OUTGROUP MODEL TO STUDY EVOLUTION OF SCAVENGER RECEPTOR CLASS B TYPE I FUNCTIONS

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*Accepted for publication in BBA - Molecular and Cell Biology of Lipids*

# Abstract

**Background and aims:** Scavenger receptor class B1 (SCARB1) - also known as the high-density lipoprotein (HDL) receptor - is a multi-ligand scavenger receptor that is primarily expressed in liver and steroidogenic organs. This receptor is known for its function in reverse cholesterol transport (RCT) in mammals and hence disruption leads to a massive increase in HDL cholesterol in these species. The extracellular domain of SCARB1 - which is important for cholesterol handling - is highly conserved across multiple vertebrates, except in zebrafish.

**Methods:** To examine the functional conservation of SCARB1 among vertebrates, two stable *scarb1* knockout zebrafish lines, *scarb1* 715delA (*scarb1* -1nt) and *scarb1* 715_716insGG (*scarb1* +2nt), were created using CRISPR-Cas9 technology.

**Results:** We demonstrate that, in zebrafish, SCARB1 deficiency leads to disruption of carotenoid-based pigmentation, reduced fertility, and a decreased larvae survival rate, whereas steroidogenesis was unaltered. The observed reduced fertility is *driven* by defects in female fertility (-50%, $p<0.001$). Importantly, these alterations were independent of changes in free (wild-type 2.4 ± 0.2 mg/ml versus scarb1$^{-/-}$ 2.0 ± 0.1 mg/ml) as well as total (wild-type 4.2 ± 0.4 mg/ml versus *scarb1*$^{-/-}$ 4.0 ± 0.3 mg/ml) plasma cholesterol levels. The uptake of HDL in the liver of *scarb1*$^{-/-}$ zebrafish larvae was reduced (-86.7%, $p<0.001$), but this coincided with reduced perfusion of the liver. No effect was observed on lipoprotein uptake in the caudal vein. SCARB1 deficient canaries, which also lack carotenoids in their plumage, similarly as *scarb1*$^{-/-}$ zebrafish, failed to show an increase in plasma free- and total cholesterol levels.

**Conclusion:** Our findings suggest that the specific function of SCARB1 in maintaining plasma cholesterol could be an evolutionary novelty that became prominent in mammals, while other known functions were already present earlier during vertebrate evolution.

# Introduction

Scavenger receptors (SRs) are evolutionarily ancient membrane bound receptors that are known to bind and internalize various ligands and are expressed in multiple cell types[1]. During the emergence of the first primitive multicellular organisms, scavenger receptor families arose that differ in structure and function. Currently, there are multiple classes of scavenger receptors, but there is little structural homology between the different members of this ancient gene family[2]. Class B scavenger receptors (SR-Bs) are characterized by a large extracellular loop flanked on either side by a transmembrane domain connected to the cytoplasmic tails[2]. SR-B-like domains were already found in sponges and cnidarians, simple marine animals, and have been shown to closely resemble vertebrate SR-Bs[3].



One important vertebrate representative that belongs to this class is scavenger receptor class B1 (SCARB1). SCARB1 is also known as the high-density lipoprotein (HDL) receptor. It is widely expressed in organs such as the liver and steroidogenic tissues, including the adrenal glands and gonads[4]. Multiple clinical studies have shown that low plasma HDL-cholesterol levels are associated with an increased risk for cardiovascular disease[5–7]. The protective role of HDL in the context of cardiovascular disease is mainly attributed to its role in the reverse cholesterol transport (RCT). During this process, HDL removes (excessive) cholesterol from peripheral tissues and transports it back to the liver for cholesterol excretion after selective delivery through SCARB1[8]. Studies in humans and mice have shown that disruption of the HDL-SCARB1 interaction is associated with elevated plasma HDL-cholesterol levels and increased risk for atherosclerosis development, the underlying pathology of cardiovascular disease[9–16].

SCARB1 has been identified in several groups of vertebrates, including amphibians, birds, and several fish species, indicating that SCARB1 has emerged during early vertebrate evolution. Multiple comparative studies of SCARB1 have shown that this gene is characterized by strong sequence conservation within vertebrates. A recent study by May *et al.* (2021) showed that, in humans, arginine-174 in the extracellular loop of the SCARB1 protein is essential for the interaction with HDL particles[17]. More specifically, a rare variant of SCARB1 in which arginine-174 is substituted with a cysteine, displayed a loss of its cholesterol transport functions, including HDL binding, cholesteryl ester uptake, and free cholesterol efflux. In general, the presence of arginine-174 within the SCARB1 protein is well conserved across mammals. Interestingly, May *et al.* found that in zebrafish arginine-174 is replaced by a lysine[17], a substitution that has (until now) never been observed in mammals, but might have similar effects

as the arginine-174 to cysteine substitution. It can therefore be hypothesized that SCARB1's contribution to HDL binding and selective cholesteryl ester uptake might be limited in zebrafish and that this cholesterol transport function of SCARB1 arose later during mammalian evolution. To test this hypothesis, we generated SCARB1 knockout zebrafish using CRISPR/Cas9 mutagenesis and determined the effect of SCARB1 deficiency on cholesterol metabolism, pigment development, steroid production, and fertility.

## Material and methods

*CRISPR/Cas9 mutagenesis and zebrafish maintenance*

Cloning-free sgRNAs targeting SCARB1 were designed and synthesized as described[18]. The genomic target sequence used for zebrafish SCARB1 was 5'-ggtcaccatccctaatatgc-3' (PAM sequence underlined). 300 pg nls-Cas9-nls mRNA and 250 pg of SCARB1 sgRNA were co-injected into a single-cell zebrafish embryo of the ABTL (wild-type; WT) *Danio rerio* strain. Mutagenesis efficacy, founder identification, and genotyping were performed using CRISPR-STAT[19]. Table 1 lists the guide RNA sequences and the genotyping primers. The following SCARB1 knockout zebrafish lines were generated: *scarb1* 715delA (*scarb1* -1nt), and *scarb1* 715_716insGG (Scarb1 +2nt). Adult zebrafish were maintained on a 14-hours-light/10-hours dark cycle at 28°C and fed Artemia (AM) and a mixture of GEMMA Micro 300 (200-500 µm, Skretting) and GEMMA Diamond M-0.5 (0.5 mm Skretting) (PM) daily. Fertilization was performed by natural spawning at the beginning of the light cycle. Eggs were raised at 28.5°C in egg water (60 µg/mL Instant Ocean sea salts). The zebrafish were bred and maintained at the faculty of Science, Leiden University (the Netherlands), and handled according to the guidelines for the protection of vertebrate animals used for experimental and other scientific purposes from the European Convention and in compliance with the directives of the local animal welfare committee of Leiden University[20].

**Table 1.** Guide RNA sequence for CRISPR/Cas9, forward and reverse primers used to induce and verify *scarb1* deficiency. Target sequence in presented in green.

| ***SCARB1*** | **Sequence** |
|---|---|
| **sgRNA** | TAATACGACTCACTATAGGTCACCATCCCTAATATGCGTTTTAGAGCTAGAAATAGC |
| **Primer Forward** | TGTAAAACGACGGCCAGTCACATTTTCTCACCGTTTTTCA |
| **Primer Reverse** | GTGTCTTTGAAGGAATTCTGTCACTCTGC |

*Fertility measurements*
Natural spawning was facilitated by placing breeder pairs in 1.5 L single breeder tanks overnight and by removal of the divider in the morning. Embryos were kept at 28.5°C in egg water (60 µg/mL Instant Ocean sea salts) and counted each day up till 3 days post fertilization (dpf).

*Zebrafish lipoprotein injections and confocal imaging*
The different SCARB1 knockout zebrafish lines were crossbred with *Tg(mpeg1:*RFP*)* *ump2* zebrafish in which the macrophages express red fluorescent protein, to be able to differentiate between lipid uptake by macrophages and other cells present in the caudal vein, i.e. endothelial cells. Human DiO high-density lipoprotein (HDL; KyvoBio, 770380-9), human Bodipy low-density lipoprotein (LDL; ThermoFisher, L3483), and human DiO oxidized low-density lipoprotein (oxLDL; KyvoBio, 770282-9) were diluted to 0.5 mg/mL in MilliQ. 3 dpf larvae were anesthetized in 0.01% tricaine and embedded in 0.4% agarose gel containing 0.01% tricaine prior to injection with either 2 nL HDL (0.5 mg/mL) or 1 nL (ox)LDL (0.5 mg/mL) using an Eppendorf Femtojet. The caudal veins of the larvae were imaged 30-60 min post injection with oxLDL and LDL using a Leica TCS SPE confocal microscope. Given the relatively slow plasma decay of HDL-cholesteryl esters as compared to LDL and oxLDL in rodents[21,22] and our pilot observation that no HDL-derived lipids accumulate in the caudal vein of zebrafish up to 3 hours after injection, 24h post injection was chosen as time point for imaging the uptake of HDL-associated lipid species, i.e. DiO, into the vessel wall of the caudal vein. DiO-HDL uptake was imaged in the liver at 3 hours after injection. The uptake of HDL in the liver was quantified by manual counting of the HDL uptake (punctae) outside the sinusoids of 3 confocal image depths per zebrafish. Images were processed using ImageJ to adjust color balance and merge channels.

*Plasma collection and lipid measurements*
Adult fish (> 3months old) were anesthetized in ice water until no response (10-15 seconds) and then held in warm water (30°C) for 5 seconds to prevent blood clotting. A small part of the tail was amputated using a surgical blade. Fish were then placed, wound down, in a 1.5 mL Eppendorf tube of which the bottom was cut off, which was placed on a Microvette CB 300 (Sarstedt Inc, 16.444.100) pre-coated with EDTA and centrifuged in a 50 mL tube for 3 minutes at 55 RCF at RT. Another 2-3 mm was amputated, and fish were centrifuged again in the same tubes. Blood was transferred to a 0.5 mL tube and spun down for 10 minutes at 0.5 RCF at RT. Plasma (3-8 µL per fish) was collected. Free cholesterol and total cholesterol levels were determined in plasma samples using an enzymatic colorimetric assay as previously described by Out *et al*[23].

We also studied lipid metabolism in canary birds (*Serinus canaria*). Canaries were housed in separate indoor cages (0.6 × 0.5 × 0.5 m) with ad libitum access to water and food (Prestige® canary seed mix and Orlux Gold® patee canary supplement, Versele-Laga, Deinze, Belgium). The photo-period varied accordingly to the season (14 h daylight – 10 h night during breeding season; and 10 h daylight - 14 h night during moulting). Temperature and humidity varied between 14-23ºC and 55–70% respectively during the year. For blood collection from canaries, twelve animals (six canaries exhibiting yellow carotenoid coloration and six white recessive canaries that lack functional SCARB1 and are devoid of carotenoids in their plumage) from the CIBIO/InBIO facilities (Portugal) were used. The brachial vein was pierced with a 25-gauge needle, and ~100 µL blood was collected into a heparinized capillary tube (Microvette CB 300; Sarstedt AG & Co., Germany). Subsequently, the blood samples were centrifuged at 10,062 G for 10 minutes and stored at −20ºC until further analysis. All experimental procedures were conducted in accordance with the Directive 2010/63/EU on the protection of animals. Animal care of birds kept in the CIBIO/InBIO facilities complied with national and international regulations for the maintenance of live birds in captivity (FELASA, Federation of European Laboratory Animal Science Associations).

*Cortisol analysis*

4 dpf larvae were stressed by means of netting. Larvae were lifted from the water for 1 minute followed by 30 seconds recovery time in the water. This cycle was repeated 3 times. Subsequently, larvae were allowed 15 minutes of free swimming before sacrifice. Cortisol levels were measured in whole zebrafish larvae using an ELISA kit (Cortisol free in saliva, Demeditec, DES6611) according to manufacturer's instructions.

*Data analysis*

Given that no visual or statistically significant differences were found between the two *scarb1* knock-out lines in terms of phenotype, the data of these groups were pooled to increase the statistical power and referred to as *scarb1*$^{-/-}$. Statistical differences between experimental groups were evaluated by a Student's t-test (2 groups), one-way ANOVA (>2 groups), or two-way ANOVA with Bonferroni post-test (2 parameter analysis) using GraphPad Prism 9. Values of $p<0.05$ were considered statistically significant. All graphs are presented as means ± SEM.

# Results

**_Scarb1_ deficiency in zebrafish is associated with loss of yellow pigment**

To examine which functions of *scarb1* are active in zebrafish, two stable *scarb1* knockout zebrafish lines, *scarb1* 715delA (*scarb1* -1nt) and *scarb1* 715_716insGG (*scarb1* +2nt), were created using CRISPR-Cas9 technology. Both mutations were theoretically associated with an early stop in protein translation in exon 4 and result in the generation of a non-functional protein (**SFig. 1A**). Crossing of heterozygous SCARB1 mutants resulted in the generation of viable offspring following the expected Mendelian ratios, i.e. with ~25% wild-type (WT) zebrafish and ~50% and ~25% heterozygous (HZ) and homozygous knockout (KO) zebrafish, respectively (**Fig. 1A**). Adult SCARB1 deficient zebrafish (*scarb1*$^{-/-}$) appeared phenotypically normal. However, in line with the report of Saunders *et al.* showing that *scarb1* in zebrafish is required for carotenoid uptake into xanthophores[24], both the homozygous *scarb1* mutants completely lacked the yellow pigment produced by xanthophores throughout the skin, and especially prominent in the fins of adult zebrafish (**Fig. 1B**). This phenotype was already evident in the blastula phase of *scarb1*$^{-/-}$ embryo's (**SFig. 2**).

**_Scarb1_ deficiency in zebrafish is associated with reduced fertility**

Since multiple studies have shown that *scarb1* deficiency impairs fertility in mammals[25,26], we examined whether this was also the case in zebrafish lacking *scarb1*. In accordance, *scarb1*$^{-/-}$ zebrafish crossings generated on average significantly less embryos as compared to WT crosses (WT 229 ± 22 versus *scarb1*$^{-/-}$ 99 ± 9 fertilized eggs at fertilization; $p<0.001$; **Fig. 1C**). Importantly, the survival rate of the *scarb1*$^{-/-}$ zebrafish larvae was also reduced after hatching at 3 dpf ($p<0.001$; **Fig. 1D**). However, embryos of both genotypes that were still alive at 3 dpf did not display evident morphological differences in their development (**Fig.1E**) and progressed normally into adulthood (data not shown). Given that the offspring derived from crosses between heterozygous individuals followed the expected Mendelian ratios, suggesting similar embryonic and larval viability among the different genotypes, the lower survival rate observed in crosses between *scarb1*$^{-/-}$ individuals suggests that *scarb1* deficiency in zebrafish is likely associated with an overall reduced oocyte or spermatocyte number or quality.

Studies have demonstrated that the reduced reproduction rate associated with SCARB1 deficiency in mammals is driven by reduced female fertility[15,16,26]. To uncover the relative contribution of gender and genotype to the diminished embryo development in *scarb1* knockout zebrafish, we performed the following outcrosses: ♀ WT x ♂ WT, ♀ WT x ♂ *scarb1*$^{-/-}$, ♀ *scarb1*$^{-/-}$ x ♂ WT, and ♀ *scarb1*$^{-/-}$ x ♂ *scarb1*$^{-/-}$. A reduction in the number of live fertilized eggs was detected when female *scarb1*$^{-/-}$ zebrafish were used in the crossings

(two-way ANOVA $p<0.001$ for female gender; **Fig. 1E**), suggesting that *scarb1* deficiency in zebrafish is indeed associated with reduced female fertility. Comparing ♀ *scarb1*$^{-/-}$ *x* ♂ WT crossings to ♀ WT x ♂ WT crossings, a 50% reduction was seen in the number of fertilized eggs. No further reduction was observed for ♀ *scarb1*$^{-/-}$ x ♂ *scarb1*$^{-/-}$ as compared to the ♀ *scarb1*$^{-/-}$ *x* ♂ WT crossings. The number of fertilized eggs observed in ♀ WT x ♂ *scarb1*$^{-/-}$ crossings was also reduced as compared to ♀ WT x ♂ WT crossings (-32%; $p<0.001$; **Fig. 1F**). Thus, part of the reduction in egg count in homozygous *scarb1*$^{-/-}$ zebrafish crossings can potentially also be partly due to diminished male fertility associated with *scarb1* deficiency.

### *Scarb1*$^{-/-}$ zebrafish exhibit normal lipoprotein-lipid uptake, plasma cholesterol levels, and cortisol synthesis

In general, lipid metabolic pathways are highly conserved between mammals and zebrafish[27]. Selective uptake of cholesteryl esters from HDL particles by the liver and for use in adrenal glucocorticoid synthesis are assumed conserved functions of SCARB1 during vertebrate evolution. Therefore, the effect of *Scarb1* deficiency on hepatic HDL uptake as well as the production of the primary glucocorticoid cortisol was assessed. Considering that SCARB1 is the established receptor for HDL in mammals, we hypothesized that the clearance of HDL from the circulation of *scarb1*$^{-/-}$ zebrafish larvae would be impaired. Injection of DiO-labeled HDL in 3dpf embryos, however, showed that a large part of the injected HDL remained in the circulation at 3 hours post injection (hpi)(**Fig. 2A**). We did observe a reduction in the appearance of the DiO-labeled particles in SCARB1 knockout livers (WT 31.7 ± 5.5 punctae/field versus *scarb1*$^{-/-}$ 4.34 ± 0.8 punctae/field)(**Fig. 2B-C**). However, this seems to coincide with a reduction in perfusion of the livers of the SCARB1 knockout larvae as evident from the less intense generalized staining. This makes it impossible to conclude if the observed reduction in uptake is a direct effect of the absence of functional SCARB1 on hepatocytes. DiO-labeled HDL uptake was also analysed in the caudal vein, a commonly studied site in the zebrafish model.

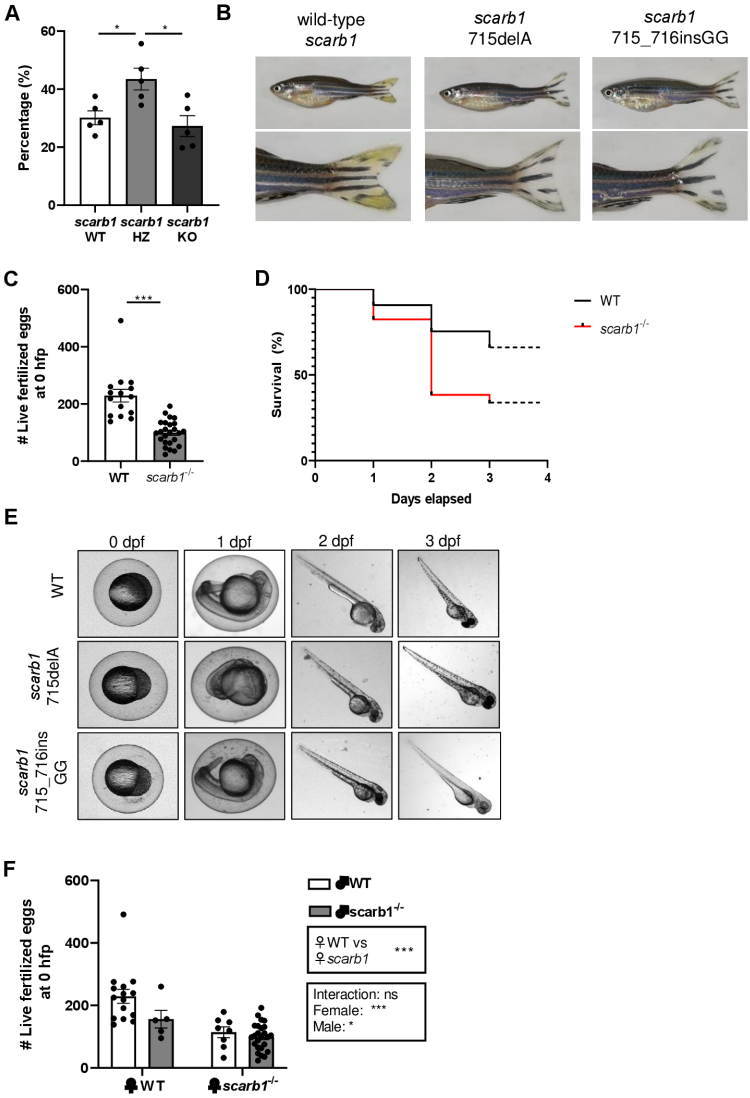



**Figure 1. SCARB1 deficiency in zebrafish is linked to reduced fertility.**

**(A)** Percentage of zebrafish that are WT, heterozygous (HZ) or knock-out (KO)(of total laid eggs) after crossing *scarb1*$^{+/-}$ fish (n=5 individual *scarb1*$^{+/-}$ crossings). **(B)** Representative pictures of adult females and their tails (*scarb1* 715delA and *scarb1* 715_716insGG homozygous and sibling controls) at 3 months post-fertilization. **(C)** Total fertilized egg count at 0 hours post fertilization (hpf) produced by WT or *scarb1*$^{-/-}$ breeding pairs ($n_{WT}$=15 breeding pairs, $n_{-1nt}$=16 breeding pairs, $n_{+2nt}$=9 breeding pairs). **(D)** Percentage of survival from 0 hpf till 3 days post fertilization (dpf) of WT and *scarb1*$^{-/-}$ zebrafish larvae. **(E)** Representative picture of the development of *scarb1*$^{-/-}$ embryo's compared to WT embryo's at 0 till 3 dpf. **(F)** Total fertilized egg count at 0 hours post fertilization (hpf) produced by outcrosses (♀ WT x ♂ WT (n=15 breeding pairs), ♀ WT x ♂ *scarb1*$^{-/-}$ (n=5 breeding pairs), ♀ *scarb1*$^{-/-}$ x ♂ WT (n=8 breeding pairs), ♀ *scarb1*$^{-/-}$ x ♂ *scarb1*$^{-/-}$ (n=25 breeding pairs)). Means ± SEM; $p$ <0.05*, $p$ <0.001*** (One-way ANOVA (multiple groups) or Student's T-test). ►

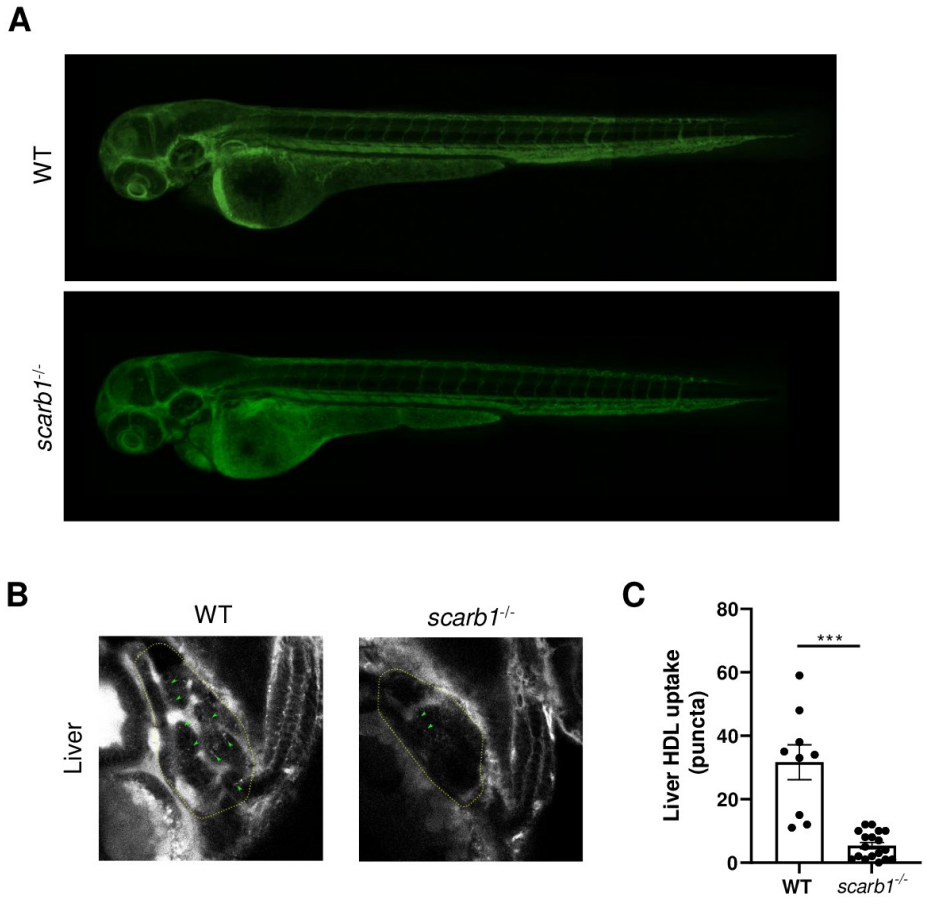



**Figure 2. Deficiency of SCARB1 affects uptake of HDL in the liver.**

**(A)** Injection of DiO-HDL (3 hours post injection)(green) in 3dpf WT and *scarb1*$^{-/-}$ zebrafish larvae. Magnification 10x. **(B)** Uptake and **(C)** quantification of DiO-HDL in livers of 3dpf WT ($n_{WT}$=3) and *scarb1*$^{-/-}$ larvae ($n_{-1nt}$=3 and $n_{+2nt}$=3). Quantification was performed by manually counting the HDL uptake (punctae) outside the sinusoids of 3 confocal image depths per zebrafish. Means ± SEM. $p$ <0.001*** (Student's T-test).

At 24 hpi, HDL-associated DiO could be detected within the wall of the caudal vein in both WT *mpeg1*:rfp and *scarb1*$^{-/-}$ *mpeg1*:rfp larvae. Although the majority of the DiO (green fluorescence) accumulated in other cells, likely vascular endothelial cells, we also observed occasional uptake by purple fluorescent macrophages (**Fig. 3A**). However, no differences were observed between WT and *scarb1*$^{-/-}$ larvae, Thus, to our surprise, cellular HDL-lipid uptake did not appear to be affected in *scarb1*$^{-/-}$ zebrafish.

Whereas SCARB1 is defined as the HDL receptor, it has been shown that mammalian SCARB1 is also able to bind other lipoproteins such as (modified) LDL[4,28]. Injection of LDL and oxidized LDL into the circulation of 3 dpf zebrafish larvae, however, also did not show any differences in lipoprotein-associated lipid accumulation within the caudal vein wall between WT and *scarb1*$^{-/-}$ variants. In line with an unaltered lipoprotein metabolism, the plasma free cholesterol levels of adult *scarb1*$^{-/-}$ zebrafish (FC; WT 2.4 ± 0.2 mg/ml versus *scarb1*$^{-/-}$ 2.0 ± 0.1 mg/ml) as well as the total cholesterol levels (TC; WT 4.2 ± 0.4 mg/ml versus *scarb1*$^{-/-}$ 4.0 ± 0.3 mg/ml) also did not differ from those of WT zebrafish (**Fig. 3B-C**). In addition, no significant difference was observed in the plasma free cholesterol to total cholesterol ratio (WT 0.6 ± 0.03 versus *scarb1*$^{-/-}$ 0.5 ± 0.02) (**Fig. 3D**).

The production of glucocorticoids from its precursor cholesterol is essential for the fine-tuning of stress responses and the corticosteroid stress axis is highly conserved between zebrafish and humans[29]. In both species, cortisol is the main stress hormone, which is respectively produced in the cortex of the adrenal gland in humans and in the inter-renal gland in zebrafish. The secretion of cortisol into the bloodstream is relatively low under unstressed (basal) conditions, while the glucocorticoid production increases rapidly in response to physiological or psychological stress. To examine whether the ability to synthesize glucocorticoids is altered in zebrafish in a similar manner as in humans and mice in absence of functional SCARB1[13,30], 5 dpf old WT and *scarb1*$^{-/-}$ zebrafish larvae were collected in the non-stressed state or stressed by means of netting[31]. Basal levels of cortisol in *scarb1*$^{-/-}$ zebrafish larvae were comparable to those in WT zebrafish larvae (WT 0.48 ± 0.04 versus *scarb1*$^{-/-}$ 0.49 ± 0.05 ng/mL; **Fig. 3E**). Netting-induced stress was evident by the associated increase in the total body cortisol levels in both WT and *scarb1*$^{-/-}$ zebrafish larvae. However, in support of an unaffected glucocorticoid production rate, also no differences were observed in stressed cortisol levels between the two zebrafish groups (WT 1.6 ± 0.1 versus *scarb1*$^{-/-}$ 1.9 ± 0.2 ng/mL).

**Altered coloration and plasma cholesterol levels in SCARB1 deficient canaries**

SCARB1 deficiency does not impact cholesterol metabolism in zebrafish in the same way as observed in mammals, indicating functional divergence of SCARB1 either in fish or during mammalian evolution. To distinguish between these two hypotheses, we used a representative of a third vertebrate lineage – the common canary. As described by Toomey *et al.*, *scarb1*$^{-/-}$ canaries display dysfunctional carotenoid transport, leading to their white appearance (**Fig. 4A**). Interestingly, plasma analysis of WT and SCARB1 knockout canaries demonstrated that canaries deficient in SCARB1 do not display higher plasma cholesterol levels, but actually significantly lower free cholesterol (WT 142.9 ± 10.7 versus SCARB1$^{-/-}$ 74.3 ± 12.7 mg/dL, $p<0.001$) and total cholesterol levels (WT 292.9 ± 12.0 versus SCARB1$^{-/-}$ 187.6 ± 28.1 mg/dL, $p<0.01$) as compared to WT canaries (**Fig. 4B**). In canaries SCARB1 thus appears to positively regulate plasma cholesterol levels, whereas in zebrafish cholesterol levels are independent of SCARB1. Since in mammals SCARB1 is a negative regulator of serum cholesterol, SCARB1 function in cholesterol metabolism appears to be complex, with negative regulation of serum cholesterol levels by SCARB1 a possible evolutionary novelty specific to the mammalian lineage.

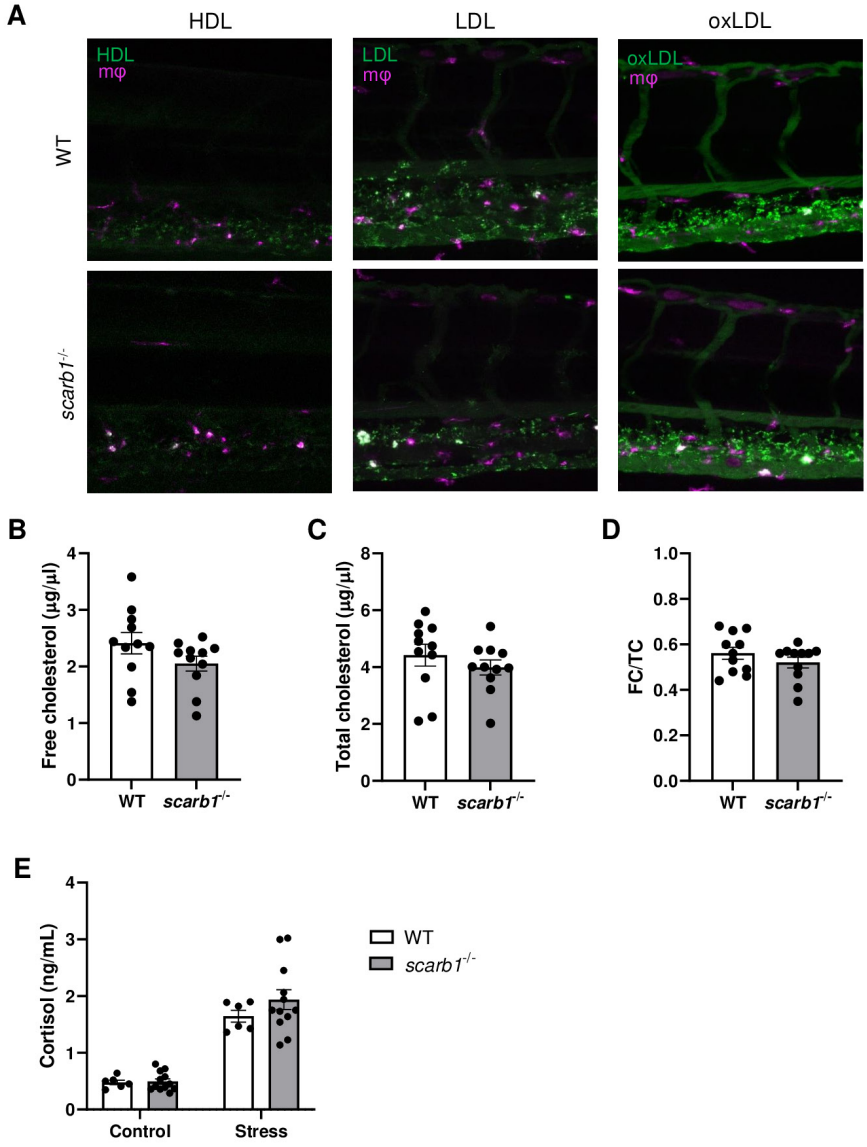
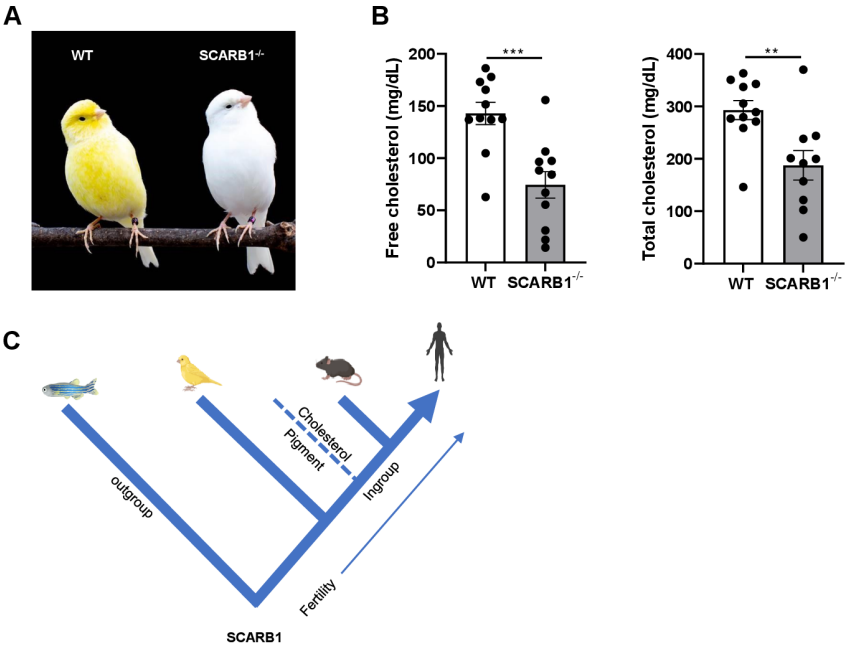



**Figure 3. Deficiency of SCARB1 does not alter fluorescently-labeled lipoprotein uptake in the caudal vein, total plasma cholesterol, and stress-induced cortisol levels in zebrafish.**

**(A)** Uptake of DiO-HDL (24 hours post injection)(green), Bodipy-LDL and DiO-oxLDL (30-60min post injection)(green) by macrophages (purple) in WT and *scarb1*$^{-/-}$ zebrafish larvae. Magnification 40x. **(B)** Free serum cholesterol (FC) **(C)** total serum cholesterol levels (TC) and **(D)** FC/TC ratio of WT sibling ($n_{WT}$=11 pooled samples of 2) and homozygous *scarb1*$^{-/-}$ zebrafish ($n_{-1nt}$=5 and $n_{+2nt}$=6 pooled samples of 2) measured with enzymatic colorimetric assay. **(E)** Cortisol levels of WT ($n_{WT}$=6 pooled samples of 30) and *scarb1*$^{-/-}$ zebrafish larvae ($n_{-1nt}$=6 pooled samples of 30, $n_{+2nt}$=6 pooled samples of 30) at baseline and after net stress.




D

| SCARB1 related function | Human | Mouse | Canary | Zebrafish |
|---|---|---|---|---|
| **Pigment/ carotenoid uptake** | Humans do not have yellow pigment.<br><br>Borel (2013)<br>- involvement of SCARB1in carotenoid uptake | Mice do not have yellow pigment | Toomey (2017)<br>- disturbed yellow pigment | Saunders (2019); This paper<br>- disturbed yellow pigment |
| **Plasma cholesterol** | Yang (2016)<br>Helgadottir (2017)<br>- higher cholesterol | Rigotti (1997)<br>Van Eck (2003)<br>- higher cholesterol | This paper<br>- lower cholesterol | This paper<br>- no change in cholesterol |
| **Adrenal steroidogenesis** | Vergeer (2013)<br>- disturbed adrenal steroidogenesis | Cai (2008)<br>Hoekstra (2012)<br>- disturbed adrenal steroidogenesis | Unknown | This paper<br>- no change in adrenal steroidogenesis |
| **Fertility** | Yates (2011)<br>- fertility issues | Miettinen (2001)<br>Jiménez (2010)<br>- fertility issues | Unknown | This paper<br>- fertility issues |

**Figure 4. Color and cholesterol levels in SCARB1 mutant canaries.**

**(A)** Representative pictures of WT and SCARB1 mutant canaries. **(B)** Free and total cholesterol levels of WT or SCARB1 mutant canaries (n=12 per genotype). **(C)** Schematic overview of the suggested functions of SCARB1 and its evolution. **(D)** Overview of known functions in SCARB1 mutant vertebrates. Means ± SEM. $p < 0.01$**; $p < 0.001$*** (Student's T-test).

**A**

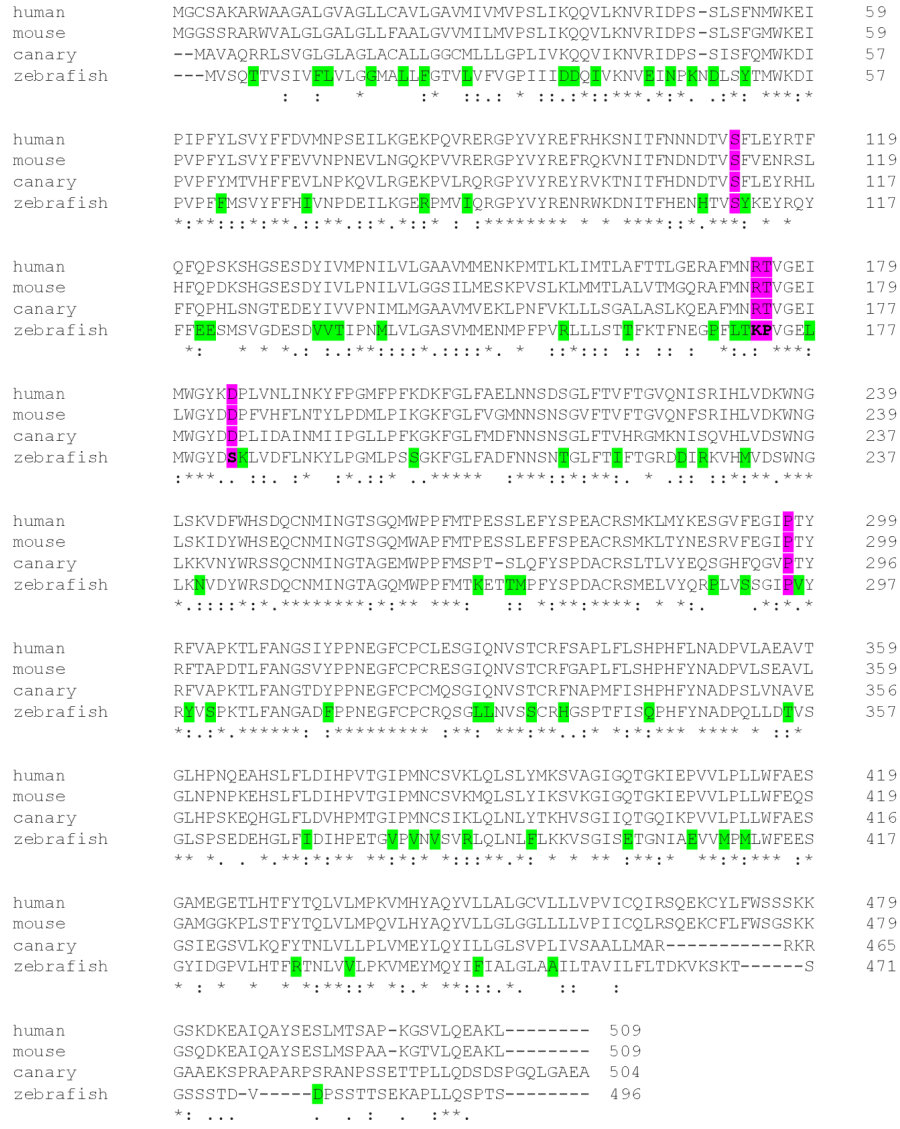



**Figure 5. Multiple species sequence alignments of SCARB1.**

**(A)** Alignments of SCARB1 sequences were generated using the Clustal Omega alignment program (EMBL-EBI). Three known human SCARB1 mutation locations (rs397514572 [p.Ser112Phe], rs187831231 [p.Thr175Ala], rs387906791 [p.Pro297Ser]) as well as the Arg-174 and Asp-185 are highlighted in magenta. The amino acids that differ in only in zebrafish are highlighted in green. An asterisk (*) indicates positions which have a fully conserved residue. A colon (:) indicates conservation between sequences that have strong similar properties. A period (.) indicates conservation between sequences that have weak similar properties.

# Discussion

For different vertebrate models, the roles of SCARB1 in carotenoid uptake, cholesterol metabolism, steroidogenesis, and fertility have been extensively described in literature (for overview, see **Fig. 4C-D**). However, how the different functions of this scavenger receptor have evolved was largely unknown. In this paper, we aimed to shed light on the evolution of SCARB1 by examining its functions in zebrafish, an outcrop model. Our study shows that SCARB1 deficiency in zebrafish is associated with reduced fertility, similarly as previously observed in mammals. However, in contrast to what has previously been established in mammals, deficiency of SCARB1 did not impact the total cholesterol levels, lipoprotein uptake by cells located in the caudal vein, nor steroidogenesis. Based on these findings, we suggest that the specific function of SCARB1 in maintaining plasma cholesterol could have evolved during vertebrate evolution.

Considering the functional differences observed between mammals and zebrafish, we have compared the sequence of SCARB1 between different species (**Fig. 5**). Several human gene variants of SCARB1, including rs397514572 [p.Ser112Phe] and rs387906791 [p.Pro297Ser] have been reported to affect SCARB1 function as well as serum cholesterol levels[32,33]. Notably, most domains in which these mutations are located are well conserved between mammals and zebrafish, excluding a potential role for these mutations in the difference between zebrafish and humans. However, the human gene variant rs187831231 [p.Thr175Ala] is not conserved between humans and zebrafish, nor is the sequence at nucleotide 174 and 185 described by May *et al*.[16]. In this context, it should be noted that May *et al*. did only mutate the human Arg-174 into the zebrafish variant Lys-174, while Asp-185 was maintained and not changed into the Ser-185 found in the zebrafish sequence. Based on our findings, it can be hypothesized that double mutation (Arg-174 into Lys-174 and Asp-185 into Ser-185) in zebrafish as compared to the human sequence has resulted in a complete SCARB1 cholesterol function impairment. To test this hypothesis, it is useful to study the HDL-cholesteryl ester uptake function of the combined Lys-174/Ser-185 zebrafish *scarb1* variant in the cell system of May *et al*. In addition, a transgenic rescue experiment of *scarb1*$^{-/-}$ of human SCARB1 and variants could further elucidate the differences in protein function.

The gene variants described above are all located in the extracellular domain of SCARB1. In line, Kartz *et al*. highlighted the importance of intact extracellular regions for the proper functioning of SCARB1[34]. Modulation of the extracellular regions by generating SCARB1/CD36 chimeric receptors affects various cholesterol transport

functions[34]. Alignment of SCARB1 sequences of different species have shown that in addition to the domains described above, multiple other domains are poorly conserved between zebrafish and humans and hence might account for the differences observed in cholesterol handling function of *SCARB1* between zebrafish and mammals (**Fig. 5**). Therefore, it would also be of interest to examine whether mutations in these poorly conserved domains can affect the cholesterol-based functions of human SCARB1.

Based on the findings from May *et al*., we expected a reduced relevance of SCARB1 in HDL-cholesteryl ester uptake in zebrafish as compared to humans. The fact that SCARB1 in zebrafish does not affect plasma cholesterol levels at all, however, is surprising at first glance. In line with this finding, no effect of SCARB1 deficiency was observed on lipoprotein uptake in the caudal vein. However, we did observe a decrease in uptake of HDL in livers of *scarb1*$^{-/-}$ larvae at 3 hours post injection, but this seemed to coincide with reduced perfusion of the liver. Of note, in healthy larvae, blood flow via liver blood sinusoids has initiated at 3 dpf[35]. Although, we did not observe any differences in overall growth of the surviving *scarb1*$^{-/-}$ larvae, we cannot exclude that (slight) delays in hepatic development in the *scarb1*$^{-/-}$ zebrafish larvae might underlie the observed reduction in perfusion and consequently reduced uptake in the livers of the larvae at the investigated time point. Single cell sequencing data from Farnsworth *et al*. on 1 to 5 dpf zebrafish embryos showed that the highest expression of SCARB1 is found in intestinal cells, in xanthophores (the yellow pigment cells that form the yellow stripes on zebrafish), and in red blood cell progenitors[36]. In contrast, in mice the highest expression is found in liver, adrenal gland, ovary, and pancreas[37–39]. Hence, evolutionary changes in SCARB1 expression profiles might also underly the differential effects on cholesterol metabolism. In zebrafish, the ratio HDL-cholesterol to total cholesterol is similar to dyslipidemic humans (0.29 vs 0.21)[40], but much lower as compared to mice, in which HDL is virtually the only cholesterol carrying lipoprotein (0.76)[40], likely due to the fact that mice lack the cholesteryl ester transfer protein (CETP)[41]. One could argue that because zebrafish do express CETP, effects of SCARB1 deficiency are less pronounced as they exhibit an alternate route for clearance of HDL cholesterol, after CETP-facilitated transfer to VLDL/LDL and subsequent clearance via the LDL receptor. However, it should be noted that expression of CETP in mice only partially normalizes HDL cholesterol[42] and also in humans, that express CETP, SCARB1 mutations do affect cholesterol metabolism.

Elevated serum cholesterol levels are considered the driving force in the reduced fertility associated with functional SCARB1 deficiency in humans as well as in mice[25,26]. More specifically, abnormally high HDL-cholesterol levels result in ovulation of dysfunctional oocytes[26]. Supplementation of probucol, a cholesterol lowering drug, effectively

restores the fertility of female SCARB1$^{-/-}$ mice, indicating that indeed the abnormal HDL cholesterol levels and impaired lipoprotein metabolism are the primary cause for the infertility seen in these animals[43]. Whereas, to date, no effect of SCARB1 deficiency on fertility of canaries has been described, we did find that loss of SCARB1 protein function in canaries is associated with lower (not higher) plasma cholesterol levels. It thus appears that a change in cholesterol phenotype in these animals seemingly does not impact fertility. Based on our finding that the fertility is substantially affected in adult (female) zebrafish lacking functional SCARB1 and that the survival of the offspring is hampered in the context of unchanged plasma cholesterol levels, we propose that SCARB1 can also exert serum cholesterol-independent effects on fertility.

Antioxidants might have a beneficial role in fertility as oxidative stress has been linked to reduced fertility[44,45]. The role of HDL as an anti-oxidant is still questionable, as described by Swertfeger *et al.*[46] However, probucol serves as a powerful antioxidant and might thus also rescue fertility of *Scarb1*$^{-/-}$ mice via this mechanism. In contrast to this assumption, the study by Showell *et al.* has shown that taking antioxidants does not positively affect subfertility in women[47]. However, further research is still needed to completely rule out the role of oxidative stress in the impaired fertility seen in *scarb1*$^{-/-}$ zebrafish. Although the role of antioxidants in fertility is not fully clear, it has been established that vitamins with antioxidant functions play a role in zygote implantation and female reproduction, linking these factors also to (mammalian) fertility[48–51]. Saunders *et al.* reported earlier that maturation and uptake of yellow xanthophores depend on SCARB1 in zebrafish[24]. In line, the tissue uptake of carotenoids, the precursor of the antioxidant vitamin A, is impaired in our *scarb1* knockout zebrafish as evidenced by the lack of yellow pigment. Also, *scarb1* deficient zebrafish embryos lacked yellow pigment at the blastula-stage; the developmental stage in which *scarb1* expression peaks[52]. In humans, mutations in SCARB1 have also been linked to impaired uptake of carotenoids and vitamins[53]. The function of SCARB1 as carotenoid transporter is conserved between different species, suggesting that impaired vitamin metabolism could be linked to the reduced fertility. An argument against this hypothesis is that canaries deficient for SCARB1 suffer from vitamin A deficiency, while to date, no fertility issues have been described. However, multiple studies have shown that supplementation of vitamin A is required during the breeding studies. Although a previous study has shown that alipochromatic white canaries absorbed less vitamin A from food compared to colored canaries and therefore need a diet containing higher levels of vitamin A[54], Preuss *et al.* found that the same diet met the requirements of alipochromatic white canaries, such as SCARB1$^{-/-}$ canaries, as well as colored canaries[55]. As a result, further research is needed to confirm if a change in vitamin homeostasis contributes to the impaired fertility observed in *scarb1*$^{-/-}$ zebrafish.

In conclusion, we have shown that SCARB1 does not play a major role in plasma cholesterol homeostasis in zebrafish, while zebrafish SCARB1 deficiency does impair fertility and carotenoid uptake, leading to loss of yellow pigment. Our study is the first to suggest that during evolution SCARB1 possibly developed a novel function in cholesterol metabolism and related steroidogenesis in mammals and that, in zebrafish, the cholesterol-based function is separated from the SCARB1 function in carotenoid uptake. The fertility issues observed appear to be the main conserved phenotype of SCARB1 deficiency during vertebrate evolution. Further research should focus on comparing the function of SCARB1 in multiple other species to establish the course of evolution of SCARB1 functionality. Our findings highlight that the function of scavenger receptors can differ significantly between species and thus can have an important consequence for the use of non-mammalian models to study diseases in which cholesterol metabolism plays an important role, such as in atherosclerosis. Because this study has provided evidence that SCARB1 does not play a key role in the zebrafish cholesterol homeostasis and we recently established that class H scavenger receptors stabilin 1 and 2 are not involved in HDL lipoprotein uptake[56], it remains to be determined which class of scavenger receptors do play a significant role in zebrafish HDL metabolism.



**Financial support**
This work was supported by the NWO grant number VICI 91813603 to MvE.

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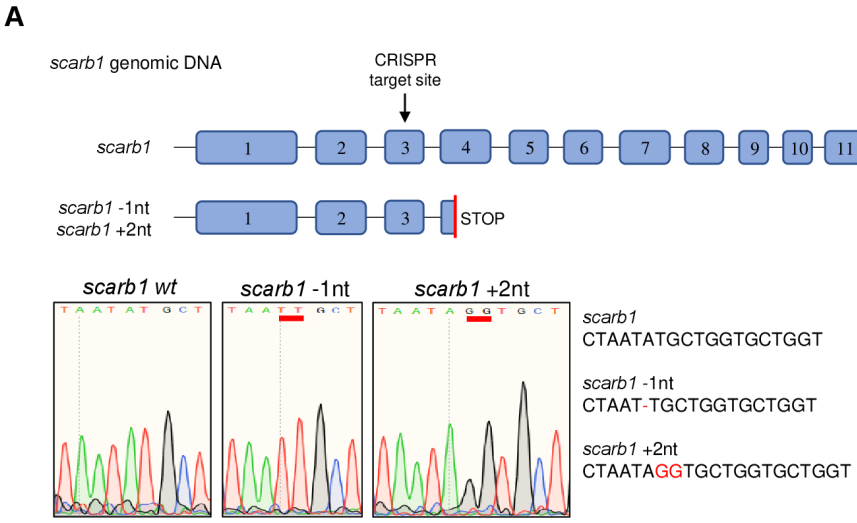
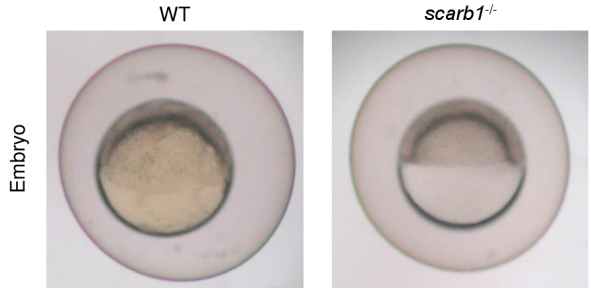



**Supplemental Figure 1. Generation of a *scarb1*$^{-/-}$ zebrafish using CRISPR/Cas9. (A)** The genomic target site of CRISPR is located in exon 3. Sequences for a selected SCARB1 knockout, showing either a 1nt deletion (715delA) or 2nt insertion (715_716insGG) that results in a frame shift and pre-stop codon.




**Supplemental Figure 2. Lack of yellow pigment in *scarb1*$^{-/-}$ zebrafish larvae.**

Representative pictures of embryos van WT and scarb1$^{-/-}$ larvae 4 hours post fertilization (blastula phase).