

Supporting Information

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Fig. S1. Alignment of zebrafish drTLR5a and drTLR5b. The amino acid sequences were aligned using the Clustal Omega server with default settings. Asterisks (*) indicate identical residues, double square dots (:) indicate highly similar residues, single square dots (.) indicate somewhat similar residues, and bars (-) indicate gaps to complete the sequence alignment. Signal peptide, NTLRR, 22 consecutive LRRs, and CTLRR are shaded in gray. Gray arrows indicate the start and end of specified regions.

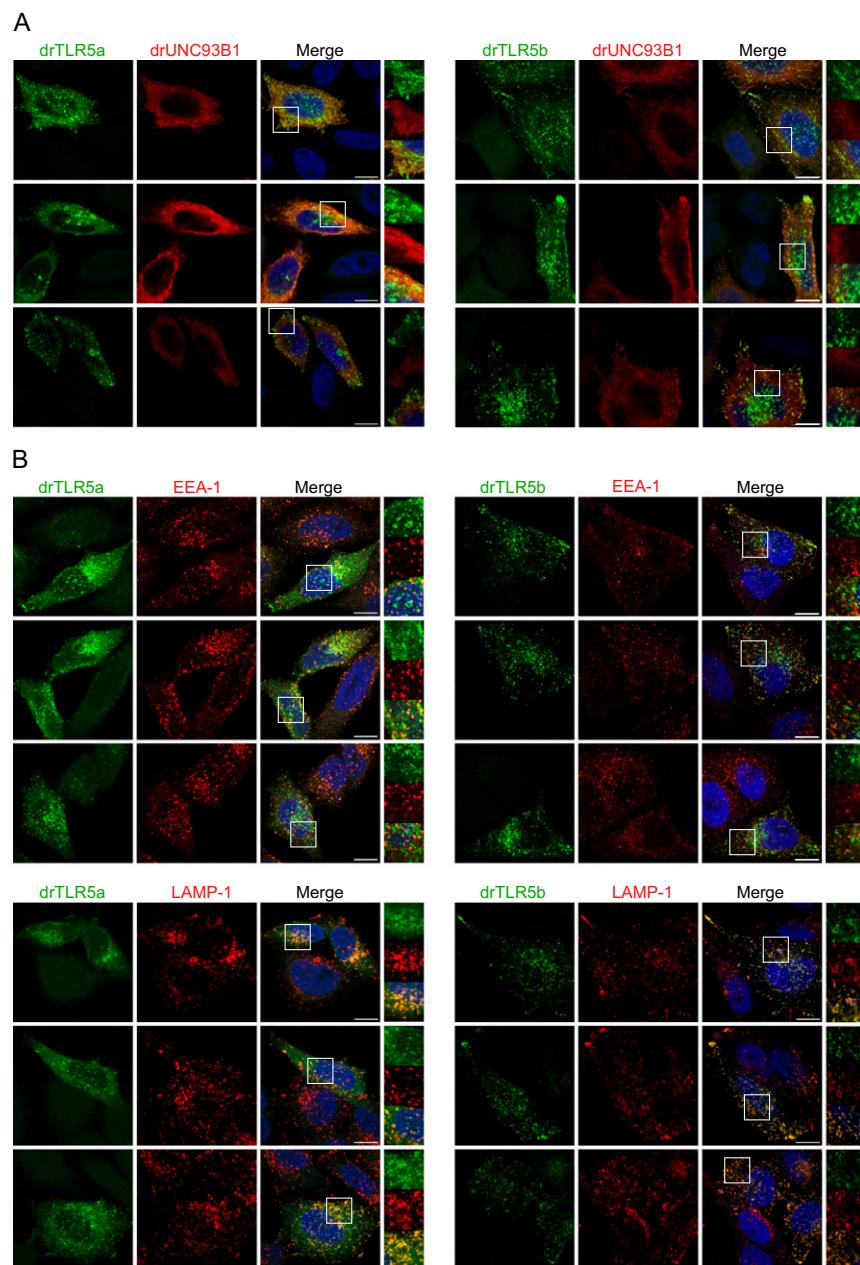


Fig. S2. Vesicular localization of drTLR5a and drTLR5b in the presence of drUNC93B1. Confocal microscopy on HeLa-57A cells expressing (A) drTLR5a-HA or drTLR5b-HA and drUNC93B1-FLAG or (B) drTLR5a-HA or drTLR5b-HA and untagged drUNC93B1 costained for EEA-1 or LAMP-1. Merge images show nuclei stained with DAPI (blue). White boxes in merge images indicate the magnified area shown for each channel on the right of merge images. Images were selected from three independent experiments, and three representative images are shown for each transfected group. (Scale bars in merge images: 10 μ m.)

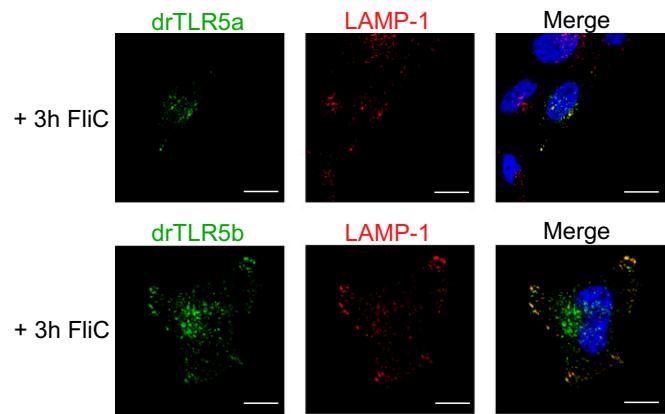


Fig. S3. Localization of zebrafish drTLR5a and drTLR5b during flagellin stimulation. HeLa-57A cells expressing drTLR5a-HA or drTLR5b-HA and untagged drUNC93B1 were stimulated for 3 h with $1 \mu\text{g mL}^{-1}$ FliC^{SE} and costained for LAMP-1. Merge images show nucleus stained with DAPI (blue). Images are representative of three independent experiments. (Scale bars: 10 μm .)

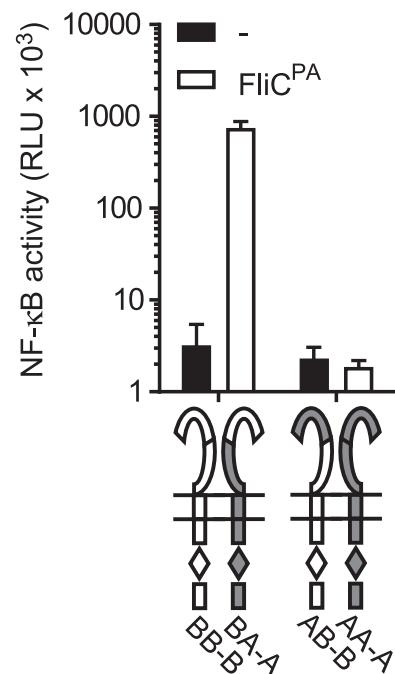


Fig. S4. Stimulation of homodimeric N-ECD of drTLR5a and drTLR5b with FliC^{PA}. HeLa-57A cells transfected with drUNC93B1 and the indicated receptor combinations were stimulated (5 h) with vehicle (-) or $1 \mu\text{g mL}^{-1}$ FliC^{PA}. Data show NF-κB activity represented by luciferase activity in RLU. Values are the mean \pm SEM of two independent experiments performed in duplicate.

A

drTLR5a-3v47 (orange) and FliC bound drTLR5b (3v47, purple)

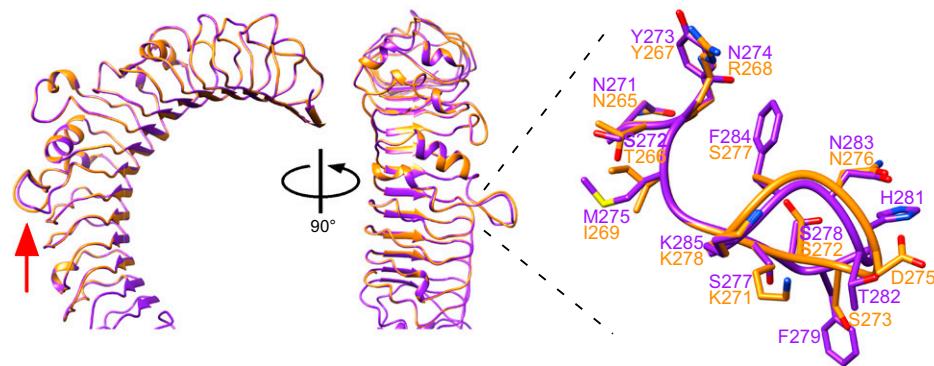
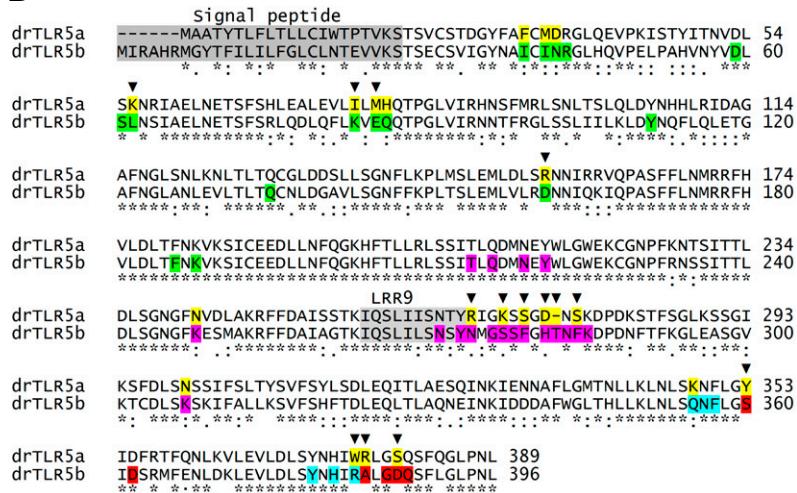
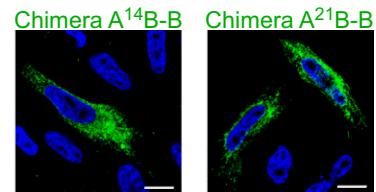
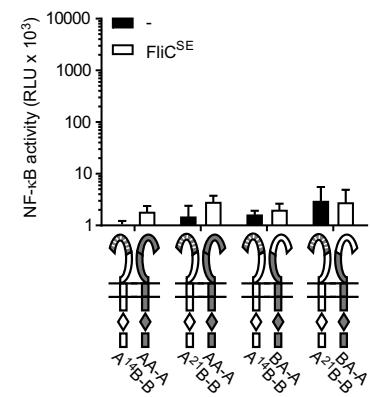
**B**

Fig. S5. (A) Superposition of FliC-bound drTLR5b (PDB ID: 3v47; purple) and a model of drTLR5a based on 3v47 (drTLR5a-3v47; orange). The red arrow indicates the flagellin-binding hotspot that forms a loop between LRR9 and LRR10. (B) Sequence alignment of the drTLR5a and drTLR5b N-ECD. The amino acid sequences were aligned using the Clustal Omega server with default settings. Asterisks (*) indicate identical residues, double square dots (:) indicate highly similar residues, single square dots (.) indicate somewhat similar residues, and bars (-) indicate gaps to complete the sequence alignment. The 45 residues in the drTLR5b crystal model responsible for flagellin binding and dimerization are color-coded according to Yoon et al. (19): green, primary interface-A; purple, primary interface-B; cyan, dimerization interface-β; red, dimerization interface-α. Green and purple residues interact with flagellin, red residues interact with the second flagellin molecule in the 2:2 stoichiometric homodimeric TLR5-flagellin crystal model, cyan residues interact with the second drTLR5b molecule, the phenylalanine (F) at position 284 interacts with flagellin and with the second drTLR5b molecule [see also Yoon et al. (19)]. Yellow-colored amino acids in the drTLR5a sequence indicate the 21 different binding or dimerization residues that were replaced in chimera AB-B to produce chimera A²¹B-B. The arrowheads indicate 14 of these 21 most deviating residues that were replaced to produce chimera A¹⁴B-B. (C) HeLa-57A cells were transfected with FLAG-tagged chimeras A¹⁴B-B or A²¹B-B and stained with α-FLAG (green) and DAPI (blue) for nuclear visualization. (Scale bars: 10 μm.) (D) HeLa-57A cells transfected with drUNC93B1 and the indicated receptor combinations were stimulated (5 h) with vehicle (-) or 1 μg mL⁻¹ FliC^{SE}. Data show NF-κB activity represented by luciferase activity in RLU. Values are the mean ± SEM of three independent experiments performed in duplicate.

C**D**

hsTLR5	-----MGD-HLDLLLGVVLMAGPVFGIPSCSFDFGRIAFYRCNLQTQPQLNT-TERLL	52
btTLR5	-----MGD-CLDLLLGVVLLTSPALGMSSCFDGWRAIYLSCNLQTQPQVPNT-TKSLL	52
mmTLR5	-----MAC-QLDLILGVIFMASPVLVISPCSSSDGRIAFFRCNLQTIPWILNTTERLL	53
ggTLR5	---MMLHQ-RLIIIVFG-IALAGDICASRSCYSEDQVSMYNSCNLTGVPPVPKD-TAKLF	53
actTLR5	--MKKMLHY-LFIFLIGMRHACREILAIPLCSEVENKIAYYDFCNLTQVPPVPED-IVLFT	56
drTLR5b	MIRAHRMGYTFILLLFGCLNTEVKSTSECSVIGYNA[CINRGHLHQVPELPAH-VNYV[59
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hsTLR5	LSFNYIRTVTASSFPFLEQLQLLELGSQLY-TPLTIDKEAFRNLPNLRILDLGSSKIYFLH	111
btTLR5	LSFNYIRTVTASFPFLEQLQLLELGSQLY-TPLTIYREAFRNLPNLRILDLGSSQINFLH	111
mmTLR5	LSFNYISMVATSPFLEROLLELGSQLY-ANLTIGPGAFRNLPNLRILDLGSSQIEVLN	112
ggTLR5	LTYNYIRQVTATSPFLLEDLFLLEIGTQRVFPFLYIGKEAFRNLPNLRVLDDGFNNILLD	113
actTLR5	LNFNSIREVKSSSFPPLKELRNLALGTQSVPVTIRRDAFRNLPNLQKLDLAGNKMTVLD	116
drTLR5b	LSUNSIASLNETSFSRQLDQLQFL[KVEQQT-PGLVIRNNNTFRGLSSLIILKLDYNOFLQLE	118
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hsTLR5	PDAFQGLFHLLFELRLYFCGLSDAVLKDGYFRNLKALTRLDLSKNQIRSLYLHPSFGKLNS	171
btTLR5	PDAFQGLPHLTKLRFCSGLSDAVLKDGYFRNLKALTRLDLSKNQIRSLYLHPSFGKLNS	171
mmTLR5	RDAFQGLPHLLELRFLFSCGLSSAFLSDGYFRNLYSLARLDLSGNQIHSRLRHSSFRELNS	172
ggTLR5	LDSFAGLQLRRTILRLFQNLLGDSILEERYFQDLRSLEELDSGNQITKLHHPHPLFYNLTI	173
actTLR5	TGAFLGLLNLRELFLYYNGNESILEGGDYFRDLISLEYDLQYNKIAARLRPHPLFFNMNS	176
drTLR5b	TGAFLGLANLEVTLTC[CNLDGAVLGSNNFFPKLTSLEMLVLR[UNIQKIQPASFFLNMR	178
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hsTLR5	LKSIDFSSNQIIFLVCHEHELEPLQGKTLSSFLSLAANSLYS--RVSVDWGKCMNPFRNMVLE	229
btTLR5	LKSIDFSSNQIPIVCEQEKFPLQGKTLSSFLSLADNQLYS--RVSVDWNKCLNPFRNMVLE	229
mmTLR5	LSDVNFNFQNIIFTICEDELEPLQGKTLSSFFGLKLTKLFS--RVSVGWETCRNPFRGVRLF	230
ggTLR5	LKAVLNFKNFKISNLCESNLTSFQGKHFFSFLSTNTLYR--TDKMIWAKCPNPFRNITFN	231
actTLR5	LGLTKLKLNQIKTICEGDLSFQGKTFELLSSLSNQLYR--DAVNWTTCGNPFKNIVIK	233
drTLR5b	FHVLDLT[TKVKSICEDLLNFQGKHTTLLRSSST[TDQMNEXWLGEKGPNFRNSSIT	238
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hsTLR5	IILDSGNGWTVTDITGNFSNAISKSQAFSLILAH-HIMGAGFGFHNIKDPDQNTFAGLARS	288
btTLR5	TLDVSGNGWVGDIMRNFSNAINGSQFSLVTR-HIMGSSFGFSNLKDPDYHTFAGLARS	288
mmTLR5	TLDLSENGWTVTDITRNSNIIQGSQISSLILKH-HIMGPFGFONIRDPDQSTFASLARS	289
ggTLR5	SLDVSENGWSTETVQYFCTAIKGQTQINYLSRS-HTMGSSGFGFNNLKNPDTDTFTGLARS	290
actTLR5	TLDVSGNGWDVATTQQFCAAVQGTPILALELSH-HIMGSSGFDNLRNPDNDTFTVGLAKS	292
drTLR5b	TLDLSGNGF[KESMAKRFDAIAGTKIQSILSNS[SYNMGSSFGHTNFKDOPDNFTFKGLEAS	298
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hsTLR5	SVRHLDSLHGFVFSLNSRVFETLKDLKVLNLAQNKKINKIADEAFYGLDNLQVLNLNSYNLL	348
btTLR5	SMIQLDISHGVIIFSLNFRIFETLQELKVLNLAQNKKINKISIRNAFYGLDNLQVLNLNSYNLL	348
mmTLR5	SVLQLDLSHGFISLNPRLFGLTKDLKMLNLAFLNKINKIGENAFYGLDLSQVLNLNSYNLL	349
ggTLR5	DHLLLDISNGFIFISLNSLNFESLRNLFRNLRNQIQKQAFFGLENEILNLSSNLL	350
actTLR5	GLKLLDLSHGSIFPLSPVVFQSLGDLLWLDLNTNKINQIGKGAFSGLLQLLNLNSYNLL	352
drTLR5b	GVKTCDSL[SKIFALLKSVFSHFTDLEQLTLAQNEINKIDDFAFWGLTHLLKLNLSQLNL[358
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hsTLR5	GELYSSNFYGLPKVAYIDLQKNHIAIIQDQTFKFLEKL	386
btTLR5	GELYNYDFDGLPKVAYIDLQKNHIGIIQDQTFKFGLKL	386
mmTLR5	GELYNSNFYGLPRVAYVDLQRNHIGI[IQDQTFRLLKTL	387
ggTLR5	GELYDYTTEGHLHSIMYIDLQKNHIGMIGEKSFSNLVNL	388
actTLR5	GEILDDYTTFVGLHNVISIDLOLQHNHIGVFGGNPEYLPKL	390
drTLR5b	G[TD[DSRMFENLDKLEVLDLS[SYNHIRAL[GDR[SFGLPNL	396
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Fig. S6. Sequence alignment of the TLR5 N-ECD of different vertebrates. Alignment of the TLR5 N-ECD of *Homo sapiens* (human; NCBI reference sequence: AAI09119.1), *Bos taurus* (bovine; ABC68311.1), *Mus musculus* (mouse; AAI25248.1), *Gallus gallus* (chicken; ACR26275.1), *Anolis carolinensis* (green anole lizard; ALT10445.1), and zebrafish. Alignment was constructed with the Clustal Omega server with default settings. Asterisks (*) indicate identical residues, double square dots (:) indicate highly similar residues, single square dots (.) indicate somewhat similar residues, and bars (-) indicate gaps to complete the sequence alignment. Residues in the drTLR5b crystal model responsible for flagellin binding and dimerization are color-coded according to Yoon et al. (19): green, primary interface-A; purple, primary interface-B; cyan, dimerization interface-β; red, dimerization interface-α. Green and purple residues interact with flagellin, red residues interact with the second flagellin molecule in the 2:2 stoichiometric homodimeric TLR5-flagellin crystal model, cyan residues interact with the second drTLR5b molecule [see also Yoon et al. (19)]. ac, *Anolis carolinensis*; bt, *Bos taurus*; gg, *Gallus gallus*; hs, *Homo sapiens*; mm, *Mus musculus*.

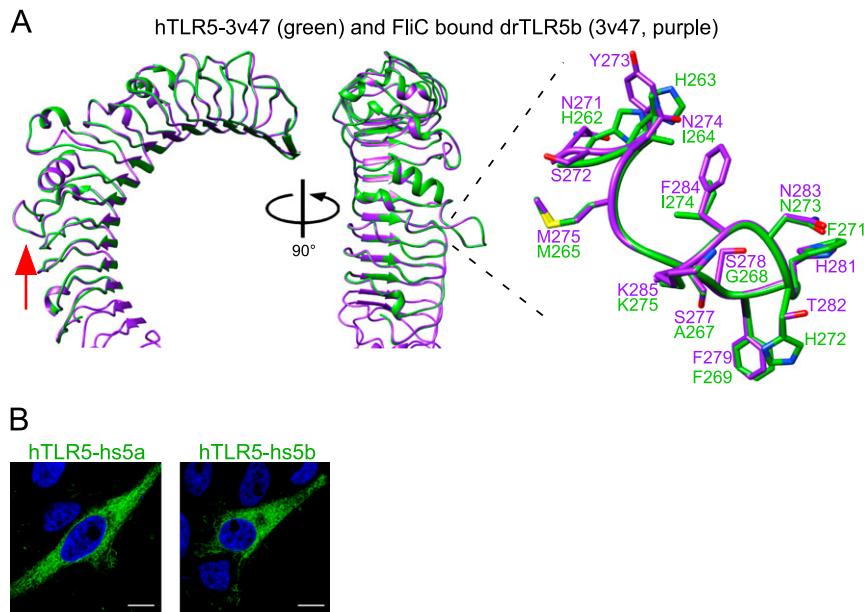


Fig. S7. (A) Structural modeling of human TLR5 onto drTLR5b. Superposition of FliC-bound drTLR5b (PDB ID: 3v47; purple) and a model of hTLR5 based on 3v47 (hTLR5-3v47; green). The red arrow indicates the flagellin-binding hotspot that forms a loop between LRR9 and LRR10. (B) Expression of hTLR5 chimeric constructs. HeLa-57A cells were transfected with FLAG-tagged hTLR5-hs5a or hTLR5-hs5b and stained with α -FLAG (green) and DAPI (blue) for nuclear visualization. Images are representative of two independent experiments. (Scale bars: 10 μ m.)

Table S1. Primers used in this study

Product	Primer	Sequence (5'-3')
BamHI-Kozak-drTLR5b-NotI	CV039 F CV054 R	CCGGATCCGCCACCATGATCCGTGCTCACAGAACATGG CCGGCGCCGCTTACTGCTGTGTTGGTGTGAAATTG
BamHI-Kozak-drTLR5a-NotI	CV095 F CV096 R	CCGGATCCGCCACCATGGCAGCTACATACACTTATTTC CCGGCGCCGCTAAGTGCACTGAGTGTCTGCTTGAAAC
KpnI-Kozak-drUNC93B1-NotI	CV159 F CV160 R	CCGGTACCGCCACCATGGCAGCACTGATCGC CCGGCGCCGCACTGTTGGACGTAGTCATCTC
KpnI-Kozak-drUNC93B1-NotI-stop codon	CV159 F CV180 R	CCGGTACCGCCACCATGGCAGCACTGATCGC CCGGCGCCGCTTAGTGTTGGACGTAGTCATCTC
BamHI-Kozak-hTLR5-NotI	CV257 F CV259 R	CCGGATCCGCCACCATGGGAGA CCGGCGCCGCTGGAGATGGTTGCTACAGTTG

Restriction enzyme sequences are in bold; the Kozak sequences in forward primers are underlined.