Supporting Information

Voogdt et al. 10.1073/pnas.1719245115

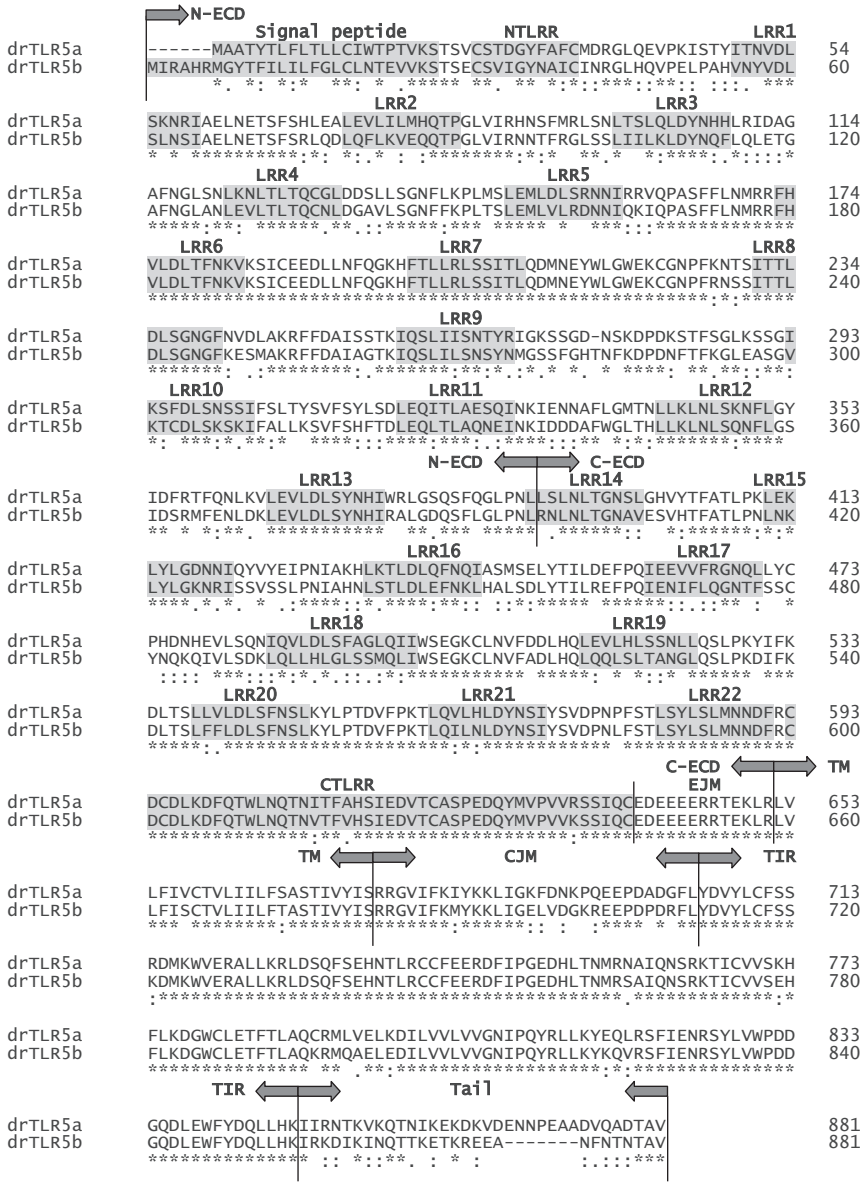

Fig. S1. Alignment of zebrafish drTLR5a and drTLR5b. The amino acid sequences were aligned using the Clustal Omega server with default settings. Asterisks (*) indicate identical residues, double square dots (:) indicate highly similar residues, single square dots (.) indicate somewhat similar residues, 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.

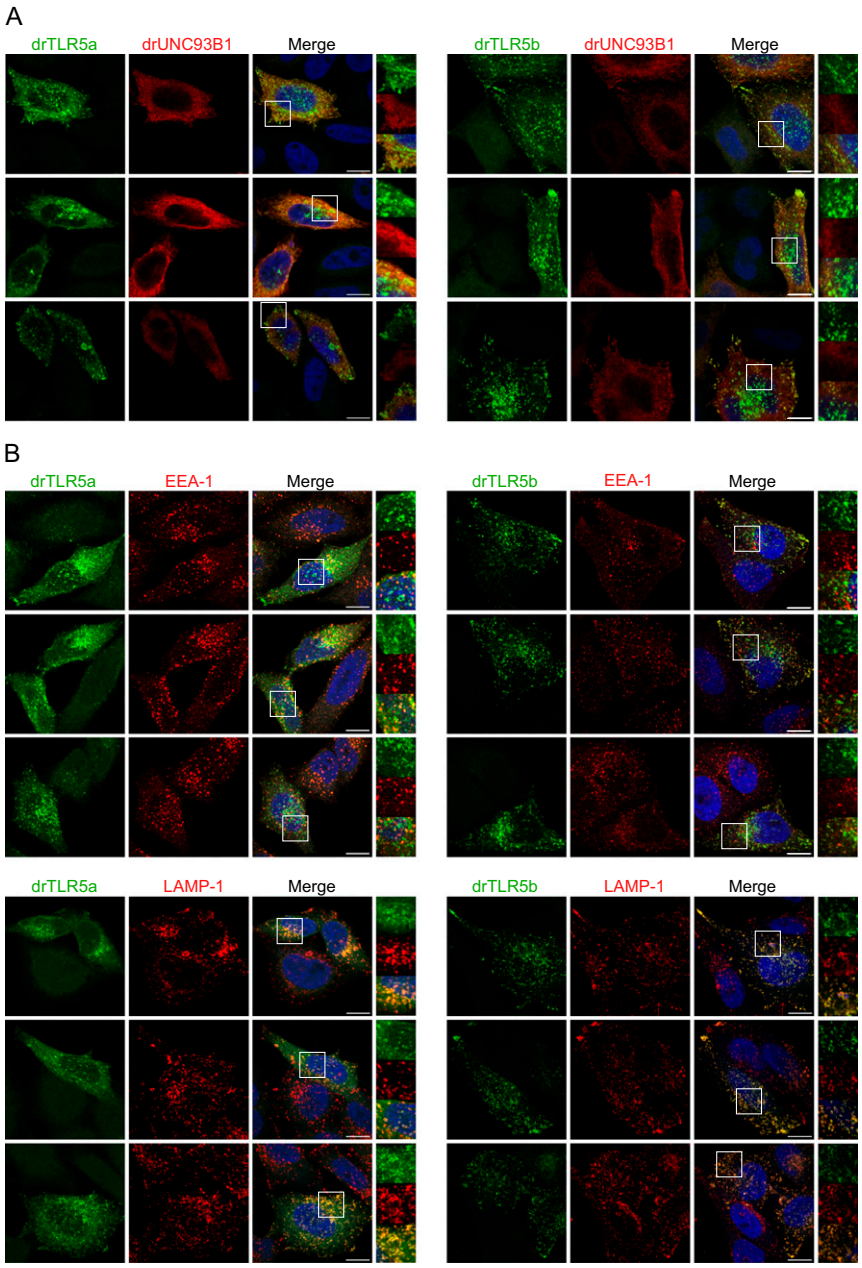

Fig. S2. Vesicular localization of drTLR5a and drTLR5b in the presence of drUNC93B1. Confocal microscopy on HeLa-57A cells expressing (*A*) drTLR5a-HA or drTLR5b-HA and drUNC93B1-FLAG or (*B*) drTLR5a-HA or drTLR5b-HA and untagged drUNC93B1 costained for EEA-1 or LAMP-1. Merge images show nuclei stained 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 μm.)

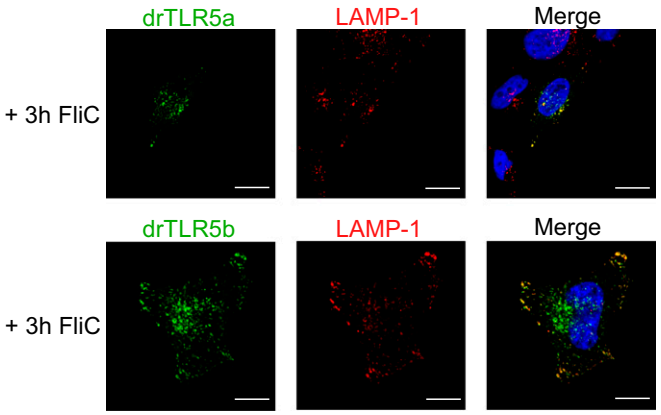

Fig. S3. Localization of zebrafish drTLR5a and drTLR5b during flagellin stimulation. HeLa-57A cells expressing drTLR5a-HA or drTLR5b-HA and untagged drUNC93B1 were stimulated for 3 h with 1 μg mL^{-1} $FliC^{SE}$ and costained for LAMP-1. Merge images show nucleus stained with DAPI (blue). Images are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

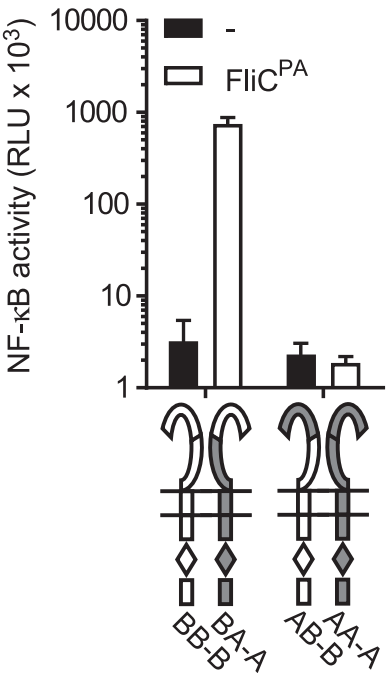

Fig. S4. Stimulation of homodimeric N-ECD of drTLR5a and drTLR5b with $FliC^{PA}$. HeLa-57A cells transfected with drUNC93B1 and the indicated receptor combinations were stimulated (5 h) with vehicle (–) or 1 μg mL^{-1} $FliC^{PA}$. Data show NF-κB activity represented by luciferase activity in RLUs.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

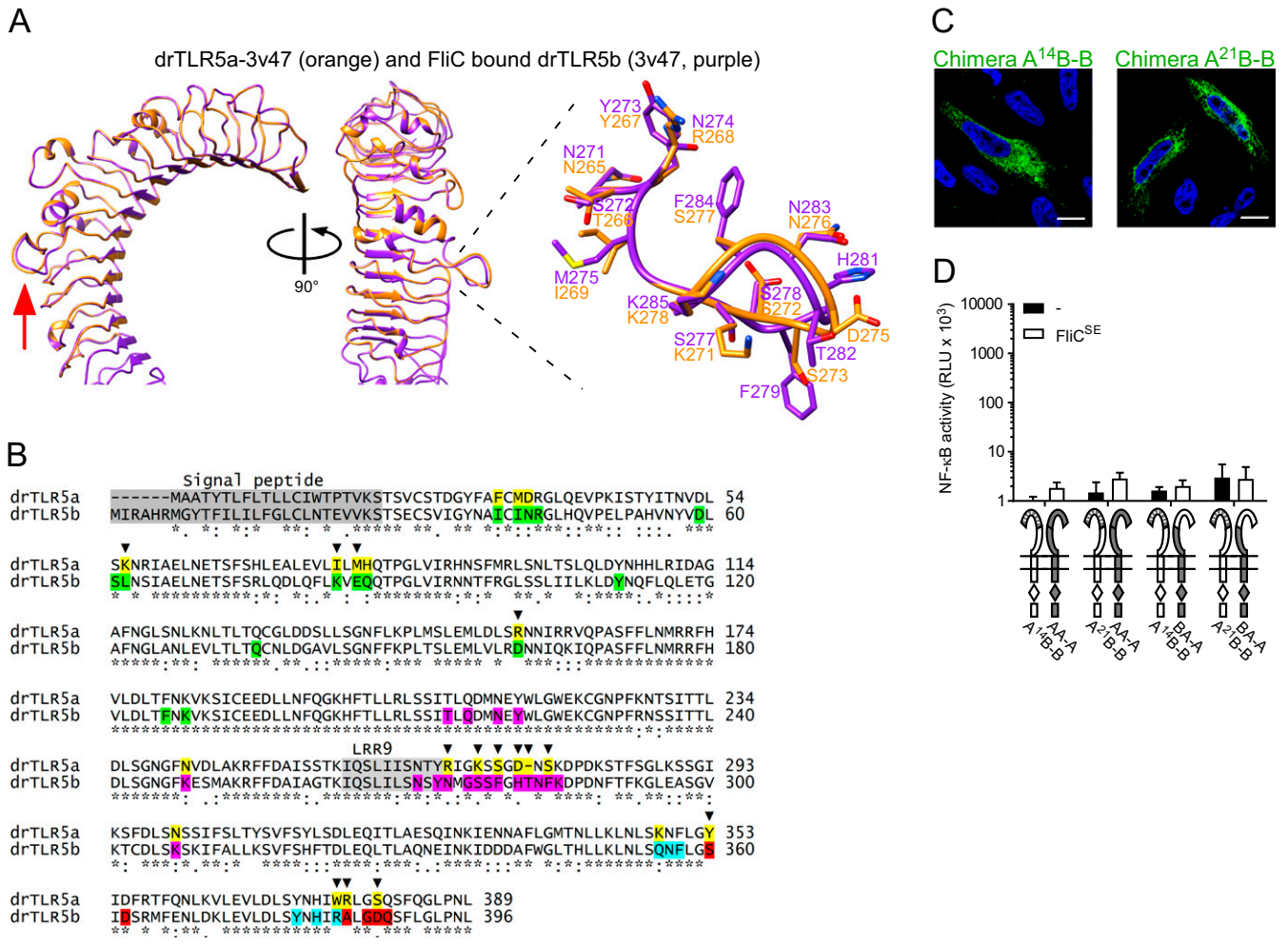

Fig. S5. (*A*) Superposition of FliC-bound drTLR5b (PDB ID: 3v47; purple) and a model of drTLR5a based on 3v47 (drTLR5a-3v47; orange). The red arrow indicates the flagellin-binding hotspot that forms a loop between LRR9 and LRR10. (*B*) Sequence alignment of the drTLR5a and drTLR5b N-ECD. The amino acid 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 A^{21}B-B. The arrowheads indicate 14 of these 21 most deviating residues that were replaced to produce chimera A^{14}B-B. (*C*) HeLa-57A cells were transfected with FLAG-tagged chimeras A^{14}B-B or A^{21}B-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 RLUs. Values are the mean ± SEM of three independent experiments performed in duplicate.

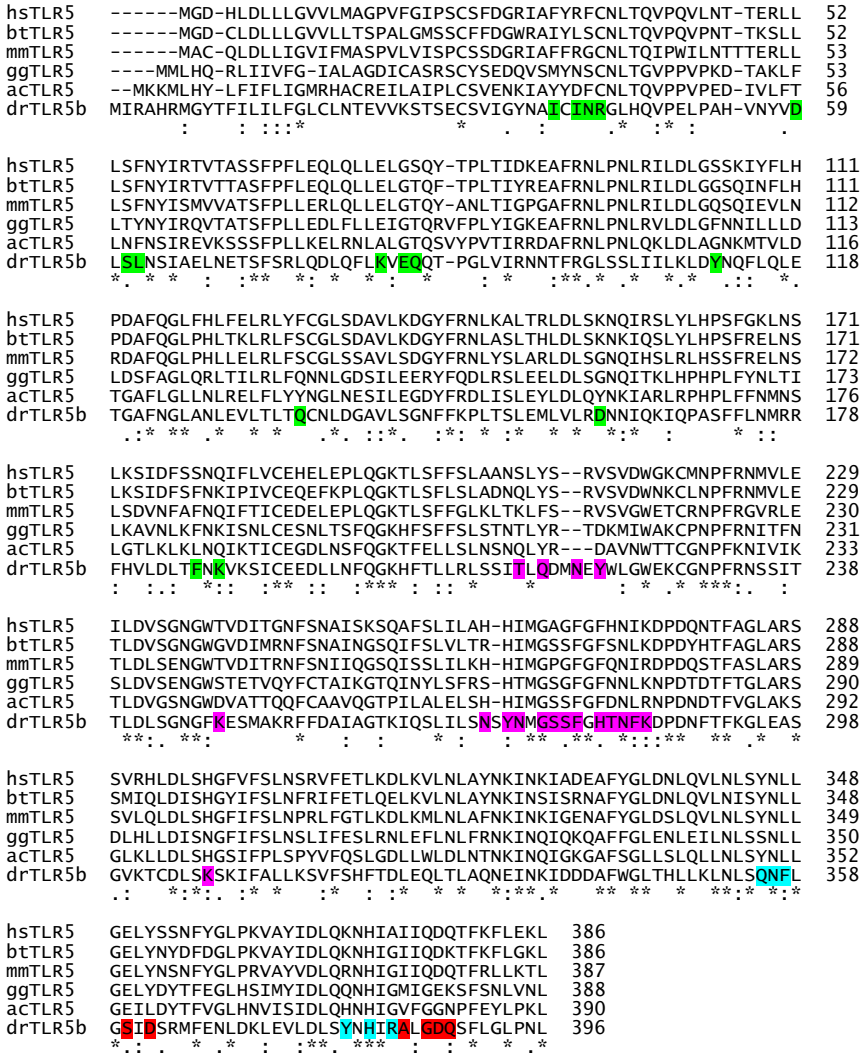

Fig. S6. Sequence alignment of the TLR5 N-ECD of different vertebrates. Alignment of the TLR5 N-ECD of *Homo sapiens* (human; NCBI reference sequence: AAI09119.1), *Bos taurus* (bovine; ABC68311.1), *Mus musculus* (mouse; AAI25248.1), *Gallus gallus* (chicken; ACR26275.1), *Anolis carolinensis* (green 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.*

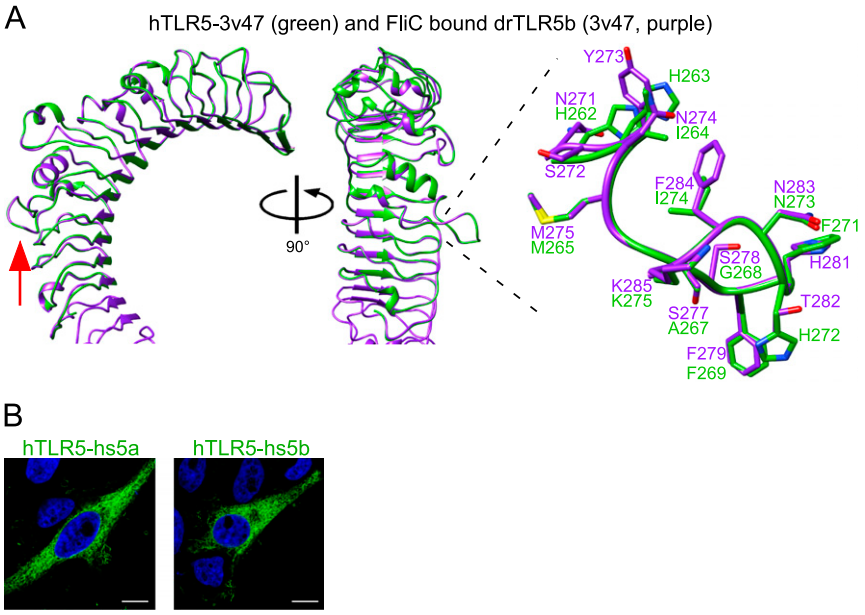

Fig. S7. (*A*) Structural modeling of human TLR5 onto drTLR5b. Superposition of FliC-bound drTLR5b (PDB ID: 3v47; purple) and a model of hTLR5 based on 3v47 (hTLR5-3v47; green). The red arrow indicates the flagellin-binding hotspot that forms a loop between LRR9 and LRR10. (*B*) Expression of hTLR5 chimeric 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 μm.)

Table S1. Primers used in this study

Product	Primer	Sequence (5′-3′)
BamHI-Kozak-drTLR5b-**NotI**	CV039 F	CCGGATCCGCCACCATGATCCGTGCTCACAGAATGG
	CV054 R	CCGCGGCCGCTTACTGCTGTGTTGGTGTTGAAATTG
BamHI-Kozak-drTLR5a-**NotI**	CV095 F	CCGGATCCGCCACCATGGCAGCTACATACACTTTATTTC
	CV096 R	CCGCGGCCGCTAACTGCAGTGTCTGCTTGAAC
KpnI-Kozak-drUNC93B1-**NotI**	CV159 F	CCGGTACCGCCACCATGGCAGCACTGATCGC
	CV160 R	CCGCGGCCGCAGTGTTGGACGTAGTCATCTC
KpnI-Kozak-drUNC93B1-**NotI**-stop codon	CV159 F	CCGGTACCGCCACCATGGCAGCACTGATCGC
	CV180 R	CCGCGGCCGCTTAGTGTTGGACGTAGTCATCTC
BamHI-Kozak-hTLR5-**NotI**	CV257 F	CCGGATCCGCCACCATGGGAGA
	CV259 R	CCGCGGCCGCTGGAGATGGTTGCTACAGTTTG

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