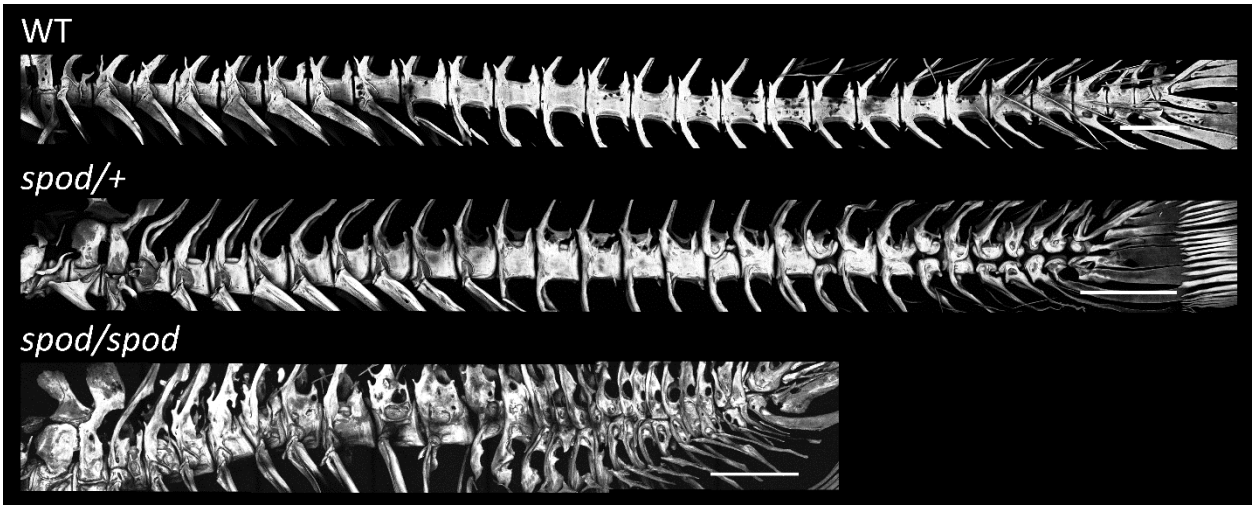


Figure S1. Dose dependent effects of *cmn*$^{spod/+}$ mutation on vertebral patterning. Related to Figure 1. Alizarin red stained skeletal preparations imaged on a Fluoview FV3000 (Olympus) confocal microscope. Wild-type and *cmn*$^{spod/+}$ fish were fixed and stained at 6 weeks old. Homozygous mutants were 4 months of age. Homozygous *spondo* mutants display highly irregular vertebral morphology.

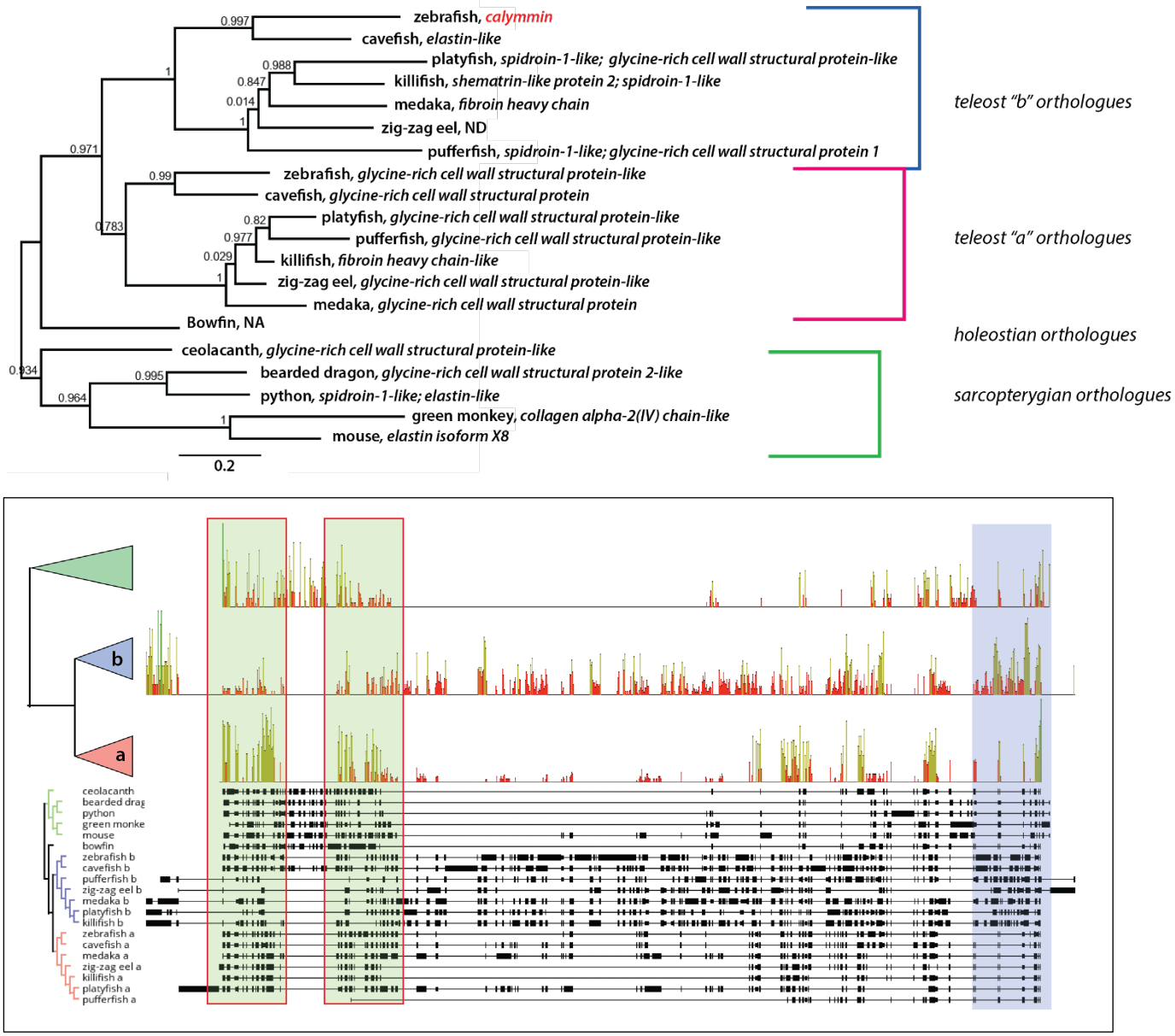
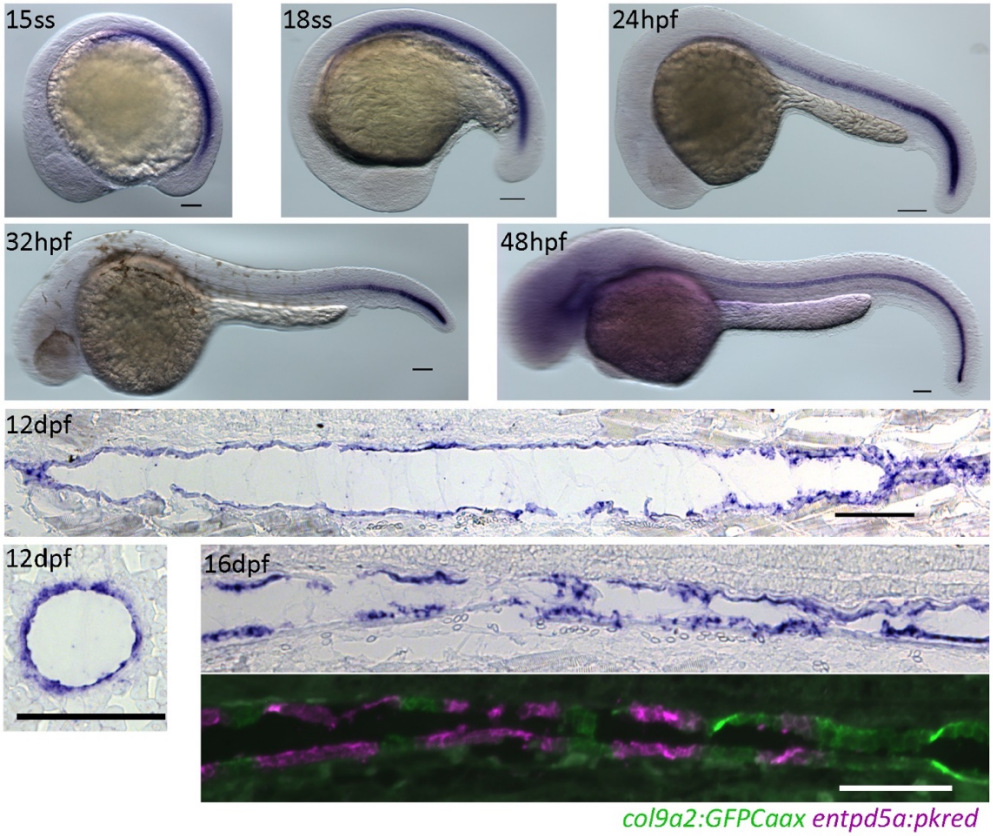


Figure S2. Phylogeny, alignment, and conservation of *calymmin* related genes. Related to Figure 3. **Top**) Maximum likelihood phylogeny of *cmn* coding regions and identified orthologues across vertebrates. Designation of 'a' and 'b' paralogous groups within teleosts based on prior notation of genes syntenic to *cmn*. No orthologues were found for gar or any bird species. Numbers indicate bootstrap support. **Bottom**) Multiple sequence alignment and percent identity of *cmn* coding regions and orthologues across vertebrates. These genes are rapidly evolving, but conservation within orthologous groups reveals both shared and unique critical regions (highlighted), suggesting neofunctionalization. Bar height and color indicate percent identity: green = 100%, yellow > 30%, red < 30%.



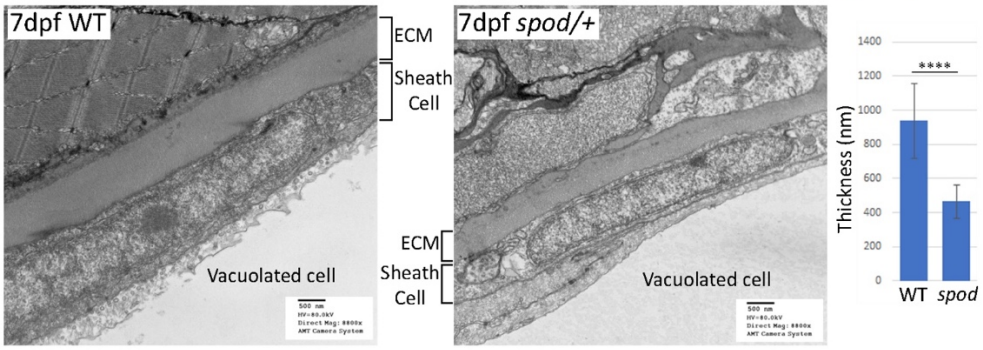


Figure S3. Expression dynamics of *calymmin* during larval development and effects of the *spondo* mutation on ECM organization and thickness. Related to Figure 3. Top: *In situ* hybridization of *cmn* expression at different stages of zebrafish development (15ss-16dpf). *15ss-48hpf*) At each stage, *cmn* expression is found throughout the notochord. During initial chordablast differentiation, expression is enriched in the posterior tail region. *12dpf)* At later larval stages, *calymmin* expression is found throughout the notochord and is restricted to the sheath cells. *16dpf*) During notochord segmentation, *cmn* expression is enriched in chondrocyte-like sheath cells as well as cells that are transitioning into mineralizing cells and express both *col9a2* and *entpd5a*. Following sheath cell differentiation, *calymmin* expression is downregulated in cells that form the chordacentra. Image at 16dpf is an uncropped version of Figure 2G and G'. 7dpf WT, 7dpf *spod/+*). **Bottom,** transmission electron microscopy was performed on 7dpf wild-type and *cmn*$^{spod/+}$ mutants. The sheath extracellular matrix in *spondo* mutants contains more loosely packed fibrils and is thinner than wild-type siblings. Quantification of matrix thickness is shown to the right. For quantification, 55 measurements per genotype were obtained (n=2 fish per genotype). Two-tailed p value = 1.67E-23

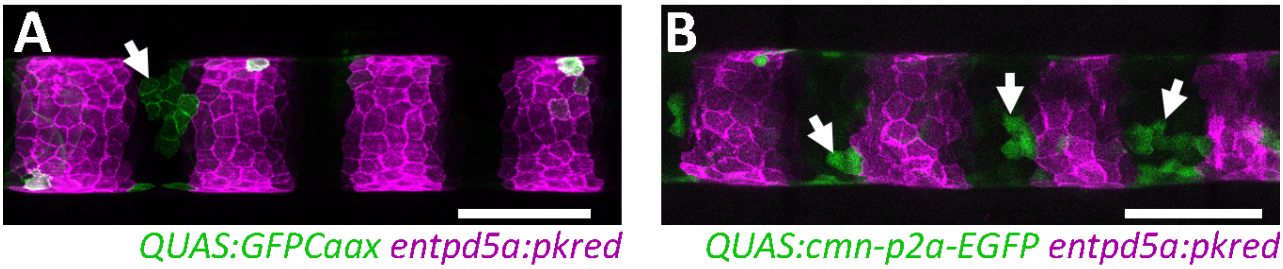


Figure S4. Overexpression of wild-type *cmn* does not lead to ectopic *entpd5a* activation in *col9a2+* domains. Related to Figure 3. **A,B**) Overexpression of either a *QUAS:GFPCaax* or *QUAS:cmn-p2a-EGFP* DNA construct in fish containing a *col9a2:QF2* transgene. Neither cells overexpressing the GFPCaax control nor the wild-type *cmn* construct began to ectopically express *entpd5a* win the prospective IVD domains. The number of GFP+/entpd5a+ cells were counted within each prospective IVD domain. An average of 4.1% of cells were double positive for GFPCaax and e*ntpd5a* in the control group. Of the cells within the IVD domains overexpressing wild-type *cmn,* 9.3% were also *entpd5a*+ (two-tailed *p* value of 0.09). These data indicate that overexpression of wild-type *cmn* is not sufficient to induce the differentiation of notochord sheath cells into mineralizing cells. Arrows point to cells overexpressing the DNA constructs that have not activated *entpd5a*

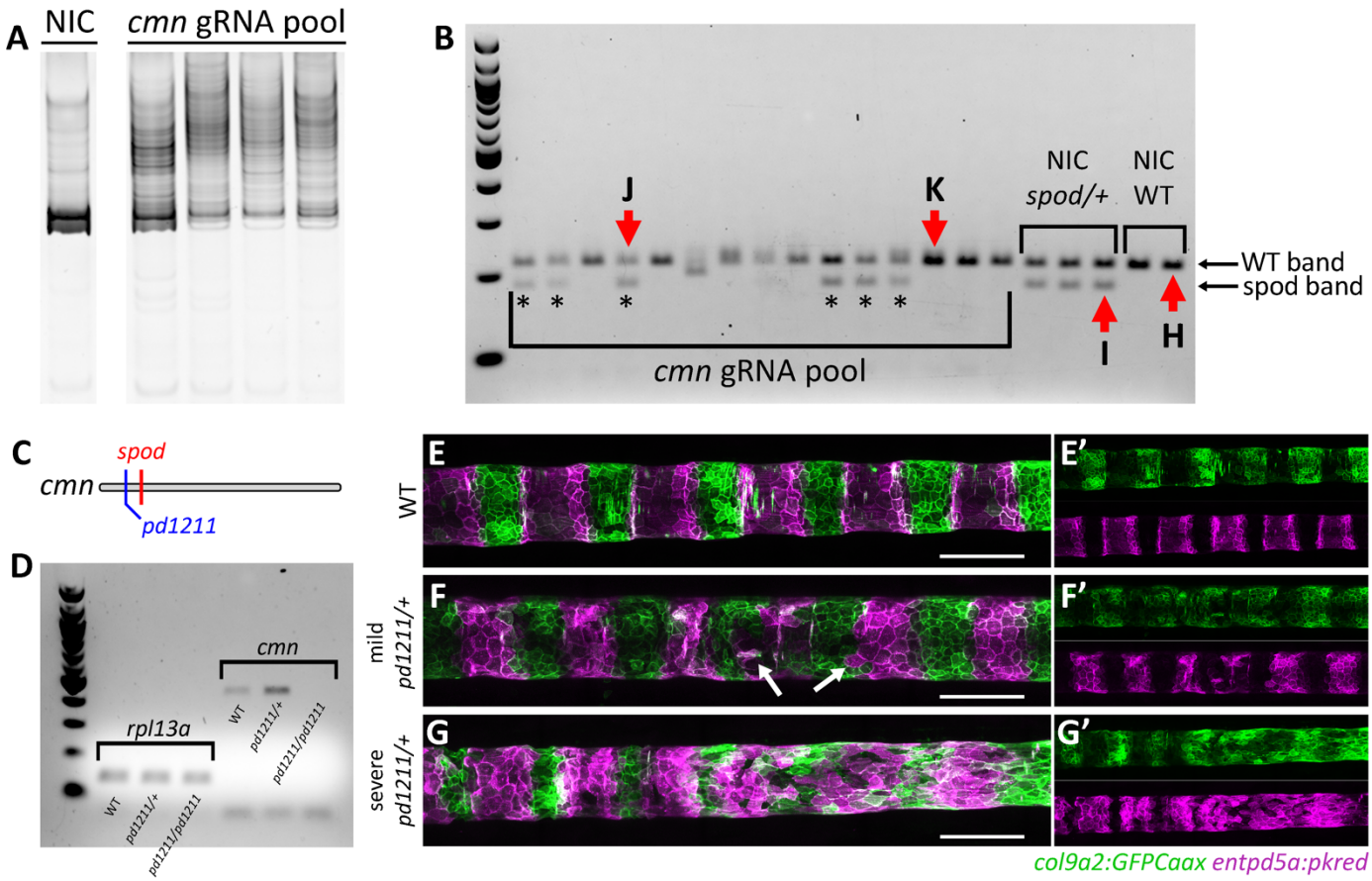


Figure S5. Loss of *cmn* function leads to irregular boundaries between notochord segments. Related to Figure 3. **A**) Heteroduplex mobility shift assay run on an acrylamide gel to visualize indels generated by injection of a gRNA pool targeting the *cmn* locus. NIC = non-injected control. **B**) Genotyping of injected and non-injected fish. Upper band is wild type *cmn* and the lower band is only present in *spondo* mutants after a restriction digest with BseRI (NEB). Arrows indicate fish depicted in Figure 3 H-K. Asterisks indicate injected *spondo* mutants. **C**) A stable mutant line (*cmn*^{pd1211}) containing a 2bp deletion upstream of the *spondo* mutation was generated using CRISPR/Cas9 genome editing. **D**) RT-PCR performed on 5dpf embryos revealed that the *cmn* transcript is degraded in *cmn*^{pd1211} homozygous mutants. **E-G'**) Loss of *cmn* function leads to irregular segment boundaries. **E**) Wild-type sibling displaying normal notochord segmentation. **F,G**) *cmn*^{pd1211} heterozygotes had a notochord segmentation phenotype that was highly variable, ranging from mild to severe. In mild cases (n=8/25 mutant fish), jagged boundaries between the *col9a2* and *entpd5a* domains were apparent similar to the pooled gRNA experiment. In more severe expressivity in heterozygous fish, the two segment domains are indistinguishable and blend into one another (n=3/25 mutant fish). Homozygous *cmn*^{pd1211} were not recovered at this stage (14-16dpf). **E'-G'**) Isolated green and red channels for each image depicted in E-G.

Homologue	Species	Source*	Annotated Name
Calymmin	*Pogona vitticeps*	https://www.ncbi.nlm.nih.gov/gene/110088379	glycine-rich cell wall structural protein 2-like
Calymmin	*Chlorocebus sabaeus*	https://www.ncbi.nlm.nih.gov/gene/103227069	collagen alpha-2(IV) chain-like
Calymmin	*Mus musculus*	https://www.ncbi.nlm.nih.gov/gene/320309	elastin isoform X8
Calymmin	*Python bivittatus*	https://www.ncbi.nlm.nih.gov/gene/103054235 https://www.ncbi.nlm.nih.gov/gene/103054480	spidroin-1-like elastin-like
Calymmin	*Latinmeria chalumnae*	https://www.ncbi.nlm.nih.gov/gene/102353231	glycine-rich cell wall structural protein-like
Calymmin	*Amia calva*	Ingo Braasch, personal communication	
calymmin b	*Danio rerio*	https://www.ncbi.nlm.nih.gov/gene/30208	*calymmin*
calymmin b	*Oryzias latipes*	https://www.ncbi.nlm.nih.gov/gene/101174706	fibroin heavy chain
calymmin b	*Mastacembelus armatus*	http://useast.ensembl.org/Mastacembelus_armatus/Transcript/Summary?db=core;pt=GENSCAN00000024764;r=OOHQ01000019.1:10867594-10914660	GENSCAN00000024764.1
calymmin b	*Astyanax mexicanus*	https://www.ncbi.nlm.nih.gov/gene/103038914	elastin-like
calymmin b	*Xiphophorus couchianus*	https://www.ncbi.nlm.nih.gov/gene/114151854 https://www.ncbi.nlm.nih.gov/gene/114161560	spidroin-1-like glycine-rich cell wall structural protein 1.8-like
calymmin b	*Kryptolebias marmoratus*	https://www.ncbi.nlm.nih.gov/gene/108247781 https://www.ncbi.nlm.nih.gov/gene/108247328	shematrin-like protein 2 spidroin-1-like
calymmin b	*Takifugu rubripes*	https://www.ncbi.nlm.nih.gov/gene/105416331 https://www.ncbi.nlm.nih.gov/gene/105416667	spidroin-1-like glycine-rich cell wall structural protein 1.8-like
calymmin a	*Danio rerio*	https://www.ncbi.nlm.nih.gov/gene/100001051	glycine-rich cell wall structural protein-like
calymmin a	*Oryzias latipes*	https://www.ncbi.nlm.nih.gov/gene/101167291	glycine-rich cell wall structural protein 1.0
calymmin a	*Xiphophorus couchianus*	https://www.ncbi.nlm.nih.gov/gene/114160123	glycine-rich cell wall structural protein-like
calymmin a	*Kryptolebias marmoratus*	https://www.ncbi.nlm.nih.gov/gene/108237682	fibroin heavy chain-like
calymmin a	*Mastacembelus armatus*	https://www.ncbi.nlm.nih.gov/gene/113140701	glycine-rich cell wall structural protein 1.0-like
calymmin a	*Takifugu rubripes*	https://www.ncbi.nlm.nih.gov/gene/105416492	glycine-rich cell wall structural protein-like
calymmin a	*Astyanax mexicanus*	https://www.ncbi.nlm.nih.gov/gene/103039514	glycine-rich cell wall structural protein

Table S1. Data sources for *calymmin* orthologues. Related to Figure 3. Annotations used to compile multiple species alignment of calymmin across vertebrates. *spliced with adjacent annotation where notated.