# A



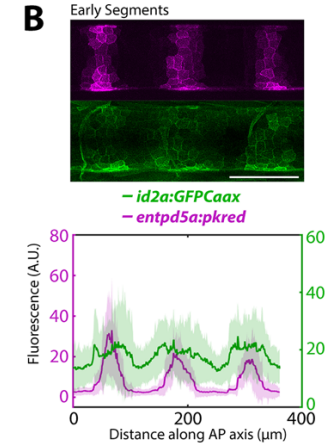| | log2 values of normalized counts | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Gene | col9a2_1_counts | col9a2_2_counts | col9a2_3_counts | double_1_counts | double_2_counts | double_3_counts | entpd5a_1_counts | entpd5a_2_counts | entpd5a_3_counts | ensID |
| bmp3 | 11.27977562 | 12.66457397 | 11.6543871 | 15.91815443 | 16.47850472 | 16.38700252 | 13.97214311 | 14.66737167 | 14.20430542 | ENSDARG00000060526 |
| bmp4 | 11.0026377 | 10.78496789 | 11.50191458 | 12.01649489 | 12.03647987 | 12.48733083 | 12.25845735 | 12.34805052 | 13.62846987 | ENSDARG00000019995 |
| bmp2a | 10.06556345 | 8.965991578 | 8.728933857 | 10.08575844 | 7.650191321 | 9.525370863 | 11.87052567 | 12.61772396 | 11.96378661 | ENSDARG00000013409 |
| bmp2b | 10.2397738 | 9.459814605 | 10.75925867 | 11.4577604 | 9.320242878 | 11.28192506 | 12.8023041 | 13.95367946 | 13.37085438 | ENSDARG00000041430 |
| bmp8a | 9.486890802 | 7.450859886 | 7.889504866 | 10.77952179 | 9.107323615 | 10.52409691 | 13.11436387 | 13.32969306 | 12.45643194 | ENSDARG00000035677 |
| bmpr1ba | 11.81129064 | 12.31382658 | 12.51580288 | 11.5442793 | 11.33377504 | 11.46085362 | 11.57216909 | 12.35347787 | 12.33115027 | ENSDARG00000104100 |
| bmpr1aa | 10.78587328 | 11.32171535 | 11.59580126 | 12.05229699 | 12.32428836 | 12.94886954 | 11.32681758 | 11.95267932 | 12.20743385 | ENSDARG00000019728 |
| bmpr1ab | 10.06167997 | 10.53396251 | 10.87448863 | 10.71017724 | 10.424513 | 11.11938179 | 11.10497627 | 11.9233329 | 12.10947803 | ENSDARG00000105045 |
| acvr1l | 10.81609148 | 11.44831177 | 11.46367475 | 11.52647421 | 12.0234621 | 12.27347295 | 11.05257965 | 11.53150728 | 11.48143615 | ENSDARG00000014986 |
| acvr1ba | 10.07066122 | 10.83662926 | 11.09986914 | 12.15450478 | 12.99577609 | 13.49715467 | 10.16555892 | 10.91644081 | 12.33538021 | ENSDARG00000018968 |
| bmpr2a | 9.431405141 | 9.088991002 | 8.430108403 | 9.952772366 | 10.420135 | 9.755059851 | 10.912777 | 10.27087552 | 9.982643397 | ENSDARG00000011941 |
| fstl1b | 13.68811267 | 14.09594879 | 14.00891585 | 14.26454748 | 14.57189534 | 14.46478497 | 15.17118561 | 15.02386405 | 14.44219354 | ENSDARG00000039576 |
| nog1 | 7.550475551 | 6.553137474 | 8.08891578 | 7.4021131 | 6.79402873 | 7.566249135 | 9.65851836 | 8.682036578 | 9.93551889 | ENSDARG00000058819 |
| nog2 | 10.23613525 | 10.83778827 | 9.266944218 | 14.01517388 | 14.26426251 | 14.4426111 | 11.53204641 | 11.28531561 | 12.14746596 | ENSDARG00000043066 |
| nog3 | 14.95812177 | 15.30940626 | 15.12671272 | 15.82951733 | 16.16907865 | 16.02517092 | 12.93856384 | 12.44630011 | 12.74173462 | ENSDARG00000053528 |
| bmper | 10.89639315 | 9.43035056 | 10.51737087 | 11.97899432 | 10.09684915 | 11.32489326 | 14.04926434 | 14.36860089 | 13.76106654 | ENSDARG00000101980 |
| id2a | 13.68227913 | 13.88144871 | 15.18928544 | 15.63863813 | 15.968894 | 16.45979076 | 13.64061764 | 14.67406185 | 14.40168461 | ENSDARG00000055283 |

# B



**Figure S1: Normalized RNA sequencing values and *id2a* data. Related to Figure 1.**
**A)** Counts were normalized based on gene lengths that were obtained through ensembl.org. Normalized counts were transformed logarithmically for ease of viewing.
**B)** Confocal image of early stage larvae containing *id2a:GFPCaax* and *entpd5a:pkred* transgenes. *id2a:GFPCaax* signal overlaps domains containing *entpd5a:pkred*. n=3 fish
**B')** Fluorescence intensity plot showing overlap of the two expression patterns in (**B**), with *id2a:GFPCaax* peaks extending more broadly along the AP axis.
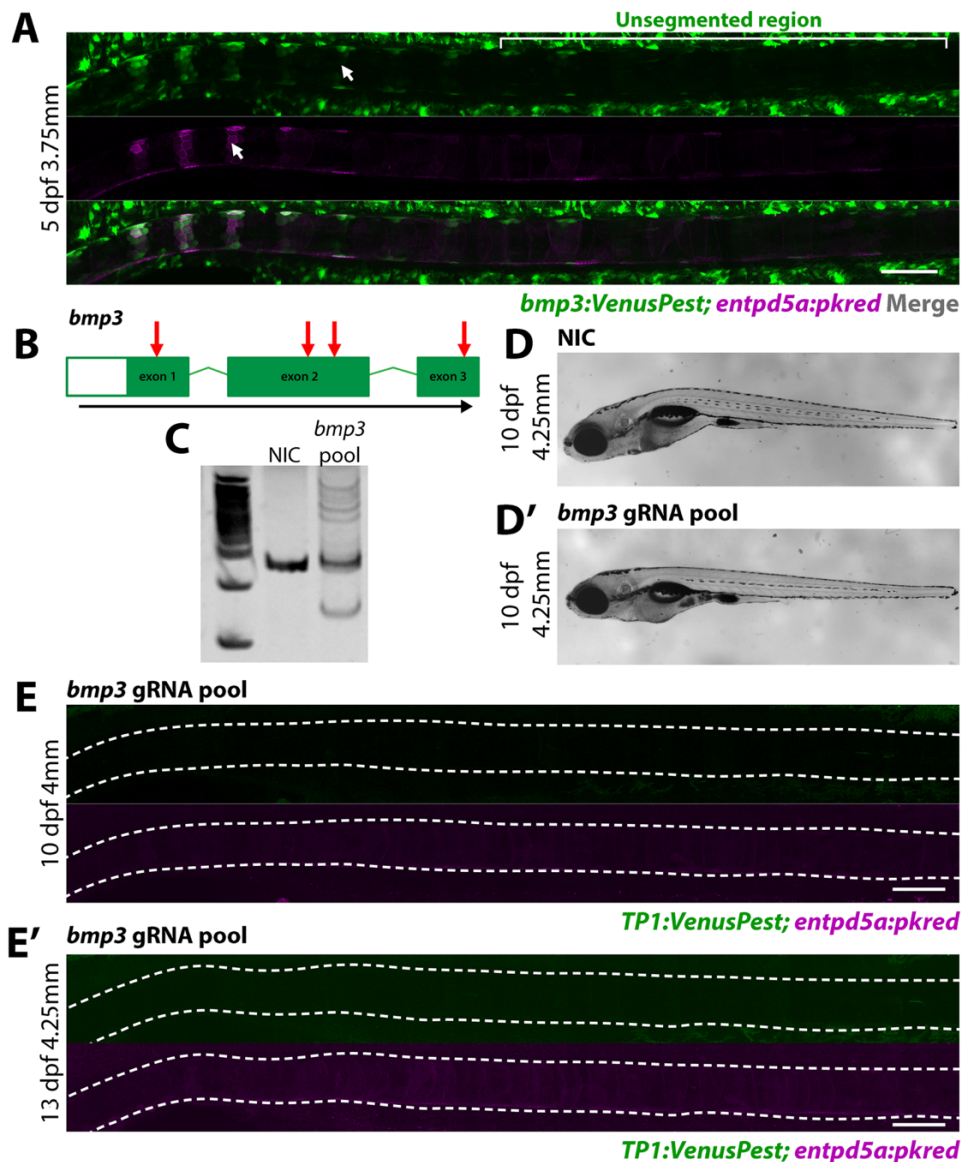
**Figure S2: Additional *bmp3* expression and loss of function data. Related to Figure 2.**
**A)** Digitally stitched confocal images of the endogenous *bmp3:VenusPest* reporter and *entpd5a:pkred* in a 5 day old larva. *bmp3* is initially off in the unsegmented sheath and gets activated prior to *entpd5a.* Arrows mark the last visible segments in the anterior. The bracket highlights the unsegmented region in which *bmp3* and *entpd5a* are not yet expressed.
**B)** Illustration depicting gRNA target sites generated for *bmp3.*
**C)** PCR analysis showing gRNA efficiency. Indels in the *bmp3* sequence are generated following pooled gRNA injection. Wild type band is 238 base pairs (bp).
**D, D')** Brightfield images of NIC and fish injected with *bmp3* gRNA pool. Injected fish do not display obvious signs of developmental delay.
**E, E')** An individual fish injected with the *bmp3* CRISPR pool imaged at 10 (**D**) and 13 dpf (**D'**). Neither *entpd5a:pkred* nor *TP1:VenusPest* have been activated, indicating segmentation was completely blocked. This phenotype was observed in 4/10 injected fish.
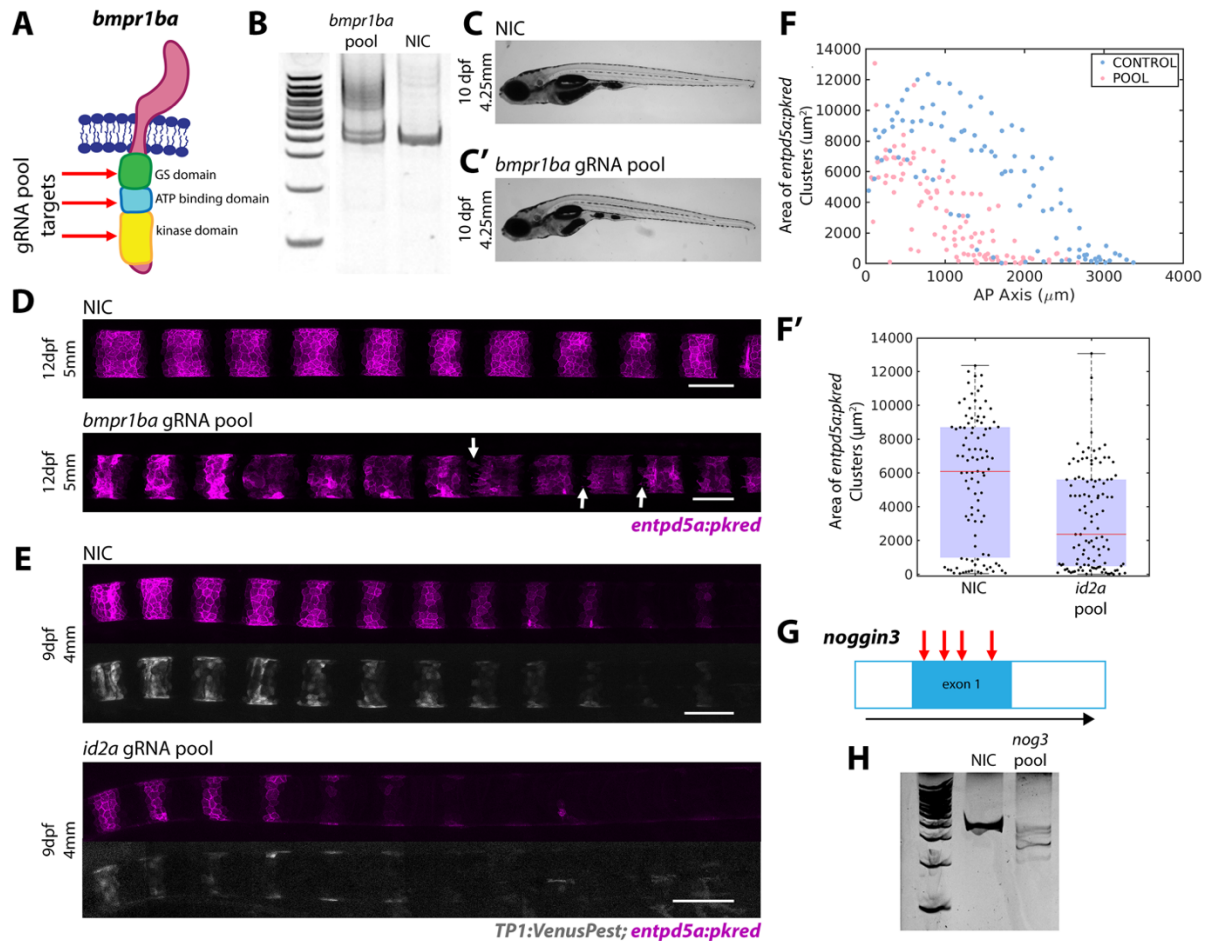
**Figure S3: Targeting of *bmpr1ba* and *id2a*. Related to Figures 3 and 4.**
**A)** Cartoon illustrating the regions targeted with gRNAs in *bmpr1ba*.
**B)** PCR analysis of gRNA efficiency. Indels in the *bmpr1ba* sequence are generated following pooled gRNA injection. Wild type band is 346 bp.
**C, C')** DIC images of NIC (**C**) and *bmpr1ba* (**C'**) gRNA injected fish at 10 dpf and 4.25mm SL.
**D)** NIC and *bmpr1ba* gRNA injected fish at similar developmental stages (12 dpf, 5mm standard length). Targeting *bmpr1ba* periodically led to the development of irregularly shaped segments with jagged boundaries (arrows).
**E)** NIC and *id2a* gRNA injected fish at similar developmental stages (9 dpf, 4mm standard length). Loss of *id2a* led to delayed segment growth.
**F)** The area of *entpd5a+* cell clusters were measured along the AP axis in larvae injected with *id2a* gRNAs and controls at 9 dpf. Loss of *id2a* led to a decrease in segment area along the AP axis. NIC n = 4, *id2a* pools n = 6.
**F')** Area distribution plot of data depicted in (**F**). NIC n = 4, *id2a* gRNA pool n = 6. p value = 2.5595e-06
**G)** Illustration depicting gRNA target sites generated for *noggin3.*
**H)** PCR analysis of gRNA efficiency. Indels in the *noggin3* sequence are generated following pooled gRNA injection. Wild type band is 387 bp.