## SUPPLEMENTARY INFORMATION

**Supplementary Table 1** Top hits of positive R-spondin 3 regulators sorted according to their z scores from the genome-wide siRNA Wnt luciferase reporter assay in HEK293T cells stimulated with Rspo3 conditioned medium. Hit list was generated after elimination of hits with high z score in the cell viability screen. Indicated are also hit z scores from the cell viability screen and the Wnt luciferase reporter screens activated with Wnt3a-conditioned medium or by transfection with the indicated Wnt1, Rspo3, LRP6, Fz8, β-catenin and LRP6ΔE1-4 constructs.

| Wnt3a | Rspo3 | β-catenin | Viability | Wnt1+ Rspo3 | Wnt1+Fz8+ LRP6 | LRP6ΔE1-4 | Gene ID | Gene Name |
|---|---|---|---|---|---|---|---|---|
| -0.62 | **-3.24** | -0.81 | -0.58 | **-3.56** | -0.75 | -0.62 | NM_003667 | **LGR5** |
| -3.04 | -3.21 | -3.73 | -1.61 | -4.12 | -2.97 | -2.89 | NM_006185 | NUMA1 |
| -4.26 | -3.2 | -5.02 | -2.69 | -4.37 | -5.73 | -5.01 | XM_373846 | LOC388639 |
| -2.39 | -3.18 | -1.27 | -0.19 | -1.97 | -1.31 | -0.89 | NM_173492 | PIP5KL1 |
| -2.15 | -3.17 | -2.49 | 0.73 | -3.29 | -1.87 | -3 | NM_138726 | ABCC13 |
| -0.47 | -3.15 | -0.34 | -1.29 | -2.6 | -1.89 | -1.55 | NM_002044 | GALK2 |
| -1.97 | -3.15 | -1.66 | -0.27 | -5.85 | -3.07 | -3.36 | NM_030628 | KIAA1698 |
| -0.96 | -3.13 | -0.45 | -1.24 | -3.99 | -1.78 | -2.52 | NM_004859 | CLTC |
| -3.65 | -3.13 | -1.92 | -1.22 | -3.87 | -4.2 | -3.99 | NM_021572 | ENPP5 |

## METHODS

**Genome-wide siRNA screens** Genome-wide siRNAs screens were performed as describe previously (Cruciat et al, 2010). The Wnt/β-catenin pathway was activated by cotransfection of the TOPFLASH reporter (10 ng) and pRenilla-CMV (4 ng) with plasmids expressing either a) mouse *Wnt1* (5 ng), human *LRP6* (3.3 ng) and mouse *Fz8* (1 ng) or b) mouse *Wnt1* (5 ng) and human *RSPO3* (2 ng) or c) the constitutive active form of human *LRP6, LRP6ΔE1-4* (10 ng) or d) *Xenopus tropicalis β-catenin* (0.1 ng). pcDNA6 vector was added to give a final DNA concentration of 50 ng per well. For the screens with recombinant proteins, 24 h later, cells were treated over night with Wnt3a or Rspo3 conditioned medium, prior firefly and luciferase activity measurement. The cell viability screen was carried out using the 'Cell Titer-Glo' detection kit (Promega) measuring cellular ATP level.

**Supplementary Table 2** LGR4 and LGR5 Morpholino (Mo) and siRNA sequences.

| *Mo/siRNA* | *Mo/siRNA sequence* | *information* |
|---|---|---|
| *LGR4* Mo1 | CAACAGCTTGCACGGTCCGACACCT | 5' upstream of ATG region (27 nucleotides) |
| *LGR4* Mo2 | CACAACCTGCACTTTATTTGGCCGC | 5' upstream of ATG region (105 nucleotides) |
| *LGR4* Mo3 | GCCATACTTACAGCGAGTGGGTGAA | splice Mo |
| *LGR5* Mo1 | AGGTGTCCATGGTGCCGATCAGATC | ATG spanning Mo |
| *LGR5* Mo2 | CGCTGCTCTAATGGTGCAGGCTAAA | 5' upstream of ATG region (31 nucleotides) |
| *LGR5* Mo3 | GCTTGAAAGGTCCTAGGGAGAAAAG | splice Mo |
| *LGR4* siRNA1 | GCAUGUCGCUUGGCUAAUC | ORF |
| *LGR4* siRNA2 | UAAGCAGCAUACCUAAUAA | ORF |
| *LGR4* siRNA3 | GUAGAAACCUGAUACAUGA | ORF |
| *LGR4* siRNA4 | UAAGAGACCUUCCAAGUUU | ORF |

| | | |
|---|---|---|
| *LGR5* siRNA1 | GAAAGAUGCUGGAAUGUUU | ORF |
| *LGR5* siRNA2 | GAACUAGGAUUUCAUAGCA | ORF |
| *LGR5* siRNA3 | CCUAGAGACUUUAGAUUUA | ORF |
| *LGR5* siRNA4 | GCUCUCAUCUUGCUCAAUU | ORF |

**REFERENCES**

Cruciat CM, Ohkawara B, Acebron SP, Karaulanov E, Reinhard C, Ingelfinger D, Boutros M, Niehrs C (2010) Requirement of prorenin receptor and vacuolar H+-ATPase-mediated acidification for Wnt signaling. *Science* **327**(5964)**:** 459-463

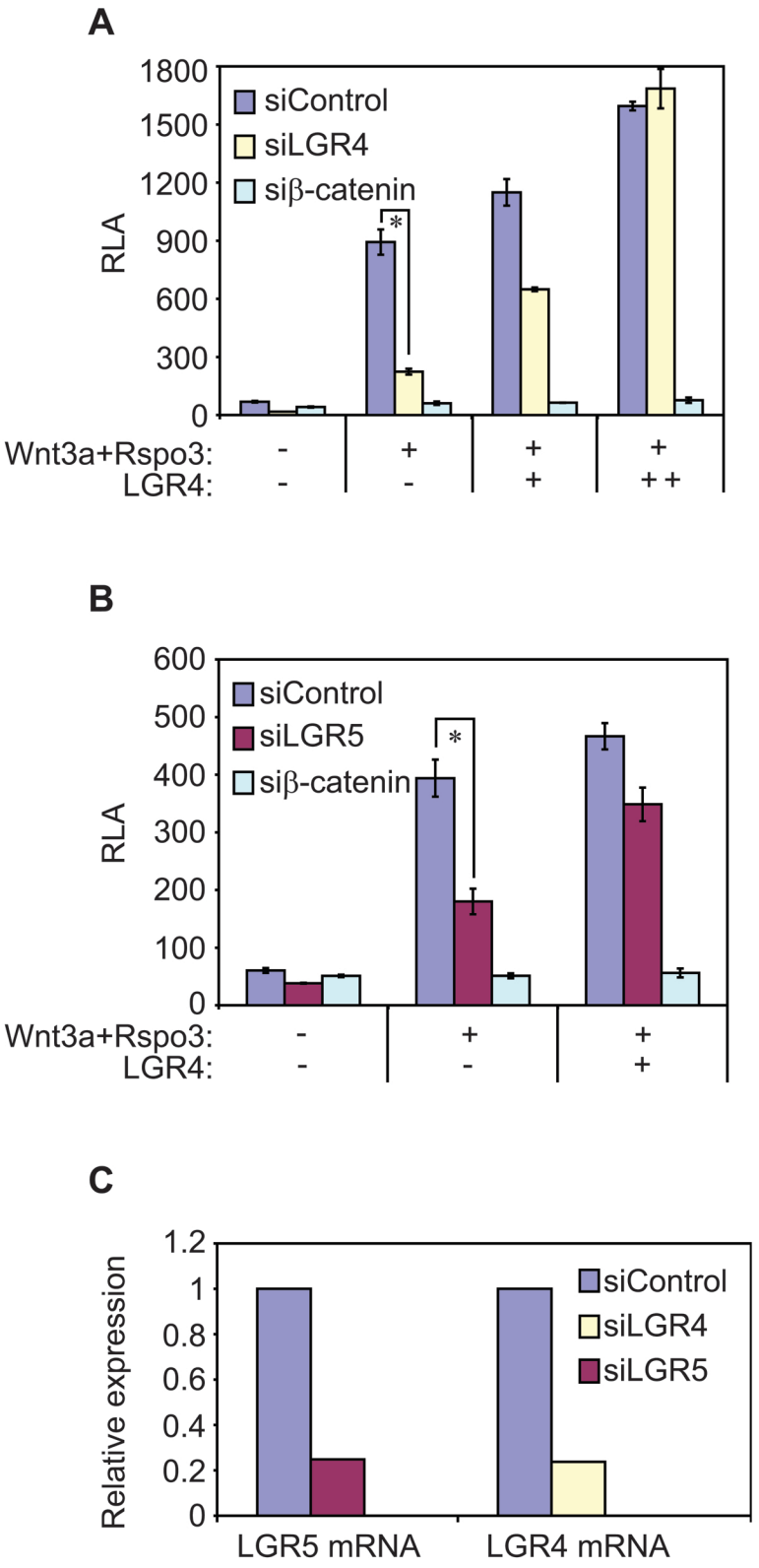


**Fig S1** Specificity of the siRNAs targeting LGR4 and LGR5. (**A**) and (**B**) Expression of *Xenopus tropicalis* LGR4 (0.01 ng (+) and 0.05 ng (++)) rescues Wnt3a and Rspo3 signalling in cells treated with LGR4 siRNA (**A**) and LGR5 siRNA (**B**), but not with β-catenin siRNA. Wnt luciferase reporter assay in HEK293T cells stimulated with control or conditioned medium containing Wnt3a and Rspo3-ΔC-Flag and transfected with the indicated siRNAs in the absence or presence of transfected LGR4. RLA, relative luciferase activity. Error bars indicate SDs; n=3, * indicates P<0.001 by Student's t-test. (**C**) qPCR analysis of the siRNAs knockdown efficiency in HEK293T cells. β-actin was used for normalization.

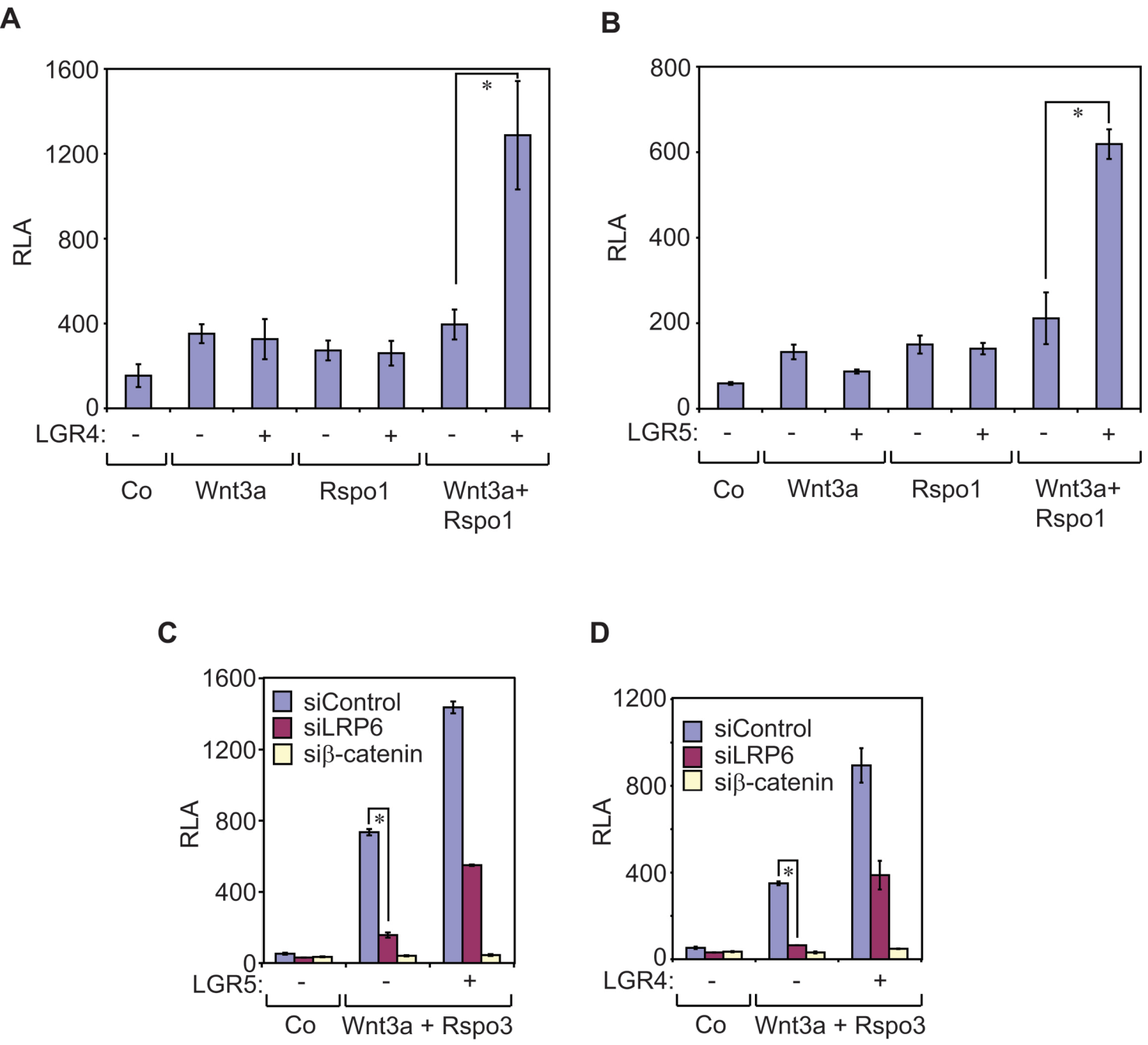


**Fig S2** LGR4 and LGR5 synergize with Wnt3a and Rspo1 in Wnt/β-catenin signalling activation (**A**, **B**) and Wnt3a-Rspo3-LGR4/5 signalling requires LRP6 (**C**, **D**). (**A**-**D**) Wnt luciferase reporter assays in HEK293T cells transfected with the indicated constructs (*Xenopus tropicalis* LGR4: 1 ng or Flag-LGR5: 5 ng), in the presence of the indicated siRNAs. Cells were stimulated with limiting doses of the indicated conditioned media. Co, control medium. RLA, relative luciferase activity. Error bars indicate SDs, n=3, * indicates $P<0.005$ by Student's t-test.

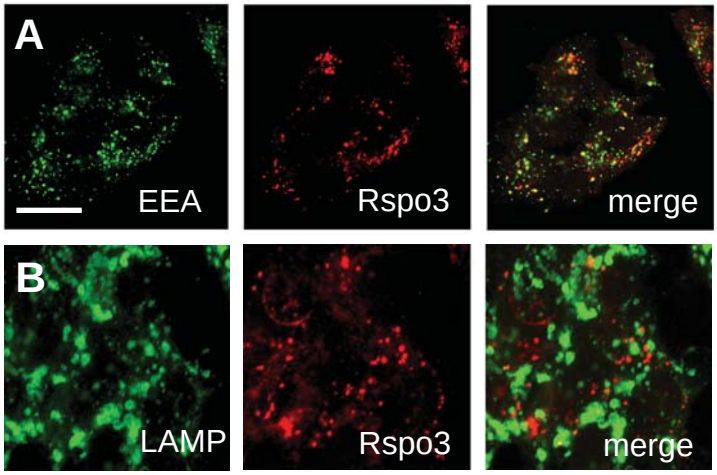


**Fig S3** Internalized Rspo3 is colocalized with EEA1 but not with LAMP1. Confocal microscopy of HepG2 cells incubated with Rspo3-ΔC-HRP, which is visualized by TSA, and followed by immunostaining of endogenous EEA1(**A**, early endosomes) or LAMP1 (**B**, late endosomes). Scale bar is 10 μm.

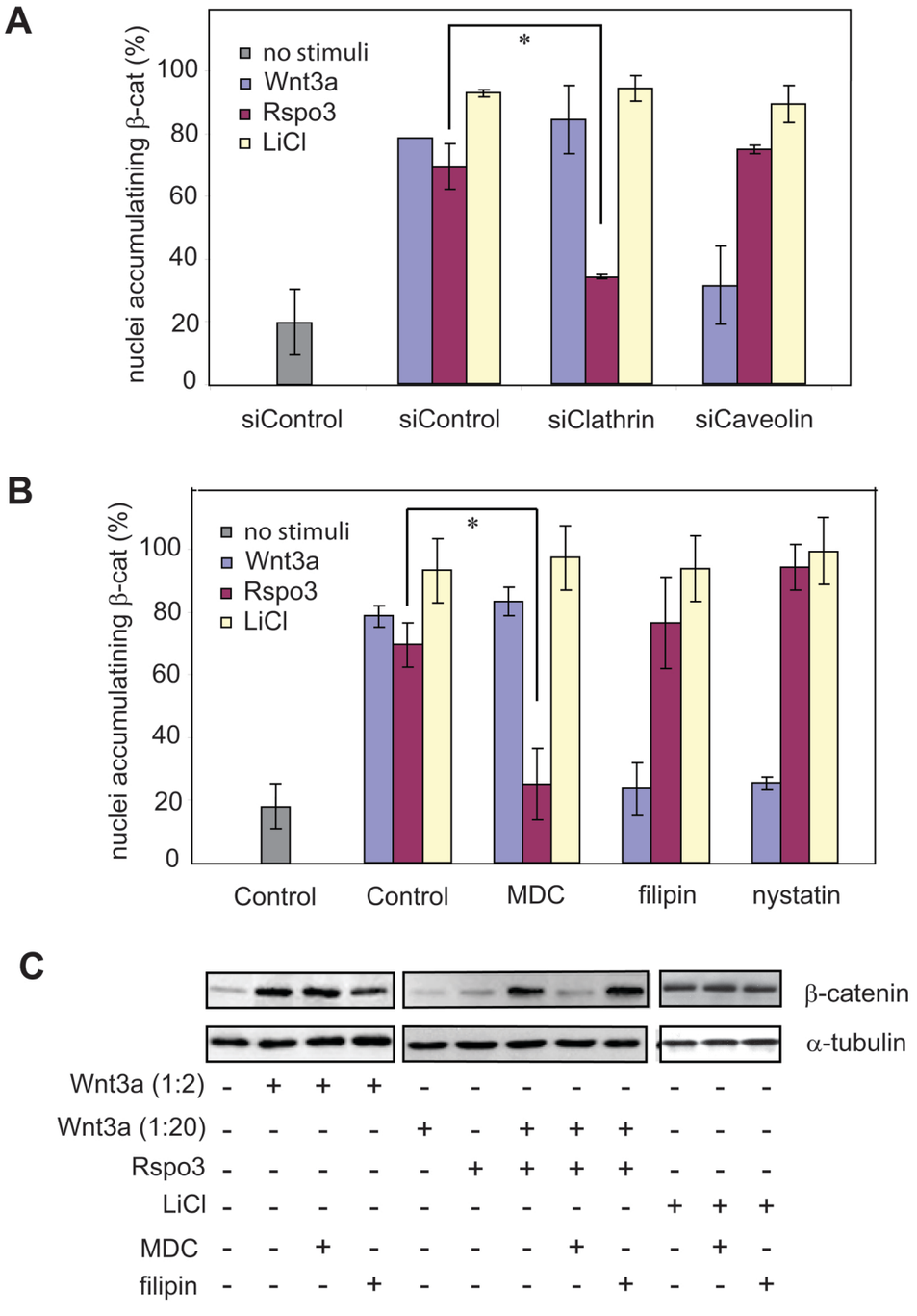


**Fig S4** Clathrin endocytosis is required for Rspo signalling. (**A**-**B**) Quantification of nuclear b-catenin accumulation in NTERA2 cells (see Fig 3). The results shown are means ± SDs from two independent experiments, * indicates $p<0.05$ by Student's t-test. (**C**) Western blot analysis of cytosolic b-catenin from HEK293T cells preincubated for 1 hour with the indicated inhibitors and treated for 4h with Wnt3a or diluted (1:20) Wnt3a together with Rspo3-DC-Flag conditioned medium or 50 mM LiCl.

**Glinka et al. Supplementary Figure 4**

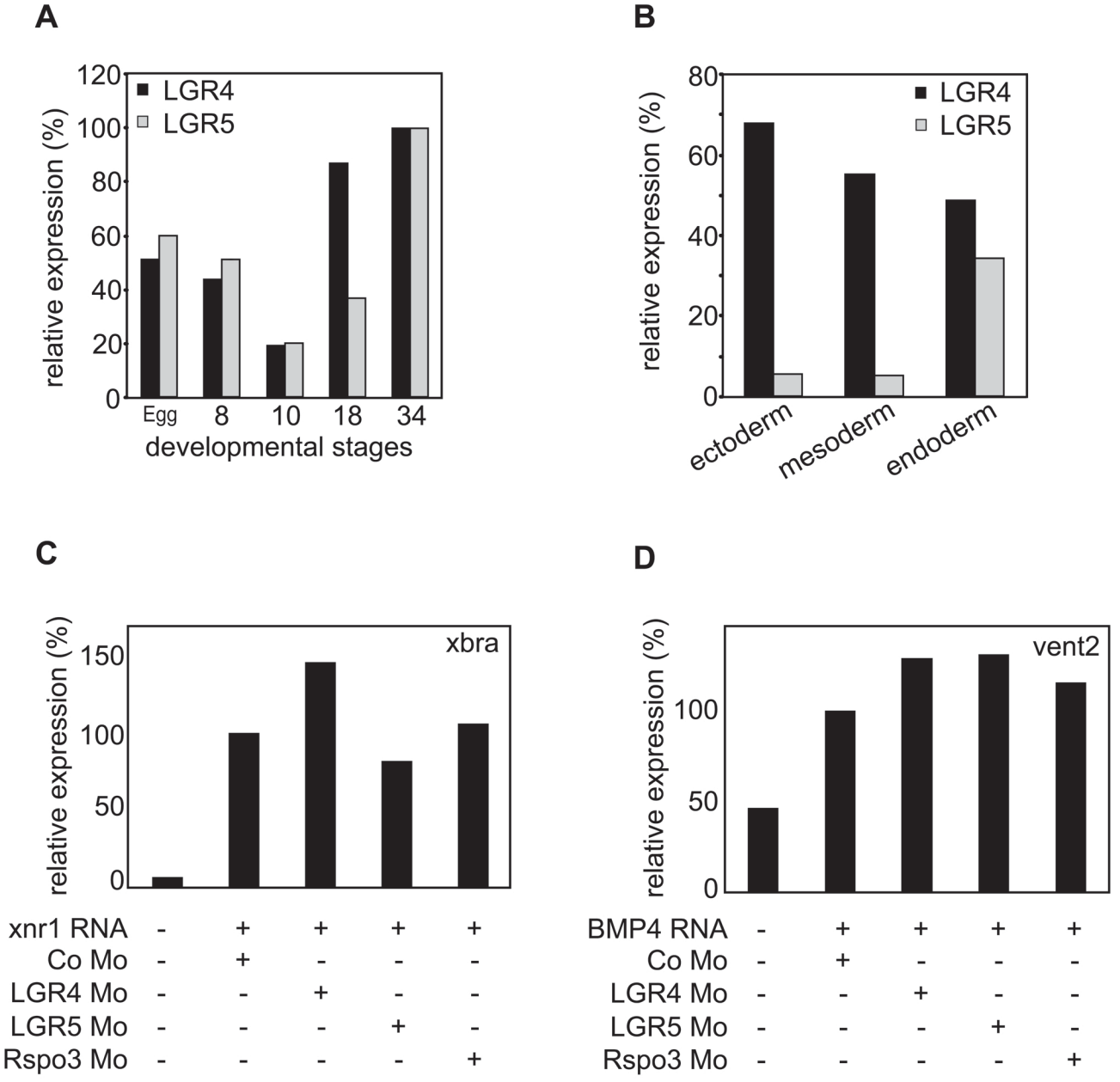


**Fig S5** Expression analysis of LGR4/5 in Xenopus embryos by qPCR analysis (**A-B**), and specificity of LGR4/5 Mo (**C-D**). (**A**) qPCR analysis of LGR4/5 in Xenopus embryos at different developmental stages. ODC was used for normalization. (**B**) Expression in explants analyzed by qPCR from st.10 embryos showing expression of LGR4 in all three germ layers. LGR5 shows its strongest expression in endodermal cells at this stage. Embryos were injected equatorially at 4-cell stage with Xenopus Rspo3 RNA (250pg) and LGR4 Mo1 (10ng [+] and 20ng[++]). (**C-D**) LGR4/5 and Rspo3 do not affect nodal or BMP signalling. Animal cap explants are injected with either xnr1 RNA to induce xbra expression (**C**) or BMP4 RNA to induce vent2 expression (**D**) analyzed by qPCR. n=1, three replicates.