**Supplemental Data**

**Suppl. Table 1.** The sev>Nemo over-rotation phenotype is dominantly modified by signaling and cell adhesion factors (only selected genes are shown out of total of >60 tested)

| genotype                        | Ommatidia with rotation over 135° (in % ± s.d.) | effects | n   |
|---------------------------------|-------------------------------------------------|---------|-----|
| control w¹¹¹⁸ (otherwise wild type) | 13.8 ± 3.4                                      | control | 976 |
| nmo¹⁰                      | 3.7 ± 2.2                                       | su**    | 623 |
| Cell adhesion and cytoskel. factors |                                               |         |     |
| arm⁵                        | 34.7 ± 7.3                                      | en**    | 542 |
| UAS-ArmS10                   | 46.5 ± 9.2                                      | en**    | 297 |
| Ncad¹⁷        | 14.6 ± 1.2                                      |         | 597 |
| UAS-Ncad¹⁷                     | 22.9 ± 7.6                                      |         | 664 |
| shg⁹⁵⁵ (null)                | 29.3 ± 5.2                                      | en**    | 800 |
| shg²³⁴ (hypom.)              | 20.5 ± 10.7                                     |         | 747 |
| dROK²³                      | 17.3 ± 4.9                                      |         | 579 |
| UAS-RhoA⁹⁸⁸¹                   | 10.1 ± 2.5                                      | su*     | 507 |
| mys¹                        | 17.9 ± 3.6                                      | su**    | 586 |
| PCP factors                   |                                                 |         |     |
| stbm¹⁰                      | 5.1 ± 1.7                                       | su**    | 799 |
| UAS-Stbm                    | 20.1 ± 5.4                                      | en*     | 1044|
| pk⁰⁵⁰¹, stbm⁵                 | 5.5 ± 4.8                                       | su*     | 729 |
| pk⁰⁵⁰¹                      | 6.6 ± 3.7                                       | su*     | 795 |
| fz²²²                       | 11 ± 5.2                                        |         | 485 |
| dsh¹                        | 17.1 ± 3.1                                      |         | 680 |
| fmi⁰³⁰¹                      | 10.0 ± 0.9                                      |         | 550 |
| dgo⁰³⁰¹                      | 10.8 ± 2.2                                      |         | 796 |
| stbm⁵, fmi⁰³⁰¹                | 5.2 ± 2.9                                       | su**    | 689 |
| N³⁰⁸¹¹                      | 6.6 ± 2.7                                       | su**    | 1161|
| D¹⁰⁵¹                       | 13.2 ± 5.0                                      |         | 604 |
| spi²⁵⁹                      | 33.7 ± 3.8                                      | en**    | 365 |
| Egfr²⁰⁰¹⁸¹                   | 28.6 ± 5.4                                      | en**    | 622 |
| pnt³⁵⁵¹                      | 16.0 ± 3.6                                      |         | 772 |
| aos¹                        | 16.7 ± 3.9                                      |         | 523 |
| Df(3)embr5(Rac1¹)            | 13.4 ± 8.7                                      |         | 693 |
| arm⁰⁵⁰¹ (wg-signaling)        | 13.7 ± 2.8                                      |         | 793 |
| sgg²³                      | 15.8 ± 9.5                                      |         | 480 |
| w¹¹¹⁸ (wild type)            | 13.8 ± 3.4                                      | control | 976 |

#: The effects highlighted as enhancers (en) or suppressors (su) show a P-value of <0.05 (*) or <0.005 (**) in student T-test. Data were collected from 3-8 adult eyes. The UAS transgenes were co-expressed with UAS-Nmo (under sevGal4 control).
Suppl. Figure 1. Molecular characterization of the $nmo^{DB}$ allele

Schematic map of the 5’ exons of $nmo$, open boxes are non-coding sequences and black box indicates coding sequence within the 2$^{nd}$ exon after the ATG. The $nmo^P$ insertion is indicated by a blue triangle and extent of the deletion in the $nmo^{DB}$ null allele by the red bar (nucleotide coordinates are as in Flybase indicated above; Flybase annotation of the $nmo$ RNA [nmo-R] and protein [nmo-P] are shown below cartoon).

Several genetic observations prompted us to determine whether $nmo^P$ was a true null allele, as suggested by Choi and Benzer (1994). RT-PCR was used to determine the presence of $nmo$ mRNA from homozygous mutant flies. While northern blotting does not detect transcript, a low-level of $nmo$ transcript is present in both pupal and adult RNA used for RT-PCR from $nmo^P$ flies. As such, the $nmo^P$ flies should not be considered null for $nmo$, but rather a hypomorphic allele.

In an attempt to make amorphic $nmo$ alleles, the P-element from the $nmo^P$ flies (blue triangle) was excised using the $\Delta2$-3 transposase source. Excision events were selected by loss of the $w^+$ eye color marker and by phenotype in trans with $nmo^{adk}$. Flies from one such excision allele, $nmo^{DB24}$ (subsequently referred to as $nmo^{DB}$), die as pharate adults, with a low frequency of escapers that show ectopic vein material. Genomic DNA was sequenced to determine the lesion in the $nmo^{DB}$ excision. A deletion was detected that spans from nt 44 to 1198 within the transcript, and removes the predicted start codon at nt 1035 and the following 54 amino acids (extent indicated by red bar). If a downstream methionine were used as a start codon in a spurious transcript, only a truncated protein missing significant parts of the kinase domain could be translated. In addition, the $nmo^{DB}$ allele is not enhanced in trans to a deficiency and thus represents a null allele, as indicated by the molecular analysis.

Suppl. Figure 2. $nmo^{DB}$ imaginal disc phenotype
Third instar eye imaginal disc (area posterior to the morphogenetic furrow) containing \( nmo^{DB} \) (\( nmo \) null) clones stained with anti-GFP (\( psq\)-GFP in green; marking R3/R4 precursors from 45° rotation angle onwards with higher staining in R3 than in R4; compare also to Figure 1B in main text), anti-Elav (blue; all R-cells) and the clonal marker \( \beta \text{Gal} \) (red; \( nmo \) mutant tissue is marked by absence of \( \beta \text{Gal} \)). \( psq\)-GFP staining indicates degree of rotation of respective clusters. Right panel shows a semi-schematic version of the disc area: white bars indicate orientation of wt and yellow bars those of mutant clusters. Note severe under-rotation in mutant tissue and the largely normal distribution of the R3/R4 staining pattern reflecting chirality.
Nmo is found throughout all imaginal disc cells analyzed, using NmoGFP transgenes (e.g. under tubulin promoter control) that fully rescued the nmo loss-of-function phenotype. (A-C) show eye discs stained for NmoGFP (anti-GFP in green, monochrome in C'), anti-Arm (red), and anti-E-cad (blue). (D) shows a wing disc stained for NmoGFP. NmoGFP is often found at the membrane (overlap with anti-E-cad staining in C and single stain in D). The respective staining pattern does not change in mutant tissue clones of E-cad/shg. (E) and (F): High resolution image ommatidial preclusters, row 8-10. NmoGFP expression under GMRshort-Gal4 in all cells posterior to the MF. NmoGFP protein is enriched in R4 cells, although its RNA is expressed evenly in all cells, suggesting that this effect is mediated by post-transcriptional regulation.
Suppl. Figure 4. The activity of Nmo does not affect the binding of E-cad and β-catenin.

Nmo activity does not affect E-cad/β-cat complex formation. Gst-pull-downs of increasing concentrations of His-β-cat in absence or presence of Nmo (marked by +). (A) His-β-cat was pre-phosphorylated and added to Gst-Cad. (B) Nmo was directly mixed with Gst-Cad and His-β-cat and incubated. Note that E-cad is only phosphorylated in panel B and the interactions are not affected by Nmo. (C) Quantification of 3 independent experiments as described for scenario in panel B; lanes 1-5 are without Nmo and 6-10 are with Nmo.
Suppl. Figure 5. ArmS10 expression and its nuclear function in Wg-signaling vs. cytoskeletal requirements.

Expression of ArmS10 at 25°C (under sev-Gal4 control) caused frequent apoptosis of R-cells due to strong activation of Wg-signaling (Brunner et al., 1997) with most ommatidia lacking several R-cells (panel A). Tangential eye section of sevGal4, UAS-ArmS10 (sev>Arm S10), anterior is left and dorsal up; ommatidial orientation is presented schematically in lower panels (arrows are as in Fig. 1, unscorable ommatidia are indicate by a dot). When ArmS10 is co-expressed with factors that can tether it to (or “trap” at) the cell membrane like an E-cadDN transgene (panel B), a dominant negative form of E-cad that binds Arm/β-catenin and has a mild under-rotation phenotype, the nuclear signaling phenotype (loss of R-cells) was suppressed (compare panels A and B). Note that the rotation defects induced by sev>ArmS10 are much...
weaker in this background (B) as compared to sev>Nmo, ArmS10 co-expression (Figures 6f,h in main text) further confirming the synergistic effects on rotation of Nmo and Arm/β-cat.

We also tested whether expression of ArmΔ, a constitutively active isoform of Arm that cannot function in adhesion (e.g. Tolwinski and Wieschaus, 2004), can be affected by Nmo in this assay: sev-driven ArmΔ causes a phenotype similar to ArmS10 (panel C; loss of R-cells), which is not suppressed by co-expression of Nmo (D) or N-cad (E) or E-cadDN (not shown). These data indicate that Nmo does not inhibit the nuclear effect of stable Arm/β-catenin in this context, but that it causes a shift of its activity to its cell adhesion function with E-cadherin in rotation.
Suppl. Figure 6. Nmo phosphorylates Arm on C-terminal residues.

An ArmΔC construct encoding amino acids 1-730 was made by cutting at a TthIII1 site within the 3' end of the Arm cDNA and a KpnI site present in the multiple cloning site of the vector. This truncated Arm protein exhibited hypo-phosphorylation by Nmo in in vitro kinase assays compared to full-length Arm (not shown) and appeared inactive for its role in rotation (not shown). Within the deleted region, three potential Nmo phosphorylation sites are encoded: Serine 786, 802 and Threonine 827 (panel B). These residues were mutated to Alanines (individually and in triple combination, and assayed for phosphorylation by Nmo (panel A)). Mutagenesis was performed on the HA-pCMV-Arm plasmid using the QuickChange site-directed mutagenesis kit according to the manufacturer’s instructions (Stratagene). The triple
mutant Arm (Arm-AAA) exhibited the weakest phosphorylation and it was determined that Nmo targets the three sites indicated in panel B. HEK293T cells were transfected with 4.0μg of pCMV-HA-Arm (wild-type/WT and AAA) and 4.0μg of pXJ-Flag-Nmo (wildtype and kinase dead [KD]). Transfected proteins were immunoprecipitated with anti-HA and anti-Flag antibodies. Immunoprecipitated proteins were subjected to in vitro kinase assays and analyzed by autoradiography. The immunoprecipitates were also immunoblotted with the indicated antibodies to confirm loading. Note that the Arm-AAA isoform is phosphorylated much less – the autophosphorylation of Nmo serving as an internal standard – as compared to Arm wild-type. The respective 3 residues are indicated in panel B. They were subsequently tested in vivo in an ArmS10 background during ommatidial rotation as outlined in the main text.

(C) The AAA mutation does not affect localization or stability in either ArmWT or the stabilized isoform ArmS10. Apical sections of eye imaginal discs are shown, note that ArmS10 is more stable and detected also in the cytoplasm of the cells as compared to ArmWT, which is only detected at the apical junctional regions (small apical tufts in R-cell precursors). There is no detectable difference between the WT and the AAA isoforms.

The kinase assays were performed as described in:
Zeng et al. (2007). *Drosophila* Nemo antagonizes BMP signaling by phosphorylation of Mad and inhibition of its nuclear accumulation. Development 134: 2061-2071.
Suppl. Figure 7. The ArmS10-AAA mutant isoform consistently blocks the Nmo gain-of-function effect.

Independent insertion lines of ArmS10 and ArmS10-AAA show very reproducible effects in the Nmo gain-of-function background: Both lines shown for ArmS10-AAA are largely neutral and do not enhance the sevGal4, UAS-Nmo (sev>Nmo) effect, unlike ArmS10-wt.
Suppl. Figure 8. Co-expression of Stbm and Nmo displays a synergistic effect in promoting ommatidial rotation.

Tangential eye section of sevGal4, UAS-Nmo, UAS-Stbm eyes (left panels), anterior is left and dorsal up; ommatidial orientation is presented schematically in lower panels (arrows are as in Fig. 1). Right panels: quantification of the genotype shown on left (top panel) and compared to sev>Nmo alone. Importantly, sevGal4, UAS-Stbm flies had no rotation or chirality defects by itself (our unpublished line), and this line was specifically selected to allow the analysis of potential synergy of the co-expression. Note that while in sev>Nmo the majority of clusters is still in the 90° range (65.9%), only one quarter (26.9%) are at a 90° angle in the sev>Nmo,>Stbm genotype, with the majority being overrotated to random degrees.
Supplemental Experimental Procedures

**Affinity purification of β-catenin with GST-Nemo**

SW480 cells (human colon carcinoma cell line from ATCC) were solubilized under non-denaturing conditions (1% NP-40, 10mM Tris-HCl pH 7.5, 150mM NaCl, 2mM EDTA) with protease inhibitors (200mU PMSF, 4ug/ml aprotinin, 1ug/ml pepstatin A, 2ug/ml leupeptin, 10ug/ml antipain, 50ug/ml benzamidine) and phosphatase inhibitors (100mM NaF, 100uM sodium vanadate, and 1uM microcystin), clarified at 14,000g for 10’ and equivalent amounts of supernatant (~500μg protein) were incubated with either GST or GST-Nemo (encoded by c5-1) (~10ug; pre-incubated with Glutathione-coupled Agaroose, Sigma, St. Louis, MO) for 3 hours at 4C. Affinity precipitated proteins were washed x3 with 1% NP-40 lysis buffer, followed by 2 washes with 0.1%NP-40 lysis buffer. Samples were boiled in sample buffer, subjected to SDS-PAGE and western analysis according to standard protocols. β-cat was detected using a monoclonal antibody (#C19220, Transduction Laboratories, Lexington, KY), goat-anti-mouse HRP (Bio-Rad, Richmond, CA) and the ECL system (Amersham, Buckinghamshire, England).

**GST-Nemo binding with recombinant β-catenin in vitro**

Recombinant Xenopus β-catenin was synthesized in SF9 cells using the Bac-N-Blue Transfection kit (Invitrogen) and isolated by binding to nickel agarose beads (Qiagen, Santa Clara, CA) as previously described [Fagotto et al., 1998]. 10μg of purified β-cat was incubated with GST or GST-Nemo in 0.1% NP-40 buffer for 3 hours at 4˚ C. Samples were processed as described above.

**Kinase reactions and pull down assays**

**In vitro** translated 35S labelled Nemo was generated using the pXJFLAG-nemo plasmid and TNT Quick Coupled Transcription/Translation kit (Promega: following manufacturer's instructions). 400μl of wash buffer with protease inhibitors was added to GST or GST-Arm bound beads along with in vitro transcribed/translated reaction. Reaction was mixed on a nutator at 4°C for 1 hour. The protein-loaded beads were washed four times with GST buffer, resuspended in 10μl SDS sample buffer, and boiled for 5 minutes. After electrophoresis, gels were fixed, dried onto Whatmann 3M paper, and subjected to autoradiography.

HEK293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% fetal bovine serum at 37°C. Cells at 70–80% confluency were subjected to transient transfection by using the Polyfect transfection reagent (Qiagen; following manufacturer's instructions). A total of 8μg DNA consisting of various expression vectors, pXJ-Flag-nemo (wild-type and kinase dead) and pCMV-HA–Arm, were used in 10cm² dishes. 24-48
hours after transfection, cells were washed in 5 mL PBS and lysed in 1ml lysis buffer at RT for
10 minutes. Lysates were sonicated and centrifuged for 2 minutes at 14,000rpm and 4°C.

Mouse anti-Flag or mouse anti-HA (Sigma) were added to supernatants at 1:1000 dilution
(nutating O/N at 4°C). 30µl of 50% slurry of protein G-sepharose beads (Sigma) were then
added for one hour at 4°C. The immunocomplexes were wash three times with lysis buffer and
boiled in 5µl SDS sample buffer, subjected to SDS-PAGE and western analysis.

Xenopus C-cad GST, mouse ß-cat-His (gift of B. Weiss) and Drosophila Nmo-His proteins
were purified under native conditions from E. coli or bacculovirus infected S2 cells using
standard procedures and dialyzed against binding buffer (50mM Tris-HCl pH 7.6, 150mMKcl,
0.5% Triton-X, 1mM DTT) containing 10% glycerol.

For non-radioactive kinase assays samples were incubated in 30µl of kinase buffer (25mM
Tris pH 7.5, 25mM MgCl₂), phosphatase inhibitor cocktail (Sigma), and 20 mM ATP (Roche).
Assays were performed for 30 min at 29°C and stopped by placing the reaction on ice.

Pull-down assays were performed in 500µl total volume of pull-down buffer (50mM Tris pH
7.5, 150 mM NaCl, 0.1% NP-40, 3mM MgCl₂, and protease inhibitor cocktail from Sigma) for 3
hrs at 4°C. Reactions were started either by adding the entire kinase reaction to pull-down
buffer with equilibrated GST-beads (GE-Healthcare) or by adding the kinase reaction containing
Nmo and ß-cat to C-cad-GST pre-incubated with beads for 1 hr at 4°C. Pull-downs were
washed 3 times and analyzed on SDS-gels as above.

**Titration experiments**

Titration experiments were performed under conditions where ß-cat is either pre-phosphorylated
with Nemo or where both ß-cat and cad-GST are pre-phosphorylated together under same
conditions as described above. Alternatively, cad-GST was added together with ß-cat for kinase
reaction. The concentrations of cad-GST and Nemo were kept constant (2.5 or 5µg for cad; 1.5
or 3µg for Nemo) with increasing concentrations of ß-cat (0.3 -1.7µg). Kinase assays were done
in 30µl of kinase buffer followed by 3-4 hr pull-down with cad preincubated with beads. The
beads were then washed in pull-down buffer and resuspended in 20µl 2X concentrated SDS
sample buffer. Samples were analyzed on 10% SDS-polyacrylamide gel, stained with
Coomassie solution to reveal protein content, and scanned with an Odyssey scanner (Licor).
Quantification of protein levels was done with Odyssey program and data was analyzed in
Excel. Western analysis was done according to standard procedures. Primary antibody was
anti- RGS-His (Quiagen).