**Supplementary Information**

# Molecular insights into intrinsic transducer-coupling bias in the CXCR4-CXCR7 system

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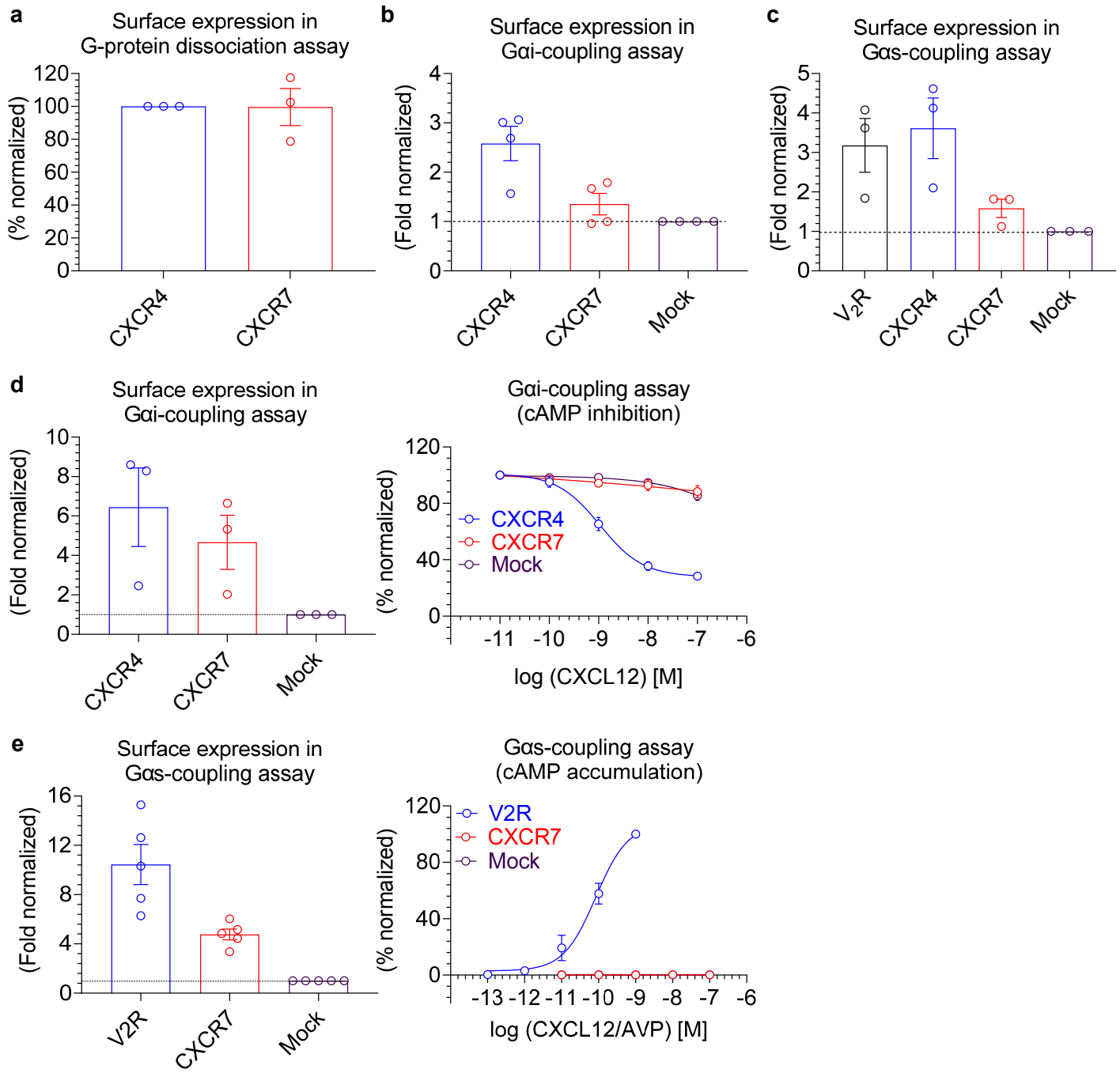
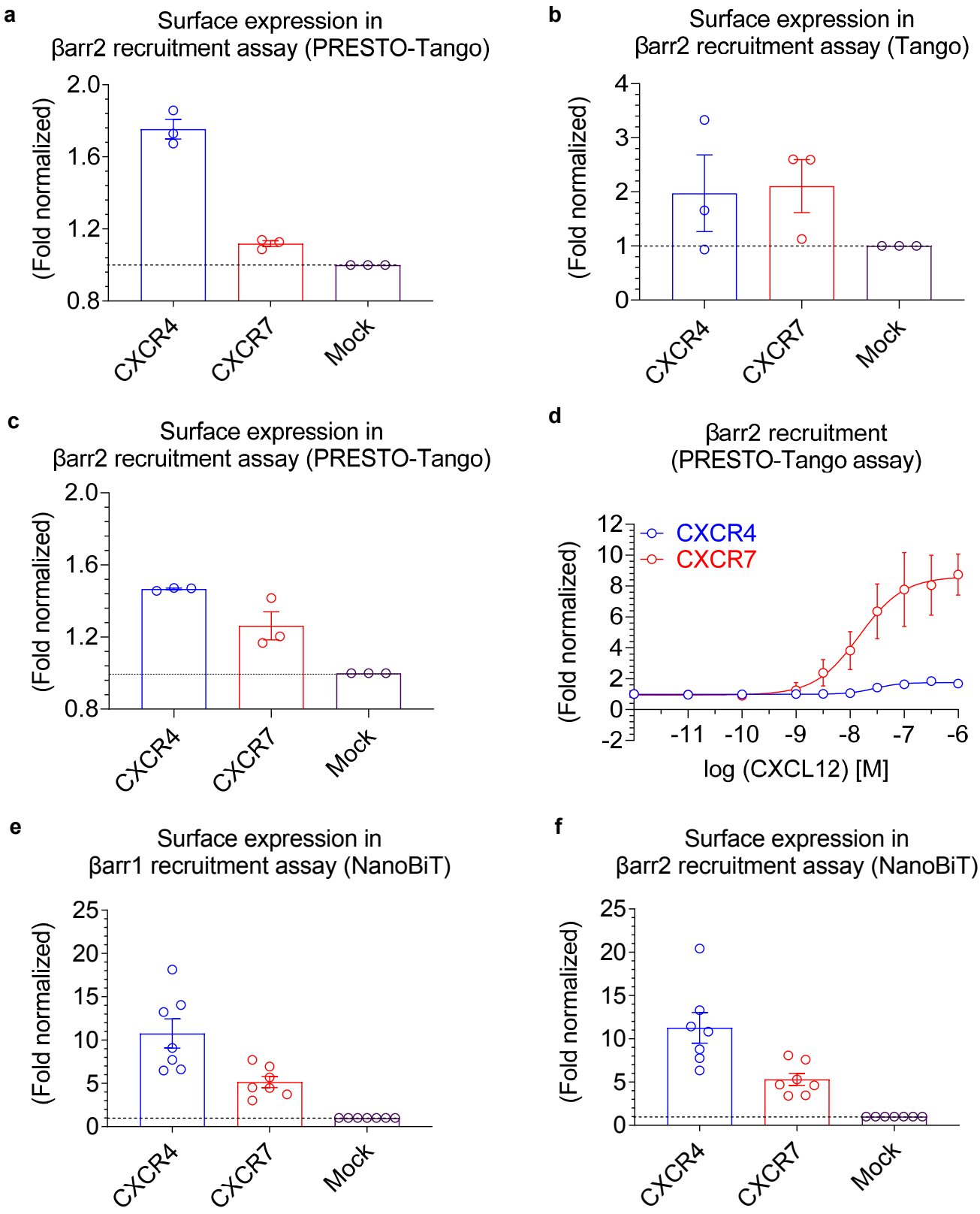
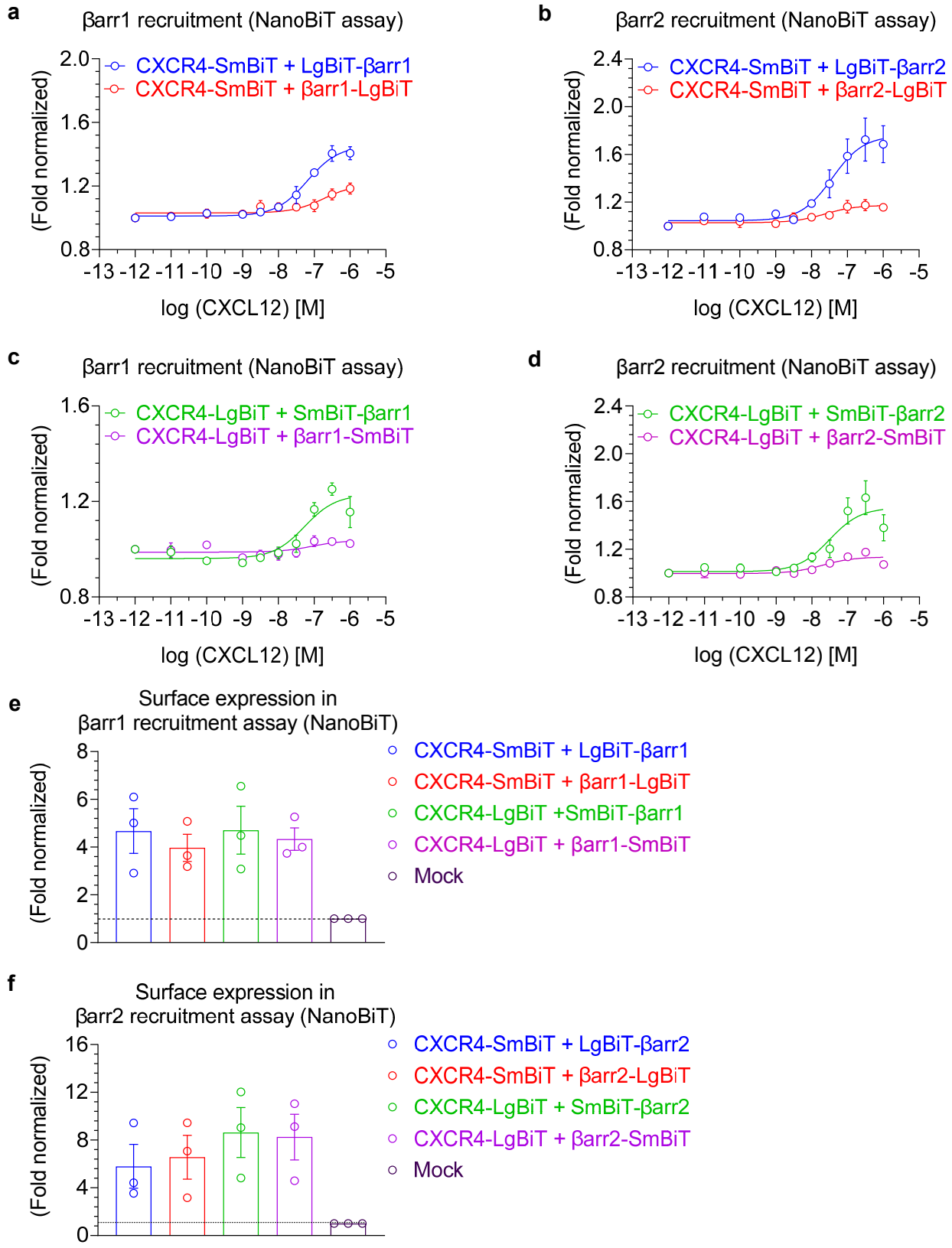


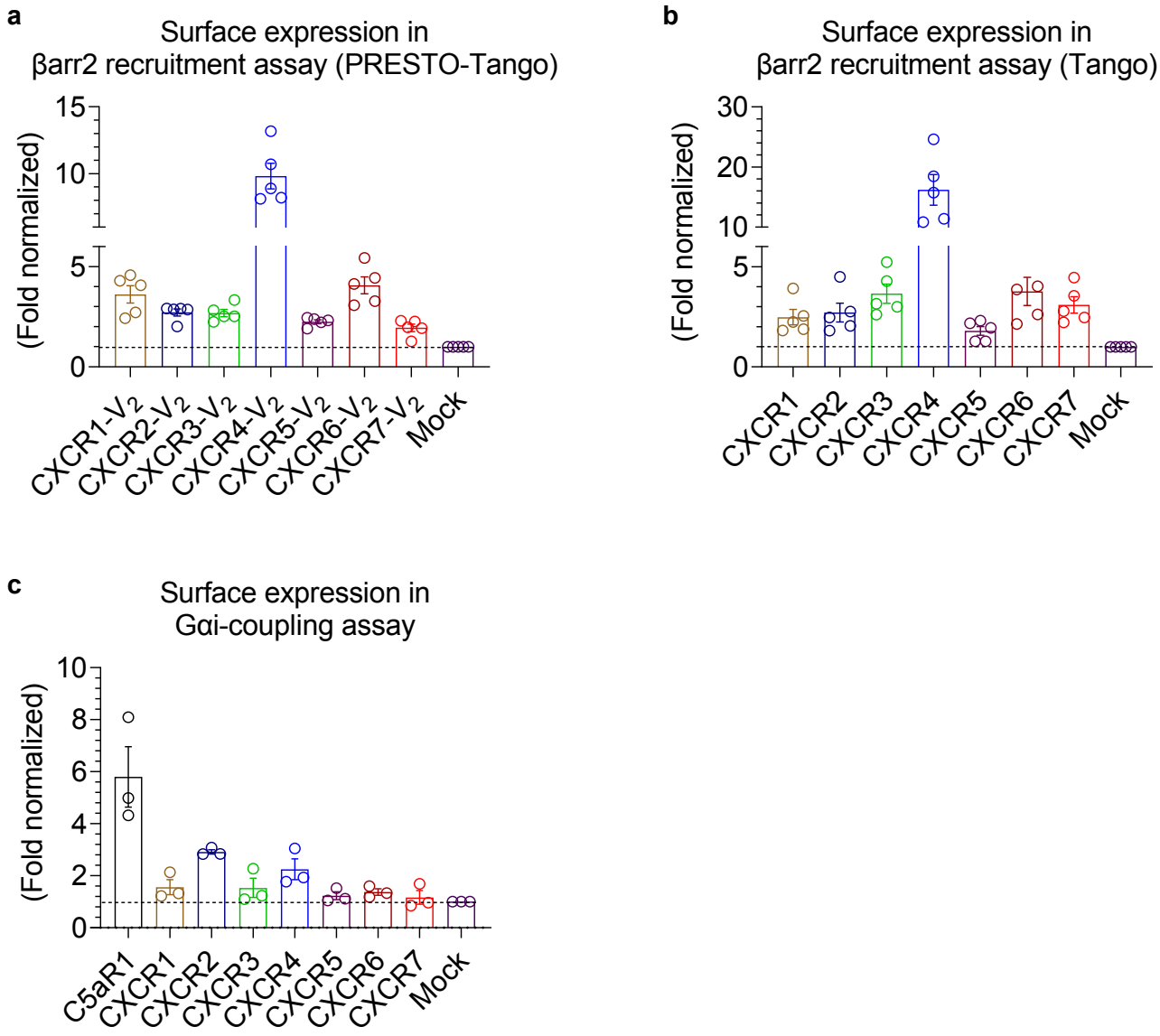

**Supplementary Fig. 1. Surface expression of CXCR4 and CXCR7 in G-protein assays. a** Surface expression of CXCR4 and CXCR7 in the G-protein dissociation assay measured using flow cytometry assay (mean±SEM; n=3 independent experiments; normalized with CXCR4 as 100%). **b-c** Surface expression of the indicated receptors in cAMP assay as measured using whole cell ELISA (mean±SEM; n=4 independent experiments for panel **b** and n=3 independent experiments for panel **c**; normalized as fold over mock (pcDNA)-transfection). **d-e** Surface expression (whole cell ELISA) and second messenger response (Gαi/s-coupling assay) under higher expression levels of CXCR4 and CXCR7 (mean±SEM; n=3 independent experiments; for panel **d** and n=5-6 independent experiments for panel **e**; for receptor surface expression: n=5 independent experiments, for Gαs-coupling assay; n=6) (Gαi/s-coupling assay in panel **d** and **e** are normalized with minimal ligand dose for CXCR4 and maximal signal for $V_2R$ treated as 100%, respectively; surface expression data are normalized as fold over mock (pcDNA)-transfection). Dotted line in the plots b, c, d, and e indicates 1 fold. Source data are provided as a source data file.



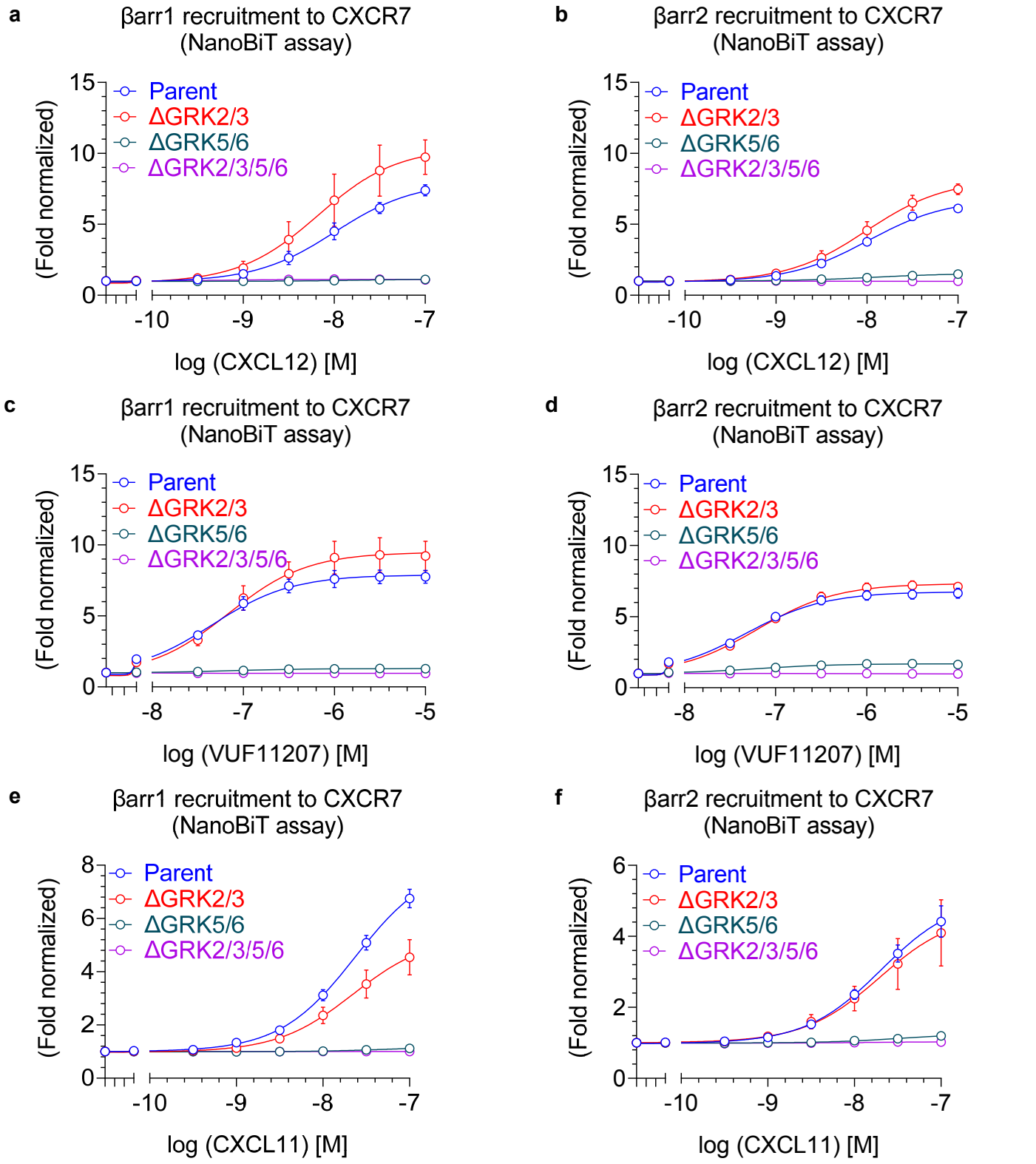

**Supplementary Fig. 2. Surface expression of CXCR4 and CXCR7 in βarr recruitment assays. a-c, e-f** Surface expression of CXCR4 and CXCR7 in βarr recruitment assays measured using whole cell ELISA (mean±SEM; n=3 independent experiments for panel a-b; n=7 independent experiments for panel e-f; n=3 independent experiments for panel c; normalized as fold over mock (pcDNA)-transfection). **d** CXCL12-induced βarr2 recruitment to CXCR4 and CXCR7 in PRESTO-Tango assay (mean±SEM; n=3 independent experiments; normalized with the luminescence signal at minimal ligand dose treated as 1). Dotted line in the plots a, b, c, e, and f indicates 1 fold. Source data are provided as a source data file.



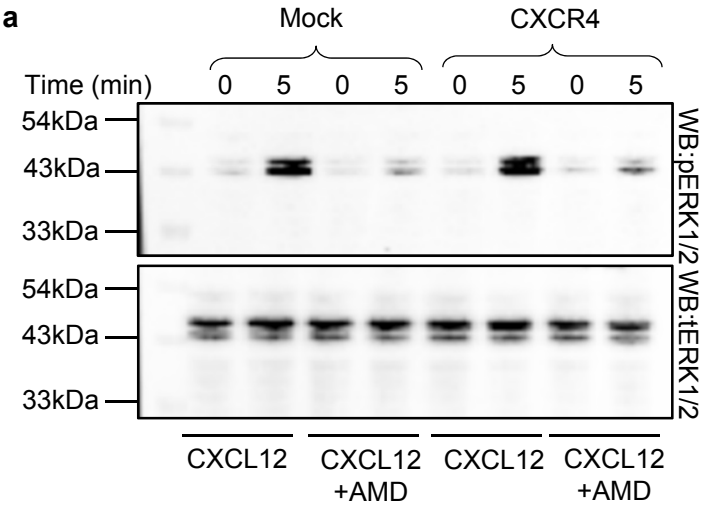

**Supplementary Fig. 3. Optimization of NanoBiT-based βarr recruitment assay for CXCR4. a-d** CXCL12-induced βarr1/2 recruitment to CXCR4 in NanoBiT assay with indicated combination of the constructs (mean±SEM; n=3 independent experiments; normalized with the luminescence signal at minimal ligand dose treated as 1). **e-f** Surface expression of CXCR4 constructs under indicated transfection conditions in the βarr recruitment assay as measured using whole cell ELISA (mean±SEM; n=3 independent experiments; normalized as fold over mock (pcDNA)-transfection). Dotted line in the plots e and f indicates 1 fold. Source data are provided as a source data file.

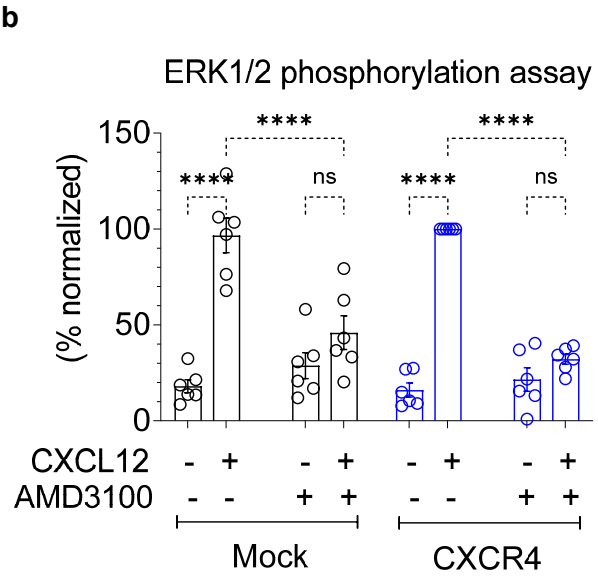


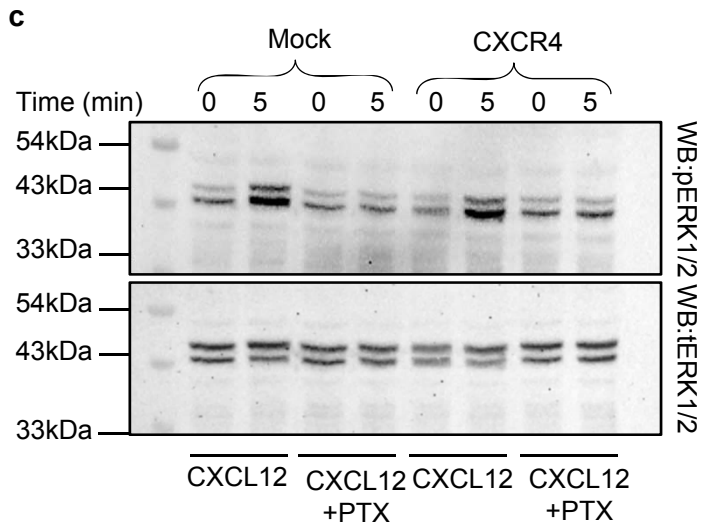

**Supplementary Fig. 4. Surface expression of CXCRs. a-b** Surface expression of indicated receptors in βarr recruitment assays measured using whole cell ELISA (mean±SEM; n=5 independent experiments for panel a-b; normalized as fold over mock(pcDNA)-transfection). **c** Surface expression of the indicated receptors in cAMP assay as measured using whole cell ELISA (mean±SEM; n=3 independent experiments; normalized as fold over mock (pcDNA)-transfection). Dotted line in plots a, b, and c indicates 1 fold. Source data are provided as a source data file.

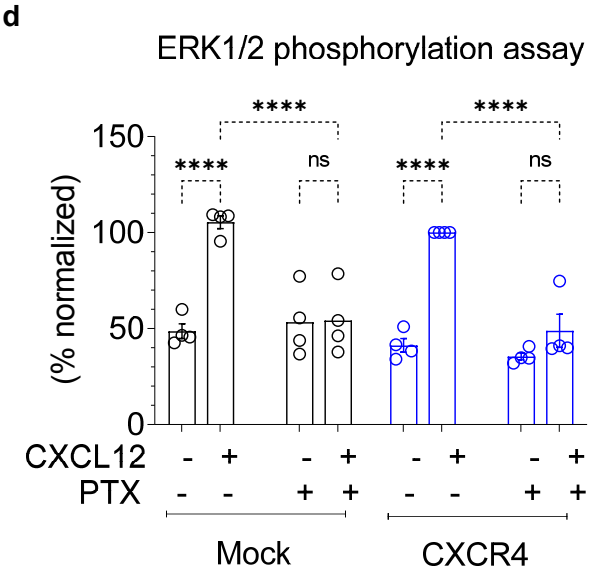


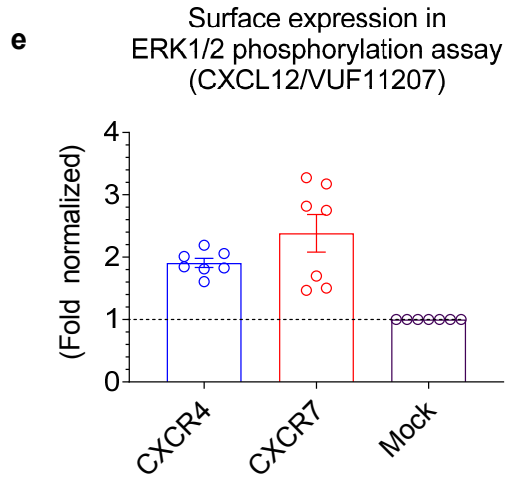

**Supplementary Fig. 5. Effect of PTX treatment on βarr recruitment. a-f** CXCL12-, VUF11207-, and CXCL11-induced βarr1 and 2 recruitment to CXCR7 in GRK knock-out cells using the NanoBiT assay with co-expression of the catalytic subunit of pertussis toxin (PTX) (Receptor-SmBiT+LgBiT-βarr1/2), respectively (mean±SEM; n=4-5 independent experiments; i.e., for CXCL12- and VUF11207-induced βarr1 recruitment: Parent, n=4; ΔGRK2/3, n=4; ΔGRK5/6, n=5; ΔGRK2/3/5/6, n=4; for CXCL12-induced βarr2 recruitment: Parent, n=5; ΔGRK2/3, n=4; ΔGRK5/6, n=4; ΔGRK2/3/5/6, n=4; for VUF11207-induced βarr2 recruitment: Parent, n=5; ΔGRK2/3, n=4; ΔGRK5/6, n=5; ΔGRK2/3/5/6, n=4; for CXCL11-induced βarr1 and βarr2 recruitment: Parent, ΔGRK2/3, ΔGRK5/6, ΔGRK2/3/5/6, n=4; normalized as fold response with respect to the unstimulated condition treated as 1). Source data are provided as a source data file.

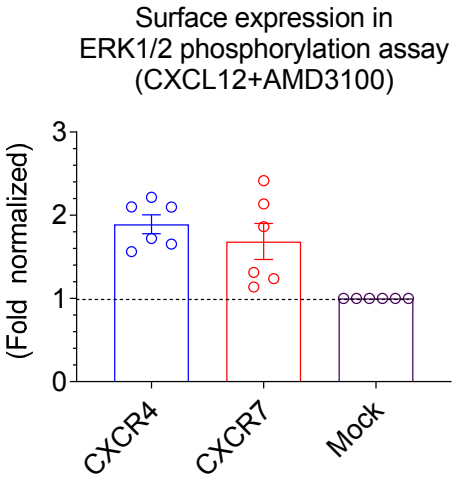

a
Mock
CXCR4
Time (min)
0 5 0 5 0 5 0 5
54kDa
43kDa
33kDa
WB:pERK1/2
54kDa
43kDa
33kDa
WB:tERK1/2
CXCL12
CXCL12 +AMD
CXCL12
CXCL12 +AMD


b
ERK1/2 phosphorylation assay
(% normalized)
150
100
50
0
****
****
****
****
****
ns
****
ns
CXCL12
AMD3100
Mock
CXCR4

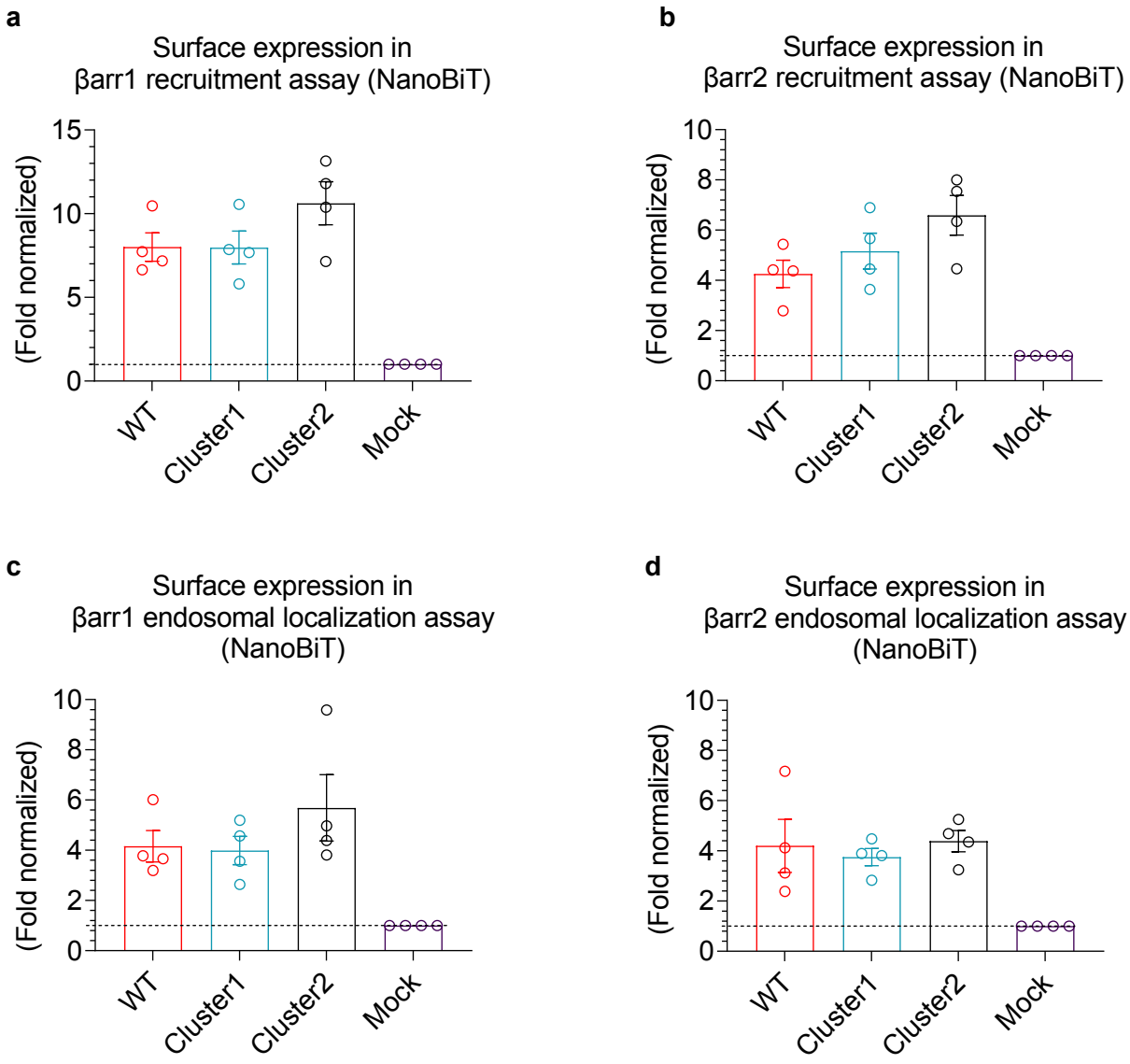

c
Mock
CXCR4
Time (min)
0 5 0 5 0 5 0 5
54kDa
43kDa
33kDa
WB:pERK1/2
54kDa
43kDa
33kDa
WB:tERK1/2
CXCL12
CXCL12 +PTX
CXCL12
CXCL12 +PTX


d
ERK1/2 phosphorylation assay
(% normalized)
150
100
50
0
****
****
****
****
****
ns
****
ns
CXCL12
PTX
Mock
CXCR4

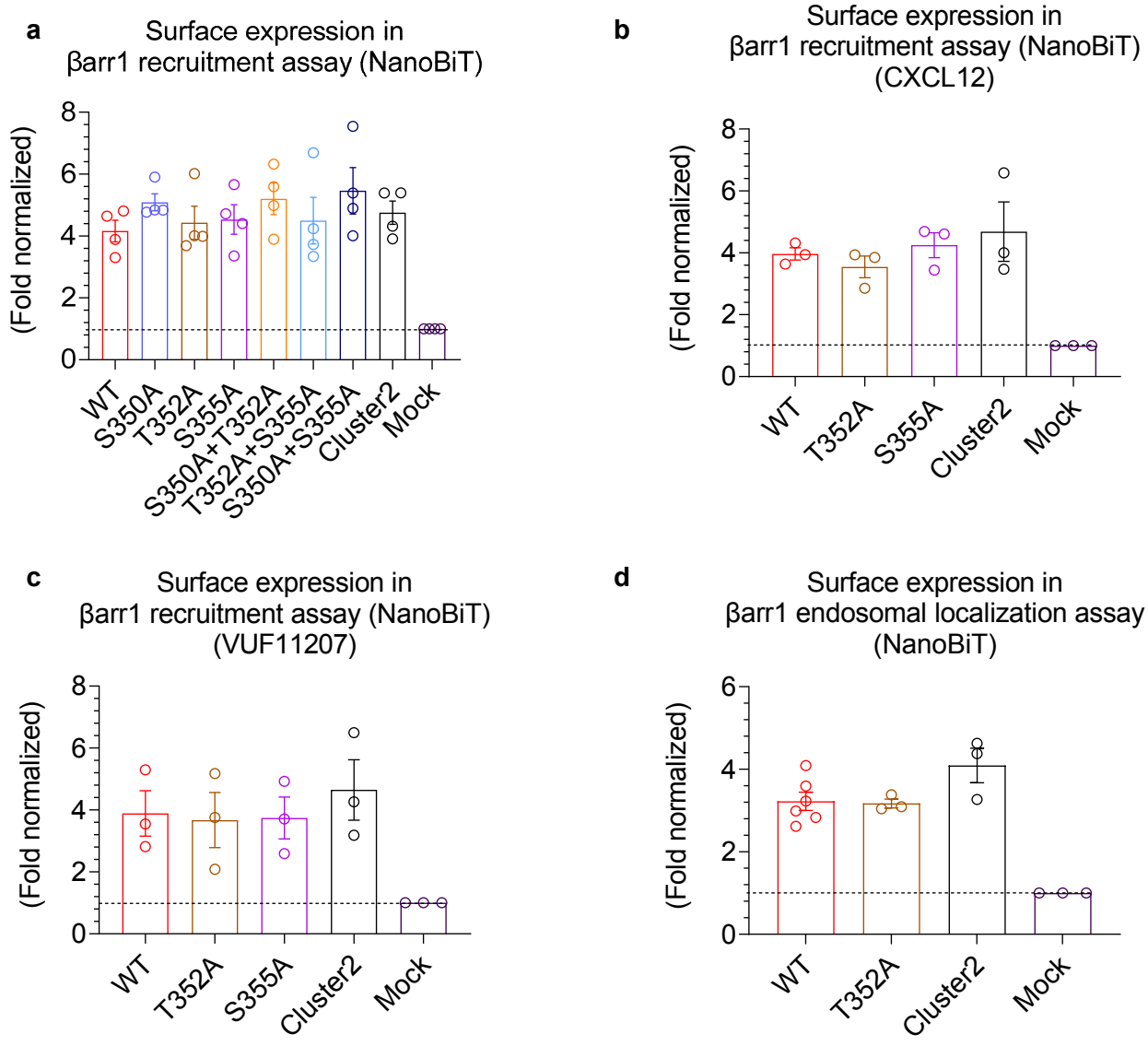

e
Surface expression in
ERK1/2 phosphorylation assay
(CXCL12/VUF11207)
(Fold normalized)
4
3
2
1
0
CXCR4
CXCR7
Mock


f
Surface expression in
ERK1/2 phosphorylation assay
(CXCL12+AMD3100)
(Fold normalized)
3
2
1
0
CXCR4
CXCR7
Mock

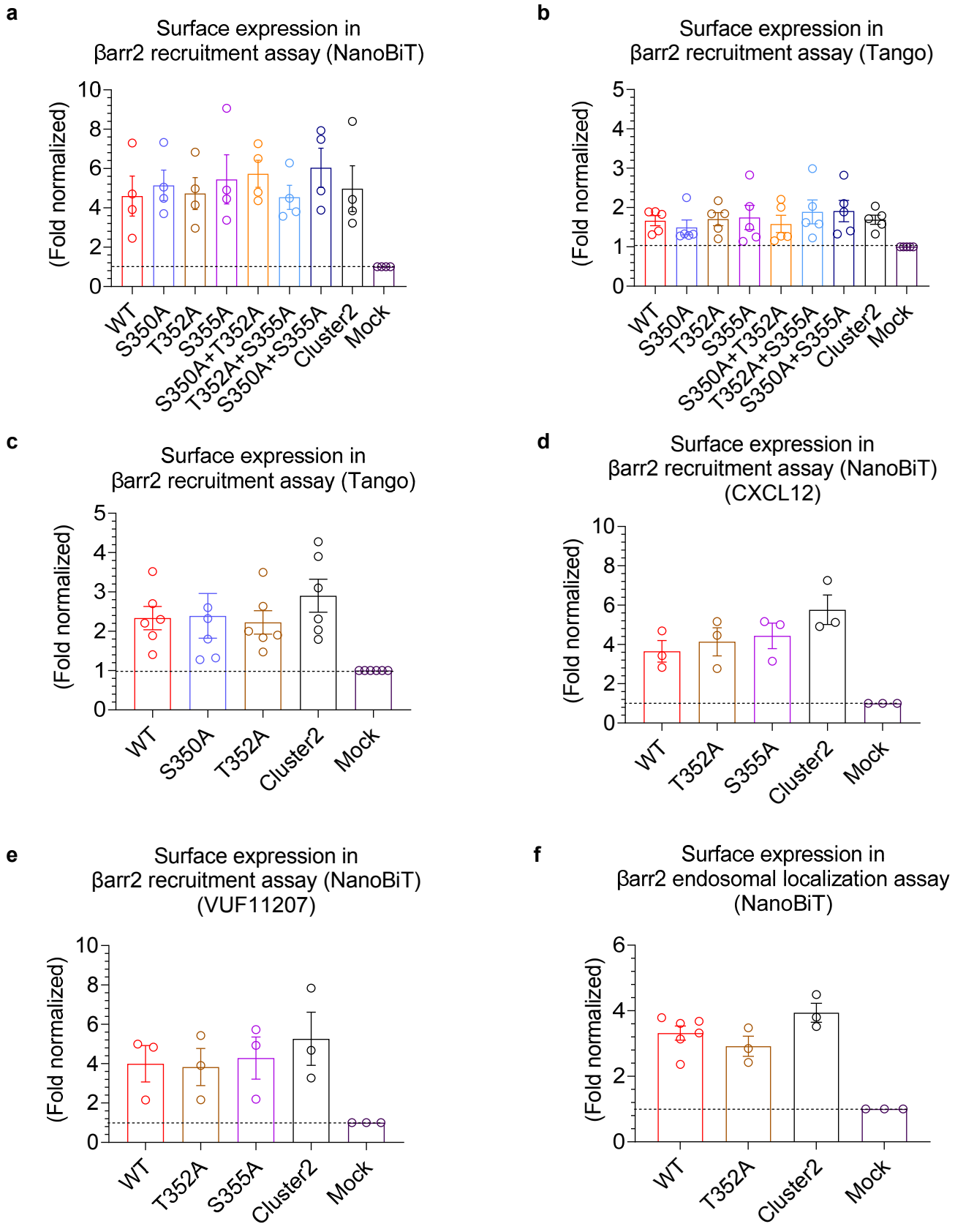
**Supplementary Fig. 6. Effect of AMD3100 and PTX treatment on ERK1/2 phosphorylation. a-d** CXC12-induced ERK1/2 phosphorylation in HEK-239 cells transfected with either pcDNA (mock) or CXCR4 plasmid with and without pre-treatment of AMD3100 and PTX as indicated. Panels b and d present densitometry-based quantification of data presented in panels a and c (mean±SEM; n=6 and n=4 independent experiments, respectively; normalized with 5 min stimulation of CXCR4 condition without AMD3100/PTX; Two-way ANOVA, Tukey's multiple comparison test. The exact *p* values for panel b are as follows: mock-AMD3100: 0 min vs. mock-AMD3100: 5 min ($p < 0.0001$), mock-AMD3100: 5 min vs. mock+AMD3100: 5 min ($p < 0.0001$), mock+AMD3100: 0 min vs. mock+AMD3100: 5 min ($p = 0.4441$), CXCR4-AMD3100: 0 min vs. CXCR4-AMD3100: 5 min ($p < 0.0001$), CXCR4-AMD3100: 5 min vs. CXCR4+AMD3100: 5 min ($p < 0.0001$), CXCR4+AMD3100: 0 min vs. CXCR4+AMD3100: 5 min ($p = 0.8975$). The exact *p* values for panel d are as follows: mock-PTX: 0 min vs. mock-PTX: 5 min ($p < 0.0001$), mock-PTX: 5 min vs. mock+PTX: 5 min ($p < 0.0001$), mock+PTX: 0 min vs. mock+PTX: 5 min ($p > 0.9999$), CXCR4-PTX: 0 min vs. CXCR4-PTX: 5 min ($p < 0.0001$), CXCR4-PTX: 5 min vs. CXCR4+PTX: 5 min ($p < 0.0001$), CXCR4+PTX: 0 min vs. CXCR4+PTX: 5 min ($p = 0.7397$) (****$p < 0.0001$, ns= non-significant). **e-f** Surface expression of CXCR4 and CXCR7 in ERK1/2 phosphorylation assay was measured using whole cell ELISA (mean±SEM; n=7 independent experiments for panel e and n=6 independent experiments for panel f, normalized as fold-over mock (pcDNA)-transfection. Dotted line in the plots e and f indicates 1 fold. Source data are provided as a source data file.




**Supplementary Fig. 7. Surface expression of CXCR7 mutants in different assays. a-e** Surface expression of indicated receptors was measured in different assays using whole cell ELISA (mean±SEM; n=4 independent experiments; normalized as fold over mock (pcDNA)-transfection). Dotted line in the plots a, b, c, and d indicates 1 fold. Source data are provided as a source data file.




**Supplementary Fig. 8. Surface expression of CXCR7 mutants in different assays. a-d** Surface expression of indicated receptors were measured in different assays using whole cell ELISA (mean±SEM; n=4 independent experiments for panel a, n=3 independent experiments for panel b and c, n=3-6 independent experiments for panel d; for βarr1 endosomal localization: WT, n=6; T352A, n=3; and Cluster2, n=3 normalized as fold over mock (pcDNA)-transfection). Dotted line in the plots a, b, c, and d indicates 1 fold. Source data are provided as a source data file.




**Supplementary Fig. 9. Surface expression of CXCR7 mutants in different assays. a-f** Surface expression of indicated receptors were measured in different assays using whole cell ELISA (mean±SEM; n=4 independent experiments for panel a, n=5 for panel b, n=6 for panel c, n=3 for panel d and e, n=3-6 for panel f; βarr2 endosomal localization: WT, n=6; T352A, n=3; and Cluster2, n=3; normalized as fold over mock (pcDNA)-transfection). Dotted line in the plots a,b,c,d, and e indicates 1 fold. Source data are provided as a source data file.

| Supplementary Table 1. List of the primers used in the study. | | |
|---|---|---|
| | | |
| **Construct** | **Primer** | **Sequence** |
| CXCR1 Tango | CXCR1_Fw | CGCGGATCC ATGTCCAATATCACCG |
| | CXCR1_Rv | CCGGAATTC CAAATTTGAAGAGACATTAAC |
| CXCR2 Tango | CXCR2_Fw | CGCGGATCCATGGAGGACTTCAACATGGAG |
| | CXCR2_Rv | CCGGAATTCCAGGGTCGTGGAGGTG |
| CXCR3 Tango | CXCR3_Fw | CGCGGATCCATGGTTCTGGAAGTTTCC |
| | CXCR3_Rv | CCGGAATTCGAGGCCAGAATAACTAGC |
| CXCR4 Tango | CXCR4_Fw | CGCGGATCCATGGAGGGGATATCAATC |
| | CXCR4_Rv | CCGGAATTCAGAGCTATGAAATGAGCTG |
| CXCR5 Tango | CXCR5_Fw | CGCGGATCCATGAACTATCCCCTGACC |
| | CXCR5_Rv | CCGGAATTCAAAGGTGGTCAGGCTAG |
| CXCR6 Tango | CXCR6_Fw | CGCGGATCCATGGCCGAACACGAC |
| | CXCR6_Rv | CCGGAATTCCAGCTGGAACATGGAGG |
| CXCR7(S350A) | CXCR7_S350A_Fw | CTCCAGAGTCGCCGAGACGGAGTAC |
| | CXCR7_S350A_Rv | GCATCGATGAGCTTGGTG |
| CXCR7(T352A) | CXCR7_T352A_Fw | AGTCTCAGAGGCCGAGTACTCTGCC |
| | CXCR7_T352A_Rv | CTGGAGGCATCGATGAGC |
| CXCR7(S355A) | CXCR7_T355A_Fw | GACGGAGTACGCCGCCTTGGAGC |
| | CXCR7_T355A_Rv | TCTGAGACTCTGGAGGCAT |
| CXCR7(S350A+T352A) | CXCR7_S350A+T352A_Fw | AGTCGCCGAGGCCGAGTACTCTG |
| | CXCR7_S350A+T352A_Rv | CTGGAGGCATCGATGAGC |
| CXCR7(T352A+S355A) | CXCR7_T352A+S355A_Fw | GGCCGAGTACGCCGCCTTGGAGC |
| | CXCR7_T352A+S355A_Rv | TCTGAGACTCTGGAGGCATC |
| CXCR7(S350A+S355A) | CXCR7_S350A+S355A_Fw | GACGGAGTACGCCGCCTTGGAGCAG |
| | CXCR7_S350A+S355A_Rv | TCGGCGACTCTGGAGGCA |
| CXCR7_Cluster1 | CXCR7_Cluster1_Fw | AGGGCTCGCCAAGCTCATCGATGCCTCC |
| | CXCR7_Cluster1_Rv | GCTTTGGCCGCGTACTTGAAGATGAAGGCC |
| CXCR7_Cluster2 | CXCR7_Cluster2_Fw | GAGTACGCTGCCTTGGAGCAGAGCACC |
| | CXCR7_Cluster2_Rv | CGCCTCTGCGACTCTGGAGGCATCGATG |
| CXCR7_cSmBiT | CXCR7_cSmBiT_Fw | CGGGGTACCGAGGAGATCTGCCACCATGGGC |
| | CXCR7_cSmBiT_Rv | TCCCCCGGGTTTGGTGCTCTGCTCCAAG |
| CXCR4_cSmBiT | CXCR4_cSmBiT_Fw | CGGGGTACCGAGGAGATCTGCCACCATGGGC |
| | CXCR4_cSmBiT_Rv | TCCCCCGGGAGAGCTATGAAATGAGCTGG |
| CXCR4_LgBiT | CXCR4_LgBiT_Fw | CGGGGTACCGAGGAGATCTGCCACCATGGGC |
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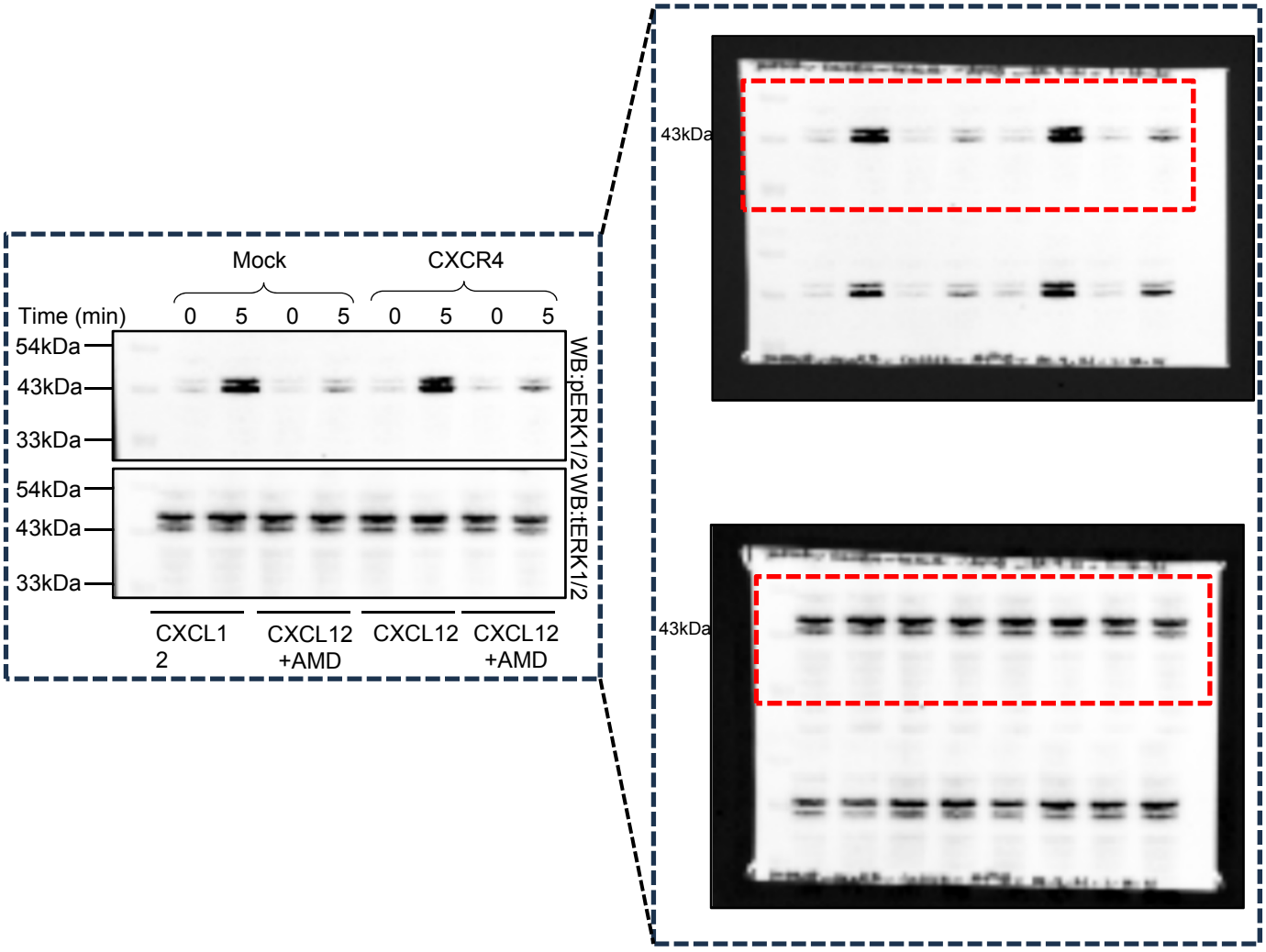
**Source Data file :Supplementary Fig. 6a**



**Source Data file :Supplementary Fig. 6c**