Supplementary Data

Time-course studies (over 45 min) were carried out in CHO-RXFP4 cells activated by either hINSL5 or mINSL5. Both INSL5 orthologues produced robust but transient ERK1/2 activation with a peak response at 5 min (hINSL5: 22.04 ± 1.73 fold/basal; mINSL5: 25.4 ± 2.56 fold/basal; n=5; Fig S1A). Akt was also activated by INSL5 peptides in a transient manner similar to ERK1/2. Peak responses were achieved at 5 min for both phosphorylation sites - Akt p-Ser473 (hINSL5: 1.54 ± 0.07 fold/basal; mINSL5: 1.56 ± 0.10 fold/basal; n=5; Fig S1D) and Akt p-Thr308 (hINSL5: 1.77 ± 0.13 fold/basal; mINSL5: 1.90 ± 0.18 fold/basal; n=5; Fig S1E). In addition, a p38MAPK response was observed after INSL5 treatment with a peak at 15 min (hINSL5: 1.46 ± 0.13 fold/basal; mINSL5: 1.44 ± 0.15 fold/basal; n=5; Fig S1B). We also observed phosphorylation of S6RP, a downstream effector of the Akt-mammalian target of rapamycin complex 1 (mTORC1) pathway (reviewed in Fingar and Blenis, 2004) and the ERK pathway (Roux et al., 2007), with the response plateauing 30 min after peptide addition (hINSL5: 5.54 ± 1.16 fold/basal; mINSL5: 5.82 ± 1.63 fold/basal; n=3; Fig S1C). None of the signalling pathways tested were activated by INSL5 in non-transfected CHO-K1 cells (n=3; Fig S2), confirming that all signalling events in response to INSL5 require the presence of RXFP4. _INSL5 increases Ca^{2+} mobilisation in HEK-293 cells transiently transfected with RXFP4 and $G\alpha_{16}$ (Luo et al., 2014), a promiscuous G protein that couples to many G protein-coupled receptors (GPCRs) that do not normally signal through Ca^{2+} (Offermanns and Simon, 1995). In CHO-RXFP4 cells without $G\alpha_{16}$, mINSL5 (100 nM) failed to increase Ca^{2+} mobilisation (n=3; Fig. 1E), indicating that RXFP4 does not normally couple to Ca^{2+} signalling.

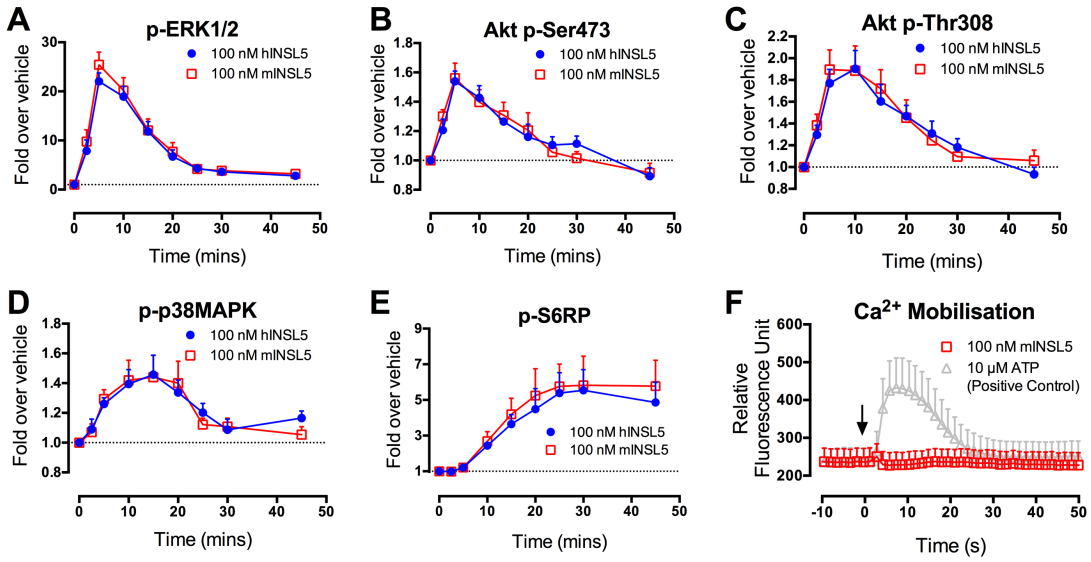

Figure S1. Time course of activation of signalling pathways by hINSL5 and mINSL5 in CHO-RXFP4 cells. In (A) phosphorylation of ERK1/2, (B) Akt Ser473, (C) Akt Thr308, (D) p38MAPK, and (E) S6RP. CHO-RXFP4 cells were treated with hINSL5 or mINSL5 (100 nM each) for up to 45 minutes, and activation of ERK1/2 (n = 5), Akt Ser473 (n = 5), Akt Thr308 (n = 5), p38MAPK (n = 5) and S6RP (n = 3) determined using phospho-specific AlphaScreen SureFire kit. In (F) there was no Ca^{2+} mobilisation detectable in response to stimulation by mINSL5 (100 nM) even though the same cells produced a substantial response to addition of ATP (10 µM; n = 3). Results are quantified as fold change in fluorescence over that of the vehicle treatment (fold over vehicle) for phosphorylation assays, or expressed as raw fluorescence counts for calcium mobilisation. All data points represent mean ± S.E.M. of independent experiments.

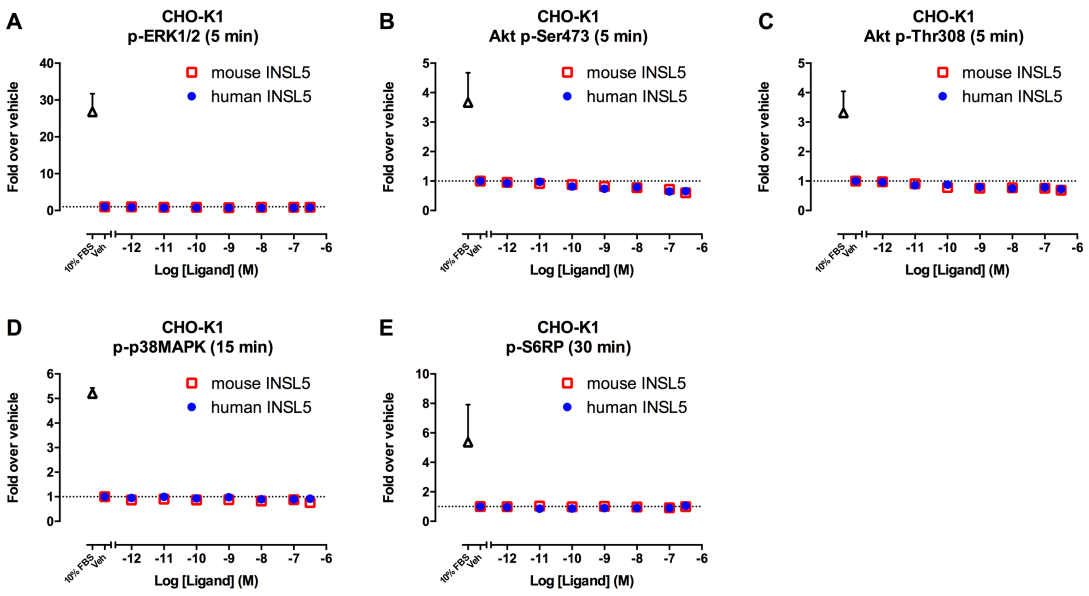

Figure S2. INSL5 does not promote signalling in CHO-K1 cells lacking RXFP4. Non-transfected CHO-K1 cells were treated with increasing concentrations of hINSL5 or mINSL5 (10^{-12} M to $10^{-6.5}$ M). Results are quantified as fold change in fluorescence over that of the vehicle treatment (fold over vehicle). Note that none of the signalling pathways tested were activated by INSL5, even though the same cells produced robust responses to 10% FBS treatment.