Supplementary Discussion.

Putative cholesterol observed in both C- and NC-state complex density maps

In the EM density maps of both C- and NC-state hNTSR1-G_{i1} complexes, a significant density is observed near the outer rims of TMs 6 and 7. The shape of the density would correspond well to cholesterol, and the residues surrounding this pocket are also suitable for cholesterol binding (Extended Data Fig. 5c). Because TMs 6 and 7 change their conformations upon activation, we would expect that this putative cholesterol molecule can only bind to NTSR1 in the active state. These observations are consistent with a previous study suggesting that cholesterol binds to NTSR1 and enhances its stability[77]. However, cholesterol was not added during the protein purification and there was no aromatic residue interacting with the sterol group, which is presumably important for cholesterol binding[78]. It is possible that cholesterol extracted from insect cell membrane was co-purified with hNTSR1. Alternatively, the density may correspond to CHS added to all purification buffers. However, given these uncertainties, we did not include this putative cholesterol or CHS molecule in the final model to avoid potentially misleading the readers.

Amino acid sequences of the NanoBiT-G protein constructs.
Coloring denotes LgBiT (red), SmBiT (blue) and 15-amino acid flexible linker (purple).

Gαs-Lg (GNAS-LgBiT)

MGCLGNSKTEDQRNEEKAQREANKKIEKQLQKDKQVYRATHRLLLLGAGESGKSTIVKQMRILHVNGFNG
EGGEEDPQAARSNSDGEKATKVQDIKNNLKEAIETIVAAMSNLGGSGGGGSGGSSSGGVFTLEDFVGDWE
QTAAYNLDQVLEQGGVSSLLQNLAVSVTPIQRIVRSGENALKIDIHVIIPYEGLSADQMAQIEEVFKVVY
PVDDHHFKVILPYGTLVIDGVTPNMLNYFGRPYEGIAVFDGKKITVTGTLWNGNKIIDERLITPDGSMLF
RVTINSGGSGGGGSGGSSSGGVPPVELANPENQFRVDYILSVMNVPDFDFPPEFYEHAKALWEDEGVRAC
YERSNEYQLIDCAQYFLDKIDVIKQADYVPSDQDLLRCRVLTSGIFETKFQVDKVNFHMFDVGGQRDERR
KWIQCFNDVTAIIFVVASSSYNMVIREDNQTNRLQEALNLFKSIWNNRWLRTISVILFLNKQDLLAEKVL
AGKSKIEDYFPEFARYTTPEDATPEPGEDPRVTRAKYFIRDEFLRISTASGDGRHYCYPHFTCAVDTENI
RRVFNDCRDIIQRMHLRQYELL

Gαi1-Lg (GNAI1-LgBiT)

MGCTLSAEDKAAVERSKMIDRNLREDGEKAAREVKLLLLGAGESGKSTIVKQMKIIHEAGYSEEECKQYK
AVVYSNTIQSIIAIIRAMGRLGGSGGGGSGGSSSGGVFTLEDFVGDWEQTAAYNLDQVLEQGGVSSLLQN
LAVSVTPIQRIVRSGENALKIDIHVIIPYEGLSADQMAQIEEVFKVVYPVDDHHFKVILPYGTLVIDGVT
PNMLNYFGRPYEGIAVFDGKKITVTGTLWNGNKIIDERLITPDGSMLFRVTINSGGSGGGGSGGSSSGGK
IDFGDSARADDARQLFVLAGAAEEGFMTAELAGVIKRLWKDSGVQACFNRSREYQLNDSAAYYLNDLDRI
AQPNYIPTQQDVLRTRVKTTGIVETHFTFKDLHFKMFDVGGQRSERKKWIHCFEGVTAIIFCVALSDYDL
VLAEDEEMNRMHESMKLFDSICNNKWFTDTSIILFLNKKDLFEEKIKKSPLTICYPEYAGSNTYEEAAAY
IQCQFEDLNKRKDTKEIYTHFTCATDTKNVQFVFDAVTDVIIKNNLKDCGLF

Gαo-Lg (GNAO1-LgBiT)

MGCTLSAEERAALERSKAIEKNLKEDGISAAKDVKLLLLGAGESGKSTIVKQMKIIHEDGFSGEDVKQYK
PVVYSNTIQSLAAIVRAMDTLGGSGGGGSGGSSSGGVFTLEDFVGDWEQTAAYNLDQVLEQGGVSSLLQN
LAVSVTPIQRIVRSGENALKIDIHVIIPYEGLSADQMAQIEEVFKVVYPVDDHHFKVILPYGTLVIDGVT
PNMLNYFGRPYEGIAVFDGKKITVTGTLWNGNKIIDERLITPDGSMLFRVTINSGGSGGGGSGGSSSGGG
IEYGDKERKADAKMVCDVVSRMEDTEPFSAELLSAMMRLWGDSGIQECFNRSREYQLNDSAKYYLDSLDR
IGAADYQPTEQDILRTRVKTTGIVETHFTFKNLHFRLFDVGGQRSERKKWIHCFEDVTAIIFCVALSGYD

QVLHEDETTNRMHESLMLFDSICNNKFFIDTSIILFLNKKDLFGEKIKKSPLTICFPEYTGPNTYEDAAA

YIQAQFESKNRSPNKEIYCHMTCATDTNNIQVVFDAVTDIIIANNLRGCGLY

Gαq-Lg (GNAQ-LgBiT)

MTLESIMACCLSEEAKEARRINDEIERQLRRDKRDARRELKLLLLGTGESGKSTFIKQMRIIHGSGYSDE

DKRGFTKLVYQNIFTAMQAMIRAMDTLGGSGGGGSGGGSSSGGVFTLEDFVGDWEQTAAYNLDQVLEQGGV

SSLLQNLAVSVTPIQRIVRSGENALKIDIHVIIPYEGLSADQMAQIEEVFKVVYPVDDHHFKVILPYGTL

VIDGVTPNMLNYFGRPYEGIAVFDGKKITVTGTLWNGNKIIDERLITPDGSMLFRVTINSGGSGGGGSGG

SSSGGKIPYKYEHNKAHAQLVREVDVEKVSAFENPYVDAIKSLWNDPGIQECYDRRREYQLSDSTKYYLN

DLDRVADPAYLPTQQDVLRVRVPTTGIIEYPFDLQSVIFRMVDVGGQRSERRKWIHCFENVTSIMFLVAL

SEYDQVLVESDNENRMEESKALFRTIITYPWFQNSSVILFLNKKDLLEEKIMYSHLVDYFPEYDGPQRDA

QAAREFILKMFVDLNPDSDKIIYSHFTCATDTENIRFVFAAVKDTILQLNLKEYNLV

Gα13-Lg (GNA13-LgBiT)

MADFLPSRSVLSVCFPGCLLTSGEAEQQRKSKEIDKCLSREKTYVKRLVKILLLGAGESGKSTFLKQMRI

IHGQDFDQRAREEFRPTIYSNVIKGMRVLVDAREKLGGSGGGGSGGGSSSGGVFTLEDFVGDWEQTAAYNL

DQVLEQGGVSSLLQNLAVSVTPIQRIVRSGENALKIDIHVIIPYEGLSADQMAQIEEVFKVVYPVDDHHF

KVILPYGTLVIDGVTPNMLNYFGRPYEGIAVFDGKKITVTGTLWNGNKIIDERLITPDGSMLFRVTINSG

GSGGGGSGGSSSGGHIPWGDNSNQQHGDKMMSFDTRAPMAAQGMVETRVFLQYLPAIRALWADSGIQNAY

DRRREFQLGESVKYFLDNLDKLGEPDYIPSQQDILLARRPTKGIHEYDFEIKNVPFKMVDVGGQRSERKR

WFECFDSVTSILFLVSSSEFDQVLMEDRLTNRLTESLNIFETIVNNRVFSNVSIILFLNKTDLLEEKVQI

VSIKDYFLEFEGDPHCLRDVQKFLVECFRNKRRDQQQKPLYHHFTTAINTENIRLVFRDVKDTILHDNLK

QLMLQ

Sm-Gγ2 (C68S) (SmBiT-GNG2 C68S)

The C68S mutation is highlighted in green.

MVTGYRLFEEILGGSGGGGSGGSSSGGASNNTASIAQARKLVEQLKMEANIDRIKVSKAAADLMAYCEAH

AKEDPLLTPVPASENPFREKKFFSAIL