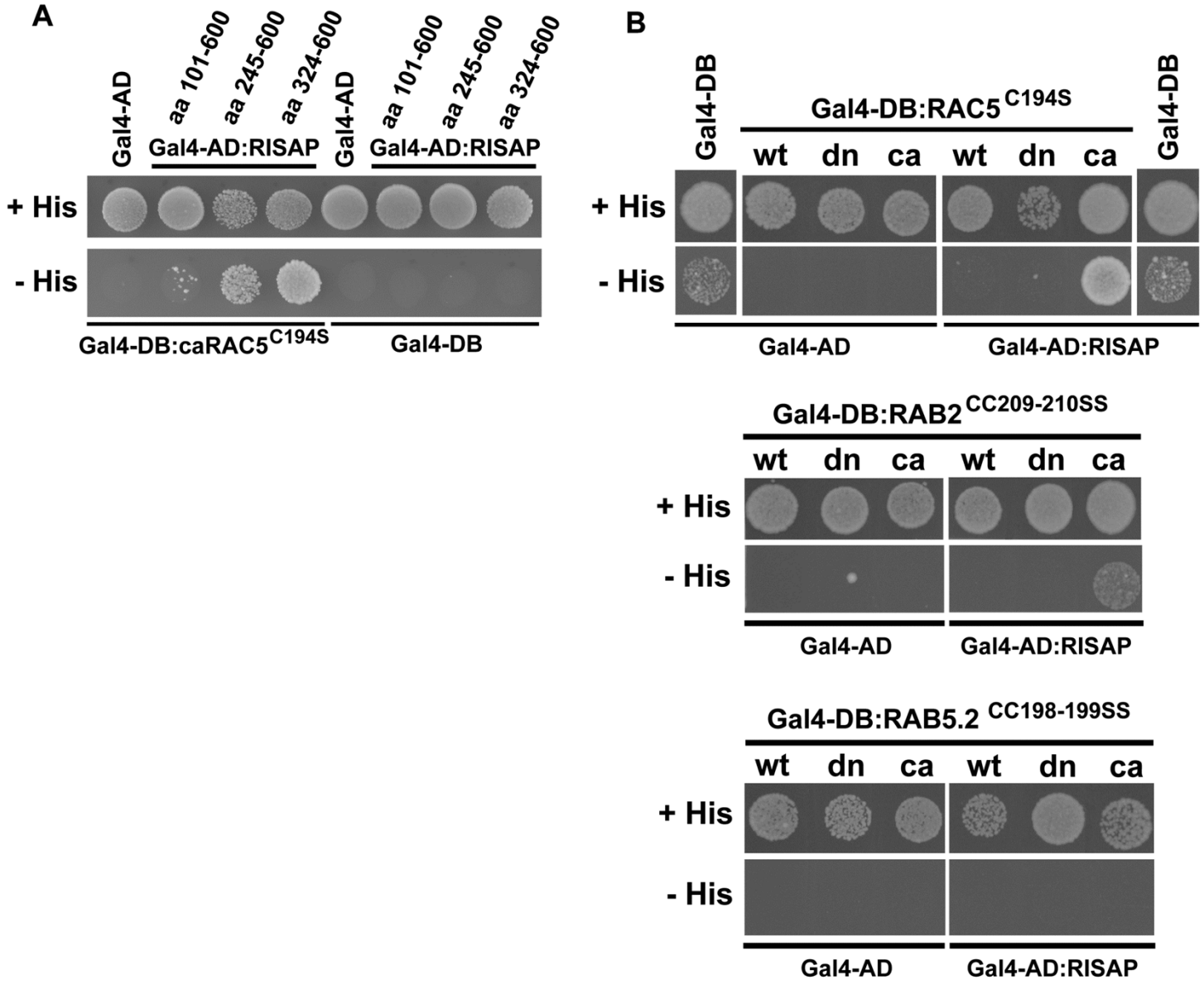
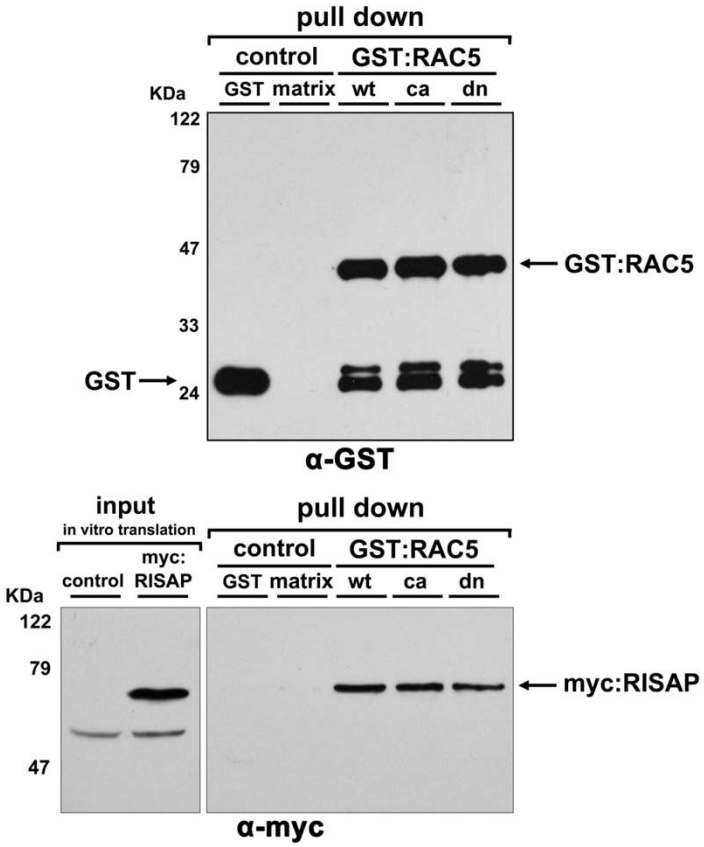


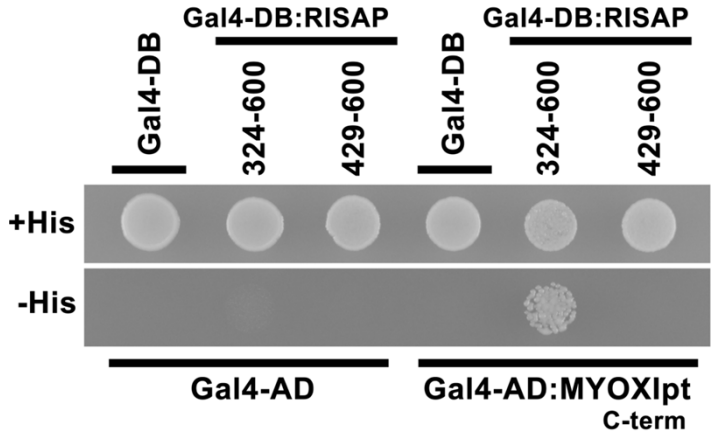
Supplemental Figure 1. Analysis of Yeast Two-Hybrid Interactions between N-Terminally Truncated RISAP Fragments and Constitutively Active RAC5, as well as between Full-Length RISAP and RAC5 Related Tobacco RAB Proteins.

(**A**) Yeast transformants co-expressing constitutively active (ca; G15V) RAC5 fused to the DNA-binding domain of the GAL4 transcription factor (GAL4-DB) together with N-terminally truncated RISAP fragments fused to the GAL4 activation domain (GAL4-AD) plated on histidine-containing (+) and on histidine-free (-) culture medium. The RAC5 bait protein carried a point mutation that enhances nuclear import by preventing posttranslational prenylation (C194S). Serving as negative controls were transformants co-expressing the RAC5 bait protein with just the GAL4-AD, or truncated RISAP prey proteins with just the GAL4-BD. Growth on histidine-free medium indicated two-hybrid interaction of all RISAP fragments with constitutively active RAC5. No two-hybrid interactions could be detected between these RISAP fragments and wild type or dominant negative (T20N) RAC5 in experiments performed under the same conditions (not shown).

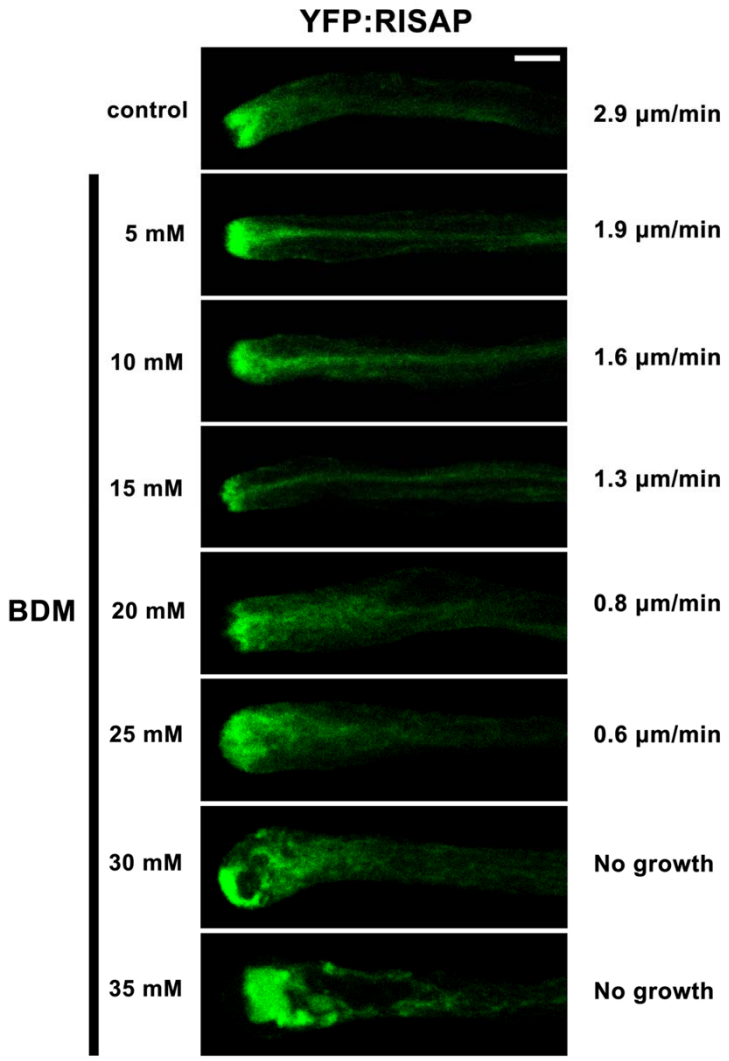
(**B**) Yeast transformants co-expressing wild type (wt), constitutively active (ca; $RAC5^{G15V}$, $RAB2^{Q65L}$, $RAB5.2^{Q69L}$) or dominant negative (dn; $RAC5^{T20N}$, $RAB2^{S20N}$, Nt-$RA5.2^{S24N}$) RAC5 (top panel), RAB2 (central panel) or RAB5.2 (bottom panel) fused to the DNA-binding domain of the GAL4 transcription factor (GAL4-DB) together with RISAP fused to the GAL4 activation domain (GAL4-AD) plated on histidine-containing (+) and on histidine-free (-) culture medium. All RAC5, RAB2 and RAB5.2 bait proteins carried point mutations that enhance nuclear import by preventing posttranslational prenylation ($RAC5^{C194S}$, $RAB2^{CC209-210SS}$, $RAB5.2^{CC198-199SS}$). Serving as negative controls were transformants co-expressing RAC5, RAB2 or RAB5.2 bait proteins with just the GAL4-AD, or the RISAP prey protein with just the GAL4-BD. Growth on histidine-free medium indicated specific two-hybrid interaction between RISAP and constitutively active RAC5 (top panel). Apart from this, only a much weaker, barely detectable interaction between constitutively active RAB2 and RISAP was observed (central panel).



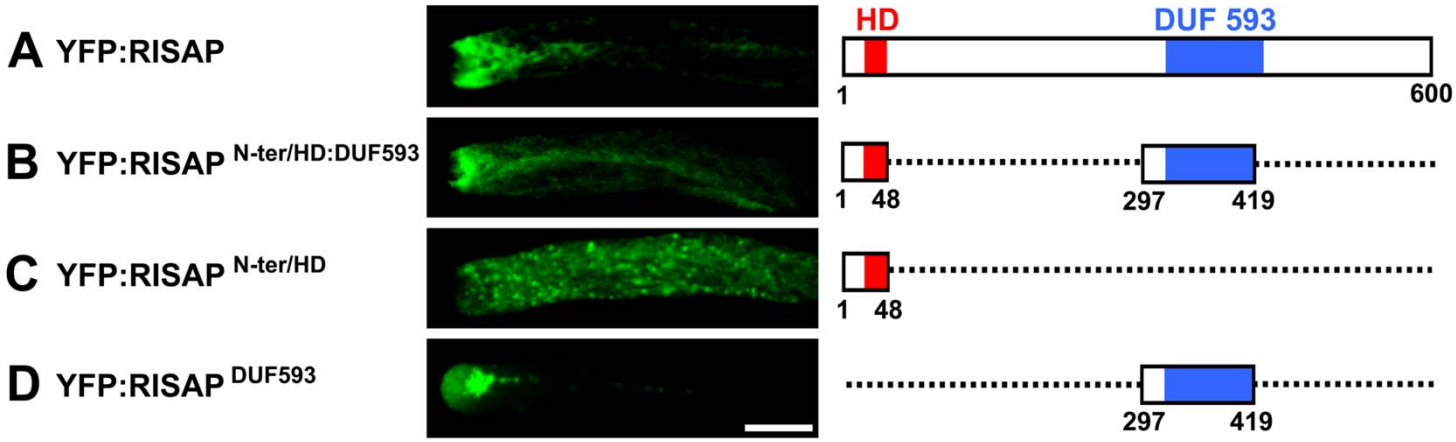
Supplemental Figure 2. Constitutive Interaction of RISAP with Wild Type, Constitutively Active and Dominant Negative RAC5 in Pull-Down Assays. *In vitro* transcribed/translated myc-tagged RISAP (lower panel, 2nd lane) was incubated with GST-tagged wild type (wt), constitutively active (ca; G15V) or dominant negative (dn; T20N) RAC5, which had been purified from *Escherichia coli* and was immobilized on magnetic beads. Beads carrying immobilized GST, as well as empty beads (matrix), were used as controls. Proteins associated with purified and washed beads were separated by SDS-PAGE and analyzed by immunoblotting using a-GST (upper panel) or a-myc (lower panel) antibodies.



Supplemental Figure 3. The DUF593 of RISAP Mediates Interaction of this Protein with the Tobacco Pollen Tube Myosin XI MYOXIpt in Yeast Two-Hybrid Assays. Yeast transformants co-expressing N-terminally truncated RISAP, either with (324-600) or without (429-600) the DUF593, fused to the DNA-binding domain of the GAL4 transcription factor (GAL4-DB) together with a 608 aa C-terminal fragment of MYOXIpt fused to the GAL4 activation domain (GAL4-AD) plated on histidine-containing (+) and on histidine-free (-) culture medium. Serving as negative controls were transformants co-expressing RISAP bait proteins, or the free GAL-DB, with just the GAL4-AD, or the MYOXIpt prey protein with just the GAL4-BD. Growth on histidine-free medium indicated specific two-hybrid interaction between the DUF593 containing RISAP fragment (324-600) and the MYOXIpt C-terminus.



Supplemental Figure 4. Treatment with the Myosin Inhibitor BDM Blocked Pollen Tube Growth and Trapped the RISAP-Associated TGN Compartment at the Tip. Single confocal optical sections through stably transformed, YFP:RISAP expressing tobacco pollen tubes ($SR1^{YFP:RISAP}$) are shown. BDM, a drug that immobilizes myosin on F-actin, inhibited pollen tube growth and altered the YFP:RISAP distribution pattern in a dose-dependent manner. YFP:RISAP labeling invaded the apex of pollen tubes showing reduced or no growth, presumably because the CZ in these cells had shrunk or disappeared. At highest BDM concentrations, pollen tube growth was completely blocked and the formation of swollen tips containing aberrant, YFP:RISAP labeled structures was induced. In all BDM treated pollen tubes, YFP:RISAP was trapped at the tip. Drug concentrations used and growth rates of individual analyzed pollen tubes are indicated next to each image. All pollen tubes treated with BDM at a specific concentration displayed essentially the same YFP:RISAP distribution pattern and similar growth rates (N=15). Scale bar: 10 µm.



Supplemental Figure 5. The RISAP N-Terminus with the Hydrophobic Domain (HD), together with the DUF593, are Necessary and Sufficient for Targeting to the Subapical TGN. Single confocal optical sections through normally elongating pollen tubes, which transiently express YFP fused to truncated (B-D) or full length RISAP (A, YFP:RISAP) 6 h after gene transfer. The different truncated forms of RISAP analyzed were composed of the N-terminus with the hydrophobic domain (amino acids 1-48) and of the DUF593 (amino acids 297-419) (B, YFP:RISAP$^{N\text{-}ter/HD:DUF593}$), of just the N-terminus with the hydrophobic domain (C, YFP:RISAP$^{N\text{-}ter/HD}$), or of just the DUF593 (D, YFP:RISAP^{DUF593}). YFP:RISAP$^{N\text{-}ter/HD:DUF593}$ (B; 87% of 16 analyzed pollen tubes) displayed essentially the same subapical localization as full length YFP:RISAP (A; 100% of 6 analyzed pollen tubes), which strongly supports the notion that RISAP association with the subapical TGN compartment is mediated by the hydrophobic domain. Interestingly, deletion of the DUF593 from YFP:RISAP$^{N\text{-}ter/HD:DUF593}$ resulted in labelling of disperse punctate structures throughout the pollen tube cytoplasm (C, YFP:RISAP$^{N\text{-}ter/HD}$; 75% of 12 analyzed pollen tubes). These structures may represent TGN fragments unable to maintain subapical positioning in the absence of DUF593-dependent myosin binding. This interpretation implies that overexpressed YFP:RISAP$^{N\text{-}ter/HD}$ out-competes endogenous RISAP with regards to membrane association at the TGN. YFP:RISAP^{DUF593} had a tendency to form aggregates at high expression levels (as indicated by bright fluorescence) and accumulated at the apex (D; 90% of 10 analyzed pollen tubes), suggesting that the RISAP DUF593 may bind particularly strongly to a myosin specifically present at this location. Scale bar: 10 µm.