

Extensive molecular tinkering in the evolution of the membrane attachment mode of the Rheb GTPase

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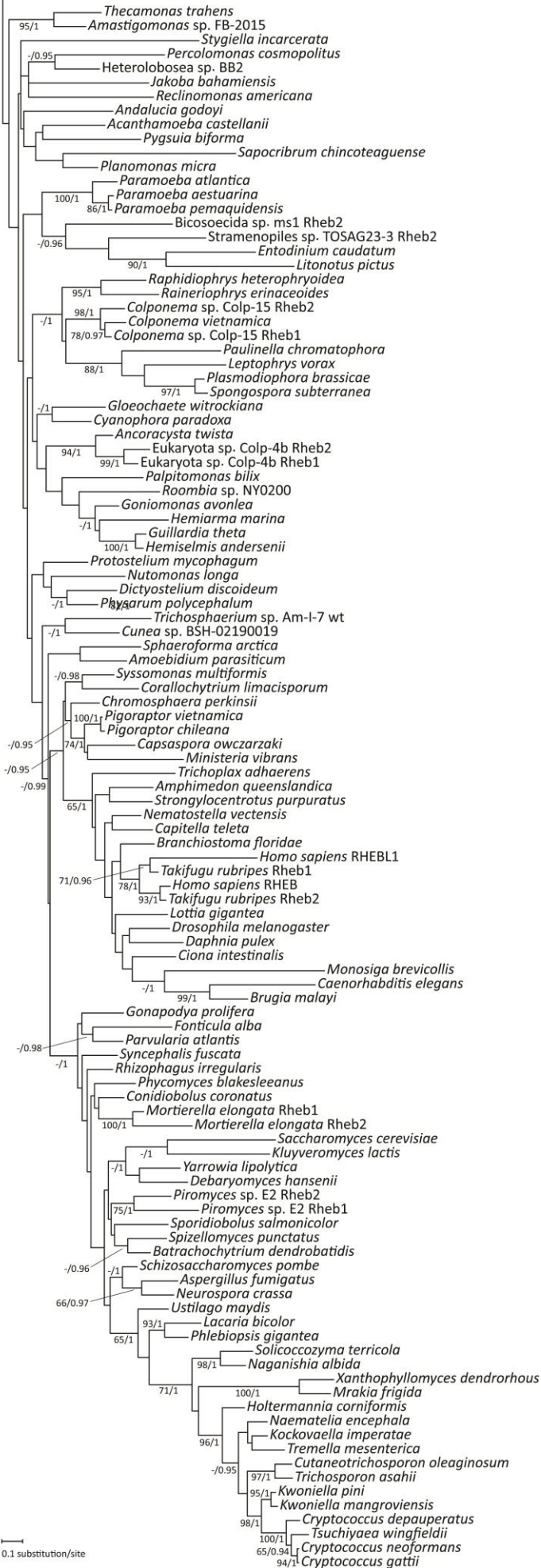
Supplementary Figures S1 to S7



Fig. S1. Phylogenetic analysis confirming identification of Rheb orthologs. The maximum likelihood tree was inferred using RAxML-HPC2 (LG4X+ Γ substitution model) based on a multiple alignment of the conserved GTPase domain (151 aligned amino acid position). The root is arbitrarily placed between the clade comprising bona fide Rhebs and other Ras family proteins. The numbers at branches refer to ML bootstrap values (shown only when $\geq 65\%$) and posterior probabilities (≥ 0.9) calculated using MrBayes (WAG+ Γ +I substitution model). If the species has more than one Rheb gene, these are distinguished by a specific gene label, otherwise only the species name is indicated. Sequence identifiers of the Rheb sequences included in the analysis is provided in table S1, those of other Ras family proteins are indicated in brackets (the respective databases are listed in table S1). Note the position of the *Naegleria gruberi* protein “61087” (among “other Ras family proteins”), which seems to have been misinterpreted as a Rheb ortholog by van Dam et al. (2011b).

B)

see part A), previous page



strain SRT308
Prokinetoplastina sp. PhM-4 Rheb2a
Prokinetoplastina sp. PhF-6 Rheb2a
Prokinetoplastina sp. PhM-4 Rheb2b
Prokinetoplastina sp. PhF-6 Rheb2b
Prokinetoplastina sp. PhM-4 Rheb1
Prokinetoplastina sp. PhF-6 Rheb1
Bodo saltans
Neobodo designis
Diplonema papillatum Rheb1
Diplonema ambulator Rheb1
Rhynchopus euleeides Rheb1
Flectonema neradi Rheb1
Hemistasia phaeocysticola Rheb1
Euglena gracilis Rheb1
Euglena longa Rheb1
Eutreptiella cf. gymnastica Rheb1
Rhabdomonas costata Rheb1
Peranema trichophorum Rheb1
Entosiphon sulcatum Rheb1
Euglena gracilis Rheb2
Euglena longa Rheb2
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Rhynchopus euleeides Rheb3
Hemistasia phaeocysticola Rheb3
Entosiphon sulcatum Rheb3
Peranema trichophorum Rheb3
Homo sapiens RHEB
Consensus/80%

```
MS-----SRRFRW---VDPREAKECTQ---CSTKFTFLKTRKHCRSCG---SVFCDSCNTYQSYLP  
M---NYLNPCEFG(5)---TTGKDVGDKKGLYHKNNDDDDIACCSSV---CKTGFSETR-RAHICSKCK---AFPCDACAASSTRT  
M---NSISFKYISECES---DRDHIDFTCMC---CKERFNGPNR-RAFVCSVCK---GGFCESCGITREGGS  
M(L)5)ASQSSST(18)SSPPTGAPHRGATPW---IPDSSTRRQCHILCSRPFGLFT-RRHCRSCG---RIFCARCTYSRRDNU  
MIF---MAVHW---HSVGTDGSASW---ISDESVSKEQ---CTTDDTTT-RRHCRSCG---HVFCSCSAHKKHEV  
MV---STPSTHS(7)SLNNNNNNNNNSVRFNW---KPDTVNACEK---CRLTFTLIR-RRHCRRCG---GIFDCASSNRRTGL  
MS-----SPPQDDNGSFVAKW---KEDSNCRKCDK---CSSEFTIFR-RKHCRRCG---NVFCCQSSQCSV  
-----MST(4)SASTAHM(30)GAAGGGATAGVRAVW---KADDSVNACER---CGAAFTFYR-RRHCRRCG---GIFCLDCARDKVPGI  
MAT-----DEAADVKAGSAQRFRW---KPRDRTKCEE---KAEFFSFFN-RRHCRRCG---GLYCNKCSSEKRVLD  
MSD-----EAGSN---GASTHFRVW---RDPSEVTNCEE---KAEFFSFFN-RRHCRRCG---GIFCNKCSSEKRVLD  
MGE-----DVG---GSSGGRFKW---RPADEGICEE---CGEFGSFFN-RRHCRRCG---GLFCGKCSAKRLLS  
MAE-----DSNSQ---SMVQRFRW---KPDSSGSTMCEV---CNAEFSFFN-RRHCRRCG---GLFCNKCSDKREL  
MA-----AQRRAV---KRDTEVSNCEE---CDAAFTEFFN-RRHCRRCG---GIFCNKCTSDRRVPI  
MTS-----AVQQAAPW---KPDSSAKACEG---CQPFSSFFRRRHRCHCG---GVFCDCGLSSARCAIP  
MTS-----AVQQAAPW---KPDSSAKVCEG---CQPFSSFFARRRHRCHCG---GVFCDCSSARCAIP  
MK-----QQPFPW---KPDSSKVCEN---CNAFFSFFRRRHRCHCG---GVFCDCPSNLRCALIP  
MP-----TAPFPW---KPDSSSVCEA---CQPFPTFFRRRHRCHCG---ALFYCACSGRSAIP  
-----MSAFPW---KPDNSVKSCES---CTLFSSFMTRRRHCRHCG---GLFCDCPSNRCALIP  
-----MKRFPW---KPNTPS---CEA---CNKSFLLV-RPHCRHCG---GVFCDECTSSRCTIP  
MN-----SSDKKPPA  
MN-----SSDKKPP  
MN-----TPDKDKQT  
ML-----GSSKDRTP  
MGI---WCCFKRAQ-----  
M---CQFSHEDKP-----  
MN---LSPKAPQ-----TEREARRPGT---QDPD-----YKRSGGNVASTG---ALSASGRNGRTARAP  
-----MNGGPDLS-K-----VTVCYSATSET  
-----MVA  
-----DQALLGLTASDGKASIT  
MPQC-----EGGASGAGTQRTLGQTRGW---PSNSSID-----GAITPMSPDSESDV  
-----SRKLTIT  
ME-----DEAPKR-----CHTDIDRLTQRTESARTIA(23)NLVTHATPASFDGP  
M-----P
```

strain SRT308
Prokinetoplastina sp. PhM-4 Rheb2a
Prokinetoplastina sp. PhF-6 Rheb2a
Prokinetoplastina sp. PhM-4 Rheb2b
Prokinetoplastina sp. PhF-6 Rheb2b
Prokinetoplastina sp. PhM-4 Rheb1
Prokinetoplastina sp. PhF-6 Rheb1
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Neobodo designis
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Diplonema ambulator Rheb1
Rhynchopus euleeides Rheb1
Flectonema neradi Rheb1
Hemistasia phaeocysticola Rheb1
Euglena gracilis Rheb1
Euglena longa Rheb1
Eutreptiella cf. gymnastica Rheb1
Rhabdomonas costata Rheb1
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Euglena longa Rheb2
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Rhabdomonas costata Rheb2
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Flectonema neradi Rheb3
Rhynchopus euleeides Rheb3
Hemistasia phaeocysticola Rheb3
Entosiphon sulcatum Rheb3
Peranema trichophorum Rheb3
Homo sapiens RHEB
Consensus/80%

```
domain  
E-----FEY---TEPQRVCQS-CHDKIAIFE(26)NNTSLPSSNPNSN(9)PPSQNGDDTQPAVARRKIVVCGSRVAKSSITIRVAK  
S-----SL---QQDMTLMCCR-CVSTNHH(16)NGTSAPLSEGLT(6)GSVPAARRMIKSVRRKICICLQHTSVCKSAMVSRPCD  
I-----TC---SPCIARQCTA-TMTDPRBP(32)EQQPPSNNGVVS(5)AIPQPKKSQORALKRRLKLCFQHTSVCKSALTIRVID  
K-----GYF-ADVAVRVCRCQ-CSGCVSFS(22)NGAGGGSNDVANN---MSMPSRSHETSRLWRKICLCLGARGCKTALTITQFVE  
R-----GY---ENTFVRVCDSS-CYKGDGM-----SPPTLSRPITQKEMIRSRKICICLQHTSVCKSALTIRVIVVE  
P-----GFPSTGPPQRVCDT-CFLMDKFL-----GEVLVNDMSVAPG-----LPYVFPENRKRERKICICLQHTSVCKSALTIRVIVVE  
P-----GY---TSPQRVCDE-CYVQ-----STVFAAEKDGGG-----QRVVFPSSQRPERRKICICLQHTSVCKSALTIRVIVVE  
P-----GY---EETLQRICGA-CXQALQ-----AMREVGKRRPERRKICICLQHTSVCKSALTIRVIVVE  
S-----GW---SGAERICRI-CQTL-----APAMALAAQPRETTVIVLQSPGVCKSALTIRVIVVE  
S-----QLGY---GHEPQRVCRQ-CARLAD-----VGTSTQANN-----IVEKKEPVLKORIKICLQHTSVCKSALTIRVIVVE  
A---EMGY---GSEQRVCKT-CAKLDL-----KGAAVAP-----HIAEKKEPVLKORIKICLQHTSVCKSALTIRVIVVE  
K---ELGY---GSEQRVCKP-CARSLDPP-----PGSVVTSGFSS-----GNSDRKDPVVKORIKICLQHTSVCKSALTIRVIVVE  
K---SMGY---GTDPPQRVCR-CCKHVD-----RGSAPKQ-----HFPPEKREPVVKORIKICLQHTSVCKSALTIRVIVVE  
G-----LDGPQRVCGD-CREQLAGGRAPTSQSSAVGAGGGGGVARGPPEPRRDSVTKORIKICLQHTSVCKSALTIRVIVVE  
K---YGY---EDETRVRCQ-CWMLYK-----DDHLRG-----TERARADSLRORIKICLQHTSVCKSALTIRVIVVE  
K---YGY---EDEARVRCQ-CWMLYK-----DDHLRG-----TERARADSLRORIKICLQHTSVCKSALTIRVIVVE  
K---YGY---EDDRVRCVA-CFKYVVKNE-----DDHLRG-----TERARADSLRORIKICLQHTSVCKSALTIRVIVVE  
K---FGF---TEEVRCVDA-CFDLLKRE-----E-HRG-----VDKEPVESELKORIKICLQHTSVCKSALTIRVIVVE  
K---FGY---ESEVRVCSQ-CODYLKRE-----EDHNKG-----TERARADSLRORIKICLQHTSVCKSALTIRVIVVE  
K---FEY---TTPVRVCPG-CFDYLKRE-----TSG-----PQRAHTDSLORIKICLQHTSVCKSALTIRVIVVE  
A-----NGASPDEAG-----TRPAHDVVKORIKICLQHTSVCKSALTIRVIVVE  
A-----NGASPDEAG-----AGPAHDVVKORIKICLQHTSVCKSALTIRVIVVE  
G-----NGGPSNEDG-----TYPFPAVKKORIKICLQHTSVCKSALTIRVIVVE  
-----TGSTDRKKN-----PATHAAPLAVKERIKIVVCGSRVAKSSITIRVIVVE  
-----EGTEHTDDGQA-----RGPASPQDMKORIKIVVCGSRVAKSSITIRVIVVE  
-----YDPLPSLNLIKPKORIKIVVCGSRVAKSSITIRVIVVE  
-----EKTPIID-----TRPADSQVCKORIKIVVCGSRVAKSSITIRVIVVE  
G-----PGSA-ANSPRG-----DYPPGVAAVTKORIKIVVCGSRVAKSSITIRVIVVE  
S-----GGKEGPGWGD-CPKAA-----NEDEVFVRKORIKICLQHTSVCKSALTIRVIVVE  
D-----ADNHLKRLPS-VERSSSED---ASL---QRNNTDGM-EQVVQRLRRHEPFRORIKICLQHTSVCKSALTIRVIVVE  
S-----HEEERKAASV-TEHSSSED---LVIGTLRDQRD-----NKTKSRPFRORIKICLQHTSVCKSALTIRVIVVE  
S-----SVAALRKAFD-----TMALAAESFVRKORIKIVVCGSRVAKSSITIRVIVVE  
K---LDF---DPNGKVCNA-HVELAEDE(37)QQQHPNSIALV(8)SFARARARASILLRRLKICLQHTSVCKSALTIRVIVVE  
S(15)FGF---DDTGKVCAAHCIDKQSLSL---QVEDQTFKISFMGG---SYSKKAVIDVPLRIVVCGSRVAKSSITIRVIVVE  
-----PQSKSRITATGYSRVCSSITIRVIVVE  
.....h+PRK1sILG.s.VGK*tls.pasp
```

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Prokinetoplastina sp. PhF-6 Rheb2a
Prokinetoplastina sp. PhM-4 Rheb2b
Prokinetoplastina sp. PhF-6 Rheb2b
Prokinetoplastina sp. PhM-4 Rheb1
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Hemistasia phaeocysticola Rheb3
Entosiphon sulcatum Rheb3
Peranema trichophorum Rheb3
Homo sapiens RHEB
Consensus/80%

```
-----Rheb GTPase domain-----  
---KH---VDIYNG---LHKFKPARIR---YREK-EFNKLLD-AGODEHSILOENYITGCHGVLYVSVVRAPEMIRVLLHDKV  
---DK---SSYHNE---LSSLQTKTSS---VGBE-QNVSITLD-AGODEYAVKQWSICGHEVLYVSVSDSTLDAALITRAHL  
---CK---SGYHNE---LSSVQVKTGT---VGBE-SNVSITLD-AGODEYTIKQWSICGHEVLYVSVNSALALSTIRNH  
---HT---SPVYLL---SWTTATKQTL---MRGQ-EFSLSVLD-AGVDECSQ-QOYSICGHEVLYVSVVAVDDAAGLEMAKQIERSAL  
---RS---VAAYSF---PATSTCSRNIT---HNGR-DVVTIVVD-AGLDECSQ-QOYALGHEVLYVSVTPPIFVTKRIRHVR  
---RQ---VEDYTF---LQATQRRVLD(16)LPHD-TYELVITDASGSSCDV-QOYSLVSGHEVLYVSVYDTRFPAEVCHEHRE  
---KS---LMEDYT---LSSTLKRVVRRD-QHDHV-DYELVITD-AGLSSCDM-KESLQCGHEVLYVSVVDRASPLEVPAIHSSL  
---DASTSHDQKVI---FGTRQMSLR---TGGEFYFISVVDI---SPTGCEM-QOYTCGHALIFMFINVDRNSFLLALPDRVR  
(25)SGAGGAGAGSS-SGASCARMLR---HRSAA-DVYVALQDAISHTHVGSPBALA-AAHLFLVVEVDDHAYLYALRPIRVE  
---DR---SPYYNE---LNHTYQRKIR---YRNO-QYLLSITLD-AGODECSM-QOYSICGHEVLYVSVNDYHPEMVKSIYDRI  
---DR---SPYYNE---LNHTYQKIR---HGNN-QYLLSITLD-AGODECSM-QOYSICGHEVLYVSVNDYHPEMVKSIYDRI  
---DR---SPYYNE---LNHTYQRQMK---HKGQ-QYLLSITLD-AGODECSM-QOYSICGHEVLYVSVNDYHPEMVKSIYDRI  
---DR---SPYYNE---LNHTYQRKIR---YSSQ-NYLLSITLD-AGODECSM-QOYSICGHEVLYVSVNDYHPEMVKSIYDRI  
---DR---SSVYNE---LNHTYQRKIR---LHGE-TYLLMIIID-AGODECSP-QOYSICGHEVLYVSVNDYHPEMVKSIYDRI  
---DR---QQAYNE---LNHTYFSKTK---VRGE-EPLMTIID-AGODETSIQOYSICGHEVLYVSVNDYHPEMVKSIYDRI  
---DR---QOYNE---LNHTYFSKTK---VRGE-EPLMTIID-AGODETSIQOYSICGHEVLYVSVNDYHPEMVKSIYDRI  
---ER---QOAYNE---LNHTYFSKTK---VRGE-EPLMTIID-AGODETSIQOYSICGHEVLYVSVNDYHPEMVKSIYDRI  
---ER---NQAYNE---LNHTYFSKTK---VRGE-EPLMTIID-AGODETSIQOYSICGHEVLYVSVNDYHPEMVKSIYDRI  
---EK---QSAYNE---LNHTYFSKTK---VRGE-EPLMTIID-AGODETSIQOYSICGHEVLYVSVNDYHPEMVKSIYDRI  
---DR---VQAYNE---LQTYAKRVK---VRGE-EPLMTIID-AGODECSP-QOYSICGHEVLYVSVNDYHPEMVKSIYDRI  
---DR---TPEYNE---VDQTHYKTRIR---IRGE-DYLLMIIID-AGODETSLQRYTAADEGVIVYSDDAYSPDIRARVYER  
---DR---TPEYNE---VDQTHYKTRIR---IRGE-DYLLMIIID-AGODETSLQRYTAADEGVIVYSDDAYSPDIRARVYER  
---EK---VPDYNE---LNHTYKTKM---FRGE-EYLLMIIID-AGADDTSLQRYTAADEGVIVYSDDAYSPDIRARVYER  
---DK---VPEYNE---LDQSHYKTKI---LRGE-EYLLMIIID-AGODETSLQRYTAADEGVIVYSDDAYSPDIRARVYER  
---DR---MAEYNE---VDQTHYKTRIR---VRGE-EYLLMIIID-AGODETSLQRYTAADEGVIVYSDDAYSPDIRARVYER  
---QR---PTIYHE---VDQTHYKTRIR---VRGE-EYLLMIIID-AGODETSLQRYTAADEGVIVYSDDAYSPDIRARVYER  
---EK---PVTYYS---VDQTHYKTRIR---VRGE-EYLLMIIID-AGODECSP-QOYSICGHEVLYVSVNDYHPEMVKSIYDRI  
---DR---EPHYNE---LNHTYTRKEH---YRGO-EYVFTIID-AGODECDV-QOYALGHEVLYVSVNDYHPEMVKSIYDRI  
---GT---SNEHLE---ATNRTSSKRTT---RNGY-EYVNVIIID-AGODECGF-DEQYTCGDEGLLYVSVNDYHPEMVKSIYDRI  
---GS---SHEHYE---ATNRTSSKRTT---RNGY-EYVNVIIID-AGODECGF-DEQYTCGDEGLLYVSVNDYHPEMVKSIYDRI  
---GS---SHEHYE---AANKTSKTIIE---RNGY-EYVNVIIID-AGODECGF-DEQYTCGDEGLLYVSVNDYHPEMVKSIYDRI  
---GT---SHEHYE---LNHTYKTKM---RNGY-EYVNVIIID-AGODECGF-DEQYTCGDEGLLYVSVNDYHPEMVKSIYDRI  
---GV---RHEHSL---LNHTYKTKM---RNGY-EYVNVIIID-AGODECGF-DEQYTCGDEGLLYVSVNDYHPEMVKSIYDRI  
---HR---IPANHE---LNHTYKTKM---RNGY-EYVNVIIID-AGODECGF-DEQYTCGDEGLLYVSVNDYHPEMVKSIYDRI  
---RK---IPETHHE---LNHTYKTKM---RNGY-EYVNVIIID-AGODECGF-DEQYTCGDEGLLYVSVNDYHPEMVKSIYDRI  
---GG---VDSYDE---LNHTYKTKM---RNGY-EYVNVIIID-AGODECGF-DEQYTCGDEGLLYVSVNDYHPEMVKSIYDRI  
.....ppF...a.P...Tlsp*b.+lp.....pGp.pa.lpl1DtsGQD-htbFpPpa*IGTCGY11laS1sd..SF-bhp.lapcl
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continues on the next page

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strain SRT308
Prokinetoplastina sp. PhM-4 Rheb2a
Prokinetoplastina sp. PhF-6 Rheb2a
Prokinetoplastina sp. PhM-4 Rheb2b
Prokinetoplastina sp. PhF-6 Rheb2b
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Bodo saltans
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Diplonema papillatum Rheb1
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Flectonema neradi Rheb1
Hemistasia phaeocysticola Rheb1
Euglena gracilis Rheb1
Euglena longa Rheb1
Eutreptiella cf. gymnastica Rheb1
Rhabdomonas costata Rheb1
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Peranema trichophorum Rheb3
Homo sapiens RHEB
Consensus/80%

```

LNA(35)SK-VVILVGNM---CDLER---NRCSNEEGEALRELGCSTFIECSKTDENLHKIETAVHNYELRFNQ---E
LECHA---PD-VPLVLCANK---CDTSE---MDROQLSDQGLELQRCDFVFECSKTGYNVPTVEKLLITRAQSS-----
LECHA---PD-VPLVLCANK---VDIPD---GIIQISEAQGREMLAKKTSFYVCSKTGEGNEMVETALLRRVAHN-----
LECYA---SG-VTVMLVGNK---ADVPA---SQRVYSSSEGHALLEMCCPFVFECSATGSCVERMVAIILLESQLQENV---R
LECHP---PG-IPVIVAGTK---ADARN---SERQVSEEGKALSAVGCDFVFECSRTGINVDKVLSSLLEKQKDD-----
REC-H---PN-APVIVGNK---SDIER---STERAITAARGAALAEELSCGFLECTETKANVATAVPTITLDMVAABEAV---V
AQC-H---ND-VPLVLCANK---IDIVE---KAAERQTSSEGAELARLGCSTFIECSRTQKAVQNVSTITLILVAASEKD---
LECGG---ME-KAMVIVGNK(10)TNGPQ---LDROTVDEVSSVGGHRVGVPHLELSSSKQRQVDEIKNTLRLVAERDHL---S
LDCGG---HF-LPILVIGTT---RG---ESRVPSDECAVAEFGAKVVELITLPGD-VAGLERRALDQVAKE-----
QDCNI---LEGVVLVGNK---NDIPE---ERQNSYDEAQQVLAASGFFVFECSKKAESVEGVNLVLEELKKAEGD---A
QDCNI---LDGVVLVGNK---SDIPA---SERQSYEEGQQVLAQS GFFVFECSKKAENIERVNLVLEELNLAEGE---G
RHCNI---LDGVVLVGNK---SDIPS---SERQSHHEGQQVLAQS GFFVFECSKQSDTVETGVNLVLEELKKAEGD---A
QDCNI---RDGVVLVGNK---NDIPA---ERQSYFEEGQQVLAAS GFFVFECSKKAENIEAVNLVLEELRAEGD---S
QDCNI---LDGVVLVGNK---MDIRE---DRKHAEAAQVLAAS GVPVFECSKKAESVELVNTVLEELVLEAP---A
HSY-A---VD-LVVLVGNK---TDIDH---ORQSHHEALKVLAQECRPLFECSKKAENISKVYTIMBELTQEDG---Q
HSY-A---VD-LVVLVGNK---TDIDH---ORQSHHEALKVLAQECRPLFECSKKAENISKVYTIMBELTQEDG---A
HSY-A---VD-VVVLVGNK---TDIEK---DRQTYDEAETLARENCFVFECSKKAENIAKVYTIMBELTQEDG---V
HSF-A---VD-VVVLVGNK---CDIER---DRQSYEEGQQVLAQRCDFVFECSKKAENIAKVYTIMBELTQEDG---G
YSY-A---VD-VVVLVGNK---SDIDK---ERQSYEEGSAIAREKCFVFECSKKAENIVRVYTIMBELTQEDG---T
FSH-A---VD-VVVLVGNK---SDIDK---DRQSYVEAGKALAKE DSBFVFECSKKAENIAKVYTIMBELTQEDG---G
HEY-V---VD-GVVVLGNK---TDIEK---DRQSYQQAAQVLAAS GCFVFECSKKAENIQRVYTIMBELTKREDT---P
HEY-V---VD-GVVVLGNK---TDIEK---ERQSYQQAAQVLAAS GCFVFECSKKAENIQRVYTIMBELTKREDT---P
HEY-V---VE-GVVVLGNK---VDIDA---ROVSHHAQQAAQVLAAS GCFVFECSKKAENIQRVYTIMBELTKREDA---P
QDSGV---VE-AVVVLGNK---TDIDA---ROVSHHAQQAAQVLAAS GCFVFECSKKAENIQRVYTIMBELTKREDA---P
HEY-V---VD-AVVVLGNK---TDIDR---ERQITTEGQQVLAQSCRTYFECSKKAENIVNRVYTIMBELTQEDG---S
CDN-V---LY-PTVVLGNK---SDRQS---HRKVTREGQAQVLAAS GCFVFECSKKAENISANVYTIMBELTQEDG---G
HCE-V---VD-AAVVLGNK---IDIEK---ORQVPTTEAQAQVLAQS GCFVFECSKKAENISANVYTIMBELTQEDG---S
RD-N---LDDVAVVGNK---SDIAG---DREPVVEAMRVAQVLAAS GCFVFECSKKAENISANVYTIMBELTQEDG---S
WAY-T---PL-VPVVLGNK---SDIKA---QVSSGEEGWNVLAAS GCFVFECSKKAENISANVYTIMBELTQEDG---S
WAY-T---PL-VPVVLGNK---VDVVD---HREVEEGEGRVLAAS GCFVFECSKKAENISANVYTIMBELTQEDG---S
WAY-T---PL-VPVVLGNK---ADITS---DREVEEGEGLVLAAS GCFVFECSKKAENISANVYTIMBELTQEDG---S
WAY-T---PL-VPVVLGNK---VDITH---ERVTEQDGRAQVLAAS GCFVFECSKKAENISANVYTIMBELTQEDG---S
WAY-T---PL-VPVVLGNK---VDIAT---QREVTAREGQVLAAS GCFVFECSKKAENISANVYTIMBELTQEDG---S
MAN-T---VG-IPVVLGNK---SDICR(10)GAGAVTEGEMRVAAS DAQVMECTVLAAS GCFVFECSKKAENISANVYTIMBELTQEDG(6)S
QEN-T---VC-PTVVLGNK---SDIP---NREVEEGEGLVLAAS GCFVFECSKKAENISANVYTIMBELTQEDG---S
LDM-V---GKVV-IPVVLGNK---KDHM---EERVISYEEGKALAEESNAALFECSKKAENISANVYTIMBELTQEDG---S
bph.s.....ssllLVtNK.....sdl.....pRpV*.pptb.LA.pw.sshEs*App.ps1.lf...llpI...b.....

```

strain SRT308
Prokinetoplastina sp. PhM-4 Rheb2a
Prokinetoplastina sp. PhF-6 Rheb2a
Prokinetoplastina sp. PhM-4 Rheb2b
Prokinetoplastina sp. PhF-6 Rheb2b
Prokinetoplastina sp. PhM-4 Rheb1
Prokinetoplastina sp. PhF-6 Rheb1
Bodo saltans
Neobodo designis
Diplonema papillatum Rheb1
Diplonema ambulator Rheb1
Rhynchopus euleeides Rheb1
Flectonema neradi Rheb1
Hemistasia phaeocysticola Rheb1
Euglena gracilis Rheb1
Euglena longa Rheb1
Eutreptiella cf. gymnastica Rheb1
Rhabdomonas costata Rheb1
Peranema trichophorum Rheb1
Entosiphon sulcatum Rheb1
Euglena gracilis Rheb2
Euglena longa Rheb2
Eutreptiella cf. gymnastica Rheb2a
Eutreptiella cf. gymnastica Rheb2b
Rhabdomonas costata Rheb2
Peranema trichophorum Rheb2
Entosiphon sulcatum Rheb2
Hemistasia phaeocysticola Rheb2
Diplonema papillatum Rheb3
Diplonema ambulator Rheb3
Flectonema neradi Rheb3
Rhynchopus euleeides Rheb3
Hemistasia phaeocysticola Rheb3
Entosiphon sulcatum Rheb3
Peranema trichophorum Rheb3
Homo sapiens RHEB
Consensus/80%

```

AYH-----QETPPT-**SLI**-----

E-----
-----V
C-----
-----D
G-----

S-----

PKQPAK-----PFWRIA-----
PKQPIK-----PFWRIG-----Q
PKQTSK-----MFWRKA-----DR
PRPSRW-----PSWLRP-----
PKKEGKD-----KAKPK-GKGLF-----
QRRLPP-----TPK-K-----
ATAGDRP-----AAKPF-WGMFQS-----G
ATAGDRP-----PIKPF-WGMFQS-----S
CCAGDRP-----QSRPF-WTGMFHK-----S
FSSGQSA-----HECSSI-WKAIF-----
GAGAKQSKGLR-----GFFGK-----P
T-----PTSGT-SSSFLHKIM-----GT
A-----RVRGS-W---FNKTP-----R

PEFAK--(4)FGTG-----
ETFIR--(4)NTAPRPS-**SAIC**-----
ETFVK--(2)SQANQPS-**SVV**-----
DTFFK--(3)--RQQQS-**SVV**-----
ETFAPTE(10)QRSKDG-**SVV**-----
TTTRNN(71)REEEPA-ASVVAEEKRSAAKRTGE
DPGVRR(14)AESEGYR-**VYI**-----
S-----QGKSS-**SVV**-----
.....

Fig. S3. Annotated multiple alignment of Rheb protein sequences from Euglenozoa. Sequence accession numbers are provided in table S1. The alignment was prepared using MAFFT v7, manually adjusted, and processed for visualization using Chroma v1.0 (numbers in brackets indicate the number of amino acid residues omitted from the display for simplicity). The *Homo sapiens* RHEB sequence represents the conventional Rheb form. Positions predicted to be N-myristoylated (see table S3) are highlighted with a blue background, positions predicted to be S-palmitoylated (see table S4) are highlighted with a pink background, the CaaX box is highlighted with a red background. Note that the sequence of the C-terminus of the Rheb3 protein from *Entosiphon sulcatum* has not been defined (the available transcript sequence is either 3'-truncated or has a retained intron), so it is unclear whether this protein has the CaaX box (as its orthologs) or lacks it. The region corresponding to the FYVE domain is boxed. Note that Pfam search (<http://pfam.xfam.org/>) failed to recognize the FYVE domain in the Rheb1 proteins from the two *Prokinetoplastina* species, but the presence of the domain (albeit possibly divergent) in these two proteins is supported by sequence conservation in the respective region and searches against the NCBI Conserved Domains database (<https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>).

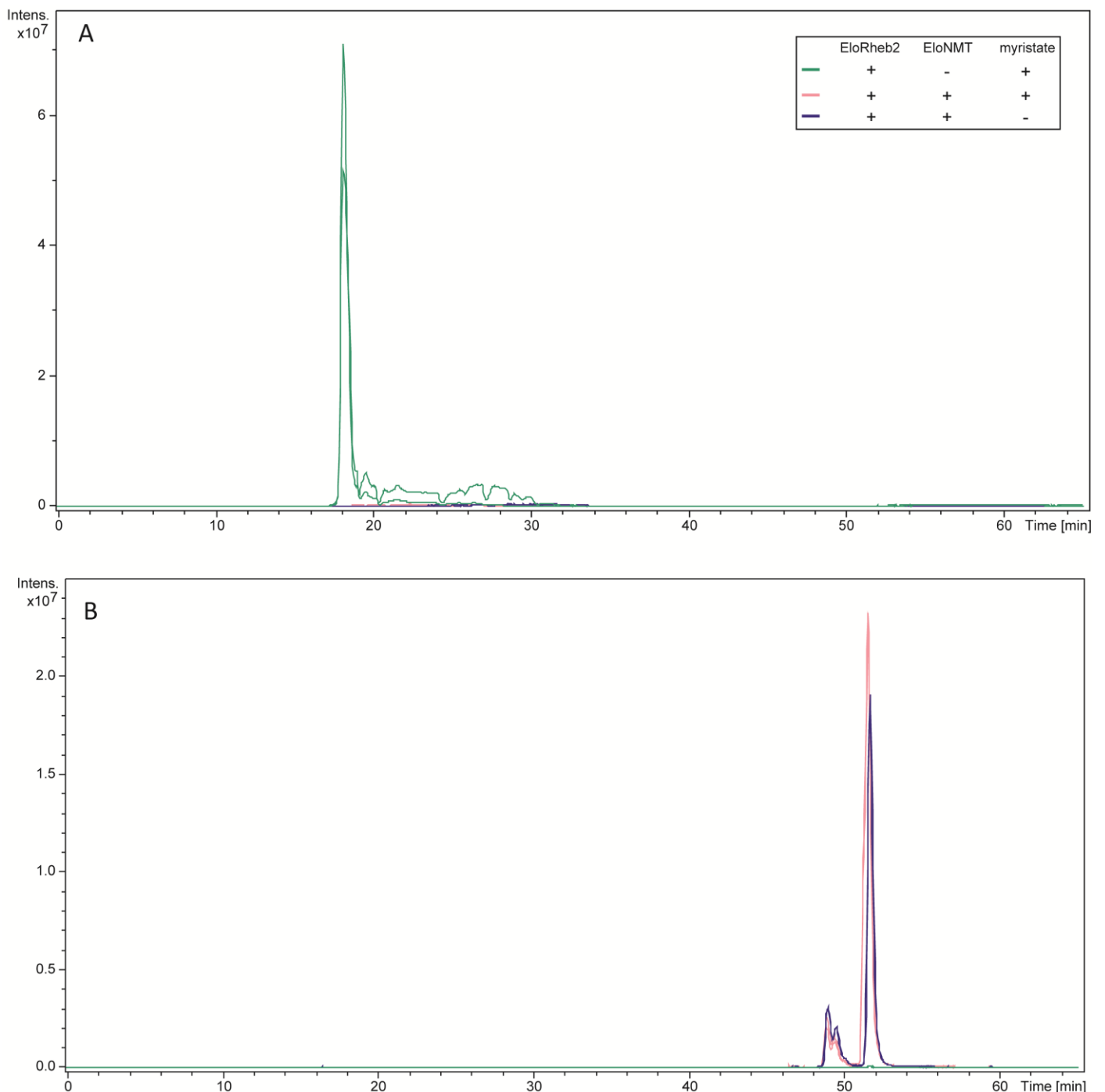


Fig. S4. N-terminal myristoylation of the *Euglena longa* Rheb2 protein detected by mass spectrometry. EloRheb2 protein was affinity purified on S-protein agarose, dissected from a gel after 12% SDS-PAGE electrophoresis, cleaved with trypsin, and the peptides were analyzed by mass spectrometry. The plots show the signal for two different variants of the N-terminal tryptic peptide (GNSSDKEKPPANGASPDEAGAGPAHDVK) of the Rheb2 protein: A) an unmodified form (2702.25 Da, a lower LC-MS retention time); B) a myristoylated form (2912.45 Da, a higher LC-MS retention time). Both peptide variants were monitored in three different samples (each sample processed twice, the results shown as two lines of the same colour): green, samples from *E. coli* expressing Rheb2, but not N-myristoyl transferase (NMT), supplemented with myristate; pink and blue, samples from *E. coli* expressing Rheb2 and NMT in the presence or absence of external myristate, respectively. Note the lack of a green signal for the myristoylated peptide (consistent with the lack of myristoylation in the absence of NMT). The lack of a pink and a blue signal for the non-myristoylated peptide indicates a high efficiency of NMT-dependent N-myristoylation in the heterologous system of *E. coli* cells, even in the absence of the external myristate.

