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Comparative proximity biotinylation implicates RAB18 in cholesterol mobilization and biosynthesis

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GEF-dependent RAB18 interactions

AUTHORS

Robert S. Kiss*[1], Jarred Chicoine[1], Youssef Khalil[2], Robert Sladek[1], He Chen[1], Alessandro Pisaturo[1], Cyril Martin[1], Jessica D. Dale[3], Tegan A. Brudenell[3], Archith Kamath[4,5], Emanuele Paci[6], Peter Clayton[2], Jimi C. Wills[4], Alex von Kriegsheim[4], Tommy Nilsson[1], Eamonn Sheridan[3], Mark T. Handley*[3,7]

AFFILIATIONS

[1]Research Institute of the McGill University Health Centre

1001 boul Decarie

Glen Site Block E

Montreal, QC

H4A 3J1

Canada

[2] Genetics and Genomic Medicine

Great Ormond Street Institute of Child Health

University College London

30 Guilford Street
London
WC1N 1EH
United Kingdom

[3]Leeds Institute of Medical Research
St James's University Hospital
Leeds
LS9 7TF
United Kingdom

[4]Cancer Research UK Edinburgh Centre
MRC Institute of Genetics & Molecular Medicine
The University of Edinburgh
Western General Hospital
Edinburgh
EH4 2XR
United Kingdom

[5]Medical Sciences Division
University of Oxford
Oxford
OX3 9DU

United Kingdom

[6]Astbury Centre for Structural Molecular Biology
University of Leeds
Leeds
LS2 9JT
United Kingdom

[7]Faculty of Biological Sciences
University of Leeds
Leeds
LS2 9JT
United Kingdom.

*authors contributed equally

CORRESPONDING AUTHORS
Robert S. Kiss: robert.kiss@mcgill.ca
Mark T. Handley: m.handley@leeds.ac.uk

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SUMMARY STATEMENT

We used proximity biotinylation together with guanine nucleotide exchange factor (GEF)-null cell lines to discriminate functional RAB18-interactions. Our data suggest that RAB18 mediates lathosterol mobilization and cholesterol biosynthesis.

ABSTRACT

Loss of functional RAB18 causes the autosomal recessive condition Warburg Micro syndrome. To better understand this disease, we used proximity biotinylation to generate an inventory of potential RAB18 effectors. A restricted set of 25 RAB18-interactions were dependent on the binary RAB3GAP1-RAB3GAP2 RAB18-guanine nucleotide exchange factor (GEF) complex. Consistent with a role for RAB18 in regulating membrane contact sites (MCSs), interactors included groups of microtubule/membrane-remodelling proteins, membrane-tethering and docking proteins, and lipid-modifying/transporting proteins. We provide evidence validating novel interactions with SEC22A and TMCO4. We also provide functional evidence that RAB18 links the Δ8-Δ7 sterol isomerase emopamil binding protein (EBP) to a molecular machinery mobilizing the products of EBP-catalysis. The cholesterol precursor lathosterol accumulates in RAB18-null cells, and *de novo* cholesterol biosynthesis is reduced. Our data demonstrate that GEF-dependent Rab-interactions are highly amenable to interrogation by proximity biotinylation and suggest that Micro syndrome is a cholesterol biosynthesis disorder.

INTRODUCTION

Rab Proteins are a large subfamily of small GTPases with discrete roles in coordinating membrane trafficking (Zhen & Stenmark, 2015). Like other small GTPases, they adopt different conformations and enter into different protein-protein interactions according to whether they are GDP-, or GTP-bound. Although they possess some intrinsic GTP-hydrolysis activity, their *in vivo* nucleotide-bound state is tightly governed in cells by two classes of regulatory proteins. Guanine-nucleotide exchange factors (GEFs) catalyse the exchange of bound GDP for GTP while GTPase-activating proteins (GAPs) promote the hydrolysis of bound GTP to GDP (Barr & Lambright, 2010, Lamber et al., 2019).

Biallelic loss-of-function variants in *RAB18*, *RAB3GAP1*, *RAB3GAP2*, or *TBC1D20*, cause the autosomal recessive condition Warburg Micro syndrome (Aligianis et al., 2005, Bem et al., 2011, Borck et al., 2011, Handley & Sheridan, 2018, Liegel et al., 2013)(MIMs 600118, 614222, 614225, 615663, 212720). *RAB3GAP1* and *RAB3GAP2* encode subunits of the binary RAB18-GEF complex, 'RAB3GAP', whereas *TBC1D20* encodes a RAB18-GAP (Gerondopoulos et al., 2014, Handley et al., 2015). Thus, the same pathology is produced when functional RAB18 is absent or when its normal regulation is disrupted. However, it is unclear how RAB18 dysfunction contributes to disease pathology at a molecular level.

Rab proteins fulfil their roles by way of protein-protein interactions with interacting partners termed 'effectors'. The identification of these proteins can therefore provide insight into these roles. However, biochemical identification of Rab effectors is challenging; Rab-effector interactions are usually GTP-dependent and are often highly transient. Immunoprecipitation, affinity purification and yeast-2-hybrid approaches have each been used, but may be more or less effective depending on the Rab isoform studied (Christoforidis et al., 1999, Fukuda et al., 2008).

One newer approach is 'BioID' proximity biotinylation utilizing Rab proteins fused to mutant forms of the biotin ligase BirA. The Rab fusion protein biotinylates proximal proteins which are then purified on streptavidin and identified through mass spectrometry (Gillingham et al., 2019, Liu et al., 2018, Roux et al., 2012). Biotin labelling occurs in a relatively physiological context and prospective effectors can be purified under high stringency conditions. However, a drawback of the technique is

that it does not distinguish between close associations resulting from functional protein-protein interactions and those resulting from overlapping localizations.

To discriminate functional RAB18 interactions, we compared BirA*-RAB18 labelling of protein in wild-type HeLa cells to that in cells in which RAB18-GEF activity was disrupted with CRISPR. Known and novel effectors were more strongly labelled in the wild-type cells. In particular, 25 RAB18-interactions were RAB3GAP-dependent. These proteins comprised several groups. Proteins within each group were clearly interrelated through involvement in connected biological processes. Moreover, gene-disease associations within the set included multiple overlapping phenotypes. We present additional evidence for several examples of RAB3GAP-dependent RAB18 interactions: SPG20/SPART; SEC22A; TMCO4; EBP; ORP2/OSBPL2 and INPP5B.

Our data elaborate an existing model suggesting that RAB18 effectors act collectively in lipid transfer at membrane contact sites (Xu et al., 2018). We identify multiple proteins already implicated in the establishment and maintenance of membrane-contacts. Importantly, we also identify novel RAB18-interactors involved in lipid biosynthesis, mobilization and metabolism. We find that RAB18 appears to coordinate generation of the cholesterol precursor lathosterol by the Δ8-Δ7 sterol isomerase enzyme EBP, and its subsequent mobilization by the lipid transfer protein (LTP) ORP2/OSBPL2. Consistently, cholesterol biosynthesis is impaired in model cell lines lacking RAB18 or its regulators. Taken together, these findings suggest that Micro syndrome is a cholesterol biosynthesis disorder. Further, that therapeutic interventions might be feasible in this disease.

RESULTS

An inventory of RAB18-GEF-dependent RAB18-associated proteins in HeLa cells

We first used CRISPR to generate a panel of clonal, otherwise isogenic, HeLa cell lines null for RAB18 and a number of its regulators (see Figure S1). We then carried out proximity labelling using transient expression of the same exogenous BirA*-RAB18 construct in RAB3GAP1-, RAB3GAP2- and TRAPPC9-null cell lines and in wild-type cells (Figure 1A). RAB3GAP1 and RAB3GAP2 are each essential subunits of a binary RAB18-GEF complex (Gerondopoulos et al., 2014). TRAPPC9 is reported to be essential for the RAB18-GEF activity of a different GEF, the multisubunit TRAPPII complex (Li et al., 2017).

Proximity-labelling, affinity purification and mass spectrometry of biotinylated proteins were carried out essentially as previously described (Roux et al., 2018, Roux et al., 2012). Prior to mass-spec analysis, samples from each of the streptavidin pull-downs were subjected to Western blotting to ensure comparable BirA*-RAB18 expression (Figure S2A). Label-free quantitative proteomics analyses were used to calculate 'LFQ intensities' for each RAB18-associated protein (Cox et al., 2014). These were then normalized in each experiment according to the quantity of RAB18 found in each sample. Samples from three independent experiments were analysed. Pull-downs from untransfected biotin-treated cells were used as controls. After filtering the data to remove known mass-spec contaminants and any protein identified at a high level in control samples, a total of 584, 483 and 506 RAB18-associated proteins were identified in each experiment. A total of 457 proteins were present in two or more of the replicate experiments (see Table S1).

Different Rab-GEF complexes may operate in distinct subcellular localizations and coordinate associations with different effectors (Carney et al., 2006). Therefore, we assessed whether non-zero intensities for each RAB18-associated protein correlated between samples (Figure 1B, Figure S2B). Very strong correlations between protein intensities from RAB3GAP1- and RAB3GAP2-null cells indicated that loss of either protein had a functionally equivalent effect (R^2=0.99, Figure 1B). In contrast, intensities from RAB3GAP1/2- and TRAPPC9-null cells were much more poorly correlated (R^2=0.73, R^2=0.74, Figure S2B). We therefore considered RAB3GAP- and

TRAPPC9-dependent RAB18-interactions separately. Intensities from wild-type and RAB3GAP-null samples correlated with an R^2=0.87, but a number of proteins showed reduced intensities in the RAB3GAP-null samples (Figure 1C).

GEF activity promotes Rab GTP binding and this is usually necessary for effector interactions. We therefore reasoned that levels of true effector proteins would be reduced in samples from GEF-null cells as compared to those from wild-type cells. We calculated GEF-null:wild-type intensity ratios for each RAB18-associated protein (Table S1). Only 25 proteins showed a RAB3GAP-null:wild-type ratio <0.5 (Figure 1D, Table 1, Table S1). 133 proteins showed a TRAPPII-null:wild-type intensity ratio <0.5 (Figure 1D, Table S1). There was only limited overlap between RAB3GAP- and TRAPPC9-dependent associations (Figure 1D).

The most comprehensive annotation of candidate RAB18 effectors thus far was made in the 2014 paper by Gillingham et al., which utilized an affinity purification-mass spectrometry (AP-MS) approach and the *Drosophila* RAB18 orthologue (Gillingham et al., 2014). In that study, a total of 456 proteins were identified as interacting with RAB18. However, only 14 of these were well represented in terms of spectral counts, exhibited low non-specific binding to GST/sepharose and showed low binding to other Rab protein isoforms. We took these 14 proteins as the most plausible physiological RAB18 interactors and searched for these in our datasets.

Orthologues of 11 of the 14 putative RAB18-interacting proteins identified by Gillingham et al. were identified in our combined dataset. 10/14 were among the 25 RAB3GAP-dependent associations (Table 1). 2/14 were among the TRAPPII-dependent associations (Table S1).

For initial validation of our dataset, we carried out an additional independent BioID experiment with wild-type and RAB3GAP1-null cells and subjected the resulting samples to Western blotting for selected RAB18-associated proteins (Figure 1E). As with the mass spectrometry, these proteins showed either complete (RAB3GAP1, RAB3GAP2, ZW10) or partial (SPG20, STX18) dependence on RAB3GAP for their RAB18 association.

We further validated our approach with additional proximity biotinylation experiments in HEK293 cells. We used cells stably expressing BirA*-tagged RAB18 fusions incorporating wild-type RAB18, GTP-hydrolysis deficient RAB18(Gln67Leu), or

nucleotide-binding deficient RAB18(Ser22Asn) mutants (Figure S3A-B). A total of 98 proteins were identified as associating with RAB18 across all samples (Table S2). Gln67Leu:wild-type intensity ratios for known RAB18-interactors ranged from 0.1-1.49 indicating that RAB18 associations were altered by the Gln67Leu variant, but not predictably so. In contrast, Ser22Asn:wild-type intensity ratios were <0.5 for the majority of these proteins. 28 nucleotide-binding-dependent RAB18 associations included 6 of the RAB3GAP-dependent associations and 7 of the TRAPPII-dependent associations seen in the HeLa cells (Figure S3C). These data confirm that the loss of GEFs has similar effects on RAB18-interactions to direct loss of nucleotide binding. In addition, they support the differing regulation of specific RAB18-interactions by different GEFs.

<u>Validation screening of RAB3GAP-dependent RAB18 associations reveals reduced levels of SPG20 in RAB18-null and TBC1D20-null cells</u>

Our continued study focused on the 25 RAB3GAP-dependent RAB18 associations identified in HeLa cells. Encouragingly, these appeared to share interconnected functions and fell into discrete groups (Table 1). Furthermore, genes encoding 11 of the 25 proteins or their homologues are associated with inherited diseases that share features with Micro syndrome (Table 2).

Given the suggestive convergences in protein function and gene-disease-associations, we examined the subcellular localizations of 12 putative effectors for which antibodies were available (Figure 2A-B). To determine whether the localization of these proteins was altered in cells lacking RAB18, we analysed wild type and RAB18-null lines in each case. In order to directly compare cells of different genotypes under otherwise identical conditions, we labelled them with CellTrace-Violet and CellTrace-Far Red reagents before seeding, immunostaining and imaging them together. Since RAB18 can localize to lipid droplets (LDs), we analysed both untreated cells (Figure 2A) and cells loaded with oleic acid and labelled with BODIPY-558/568-C12 (Figure 2B).

The putative effector proteins showed various staining patterns. These ranged from staining that was enriched at the perinuclear region of cells, to staining that appeared reticular, to staining that appeared more diffuse. Staining patterns were similar in the

HeLa cells and also in RPE1 cells generated to provide biological replicates (Figure S4A). Each pattern was compatible with the known localization of RAB18, which is distributed between *cis*-Golgi, ER and cytosolic compartments (Handley et al., 2015). In lipid-loaded cells, localizations of proteins with reticular staining patterns overlapped with LDs but they did not obviously shift to adopt a LD localization. Two proteins that showed diffuse staining patterns in untreated cells - ZW10 and SPG20 - appeared enriched in the vicinity of LDs (Figure 2B, bottom right panels).

We saw no evidence for dramatic changes in protein localizations in RAB18-null cells as compared to their wild-type counterparts. Fluorescence intensities in RAB18-null and wild-type cells were also generally similar, except in the case of staining for SPG20, which appeared lower in RAB18-null HeLa cells than in wild-type cells (Figure 2A, bottom right panels).

To confirm the reduction in levels of SPG20 we observed in RAB18-null HeLa cells and to determine the effects of other genotypes, we used quantitative fluorescence microscopy (Figure 2C). To establish SPG20 antibody specificity we first analysed SPG20-null cells (Figure 2D, left panels). Measured fluorescence intensity of these cells also provided a baseline level, above which fluorescence levels are proportional to levels of SPG20 (Figure 2E). In RAB18-null cells, SPG20 fluorescence was reduced to 67.16±3.77% ($p<0.001$) of that in wild-type cells (Figure 2F). Loss of the RAB18-GEF subunits RAB3GAP1 or RAB3GAP2 did not significantly affect levels of SPG20, whereas loss of the RAB18-GAP TBC1D20 led to a reduction comparable to that in RAB18-null cells (57.48%±2.57, $p<0.00005$)(Figure 2F).

We analysed levels of SPG20 in the corresponding panel of RPE1 cell lines using LFQP analysis of whole cell lysates (Figure S4B, Table S3). As in the HeLa cells, levels of SPG20 were significantly reduced in RAB18- and TBC1D20-null RPE1 cells compared to wild-type controls ($p<0.05$ following FDR correction), but not in the other genotypes tested. These data suggest that reduced SPG20 levels result from specific genotypes and are not the result of clonal variation. A comparison between LFQP data from wild-type and TBC1D20-null RPE1 and HeLa cells (Tables S3 and S4) showed limited overlap between differentially expressed proteins. This indicates that reduced SPG20 levels are unlikely to have resulted from widespread dysregulation of proteostasis. The RAB18-SPG20 interaction has been previously

reported (Gillingham et al., 2014), and our findings (above) provide further support for a physiological relationship between these proteins.

SEC22A associates with RAB18 and its knockdown causes altered LD morphology

The most studied group of RAB18 effector proteins to date are the tethering factors that comprise the NRZ/Dsl complex (ZW10, NBAS and RINT1), and the ER SNARE proteins that comprise the Syntaxin18 complex (STX18, BNIP1, USE1 and SEC22B) (Gillingham et al., 2014, Li et al., 2019, Xu et al., 2018, Zhao & Imperiale, 2017). Although SNARE complexes typically mediate membrane fusion, it has been proposed that RAB18 interacts with these proteins to mediate the close apposition of membranes to facilitate lipid transfer (Xu et al., 2018). It has also been suggested that SEC22B is dispensable for this function (Xu et al., 2018).

Our screen for RAB3GAP-dependent RAB18-interactors identified all of the NRZ complex components, as well as the SNARE proteins STX18 and BNIP1 (Table 1). Interestingly, we did not identify SEC22B but did identify SEC22A among these proteins. SEC22A is one of the two SEC22B homologues in humans that lack the central coiled-coil SNARE domain through which SEC22B mediates membrane fusion (Rossi et al., 2004). Since it had not been previously described as a RAB18-interacting protein, we investigated this further.

In the absence of commercially available antibodies for SEC22A, we examined its localization through expression of a mEmerald-SEC22A fusion protein (Figure 3A). mEmerald-SEC22A produced a characteristic reticular staining pattern and colocalized with an exogenous ER marker suggesting that SEC22A localizes to the ER. We next sought to compare the localization of SEC22A and RAB18 and to determine whether they interact. However, coexpression of mEmerald-SEC22A and mCherry-RAB18 disrupted normal ER morphology produced vesicular structures and inclusions positive for both proteins (Figure S5). Although this was not inconsistent with a protein-protein interaction, it precluded the use of coexpressed exogenous proteins in continued testing.

As an alternative means of assessing SEC22A-interactions, we used proximity biotinylation with a BirA*-SEC22A fusion protein in the HeLa cell panel. To minimize potential toxicity while increasing biotin-ligase activity, we used BioID2 (Kim et al.,

2016) with a p.Gly40Ser active site modification (Branon et al., 2018) and reduced biotin incubation time. Despite a low level of BioID2(Gly40S)-SEC22A expression, the construct appeared to label RAB18 in a RAB3GAP-dependent manner (Figure 3B). 55 SEC22A-associated proteins were present in samples from wild-type cells in >2 replicate experiments and represented by >3 spectral counts (Table S5). Further, a subset of 9 SEC22A-associations were attenuated (intensity ratios <0.5) in samples from both RAB18-null and RAB3GAP-null cells. These data are consistent with a physiological SEC22A-RAB18 interaction.

A phenotype of altered LD morphology in lipid-loaded cells has been widely reported in cells deficient in RAB18 (Bekbulat et al., 2019, Carpanini et al., 2014, Gerondopoulos et al., 2014, Li et al., 2017, Liegel et al., 2013, Xu et al., 2018). Similar observations have been made in cells deficient in some components of the NRZ or Syntaxin18 complexes, but not in cells deficient in SEC22B (Xu et al., 2018). To test whether SEC22A expression influences LD morphology, we examined the effects of its silencing in oleic acid-loaded induced human hepatocyte (IHH) cells (Figure 3C). ZW10 and NBAS silencing provided positive controls in our experiments. ZW10 silencing led to a significant reduction in LD number ($p<0.001$) compared to controls, whereas NBAS silencing led to both a significant reduction in LD number and a significant increase in LD size ($p<0.001$ in each case). The effects of SEC22A silencing mirrored those of NBAS silencing, producing a significant reduction in LD number ($p<0.001$) and a significant increase in LD size ($p<0.001$). Together, these data implicate SEC22A as involved in the same RAB18-mediated process(es) as the NRZ and SNARE proteins.

RAB18 recruits the orphan lipase TMCO4 to the ER membrane in a RAB3GAP-dependent manner

The most novel group of putative RAB18 effectors identified in our study were the lipid modifying/mobilizing proteins, none of which had been reported to associate with RAB18 previously. Among these, TMCO4 was identified in all three replicate experiments and its association with RAB18 was highly RAB3GAP-dependent (intensity ratio 0.06). Although annotated as containing transmembrane and coiled-coil domains, it is orthologous to the Yeast protein Mil1/ Yfl034w, and likely to be a

partly soluble lipase (Whitfield et al., 2016). Consistently, TMCO4-EGFP expressed in HeLa cells showed a diffuse localization. In contrast, EGFP-RAB18 partly localizes to the ER, as shown by its colocalization with an ER marker (Figure 4A).

To assess the potential RAB18-TMCO4 interaction, we coexpressed mCherry-RAB18 and TMCO4-EGFP (Figure 4B). As in our previous experiments, Celltrace reagents were used to distinguish cells of wild-type and mutant genotypes. In wild-type HeLa cells, coexpression of mCherry-RAB18 led to a dramatic redistribution of TMCO4-EGFP to the ER membrane suggesting that RAB18 mediates recruitment of TMCO4 to this compartment. Redistribution was completely absent in RAB3GAP1- and RAB3GAP2-null cells but unaffected in TRAPPC9-null cells, consistent with the BioID data.

As a means of verifying the RAB18-TMCO4 interaction, we carried out immunoprecipitation experiments using exogenous HA-RAB18 and TMCO4-EGFP (Figure 4C). As expected, TMCO4-EGFP copurified with HA-RAB18 when expressed in wild-type or TRAPPC9-null cells, but not when expressed in RAB3GAP1-null cells. These data indicate that RAB18 and TMCO4 interact directly or indirectly as part of a protein complex in a RAB3GAP-dependent manner. Further, both the microscopy and the immunoprecipitation data support the suggestion that different GEFs can promote different RAB18-interactions.

<u>RAB18 is involved in cholesterol mobilization and biosynthesis</u>

Other putative RAB18 effectors with lipid-related functions included ORP2/OSBPL2, INPP5B and EBP. ORP2 and INPP5B are robustly linked to a role in cholesterol mobilization. ORP2 is thought to function as a lipid transfer protein that exchanges cholesterol and PI(4,5)P_2 (Wang et al., 2019). INPP5B is implicated in the hydrolysis of ORP2-bound PI(4,5)P_2, presumably driving the exchange process (Wang et al., 2019). EBP is involved in *de novo* cholesterol biosynthesis (Silve et al., 1996). In the Bloch pathway, it catalyses the conversion of 5α-cholesta-8, 24-dien-3β-ol (zymosterol) to 5α-cholesta-7, 24-dien-3β-ol (24-dehydrolathosterol). In the Kandutsch-Russel pathway, it catalyses the conversion of 5α-cholest-8(9)-en-3β-ol to 5α-cholest-7-en-3β-ol (lathosterol)(Platt et al., 2014). On the basis of these

findings, we investigated the potential role of RAB18 in cholesterol uptake, efflux and biosynthesis.

We first performed loading and efflux experiments to measure the flux of cholesterol/cholesteryl ester (CE) while modifying the activity of RAB18. Chinese hamster ovary (CHO) cells were generated to stably express RAB18(WT), RAB18(Gln67Leu), or RAB18(Ser22Asn) (Figure S6A). In cells labelled with [^{14}C]-oleate, but cholesterol depleted with lipoprotein-depleted serum (LPDS), levels of CE were comparable in RAB18(Ser22Asn) and RAB18(WT) cells, whereas RAB18(Gln67Leu) cells stored significantly more (Figure 5A, left panel). In cells labelled with [^{14}C]-oleate and cholesterol-loaded FBS, levels of CE in RAB18(Ser22Asn) remained unchanged, whereas its storage was elevated in RAB18(WT) cells and RAB18(Gln67Leu) cells (Figure 5A, right panel). Interestingly, in both [^{14}C]-oleate/LPDS and [^{14}C]-oleate/FBS cells, the addition of high density lipoprotein (HDL, a vehicle mediating removal of cellular cholesterol) led to rapid depletion of CE in RAB18(Gln67Leu) cells, but not in RAB18(Ser22Asn) or RAB18(WT) cells (Figure 5A). Consistently, RAB18(Gln67Leu) cells also effluxed significantly more [^{3}H]-cholesterol upon their incubation with apolipoprotein (apo) A-I than the other cell types (Figure 5B). These observations were not explained by altered expression of ABCA1, the transporter responsible for the rate-limiting step of cholesterol efflux (Figure S6B). These data suggest that ‘activated’ GTP-bound RAB18 strongly promotes the storage, turnover and mobilization of CE stored in LDs. A plausible explanation for this is that active RAB18 promotes cholesterol mobilization via ORP2 and INPP5B.

Given that ORP2 and INPP5B function in sterol mobilization whereas EBP functions in sterol biosynthesis, we reasoned that RAB18 might coordinate their activities; that ORP2 might act as an exchanger for the substrates or products of EBP-catalysis as well as for cholesterol. In this case, defective mobilization of cholesterol precursors might lead to their accumulation in RAB18-null cells. To test this hypothesis, we incubated wild-type and RAB18-null cells for 48 hours in media supplemented with LPDS, then subjected samples to analysis by GC-MS (Figure 5C-D). In RAB18-null cells, we found that levels of the EBP-substrate cholest-8(9)-en-3β-ol were not significantly different from those in wild-type cells. In contrast, levels of EBP-product lathosterol were significantly increased ($p<0.01$). This suggests that RAB18

facilitates ORP2-mediated mobilization of lathosterol away from the site of its generation by EBP. Consistently, levels of desmosterol - downstream of 24-dehydrolathosterol in the Bloch pathway - were significantly reduced in RAB18-null cells ($p<0.01$). These data suggest that delivery of substrates to post-EBP biosynthetic enzymes is impaired.

To further test our hypothesis, we used molecular dynamics (MD) simulations to model lathosterol binding to ORP2. We compared simulations of the ORP2 OSBP-related domain (ORD) incorporating cholesterol and lathosterol over 1µs (Figure 5E-G, Figure S7, Videos S1-2). Over this time, both simulations remained comparatively stable in terms of positional root-mean-square deviation (RMSD) (Figure S7A), with both termini highly dynamic. The pattern of positional root-mean-square fluctuations (RMSFs) for atoms in each sterol showed higher conformational heterogeneity for bound lathosterol than bound cholesterol, suggesting a less constrained interaction (Figure S7B). RMSFs for the ORP2-ORD amino acid residues show similar patterns for the two systems, although Tyr52 (Tyr110 in NP_653081), proximal to the sterol 3-hydroxyl, fluctuated less in the presence of lathosterol (Figure 5E). This residue appeared differently oriented with respect to the sterol in each simulation (Figure 5F, Videos S1-2). Indeed, for a large proportion of the span of the simulation with lathosterol, the Tyr52 hydroxyl and the sterol 3-hydroxyl were located close together and this distance remained relatively stable (Figure 5G).

In crystal structures of sterols with the OSBP-related protein Osh4, water-mediated interactions were prominent (Im et al., 2005). Therefore, we explored whether positioning of water molecules might differ between the two simulations. Interestingly, we found that water molecules were more frequently found in close proximity to lathosterol than the cholesterol (Figure S7C). Together, the MD simulations suggest that the ligand-binding tunnel of the ORP2-ORD adopts distinct conformations when bound to lathosterol or cholesterol despite their minor structural differences. The interactions appear relatively stable in each case, but speculatively, the interaction with lathosterol may be more entropically favourable.

We next reasoned that impaired delivery of substrates to post-EBP enzymes might reduce cholesterol biosynthesis in cells in which RAB18 is absent or dysregulated. To explore this possibility, we cultured the panel of HeLa cell lines for 24 hours in media supplemented with LPDS, treated them for 24 hours with [^{3}H]-mevalonate or

[^{3}H]-acetate, then quantified labelled cholesterol (Figure 5H). Under both conditions, labelling in two clonal wild-type controls was comparable, but was reduced in RAB18-, RAB3GAP1-, RAB3GAP2-, TBC1D20- and TRAPPC9-null cells. Labelling was lowest in the RAB18-null cells (39.5±2.5% and 6.8±0.5% of controls for [^{3}H]-mevalonate and [^{3}H]-acetate respectively). Levels in the cells of other genotypes were between 46±2.5%-73±5% for [^{3}H]-mevalonate and 23±2%-43±3%±.0.5% for [^{3}H]-acetate. These data strongly suggest that RAB18 and its regulators are required for normal cholesterol biosynthesis.

DISCUSSION

In this study, we have complemented previous work showing that proximity biotinylation is a powerful means of identifying candidate Rab effectors (Gillingham et al., 2019). Further – at least in the case of RAB18 - we have found that comparing biotin-labelling produced by a BirA*-Rab in wild-type and GEF-deficient cells can be particularly informative. We found that marked reductions in RAB18-association in RAB3GAP-null cells were restricted to only 25 proteins and that these comprised known and/or plausible effectors. We were able to exclude ~95% of RAB18-associations from consideration as more likely to represent 'noise' from bystander proteins.

Prior evidence supported 12 of the 25 interactions we detected. Independent experiments with a mutant RAB18 fusion protein confirmed nucleotide-binding-dependence of several interactors, and immunofuorescence confirmed compatible localizations of several more. The known functions of the proteins were consistent with previous work implicating RAB18 in coordination of lipid exchange between apposed membranes (Xu et al., 2018). Further, gene-disease associations showed substantial overlap with RAB18-deficiency/Warburg Micro syndrome. We have presented additional validation for six interactions, five of which are novel.

Our protein-interaction data implicate RAB18 in regulation of a stepwise process in which membrane/cytoskeletal remodelling precedes the engagement of tethering proteins and then SNAREs to establish membrane contact sites. At these sites, it then appears to couple the generation of lathosterol by EBP and its subsequent mobilization and delivery to downstream biosynthetic enzymes. The effectors most directly responsible for sterol mobilization are likely to be ORP2 and INPP5B. We propose that RAB18, ORP2 and INPP5B function in an analogous manner to the well studied ARF1-OSBP-SACM1L axis (Antonny et al., 2018). The GTPase (RAB18/ARF1) facilitates ORP (ORP2/OSBP)-mediated exchange of sterol (lathosterol/cholesterol) and phosphatidylinositde (PI(4,5)P_2/PI(4)P) between apposed membranes. The phosphatase (INPP5B/SACM1L) acts to maintain the necessary phosphoinositide concentration gradient (Figure 6).

The role of Rab proteins in mediating vesicular membrane trafficking is well known. Emerging evidence also suggests that regulation of nonvesicular lipid exchange at

membrane contact sites may likewise be a broadly conserved function. Several Rab isoforms are shown to interact with ORPs (Gillingham et al., 2019, Levin-Konigsberg et al., 2019, Rocha et al., 2009, Sobajima et al., 2018). Many are also found to interact with phospholipid phosphatases, including phosphatases from distinct protein families (Fukuda et al., 2008, Gillingham et al., 2019, Williams et al., 2007). Thus, the large number of possible GTPase-OSBP-phosphatase combinations provides a potential means to orchestrate mobilization of a multitude of lipid species. In this context, our finding that RAB18 also interacts with a biosynthetic enzyme and mediates mobilization of a specific sterol intermediate is suggestive. The connection between the enzyme and the mobilization machinery provides a mechanism conferring increased selectivity for the lipid cargo (Nishimura & Stefan, 2020).

Resolving the functional and compositional diversity of membrane contact sites is a challenge for future research. Similarly, the scope and precise mechanics of RAB18-regulated lipid exchange are not yet understood. Although our experiments have helped to validate our proximity-labelling approach, the roles of most of the RAB18-effectors identified require additional analyses. Among the lipid-related proteins, TMCO4 may potentially be directly or indirectly associated with sterol metabolism. Although its substrate(s) are unknown, its expression is found to be upregulated in hypercholesterolemia (Ong et al., 2013) and it is present on lipid rafts (Jin et al., 2012). The remaining lipid-related proteins, C2CD2L/TMEM24 and C2CD2, might potentially function in concert with ORP2 and/or INPP5B, since C2CD2L is found to mediate PI transport and to facilitate generation of PI(4,5)P_2 (Lees et al., 2017).

The possible substitution of SEC22B for SEC22A in a RAB18-regulated Syntaxin18 SNARE complex (Figure S8A), and a possible role for this complex in promoting membrane contacts rather than membrane fusion, is consistent with previous data (Xu et al., 2018). Further, it would be in keeping with roles for the NRZ/Dsl1 complex and SCFD2/Sly1 in dynamically orchestrating SNARE complex assembly (Ren et al., 2009, Spang, 2012, Yamaguchi et al., 2002). More ambiguously, the RAB18-interacting microtubule binding proteins have not previously been reported to work together but do function in compatible locations (Figure S8B). SPG20 and CAMSAP1 each associate with mitotic spindle poles, REEP4 participates in spindle-dependent ER clearance from metaphase chromatin and BICD2 is a component of the minus-end-directed dynein-dynactin motor complex (Hendershott & Vale, 2014,

Hueschen et al., 2017, Kumar et al., 2019, Lind et al., 2011, Nicholson et al., 2015, Schlaitz et al., 2013, Urnavicius et al., 2015). Our TRAPPII-dependent RAB18 interaction data indicate that different GEF complexes affect largely distinct subsets of interactions. However, more work will be required to determine whether these regulators mediate independent or interdependent functions.

Our objective in studying RAB18 was to better understand the molecular pathology of Warburg Micro syndrome. Though our protein-interaction data are relatively preliminary, our functional findings represent good progress towards this goal. Our key finding is that disrupted *de novo* cholesterol biosynthesis may contribute to disease pathogenesis. Strongly supporting this suggestion, genes encoding multiple cholesterol biosynthesis enzymes are linked to similar disorders (Platt et al., 2014). For example, pathogenic variants in the lathosterol oxidase gene, *SC5D*, cause lathosterolosis, which is associated with microcephaly, intellectual disability, micrognathia, high arched palate and cataract (Anderson et al., 2019, Brunetti-Pierri et al., 2002, Ho et al., 2014, Krakowiak et al., 2003, Rossi et al., 2007). Pathogenic variants in the 7-dehydrocholesterol reductase gene, *DHCR7*, cause Smith-Lemli-Opitz syndrome (SLOS), which has a similar spectrum of features and is among the top differential diagnoses for Micro syndrome (Handley & Sheridan, 2018, Nowaczyk & Wassif, 1998). Indeed, the similarities with SLOS were noted in the report first identifying RAB18 as a disease-associated gene nearly a decade ago (Bem et al., 2011).

Therapeutics have been trialled in lathosterolosis and SLOS. Cholesterol supplementation has been widely used and more recently is being combined with antioxidants with the aim of reducing toxicity from aberrant cholesterol metabolites (Fliesler et al., 2018, Korade et al., 2014, Svoboda et al., 2012). Additionally, paradoxical treatment with statins may increase the expression of sterol synthesizing enzymes (Correa-Cerro et al., 2006, Ho et al., 2014, Jira et al., 1997, Wassif et al., 2017). Careful clinical research will be required to determine the safety and efficacy of such interventions in Warburg Micro syndrome.

MATERIALS AND METHODS

Plasmids

The EGFP-RAB18 construct has been described previously (Gerondopoulos et al., 2014). The RAB18 sequence was excised from this construct using BamHI and HindIII restriction enzymes (New England Biolabs, Hitchin, UK), and used to generate constructs encoding mEmerald-RAB18 and mCherry-RAB18 by ligation into mEmerald-C1 and mCherry-C1 vectors (Addgene, Watertown, MA) using HC T4 Ligase and rapid ligation buffer (Promega, Southampton, UK). Constructs encoding BirA*-RAB18, BioID2(Gly40Ser)-SEC22A and mEmerald-SEC22A were generated following PCR amplification from template and subcloning into an intermediate pCR-Blunt II-TOPO vector using a Zero Blunt TOPO PCR Cloning Kit (ThermoFisher Scientific, Waltham, MA) according to manufacturer's instructions. Fragments were excised from intermediate vectors and then subcloned into target vectors using restriction-ligation, as above. A construct encoding mCherry-ER was obtained from Addgene, and a construct encoding TMCO4-EGFP was synthesised and cloned by GeneWiz (Leipzig, Germany). Generation of recombinant pX461 and pX462 plasmids for CRISPR gene-editing, and recombinant pCMV vectors for preparation of stable CHO cell lines are described below. Generation of recombinant pcDNA5 FRT/TO FLAG-BirA(Arg118Gly) vectors for preparation of stable T-Rex-293 cell lines is described in supplementary methods. Details of PCR templates, primers and target vectors are listed in Table S6.

Antibodies and reagents

A custom polyclonal antibody to RAB18 generated by Eurogentec (Southampton, UK) has been described previously (Handley et al., 2015). An antibody to RAB3GAP1 was obtained from Bethyl Labs (Montgomery, TX), an antibody to GFP was obtained from Takara Bio (Saint-Germain-en-Laye, France), an antibody to β-Tubulin was obtained from Abcam (Cambridge, UK) and an antibody to β-Actin was obtained from ThermoFisher. Antibodies to hemagglutinin (HA), RAB3GAP2 and TBC1D20 were obtained from Merck (Gillingham, UK). Antibodies to ZW10, STX18, SPG20, RINT1, REEP4, BNIP1, C2CD2, TRIM13, WFS1, INPP5B, OSBPL2 and

NBAS were obtained from Proteintech (Manchester, UK). Antibody catalogue numbers and the dilutions used in this study are listed in Table S6.

Cell culture

HeLa, T-REx-293 and IHH cells were maintained in DMEM media, RPE1 cells in DMEM/F12 media and CHO cells in alpha-MEM media (ThermoFisher). In each case, media was supplemented with 10% foetal calf serum (FCS) and 1% penicillin-streptomycin (PS). Cells were maintained at 37°C and 5% CO_2.

Generation of clonal 'knockout' HeLa and RPE1 cell lines

CRISPR/Cas9 gene-editing was carried out essentially as described in Ran et al., 2013 (Ran et al., 2013). Guide RNA (gRNA) sequences are shown in Table S6. A list of the clonal cell lines generated for this study, together with the loss-of-function variants they carry is shown in Figure S1A. Western blot validation is shown in Figure S1B-E. Briefly, for each targeted exon, pairs of gRNA sequences were selected using the online CRISPR design tool (http://crispr.mit.edu/). Oligonucleotide pairs incorporating these sequences (Sigma) were annealed (at 50mM ea.) in 10mM Tris pH8, 50mM NaCl and 1mM EDTA by incubation at 95ºC for 10 minutes followed by cooling to room temperature. Annealed oligonucleotides were diluted and ligated into BbsI-digested pX461 and pX462 plasmids (Addgene) using HC T4 Ligase and rapid ligation buffer (Promega). Sequences of all recombinant plasmids were verified by direct sequencing. Pairs of plasmids were contransfected into cells using Lipofectamine 2000 reagent according to manufacturer's instructions. Cells were selected for puromycin resistance (conferred by pX462) using 24 hours puromycin-treatment. Following 12 hours recovery, they were selected for GFP fluorescence (conferred by pX461) and cloned using FACSAria2 SORP, Influx or FACSMelody instruments (BD, Wokingham, UK). After sufficient growth, clones were analysed by PCR of the targeted exons (Primers are listed in Table S6). In order to sequence individual gene-edited alleles, PCR products from each clone were first cloned into ZeroBlunt TOPO vector (ThermoFisher) and then subjected to colony PCR. These PCR products were then analysed by direct sequencing. Sequencing data was assessed using BioEdit software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html).

BirA*/BioID proximity labelling (HeLa cells)

Proximity-labelling in HeLa cells was carried out largely as described by Roux et al. (Roux et al., 2012), but with minor modifications. HeLa cells were grown to 80% confluence in T75 flasks and then each flask was transfected with 1-1.5µg of the BirA*-RAB18 construct or 1µg of the BioID2(Gly40Ser)-SEC22A construct using Lipofectamine 2000 reagent in Optimem serum-free medium (ThermoFisher) for 4 hours, according to manufacturer's instructions. 24 hours post-transfection, media was replaced with fresh media containing 50µM Biotin (Merck) and the cells were incubated for a further 24 or 6 hours (for BirA*-RAB18 and BioID2(Gly40Ser)-SEC22A experiments respectively). Cells were then trypsinised and washed twice in PBS before pellets were transferred to 2ml microcentrifuge tubes and snap-frozen. For each pellet, lysis was carried out in 420µl of a buffer containing 0.2% SDS, 6% Triton-X-100, 500mM NaCl, 1mM DTT, EDTA-free protease-inhibitor solution (Expedeon, Cambridge, UK), 50mM Tris pH7.4. Lysates were sonicated for 10 minutes using a Bioruptor device together with protein extraction beads (Diagenode, Denville, NJ). Each lysate was diluted with 1080µl 50mM Tris pH7.4, and they were then clarified by centrifugation at 20 000xg for 30 minutes at 4ºC. Affinity purification of biotinylated proteins was carried out by incubation of clarified lysates with streptavidin-coated magnetic Dynabeads (ThermoFisher) for 24 hours at 4ºC. Note that a mixture of Dynabeads - MyOne C1, MyOne T1, M270 and M280 – was used to overcome a problem with bead-clumping observed when MyOne C1 beads were used alone. Successive washes were carried out at room temperature with 2% SDS, a buffer containing 1% Triton-X-100, 1mM EDTA, 500mM NaCl, 50mM HEPES pH7.5, a buffer containing 0.5% NP40, 1mM EDTA, 250mM LiCl, 10mM Tris pH7.4, 50mM Tris pH7.4, and 50mM ammonium bicarbonate.

Mass spectrometry

Washed beads from BioID experiments with HeLa cells were subjected to limited proteolysis by trypsin (0.3 µg) at 27°C for 6.5hours in 2mM urea, 1mM DTT, 75mM Tris, pH=8.5, then supernatants were incubated overnight at 37°C. Samples were alkylated with 50mM iodoacetamide (IAA) in the dark for 20minutes, then acidified by addition of 8µl 10% trifluoroacetic acid (TFA).

Peptides were loaded on to activated (methanol), equilibrated (0.1% TFA) C18 stage tips before being washed with 0.1% TFA and eluted with 0.1% TFA/80 acetonitrile. The organic was dried off, 0.1% TFA added to 15 µl and 5 µl injected onto LC-MS. Peptides were separated on an Ultimate nano HPLC instrument (ThermoFisher), and analysed on either an Orbitrap Lumos or a Q Exactive Plus instrument (ThermoFisher). After data-dependent acquisition of HCD fragmentation spectra, data were analysed using MaxQuant and the uniprot human reference proteome. Versions, releases, parameters and gradients used for separation are provided in Table S6.

Cell labelling

In order to distinguish cells of different genotypes within the same well/on the same coverslip, CellTrace Violet and CellTrace Far Red reagents (ThermoFisher) were used to label cells before they were seeded. Cells of different genotypes were first trypsinised and washed with PBS separately. They were then stained in suspension by incubation with either 1µM CellTrace Violet or 200nM CellTrace Far Red for 20 minutes at 37°C. Remaining dye was removed by addition of a ten-fold excess of full media, incubation for a further 5 minutes, and then by centrifugation and resuspension of the resulting pellets in fresh media. Differently-labelled cells were combined prior to seeding.

Immunofluorescence microscopy

Cells were seeded in 96-well glass-bottom plates (PerkinElmer, Waltham, MA) coated with Matrigel (Corning, Amsterdam, Netherlands) according to manufacturer's instructions, and allowed to adhere for 48 hours prior to fixation. In lipid-loading experiments, cells were treated with 200µM oleic acid complexed to albumin (Merck) and 1µg/ml BODIPY-558/568-C12 (ThermoFisher) for 15 hours prior to fixation. Cells were fixed using a solution of 3% deionised Glyoxal, 20% EtOH, 0.75% acetic acid, pH=5 (Richter et al., 2018), for 20 minutes at room temperature. They were then washed with PBS containing 0.9mM $CaCl_2$ and 0.5mM $MgCl_2$ and blocked with a sterile-filtered buffer containing 1% Milk, 2% donkey serum (Merck), 0.05% Triton-X-100 (Merck), 0.9mM $CaCl_2$ and 0.5mM $MgCl_2$ in PBS pH=7.4 for at least 1 hour prior

to incubation with primary antibody. Primary antibodies were added in blocking buffer without Triton-X-100, and plates were incubated overnight at 4ºC. Antibody dilutions are listed in Table S6. Following washing in PBS, cells were incubated with 1:2000 Alexa 488-conjugated secondary antibody (ThermoFisher) in blocking buffer at room temperature for 1-2 hours. Following further washing in PBS, cells were imaged using an Operetta High Content Imaging System (PerkinElmer) equipped with Harmony software. In comparative fluorescence quantitation experiments, at least 18 frames – each containing >5 wild-type and >5 mutant cells – were analysed per genotype. ImageJ software was used to produce regions of interest (ROIs) corresponding to each cell using thresholding tools and images from the 405nm and 645nm channels. Median 490nm fluorescence intensity was measured for each cell and mutant fluorescence intensity (as %wild-type) was calculated for each frame and combined for each genotype.

Confocal microscopy – Live cell imaging

HeLa or RPE1 cells were seeded on glass-bottom dishes (World Precision Instruments, Hitchin, UK) coated with Matrigel (Corning) and allowed to adhere for 24 hours prior to transfection. Transfections and cotransfections were carried out with 0.5µg of each of the indicated constructs using Lipofectamine 2000 reagent in Optimem serum-free medium for 4 hours, according to manufacturer's instructions. Media were replaced and cells were allowed to recover for at least 18 hours prior to imaging. Imaging was carried out on a Nikon A1R confocal microscope equipped with the Nikon Perfect Focus System using a 60× oil immersion objective with a 1.4 numerical aperture. The pinhole was set to airy1. CellTrace Violet was excited using a 403.5nm laser, and emitted light was collected at 425–475nm. EGFP and mEmerald were excited using a 488 nm laser, and emitted light was collected at 500–550 nm. mCherry was excited using a 561.3 nm laser, and emitted light was collected at 570–620 nm. CellTrace Far Red was excited using a 638nm laser, and emitted light was collected at 663-738nm.

Immunoprecipitation

HeLa cells were seeded onto 10cm dishes and allowed to adhere for 24 hours prior to transfection. Transfections and cotransfections were carried out with 0.5µg of each of the indicated constructs using Lipofectamine 2000 reagent in Optimem serum-free medium for 4 hours, according to manufacturer's instructions. 24 hours post-transfection cells were trypsinised, washed with PBS, then lysed in a buffer containing 150mM NaCl, 0.5% Triton-X-100 and EDTA-free protease-inhibitor solution (Expedeon), 10mM Tris, pH=7.4. Lysates were clarified by centrifugation, input samples taken, and the remaining supernatants then added to 4µg rabbit anti-HA antibody (Merck). After 30 minutes incubation at 4ºC on a rotator, 100µl washed protein G-coupled Dynabeads (ThermoFisher) were added and samples were incubated for a further 1 hour. The Dynabeads were washed x3 with buffer containing 150mM NaCl, 0.1% Triton-X-100, 10mM Tris, pH=7.4, then combined with a reducing loading buffer and subjected to SDS–PAGE.

<u>Generation of stable CHO cell lines</u>

A PCR product encoding mouse RAB18 was subcloned into an intermediate TOPO vector using a TOPO PCR Cloning Kit (ThermoFisher) according to manufacturer's instructions. The RAB18 fragment was then excised and subcloned into the pCMV vector. PCR-based site-directed mutagenesis using a GeneArt kit (ThermoFisher) was then used to generate pCMV-RAB18(Gln67Leu) and pCMV-RAB18(Ser22Asn) constructs. CHO cells were transfected using Lipofectamine 2000 reagent (ThermoFisher) and cells stably-expressing each construct were selected-for with blasticidin. Under continued selection, clonal cell-lines were grown from single cells and then RAB18 protein expression was assessed. Cell lines comparably expressing RAB18 constructs at levels 2.5-5x higher than those wild-type cells were used in subsequent experiments.

<u>Lipid loading experiments</u>

For LD number and diameter measurements, IHH cells were seeded onto glass coverslips. siRNA transfections were carried out using FuGene reagent (Promega) according to manufacturer's instructions. siRNAs targeting ZW10 and NBAS were

obtained from IDT, Coralville, IA; siRNA targeting SEC22A was obtained from Horizon Discovery, Cambridge, UK. 48 hours following transfection, cells were treated with 200nM BSA conjugated oleate for 24 hours. Coverslips were washed, fixed with 3% paraformaldehyde and stained with 1µg/mL BODIPY and 300nM DAPI. Fluorescence images were captured on a Zeiss LSM 780 confocal microscope equipped with a 100x objective. Images were analysed using ImageJ software. Data are derived from measurements from >100 cells/condition and are representative of three independent experiments.

For cholesterol storage and efflux experiments with [^{14}C]-oleate, CHO cell lines (described above) were seeded onto 12-well plates and then grown to 60-75% confluence in Alpha media supplemented with 10% LPDS. Cells were grown in the presence of 10% LPDS for at least 24 hours prior to the addition of oleate. 1 µCi/ml [^{14}C]-oleate (Perkin Elmer) was added in the presence of 10% LPDS or 10% FBS for 24 hours. Cells were then washed and incubated with 50µg/ml HDL for 0, 4 or 8 hours. Cellular lipids were extracted with hexane. Lipids were then dried-down and separated by thin layer chromatography (TLC) in a hexane:diethyl ether:acetic acid (80:20:2) solvent system. TLC plates were obtained from Analtech, Newark, NJ. Bands corresponding to cholesteryl ester (CE) were scraped from the TLC plate, and radioactivity was determined by scintillation counting in a Beckman Coulter LS6500 Scintillation Counter using BetaMax ES Liquid Scintillation Cocktail (ThermoFisher). Three independent experiments were carried out, each with four replicates of each condition. Data from a representative experiment are shown.

For cholesterol efflux experiments with [^{3}H]-cholesterol, CHO cells were seeded onto 12-well plates and then grown to 60% confluence in Alpha media supplemented with 10% FBS. 5 µCi/ml [^{3}H]-cholesterol (PerkinElmer) was added in the presence of 10% FBS. After 3x PBS washes, cells were incubated with serum-free media containing 25µg/ml of human apolipoprotein A-I (ApoA-I) for 5 hours. ApoA-I was a kind gift of Dr. Paul Weers (California State University, Long Beach). Radioactivity in aliquots of media were determined by scintillation counting in a Beckman Coulter LS6500 Scintillation Counter using LSC Cocktail (PerkinElmer). Cell lysates were produced by addition of 0.1N NaOH for 1 hour, and their radioactivity was determined as above. Cholesterol efflux was calculated as an average (+/- SD) of the % cholesterol efflux (as a ratio of the media cpm/(media + cellular cpm) x 100%).

Sterol analysis

HeLa cells were grown to 80% confluence in T75 flasks, washed twice in PBS and then grown for a further 48 hours in DMEM supplemented with 10% LPDS. They were then trypsinised and washed twice in PBS before pellets were transferred to microcentrifuge tubes and snap-frozen. Pellets were resuspended in 200µl deionised water, sonicated for 20 seconds using an ultrasonic processor (Sonics & Materials Inc., CT, USA), then placed on ice. 750 µl of isopropanol containing 4 µmol/L 5α-cholestane as an internal standard was added to each sample, and then each was sonicated for a further 10 seconds. Lysates were transferred to 7ml glass vials and mixed with 250 µl tetramethylammonium hydroxide for alkalyine saponification at 80°C for 15 min, then cooled down for 10 minutes at room temperature. Sterols were extracted by addition of 500 µl tetrachloroethylene/methyl butyrate (1:3) and 2 ml deionised water, then thorough mixing. Samples were centrifuged for 10 minutes at 3000 rpm, and the organic phase containing the sterols was transferred to 300 µl GC vials. Extracts were dried under a stream of nitrogen, then sterols were silylated with 50 µL Tri-Sil HTP (HDMS:TMCS:Pyridine) Reagent (ThermoFisher) at 60°C for 1 hour.

Chromatography separation was performed on an Agilent gas chromatography-mass spectrometry (GC-MS) system (6890A GC and 5973 MS) (Agilent Technologies, Inc., CA, USA) with an HP-1MS capillary column (30 m length. x 250 µm diameter x 0.25 µm film thickness). The GC temperature gradient was as follows: Initial temperature of 120°C increased to 200°C at a rate of 20°C/min, then increased to 300°C at a rate of 2°C/min with a 15 minute solvent delay. Injection was at 250°C in splitless mode with ultrapurified helium as the carrier gas and the transfer line was 280°C. The mass spectra were acquired by electron impact at 70 eV using selected ion monitoring as follows: Lathosterol-TMS, cholesterol-TMS, and cholest8(9)-enol-TMS: m/z 458; 5α-cholestane and desmosterol-TMS: m/z 372; Lanosterol-TMS: m/z 393; and 7-dehydrocholesterol-TMS: m/z 325. The data were analysed using MassHunter Workstation Quantitative Analysis Software (Agilent Technologies, Inc.) and OriginPro 2017 (OriginLab Corp, MA, USA).

Molecular dynamics

Simulations were constructed as previously described, starting from a modified version of the OSBPL2 lipid-binding domain structure (PDB 5ZM8)(Wang et al., 2019). Parameters for lathosterol were obtained by modifying those of cholesterol to substituting the double bond between C5-C6 with one between C7-C8. Simulations were performed with the CHARMM36 force field.

Western blotting

Cell lysates were made with a buffer containing 150mM NaCl, 0.5% Triton-X-100 and EDTA-free protease-inhibitor solution (Expedeon), 50mM Tris, pH=7.4. Cell lysates and input samples from BioID and immunoprecipitation experiments were combined 1:1 with a 2x reducing loading buffer; a reducing loading buffer containing 10mM EDTA was added directly to Dynabead samples. SDS–PAGE and Western blotting were carried out according to standard methods.

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COMPETING INTERESTS

No competing interests declared.

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DATA AVAILABILITY

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Perez-Riverol et al., 2019) partner repository with the dataset identifiers PXD016631, PXD016336, PXD016326, PXD016233 and PXD016404.

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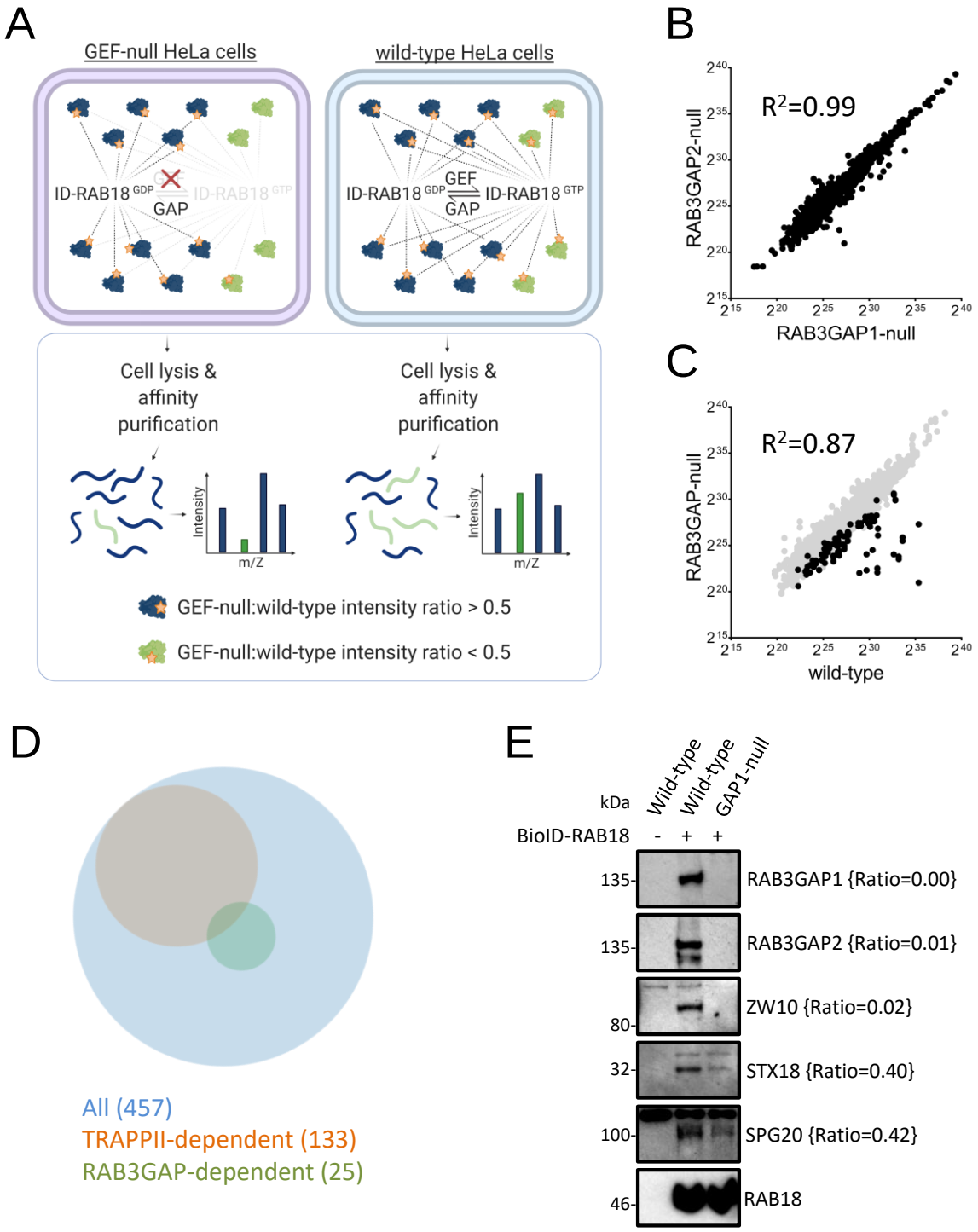

Figure 1. RAB3GAP-dependent RAB18-interactions in HeLa cells. (A) Schematic to show experimental approach. Proximity biotinylation of guanine nucleotide exchange factor (GEF)-dependent interactors by BirA*-RAB18 (ID-RAB18) is disrupted in GEF-null cells. GEF-independent interactors are biotinylated in both GEF-null and wild-type cells. Following affinity purification, GEF-dependent

interactions are determined by LFQ intensity ratios. (B) Plot to show correlation between Log_2 LFQ intensities of individual proteins identified in samples purified from RAB3GAP1- and RAB3GAP2-null cells. (C) Plot to show correlation between Log_2 LFQ intensities of individual proteins identified in samples purified from wild-type and RAB3GAP-null cells. Highlighted datapoints correspond to proteins later found to have RAB3GAP-null:wild-type intensity ratios <0.5. (D) Venn diagram to show overlap between all RAB18-associations, TRAPPII-dependent interactions (TRAPPC9-null:wild-type intensity ratios <0.5) and RAB3GAP-dependent associations (RAB3GAP-null:wild-type intensity ratios <0.5). (E) Western blotting of samples purified from wild-type and RAB3GAP1-null cells in an independent BioID experiment. Levels of selected proteins are consistent with RAB3GAP-null:wild-type intensity ratios {braces}.

Protein	n	Ratio	Orthologue PPI (Gillingham et al., 2014)	Additional evidence	
CAMSAP1	3	0.26			**Microtubule/membrane remodelling**
REEP4	3	0.35		Tinti et al., 2012*	
BICD2	2	0.25	BicD	Gillingham et al., 2019	
SPG20	**2**	**0.42**	**CG12001**	**This study (Figure 2)**	
ZW10	3	0.02	mit(1)15	Xu et al., 2018; Gillingham et al., 2019	**Membrane tethering/docking**
RINT1	3	0.16	CG8605	Xu et al., 2018	
NBAS	3	0.17	rod	Xu et al., 2018; Gillingham et al., 2019	
SCFD2	3	0.41	Slh	Gillingham et al., 2019	
SEC22A	**3**	**0.46**		**This study (Figure 3)**	
BNIP1	2	0.36		Xu et al., 2018	
STX18	2	0.40	Syx18	Xu et al., 2018	
TMCO4	**3**	**0.06**		**This study (Figure 4)**	**Lipid modifying/ mobilising**
OSBPL2	**3**	**0.35**		**This study (Figure 5)**	
EBP	**3**	**0.38**		**This study (Figure 5)**	
INPP5B	**2**	**0.00**		**This study (Figure 5)**	
C2CD2L	2	0.22			
C2CD2	2	0.36			
TRIM13	2	0.29			**Autophagy receptors**
FAM134B	2	0.46			
RAB3GAP2	3	0.00	rab3-GAP	Gerondopoulos et al., 2014	**Other**
RAB3GAP1	3	0.01	CG31935	Gerondopoulos et al., 2014	
SSR3	3	0.49			
MFHAS1	2	0.00	lrrk		
WFS1	2	0.00			
SCARA3	2	0.42			

Table 1. RAB3GAP-dependent RAB18-interactions in HeLa cells. 25 proteins with mean RAB3GAP-null:wild-type intensity ratios <0.5, identified in two or more independent proximity biotinylation experiments. Orthologous proteins identified by Gillingham et al., 2014, and other studies providing supporting evidence for interactions are shown. Proteins are grouped according to their reported functions. The full dataset is provided in Table S1.

Gene(s)	Homologue(s)	Syndrome(s)	Inheritance	OMIM	Overlapping features
RAB3GAP1, RAB3GAP2, RAB18, TBC1D20	-	Warburg Micro syndrome; Martsolf syndrome	AR	600118, 614222, 614225, 615663; 212720	intellectual disability (ID), microcephaly (M), ascending spastic paraplegia (ASP), cataract (C), microphthalmia (Mo), microcornea (Mc), optic atrophy (OA), seizures (S), corpus callosum hypogenesis (CCH), cerebellar vermis hypoplasia (CVH), genital abnormalities (GA), Neuropathy (N)
EBP	-	CDPX2; MEND syndrome	XLD; XLR	302960; 300960	ID, M, C, Mo, Mc, S, CCH, CVH, GA
INPP5B	*OCRL*; *INPP5K*	Lowe syndrome; MDCCAID	XLR; AR	309000; 607875	ID, M, C, S
SSR3	-	Congenital disorder of glycosylation	AR	Ng et al., 2019	ID, M, CCH, GA
WFS1	-	Wolfram syndrome; CTRCT41	AR; AD	222300; 116400	C, OA, GA
SPG20	-	Troyer syndrome (SPG20)	AR	275900	ID, M, ASP
BICD2	-	Spinal muscular atrophy	AD	615290; 615291	ASP
REEP4	*REEP1*; *REEP2*	SPG31; SPG72	AD	610250; 615625	ASP
NBAS	-	SOPH syndrome	AR	614800	OA
FAM134B	-	HSAN IIB	AR	613115	N

Table 2. Genes encoding putative RAB18 effectors or their homologues are associated with diseases that share overlapping features with Warburg Micro syndrome. AR, autosomal recessive; XLD, X-linked dominant; XLR, X-linked recessive; AD, autosomal dominant.

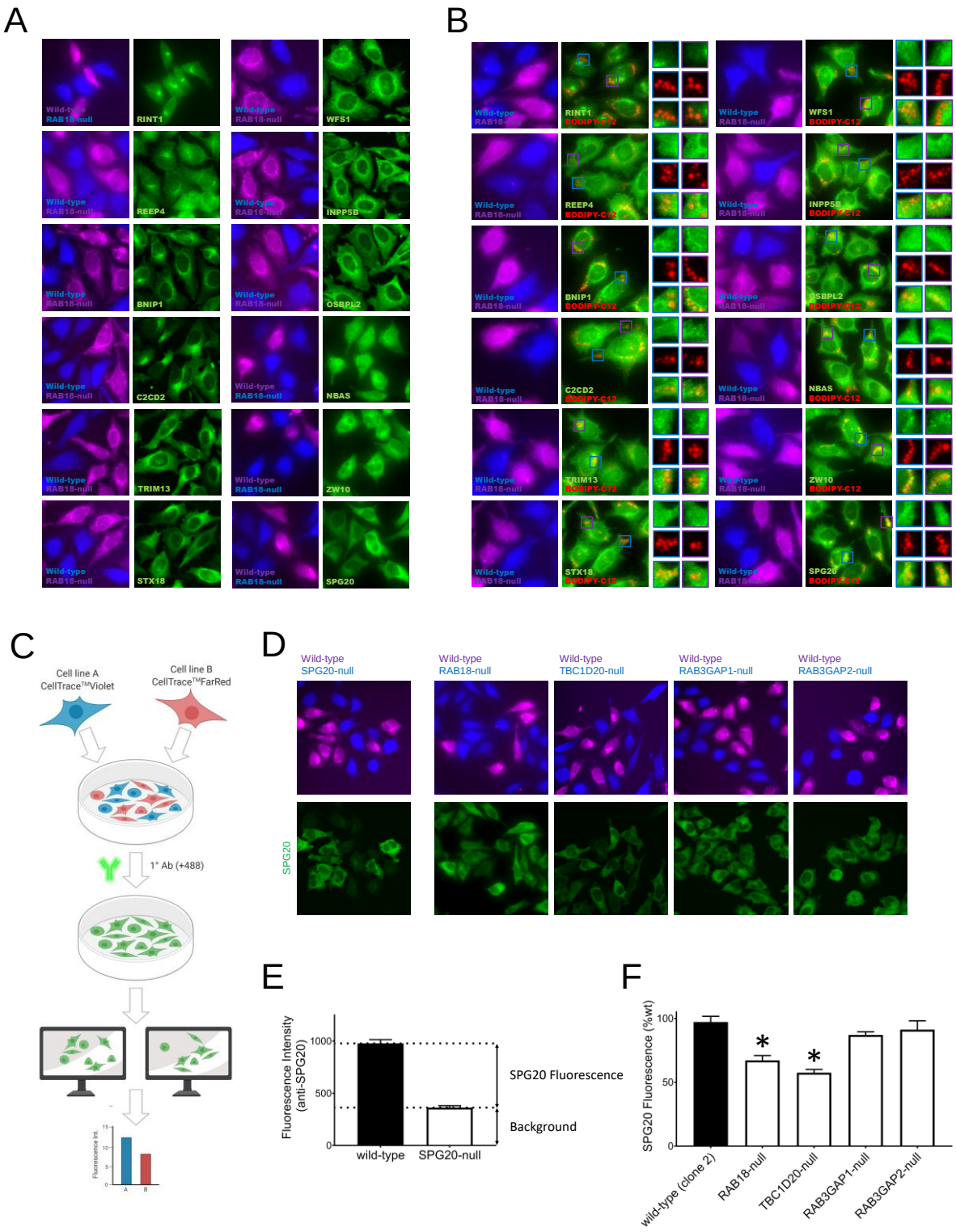

Figure 2. Initial screening of putative RAB18 effectors reveals that levels of SPG20 are significantly reduced in RAB18-null and TBC1D20-null cells. (A) Comparative fluorescence microscopy of selected RAB18-associated proteins in wild-type and RAB18-null HeLa cells. Cells of different genotypes were labelled with

CellTrace-Violet and CellTrace-Far Red reagents, corresponding to blue and magenta channels respectively. Cells were stained with antibodies against indicated proteins in green channel panels. (B) Comparative fluorescence microscopy of selected RAB18-associated proteins in lipid-loaded wild-type and RAB18-null HeLa cells. Cells were stained as above but were treated for 15 hours with 200µM oleic acid, 1µg/ml BODIPY-558/568-C12 (Red channel) prior to fixation. (C) Schematic to show method for quantification of protein levels by fluorescence intensity. In each frame, cell areas for each genotype are generated by thresholding CellTrace channels, intensity of antibody-staining is measured for each cell in multiple frames. (D) Example frames showing wild-type and mutant cells of the indicated genotypes, labelled with CellTrace-Far Red and CellTrace-Violet and reagents respectively, then stained for SPG20. (E) Quantification of SPG20-specific fluorescence in wild-type cells by direct comparison with SPG20-null cells. (F) Quantification of SPG20 fluorescence (%wt) in cells of different genotypes. Data were derived from analysis of at least 18 frames – each containing >5 wild-type and >5 mutant cells – per genotype. Error bars represent s.e.m. *$p<0.001$.

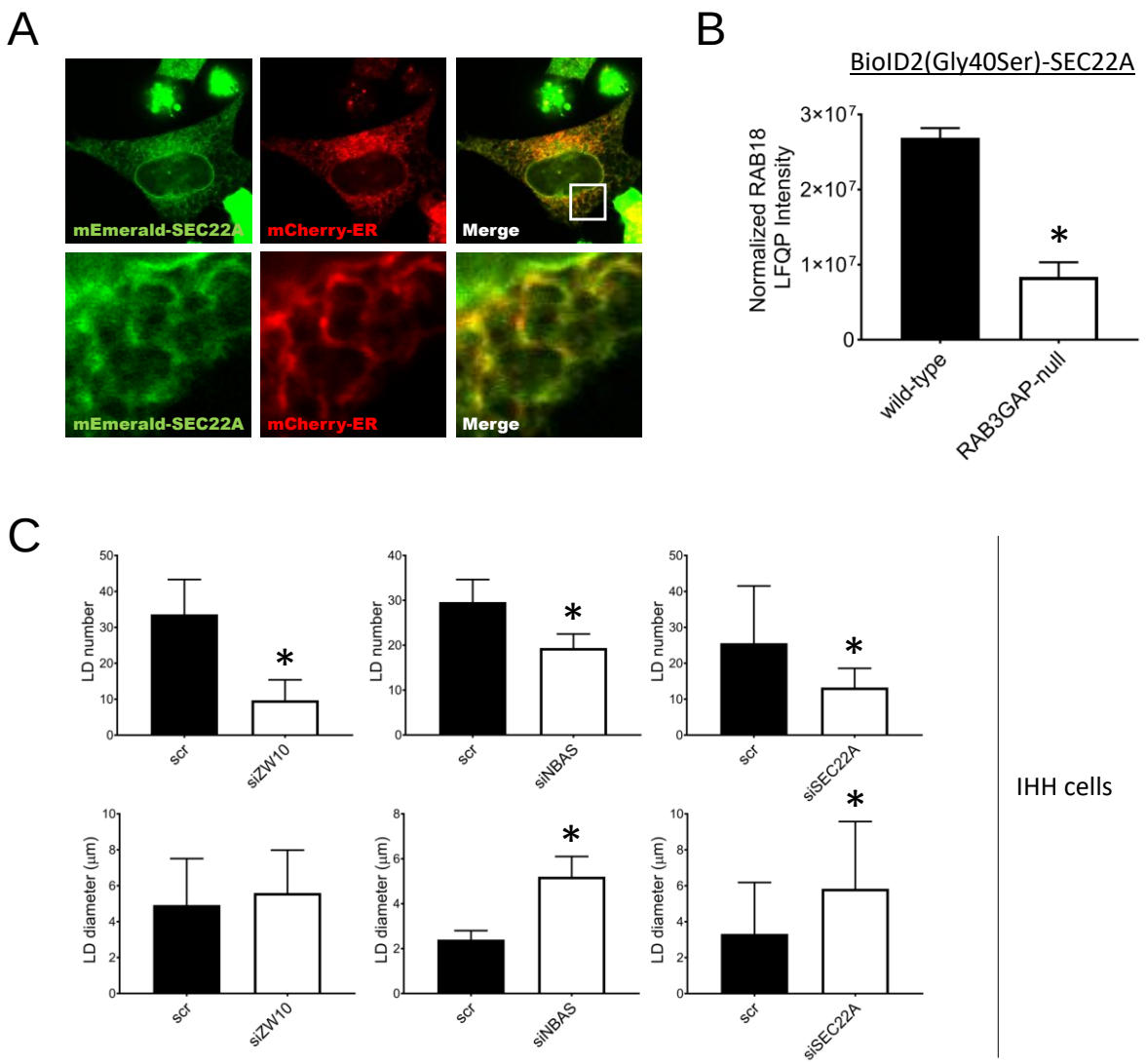

Figure 3. SEC22A associates with RAB18 and influences LD morphology. (A) Confocal micrograph to show overlapping localization of exogenous mEmerald-SEC22A (Green) and mCherry-ER (Red) in HeLa cells. (B) RAB18 LFQ intensities from a reciprocal BioID experiment showing a reduced association between BioID2(Gly40Ser)-SEC22A and endogenous RAB18 in RAB3GAP-null compared to wild-type HeLa cells. Data were adjusted to account for non-specific binding of RAB18 to beads and normalized by SEC22A LFQ intensities in each replicate experiment. Error bars represent s.e.m. Data for other BioID2(Gly40Ser)-SEC22A-associated proteins are provided in table S5. (C) Bar graphs to show effects of ZW10, NBAS and SEC22A knockdowns on lipid droplet number and diameter. siRNA-treated IHH cells were loaded with 200nM BSA-conjugated oleate, fixed and stained with BODIPY and DAPI, and imaged. Images were analysed using ImageJ.

Data are derived from measurements from >100 cells/condition and are representative of three independent experiments. Error bars represent SD. *p<0.001

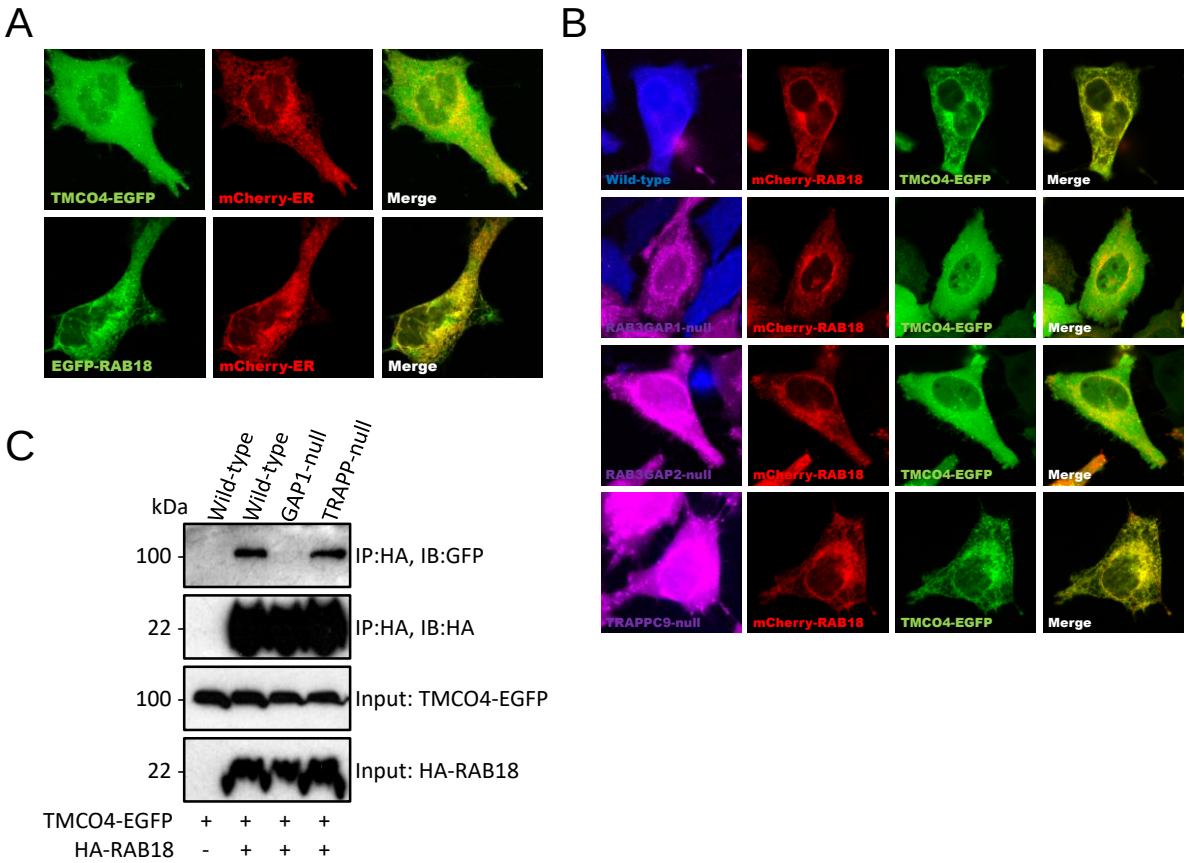

Figure 4. mCherry-RAB18 recruits TMCO4-EGFP to the ER membrane in a RAB3GAP-dependent manner. (A) Confocal micrographs to show diffuse localization of exogenous TMCO4-EGFP (Green) compared to mCherry-ER (Red) and overlapping localization of exogenous EGFP-RAB18 (Green) and mCherry-ER in HeLa cells. (B) Confocal micrographs to show localization of exogenous mCherry-RAB18 and TMCO4-EGFP in wild-type cells and in mutant cells of different genotypes. Wild-type and mutant cells of the indicated genotypes were labelled with CellTrace-Violet and CellTrace-Far Red reagents respectively (magenta and blue channels). (C) Immunoprecipitation of exogenous HA-RAB18 from HeLa cells of different genotypes. Cells were transfected with the indicated constructs and lysed 24 hours post-transfection. Anti-HA immunoprecipitates and input samples were subjected to SDS-PAGE and immunostaining for HA and GFP.

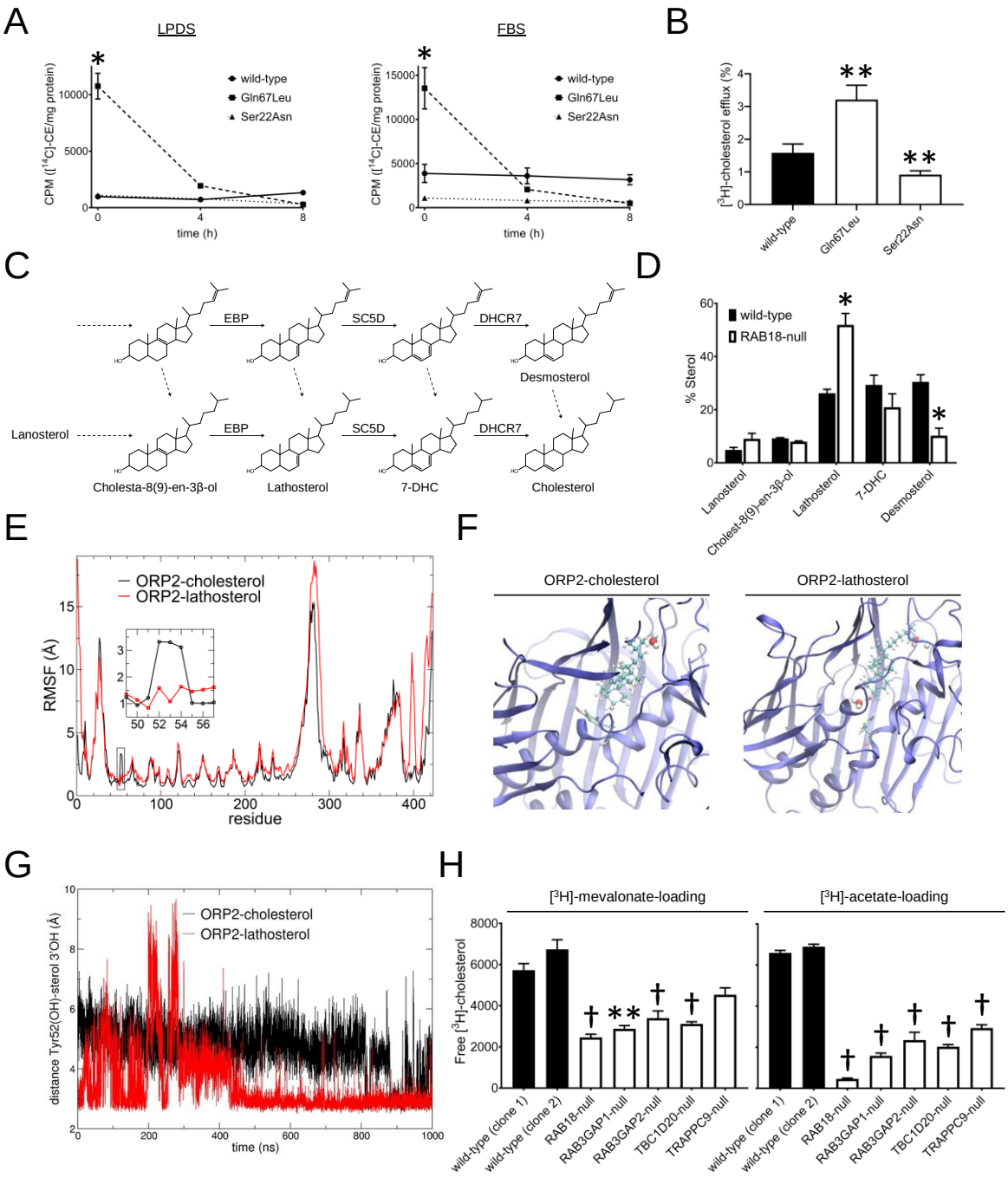

Figure 5. RAB18 is involved in the mobilization and biosynthesis of cholesterol. (A) Plots to show cholesteryl ester (CE) loading and efflux. CHO cells, stably expressing RAB18(WT), RAB18(Gln67Leu) and RAB18(Ser22Asn), were incubated with [^{14}C]-oleate, for 24 hours, in the presence of lipoprotein depleted serum (LPDS)(Left panel) or FBS (Right panel). Following lipid extraction, thin layer chromatography (TLC) was used to separate CE, and radioactivity was measured by scintillation counting. Measurements were made at t=0 and at 4 and 8 hours

following the addition of 50µg/ml high density lipoprotein (HDL) to the cells. (B) Bar graph to show cholesterol efflux. CHO cells were incubated with [^{3}H]-cholesterol, for 24 hours, in the presence of FBS. After washing, they were incubated with 25µg/ml apolipoprotein A-I for 5 hours. The quantity of [^{3}H]-cholesterol in the media is shown as a percentage of the total cellular radioactivity (mean±SD). (C) Schematic of post-squaline cholesterol biosynthesis pathway with the sterols quantified by GC-MS named. Solid arrows indicate biosynthetic steps catalysed by EBP, SC5D and DHCR7. (D) Bar graph of sterols profile in wild-type and RAB18-null HeLa cells. Cells were grown in media supplemented with LPDS for 48 hours. Extracted sterols were analysed by gas chromatography-mass spectrometry (GC-MS). % Sterol was calculated as a proportion of total quantified sterols, excluding cholesterol, following normalization to a 5α-cholestane internal standard. n=3; ±s.e.m. (E) Positional root mean square fluctuations (RSMFs) for amino acid residues in simulations of ORP2-ORD complexed with cholesterol and lathosterol. Box and arrow show position of Tyr52 (Tyr110 in NP_653081). Simulations used a structure of the ORP2-ORD based on PDB 5ZM8. Parameters for lathosterol substituted the cholesterol double bond between C5-C6 with one between C7-C8. Simulations were performed with the CHARMM36 force field. (F) Still images from molecular dynamics simulations of the ORP2-ORD complexed with cholesterol (left) and lathosterol (right). Position of ORP2 Tyr110 and sterol-proximal water molecules are shown. (G) Plot to show the distance between Tyr52 and sterol 3’ hydroxyl groups over time in each simulation. (H) Bar graphs to show incorporation of [^{3}H]-mevalonate and [^{3}H]-acetate into cholesterol in a panel of HeLa cell lines. Cells were grown in media supplemented with LPDS for 24 hours, then incubated with 5µCi/well [^{3}H]-mevalonate or 10µCi/well [^{3}H]-acetate for 24 hours. TLC was used to separate free cholesterol and radioactivity was quantified by scintillation counting (n=4; mean±SD). *$p<0.01$, **$p<0.001$, †$p<0.0005$.

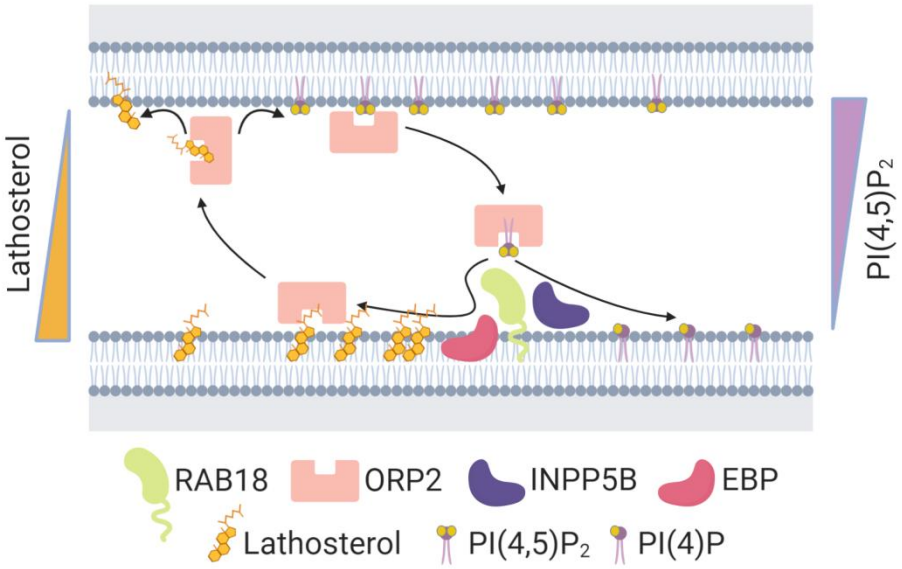

Figure 6. Model for lathosterol mobilization mediated by RAB18. ORP2 binds PI(4,5)P2 on an apposed membrane. RAB18 interacts with ORP2 and INPP5B promoting the hydrolysis of PI(4,5)P2 to PI(4)P and maintaining a PI(4,5)P2 concentration gradient. RAB18 then coordinates the biosynthesis of lathosterol by EBP and subsequent lathosterol mobilization by ORP2.