LETTERS

Functional genomics reveals genes involved in protein secretion and Golgi organization

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Yeast genetics and *in vitro* biochemical analysis have identified numerous genes involved in protein secretion[1,2]. As compared with yeast, however, the metazoan secretory pathway is more complex and many mechanisms that regulate organization of the Golgi apparatus remain poorly characterized. We performed a genome-wide RNA-mediated interference screen in a *Drosophila* cell line to identify genes required for constitutive protein secretion. We then classified the genes on the basis of the effect of their depletion on organization of the Golgi membranes. Here we show that depletion of class A genes redistributes Golgi membranes into the endoplasmic reticulum, depletion of class B genes leads to Golgi fragmentation, depletion of class C genes leads to aggregation of Golgi membranes, and depletion of class D genes causes no obvious change. Of the 20 new gene products characterized so far, several localize to the Golgi membranes and the endoplasmic reticulum.

Drosophila S2 tissue culture cells were transformed to stably express horseradish peroxidase fused to a signal sequence (ss-HRP) on an inducible promoter (Fig. 1a). Addition of Cu^{2+} ions to the cells induced the production of ss-HRP, which is translocated into the endoplasmic reticulum (ER), transported to the Golgi apparatus and then secreted into the medium. An aliquot of the medium was removed to measure peroxidase activity by chemiluminescence, thereby providing a robust assay to monitor secretion in a high-throughput format (Fig. 1b). Two controls were used to verify that secretion of HRP occurred through the generic secretory pathway: knockdown of Syntaxin 5 (a t-SNARE) and β-COP (a component of the COP1 coat)[3–5] each caused a 100-fold reduction in HRP secretion (Fig. 1c).

We used a genome-wide library of ~22,000 double-stranded RNAs (dsRNAs) that have been previously used in several screens (refs 6–9 and http://www.flyrnai.org). Cells secreting ss-HRP were plated in 384-well plates containing dsRNA. After 5 d, ss-HRP production was induced, and 12 h later peroxidase activity released into the supernatant was measured by chemiluminescence (Fig. 2a). Each plate included wells with dsRNA encoding Syntaxin 5 and GFP as positive and negative controls. The screen was carried out in duplicate. Because most dsRNAs did not inhibit HRP secretion, the average for a given plate was very close to that of non-treated wells. Therefore, the *z*-score of each well, equal to the value of the well (peroxidase activity) minus the average of the plate divided by the standard deviation for the plate, was used to compare the effects of each dsRNA on secretion across the whole set of plates. The average of the two *z*-scores for each dsRNA is shown in Fig. 2b. The scatter plot of the duplicated assay shows that most dsRNAs did duplicate with a correlation coefficient of 0.63 (Fig. 2c). A few of the dsRNAs did not duplicate, but were included in the next round of selection to recover potential positives. On the basis of this analysis, 1,133 dsRNAs were selected (Supplementary Table S1).

The genes corresponding to the 1,133 dsRNAs were analysed with Flybase (www.flybase.org). Genes that could affect secretion indirectly through their roles in apoptosis, transcription, protein translation, protein degradation and basic metabolism were discarded from further analysis (Supplementary Table S1). In addition, dsRNAs that scored positively in previous cell survival screens were removed[6]. Known components of the secretory pathway did not score in those cell survival screens[6]. This selection reduced the number to 284 dsRNAs that we tested further in two additional HRP secretion assays in a 96-well plate format. The DNA-binding dye Hoechst was used to exclude dsRNAs that could affect cell number (Fig. 2d). The list of 284 dsRNAs tested and our reasons for excluding 154 from further analysis are given in Supplementary Table S2.

We generated a S2 cell line stably expressing mouse Mannosidase II, a marker of the *cis* and medial Golgi cisternae, coupled to GFP (MannII–GFP) to test the effect of 130 selected genes on Golgi

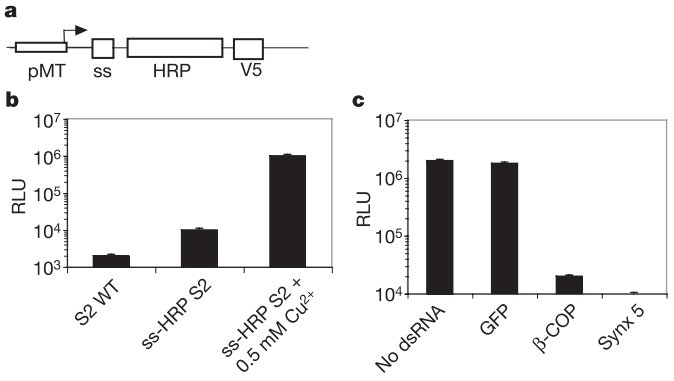

Figure 1 | HRP secretion in *Drosophila* S2 cells. a, S2 cells transfected with a plasmid containing the signal sequence (ss) of *Drosophila* Bip appended to HRP and a V5 tag under the influence of an inducible metallothionine promoter (pMT). **b**, The peroxidase activity in the supernatant from S2 cell culture is HRP. The supernatant from wild-type cells, cells expressing HRP but not induced, and cells induced to produce HRP was analysed for peroxidase activity by chemiluminescence. RLU, relative light units. **c**, RNAi of known effectors of trafficking effectively blocks HRP secretion. RNAi of the *bona fide* transport components β-COP and Syntaxin 5 was used to monitor effects on the secretion of HRP. Cells induced to produce HRP secrete considerable peroxidase activity, which is inhibited on depletion of β-COP and Syntaxin 5. Error bars in **b** and **c** represent the s.d. of triplicate measurements from representative experiments.

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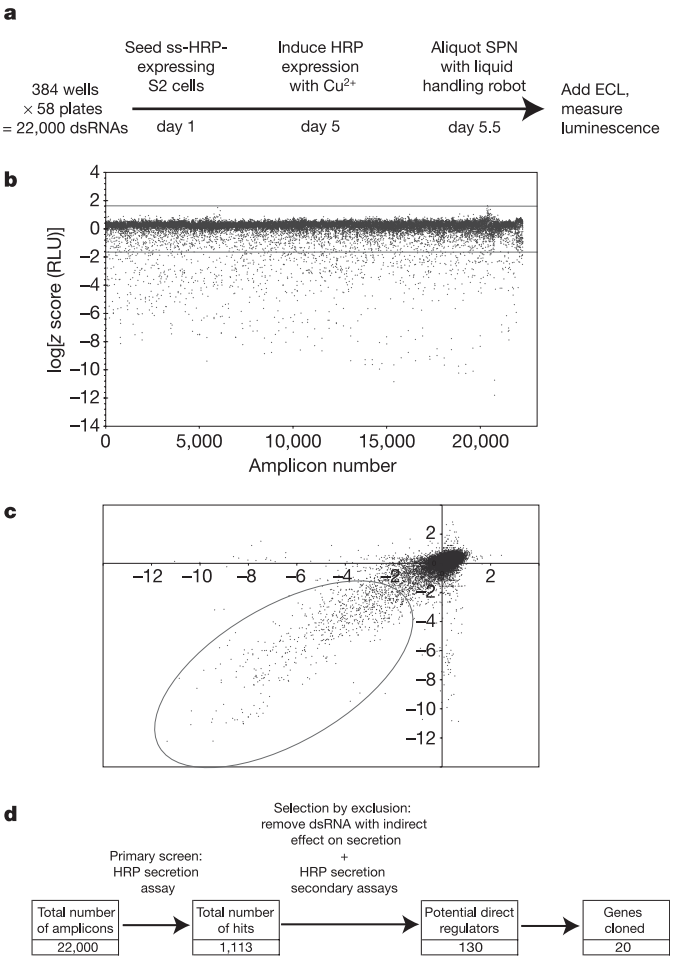

Figure 2 | Identification of the genes involved in HRP secretion. a, Each well of each plate containing a dsRNA corresponding to a specific *Drosophila* gene was seeded with 1×10^4 S2 cells stably expressing ss-HRP. After 5 d of incubation, cells were induced to synthesize HRP. After 12 h, 10 μl of culture supernatant was transferred to another well, the HRP substrate was added, and luminescence was measured. **b**, Whole-genome assay for HRP secretion. The *z*-score was derived from the luminescence of each well. All dsRNAs that inhibited secretion with a *z*-score of less than −1.5 (lower red line) were selected as positive hits for further analysis. **c**, Scatter plot for the duplicate screen. The two *z*-scores derived for each dsRNA are plotted on the *x* and *y* axis to show the overall reproducibility. **d**, Flow chart showing the selection of genes involved in secretion. From an initial collection of 1,133 genes (Supplementary Table S1), 130 (Supplementary Table S3) were found to be highly specific for HRP secretion. The subcellular localization of 20 of these genes was assessed after cloning and transient transfection.

organization. As previously reported[10], Golgi membranes in S2 cells are organized as several unconnected stacks of cisternae (Fig. 3a). We incubated S2 cells expressing MannII–GFP with dsRNAs by the procedure described above for HRP-secreting S2 cells, and then imaged them by high-resolution deconvolution fluorescence microscopy. The genes were classified into four groups on the basis of the effect of their depletion on Golgi membranes in greater than 50% of the total cells. RNA-mediated interference (RNAi) of class A genes fused Golgi membranes with the ER, as shown by the relocation of MannII–GFP in a ring around the nucleus and a diffuse reticular network (Fig. 3b). RNAi of class B genes fragmented the Golgi membranes into smaller elements, RNAi of class C genes caused aggregation and swelling, and RNAi of class D genes had no apparent effect on Golgi organization (Fig.3b). The complete list of the four classes of genes, their potential human orthologues and their domains are given in Supplementary Table S3. We found that 77 out of the 130 genes had potential human orthologues and 26 had homologues identified previously as trafficking components. The dsRNAs that have potential off-target effects on the basis of the presence of a 21-base-pair overlap with other genes, and which therefore need further validation, are also listed in Supplementary Table S3.

In mammalian cells, the Golgi membranes are fragmented and protein transport is blocked during mitosis[11]. The list of 130 genes includes *separase*, *klp61F* and *microtubule star*, which have been linked to mitosis-specific events[12–14]. It is therefore possible that effects on trafficking and Golgi organization in S2 cells depleted of these genes are due to arrest in mitosis. RNAi-treated MannII–GFP cells were visualized with antibody against tubulin and mitosis-specific antibody against phosphorylated histone H3. We could detect mitotic cells in control wells (Supplementary Fig. S1), but there was no increase in the mitotic index of cells depleted of the genes mentioned above. Consistently, the phenotypes of class B or C genes were not associated with DNA condensation, microtubule reorganization or increase in staining for phosphorylated histone H3 (Supplementary Fig. S1). Therefore, the fragmented Golgi phenotype and inhibition in secretion of HRP in these cells are not due to an arrest in mitosis. However, other caveats remain; for example, we found that the knockdown of genes involved in fatty acid and cholesterol biosynthesis results in a block of HRP secretion and a class A Golgi membrane phenotype (Supplementary Table S2). To confirm their direct role in membrane trafficking, therefore, the 104 candidate genes need further functional tests and, notably, characterization of the intracellular localization of their products.

To this end, 20 randomly selected genes including *CG14181*, a likely orthologue of *Use1* (encoding a t-SNARE localized to the ER in *Saccharomyces cerevisiae*[15]), were cloned in an inducible expression vector with a V5 tag. S2 cells expressing MannII–GFP were transfected with the tagged cloned genes, and the gene products were

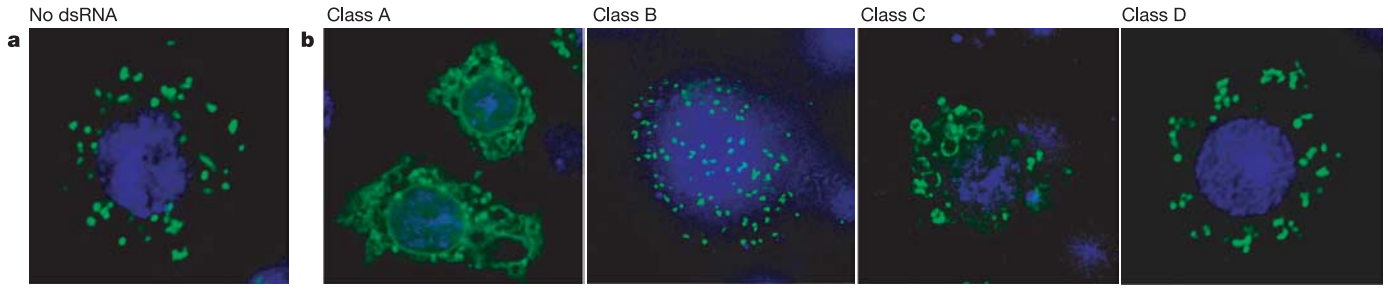

Figure 3 | *Drosophila* genes involved in Golgi organization. S2 cells stably expressing MannII–GFP, a marker of medial Golgi, were subjected to RNAi for each of the 130 genes identified to be essential for secretion. **a**, Organization of the untreated control MannII–GFP in S2 cells. MannII appears in discrete units around the nuclear periphery. **b**, On the basis of their effects on organization of the Golgi membranes, the 130 secretory components are grouped into four classes (Supplementary Table S3). Their depletion caused the Golgi to fuse with the ER (class A), fragmented the Golgi (class B), induced Golgi membranes to aggregate and swell (class C) or had no effect on Golgi organization (class D).

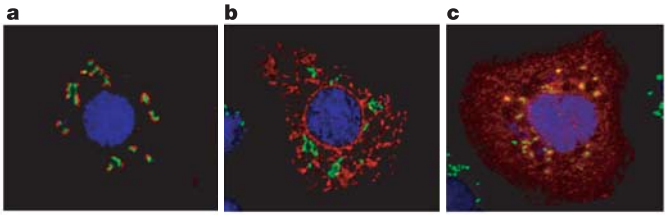

Figure 4 | Localization of the products of new genes regulating secretion. Candidate genes were cloned in an inducible vector with a V5 tag and transiently transfected into S2 cells expressing MannII–GFP. After fixation, cells were labelled with Hoechst (to stain DNA) and an anti-V5 antibody coupled to Texas red. MannII–GFP is green, DNA is blue and the gene product is red. **a**, *CG11098* (Golgi). **b**, *CG32675* (ER). **c**, *CG8309* (Golgi and cytosol).

visualized with an antibody against V5 and imaged by deconvolution. The localization of the gene products was compared with that of the Golgi marker (MannII–GFP), the ER pattern, and the diffuse cytosolic pattern of a soluble protein (examples of typical localization are shown in Fig. 4). Of the 20 cloned genes products, 4 localized to the Golgi membranes and 7 (including the *CG14181* product) localized to the ER, suggesting that they have a direct role in membrane trafficking (Table 1). The gene product of *CG11098*, named *TANGO1* for transport and Golgi organization (Table 1), is perfectly juxtaposed to the MannII–GFP-containing Golgi membranes (Fig. 2a). TANGO1 contains an amino-terminal Src homology 3 (SH3) domain followed by two coiled-coil domains, two transmembrane domains and a proline-rich domain. The coiled-coil domains have homology to the yeast protein Uso1p and the golgin p115. TANGO1, however, is not the *Drosophila* orthologue (encoded by *CG1422*) of Uso1p or p115 and has a human but not an *S. cerevisiae* homologue.

Another example of a metazoan-specific gene is *CG1098*, whose product localizes to the cytoplasm and Golgi membranes. *CG1098* has a human orthologue, *NRBP*, which encodes a serine/threonine kinase that is recruited to Golgi membranes on viral infection[16] and that, when overexpressed, perturbs early Golgi membranes in mammalian cells[17]. Among the candidates with a yeast homologue, *TANGO2* (*CG11176*) contains a domain, DUF833, of unknown function that is widely conserved, even in some bacteria. *TANGO4* (*CG1796*) also has a potential homologue in yeast, the splicing factor gene *Prp46*. Contrary to other splicing factors, however, RNAi of *TANGO4* resulted in a marked effect on Golgi membranes (class A phenotype) and its gene product localized to the cytosol and not the nucleus, suggesting that *TANGO4* regulates secretion independently from its potential role in RNA splicing. The challenge now is to understand how these and the other identified components regulate protein secretion and whether they regulate Golgi membrane organization directly. Their characterization will hopefully help to resolve the emerging complexities of the metazoan secretory pathway.

Table 1 | Intracellular localization of the gene products involved in HRP secretion

CG number	Gene name	Golgi phenotype	Localization of gene product
CG11098	*TANGO1*	A	Golgi
CG11176	*TANGO2*	A	cyto + Golgi
CG12444	*TANGO3*	A	ER
CG14181	*Use1*	A	ER
CG1796	*TANGO4*	A	cyto
CG32675	*TANGO5*	A	ER
CG18398	*TANGO6*	B	cyto + Golgi
CG8309	*TANGO7*	B	cyto + Golgi
CG14503	*TANGO8*	C	ER
CG9191	*Klp61F*	C	ER + Golgi + microtubules
CG10007	*TANGO9*	D	Golgi
CG1098	*Madm*	D	cyto + Golgi
CG1841	*TANGO10*	D	cyto
CG30404	*TANGO11*	D	ER
CG31052	*TANGO12*	D	Golgi
CG32632	*TANGO13*	D	Golgi
CG33553	*Doa*	D	ER
CG4775	*TANGO14*	D	ER
CG7850	*puckered*	D	ER + Golgi
CG8588	*pastrel*	D	cyto

Twenty genes were cloned and tagged with V5, and their subcellular localization was monitored in S2 cells expressing MannII-GFP. The localization of the gene product is abbreviated as follows: cyto, cytosolic; Golgi, Golgi membranes; ER, endoplasmic reticulum. Computed genes without a previous name are labelled *TANGO* for transport and Golgi organization.

METHODS

Constructs and cell lines. The *HRP-C* gene was cloned by PCR from a construct[18] provided by D. Cutler (MRC, University College London) and inserted into pMT/BiP/V5–His (Invitrogen). The sequence corresponding to the 100 N-terminal amino acids of mouse MannII was fused with the GFP sequence and inserted into pAC5/V5–His. S2 cells were cotransfected with pHygro (Invitrogen) in a ratio of 20 to 1 and selected with 0.3 mg ml^{-1} of Hygromycin.

Primary screen and analysis. Two sets of 58 plates containing 0.25 μg of dsRNA per well were provided by the *Drosophila* RNAi Screening Center (DRSC; http://flyrnai.org). 1×10^4 ss-HRP cells were seeded in each well with a Multidrop 384. After incubation for 2 h, 20 μl of fetal bovine serum containing medium was added. After 5 d, the culture medium was replaced with 50 μl of medium containing 500 μM copper and the cells were incubated overnight. We transferred 10 μl of supernatant into a receptacle plate with a Cybio CyBi-Well vario system and 50 μl of ECL reagent (Perkin-Elmer Western Lightning) was added. Luminescence was measured with an Analyst plate reader. Each receptacle plate was assigned a number and a barcode similar to the initial set for automatic identification by the plate reader.

Analysis of primary screen. *z*-Scores were derived from the log value of luminescence and genes with a score below −1.5 were selected. By using the DSRC database, genes that scored in cell survival screens[6], as well as genes involved in transcription, RNA splicing, protein translation and proteasome function, were excluded from further analysis. The raw data for the whole screen will be made available (http://flyrnai.org.). Identification of potential orthologues in humans was based on information available in Flybase (http://flybase.bio.indiana.edu/) and on the reciprocal best blast searches with the InParanoid algorithm.

Secondary screens and morphological effects on Golgi membranes. Using PCR templates provided by the DRSC, we resynthesized dsRNAs and tested their effect on HRP secretion with a protocol similar to the primary screen. To measure cell number, Hoescht was added at 5 μg ml^{-1} to cells for 90 min, the cells were washed, and ultraviolet fluorescence was measured with a plate reader (Tecan). dsRNAs resulting in greater than 50% inhibition of HRP secretion without affecting cell number were selected as positives. MannII–GFP cells were incubated with dsRNAs as described above; after 5 d, the cells were transferred to concanavalin-treated 96-well glass-bottom plates, allowed to spread for 2 h and then fixed and labelled with Hoescht at 2 μg ml^{-1}. Image stacks for five fields in each well were acquired with a 60 × objective and treated for deconvolution.

Cloning of genes for expression in S2 cells. Genes were cloned by RT–PCR from a library of poly(A) RNA from *Drosophila* larvae (Clontech) using the Gateway system (Invitrogen) and subcloned into pDEST48. MannII–GFP S2 cells were transfected 2 d before gene expression was induced with Cu^{2+} for 4 h, fixed, labelled with antibody against V5 (Invitrogen), and processed for imaging as described above.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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