Shared and specific functions of Arfs 1–5 at the Golgi revealed by systematic knockouts

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ADP-ribosylation factors (Arfs) are small GTPases regulating membrane traffic in the secretory pathway. They are closely related and appear to have overlapping functions, regulators, and effectors. The functional specificity of individual Arfs and the extent of redundancy are still largely unknown. We addressed these questions by CRISPR/Cas9-mediated genomic deletion of the human class I (Arf1/3) and class II (Arf4/5) Arfs, either individually or in combination. Most knockout cell lines were viable with slight growth defects only when lacking Arf1 or Arf4. However, Arf1+4 and Arf4+5 could not be deleted simultaneously. Class I Arfs are nonessential, and Arf4 alone is sufficient for viability. Upon Arf1 deletion, the Golgi was enlarged, and recruitment of vesicle coats decreased, confirming a major role of Arf1 in vesicle formation at the Golgi. Knockout of Arf4 caused secretion of ER-resident proteins, indicating specific defects in coatomer-dependent ER protein retrieval by KDEL receptors. The knockout cell lines will be useful tools to study other Arf-dependent processes.

Introduction

The secretory pathway is a major route of membrane traffic in the cell, transporting soluble and membrane proteins from their site of synthesis, which is the rough ER, to their final destinations. On the way, cargo proteins pass through successive compartments, where they acquire modifications and undergo multiple rounds of sorting and packaging into transport carriers. This anterograde traffic is counterbalanced by retrograde transport of membranes and proteins to maintain organelle identity and homeostasis, and retain specific proteins in defined compartments. Key players in these processes are the small GTPases of the ADP-ribosylation factor (Arf) family.

The Arf family is composed of 30 members: the six “true” Arfs, 21 Arf-like proteins (Arls), 2 Sars, and Trim23 (Li et al., 2004; Kahn et al., 2006). The Arfs are closely related, while the other members are more divergent in sequence and cellular functions (reviewed in Gillingham and Munro, 2007; Donaldson and Jackson, 2011; Sztul et al., 2019). The five human Arfs (humans lack Arf2) are assigned to three classes based on sequence homology: Arf1 and 3 belong to class I, Arf4 and 5 to class II, and Arf6 is the only member of class III. Class I and II Arfs mainly localize to the Golgi, but also to endosomes and/or the ER–Golgi intermediate compartment (ERGIC), whereas Arf6 is found in the cell periphery. Arfs are ubiquitously expressed, but vary in their abundance (Cavenagh et al., 1996; Itzhak et al., 2016). In the widely used HeLa cells, Arf1 is the most abundant Arf, followed by Arf4 (~1/3), Arf3 and Arf6 (~1/10), and Arf3 (~1/100; Itzhak et al., 2016).

Arfs are N-myristoylated, which allows them to loosely associate with membranes already in the GDP-bound state. Binding of a guanine nucleotide exchange factor (GEF) and subsequent activation via GDP–GTP exchange lead to displacement of the N-terminal amphipathic helix from the hydrophobic binding pocket, resulting in tight membrane association (Antonny et al., 1997; Renault et al., 2003). Concomitant conformational changes enable binding of effectors.

The interplay of Arfs and their various effectors contributes to diverse cellular processes throughout the cell (reviewed in Donaldson and Jackson, 2011; Jackson and Bouvet, 2014; Sztul et al., 2019). The most prominent function is the contribution of the Golgi-localized Arfs (Arf1–5) to transport carrier formation in intracellular traffic, especially in the secretory pathway. Two major aspects are linked to Arf activity in this context: the modification of membrane lipids (reviewed in De Matteis and Godi, 2004; Donaldson and Jackson, 2011) and the recruitment of coat components. The best-characterized coat complexes are the coat protein complex I (COP1) at the ERGIC and the Golgi mainly for retrograde transport back to the ER, and the adaptor protein complex I (AP1) and the Golgi-localized, γ-ear-containing, Arf-binding proteins (GGAs) at the Golgi and on endosomes for transport from the TGN to endosomes and back.
The activity of Arfs is tightly regulated spatially and temporally by their GEFs and GTPase-activating proteins (GAPs). All 15 known GEFs share a common Sec7 domain to catalyze nucleotide exchange, but in addition possess diverse domains regulating their own membrane association and activity (reviewed in Nawrottek et al., 2016; Sztul et al., 2019). Also, the 28 ArfGAPs share a common GAP domain and are increasingly perceived to be more than simple terminators of Arf activity, but rather effectors themselves (Donaldson and Jackson, 2011; Sztul et al., 2019).

Originally discovered as a factor required for cholera toxin-mediated stimulation of adenylate cyclase by ADP-ribosylation of the stimulatory heterotrimeric G protein Gs (Kahn and Gilman, 1984, 1986), the role of Arfs in intracellular traffic by recruiting coat proteins was uncovered a few years later (Serafini et al., 1991; Stamnes and Rothman, 1993; Palmer et al., 1993; Traub et al., 1993). Early approaches to identify Arf functions were based on the manipulation by dominant-negative and dominant-active mutants (Teal et al., 1994; Zhang et al., 1994; Dascher and Balch, 1994) and by the fungal macrolide Brefeldin A. However, these approaches lacked specificity for individual Arfs, since Arf mutants and Brefeldin A sequester shared GEFs or GAPs and hence influence the activity of various Arfs simultaneously. Direct interactions between Arfs and coat components were analyzed in the presence of GTPγS by in vitro and in vivo experiments, which suggested that both class I and II Arfs can recruit COP, API, and GGA proteins to Golgi membranes (Liang and Kornfeld, 1997; Boman et al., 2000; Austin et al., 2002; Takatsu et al., 2002).

Volpicelli-Daley and colleagues were the first to systematically dissect the role of individual Arfs in the secretory and endocytic pathways by siRNA-mediated knockdown. They proposed that no single Arf is required for any transport step, since only pairwise knockdowns resulted in specific phenotypes, implying that no single Arf is required for viability and cell growth (Volpicelli-Daley and colleagues, 2002).

Later studies provided a glimpse into the complex interactome surrounding the Arfs involving GEFs, GAPs, effectors, and other GTPases, and highlighted that Arfs do not act in isolation but in complex networks (reviewed in Donaldson and Jackson, 2011; Mizuno-Yamasaki et al., 2012; Baschieri and Farhan, 2012; Thomas and Fromme, 2016). However, fundamental questions remained unanswered, such as the major contributions of individual Arfs, their specificities and redundancies, and their regulation and coordination (Sztul et al., 2019).

Here, we revisited basic questions concerning the functions of Arf1–5 in the secretory pathway using CRISPR/Cas9 genome editing, generating Arf knockout (KO) cell lines by genomic deletion. We found that cells lacking any single Arf are viable, as well as cells deleted for certain double or triple combinations. In fact, Arf4 is able to sustain all essential functions in the absence of all other class I and class II Arfs. Yet we observed distinct phenotypes already in single-KO cell lines: deletion of Arf1 caused an increased Golgi volume, altered Golgi morphology, and reduced recruitment of vesicle coats to the Golgi, while the KO of Arf4 produced a specific defect in retrieval of ER resident proteins.
exception of Arf4 in combination with Arf1 or Arf5. Simultaneous deletion of these Arfs appears to be lethal, since the respective KO cell lines could not be generated. Remarkably, Arf4 alone is sufficient for cell viability in the absence of all other class I and II Arfs. This highlights that class I Arfs are not essential.

Golgi morphology is altered and volume increased in cells deleted for specific Arfs

First, we assessed the impact of Arf deletions on the morphology of the Golgi complex by confocal microscopy acquiring serial z-stack images of cells immunostained for the cis-Golgi golgin GM130. In maximum intensity projections, the Golgi of parental HeLa cells appeared as a perinuclear compact tangle of ribbons (Fig. 2 A). In all cell lines lacking Arf1 (Arf1ko, Arf1+5ko, Arf3+1ko, and Arf3+1+5ko), however, this shape was altered to a more diffuse and less compact pattern that appeared swollen and enlarged. Subtle changes were also suspected for cell lines lacking Arf4 (Arf4ko and Arf3+4ko), as the GM130-positive ribbons appeared to be more densely packed. No alteration was observed by eye in Golgi morphology of the other Arf KO cell lines.

Golgi volume and Feret diameter (the largest distance between two contour voxels) were measured after 3D reconstruction and confirmed the visual evaluation (Fig. 2, B and C). All cell lines deleted for Arf1 displayed a significant increase in

Figure 1. Characterization of viable Arf KO cell lines. (A) Arf KO HeLa cell lines were generated by CRISPR/Cas9 as described in Materials and methods and analyzed for partial deletion of exon 1 by genomic PCR. Uniform shortening of PCR fragments in KO cell lines indicated successful genomic deletion in all corresponding Arf alleles. (B) The deletion of Arfs on the protein level was verified by immunoblot analysis. For each cell line, three biological replicates were analyzed on the same gel. Molecular weight markers are indicated in kilodaltons. (C) Growth curves of Arf KO cell lines were derived from live cell counts obtained in three independent experiments (mean ± SD). Cells were seeded at the same density and monitored for 5 consecutive days. (D) Doubling times were calculated from the growth curves displayed in C. Bars, mean ± SD. Unpaired one-way ANOVA versus parental (ns, P > 0.05; *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001).
Golgi volume (on average 1.9-fold) and in the Feret diameter (on average 1.3-fold). In cell lines lacking Arf4, the Golgi volume was also significantly increased (on average 1.3-fold), whereas the Feret diameter remained at the levels of parental HeLa α cells.

The other KO cell lines (Arf3ko, Arf5ko, and Arf3+5ko) did not exhibit a change in either parameter.

How these changes manifest themselves in the 3D structure of the Golgi was addressed by super-resolution microscopy (3D structured illumination microscopy [3D-SIM]; Fig. 2 D). In maximum intensity projections of 3D-SIM z-stacks, the GM130-labeled Golgi of parental HeLa α cells presented itself as a network of ribbons. In Arf4ko cells, this pattern was altered and...
appeared less defined and more uniformly distributed. Arf4ko Golgi displayed an intermediate phenotype. Tomographic 2D slices showed individual ribbons that were increased in the diameter in Arf1ko cells. In Arf4ko Golgi, the ribbons seemed to be more intertwined than in parental cells.

Golgi surface reconstruction showed a marked difference between a tangle of tubular structures in parental cells, clusters of more planar sheets in Arf1ko cells, and more densely packed Golgi ribbons in Arf4 KO cells. Taken together, the 3D analysis of the Golgi in KO cell lines confirmed an increase in Golgi volume for Arf1ko and Arf4ko cell lines and linked it to a broadening or higher number of Golgi ribbons, respectively.

Maintenance of Golgi polarity in Arf1ko cells, which are the morphologically most affected KO cells, was assessed by super-resolution microscopy of coimmunostained cells. The separation of the cis-Golgi marker GM130 and the TGN marker TGN46 in Arf1ko Golgi complexes was comparable to parental cells, thus indicating that Golgi polarity is not perturbed (Fig. S2).

**Morphological changes in the ultrastructure of Arf1 KO cells**

We further analyzed the ultrastructure of the Golgi by thin-section transmission electron microscopy (Fig. 3 A). The only difference in the appearance of Golgi structures was an increase in the length of individual stacks in cell lines lacking Arf1 compared with parental HeLa cells. Quantitation revealed a significant increase in stack length of ~65% in Arf1ko cells, while stack thickness and the number of cisternae per stack remained unchanged (Fig. 3, B–D). This is consistent with the increased ribbon diameter observed by super-resolution microscopy. No indication of morphological changes in Golgi structure was seen in other cell lines.

Interestingly, in the triple-KO cell line (Arf3+1+5ko), the tubular elements of the ER, as identified by bound ribosomes, appeared frequently dilated (Fig. 3 A, arrowheads).

**KO of specific Arfs affects steady-state localization of coats**

Next we examined the impact of Arf deletions on the recruitment of the Golgi-associated, Arf-dependent coats, COP1, API1, and GGA2, by analyzing their steady-state localization by immunofluorescence microscopy in cells costained for the Golgi marker GM130.

The signal for βCOP mostly colocalized with GM130 (Fig. 4 A). However, in all Arf1 KO cell lines, the fluorescent βCOP signal appeared to be reduced at the Golgi compared with parental HeLa cells. No change was observed in the other Arf KO cell lines.

To substantiate this visual assessment, we quantified the signal intensity of βCOP and GM130 at the Golgi. Total fluorescence intensity revealed a significant increase of Golgi-localized GM130 in cells lacking Arf1 or Arf4 (Fig. 4 B), replicating the increase in Golgi volume described above. The total signal of βCOP, on the contrary, did not differ between cell lines (Fig. 4 C). As a consequence, the ratio of βCOP to GM130, i.e., the βCOP density at the Golgi, was reduced by an average of 40–50% in the Golgi region of all Arf1 KO cell lines compared with parental cells (Fig. 4 D). For cell lines lacking Arf4, a slight, although mostly not significant, decrease of Golgi-localized βCOP was observed. Since the total protein level of βCOP remained unchanged in Arf KO cells (Fig. S3), the results indicate reduced βCOP recruitment per Golgi unit.

The TGN coats API1 and GGA2 were analyzed following the same procedure using GM130 as a Golgi marker (Fig. 4, E and F) or TGN46 as a TGN marker (Fig. S4, A and B). As for βCOP, Golgi-localized API1 was reduced relative to GM130 by an average of 30–40% in all Arf1 KO cell lines compared with parental cells (Fig. 4 G). Similarly, the density of GGA2 at the Golgi was also reduced in all Arf1-deleted cell lines (Fig. 4 H). Remarkably, the additional absence of Arf3 in Arf3+1ko and Arf3+1+5ko cells even enhanced the loss of GGA2 at the Golgi compared with Arf1ko and Arf1+5ko cells from ~30 to ~60%. For cell lines lacking Arf4, a slight, although mostly not statistically significant, decrease in Golgi density of API1 and GGA2 was observed.

These findings suggest that normally, Arf1 is the main mediator of coat recruitment at the Golgi. In its absence, the rate of coat recruitment and thus of the formation of Golgi-exit carriers per Golgi unit is reduced, which results in expansion of the Golgi to match influx and efflux of proteins and membranes in a new steady-state.

**KO of Arf4 causes defective retrieval of ER resident proteins**

To assess the functionality of the secretory pathway upon deletion of Arfs, we analyzed total secretion by visualizing secreted proteins collected from the media by SDS-gel electrophoresis and Coomassie staining (Fig. 5 A). Strikingly, in the media of Arf4-deleted cell lines, additional bands were detected. For identification of these additionally secreted proteins, media were collected from Arf4ko, Arf3+4ko, and parental HeLa cells and analyzed by mass spectrometry. This approach identified 75 and 87 proteins to be significantly up-regulated (as defined by fold change >2, q-value <0.01) in the secretomes of Arf4ko and Arf3+4ko cell lines, respectively (Fig. 5 B, Table S1, and Table S2). 70 of these were shared between the two KO cell lines. Among the top hits, we found ER chaperones, such as BiP, calreticulin, and GRP94, peptidyl-prolyl cis/trans isomerases, and protein disulfide isomerasers. Gene ontology term (GOterm) enrichment analysis identified the ER as the main compartment of origin (Table S3 and Table S4). Secretion of BiP and calreticulin was verified by immunoblot analysis (Fig. 5 C). Both chaperones were found to be strongly secreted specifically in the two cell lines lacking Arf4.

 aberrant secretion of ER resident proteins in Arf4-deleted cells indicates a defect in their retrieval from the Golgi back to the ER. The most prominent mechanism is retrograde transport by the KDEL receptors (Lewis and Pelham, 1990, 1992; Hsu et al., 1992; Raykhel et al., 2007). Indeed, ~30% of the proteins whose secretion was increased in Arf4 KO cells contain a KDEL motif or a variant thereof at their C terminus according to the PROSITE database [https://prosite.expasy.org] consensus sequence [KRHQS–][DENQ]E-L> (entry PDOC00014). However, this might be an underestimation, as studies suggested that not all motifs recognized by the KDEL receptors are included in this consensus pattern (Raykhel et al., 2007).

Functionality of KDEL-mediated retrieval in Arf KO cell lines was tested by transient expression of a signal sequence-GFP-KDEL (GFP-KDEL) construct and examination of its steady-state
localization (Fig. 5 D). In parental HeLa cells, the GFP signal was visible in the reticular pattern of the ER and a number of puncta. Exclusively in cells lacking Arf4, the GFP-KDEL–positive puncta were completely lost and the reticular staining reduced. Instead, a perinuclear accumulation colocalizing with GM130 was detected, consistent with a defective retrieval from the Golgi back to the ER in the absence of Arf4.

Therefore, we determined the localization of the KDEL receptors in Arf1ko and Arf4ko cells, which showed the strongest defects in βCOP recruitment and GFP-KDEL transport, respectively (Fig. S5 A). In parental HeLa and Arf1ko cells, the KDEL receptor staining exhibited a faint perinuclear staining colocalizing with the GM130 signal and puncta distributed throughout the cell. In Arf4ko cells, the signal appeared more intense in the perinuclear region. Quantitation of Golgi-localized KDEL receptor signal relative to that of GM130 (Fig. S5 B) confirmed a significant increase of KDEL receptors at the Golgi in Arf4ko cells compared with parental cells. This change was caused by altered transport, since the intracellular levels of KDEL receptors remained unchanged upon Arf4 KO (Fig. S5 C).
Figure 4. Immunostaining for Golgi associated coat proteins. (A) All Arf KO cell lines as well as parental HeLa α cells were coimmunostained for the Golgi marker GM130 in magenta and the coat component βCOP in green to examine their steady-state localization. DAPI signal is shown in blue. Lower panels show

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https://doi.org/10.1083/jcb.202106100
Defective retrieval and aberrant secretion of proteins with a KDEL motif and accumulation of KDEL receptors at the Golgi in cells lacking Arf4 indicate an important role for Arf4 in retrograde transport of KDEL receptors and their cargo from the Golgi to the ER.

**Rescue experiments to define Arf specificity**

To assess the specificity of the observed Arflko and Arf4ko phenotypes and the rescue potential of different Arfs, stable cell lines were generated by lentiviral transduction to overexpress individual Arfs in parental HeLa, Arflko, and Arf4ko cells. Compared with the endogenous Arf levels in parental cells, Arfl and Arf4 were moderately, and Arf3 and Arf5, which have low endogenous levels, were highly overexpressed (Fig. 6, A and B; and Fig. 7, A and B).

Rescue of the Arflko phenotype was assessed based on Golgi morphology visualized by immunofluorescence staining of GM130. In parental HeLa cells, overexpression of Arfl or the other class I member, Arf3, showed an opposite effect of Arfl deletion: the Golgi condensed onto individual filaments and experiment are shown as filled circles together with the values from individual cells as small dots colored by experiment. Mean ± SD of the three experiments are indicated. Unpaired one-way ANOVA versus parental, unless indicated otherwise (ns, P > 0.05; *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001).

Discussion

No single Arf is essential and Arf4 is sufficient for cell survival

Members of the Arf family are found in all known eukaryotes from yeast to human. Among the “true” Arfs, class I orthologues are present more frequently, while class II Arfs arose later in evolution (Gillingham and Munro, 2007; Donaldson and Jackson, 2011). In yeast, for instance, three Arfs exist, which are homologous to class I (Arfl and Arf2) and class III (Arf3) Arfs, but none of class II (Lee et al., 1994). None of the three Arfs is essential, but the simultaneous deletion of both class I Arfs is lethal (Stearns et al., 1990).

Arf GTPases are important regulators of a range of cellular processes, especially within the secretory pathway. For this reason, their misregulation is associated with diverse human diseases (reviewed in Casalou et al., 2016; Sztul et al., 2019). In cancer, for instance, Arf signaling was reported to be altered, most commonly by gene amplification. Moreover, Arf4 and Arf6 were linked to cancer cell migration, invasion, and metastasis (Sztul et al., 2019). Hence, it is important to decipher and understand shared and specific functions of individual Arfs. Their identification has been complicated, for instance, by apparent functional redundancy, shared GEFs and GAPs, and a complex interactome, as well as technical difficulties (Sztul et al., 2019).

Previous cellular analyses employed shRNA- or siRNA-mediated knockdowns, potentially prone to incomplete depletion and off-target effects, or (over)expression of mutant Arfs. Dominant-active (GTPase-deficient) or dominant-negative (GDP-locked) mutants may interfere with the activity of other Arfs by outcompeting them or by blocking shared GEFs. Furthermore, epitope-tagging was shown to change the Arfs’ properties in subtle ways (Jian et al., 2010).

Our approach to gain insight into Arf functions was based on genomic deletions of individual Arfs and Arf combinations and subsequent observation of defects in cell growth, Golgi morphology, and coat recruitment to the Golgi. We successfully generated nine genomic Arf KO cell lines, composed of four single-KOs of each human class I (Arfl and Arf3) and class II members.
(Arf4 and Arf5) Arf, and four double-KO and one triple-KO combination. These cells are permanently depleted for the respective Arfs, which may lead to compensatory effects. In this line, we only observe a up-regulation of Arf4 in all cell lines lacking Arf1 (Fig. 1B), but no further changes in Arf expression levels.

In agreement with knockdown based studies, we found that cultured cells can cope rather well with the loss of any single Arf and even certain combinations. However, the single-KO of Arf1 or Arf4 exhibited significant and distinct phenotypes, for example, either deletion reduced the growth rate and increased the fraction of cells in G0/1 phase of the cell cycle. The combined Arf1+4 double-KO was not viable. Interestingly, the same is true for simultaneous deletion of both class II Arfs (4+5), whereas cells knocked out for both class I Arfs (1+3) grew as well as...
Arf1ko cells. In addition, Arf4 was found to be able to sustain all essential Arf functions alone in cultured cells lacking the other three Arfs. Results may differ in other cell lines, as is documented by the loss-of-function screens in DepMap (http://www.depmap.org), which show that several cell lines depend on Arf1 and/or Arf4. Nontransformed cells may also be more sensitive to the lack of individual Arfs. In mice, KOs of Arf1 or Arf4 (as well as of Arf6) were embryonically lethal, and conditional KOs in specific tissues led to various dysfunctions (Sztul et al., 2019; International Mouse Phenotyping Consortium [http://www.mousephenotype.org]).

Deletion of Arf1 increases Golgi volume and reduces coat recruitment
The most striking phenotype of cells deleted for Arf1, alone or in combination with Arf3 and/or Arf5, was an enlargement of the Golgi, apparent in a more expanded GM130 staining, a higher volume in 3D Golgi reconstructions, and longer Golgi stack cross-sections in electron microscopy. Furthermore, steady-state densities of COPI, API, and GGA2 vesicle coat components at the Golgi were reduced. This indicates a lower rate of coat recruitment and thus of formation of COPI and AP1/GGA/clathrin transport vesicles per Golgi area, causing a reduction of cargo and membrane traffic originating from the Golgi in retrograde and anterograde direction. The resulting imbalance of influx and efflux might explain the increase in Golgi size, as proposed by Sengupta and Linstedt (2011). Indeed, the reduction in coat density was found to be compensated by the increase in Golgi size restoring the equilibrium between incoming and outgoing material.

An increase in Golgi volume has also been observed physiologically upon an increased demand for cargo transport,

Figure 6. Rescue experiments of the Arf1ko phenotype by overexpressing Arf1, Arf3, or Arf4. Based on parental HeLa and Arf1ko cells, stable cell lines were generated by lentiviral transduction, either expressing the empty vector (+empty) as a control or overexpressing the indicated untagged Arf. (A and B) Expression levels of Arfs in clonal (A) and pooled cell lines (B) were analyzed by immunoblotting. Ten and five times less lysate were loaded for Arf-overexpressing cell lines in A and B, respectively. Actin served as a loading control. (C and D) Parental HeLa and Arf1ko cell lines transduced with the empty vector or overexpressing class I Arfs Arf1 and Arf3 (C) or class II Arf Arf4 (D) were immunostained for GM130 to examine Golgi morphology. Magnified sections are shown in the lower panels. Scale bars, 10 µm.
processing, and sorting at the Golgi (Sengupta and Linstedt, 2011). As cells grow during interphase, increased cargo load causes a Golgi volume increase. Golgi growth was shown in HeLa cells to occur by cisternal elongation of existing Golgi stacks rather than by addition of new stacks (Sin and Harrison, 2016). Thus, the observed length increase in Golgi stacks of our Arf1 KO cells is likely to result from increased cargo content due to reduced export rates. Taken together, Arf1 appears to be the major mediator of vesicle traffic originating from the Golgi. Similar observations were made in yeast, where KO of Arf1 also

Figure 7. Rescue experiments of the Arf4ko phenotype by overexpressing Arf1, Arf4, or Arf5. Stable cell lines were generated from parental HeLa and Arf4ko cells by lentiviral transduction, either expressing the empty vector (+empty) as a control or overexpressing the indicated untagged Arf. (A and B) Expression levels of Arfs in clonal (A) and pooled (B) cell lines were analyzed by immunoblotting. Ten and five times less lysate were loaded for Arf-overexpressing cell lines in A and B, respectively. In B, two irrelevant lanes were removed as indicated by the black line. Actin served as a loading control. (C and D) Immunoblot analysis probing the media (M) and cell lysates (L) for the chaperones BIP and calreticulin (Calret.) of parental HeLa and Arf4ko cell lines transduced with the empty vector or overexpressing class II Arfs (C) or class I Arf Arf1 (D). Actin served as a loading control. (E and F) Immunofluorescent microscopy revealed the steady-state localization of transiently expressed signal sequence–GFP–KDEL (green) coimmunostained with GM130 (magenta) in parental HeLa and Arf4ko cell lines transduced with the empty vector or overexpressing class II Arfs Arf4 or Arf5 (E) or overexpressing class I Arf Arf1 (F). DAPI signal is shown in blue. Lower panels show magnified image sections. Asterisks mark images with enhanced intensity. Scale bars, 10 µm.
KO of Arf4 specifically disrupts retrieval of ER proteins

The deletion of Arf4, alone or in combination with Arf3, caused a slight increase in Golgi volume and a reproducible but not statistically significant decrease of coat components at the Golgi. Arf4 thus appears to contribute to the recruitment of COPI, AP1, and GGA2, and the consequential mild reduction of Golgi exit might explain the observed slight increase in Golgi volume in Arf4ko cell lines.

Most strikingly, however, Arf4ko cells manifest an aberrant secretion of ER resident proteins that are normally retrieved from the Golgi back to the ER by the KDEL receptors. The phenotype resembles the one reported for knockdowns of multiple KDEL receptors (Li et al., 2015). However, this defect upon Arf4 deletion cannot be simply linked to a general defect in retrograde transport due to reduced COPI recruitment, since this is more strongly observed in Arf1ko cells without causing ER protein secretion. Moreover, overexpression of Arf1, the main COPI-recruiting Arf, is unable to compensate for the loss of Arf4 in this function. Thus, a more specific function in ER protein retrieval must be defective in Arf4ko cells. It has previously been shown that in addition to Arf1, the class II Arfs, but not Arf3, can competitively support COPI vesicle formation (Popoff et al., 2011). For COPI, it has been shown that two paralogs of γ-COP (γ1/2Y) and of γ'-COP (γ3/2ζ) can form three distinct COPI complexes (γ1γ1, γ2γ3, or γ2ζ) with potentially different functions (Moelleken et al., 2007; Wegmann et al., 2004). Furthermore, Scy1, a member of the Scy1-like family of catalytically inactive protein kinases, was identified as an interactor of COPI at the cis-Golgi and ERGIC that causes reduced retrograde traffic of the KDEL receptors when inactivated (Burman et al., 2008). Subsequently, Scy1 was shown to bind to class II Arfs, preferentially Arf4, and to interact directly with the COPI subunit γ2 (Hamlin et al., 2014). This interaction was recently shown to depend on arginine methylation of Scy1 by PRMT1 (Amano et al., 2020). A tripartite Scy1–Arf4–γ2ζ-COP complex thus was proposed to specifically mediate KDEL receptor traffic. This mechanism may thus account for the specific phenotype we observe upon Arf4 deletion.

However, in other studies, Scy1 was also reported to bind preferentially to Arf1 and to GORAB, a protein associated with gerodermia osteodysplastica, at the trans-Golgi to promote COPI recruitment (Witkos et al., 2019). Pathogenic GORAB mutations cause impairment of COPI-mediated retrieval of trans-Golgi enzymes and a deficit in glycosylation of secretory proteins. Based on their results, the authors suggest that there might be two separate pools of Scy1, a GORAB-dependent one at the trans-Golgi and a pool at the cis-Golgi/ERGIC for distinct COPI functions.

The situation is further complicated by the recent finding that a mutation in γ1-COP (K652E), shown to cause defective humoral and cellular immunity, disrupted KDEL receptor binding to COPI, thus affecting KDEL receptor localization, increasing ER stress in activated T and B cells and apoptosis in activated T cells (Bainter et al., 2021). Thus, other, γ1-containing COPI complexes also appear to contribute to KDEL receptor sorting in these cells. How Arf4 specifically mediates KDEL receptor retrieval is therefore not entirely clear yet.

Arf KO combinations

In the majority of viable Arf double- or triple-KO cell lines, no additional phenotypes were detected beyond those of Arf1 or Arf4 single deletion regarding Golgi size and morphology, coat recruitment, or secretion of ER resident proteins. However, in Arf3+ko cells, the loss of GGA2 from the Golgi was more pronounced than in Arf1ko cells. This suggests a functional overlap of Arf1 and Arf3 in the recruitment of GGA2, which is consistent with Arf3’s known preferential localization to the TGN and activation by the trans-Golgi GEF BIG1 (Manolea et al., 2010) and its ability to bind GGAAs (Boman et al., 2000). The only exception is the additional observation of a dilated ER in the Arf3+1+5 KO cell line.

Previous knockdown-based studies reported phenotypes only upon simultaneous silencing of two Arfs (Volpicelli-Daley et al., 2005; Kondo et al., 2012; Nakai et al., 2013). KDEL-receptor localization, for example, was described to be enhanced at the Golgi upon double knockdown of Arf3+Arf4 (Volpicelli-Daley et al., 2005) and of Arf4+Arf5 (Volpicelli-Daley et al., 2005; Li et al., 2015). Our results attribute this phenotype solely to the deletion of Arf4. The same applies to a slight compaction of the Golgi observed upon knockdown of Arf4+Arf5 (Nakai et al., 2013).

In other cases, the reported phenotype, for instance the peripheral β-COP puncta observed upon knockdown of Arf1+Arf3 and Arf1+Arf5 (Volpicelli-Daley et al., 2005; Kondo et al., 2012), cannot be rationalized by our KOs. Knockdown of Arf combinations for which no KO cell lines could be generated could provide information on the defects that lead to growth arrest or cell death. In this line, the simultaneous knockdown of Arf1+Arf4 severely impacted Golgi morphology and AP1 and COPI localization (Volpicelli-Daley et al., 2005; Nakai et al., 2013).

In the present study, we established Arf KO cell lines as tools to study shared and specific functions of Arfs at the Golgi. Of course, these cell lines offer themselves to investigate Arf-dependent processes that do not require specialized cell types.

Materials and methods

Cell culture

Hela cells were grown in DMEM (high glucose; Sigma-Aldrich) with 10% FCS (FCS premium, VWR), 2 mM L-glutamine, 100 U/ml penicillin G, and 100 ng/ml streptomycin at 37°C and 7.5% CO2.

HeLa KO cell lines

Two gRNAs were designed for each targeted Arf gene as listed in Table S5 to facilitate genomic deletion of exon 1 using several online tools (e.g., CRISPOR; Concordet and Haeussler, 2018). gRNAs were cloned in the pSpCas9(BB)-2A-GFP (Addgene plasmid #48138; a gift from Feng Zhang, Broad Institute, Cambridge, MA), and pSpCas9(BB)-2A-mCherry, which was derived the former by exchanging GFP to mCherry. Target cells were
transfected simultaneously with the corresponding plasmids using jetPRIME (Polyplus Transfection). After 24 h, double-fluorescent cells were selected by FACS (FACS AriaIII; BD Biosciences). After 7 d, double-negative cells were selected by FACS and single cells sorted into 96-well plates with growth medium containing 10% conditioned medium. After ~14 d, clonal cell lines were expanded and analyzed.

Genomic PCR
Trypsinized cells were pelleted, resuspended in 10 mM Tris (pH 8.7), heated at 95°C for 10 min, incubated with proteinase K (0.5 µg/µl) for 20 min at 37°C, inactivated at 95°C for 15 min, and used as DNA template for PCR with Phusion Polymerase (NEB) or Q5 Polymerase (NEB, for Arf5 KO), following the manufacturer’s protocol for high GC content. Primers were designed to amplify the genomic region surrounding the site of deletion are listed in Table S5.

Immunoblot analysis
Cell lysates were denatured in SDS-sample buffer for 5 min at 95°C (10 min at 37°C to analyze KDEL receptors), separated by SDS-gel electrophoresis (15% polyacrylamide for Arfs), and transferred to Immobilon-P PVDF membranes (Millipore). Membranes were blocked with TBST (20 mM Tris, 150 mM NaCl, pH 7.6, and 0.1% Tween20) with 3% nonfat dry milk for 1 h and incubated with primary antibody in TBST with 1% milk overnight at 4°C: anti-Arf1 (1:2,500; MAB10011; Abnova), anti-Arf3 (1:1,000; 610784; BD Bioscience), anti-Arf4 (1:1,000; 11673–1-AP; Proteintech), anti-Arf5 (1:750; H00000381-M01; Abnova), anti-actin (1:100,000; MAB1501; Sigma-Aldrich), anti-calreticulin (1:2,500; 27298–1-AP; Proteintech), anti-Grp78/BIP (1:10,000; GTX13340-10; Genetex), and anti-KDEL receptor antibody (1:1,000; 69659; Abcam). After washing, the membranes were incubated with HRP-conjugated secondary antibody (1:10,000, anti-rabbit, A0545, Sigma-Aldrich; anti-mouse, A0168, Sigma-Aldrich) in TBST with 1% milk. Chemiluminescence signals were detected using Immobilon Western HRP Substrate (Millipore) or Radiance Plus (Azure Biosystems) and imaged using a FusionFX (Vilber Lourmat).

Growth assay
Cells were seeded in 12-well plates at a density of 5,500 cells/well, which was confirmed by recounting. Every 24 h for 5 consecutive days, cells from one well for each cell line were trypsinized, resuspended in PBS, and counted. Doubling times were estimated by exponential fitting of the growth curves.

Flow cytometry
The procedure was adapted from Sin and Harrison (2016). Trypsinized cells were collected in ice-cold 10% FCS in PBS, pelleted at 50 rcf (relative centrifugal force) for 5 min at 4°C, washed once with PBS, and resuspended in PBS. Cells were fixed with ice-cold 70% ethanol on ice for at least 2 h. Afterwards, cells were incubated in PBS for 15 min at room temperature and counted. Per condition, 1.5 × 10^6 cells were used. Cells were incubated in PBS with 200 µg/ml RNase A, 0.1% Triton X-100, and 2 µg/ml propidium iodide (Sigma-Aldrich). Fluorescence signals were measured by flow cytometry using the LSR Fortessa (BD Biosciences). Data were analyzed with the FlowJo software, first gating for single cells and subsequently applying the built-in cell cycle fitting model.

Immunofluorescence staining
Cells were grown on glass coverslips for 1 d, then fixed with 3% PFA for 10 min, quenched with 50 mM NH4Cl in PBS for 5 min, permeabilized with 0.2% Triton X-100 in PBS for 10 min, blocked with 1% BSA in PBS for 1 h, and incubated with primary antibodies diluted in 1% BSA in PBS for 1 h: anti-Arfl (1:1,000; self-made from hybridoma cells), anti-βCOP (1:500; CM1; hybridoma supernatant; gift from Dr. Felix Wieland, Heidelberg University, Heidelberg, Germany), anti-GGA2 (1:500; 612613; BD Bioscience), anti-GM130 (1:1,000; 12480S; Cell Signaling), and anti-TGN46 (1:1,000; AHP500G; Bio-Rad). Samples were washed and incubated with fluorescent secondary antibodies diluted in 1% BSA in PBS for 1 h (1:400, anti-mouse-Alexa488, A21202, Invitrogen; anti-rabbit-Alexa568, A10042, Invitrogen; anti-sheep–Cy3, 713–165–147, Jackson ImmunoResearch). For KDEL receptor staining, cells were fixed and quenched as described above, permeabilized with 0.1% saponin in PBS for 20 min, and blocked in 2% BSA and 0.1% saponin in PBS for 30 min. KDEL receptor antibody (1:1,000; 69659; Abcam) and the secondary antibody were diluted in blocking solution and incubated for 1 h and 30 min, respectively. Coverslips were mounted in FluormountG (SouthernBiotech) supplemented with 0.5 ng/ml DAPI (Sigma-Aldrich) for confocal microscopy and in Vectashield (Vector Laboratories) for super-resolution microscopy, and stored in the dark at 4°C. For localization of GFP-KDEL, cells were grown on coverslips for 1 d, transfected with pcDNA3-ssGFP-KDEL, and fixed and stained a day later.

Confocal microscopy and quantitation of coat localization and Golgi volume
Images were acquired using a LSM700 Upright confocal laser-scanning microscope with the Zen 2010 software (Zeiss) equipped with a Plan-Apochromat 63×/1.4 oil-immersion objective lens and two photomultiplier tubes. Imaging parameters were kept constant throughout each experiment. For quantification of coat proteins at the Golgi, the GM130-stained area was selected in Fiji using the freehand tool, and the mean fluorescence intensity was measured for GM130 and the coat protein. To measure Golgi volume, z-stacks at 0.13 µm per slice were acquired and analyzed in Fiji using the “3DGolgiCharacterization” script (https://doi.org/10.5281/zenodo.4068393).

Super-resolution microscopy
3D-SIM was performed on a DeltaVision OMX-Blaze V4 system (Cytiva) equipped with solid-state lasers. Images were acquired using a Plan Apo N 60×, 1.42 NA oil-immersion objective lens (Olympus), and four liquid-cooled sCMOS cameras (pc.edge 5.5, full frame 2,560 × 2,160; PCO). Exciting light was directed through a movable optical grating to generate a fine-striped interference pattern on the sample plane. The pattern was shifted laterally through five phases and three angular rotations of 60° for each z-section. The 405-, 488-, and 568-nm laser lines

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DOI: 10.1083/jcb.202106100

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were used during acquisition, and the optical z-sections were separated by 0.125 µm. For the acquisition at 405 nm, laser power was attenuated to 50% with an exposure time of 40 ms, for 488 nm to 10% and 6 ms, and for 568 nm to 10% and 50 ms. Settings were adjusted to achieve optimal intensities of between 5,000 and 8,000 counts in a raw image of 15-bit dynamic range at the lowest laser power possible to minimize photobleaching. Multichannel imaging was achieved through sequential acquisition of wavelengths by separate cameras.

Raw 3D-SIM images were processed and reconstructed using the DeltaVision OMX SoftWoRx software package (v6.1.3; Cytiva). The resulting size of the reconstructed images was of 512×512 pixels from an initial set of 256×256 raw images. The channels were aligned in the image plane and around the optical axis using predetermined shifts as measured using a target lens and the SoftWoRx alignment tool. The channels were then carefully aligned using the alignment parameter from control measurements with 0.5-µm-diameter multi-spectral fluorescent beads (Invitrogen, Thermo Fisher Scientific). For visualization of the Golgi surface, we used the surface tool of the Imaris Cell Imaging software (Oxford Instruments).

Electron microscopy

Cells were fixed in serum-free medium with 2.5% glutaraldehyde and 3% formaldehyde for 2 h at room temperature, washed with PBS, and incubated with 2% osmium tetroxide and 1% K-hexacyanoferrate in H₂O for 1 h at 4°C. After washing with H₂O, uranyl-acetate (2% in H₂O) was added and incubated at 4°C overnight. Cells were scraped after washing with H₂O, pelleted, dehydrated by sequential incubation in 20%, 50%, 70%, and 90%, and three times 100% acetone/H₂O for 30 min each, infiltrated with EMbed-812 (Electron Microscopy Science) according to the manufacturer’s protocol, and allowed to polymerize for 24 h at 60°C. The embedded cell pellets were cut into 60–70-µm-thin sections using an ultramicrotome (UltracutE, Reichert-Jung), collected on carbon-coated Formvar-Ni grids (Electron Microscopy Science), and stained for 10 min in 4% uranyl acetate – 0.01 M acetic acid and subjected to liquid chromatography with tandem mass spectrometry analysis using a Q Exactive Plus Mass Spectrometer coupled with an EASY-nLC 1000 (both Thermo Fisher Scientific) and a custom-made column heater set to 60°C. Peptides were resolved using a reverse-phase HPLC column (75 µm × 30 cm) packed in-house with C18 resin (ReproSil-Pur C18–AQ, 1.9 µm resin; Dr. Maisch GmbH) at a flow rate of 0.2 µl/min. The following stepwise gradient of buffers A (0.1% formic acid in water) and B (80% acetonitrile, 0.1% formic acid in water) was used for peptide separation: 5–10% buffer B over 5 min, 10–35% over 45 min, 35–50% over 10 min, and finally 50–95% B over 2 min, followed by 18 min at 95% B.

The mass spectrometer was operated in data dependent acquisition mode with a total cycle time of ∼1 s. Each MS1 scan was followed by high-collision dissociation of the 10 most abundant precursor ions with dynamic exclusion set to 45 s. For MS1, 3×10⁶ ions were accumulated in the Orbitrap over a maximum time of 100 ms and scanned at a resolution of 70,000 full width at half maximum (at 200 m/z). MS2 scans were acquired at a target setting of 10⁵ ions, maximum accumulation time of 100 ms, and a resolution of 35,000 full width at half maximum (at 200 m/z). Singly charged ions and ions with unassigned charge state were excluded from triggering MS2 events. The normalized collision energy was set to 27% and the mass isolation window to 1.4 m/z, and one microscan was acquired for each spectrum.

Acquired raw files were imported into Progenesis QI software (v2.0, Nonlinear Dynamics Limited) to extract peptide precursor ion intensities across all samples applying the default parameters. The generated mgf file was searched using MASCOT against a human database (consisting of 41,484 forward and reverse protein sequences downloaded from UniProt on 20200417) and 392 commonly observed contaminants using the following search criteria: full tryptic specificity was required (cleavage after lysine or arginine residues, unless followed by proline); three missed cleavages were allowed; carbamidomethylation was set as fixed modification; oxidation and protein N-terminal acetylation were applied as variable modifications; mass tolerance of 10 ppm (precursor) and 0.02 D (fragments). The database search results were filtered using the ion score to set the false discovery rate to 1% based on the number of reverse protein sequence hits in the dataset. Results from label-free quantitation were processed using the SafeQuant R package v.2.3.2 (PMID: 27345528) to obtain peptide relative abundances.

This analysis included global data normalization by equalizing the total peak/reporter areas across all liquid chromatography with mass spectrometry runs, data imputation using the knn algorithm, summation of peak areas per protein and liquid chromatography with tandem mass spectrometry run, followed...
by calculation of peptide abundance ratios. Only isoform specific peptide ion signals were considered for quantification. To meet additional assumptions (normality and homoscedasticity) underlying the use of linear regression models and tests, mass spectrometry–intensity signals were transformed from the linear to the log-scale. The summarized peptide expression values were used for statistical testing of between condition differentially abundant peptides. Here, empirical Bayes-moderated tests were applied, as implemented in the R/Bioconductor limma package (PMID: 25605792). The resulting per protein and condition comparison P values were adjusted for multiple testing using the Benjamini–Hochberg method. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD028846 (https://www.ebi.ac.uk/pride/archive/projects/PXD028846). G0term enrichment analysis was performed using GORILLa (Eden et al., 2007; 2009).

**Lentiviral transduction**

RNA was isolated from HeLaA cells using the RNeasy Mini Kit (Qiagen) and the RNase-Free DNase Set (Qiagen), and cDNA was reverse-transcribed using the iScript cDNA Synthesis Kit (Bio-Rad). The coding sequences of Arfs were amplified by PCR (primers listed in Table S5), inserted into the pQXCI plasmid (Takara Bio) using the Agel and BamHI restriction sites, and sequenced. Plasmids were transfected into the packaging cell line Phoenix-ampho (Nolan laboratory, Stanford University, Stanford, CA) using jetPrime (Polyplus Transfection). After 24 h, medium was exchanged to a smaller volume of medium supplemented with 1 mM pyruvate. Medium containing the viral particles was collected after 36 h and cleared by filtration through a 0.45-µm filter. After addition of polybrene to 20 µg/ml, the supernatant was transferred onto target cells. Selection was started 48 h after transduction using medium containing 1.5 mg/ml puromycin (InvivoGen). After 10 d, single cells were sorted by FACS (FACS Diva) and expanded to a smaller volume of medium supplemented with 1 mM pyruvate. Medium containing the viral particles was collected after 36 h and cleared by filtration through a 0.45-µm filter. After addition of polybrene to 20 µg/ml, the supernatant was transferred onto target cells. Selection was started 48 h after transduction using medium containing 1.5 mg/ml puromycin (InvivoGen). After 10 d, single cells were sorted by FACS (FACS Diva) and expanded to a smaller volume of medium supplemented with 1 mM pyruvate. Medium containing the viral particles was collected after 36 h and cleared by filtration through a 0.45-µm filter.

**Statistics**

SuperPlots were generated according to Lord et al. (2020), and statistical analysis was done with Prism8 (GraphPad) using unpaired or paired one-way ANOVA and unpaired, two-tailed t tests, respectively. Data distribution was assumed to be normal, but this was not formally tested.

**Online supplemental material**

Fig. S1 shows the cell cycle distribution of HeLaA, Arfliko, and Arf4ko cells. Fig. S2 shows Golgi polarity in parental and Arfliko cells by super-resolution microscopy. Fig. S3 displays the levels of BCOP in Arf KO cell lines by immunoblotting. Fig. S4 shows immunofluorescence colocalization of APTY1 and GGA2 with TGN46. Fig. S5 documents the localization and levels of the KDEL receptors in Arf KO cells. Table S1 lists gRNAs and primers. Table S2 lists significantly up-regulated hits of Arf4ko versus HeLa. Table S3 lists significantly up-regulated hits of Arf3+4 versus HeLa. Table S4 lists G0term enrichment analysis of Arf4ko versus HeLaA. Table S5 lists G0term enrichment analysis of Arf3+4ko versus HeLaA.

**Acknowledgments**

We thank the Imaging Core Facility (University of Basel), in particular Dr. Kai Schleicher, Dr. Alexia Ferrand, and Laurent Guerard, for assistance at the microscopes and with data analysis, and Janine Bügli and Stella Stefanova of the FACS Core Facility for their support.

This work was supported by the Swiss National Science Foundation (grant 31003A-182519).

The authors declare no competing financial interests.

Author contributions: M. Pennauer: conceptualization, investigation, validation, visualization, writing – original draft/review, and editing. K. Buccak: investigation (mass spectrometry analysis). C. Prescianotto-Baschong: investigation (electron microscopy). M. Spiess: funding acquisition, conceptualization, project administration, resources, supervision, writing – original draft/review, and editing.

Submitted: 16 June 2021
Revised: 14 September 2021
Accepted: 4 October 2021

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Figure S1. Distribution of parental Hela, Arf1ko and Arf4ko cells in the interphase. Cells were assigned to G0/1, S, or G2 phase based on their DNA content measured by Hoechst staining and flow cytometry (100,000 cells/experiment; \( n = 3 \)). The fraction of cells in the respective phase is shown in percentage of the total. Mean ± SD of three experiments. Statistical significance was calculated for the G0/1 fraction, using paired one-way ANOVA versus parental (*, \( P < 0.05 \)).
Figure S2. **Golgi polarity is maintained in Arf1ko cells.** Golgi complexes from parental HeLa and Arf1ko cells were immunostained for the cis-Golgi marker GM130 (green) and the TGN marker TGN46 (magenta). Images were acquired as confocal z-stack images using super-resolution microscopy and displayed as maximum intensity projections or tomographic 2D slices. The lower panels show magnified image sections. Scale bars, 3 µm.

Figure S3. **Intracellular βCOP levels.** Levels of βCOP in parental HeLa and Arf KO cell lines were analyzed by immunoblot analysis with actin as a loading control. For each cell line, three biological replicates were analyzed on the same gels with parental lysates.
Figure S4. Coimmunostaining for the TGN marker TGN46 and coat proteins. Parental HeLa cells and all Arf KO cell lines were coimmunostained for TGN46 in magenta and AP1y1 (A) or GGA2 (B) in green, respectively. DAPI signal is shown in blue. Lower panels show magnified image sections. Scale bars, 10 µm.

Figure S5. Intracellular localization and protein level of KDEL receptors. (A) Parental HeLa, Arf1ko, and Arf4ko cells were coimmunostained for KDEL receptors (KDELRS) in green and GM130 in magenta. DAPI signal is shown in blue. Lower panels show magnified image sections. Scale bars, 10 µm. (B) Perinuclear localization of the KDEL receptors was quantified as a ratio to GM130. Average values obtained from three independent experiments with >40 Golgi analyzed per cell line and experiment are shown as filled circles together with the values from individual cells as small dots colored by experiment. Mean ± SD of the three experiments are indicated. Unpaired one-way ANOVA versus parental (***, P < 0.001). (C) KDEL receptor levels in parental HeLa and Arf KO cell lines were analyzed by immunoblot analysis with actin as a loading control. For each cell line, three biological replicates were analyzed on the same gels with parental lysates.
Five supplemental tables are provided as separate Excel files. Table S1 lists gRNAs and primers. Table S2 lists significantly up-regulated hits of Arf4ko versus HeLa. Table S3 lists significantly up-regulated hits of Arf3+4 versus HeLaα. Table S4 lists GOterm enrichment analysis of Arf4ko versus HeLaα. Table S5 lists GOterm enrichment analysis of Arf3+4ko versus HeLaα.