Dominantly acting variants in ARF3 have disruptive consequences on Golgi integrity and cause microcephaly recapitulated in zebrafish

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Abstract

Vesicle biogenesis, trafficking and signaling via the ER-Golgi network support essential processes during development and their disruption can lead to neurodevelopmental disorders and neurodegeneration. We report that de novo missense variants in ARF3, encoding a small GTPase regulating Golgi structure and function, cause a neurodevelopmental disease showing microcephaly and progressive cortical atrophy, with microsomia and rib anomalies in severely affected subjects, suggesting a pleiotropic effect. All microcephaly-associated variants clustered in the guanine nucleotide binding pocket and perturbed the biochemical behavior of the protein by stabilizing it in a GTP-bound state. Functional analysis proved the disruptive consequences of the variants on Golgi integrity, and brain and body plan formation. In-depth analysis in zebrafish embryos expressing ARF3 mutants traced back the developmental alterations to defective gastrulation cell movements as the earliest detectable effect. Our findings document a role of ARF3 in Golgi homeostasis and demonstrate an obligate dependence for early development.
Introduction

The Golgi apparatus (GA) is a polarized, membrane network-built organelle responsible for transporting, modifying, and packaging proteins and lipids into vesicles for their delivery to targeted destinations\(^1,2\). It is organized as a series of flattened, stacked pouches (known as *cisternae*) that are held together by matrix proteins and microtubules, and are structured into two major networks, the *cis* and *trans*-Golgi compartments, coordinating proper sorting of proteins and lipids received from the endoplasmic reticulum and directing their transport toward the cell membrane\(^3,4\). GA organization is highly dynamic and undergoes rapid remodeling in response to different physiological and pathological stimuli via various tightly regulated processes involving ribbon disassembly and tubulovesicular conversion as well as repositioning of Golgi stacks\(^5\). Besides the role of GA in posttranslational modification and sorting of proteins, a large body of studies recently revealed that GA membranes also provide signaling platforms for the regulation of a wide range of cellular processes (e.g., cell polarization, directed migration, stress response, mitosis, and autophagy) orchestrating animal development, suggesting that GA can act as a cell sensor and regulator similarly to other intracellular organelles\(^4\). Not surprisingly, in the last years, a number of Mendelian disorders have been causally related to the defective or aberrant function of component of the GA-related transport machinery and disrupted GA function and organization, most of which sharing altered neurodevelopment and early-onset neurodegeneration\(^6\text{-}^8\). In these disorders, which have collectively been termed as “Golgipathies”, recurrent features include microcephaly, central nervous system (CNS) defects (e.g., delayed myelination, cortical atrophy, abnormal corpus callosum, and pontocerebellar hypoplasia) and developmental delay (DD)/intellectual disability (ID)\(^8,9\).
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The five members of the ADP-ribosylation factors (ARF) family of small GTPases (ARF1, ARF3-6) regulate key events in vesicular biogenesis, transport and various GA functions, and participate in the control of bidirectional membrane trafficking required for secretion, endocytosis and recycling\textsuperscript{10–13}. These proteins bind to guanine nucleotides with high affinity and specificity, and have a slow intrinsic competence to hydrolyze GTP to GDP\textsuperscript{12,14}. ARF proteins are characterized by a unique myristoylated N-terminal region, a GTP/GDP-interacting pocket, and two domains mediating binding with regulators and effectors (\textit{i.e.}, switch 1 [SW1] and switch 2 [SW2] regions) that undergo a GTP/GDP-dependent structural rearrangement, allowing the GTPase to interact with effectors in its GTP-bound state\textsuperscript{15–17}. Similarly to other members of the RAS superfamily, release of GDP is stimulated by specific guanine nucleotide exchange factors (ARFGEFs), which indirectly favor binding to GTP\textsuperscript{12,18,19}. As a consequence of the conformational change promoted by GTP, the N-terminal myristoylated region is exposed, allowing anchoring of the GTPase to the cytoplasmic leaflet of membranes of different organelles, including \textit{cis} and \textit{trans}-Golgi, plasma membrane (PM) and endosomes, where these proteins exert their function\textsuperscript{12,14,17,20,21}. The intrinsic slow GTPase activity of ARFs is substantially accelerated by specific GTPase-activating proteins (ARFGAPs), which result in protein inactivation and release from membranes\textsuperscript{12,21–23}.

To exert their function, ARF proteins interact with a number of effectors, most of which are coat proteins and adaptors\textsuperscript{1,12}; they can also recruit non-coat GA-specific proteins to membranes (\textit{e.g.}, golgin-160 and GCC88)\textsuperscript{24}, which are important for GA structure homeostasis\textsuperscript{25,26}. Evidence shows that ARF proteins actually contribute to the control of GA and organelles structural organization and function\textsuperscript{13,27,28}, and GA dynamics.
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151 during cell division and cytokinesis in precursor cells. By controlling GA structure, function, cargo sorting and ER-GA targeted trafficking, ARFs actively participate to the fine regulation of key events during embryogenesis (i.e., cell polarity establishment and migration in early gastrulation, neuronal maturation and tissue morphogenesis). Indeed, hyperactive arf1 function in zebrafish results in shortening of the anterior-posterior (AP) axis, a likely consequence of an altered planar cell polarity (PCP)-dependent cell movements, which was also shown in a fly wing morphogenesis model. Nevertheless, the underlying mechanisms by which the activity of several ARF proteins on GA organization and trafficking contributes to developmental processes are yet not fully understood.

Notwithstanding the pivotal roles of ARF proteins in development, mutations in ARF genes have only recently been linked to human disease, with activating missense variants of ARF1 (MIM: 103180) causing a rare dominant neurodevelopmental disorder (NDD) resulting from defective neuronal migration (MIM: 618185). Here, we report de novo missense variants in ARF3 underlying a disorder affecting neurodevelopment and causing neurodegeneration in five patients. In silico and in vitro analyses strengthened by in vivo morphometric and live cell behavioral analyses in fish embryos expressing ARF3 mutants provide evidence of a variable impact of mutations on protein function and disruptive consequences on GA integrity, brain and body axes development and PCP-dependent gastrulation processes. These findings demonstrate the relevance of ARF3 function on organelle homeostasis and development.
Results

**ARF3 mutations cause a developmental disorder characterized by microcephaly and cortical atrophy**

In the frame of a research program dedicated to subjects affected by unclassified diseases, trio-based exome sequencing allowed us to identify a previously unreported *de novo* ARF3 variant, c.379A>G (p.Lys127Glu; NM_001659.2), as the putative disease-causing event in a girl (Subject 1) with severe syndromic NDD characterized by growth restriction, microcephaly, progressive diffuse cortical atrophy and other brain anomalies at MRI (*i.e.*, lateral ventricular enlargement, progressive pontocerebellar hypoplasia with major involvement of the cerebellar vermis and hypoplasia of corpus callosum), seizures, inguinal hernia, congenital heart defects (CHD) and skeletal involvement (*i.e.*, 11 rib pairs and scoliosis) ([Table 1, Supplementary Figure 1, Supplementary clinical reports](#)). WES data analysis excluded presence of other relevant variants compatible with known Mendelian disorders based on their expected inheritance model and associated clinical presentation, and high-resolution SNP array analysis excluded occurrence of genomic rearrangements. The missense change, which had not previously been reported in population databases, affected an invariantly conserved residue among orthologs, paralogs and other structurally related GTPases of the RAS family ([Supplementary Figure 2a](#)).

Through networking and GeneMatcher<sup>35</sup>, we identified four additional subjects with *de novo* ARF3 missense variants, which had not been reported in ExAC/gnomAD and involved amino acid residues located in regions highly constrained for variation ([Supplementary Table 1, Supplementary Figure 2b](#)). No additional candidate variants in clinically associated genes were identified in any patients (WES statistics and data...
output are reported in Supplementary Tables 2-6). All affected residues were conserved among ARF3 orthologs and paralogs and three of them were also conserved among other RAS GTPases (Supplementary Figure 2a). The identified missense variants affected residues whose corresponding positions in other GTPases of the RAS superfamily had previously been associated with human disease (Supplementary Table 7). The same Lys-to-Glu substitution at codon 127 in Subject 1 was recently reported to affect the corresponding residue in ARF1 in a patient with DD, microcephaly, periventricular heterotopia, progressive cerebral atrophy and epilepsy. Of note, this amino acid is homologous to Lys in HRAS (MIM: 190020), and an activating missense variant of this residue in HRAS was reported in Costello syndrome (CS [MIM: 218040]). Pro (mutated in Subject 3) is homologous to Pro in HRAS, KRAS and NRAS (Supplementary Figure 2a). The same Pro-to-Ser change have previously been reported as a somatic event in HRAS in vascular tumors, and changes affecting Pro in KRAS, HRAS and NRAS have been described in RASopathies (ClinVar). Moreover, a missense change affecting the adjacent residue in ARF1 (p.Thr48Ile) was observed in a patient with clinical features overlapping with the present series. In HRAS and KRAS, mutations affecting Thr, which is adjacent to the aspartic acid residue homologous to Asp in ARF3 (mutated in Subject 2), have causally been linked to RASopathies. Finally, amino acid substitutions at these residues in ARF3, its paralogs and members of the RAS subfamily have been observed as somatic events in malignancies, providing further evidence of their functional and clinical relevance (Supplementary Table 7).

Affected subjects showed variable degree of DD/ID (Table 1, Supplementary clinical reports). No characteristic craniofacial gestalt was noted, with only minor craniofacial
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features reported, which were mainly related to microcephaly. Likewise Subject 1, Subject 2 showed microcephaly, profound DD, progressive diffuse cortical atrophy with diminished hemispheric white matter, with a thin corpus callosum, progressive pontocerebellar hypoplasia, hypotonia, microsomia, and skeletal defects. A comparable condition was also observed in Subjects 3 and 4, who manifested hypotonia, variable DD/ID, microcephaly, and various MRI anomalies (Table 1). In Subject 5, global DD and cognitive deficits were associated with hypoplasia of the corpus callosum and mild white matter involvement in periventricular and supraventricular areas, with normal head circumference (Supplementary Figure 1).

Early-onset seizures had been reported in Subjects 1 and 3.

**Disease-associated ARF3 variants variably affect protein stability and function**

The identified disease-associated variants affected residues spotted throughout the coding sequence with exception of the C-terminus region (Figure 1a). First, we investigated the functional consequences of each amino acid substitution by using a three-dimensional structure of the GTPase recently solved by X-ray diffraction. We noted that all residues cluster within or close to the GTP/GDP binding pocket (Figure 1b). Specifically, Lys\(^{127}\) is one of the four residues of the NKXD motif directly mediating binding to GTP/GDP by binding to the ribose ring\(^{16}\), and substitution of the positively charged residue with a negatively charge glutamate was predicted to affect proper nucleotide binding (Figure 1c). Similarly, Thr\(^{32}\) contributes to stabilize the GTP/GDP binding via direct hydrogen bonding with one oxygen atom of the \(\alpha\) phosphate (Figure 1c). While conservative, the Thr to Asn substitution was predicted to result in a steric hindrance. Asp\(^{93}\) does not directly contact GTP, even though it participates to the overall general structure of the nucleotide binding pocket by a direct hydrogen bond.
with the lateral chain of Lys\textsuperscript{127} (Figure 1d). Since the high GTP:GDP ratio within cells, these three changes were anticipated to favor an active, GTP-bound state of the GTPase, bypassing the requirement for a GEF, as previously reported for pathogenic mutations affecting RAS proteins\textsuperscript{44,45}. On the other hand, Pro\textsuperscript{47} and Asp\textsuperscript{67} were predicted to affect ARF3 GTPase activity. Specifically, Pro\textsuperscript{47} is located within the switch 1 region, which plays a key role in the catalytic activity of the GTPase and the conformational rearrangement mediating binding to effectors\textsuperscript{12,16}. Substitution of this non-polar residue with a polar serine is expected to strongly perturb the functional behavior of the protein. Similarly, Asp\textsuperscript{67} participates in the coordination of the Mg\textsuperscript{2+} ion through direct hydrogen bonds with a water molecule\textsuperscript{15} (Figure 1c), and contributes to the regulation of GDP/GTP binding upon the “inter-switch toggle” mechanism\textsuperscript{46}; its substitution with valine was predicted to considerably perturb the GTP/GDP binding switch\textsuperscript{17} and the overall organization of the nucleotide binding pocket. No obvious consequence was hypothesized for the substitution involving Leu\textsuperscript{12} (found in cis with p.Asp67Val in Subject 2) which resides in the flexible N-terminal domain of the GTPase. Of note, while Thr\textsuperscript{32}, Asp\textsuperscript{93} and Lys\textsuperscript{127} map regions of the GTPase not directly involved in intermolecular contacts, Pro\textsuperscript{47} and Asp\textsuperscript{67} lie in regions close to the surface of the GTPase interacting with effectors/regulators\textsuperscript{47}, which does not rule out the possibility of a more complex functional behavior of the p.Pro47Ser and p.Asp67Val changes. To explore the structural and functional consequences of these two amino acid substitutions, we built a model of ARF3 interacting with the cytosolic coat protein complex (COP) formed by γ-COP (COPG1) and ζ-COP (COPZ1) starting from an available GTP-bound ARF1:COPG1-COPZ1 complex (PDB: 3TJZ) as template\textsuperscript{48}. The model for the wild-type (WT) ARF3 protein was validated by a 500-ns molecular dynamics (MD) simulation, documenting conservation of all known interactions with
GTP and Mg2+ (Figure 1d,e; Supplementary Table 8). The ARF3:COPG1 interface is stabilized by an intermolecular hydrogen bonding network involving Arg\textsuperscript{19}, Thr\textsuperscript{48} and Asn\textsuperscript{84} ARF3 residues (Supplementary Table 9). We assessed the structural perturbations due to the introduced p.Pro47Ser and p.Asp67Val changes using the same time-frame. A minor impact on the ARF3 surface interacting with COPG1 was evident in the simulation when introducing the Asp67Val substitution (Figure 1f; Supplementary Table 9). As predicted by the structural inspection, this change resulted instead in a significant rearrangement of the nucleotide binding pocket with a reduction of the interactions of Lys\textsuperscript{127} and Thr\textsuperscript{45} with GTP (Supplementary Table 8). The Pro-to-Ser substitution at codon 47 did not significantly affect ARF3 binding to GTP (Supplementary Table 8), while a dramatic perturbation of the intermolecular binding network with COPG1 due to a substantial rearrangement of the Switch 1 region was observed (Figure 1g; Supplementary Table 9). Consistently, essential dynamics analysis documented a major effect of Pro47Ser in terms of global fluctuations and long range correlated movements, compared to the other simulations (Supplementary Figure 3). Overall, the structural analyses predicted that all variants but p.Lys12Val affect GTP/GDP binding and/or the GTPase activity of the protein, promoting an overall stabilization of ARF3 in its catalytically active conformation. A more articulated impact on conformational rearrangements mediating binding to effectors was suggested for p.Pro47Ser.

To experimentally validate the predicted consequences of the identified variants on ARF3 function, we investigated the biochemical behavior of a subset of mutants (i.e., ARF3\textsuperscript{P47S}, ARF3\textsuperscript{L12V,D67V} and ARF3\textsuperscript{K127E}) \textit{in vitro}. First, we examined the protein levels of the selected ARF3 mutants in transiently transfected COS-1 cells. Immunoblotting
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Analysis documented that ARF3<sup>P47S</sup> level was comparable to the WT protein, while we observed a considerably reduced level for both the ARF3<sup>K127E</sup> and ARF3<sup>L12V:D67V</sup> mutants (Figure 2a), which was confirmed also in vivo (Supplementary Figure 4). Treatment with the proteasome inhibitor MG132 and the autophagy inhibitor bafilomycin A1 partially restored the levels of both mutants, indicating a reduced stability and accelerated degradation (Figure 2b).

In its active GTP-bound state, ARF3 is able to bind to the Golgi-associated gamma-adaptin ear-containing ARF-binding protein 3 (GGA3) to regulate downstream events controlling trans-Golgi function and intracellular trafficking<sup>49</sup>. Thereby, we performed pull-down experiments using the GGA3 protein-binding domain (PBD) on cell lysates to compare the relative amounts of GTP-bound fraction of WT and mutant ARF3 proteins and their ability to transduce signaling through proper binding to effectors. Immunoblot analysis revealed a basal increase of the GTP-bound fraction for all mutants compared to WT ARF3, which was statistically significant for ARF3<sup>K127E</sup> and ARF3<sup>L12V:D67V</sup> (Figure 2c). These data are consistent with the MD predictions and indicate a stabilized GTP-bound conformation and an overall hyperactive behavior of the microcephaly-associated variants.

**Golgi integrity is altered in cells expressing the disease-associated ARF3 mutants**

The intracellular localization of ARF proteins is highly dynamic. GTP-bound ARF3 specifically localizes at the trans-Golgi, where it coordinates vesicle budding<sup>14,50</sup>. Based on these considerations and previous observations indicating the relevant role of ARF proteins in maintaining proper organization and function of GA<sup>4,28,51</sup>, we examined the subcellular distribution of C-terminal mCherry-tagged ARF3 mutants and evaluated the
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...morphology of the trans-Golgi compartment using EGFP-tagged galactosidase T (GalT) as a marker, in transfected COS-1 cells and zebrafish embryos (Figure 3a).

As expected, live cell imaging by confocal microscopy documented a diffuse localization of WT ARF3 in cells (GDP-bound ARF3) already from ~4 hours after transfection, and its co-localization with EGFP-GalT (GTP-bound ARF3), which showed a compact morphology of the intact trans-Golgi (Figure 3b; Supplementary Video 1). In striking contrast, cells overexpressing ARF3\textsuperscript{K127E} were characterized by a dispersed EGFP-GalT signal over time (Figure 3c; Supplementary Video 1), indicating a fragmentation of the trans-Golgi structure. This finding was confirmed by analyzing fixed cells overexpressing ARF3\textsuperscript{K127E} also 24 hours after transfection by immunofluorescence analysis against golgin 97, whose staining appeared weak and dispersed compared to what observed in cells overexpressing WT ARF3 (Supplementary Figure 5a,b). Double labeling experiments and quantitative analysis were extended to other ARF3 mutants, which confirmed a variable scattered/absent golgin 97 signal, compared to the perinuclear compact and intense staining documented in cells expressing the WT protein (Supplementary Figure 5c,d). Fragmented trans-Golgi was observed in all the examined cells expressing ARF3\textsuperscript{K127E} and ARF3\textsuperscript{L12V,D67V} and in a relevant proportion of cells expressing ARF3\textsuperscript{P47S} (approximately 40%) (Supplementary Figure 5e). To further validate these findings in vivo, we microinjected WT and mutant mCherry-tagged ARF3 mRNAs in zebrafish embryos at the one-cell stage and assessed protein intracellular localization in superficial cells (envelope layer cells, ELC) of early gastrula (Figure 3a,d,e). Consistent with the in vitro findings, we observed a diffused distribution of WT ARF3 in cells, which partially overlapped with EGFP-GalT staining (Figure 3d). Conversely, ARF3\textsuperscript{K127E} distribution in cells appeared markedly restricted, organized in small puncta, and co-
localized with EGFP-GalT (Figure 3e), providing evidence of a strict localization at the
trans-Golgi and thereby of a stabilized GTP-bound state of the mutant protein. 
Confirming the in vitro observations, differently from what observed in ELC cells in
embryos overexpressing WT mCherry-tagged ARF3, EGFP-GalT staining appeared
reduced and more scattered (with an increase of cells showing “puncta” morphology)
in embryos injected with ARF3K127E mRNA, suggesting trans-Golgi fragmentation
(Figure 3e, yellow arrows, Supplementary Figure 6). Overall, these data suggest that
the p.Lys127Glu and p.Leu12Val;p.Asp67Val changes force ARF3 into a GTP-locked
(active) state, promoting a stable binding of the GTPase to the trans-Golgi membrane
that, in turn, affects Golgi integrity, resembling what previously observed for a
constitutively active (GTP-bound) ARF1 mutant. To a minor extent, this effect was
also documented for the ARF3P47S mutant.

Expression of ARF3K127E and ARF3L12V:D67V in zebrafish embryos causes
pleiotropic effects on development and recapitulates the microcephalic trait
Next, we analyzed the impact of the disease-associated missense changes in early
zebrafish development. WT and mutant myc-tagged ARF3 mRNAs were microinjected
together with the membrane maker GFP-CAAX mRNA at one cell stage
(Supplementary Figure 7a). Few hours after injection, embryos in their cleavage period
were sorted based on the expression of GFP-CAAX (Supplementary Figure 7b, left),
and developmental progression was followed from early time points of gastrulation till
48 hpf embryos (long-pec stage), when morphogenesis is nearly completed and sub-
compartmentalization of different neural structures can be appreciated. Upon
standard rearing conditions, we observed a statistically significant decrease in the
survival rate of embryos expressing each mutant at 24 hpf and 48 hpf, compared to
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those expressing WT ARF3 and their not-injected siblings (Supplementary Figure 7b, right), with the strongest effect observed for ARF3^{K127E}. Development appeared perturbed both at the level of the head and trunk (Supplementary Figure 7c-e). At 24 hpf, the severe phenotype (presence of microcephaly, microphthalmia, defective body elongation and severe lateral bending) was highly prevalent in embryos expressing ARF3^{K127E} (45.4%) (Supplementary Figure 7c',e), and occurred in a small proportion of embryos expressing the ARF3^{L12V;D67V} mutant (3.8%) (Supplementary Figure 7c'',e). While a negligible fraction of embryos expressing the WT protein (4.3%) was mildly affected, this proportion was significantly higher in fish expressing each mutant (74%, ARF3^{K127E}; 27%, ARF3^{L12V;D67V}) (Supplementary Figure 7e). A similar incidence was observed even halving the concentration of injected ARF mutant mRNAs (Supplementary Figure 8a). The phenotype worsened with time for both mutants, with the most severe cases reaching 57% for ARF3^{K127E} and 17% for ARF3^{L12V;D67V} by the time of hatching (48 hpf) (Supplementary Figure 8b, c).

When analyzed at 48 hpf, microcephaly was evident in zebrafish expressing mutant ARF3 alleles (Figure 4a-c'). Indeed, embryos injected with ARF3^{K127E} and ARF3^{L12V;D67V} mRNAs showed a significant reduction of the head area compared to animals expressing WT ARF3 and not injected controls, with the most severe cases lacking the frontal part of the brain and eyes (Figure 4a,c), as observed already at 24 hpf (Supplementary Figure 7d,d'). To validate the significance of the observed head reduction, next we injected ARF3^{WT} and ARF3^{K127E} mRNAs in the transgenic NBT:dsRed line, labeling differentiated neurons (Figure 4b). Volumetric reconstructions and measurements from live confocal z-stack acquisitions at 48 hpf confirmed a significant reduction of the brain volume (Figure 4b,c'). Additional
volumetric measurements obtained from fixed specimens at 48 hpf by labeling mature brain structures using anti-acetylated alpha-tubulin and anti-HuC/Elav antibodies (Supplementary Figure 9) further confirmed the deleterious effect of the ARF3^K127E allele on brain development.

Next, to investigate the developmental processes implicated in the body curvature defects observed in the embryos expressing the ARF3 mutants, we analyzed the morphology and development of the notochord, which is crucial to support the body elongation along the anterior to posterior axis (AP) and for subsequent spine formation. At 30-35 hpf, when notochord morphogenesis is almost concluded, confocal imaging analysis documented the occurrence of multiple notochord curvatures per embryo in animals expressing each of the two mutant mRNAs, which were distributed throughout the AP axis and were characterized by bending of variable degrees (Figure 4d-f). In agreement with the overall severity of the observed phenotype, ARF3^K127E showed a significant higher number of notochord curvatures. On the other hand, quantification of the degree of bending (180°: normal; 179° ≥ angle ≥ 110°: mild; angle ≤ 109°: severe) documented equivalent involvement in embryos expressing the two ARF mutants. No gross phenotype related to either brain or body axis morphology and size was observed in embryos expressing WT ARF3 and in non-injected control siblings. Overall, these data indicate that the severe reduction of the head in animals expressing mutant ARF3 proteins is caused by an impaired brain development, recapitulating patients’ microcephaly and that the observed embryo curvatures are due to a perturbed notochord development. ARF3^K127E and ARF3^L12V;D67V alter PCP-dependent processes in early zebrafish development.
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We investigated further the perturbed head-trunk morphogenesis by tracing back axes establishment in embryos expressing WT and mutant ARF3 proteins. First, we examined patterning and morphogenesis in animals in their segmentation period (15 hpf, 13-14 somites). During this period, forebrain, midbrain and hindbrain are discernible, the embryo AP and ML axes are already established, and the embryo actively elongates with the tail bud protruding together with somites formation. mRNA levels of *Krox20*, which is expressed in anterior cephalic domain (hindbrain rhombomeres) and *MyoD*, expressed at the level of the trunk paraxial mesoderm (developing somites), was assessed in whole-mount embryos by *In situ* hybridization (ISH) (*Figure 5a*). While proper patterning of cephalic hindbrain region and paraxial mesoderm was in place, we observed a significant perturbation of the AP and ML axes (*Figure 5b-f*), which are defects typically linked to alteration of planar cell polarity (PCP)-controlled processes\(^{55,56}\) and consistent with previous observations reported in fish embryos expressing mutant ARF1 proteins\(^33\). In detail, compared to their control siblings, mutant fish showed a clear shortening of the AP length and an expansion of the paraxial tissue in the ML axis (*Figure 5b*). Morphometric analysis of the *Krox20* and *MyoD* mRNA spatial expression profiles revealed a significant reduction of the AP length in the mutant embryos (*Figure 5b,c*) and a significant enlargement of the ML axis was evident in embryos expressing ARF3\(^{K127E}\) (*Figure 5b,d,e*). Consistently, the number of somites in mutant embryos was also reduced (*Supplementary Figure 10*). For both AP and ML axes defects, severely affected embryos were more prevalent among those expressing ARF3\(^{K127E}\) (*Figure 5f*).

Shortening and alteration of the body axes normally depend upon defective gastrulation occurring early during embryogenesis. At this developmental stage, cells
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undergo a complex series of movements converging towards the dorsal midline and extending anteriorly that are controlled by trafficking and asymmetrical positioning of PCP components. To clarify the mechanism by which overexpression of activating ARF3 proteins causes severe axes perturbation in zebrafish development, first we investigated possible extension defects by measuring animal elongation in embryos at around 13 hpf (Figure 6a). At this stage, as a result of correctly orchestrated gastrulation movements, brain thickenings and tail bud are visible at the very anterior and posterior end of the embryo, respectively. Compared to embryos injected with WT ARF3 mRNA, a statistically significant increase of the angle between the developed cephalic and caudal structures was observed in embryos expressing ARF3\(^{K127E}\) (Figure 6b,c), which is indicative of delayed extension cell movements. Of note, embryos expressing the two mutants showed a statistically significant difference in the extent of early body elongation, which was in line with the difference observed during later segmentation stage (15 hpf) and at 24 hpf. In addition, live confocal imaging performed during gastrulation in individual ARF3\(^{WT}\) or ARF3\(^{K127E}\)-mCherry expressing embryos documented delayed epiboly in animals expressing the ARF3 mutant (Figure 6d,e). We also observed defective gastrulation movements in embryos expressing ARF3\(^{K127E}\) during the entire time-lapse, resulting in defective head and tail bud formation by the end of the acquisition.

These findings suggest that compromised convergent extension movements resulting from ARF3\(^{K127E}\) overexpression during gastrulation lead to an impairment of PCP-dependent body axes establishment. Cells expressing the mutant appeared mostly round in shape, with a reduced number of protrusions, with respect to cells expressing the WT protein (close-up in Figure 6d,e and Figure 6f), suggesting the possibility of an
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altered polarity establishment and cytoskeletal organization as a molecular event contributing to the defective cell motility.

Discussion

Here we identify de novo activating missense variants of ARF3 as the molecular event underlying a neurodevelopmental condition characterized by microcephaly, progressive cerebral atrophy, and DD/ID, with short stature and skeletal abnormalities variably occurring as associated features. The phenotype of this disorder is reminiscent of the condition caused by activating mutations in ARF1. Consistently, we show that disease-causing ARF3 variants are variably activating, stabilizing the protein in its GTP-bound conformation. Importantly, we also document that these dominantly acting amino acid substitutions affect Golgi integrity, and perturb brain formation and embryonic axes development in a vertebrate model, tracing back their first assessable effect to defective PCP-mediated cell motility in early precursor cells.

The small GTPases of the ARF family are highly conserved across eukaryotes, occupy different subcellular compartments involved in ER-GA network, and have both redundant and distinct functions. These proteins control key molecular and cellular processes, including targeted intracellular trafficking of signaling proteins, cell migration and division, lipid metabolism, and signaling, which are important during animal development. The intracellular localization and activity of ARF3 at the trans-Golgi, where it promotes recruitment of coat complexes and vesicle formation, is tightly regulated via a conformational switch controlled by reversible GDP-to-GTP binding. By structural inspection, we demonstrated that disease-causing ARF3 mutations affect conserved residues involved in GDP/GTP binding/exchange, and pull-
down assays experimentally confirmed that two of the three tested mutants stabilize
the GTP-bound conformation of the protein, similarly to what has previously been
observed for other small monomeric GTPases of the RAS superfamily (e.g., ARF1, HRAS, KRAS, NRAS, MRAS, RRAS, RRAS2) implicated in human disease (COSMIC database). The functional relevance of this perturbed equilibrium between the inactive and active state of the GTPase was further evidenced in vivo by documenting a strict localization of the ARF3K127E protein at the trans-Golgi in zebrafish embryos. It should be mentioned that two mutations (p.Pro47Ser and p.Asp67Val) affected a conserved hydrophobic region involved in effector binding, with molecular dynamics simulations suggesting a major perturbation exerted by the p.Pro47Ser substitution on ARF3 binding to effectors. These considerations stimulate future studies aimed to demonstrate whether effector binding in these mutants is qualitatively and/or quantitatively altered.

ARF1 and ARF3 are known to play a key role in maintaining organelle integrity. Xiang et al. (2007) showed that physiological GA fragmentation in mitotic cells is principally mediated by the active form of ARF1 (GTP-bound), which triggers the continued budding of COPI vesicles. Similarly, while depletion of ARF1 was not reported to affect GA function, disruption of the GA network was observed in cells expressing a constitutively active ARF1 mutant, causing GA swelling with sustained vesiculation. Consistent with the role of these proteins in maintaining organelles structural organization, live imaging in cells expressing the ARF3K127E protein both in vitro and during early zebrafish gastrulation documented a strong and rapid effect on GA integrity, indicating that a shift of the equilibrium towards the GTP-bound form of ARF3 dramatically affect trans-Golgi morphology and embryo development. The
molecular mechanism by which aberrant ARF3 function causes GA fragmentation and
whether this impacts vesicle biogenesis and trafficking remains to be determined.

The finding of fragmented GA in cells and embryos expressing the disease-associated
ARF3 mutants assigns this disorder to the recently defined family of “Golgipathies”, a
group of neurodevelopmental disorders clinically characterized by a wide spectrum of
central nervous system abnormalities\(^8,9\). Similar to what was observed in the present
cohort, patients with activating \textit{ARF1} pathogenic variants show DD/ID, microsomia,
microcephaly and brain abnormalities, including delayed myelination, cortical and
cerebellar atrophy, and seizures as major features. Different from what observed in the
present series, periventricular heterotopia is a recurrent feature in patients with
activating \textit{ARF1} variants. A related but distinct neurodevelopmental disorder,
periventricular heterotopia with microcephaly (ARPHM, MIM: 608097), has been linked
to biallelic inactivating variants of \textit{ARFGEF2}. This recessive disorder is characterized
by severe DD/ID, epilepsy, brain atrophy and delayed myelination associated with thin
corpus callosum\(^67\). Together with previous findings, our work documents the critical
role of GA for diverse neurodevelopmental processes as well as for neuronal function.

Our \textit{in vivo} results deepen the understanding on the impact of dominant \textit{ARF3}
mutations on development. Post-Golgi cell trafficking and correct sorting of polarity
components are required for asymmetric cell division and migration in the vertebrate
brain, underlying neurogenesis, axon arborization and sustained synaptogenesis\(^32,68\).

In line with this evidence and the features documented in patients with mutated \textit{ARF3}
alleles, transient overexpression of the ARF3\(^{K127E}\) protein coupled to volumetric
imaging reconstruction and live cell analysis in zebrafish embryos revealed a clear
microcephalic trait and occurrence of defects linked to altered PCP signaling during
early gastrulation.

Convergence and extension movements, which require a fine tuning of cell polarity mechanisms and are needed to shape the AP and ML axes and proper head and trunk domains\(^56\), were evidently affected in animals expressing the ARF3 mutants. Biosynthetic trafficking and a correct function of ER and GA is essential during animal development for regulating morphogens' distribution\(^32,33,69\), which include components of the PCP pathway and cadherins required for cell polarity establishment and migration, as shown \textit{in vitro}\(^70\), nematodes\(^71\) and zebrafish\(^72\). Consistent with our findings, fish expressing the hyperactive microcephaly-associated ARF1 mutant show PCP-related axial defects\(^33\), and fish mutants for ARF-interacting COPI/COPII coat components exhibit skeletal and notochord abnormalities with GA disruption\(^73,74\). Of note, the impaired function of ARFGEF2 underlying the microcephalic traits observed in patients with ARPHM, has been linked to proliferative and migratory defects due to \textit{trans}-Golgi to membrane trafficking of E-cadherins and beta-catenin\(^75\). Altogether, this body of evidence points to an important role of ARF-mediated trafficking of signaling components during brain and body plan development deserving further investigation.

In conclusion, our findings highlight a role of ARF3 in the maintenance of \textit{trans}-Golgi integrity, and document an obligate dependence of early developmental processes and brain morphogenesis on proper function of this GTPase, identifying ARF3 as a novel gene implicated, when mutated, in a neurodevelopmental disorder belonging to the emerging class of “Golgipathies”\(^8,9\). Further studies are required to understand how ARF3-driven disruption of GA integrity impacts developmental signaling homeostasis throughout embryogenesis, and thereby the cellular processes underlying brain formation.
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Methods

Subjects

The study has been approved by the local Institutional Ethical Committee of the Ospedale Pediatrico Bambino Gesù IRCCS (OPBG), Rome (1702_OPBG_2018).

Subject 1 was analyzed in the frame of a research project dedicated to undiagnosed disorders (Undiagnosed Patients Program, OPBG), while the other subjects were referred for diagnostic genetic testing. Clinical data and DNA samples were collected, stored and used following procedures in accordance with the ethical standards of the declaration of Helsinki protocols, and after signed consents from the participating families. Permission was obtained to publish photographs of Subjects 1 and 4.

Exome sequencing analysis

In all families, whole-exome sequencing (WES) was performed using DNA samples obtained from leukocytes and a trio-based strategy was used. Target enrichment kits, sequencing platforms, data analysis, and WES statistics are reported in Supplementary Table 2-6 and in the Supplementary Methods. WES data processing, read alignment to the GRCh37/hg19 version of genome assembly, and variant filtering and prioritization by allele frequency, predicted functional impact, and inheritance models were performed as previously reported. WES data output is summarized in Supplementary Table 2-6. Cloning of the genomic portion encompassing the c.34C>G and c.200A>T missense substitutions (p.Leu12Val and p.Asp67Val; Subject 3) was used to confirm that both variants were on the same allele. Variant validation and segregation were assessed by Sanger sequencing in all the subjects included in the study.
**Structural analysis and molecular dynamics simulations**

The structural impact of the disease-associated missense changes was assessed using the available three-dimensional structures of human ARF3 complexed with GTP and *V. vulnificus* multifunctional-autoprocessing repeats-in-toxin (MARTX) (PDB 6ii6). The structure was visualized using the VMD visualization software.

A model of GTP-bound ARF3 interacting with the cytosolic coat protein complex subunits γ-COP (COPG1) and ζ-COP (COPZ1) was built using the SWISS-MODEL automated protein structure homology modelling server (http://swissmodel.expasy.org) using the 2.90 Å resolution X-ray structure (PDB 3TJZ). Alignment of template and model amino acid sequences is reported in Supplementary Figure 11. The p.Asp67Val and p.Pro47Ser mutations were introduced using the UCSF Chimera package. The side-chain orientations were obtained with the Dunbrack backbone-dependent rotamer library, choosing the best rotamer with minimal/no steric clashes with neighboring residues. Following protonation of titratable amino acids at pH=7, proteins were added in cubic boxes and solvated in water. Counter-ions were added to neutralize the charges of the system with the genion GROMACS tool. After energy minimizations, the systems were slowly relaxed for 5 ns by applying positional restraints of 1000 kJ mol⁻¹ nm⁻² to atoms. Unrestrained simulations were carried out for a length of 500 ns with a time step of 2 fs using GROMACS 2020.2. The CHARMM36 all-atom force field was used for the protein description and water molecules were described by TIP3P model. V-rescale temperature coupling was employed to keep the temperature constant at 300 K. The Particle-Mesh Ewald method was used for the treatment of the long-range electrostatic interactions.
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interactions\textsuperscript{88}. The first 5 ns portion of the trajectory was excluded from the analysis.

All analyses were performed using GROMACS utilities.

**Expression constructs and *in vitro* mRNA synthesis**

The full-length coding sequence of WT human ARF3 (NM\_001659.3) was obtained by PCR and cloned into the pcDNA3.1/myc-6His eukaryotic expression vector (Life Technologies). The disease-associated substitutions were introduced by site-directed mutagenesis (QuikChange II Site-Directed Mutagenesis Kit, Agilent Technologies). For zebrafish expression experiments, the myc-tagged (C-terminus) WT and mutant ARF3 sequences were subcloned into the pCS-Dest vector (plasmid 22423, Addgene)\textsuperscript{89} via LRII clonase-mediated recombination (ThermoFisher). pCS-Dest-mKOFP2-CAAX and pCS-Dest-EGFP-GalT were generated by subcloning the Addgene plasmids 75155\textsuperscript{90} and 11929\textsuperscript{91}, respectively. Plasmids were digested and linearized with *Kpn*I (NEB New England Biolabs), and mRNA was produced using mMessage mMachine SP6 transcription kit and poly(A) tailing kit (Thermo Fisher). The GFP-CAAX expressing plasmid was a gift from Dr. M. Handberg Thorsager. For live confocal experiments, pcDNA3/hARF3(WT)-mCherry (plasmid 79420, Addgene)\textsuperscript{92} was used and subcloned into zebrafish pCS-Dest vector. All cloned sequences were confirmed by bidirectional DNA sequencing.

**COS-1 cell culture and transient transfection assays**

COS-1 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal bovine serum (GIBCO), 1x sodium pyruvate and 1x penicillin-streptomycin, at 37 °C with 5% CO\textsubscript{2}. Subconfluent cells were transfected with myc- or mCherry-tagged WT and mutant ARF3 expressing vectors using FuGENE 6 (Promega), according to the manufacturer’s instructions.
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**ARF3-myc immunoblotting**

Transfected COS-1 cells were lysed in radio-immunoprecipitation assay (RIPA) buffer, pH 8.0, containing phosphatase and protease inhibitors (Sigma-Aldrich). Lysates were kept on ice for 30 min and centrifuged at 16,000 g for 20 min at 4 °C. Samples containing an equal amount of total proteins (15 μg) were resolved by 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel (Biorad). Proteins were transferred to nitrocellulose membrane using a dry transfer system (Biorad), and blots were blocked with 5% non-fat milk powder (Biorad) in Phosphate-buffered saline (PBS) containing 0.1% Tween-20 for 1 h at 4 °C and incubated with mouse monoclonal anti-myc (1:1000, Cell Signaling), mouse monoclonal anti-β-tubulin (1:1000, Sigma-Aldrich) and anti-mouse HRP-conjugated secondary antibody (1:3000; Sigma). Pools of zebrafish embryos (n = 30) were collected stored dry (-80 °C) at gastrula (6 hpf) and segmentation stage (15 hpf). Lysates from non-injected control embryos and those injected with myc-tagged ARF3\(^\text{WT}\), ARF3\(^{K127E}\) and ARF3\(^{L12V;D67V}\) were obtained by syringe homogenization in lysis buffer (Tris HCl 10 mM pH 7.4; EDTA 2 mM; NaCl 150 mM; Triton X-100 1% with 1X protein inhibitors cocktail (Roche) and equal amounts of protein extracts (40 μg) were separated on a 12% Sodium dodecyl sulfate (SDS)-polyacrylamide gel. The total protein concentration was determined by the Bradford assay (Bio-Rad) using Cary 100 UV-Vis (Agilent Technologies). After electrophoresis, the proteins were transferred to PVDF membrane (Bio-Rad) using a wet transfer system (Biorad). Blots were blocked with 5% non-fat milk powder (Biorad) or bovine serum albumin (Sigma-Aldrich) in PBS containing 0.1% Tween-20 overnight at 4°C constantly shaking and incubated with primary antibody in blocking solution. The following primary antibodies were used: mouse monoclonal anti-myc (Cell Signaling,
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Dilation 1:1000), rabbit polyclonal anti-GAPDH (Genetex, dilution 1:1000). Following washes in PBST 0.1%, membranes were incubated with anti-mouse and anti-rabbit-HRP-conjugated secondary antibodies (1:3000) (Sigma). Immunoreactive proteins were detected by an enhanced chemiluminescence (ECL) detection kit (Thermo Fisher) according to the manufacturer’s instructions, and an Alliance Mini HD9 was used for chemiluminescence detection (Uvitec).

**ARF3 protein stability assays**

COS-1 cells were seeded at $3 \times 10^5$ in 6-well plates and the following day were transfected with WT or mutant myc-tagged ARF3 expression constructs for 24 hours. A subset of transfected cells was then treated with proteasome inhibitor MG132 (100 μM) or with the autophagy inhibitor bafilomycin A1 (200 nM) (Sigma-Aldrich) for 6 hours.

**ARF3 activation assay**

COS-1 cells ($1 \times 10^6$) were seeded in 100 mm petri dishes and transfected with myc-tagged ARF3 expression constructs. Twenty-four hours after transfection, cells were washed twice with ice cold PBS and collected in 50 mM Tris (pH 7.4), 150 mM NaCl, 10 mM MgCl$_2$, 10% Glycerol, 1% NP-40 with proteases and phosphatase inhibitors (Sigma-Aldrich). Cell lysates were further subjected to pull-down using GGA3-conjugated agarose beads (Cell Biolabs) and incubated at 4 °C for 60 min. For immunoblotting analyses, pulled down samples and whole cell lysates were combined with a 2x sample buffer and denatured at 95 °C for 5 min. Samples were then separated by SDS-PAGE and incubated with anti-myc and anti-β-tubulin antibodies. GTP-bound protein level was detected by an ECL detection kit (Thermo Fisher).
ARF3 protein localization and Golgi morphology assessment in fixed COS-1 cells

COS-1 cells (30 x 10^3) were seeded in 24-well cluster plates onto 12-mm cover glasses and transfected with WT or mutant mCherry-tagged ARF3 expression constructs for 24 hours. Cells were then fixed with 3% paraformaldehyde for 30 minutes at 4 °C, followed by permeabilization with 0.5% Triton X-100 for 5 minutes at room temperature. Cells were stained with mouse monoclonal anti-Golgin 97 antibody (1:50, Abcam) for 1 hour at room temperature, rinsed twice with PBS and incubated with Alexa Fluor 488 goat anti-mouse secondary antibody (1:200, Molecular Probes) for 1 hour at room temperature. After staining, coverslips were mounted on slides by using Vectashield Antifade mounting medium (Vector Laboratories) containing 1.5 μg/ml DAPI (Sigma). Images were acquired using Olympus Fluoview FV1000 confocal microscope using 60x/1.42 oil objective and signals from different fluorescent probes were taken in sequential scanning mode. Cells were screened for incidence of Golgi phenotype.

Time-lapse imaging of Golgi dynamics in COS-1 cells expressing ARF3

For live imaging, COS-1 cells (10 x 10^4) were seeded into μ-dishes 35 mm (Ibidi) 24 hours before transfection. The day after, cells were co-transfected with WT or mutant mCherry-tagged ARF3 and EGFP-GalT constructs. Four hours post-transfection, time-lapse acquisitions were performed with a Leica TCS-SP8X confocal microscope (Leica Microsystems) with a PLAp0 CS2 20x/0.75 objective. Z-reconstructions of serial single optical sections were obtained every 15 min and carried out with a 1024x1024 format, scan speed of 600Hz, a zoom magnification up to 1.5 and z-step size of 0.7 μm. Live imaging of samples was performed simultaneously using the Mark & Find mode of the LAS X software. Time-lapse microscopy was
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performed with a stage incubator (OkoLab) allowing to maintain stable conditions of temperature, CO₂ and humidity during live cell imaging.

Zebrafish husbandry

Zebrafish NHGRI-1 and Tg(NBT: dsRed) were cultured following standard protocols. Fish were housed in a water circulating system (Tecniplast) under controlled conditions (light/dark 14:10, 28 °C, 350-400 uS, pH 6.8-7.2) and fed daily with dry and live food. All experiments were approved by the Italian Ministry of Health (23/2019-PR).

Zebrafish mRNA microinjection

Injection of in vitro synthesized capped mRNAs encoding myc-tagged ARF3 (7.5 and 15 pg), mCherry-tagged ARF3 (15 pg), mKOFP-CAAX (15 pg), H2A-mCherry (15 pg), EGFP-GalT (15 pg), EGFP-CAAX (15 pg) and EGFP-GalT (50pg) was performed in one-cell stage zebrafish embryos using FemtoJet 4x microinjection system (Eppendorf). Injected embryos were cultured under standard conditions at 28 °C in fresh E₃ medium and for each batch, non-injected fish were used as controls together with fish injected with the WT form of ARF3 mRNA. Embryos were monitored every day and survival rate was analyzed at 24 and 48 hours post-fertilization (hpf).

Zebrafish body axis, notochord and head phenotyping

Embryos were screened for gross phenotype penetrance classified as normal, mild and severe at 24 hpf. For detailed analysis, not-injected controls and injected fish at 12 and 15 hpf (for body axis), 24 hpf (for notochord) and 48 hpf (for head size) were embedded in 2% low melting agarose dissolved in E₃ medium. Bright-field images were acquired at Leica M205FA microscope with 0.63x magnification (Leica Microsystems).
These parameters were assessed: i) angle between the antero-posterior ends (body axis); ii) number of notochord curvatures and degree of the notochord angles (plotted in a Rose diagram using Oriana)\(^6\); iii) head size measured by the area surface between the rostral most part of the head and the optic vesicle.

**Whole-mount immunofluorescence for acetylated α-tubulin and HuC/Elav in zebrafish embryos**

Whole-mount samples were fixed in 4% paraformaldehyde (Thermo Fisher), washed in PBS-Triton 0.8% (PBSTr 0.8%), permeabilized with proteinase K treatment (1 µg/ml) and incubated in 2% blocking reagent (Roche) for 2 hours. Samples were then incubated with the primary antibody in 2% blocking reagent overnight at 4 °C (anti acetylated α-tubulin, 1:500, Sigma-Aldrich). After several washes in PBSTr 0.8%, samples were incubated with the Alexa Fluor 488 goat anti-mouse secondary antibody (1:1000, Thermo Fisher) overnight at 4 °C with gentle shaking. Whole-mount larval samples were mounted in 90% glycerol. Dorsal z-stacks (volumes) of embryos stained for acetylated α-tubulin were acquired with a Leica TCS-SP8X confocal microscope with PLApO CS2 20x/0.75 objective scanning with 1024 x 1024 format, speed of 400 Hz and z-step size of 2 µm. Embryos stained for HuC/Elav were imaged as above or using Olympus FV1000, objective 20x/0.75 dry, with same parameters.

**Zebrafish live brain volume imaging and 3D rendering from Tg(NBT:dsRed) fish**

Live confocal acquisitions from 48 hpf injected Tg(NBT:dsRed) fish embedded in 2% low melting agarose in E\(_3\) medium were obtained with Leica Stellaris 5 confocal microscope using hybrid detectors and keeping minimal laser power. Scans were obtained with 512x 512 resolution, 400 Hz. Live z-stacks were acquired with Fluotar 25x/0.95 water-immersion objective and with a z-step size of 2.5 µm. Volumetric brain
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reconstructions from representative 48 hpf injected Tg(NBT:dsRed) fish were obtained using 3D Volume (Blend model) and Surfaces rendering functions of Imaris (Bitplane), employing the same parameters for the different individuals.

Zebrafish brain volume analysis

Anterior brain volume was measured on both live Tg(NBT:dsRed) embryos and fixed specimens stained with antibodies against HuC/Elav and acetylated α-tubulin. A region of interest (ROI) comprising the most anterior part of the NBT:dsRed+ neurons and the cerebellum was selected along the whole z-stacks of the confocal 3D data using Fiji analysis tools. Image data were binarized employing Threshold/Otsu and B&W/Dark background algorithms. For every z-stack layer the surface area measurement was extrapolated using a custom-made macro. Volumetric measurements were obtained taking into account the x Z-step value.

Whole-mount in situ hybridization of Krox20 and MyoD mRNA

The fragments of Krox-20 and MyoD cDNA used for riboprobe synthesis were amplified from a zebrafish cDNA preparation by PCR using One Taq DNA polymerase (NEB New England Biolabs) and the primers listed in Supplementary Table 10. The PCR fragments were cloned into pGEM-T Easy vector (Promega) and sequences were confirmed by DNA sequencing. The digoxigenin-labeled antisense riboprobes were synthesized by in vitro transcription with DIG RNA labeling kit SP6/T7 (Roche). In situ hybridization analysis in whole-mount zebrafish embryos at 15 hpf was performed as previously described⁹⁷. Briefly, samples were permeabilized with proteinase K treatment (1 µg/ml) for 2 minutes, pre-incubated in 2% blocking reagent (Roche) and incubated with riboprobes (2 ng/µl) in hybridization mix (50% formamide, 1.3x SSC, 100 g/ml heparin, 50 µg/ml yeast RNA, 0.2% Tween-20, 0.5% CHAPS, 5 mM EDTA
pH 8) at 65 °C for at least 15 hrs. Afterwards, samples were rinsed with scalar dilutions
of SSC solutions and incubated with anti-alkaline phosphatase (AP)-conjugated
antibody (1:5000, Roche) for 2 hours at room temperature. Chromogenic staining was
developed via BM Purple substrate (Roche) according to manufacturer’s instructions.
Specimens were mounted in 90% glycerol and dorsal images were acquired from
Olympus TH4-200 microscope (Olympus Life Science) with 10x objective.

Confocal live imaging of zebrafish embryos during gastrulation
For in vivo imaging, embryos at mid-gastrula stage were embedded in 2% low melting
agarose dissolved in E3 medium. 4D fluorescent data were acquired using Leica TCS-
SP8X confocal microscope using hybrid detectors, keeping minimal laser power.
Scans were obtained in a 1024 x 1024 format, with a speed of 400Hz. Fluorochromes
unmixing was performed by acquisition of automated-sequential collection of multi-
channel images, in order to reduce spectral crosstalk between channels, and the same
setting parameters were used for all examined samples. For ARF3 localization and
Golgi detection sequential confocal images were acquired with Fluotar 25x/0.95 water-
immersion objective with a z-step size of 2.5 μm. Gastrulation time-lapses were
acquired with a PIAPo CS2 20x/0.75 objective. Z-reconstructions of serial single optical
sections were obtained every 30 min with a z-step size of 3 μm. Embryo live imaging
was performed simultaneously using the Mark & Find mode of the LAS X software.

Trans-Golgi fragmentation analysis in live zebrafish embryos
Fragmented trans-Golgi morphology was assessed from single confocal images of the
animal pole of at 12 hpf embryos injected with ARF3\textsuperscript{WT} and ARF3\textsuperscript{K127E} GalT-GFP and
mKOFP-CAAX mRNA. Trans-Golgi morphology (GalT-GFP+) in each cell was scored
as “ribbon” if it displayed a recognizable circular and compact structure or “puncta” if it
displayed a clear fragmented pattern. Circa 30-40 cells per embryo were counted, n= 4 (ARF3^{WT}) and n = 5 (ARF3^{K127E}) embryos. Fisher’s exact test in a 2×2 contingency table (puncta vs. ribbon) (**** p < 0.0001) is used to assess statistical significance.

**Statistical analysis and Image processing**

Data were analyzed independently by at least two researchers and statistical assessments were performed using GraphPad Prism. Log-rank (Mantel-Cox) test was used to assess survival in zebrafish mutants. For phenotype penetrance assessment Fisher’s exact test in a 2x2 contingency table was used, performed as pairwise statistical comparisons across experimental conditions (normal versus mild or severe in vivo and compact versus fragmented Golgi morphology phenotype for in vitro analyses). Normality tests (Anderson-Darling, D’Agostino & Pearson, Shapiro-Wilk and Kolmogorov-Smirnov tests) were run to assess normal distribution of the data. Parametric data with more experimental groups were analyzed with Anova test, non-parametric data with Kruskal-Wallis test and specific post hoc tests were always used as indicated in the figure legends. Whenever multiple measurements in zebrafish were performed on the same individual (e.g., different cells from the same embryo), analysis with a mixed-effects model were used to take account of resampling from the same cluster (embryo). All the analyses were two-tailed. Outliers identification were assessed by ROUT method (Q = 1%). An overview of the statistical analysis for each dataset is shown in Supplementary Table 11. Raw images were analyzed with Fiji^98, LAS X Life Science imaging software (Leica Microsystem), FV10-ASW software, Olympus CellSens Standard imaging software (Olympus Life Science) and Imaris (Bitplane) and processed using Photoshop or Illustrator (Adobe Systems Incorporated).
for figure assembly. Brightness and contrast were adjusted equally across the whole image.

**Supplemental data description**

The article includes the following supplemental data:

- Supplementary Methods and clinical reports, 11 figures, 11 tables and 1 video (.mp4).

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**Authors’ contribution**

G.F. designed and performed the *in vivo* experiments and contributed to write the manuscript. V.M. designed and performed the *in vitro* experiments and contributed to write the manuscript. F.C.R. coordinated the clinical data collection and phenotyping, analyzed the clinical data and contributed to write the manuscript. L.A.C., S.P. and S.C. performed the confocal scanning experiments. M.V. performed the *in situ* hybridization assays. A.Z., A.V., F.P., S.P., A.B., and M.I. generated and analyzed the
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... genomic data. G.C., I.G.P. and B.C. performed the structural analyses and molecular dynamics simulations. M.B., C.B., D.M., A.S., M.M., M.V.G., A.B., R.G., A.S. and B.D. identified the patients, and collected and analyzed the clinical data. A.L. and M.T. conceived, designed, supervised the project, analyzed the data, and wrote the manuscript.

Declaration of Interests

The authors declare no competing interests.

Data availability

The data generated in this work are available upon request from the corresponding authors.
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**Figures**

**Figure 1.** Structural organization of ARF3, location of mutated residues and molecular dynamics analyses. (a) Domain organization of ARF3 excluding the
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unstructured C-terminal tail. Switch 1, switch 2 and the NKXD fingerprint motif are highlighted in pink, green and yellow, respectively. The variants identified in affected subjects are also reported; (b-c) 3D structure in two different orientations of GTP-bound ARF3 interacting with the MARTX toxin (PDB 6ii6). Side chains of the ARF3 residues mutated in the affected subjects and GTP are in cyan and red, respectively. Main chain of residues belonging to switch 1, switch 2 and NKXD fingerprint motif are colored as above; (d) Enlargement of the ARF3 GTP binding pocket with the five mutated residues. The direct hydrogen bond between the N atom in the Lys127 lateral chain and the oxygen atom of the GTP ribose ring is highlighted in dashed line. The Mg$^{2+}$ ion is colored in magenta, while the oxygen atom of the water molecule, mediating the interaction between Asp67 and the manganese ion, is shown in light blue color. The two hydrogen bonds between Asp67 and the water molecule are highlighted with dotted lines. (e) Homology model of GTP-bound ARF3 interacting with the cytosolic coat protein complex COPG1-COPZ1 (PDB: 3TJZ) validated by a 500-ns molecular dynamics (MD) simulation. The region of contact between ARF3 and COPG1 (orange color) is shown in e. (f-h) MD simulations of wild-type (f), p.Asp67Val (g) and p.Pro47Ser (h) ARF3 complexed with COPG1-COPZ1. Residues involved in the contact are shown with their side chain and colored as the respective protein/region. ARF3 backbone is represented with a diameter proportional to its per-residue fluctuations (RMSF).
Figure 2. Expression, stability and GTPase activity of WT and mutant ARF3 protein in COS-1 cells. (a) Expression levels of each mutant myc-tagged ARF3 protein assessed in transiently transfected COS-1 cells via immunoblot. (b) Western blot analysis shows the protein levels of WT ARF3 and ARF3^K127E and ARF3^L12V,D67V mutants in transfected COS-1 cells, basally and after treatment with MG132 (100 µM)
and bafilomycin A1 (200 nM) for six hours. (c) Pull-down assay using GGA3-conjugated beads shows ARF3 activation in COS-1 cells transiently expressing WT or mutant myc-tagged ARF3 proteins. Active and total ARF3 levels are monitored using anti-myc antibody. Representative blots and mean ± standard error (SEM) of three independent experiments are shown. Statistical differences are obtained by one-way Anova (a,c) followed by Dunnet post hoc test and two-way Anova (b) (ns = not significant, * p < 0.05, **p < 0.01, ***p < 0.001, **** p < 0.0001). For the ARF3L12V:D67V mutant the statistical comparison between no treatment (-) and treatment with MG132 results in p=0.07 indicating an overall trend towards increased expression levels of the mutant ARF3 following the treatment.
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Figure 3. Trans-Golgi fragmentation in cells and zebrafish embryos expressing the mutant mCherry-tagged ARF3\textsuperscript{K127E}. (a) Schematic representation of the experimental set up in both \textit{in vitro} and \textit{in vivo} systems. COS-1 cells are transfected with DNA constructs expressing WT and mutant ARF3-mCherry (magenta) and EGFP-GaIT (\textit{trans}-Golgi maker, green) and analyzed by live confocal microscopy between 4-6 hours post transfection. Zebrafish embryos are injected at 1 cell stage with WT and mutant ARF3-mCherry and EGFP-GaIT mRNAs. mKOFP CAAX mRNA is used as membrane marker (cyan). Animals are analyzed by live confocal microscopy during gastrulation (~6-7 hpf). (b, c) Maximum intensity projections of representative confocal images from a time-lapse experiment (Video S1) performed in transfected COS-1 cells at 15 min (~4 hours post transfection) and 120 min later (~6 hours post transfection) from the start of the time-lapse experiment. The images show diffused EGFP-GaIT signal (\textit{trans}-Golgi fragmentation) in ARF3\textsuperscript{K127E} over time (white arrows). Scale bar = 20μm. (d,e) Representative 3D image reconstructions from live confocal z-stack acquisitions of the animal pole in developing zebrafish embryos expressing ARF3\textsuperscript{WT} and ARF3\textsuperscript{K127E} at the mid-gastrulation stage (between 6-7 hpf). White arrowheads indicate a compact \textit{trans}-Golgi morphology surrounding the nucleus (“ribbon”) in the envelope layer cells. Yellow arrowheads indicate cells showing “punta” morphology of the TG dispersed throughout the cytosol. Scale bar = 500μm.
Figure 4. Zebrafish embryos expressing ARF3 mutant proteins show microcephaly and axial defects with notochord curvatures of variable severities. (a) Representative bright field images of the head (purple dashed line) in fish injected with ARF3 WT or ARF3\textsuperscript{K127E} and ARF3\textsuperscript{L12VD67V} mutants at 48 hpf, scale bar = 250μm. (b) Volumetric reconstructions from live confocal acquisitions of whole brains in 48 hpf Tg(NBT:dsRed) fish injected with ARF3\textsuperscript{WT} and ARF3\textsuperscript{K127E} show examples of mild and...
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severe microcephaly, scale bar = 50μm. Te: telencephalon, OT: optic tectum, ce: cerebellum, hind: hindbrain. (c) Head area (indicated by the purple dashed contour in a) in ARF3^K127E (n = 22) and ARF3^L12V;D67V (n = 25) not injected controls (n = 25) and ARF3^WT (n = 26) expressing fish. (c') Brain volume in Tg(NBT:dsRed) fish at 48hpf, n =4. (d) Representative bright filed images of notochord curvatures (red angle calculated on single notochords), classified as normal (angle = 180°), mild (179° >angle>110°) and severe (109°>angle>0°), in fish expressing ARF3^WT, ARF3^K127E and ARF3^L12V;D67V at 30 hpf. The rose diagrams show the distributions of angles for each set of notochordal observations. (e) Number of notochord curvatures in embryos expressing mutant or WT ARF3. (f) Incidence of embryos showing notochord curvatures. In e and f n = 14, 17, 15, 11 for not injected, ARF3^WT, ARF3^K127E and ARF3^L12V;D67V, respectively. Data are plotted as mean ± SEM (c,c'), median with interquartile range (e) or percentage of embryos (f) from two (c,c'), or three (e, f) batches of embryos. One-way ANOVA followed by Tukey’s post hoc test (c), unpaired t-test with Welch’s correction (c’) non-parametric Kruskal-Wallis test followed by Dunn’s post hoc test (e) and Fisher’s exact test (f) are used to assess statistical significance (ns= not significant, ** p < 0.01, *** p < 0.001, **** p < 0.0001). Multiplicity adjusted P values are reported for (c,c’) and (e).
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Figure 5. Zebrafish embryos expressing ARF3 mutant proteins exhibit defective axes formation during segmentation. (a) Schematic representation of Krox20 and
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MyoD mRNA localization (light violet), labeling rhombomeres 3 and 5 (hindbrain) and developing somites (in the paraxial mesoderm), respectively, at 15 hpf. Whole-mount in situ hybridization is used to measure AP and ML embryo extension and ML extension of somites (1, 2, 3 indicate the anterior, medial and posterior somites, respectively). (b) Representative bright field images showing Krox20 and MyoD in situ mRNA staining in fish expressing WT and mutant ARF3, aberrant AP and ML axes and somite width are indicated by black square brackets, scale bar = 200μm. (c-e) AP embryo extension is reduced (c) while ML embryo (d) and somite (e) extension are increased in fish expressing ARF3 mutants, n = 10. (f) Frequency of AP and ML extension defects, n =44, 48, 37 and 33 for not injected, ARF3WT, ARF3K127E and ARF3L12V,D67V, respectively. Unaffected embryos were characterized by an unaltered MyoD and Krox20 mRNA expression compared to not-injected controls, while the mildly and severely affected embryos showed a reduced expression of the two markers along the AP axis and expansion of the ML axis. Severe cases also showed pronounced ML convergence defects, which were evident by the widely separated left and right MyoD-positive domains. Data are plotted as mean + SEM (c, d), median with interquartile range (e) or percentage of the embryos (f) from two (c-e) and three (f) batches of embryos. One-way Anova with Tukey’s post hoc test (c, d), non-parametric Kruskal-Wallis with Dunn’s post hoc test (e) or Fisher’s exact test in a 2×2 contingency table (f; severe vs. normal) (ns= not significant, * p < 0.05, *** p < 0.001, **** p < 0.0001) are used to assess statistical significance. For c, d and e the multiplicity adjusted P values are reported.
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Figure 6. Overexpression of activating ARF3\textsuperscript{K127E} in zebrafish embryos impairs convergence and extension movements during gastrulation. (a) Schematics of the experimental design. mCherry-tagged ARF3 and GFP-tagged CAAX are injected in one-cell stage embryos and morphometric measurements are performed between 6
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hpf (gastrulation) and 13 hpf (segmentation). (b) Representative bright field images from live embryos injected with ARF3\textsuperscript{WT} and ARF3 mutant mRNAs (K127E and L12V;D67V) at 13 hpf. Lateral view with anterior region on the top is shown. The angle (alpha, black) between the anterior end and posterior end \textit{anlage}, reflecting the extent of antero-posterior (AP) axis extension, is shown. (c) Values of the angle alpha showing the extension of the AP axis and measured in ARF3\textsuperscript{WT} and ARF3\textsuperscript{K127E} and ARF3\textsuperscript{L12V;D67V} embryos are shown, with a significant increase recorded in ARF3\textsuperscript{K127E} mutants, n = 30, 29, 26, 31 for not injected, ARF3\textsuperscript{WT}, ARF3\textsuperscript{K127E} and ARF3\textsuperscript{L12V;D67V}, respectively. (d-e) Confocal images (maximum z-projections) from a live time-lapse experiment showing lateral view of the embryos at different time points during gastrulation. Images show cells labelled with GFP-tagged CAAX protein expression labeling cellular outlines. Expression of mCherry-tagged ARF3\textsuperscript{WT} in the injected embryos is shown in the upper inset. Higher magnifications on the right (black dashed boxes) show cell protrusions (some are indicated with yellow arrows) of medial cells at circa 8 hpf in ARF3\textsuperscript{WT} and ARF3\textsuperscript{K127E} embryos. (f) Number of cell protrusions counted for 10 cells located in the medial portion (centre) of the embryo (between 7 and 8 hpf), n = 1. Data are plotted as median with interquartile range from two batches of embryos (c) and mean value of the cells (f). Nonparametric Kruskal-Wallis with Dunn’s post hoc test (c) or Two-way Anova, with mixed-effects model analysis and Sidak’s post hoc test (f) (* p < 0.05, ** p < 0.01; **** p < 0.0001) are used to assess statistical significance. For c the multiplicity adjusted P values are reported.
References

1. D’Souza-Schorey, C. & Chavrier, P. ARF proteins: roles in membrane traffic and beyond. *Nat. Rev. Mol. Cell Biol.* 7, 347–358 (2006).

2. Cockcroft, S. *et al.* Phospholipase D: a downstream effector of ARF in granulocytes. *Science* 263, 523–526 (1994).

3. Guo, Y., Sirkis, D. W. & Schekman, R. Protein Sorting at the trans-Golgi Network. *Annu. Rev. Cell Dev. Biol.* 30, 169–206 (2014).

4. Makhoul, C., Gosavi, P. & Gleeson, P. A. Golgi Dynamics: The Morphology of the Mammalian Golgi Apparatus in Health and Disease. *Front. Cell Dev. Biol.* 7, 112 (2019).

5. Wei, J.-H. & Seemann, J. Golgi ribbon disassembly during mitosis, differentiation and disease progression. *Curr. Opin. Cell Biol.* 47, 43–51 (2017).

6. Seifert, W. *et al.* Cohen syndrome-associated protein, COH1, is a novel, giant Golgi matrix protein required for Golgi integrity. *J. Biol. Chem.* 286, 37665–37675 (2011).

7. Shamseldin, H. E., Bennett, A. H., Alfadhel, M., Gupta, V. & Alkuraya, F. S. GOLGA2, encoding a master regulator of golgi apparatus, is mutated in a patient with a neuromuscular disorder. *Hum. Genet.* 135, 245–251 (2016).

8. Rasika, S., Passemard, S., Verloes, A., Gressens, P. & El Ghouzzi, V. Golgipathies in Neurodevelopment: A New View of Old Defects. *Dev. Neurosci.* 40, 396–416 (2018).

9. Dupuis, N. *et al.* Dymeclin deficiency causes postnatal microcephaly, hypomyelination and reticulum-to-Golgi trafficking defects in mice and humans. *Hum. Mol. Genet.* 24, 2771–2783 (2015).
10. Chavrier, P. & Goud, B. The role of ARF and Rab GTPases in membrane transport. 
   *Curr. Opin. Cell Biol.* **11**, 466–475 (1999).

11. Volpicelli-Daley, L. A., Li, Y., Zhang, C.-J. & Kahn, R. A. Isoform-selective Effects of 
   the Depletion of ADP-Ribosylation Factors 1–5 on Membrane Traffic. *Mol. Biol. Cell* **16**, 4495–4508 (2005).

12. Donaldson, J. G. & Jackson, C. L. ARF family G proteins and their regulators: roles in 
   membrane transport, development and disease. *Nat. Rev. Mol. Cell Biol.* **12**, 362–375 (2011).

13. Zhang, C. J. *et al.* Expression of a dominant allele of human ARF1 inhibits membrane 
   traffic in vivo. *J. Cell Biol.* **124**, 289–300 (1994).

14. Gillingham, A. K. & Munro, S. The small G proteins of the Arf family and their 
   regulators. *Annu. Rev. Cell Dev. Biol.* **23**, 579–611 (2007).

15. Amor, J. C., Harrison, D. H., Kahn, R. A. & Ringe, D. Structure of the human ADP- 
   ribosylation factor 1 complexed with GDP. *Nature* **372**, 704–708 (1994).

16. Kjeldgaard, M., Nyborg, J. & Clark, B. F. The GTP binding motif: variations on a 
   theme. *FASEB J. Off. Publ. Fed. Am. Soc. Exp. Biol.* **10**, 1347–1368 (1996).

17. Goldberg, J. Structural basis for activation of ARF GTPase: mechanisms of guanine 
   nucleotide exchange and GTP-myristoyl switching. *Cell* **95**, 237–248 (1998).

18. Donaldson, J. G., Finazzi, D. & Klausner, R. D. Brefeldin A inhibits Golgi membrane- 
   catalysed exchange of guanine nucleotide onto ARF protein. *Nature* **360**, 350–352 
   (1992).

19. Helms, J. B. & Rothman, J. E. Inhibition by brefeldin A of a Golgi membrane enzyme 
   that catalyses exchange of guanine nucleotide bound to ARF. *Nature* **360**, 352–354 
   (1992).
20. Randazzo, P. A., Yang, Y. C., Rulka, C. & Kahn, R. A. Activation of ADP-ribosylation factor by Golgi membranes. Evidence for a brefeldin A- and protease-sensitive activating factor on Golgi membranes. *J. Biol. Chem.* **268**, 9555–9563 (1993).

21. Antonny, B., Huber, I., Paris, S., Chabre, M. & Cassel, D. Activation of ADP-ribosylation factor 1 GTPase-activating protein by phosphatidylcholine-derived diacylglycerols. *J. Biol. Chem.* **272**, 30848–30851 (1997).

22. Randazzo, P. A. Resolution of two ADP-ribosylation factor 1 GTPase-activating proteins from rat liver. *Biochem. J.* **324** (Pt 2), 413–419 (1997).

23. Reinhard, C., Schweikert, M., Wieland, F. T. & Nickel, W. Functional reconstitution of COPI coat assembly and disassembly using chemically defined components. *Proc. Natl. Acad. Sci. U. S. A.* **100**, 8253–8257 (2003).

24. Gilbert, C. E., Sztul, E. & Machamer, C. E. Commonly used trafficking blocks disrupt ARF1 activation and the localization and function of specific Golgi proteins. *Mol. Biol. Cell* **29**, 937–947 (2018).

25. Munro, S. The golgin coiled-coil proteins of the Golgi apparatus. *Cold Spring Harb. Perspect. Biol.* **3**, a005256 (2011).

26. Kulkarni-Gosavi, P., Makhoul, C. & Gleeson, P. A. Form and function of the Golgi apparatus: scaffolds, cytoskeleton and signalling. *FEBS Lett.* **593**, 2289–2305 (2019).

27. Kondo, Y. *et al.* ARF1 and ARF3 are required for the integrity of recycling endosomes and the recycling pathway. *Cell Struct. Funct.* **37**, 141–154 (2012).

28. Dascher, C. & Balch, W. E. Dominant inhibitory mutants of ARF1 block endoplasmic reticulum to Golgi transport and trigger disassembly of the Golgi apparatus. *J. Biol. Chem.* **269**, 1437–1448 (1994).
Dominantly acting variants in ARF3 have disruptive consequences on Golgi integrity and cause microcephaly recapitulated in zebrafish
Fasano, Muto, Radio, et al.

29. Altan-Bonnet, N., Phair, R. D., Polishchuk, R. S., Weigert, R. & Lippincott-Schwartz, J. A role for Arf1 in mitotic Golgi disassembly, chromosome segregation, and cytokinesis. Proc. Natl. Acad. Sci. U. S. A. 100, 13314–13319 (2003).

30. Hanai, A. et al. Class I Arfs (Arf1 and Arf3) and Arf6 are localized to the Flemming body and play important roles in cytokinesis. J. Biochem. (Tokyo) 159, 201–208 (2016).

31. Nakayama, K. Regulation of cytokinesis by membrane trafficking involving small GTPases and the ESCRT machinery. Crit. Rev. Biochem. Mol. Biol. 51, 1–6 (2016).

32. Rodrigues, F. F. & Harris, T. J. C. Key roles of Arf small G proteins and biosynthetic trafficking for animal development. Small GTPases 10, 403–410 (2019).

33. Carvajal-Gonzalez, J. M. et al. The clathrin adaptor AP-1 complex and Arf1 regulate planar cell polarity in vivo. Nat. Commun. 6, 6751 (2015).

34. Ge, X. et al. Missense-depleted regions in population exomes implicate ras superfamily nucleotide-binding protein alteration in patients with brain malformation. NPJ Genomic Med. 1, (2016).

35. Sobreira, N., Schiettecatte, F., Valle, D. & Hamosh, A. GeneMatcher: a matching tool for connecting investigators with an interest in the same gene. Hum. Mutat. 36, 928–930 (2015).

36. Denayer, E. et al. Mutation analysis in Costello syndrome: functional and structural characterization of the HRAS p.Lys117Arg mutation. Hum. Mutat. 29, 232–239 (2008).

37. Lim, Y. H. et al. Somatic Activating RAS Mutations Cause Vascular Tumors Including Pyogenic Granuloma. J. Invest. Dermatol. 135, 1698–1700 (2015).

38. Schubbert, S. et al. Germline KRAS mutations cause Noonan syndrome. Nat. Genet. 38, 331–336 (2006).
39. Zenker, M. et al. Expansion of the genotypic and phenotypic spectrum in patients with KRAS germline mutations. *J. Med. Genet.* **44**, 131–135 (2007).

40. Landrum, M. J. et al. ClinVar: improving access to variant interpretations and supporting evidence. *Nucleic Acids Res.* **46**, D1062–D1067 (2018).

41. Nava, C. et al. Cardio-facio-cutaneous and Noonan syndromes due to mutations in the RAS/MAPK signalling pathway: genotype-phenotype relationships and overlap with Costello syndrome. *J. Med. Genet.* **44**, 763–771 (2007).

42. Gripp, K. W. & Lin, A. E. Costello syndrome: a Ras/mitogen activated protein kinase pathway syndrome (rasopathy) resulting from HRAS germline mutations. *Genet. Med. Off. J. Am. Coll. Med. Genet.* **14**, 285–292 (2012).

43. Lee, Y. et al. Makes caterpillars floppy-like effector-containing MARTX toxins require host ADP-ribosylation factor (ARF) proteins for systemic pathogenicity. *Proc. Natl. Acad. Sci. U. S. A.* **116**, 18031–18040 (2019).

44. Carta, C. et al. Germline missense mutations affecting KRAS Isoform B are associated with a severe Noonan syndrome phenotype. *Am. J. Hum. Genet.* **79**, 129–135 (2006).

45. Zampino, G. et al. Diversity, parental germline origin, and phenotypic spectrum of de novo HRAS missense changes in Costello syndrome. *Hum. Mutat.* **28**, 265–272 (2007).

46. Pasqualato, S., Renault, L. & Cherfils, J. Arf, Arl, Arp and Sar proteins: a family of GTP-binding proteins with a structural device for ‘front-back’ communication. *EMBO Rep.* **3**, 1035–1041 (2002).

47. Ménétrey, J. et al. Structural basis for ARF1-mediated recruitment of ARHGAP21 to Golgi membranes. *EMBO J.* **26**, 1953–1962 (2007).
Dominantly acting variants in ARF3 have disruptive consequences on Golgi integrity and cause microcephaly recapitulated in zebrafish
Fasano, Muto, Radio, et al.

48. Yu, X., Breitman, M. & Goldberg, J. A Structure-Based Mechanism for Arf1-Dependent Recruitment of Coatomer to Membranes. *Cell* **148**, 530–542 (2012).

49. Ratcliffe, C. D. H., Sahgal, P., Parachoniak, C. A., Ivaska, J. & Park, M. Regulation of Cell Migration and β1 Integrin Trafficking by the Endosomal Adaptor GGA3. *Traffic Cph. Den.* **17**, 670–688 (2016).

50. Adolf, F. *et al.* Proteomic Profiling of Mammalian COPII and COPI Vesicles. *Cell Rep.* **26**, 250-265.e5 (2019).

51. Xiang, Y., Seemann, J., Bisel, B., Punthambaker, S. & Wang, Y. Active ADP-ribosylation factor-1 (ARF1) is required for mitotic Golgi fragmentation. *J. Biol. Chem.* **282**, 21829–21837 (2007).

52. Manolea, F. *et al.* Arf3 is activated uniquely at the trans-Golgi network by brefeldin A-inhibited guanine nucleotide exchange factors. *Mol. Biol. Cell* **21**, 1836–1849 (2010).

53. Kimmel, C. B., Ballard, W. W., Kimmel, S. R., Ullmann, B. & Schilling, T. F. Stages of embryonic development of the zebrafish. *Dev. Dyn. Off. Publ. Am. Assoc. Anat.* **203**, 253–310 (1995).

54. Bagwell, J. *et al.* Notochord vacuoles absorb compressive bone growth during zebrafish spine formation. *eLife* **9**, (2020).

55. Jessen, J. R. *et al.* Zebrafish trilobite identifies new roles for Strabismus in gastrulation and neuronal movements. *Nat. Cell Biol.* **4**, 610–615 (2002).

56. Roszko, I., Sawada, A. & Solnica-Krezel, L. Regulation of convergence and extension movements during vertebrate gastrulation by the Wnt/PCP pathway. *Semin. Cell Dev. Biol.* **20**, 986–997 (2009).

57. Wallingford, J. B., Fraser, S. E. & Harland, R. M. Convergent extension: the molecular control of polarized cell movement during embryonic development. *Dev. Cell* **2**, 695–706 (2002).
Dominantly acting variants in ARF3 have disruptive consequences on Golgi integrity and cause microcephaly recapitulated in zebrafish
Fasano, Muto, Radio, et al.

58. Tate, J. G. et al. COSMIC: the Catalogue Of Somatic Mutations In Cancer. *Nucleic Acids Res.* **47**, D941–D947 (2019).

59. Tartaglia, M. & Gelb, B. D. Disorders of dysregulated signal traffic through the RAS-MAPK pathway: phenotypic spectrum and molecular mechanisms. *Ann. N. Y. Acad. Sci.* **1214**, 99–121 (2010).

60. Motta, M. et al. Activating MRAS mutations cause Noonan syndrome associated with hypertrophic cardiomyopathy. *Hum. Mol. Genet.* **29**, 1772–1783 (2020).

61. Flex, E. et al. Activating mutations in RRAS underlie a phenotype within the RASopathy spectrum and contribute to leukaemogenesis. *Hum. Mol. Genet.* **23**, 4315–4327 (2014).

62. Niihori, T. et al. Germline-Activating RRAS2 Mutations Cause Noonan Syndrome. *Am. J. Hum. Genet.* **104**, 1233–1240 (2019).

63. Capri, Y. et al. Activating Mutations of RRAS2 Are a Rare Cause of Noonan Syndrome. *Am. J. Hum. Genet.* **104**, 1223–1232 (2019).

64. Khan, A. R. & Ménétrey, J. Structural biology of Arf and Rab GTPases’ effector recruitment and specificity. *Struct. Lond. Engl.* **1993 21**, 1284–1297 (2013).

65. Merithew, E. et al. Structural Plasticity of an Invariant Hydrophobic Triad in the Switch Regions of Rab GTPases Is a Determinant of Effector Recognition*. *J. Biol. Chem.* **276**, 13982–13988 (2001).

66. Kuai, J. & Kahn, R. A. Residues forming a hydrophobic pocket in ARF3 are determinants of GDP dissociation and effector interactions. *FEBS Lett.* **487**, 252–256 (2000).

67. Banne, E. et al. West syndrome, microcephaly, grey matter heterotopia and hypoplasia of corpus callosum due to a novel ARFGEF2 mutation. *J. Med. Genet.* **50**, 772–775 (2013).
68. Horton, A. C. et al. Polarized Secretory Trafficking Directs Cargo for Asymmetric Dendrite Growth and Morphogenesis. *Neuron* **48**, 757–771 (2005).

69. Lauri, A., Fasano, G., Venditti, M., Dallapiccola, B. & Tartaglia, M. In vivo functional genomics for undiagnosed patients: the impact of small GTPases signaling dysregulation at pan-embryo developmental scale. *Front. Cell Dev. Biol.* **9**, (2021).

70. Prigozhina, N. L. & Waterman-Storer, C. M. Decreased polarity and increased random motility in PtK1 epithelial cells correlate with inhibition of endosomal recycling. *J. Cell Sci.* **119**, 3571–3582 (2006).

71. Winter, J. F. et al. Caenorhabditis elegans screen reveals role of PAR-5 in RAB-11-recycling endosome positioning and apicobasal cell polarity. *Nat. Cell Biol.* **14**, 666–676 (2012).

72. Ulrich, F. et al. Wnt11 Functions in Gastrulation by Controlling Cell Cohesion through Rab5c and E-Cadherin. *Dev. Cell* **9**, 555–564 (2005).

73. Sarmah, S. et al. Sec24D-dependent transport of extracellular matrix proteins is required for zebrafish skeletal morphogenesis. *PloS One* **5**, e10367 (2010).

74. Coutinho, P. et al. Differential requirements for COPI transport during vertebrate early development. *Dev. Cell* **7**, 547–558 (2004).

75. Sheen, V. L. et al. Mutations in ARFGEF2 implicate vesicle trafficking in neural progenitor proliferation and migration in the human cerebral cortex. *Nat. Genet.* **36**, 69–76 (2004).

76. Pezzani, L. et al. Atypical presentation of pediatric BRAF RASopathy with acute encephalopathy. *Am. J. Med. Genet. A.* **176**, 2867–2871 (2018).

77. Ziegler, A. et al. Confirmation that variants in TTI2 are responsible for autosomal recessive intellectual disability. *Clin. Genet.* **96**, 354–358 (2019).
Dominantly acting variants in ARF3 have disruptive consequences on Golgi integrity and cause microcephaly recapitulated in zebrafish

Fasano, Muto, Radio, et al.

78. Radio, F. C. et al. SPEN haploinsufficiency causes a neurodevelopmental disorder overlapping proximal 1p36 deletion syndrome with an episignature of X chromosomes in females. *Am. J. Hum. Genet.* **108**, 502–516 (2021).

79. Lin, Y.-C. et al. SCUBE3 loss-of-function causes a recognizable recessive developmental disorder due to defective bone morphogenetic protein signaling. *Am. J. Hum. Genet.* **108**, 115–133 (2021).

80. Vetro, A. et al. ATP1A2- and ATP1A3-associated early profound epileptic encephalopathy and polymicrogyria. *Brain J. Neurol.* (2021) doi:10.1093/brain/awab052.

81. Humphrey, W., Dalke, A. & Schulten, K. VMD: visual molecular dynamics. *J. Mol. Graph.* **14**, 33–38, 27–28 (1996).

82. Waterhouse, A. et al. SWISS-MODEL: homology modelling of protein structures and complexes. *Nucleic Acids Res.* **46**, W296–W303 (2018).

83. Pettersen, E. F. et al. UCSF Chimera--a visualization system for exploratory research and analysis. *J. Comput. Chem.* **25**, 1605–1612 (2004).

84. Dunbrack, R. L. Rotamer libraries in the 21st century. *Curr. Opin. Struct. Biol.* **12**, 431–440 (2002).

85. Páll, S. et al. Heterogeneous parallelization and acceleration of molecular dynamics simulations in GROMACS. *J. Chem. Phys.* **153**, 134110 (2020).

86. Huang, J. & MacKerell, A. D. CHARMM36 all-atom additive protein force field: validation based on comparison to NMR data. *J. Comput. Chem.* **34**, 2135–2145 (2013).

87. Bussi, G., Donadio, D. & Parrinello, M. Canonical sampling through velocity rescaling. *J. Chem. Phys.* **126**, 014101 (2007).
88. Darden, T., York, D. & Pedersen, L. Particle mesh Ewald: An N·log(N) method for Ewald sums in large systems. *J. Chem. Phys.* **98**, 10089–10092 (1993).

89. Villefranc, J. A., Amigo, J. & Lawson, N. D. Gateway compatible vectors for analysis of gene function in the zebrafish. *Dev. Dyn. Off. Publ. Am. Assoc. Anat.* **236**, 3077–3087 (2007).

90. Don, E. K. *et al.* A Tol2 Gateway-Compatible Toolbox for the Study of the Nervous System and Neurodegenerative Disease. *Zebrafish* **14**, 69–72 (2017).

91. Cole, N. B. *et al.* Diffusional mobility of Golgi proteins in membranes of living cells. *Science* **273**, 797–801 (1996).

92. Makyio, H. *et al.* Structural basis for Arf6-MKLP1 complex formation on the Flemming body responsible for cytokinesis. *EMBO J.* **31**, 2590–2603 (2012).

93. LaFave, M. C., Varshney, G. K., Vemulapalli, M., Mullikin, J. C. & Burgess, S. M. A defined zebrafish line for high-throughput genetics and genomics: NHGRI-1. *Genetics* **198**, 167–170 (2014).

94. Peri, F. & Nüsslein-Volhard, C. Live Imaging of Neuronal Degradation by Microglia Reveals a Role for v0-ATPase a1 in Phagosomal Fusion In Vivo. *Cell* **133**, 916–927 (2008).

95. Westerfield, M. *The zebrafish book. A guide for the laboratory use of zebrafish (Danio rerio).* (2000).

96. Kovach, W. L. *Oriana – Circular Statistics for Windows.* (2011).

97. Thisse, C. & Thisse, B. High-resolution in situ hybridization to whole-mount zebrafish embryos. *Nat. Protoc.* **3**, 59–69 (2008).

98. Schindelin, J. *et al.* Fiji: an open-source platform for biological-image analysis. *Nat. Methods* **9**, 676–682 (2012).
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