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Expanding the Role of the Dynein Regulatory Complex to Non-Axonemal Functions: Association of GAS11 with the Golgi Apparatus

Jessica R. Colantonio[1], Janine M. Bekker[2], Sarah J. Kim[1], Kari M. Morrissey[3], Rachelle H. Crosbie[2,3] and Kent L. Hill[1,2,*]

[1]Department of Microbiology, Immunology and Molecular Genetics, University of California, Los Angeles, CA 90095, USA
[2]Molecular Biology Institute, University of California, Los Angeles, CA 90095, USA
[3]Department of Physiological Sciences, University of California, Los Angeles, CA 90095, USA
**Corresponding author: Kent L. Hill, kenthill@mednet.ucla.edu*

The mammalian GAS11 gene is a candidate tumor suppressor of unknown function that was previously identified as one of several genes upregulated upon growth arrest. Interestingly, although GAS11 homologs in *Trypanosoma brucei* (trypanin) and *Chlamydomonas reinhardtii* (PF2) are integral components of the flagellar axoneme and are necessary for regulating flagellar beat, the GAS11 gene was discovered based on its expression in cells that do not assemble a motile cilium. This suggests that GAS11 function might not be restricted to the cilium. To investigate this possibility, we generated GAS11-specific antibodies and demonstrate here that GAS11 is expressed in a variety of mammalian cells that lack a motile cilium. In COS7 cells, GAS11 is associated with the detergent-insoluble cytoskeleton and exhibits a juxtanuclear localization that overlaps with the pericentrosomal Golgi apparatus. This localization is dependent upon intact microtubules and is cell-cycle regulated, such that GAS11 is dispersed throughout the cytoplasm as cells progress through mitosis. GAS11 remains associated with Golgi fragments following depolymerization of cytoplasmic microtubules but is dispersed upon disruption of the Golgi with brefeldin A. These data suggest that GAS11 is associated with the Golgi apparatus. In support of this, recombinant GAS11 binds Golgi membranes *in vitro*. In growth-arrested mIMCD3 cells, GAS11 co-localizes with γ-tubulin at the base of the primary cilium. The pericentrosomal Golgi apparatus and base of the cilium both represent convergence points for microtubule minus ends and correspond to sites where dynein regulation is required. The algal GAS11 homolog functions as part of a dynein regulatory complex (DRC) in the axoneme (Rupp and Porter. J Cell Biol 2003;162:47–57) and our findings suggest that components of this axonemal dynein regulatory system have been adapted in mammalian cells to participate in non-axonemal functions.

Key words: cytoskeleton, dynein, GAS11, Golgi, microtubules, trypanin

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Cytoplasmic and axonemal dyneins are microtubule-based molecular motors that harness the energy of ATP hydrolysis to drive translocation of cellular cargoes along microtubules (1,2). Cytoplasmic dynein operates in concert with kinesin to mediate intracellular trafficking and positioning of proteins, organelles, and nucleic acids (2). Within the eukaryotic flagellum/cilium (eukaryotic flagella and cilia are structurally analagous organelles and we will use these two terms interchangeably throughout this article), axonemal dyneins on adjacent outer doublet microtubules drive sliding of these microtubules past one another to initiate and propagate flagellar beat (3–5). Proper cellular function of both axonemal and cytoplasmic dynein is dependent upon correct spatial and temporal regulation of motor activity. The activity of axonemal dyneins must be co-ordinated with one another along the length and around the circumference of the axoneme, while cytoplasmic dynein activity must be co-ordinated with the plus-end-directed kinesin motor in order to achieve productive, bidirectional traffic of cargoes bound to both motors (6–11). Identification of proteins and mechanisms underlying dynein regulation therefore represents an important yet poorly understood aspect of dynein function.

Genetic, biochemical, and structural studies in the green alga *Chlamydomonas reinhardtii* have led to the identification of a dynein regulatory complex (DRC), which functions as part of a dynein control system transmitting regulatory signals to dynein from the central pair (CP) and radial spoke (RS) complexes of the axoneme (12–14). Central pair microtubules and radial spokes are critical for flagellar motility in *C. reinhardtii*, as loss of either structure results in flagellar paralysis under physiological conditions (15). The DRC was originally identified genetically, through the isolation of extragenic suppressors that restore flagellar beat to CP/RS mutants without restoring the missing structures (12). One group of suppressor loci ($sup_{pf}3$, $sup_{pf}4$, $sup_{pf}5$, *pf2*, and *pf3*) is required for assembly of the DRC (12–14,16,17). In the current distributor model for axonemal dynein regulation, the DRC acts as a reversible inhibitor of dynein, the CP apparatus distributes signals through the radial spokes to the DRC to release dynein inhibition (14,18). In the absence of CP/RS signals, the DRC constitutively inhibits dynein, leading to paralysis of CP/RS mutants.

The DRC is composed of an estimated seven polypeptides, and genetic screens in *C. reinhardtii* have identified five genetic loci that are required for DRC assembly – *pf2*, *pf3*, *pf5*, *sup-pf3*, and *sup-pf4* (12,13,16). Until recently, the identities of these genes were unknown. A critical advance in the molecular characterization of this complex was made by Rupp and Porter (19), who isolated a new *pf2* allele using insertional mutagenesis and discovered that the PF2 gene encodes a 54-kDa coiled-coil protein that is homologous to trypanin from *Trypanosoma brucei* (20,21). Trypanin is an integral component of the *T. brucei* flagellar apparatus and is required for directional cell motility and flagellum attachment (20,21). We previously reported that trypanin represents a broadly conserved protein family, with homologs in organisms as diverse as protozoa, algae, and humans (21). Aside from trypanin and PF2, the function of these proteins has not been investigated.

The mammalian trypanin/PF2 homolog was originally isolated as one of several genes that are upregulated upon growth arrest and thus referred to as GAS11 (GAS11 has been interchangeably referred to as GAS11 in humans and GAS8 in mice; for clarity, we will use GAS11 throughout this article), for growth arrest specific 11 (22–25). Although there are no sequence similarities or common structural domains among gas genes, many are now known to function in pathways that positively or negatively influence cell-cycle progression (25–31). Gas genes have also been linked to control of cytoskeletal organization, cellular differentiation, and tyrosine receptor kinase signaling (27,32,33). GAS11 is the first such gene to be implicated in a dynein-dependent process. However, the function of this protein in mammalian cells is unknown and its cell-type distribution and subcellular location are uncertain. Yeh and colleagues recently reported that GAS11 is localized along the axoneme of motile cilia (25) and the protein was identified in a proteomic analysis of cilia from human bronchial epithelial cells (34). This is consistent with a function analogous to that of trypanin and PF2 in the flagellum. However, the GAS11 gene was isolated based on its expression in murine fibroblasts, which do not assemble a motile cilium and therefore do not require an axonemal dynein regulatory complex (DRC). This suggests that GAS11 function might not be restricted to the regulation of axonemal dynein but rather may have additional functions in cells that assemble a non-motile cilium (e.g. primary cilium) or in cells that do not assemble a cilium at all.

In order to explore the possibility that GAS11 has axonemal functions in the absence of dynein arms or functions outside the axoneme, we have generated GAS11-specific antibodies to directly investigate GAS11 in mammalian cells. We demonstrate that GAS11 is not restricted to cells with motile cilia and that it is associated with the Golgi apparatus at the centrosome of non-polarized cells without a cilium and at the base of the primary cilium in growth-arrested kidney cells. Previous analogies have been made between systems that regulate axonemal and cytoplasmic dynein motors (14,35), and our results suggest that components of an axonemal dynein regulatory system have been adapted in mammalian cells to participate in non-axonemal functions.

Results

GAS11 is expressed in cells without motile cilia

GAS11 homologs in trypanosomes (trypanin) and algae (PF2) function in flagellum-based cell motility (19,20). Multiple tissue Northern blots suggested, however, that GAS11 is ubiquitously expressed in mice and humans (36), suggesting a role outside of the motile axoneme. To address this possibility, we raised antibodies against GAS11 to determine whether this protein is indeed present in cells without a motile cilium. Affinity-purified antibodies raised against the C-terminal 16 amino acids of GAS11 were used in immunoblot analysis of lysates from several mammalian cell lines (Figure 1A). These antibodies recognize an approximately 56-kDa protein in cells that lack a motile cilium, including HeLa, COS7, HEK293, and MDCK cells (Figure 1A), as well as in motile human ciliary axonemes (Figure 1B). This size is consistent with the size predicted by the full-length GAS11 open reading frame (25,36). The specificity of these antibodies is demonstrated by peptide competition (Figure 1A), as well as the fact that they recognize a GAS11–GFP fusion protein (Figure 1C). Therefore, GAS11 is expressed in several cultured mammalian cell lines and is not restricted to cells with motile cilia.

Trypanin and PF2 are associated with detergent-insoluble microtubules of the flagellar cytoskeleton (19–21). To determine whether GAS11 is similarly associated with the cytoskeleton in cells without a motile cilium, COS7 cells were extracted with non-ionic detergent in a microtubule-stabilizing buffer. As shown in Figure 1D, GAS11 remains associated with the detergent-insoluble cytoskeleton, although a small but reproducible portion is found in the detergent-soluble fraction. Analysis of lamin A/C verifies that this is not due to release of whole cells into the detergent-soluble fraction.

GAS11 is localized to the pericentrosomal MTOC in a microtubule-dependent and cell-cycle-dependent manner

The finding that GAS11 is expressed in cells without a motile cilium and is associated with the cytoskeleton suggests that in addition to its suspected role in the regulation of axonemal dynein, it also participates in microtubule/dynein-dependent processes outside the axoneme. As a first step toward elucidating this function, we determined the subcellular distribution of GAS11 via immunofluorescence. The majority of the protein was found to be located just outside the nucleus, with a lighter, punctate distribution in the cytoplasm (Figure 2A). Co-staining for β-tubulin (Figure 2A–C) demonstrated that GAS11 overlaps with the brightest area of microtubule staining at the microtubule organizing center (MTOC). To unequivocally identify the

MTOC, cells were co-stained for GAS11 and γ-tubulin as a marker for the centrosome (Figure 2D–F). In some cases, there is overlap of GAS11 with γ-tubulin (Figure 2F, arrow), while in other cases, GAS11 surrounds but does not necessarily overlap with γ-tubulin (Figure 2F, arrowhead). In addition to being distributed along the axoneme, both trypanin and PF2 are located near the flagellar basal body (19,20), which is analogous to the centrosome in mammalian cells and corresponds to the MTOC of the flagellar axoneme. Therefore, even in non-ciliated cells, GAS11 is associated with microtubule-based structures and is specifically concentrated at the MTOC.

In previous work, we showed that a GFP fusion protein containing GAS11 amino acids 115–258 co-localizes with microtubules in COS7 cells (21). Subsequent studies have demonstrated that this region represents a novel microtubule-binding domain that interacts directly with microtubules *in vitro* (Bekker, Hill and Crosbie, unpublished observation). We therefore asked whether the accumulation of GAS11 at the centrosome depends upon intact microtubules (37). As shown in Figure 3A,B, depolymerization of cytoplasmic microtubules with nocodazole resulted in GAS11 being completely dispersed into multiple cytoplasmic puncta (Figure 3A,B).

In our immunofluorescence assays, we occasionally observed control cells in which GAS11 was not concentrated at the MTOC but was dispersed throughout the cytoplasm. Given the dependence of GAS11 localization on intact microtubules (Figure 3), we considered the possibility that this might be a consequence of cell-cycle-dependent reorganization of microtubules. To test this, we used β-tubulin staining to identify mitotic versus non-mitotic cells. Representative examples are shown in Figure 3C,D. In these samples, the β-tubulin image was captured at a short exposure time to allow clear visualization of the mitotic spindle. As discussed above, GAS11 is concentrated at the MTOC in most cells (asterisks in Figure 3C,D). However, in mitotic cells, i.e. those in which a clear mitotic spindle is visible (arrows in Figure 3D), GAS11 is dispersed in the cytoplasm. During late stages of cytokinesis, when midbody microtubules are visible, two populations of GAS11 are observed. In these cells, the protein begins to reassemble into a discrete spot outside the nucleus, opposite the site of cell–cell contact (filled arrowheads, Figure 3D), which presumably corresponds to the centrosome. However, it is also concentrated near the ends of the midbody microtubules (open arrowhead in Figure 3D, and data not shown).

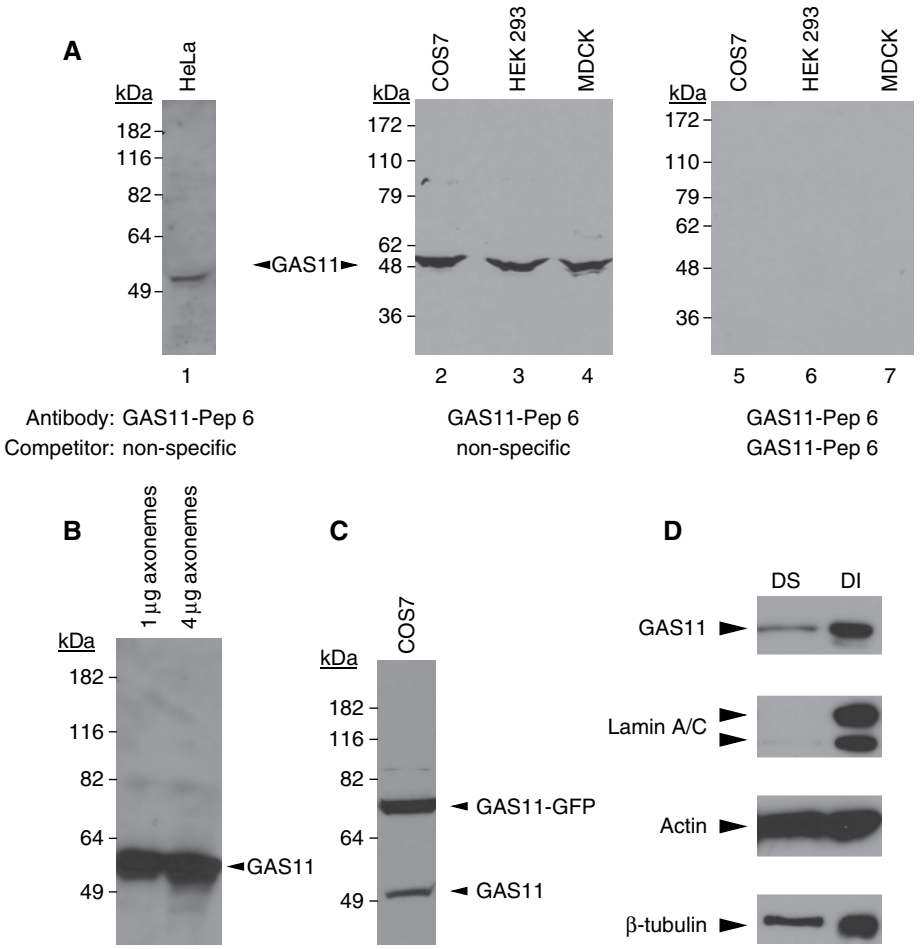

Figure 1: GAS11 is expressed in cells that lack a motile cilium. (A) Lysates from HeLa cells (lane 1), COS7 (lanes 2 and 5), HEK293 (lanes 3 and 6), and MDCK (lanes 4 and 7) cells were immunoblotted with GAS11 antibody. To verify specificity, antibodies were preincubated with non-specific (lanes 1–4) or specific (lanes 5–7) competitor peptide. Molecular weight markers are shown on the left in kDa. (B) Human ciliary axonemes were immunoblotted with antibodies against GAS11. (C) Lysates from COS7 cells transfected with GAS11–GFP were immunoblotted with GAS11 antibody. (D) Detergent-soluble (DS) and detergent-insoluble (DI) fractions from COS7 cells were immunoblotted with antibodies against GAS11, lamin A/C, actin, or β-tubulin.

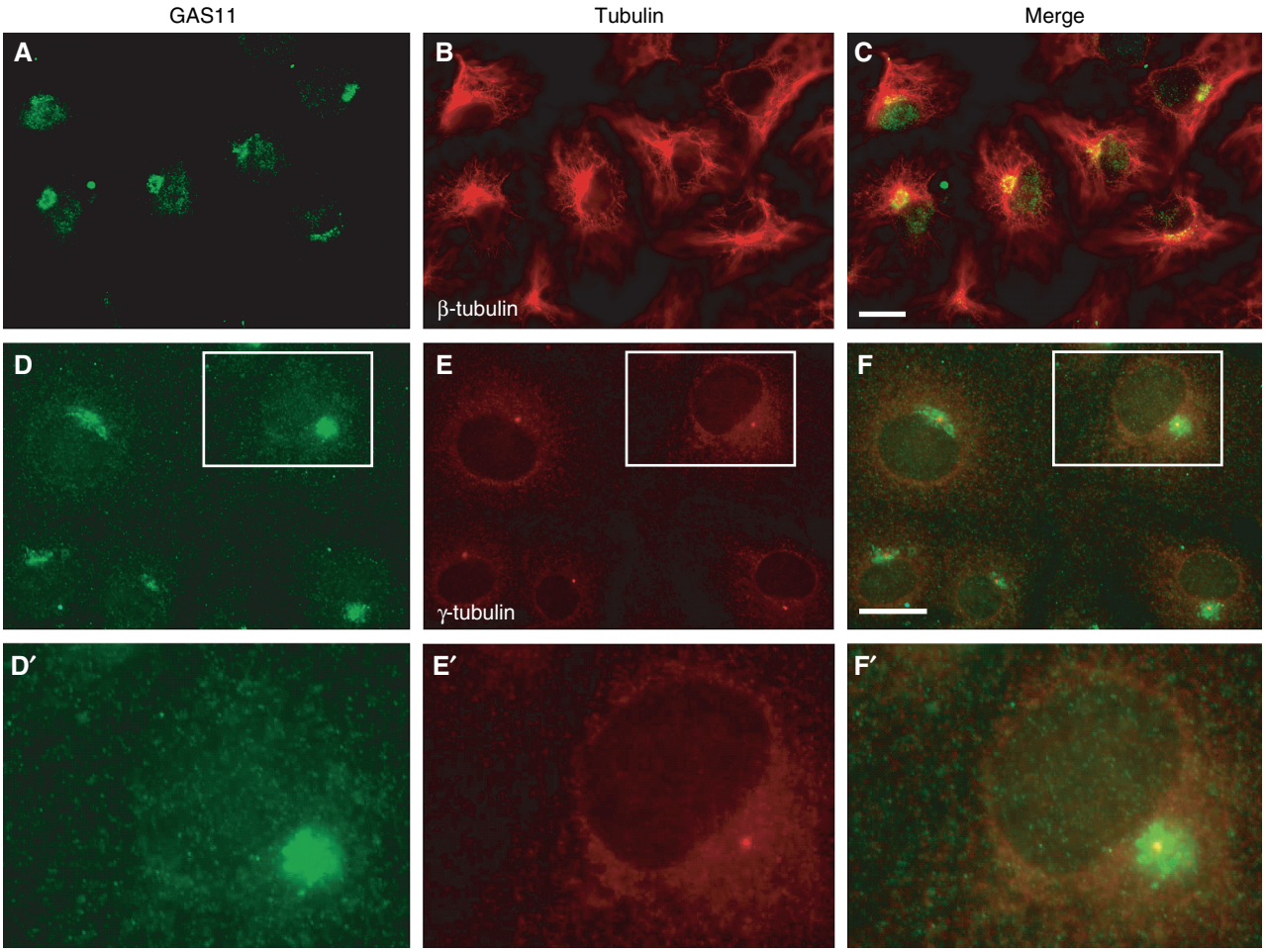

Figure 2: GAS11 localization is pericentrosomal. COS7 cells were examined by immunofluorescence using antibodies against GAS11 (green, A, D, and G), β-tubulin (red, B), γ-tubulin (red, E), and acetylated α-tubulin (red, G). Merged images are shown (C, F, and I). (D′–F′). Enlarged image of the cells outlined in (D–F). The specificity of GAS11 antibody staining was verified by preincubation of antibody with competitor peptide (not shown). Scale bars = 20 μm.

Therefore, the subcellular distribution of GAS11 is subject to cell-cycle regulation and its redistribution from the MTOC correlates with reorganization of cytoplasmic microtubules during mitosis and cytokinesis.

GAS11 interacts with the Golgi apparatus

Localization of GAS11 around the centrosome of interphase cells, in addition to the dependence of this localization on the presence of intact microtubules and cell-cycle events, is similar to what is observed for the Golgi apparatus in fibroblasts (38). We therefore looked for co-localization with Golgi markers. Co-immunofluorescence for GAS11 and the Golgi marker GM130 demonstrated that GAS11 overlaps extensively with the Golgi (Figure 4A–C). This might result from an interaction between GAS11 and the Golgi apparatus or might simply reflect coincident localization at the minus ends of cytoplasmic microtubules. To distinguish between these possibilities, cells were treated with nocodazole prior to staining for GAS11 and Golgi. Maintenance of the Golgi apparatus at the centrosome is dependent upon dynein-driven transport of ER-derived vesicles along microtubules (39,40), and depolymerization of microtubules with nocodazole disperses the Golgi into multiple cytoplasmic fragments that remain functional (38). As shown in Figure 4D–F, most of the scattered Golgi fragments in nocodazole-treated cells remain closely associated with GAS11 even after depolymerization of cytoplasmic microtubules, suggesting that co-localization in untreated cells represents a functional association with the Golgi. As a further test, cells were treated with brefeldin A, which causes resorption of Golgi membranes into the ER without affecting microtubules. If GAS11 localization depends on functional association with the Golgi apparatus, then treatment with brefeldin A should result in a concomitant loss of GAS11 from the MTOC. Indeed, GAS11 is dispersed by brefeldin A treatment and is no longer co-localized with the centrosome or Golgi markers (Figure 4G–I).

Interaction between GAS11 and Golgi membranes was tested biochemically using *in vitro* Golgi spin-down

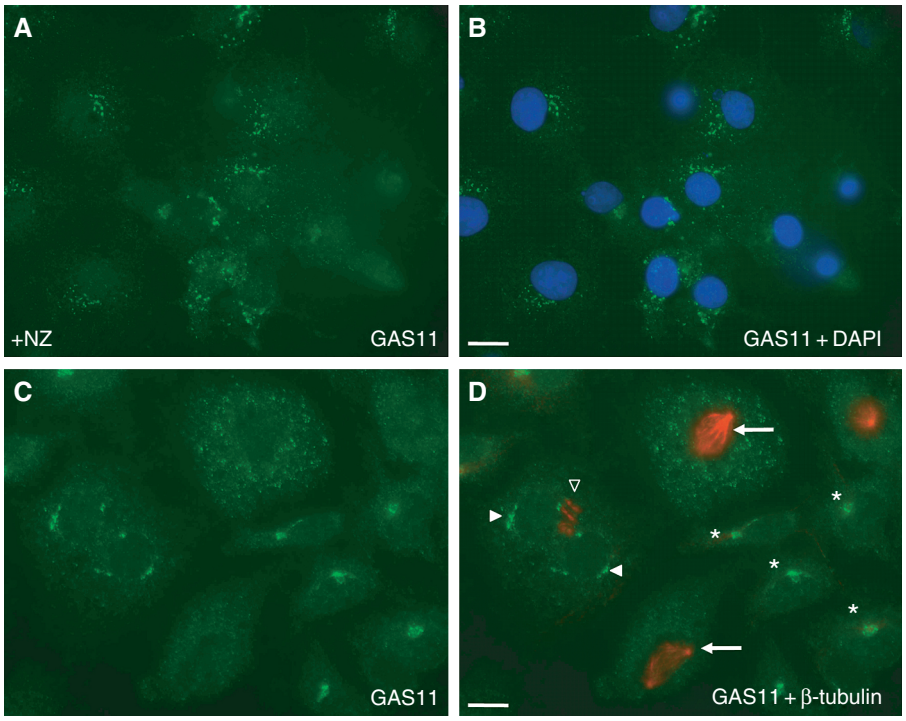

Figure 3: GAS11 localization is microtubule- and cell-cycle dependent. COS7 cells were examined by immunofluorescence using the antibodies against GAS11 (green, A–D) and β-tubulin (red, D). Cells were treated with nocodazole (A and B) prior to immunofluorescence. DAPI (blue, B) was used to visualize the nucleus. In (D), the β-tubulin image was underexposed in order to capture a clear image of the mitotic spindle. The mitotic spindle apparatus in two different dividing cells is labeled with an arrow. Midbody microtubules in a cell undergoing cytokinesis are labeled with an arrowhead. Scale bars = 20 μm.

experiments. Salt-washed Golgi membranes were incubated with purified recombinant GAS11 protein (rGAS11). Golgi membranes were isolated by centrifugation through a sucrose cushion. Immunoblot analysis of pellet fractions demonstrates that rGAS11 pellets only when Golgi membranes are added (Figure 5A), indicating that GAS11 directly interacts with Golgi membranes. Control Golgi spin-down experiments using a known Golgi-interacting protein, p115 (41), and a non-Golgi-interacting microtubule-associated protein, MAP2 (42), demonstrate the specificity of the GAS11–Golgi interaction (Figure 5B,C). Taken together, these *in vivo* and *in vitro* data indicate that GAS11 is associated with the Golgi apparatus at the centrosome and this localization is mediated by direct interaction with the Golgi.

As GAS11 is also postulated to function in the axoneme, we next examined GAS11 in cells containing a non-motile primary cilium. Most cultured mammalian cells form a non-motile primary cilium upon induction of growth arrest through serum starvation (43). The pattern of GAS11 staining did not change in serum-starved COS7 cells (Figure 6A–C). We were unable to identify a recognizable primary cilium in these cells, based on staining with antibody to acetylated tubulin (Figure 6A–C). We therefore used murine intermedullary collecting duct (mIMCD3) cells, which were grown to confluence then serum-starved to induce primary cilium formation. As shown in Figure 6, GAS11 exhibits a faint and punctate distribution but is concentrated in a bright spot near the nuclear periphery. Co-staining for acetylated tubulin as a marker for the primary cilium revealed that this focal point of GAS11 corresponds to the base of the primary cilium and overlaps with γ-tubulin at the basal body (Figure 6D–I). Localization of GAS11 to the base of the primary cilium is observed in almost all cells containing a cilium. In some but not all cases, this overlaps with Golgi (not shown). Notably, we do not see any GAS11 staining within the axoneme. We also did not observe significant upregulation of GAS11 protein levels in serum-starved cells (not shown).

Discussion

GAS11 belongs to a broadly conserved family of proteins that function in the regulation of flagellar motility in algae and trypanosomes (19–21). The *C. reinhardtii* homolog, PF2, is part of a signal transduction network that regulates dynein activity on outer doublet microtubules of the axoneme (19). Here, we report the surprising finding that the mammalian GAS11 protein is expressed in several cell types that lack a motile axoneme, indicating that the function of this protein in mammalian cells is not restricted to motile flagella.

The human GAS11 gene spans approximately 25 kb of genomic DNA on chromosome 16 and consists of 11 exons (36). The predicted full-length human ORF encodes a protein of approximately 56 kDa (36), which is consistent with the size of the GAS11 protein identified in this study in all cell types tested, as well as the size predicted by one of two full-length mouse cDNAs (25). Moreover, 56 kDa corresponds well with the size of characterized GAS11 homologs in *C. reinhardtii* (55 kDa) (19) and *T. brucei*

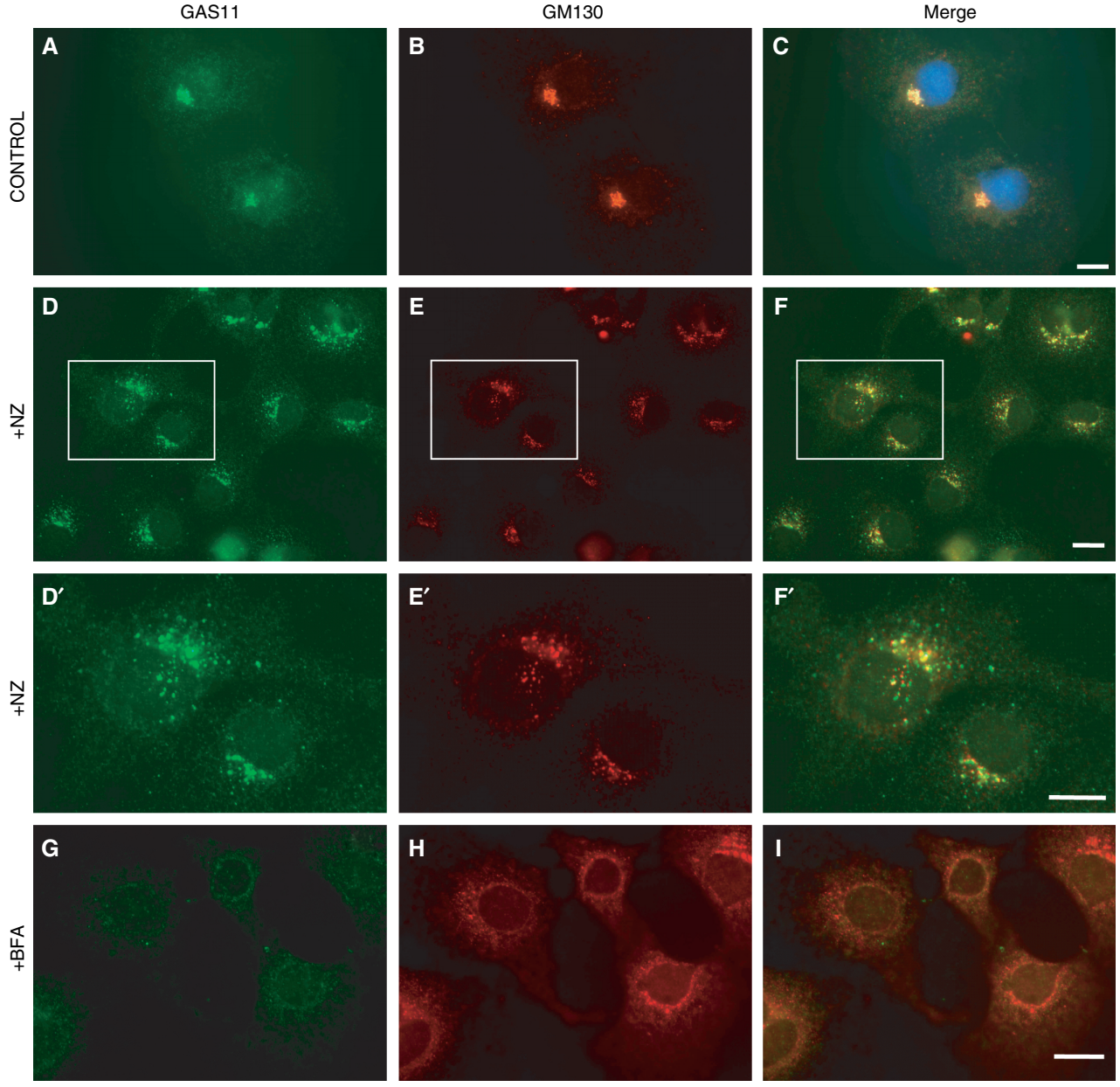

Figure 4: GAS11 co-localizes with the Golgi apparatus. COS7 cells were examined by immunofluorescence using antibodies against GAS11 (green, A, D, and G) and GM130 (red, B, E, and H). Merged images are shown (C, F, and I). DAPI (blue, C) is used to visualize the nucleus. Cells were untreated (A–C), treated with nocodazole (D–F), or treated with brefeldin A (G–I) prior to immunofluorescence. (D′–F′) Enlarged image of the cells outlined in (D–F). Scale bars = 20 μm.

(54 kDa) (20,21). As Northern blots using RNA from human tissues show multiple GAS11 transcripts, some of which are tissue specific (36), and as two alternatively spliced mouse cDNAs have been identified (25), it is possible that other isoforms of GAS11 also exist. The second cDNA isolated by Yeh *et al.* (25) does not include the peptide use for production of our antibodies.

Yeh and colleagues recently reported that GAS11 is present as a 65-kDa protein in mammalian sperm and ciliated epithelial cells, both of which contain motile axonemes (25). In this milieu, the protein likely functions in the regulation of flagellar motility as reported for homologs in other organisms (19,20), although this remains to be tested experimentally. The reason for the size difference between the protein identified in the present study and that reported by Yeh (25) is not clear. Yeh and colleagues did not observe a 56-kDa protein in the previous study. However, the authors employed immunoprecipitation followed by Western blot to assess protein expression and the presence of IgG heavy chain in the immunoprecipitate (Figure 2B) (25) would preclude detection of other

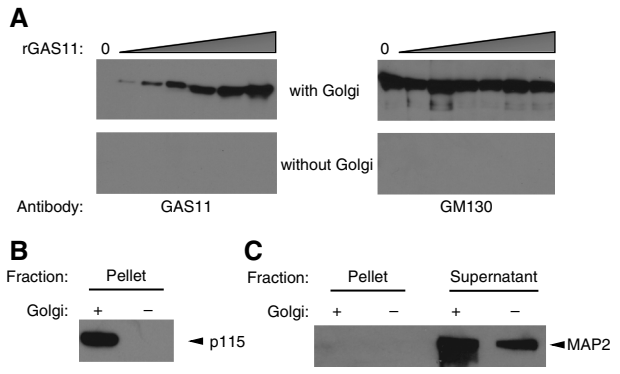

Figure 5: GAS11 binds to Golgi membranes. Increasing amounts of recombinant GAS11 (rGAS11) (A) or 0.5 μg recombinant p115 (B) or MAP2 (C) were incubated at 4 °C for 10 min with or without salt-washed Golgi membranes as indicated. Following centrifugation through sucrose cushions, the pellet fractions (A and B) or pellet and supernatant fractions (C) were analyzed by Western blot analysis for GAS11, p115, and MAP2 as indicated. The presence of Golgi membranes in the pellet was verified by immunoblotting with GM130 antibody (A, right panels).

proteins in the 56-kDa size range. Therefore, we obtained isolated ciliary axoneme preparations from human airway epithelia and subjected these to Western blot analysis with our affinity-purified anti-GAS11 antibody. These antibodies detected a single protein of approximately 56 kDa, suggesting that the 56-kDa protein observed in non-ciliated cells is also present in motile flagella of mammalian cells. In human ciliary axonemes, we did not observe any proteins in the 65-kDa range, despite using excessive amounts of protein. The 65-kDa protein observed by Yeh (25) could be an alternative isoform that is not recognized by our antibody. In the present study, we have restricted our analysis to the protein recognized by our antibody, which is a 56-kDa protein in all cells examined.

Unlike the case in motile axonemes, where a role in axonemal dynein regulation is inferred by analogy to characterized GAS11 homologs in algae and trypanosomes, it is less clear what role GAS11 might play in non-polarized cells without a motile cilium. In COS7 (Figures 2 and 4) and HeLa (data not shown) cells, GAS11 is associated with the Golgi apparatus at the microtubule organizing center. As GAS11 targeting is sensitive to BFA, remains associated with Golgi fragments following microtubule depolymerization, and binds Golgi membranes *in vitro*, we propose that this represents a functional association with the Golgi apparatus, rather than simple coincident localization at the centrosome. Redistribution of GAS11 from the centrosome to cytoplasmic puncta during mitosis is also observed for the Golgi (37).

Positioning of the Golgi apparatus at the centrosome of animal cells is dependent upon intact microtubules and dynein (37–39,43,44). This arrangement is thought to be mediated in part by Golgi-localized microtubule-associated proteins (MAPs) that tether Golgi membranes to microtubules (45). GAS11 amino acids 115–258 correspond to a novel microtubule-binding domain that co-localizes with cytoplasmic microtubules and interacts directly with purified microtubules *in vitro* (21 and JB, JC, KH and RC, unpublished observation). In this regard, GAS11 shares features with another group of proteins – hHook3, GMAP210, and CLIPR-59 – all of which are Golgi-localized microtubule-binding proteins (46–48). Although the function of these proteins is not entirely clear, they have each been suggested to participate in interactions between microtubules and Golgi membranes. Both hHook3 and GMAP210 are postulated to function in direct tethering of the Golgi apparatus to microtubules (46,48,49), although recent work on GMAP210 has produced conflicting results with respect to the position of this protein's microtubule-binding and Golgi localization domains (45,50,51). The presence of a microtubule-binding domain in GAS11 that is functional *in vivo* and *in vitro* (21), together with our finding that GAS11 interacts directly with Golgi membranes, suggests that one possible function might be to participate in Golgi attachment to microtubules.

Can localization of GAS11 at the Golgi apparatus be reconciled with its suspected role as part of a DRC (19)? In fibroblasts, assembly and maintenance of the Golgi are mediated by dynein-dependent transport of membrane vesicles from the ER toward the minus ends of microtubules, which converge upon the pericentrosomal Golgi (37–39,43). Once dynein arrives at its destination, it must be inactivated and/or released from its cargo in order to allow subsequent repositioning of motor and cargo (6,7,52). Like GAS11, cytoplasmic dynein 2 (DHC2) and the dynein 2 light intermediate chain (D2LIC) co-localize with the Golgi apparatus in a microtubule-dependent and cell-cycle-dependent manner (44,53). Cytoplasmic dynein 2 is also expected to function in IFT within the ciliary axoneme and its role in Golgi assembly is not entirely clear (53,54). Nonetheless, a requirement for dynein in the assembly and maintenance of the Golgi is well established (37–39,43). Dynein also appears to travel as cargo in kinesin-directed plus-end transport away from the Golgi (7), and co-ordination of oppositely directed dynein and kinesin motors requires reversible inactivation of each motor to allow net transport of the other (11,55). Notably, the DRC in *C. reinhardtii* performs exactly this function, i.e. reversible inhibition of axonemal dynein (19). Thus, one possibility is that GAS11 functions in a manner analogous to PF2, allowing reversible inactivation of dynein activity at the minus end of microtubules converging upon the Golgi. Although axonemal and cytoplasmic dyneins are distinct motor proteins, there are commonalities in the regulatory strategies and cargo-binding mechanisms employed by these motors (14,35). Thus far, however, we have not detected a stable interaction between GAS11 and cytoplasmic dynein 2 (Colantonio and Hill, unpublished).

The GAS11 gene was originally identified as one of several genes that are upregulated upon growth arrest of NIH 3T3

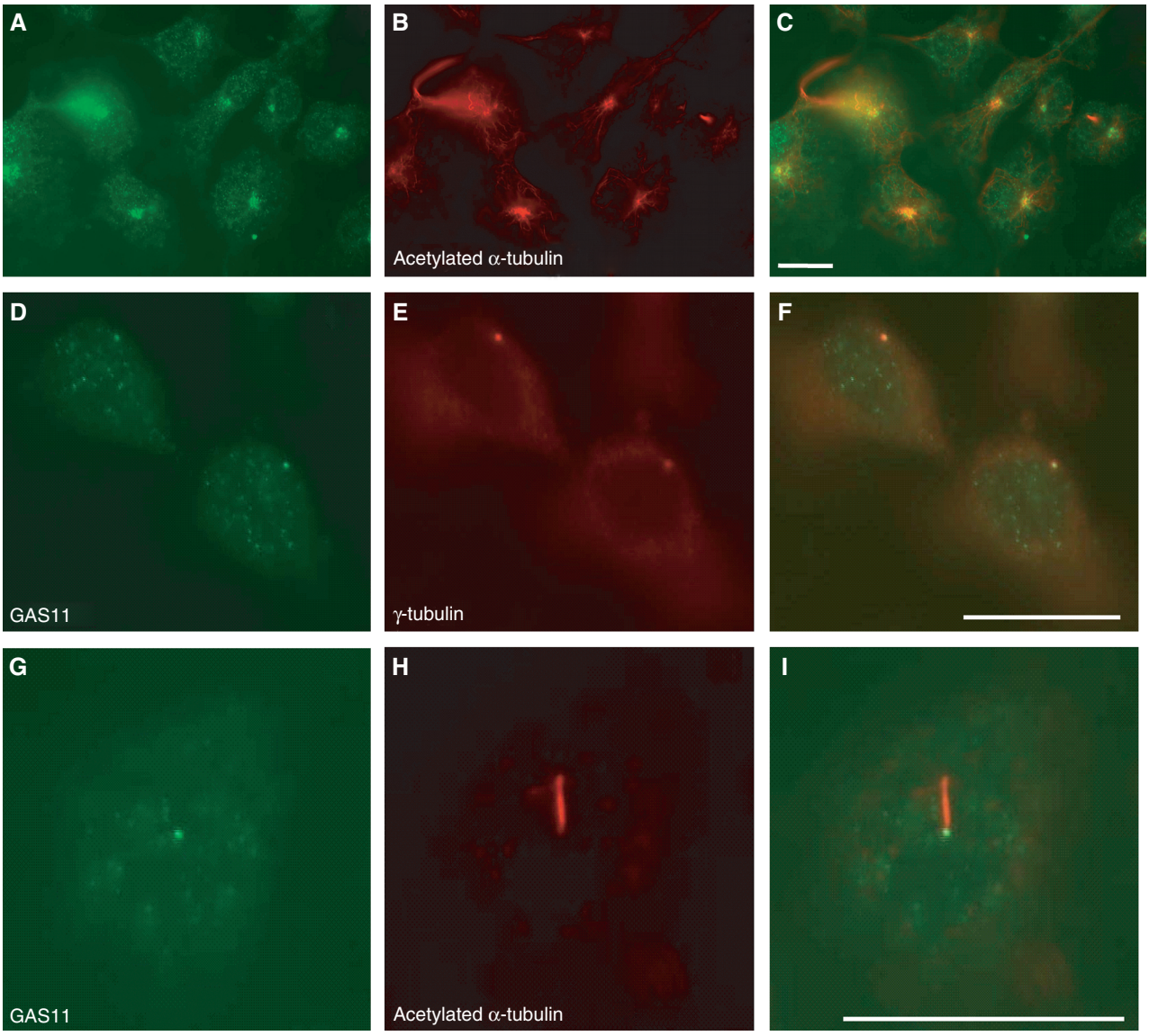

Figure 6: GAS11 is localized to the base of the primary cilium. (A–C) COS7 cells were grown to confluence, serum-starved, and subjected to immunofluorescence using GAS11 (A) or acetylated tubulin (B) antibodies. Merged image is shown in panel C. (D–I) mIMCD3 cells were grown to confluence, serum-starved, and examined by immunofluorescence using antibodies against GAS11 (green, D and G), γ-tubulin (red, E), or acetylated tubulin (red, H). Merged images are shown in F and I. Scale bars = 20 μm.

cells (22,56). Although there is no common sequence or domain structure to these 'growth arrest specific' (gas) genes, many have been found to function in pathways that control cell-cycle progression (26–31). At least one other gas gene, GAS2, has been linked to the cytoskeleton, as this protein is associated with microfilaments (27). At present, it is not clear whether there is a connection between GAS11's suspected role in microtubule/dynein-dependent processes and its upregulation in growth-arrested 3T3 cells. Our study results demonstrate that growth arrest is not required for expression of GAS11. We did however find that GAS11 is concentrated at the base of the primary cilium that is formed following growth arrest of mIMCD3 cells. Interestingly, formation of this primary cilium is postulated to require a regulatory activity that reversibly inhibits dynein at the base of the cilium (55), which is precisely the role of the DRC within the axoneme.

Differentiating between the possible roles for GAS11 expression outside the axoneme awaits further investigation and at present, we cannot rule out the possibility that GAS11 at the Golgi apparatus and ciliary basal body is simply en route to the axoneme. However, if this is the case, there must be stringent control mechanisms that prevent the protein from being incorporated into the axoneme once the cilium is formed. Defects in primary cilia assembly are now recognized as important contributors to

polycystic kidney disease, which is characterized by uncontrolled proliferation of kidney epithelia (57,58). Thus, the possibility that GAS11 might play a role in the regulation of dyneins required for primary cilium formation following growth arrest represents an interesting area for further investigation.

Materials and Methods

Cell culture and immunofluorescence

COS7, HEK293, MDCK, HeLa, and mIMCD3 cells were obtained from ATCC (Manassas, VA, USA) and cultured as recommended. Cells were grown on coverslips in complete medium, fixed with 100% methanol at −20 °C, blocked with 2% normal donkey serum (NDS), then incubated with indicated antibodies in 0.1% NDS. For COS7 and mIMCD3 serum starvation immunofluorescence assays, cells were cultured to confluence in complete medium; then they were cultured in medium containing 0.1% fetal bovine serum for an additional 48–72 h prior to fixation. Immunofluorescence microscopy was performed on a Zeiss Axioskop 2 with a 63X Plan Neofluor oil-immersion objective. Images were acquired with a Zeiss Axiocam CCD camera using AXIOVISION software (Zeiss, Thornwood, NY, USA). Cell transfections were performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the vendor's recommendations. Nocodazole and brefeldin A were used at a final concentration of 33 and 10 μM, respectively, for 45 min.

Plasmid constructs

The GAS11 ORF (36) was PCR-amplified from a GAS11 cDNA (accession number AA019846) with primers designed to introduce HindIII and XbaI sites flanking the start and stop codons. This HindIII/XbaI fragment was cloned in-frame with GFP in pcDNA3-GAS11$_{115\text{-}258}$-GFP (21) to generate pKH3, which contains the entire GAS11 ORF fused to GFP. DNA sequence was verified by direct DNA sequencing.

Antibodies

Production of polyclonal antibodies against the C-terminal 16 amino acids of human GAS11 ('Pep6', CAAGQTLGQGPAGLVGTPT) as described previously (20). This peptide is unique to GAS11. There are no other sequences in the NCBI human genome database giving a match to this peptide with an Expect value of <10. Antibodies were affinity-purified using Pep6 peptide as described (59) and used for all immunoblots as described (21). Monoclonal antibody against Pep6 was generated by Cell Essentials, Inc. (Boston, MA, USA) and used for immunoprecipitation. The E7 monoclonal antibody against β-tubulin (60) was obtained from the University of Iowa Developmental Studies Hybridoma Bank (DSHB, Iowa City, IA, USA). Monoclonal antibodies against γ-tubulin (Sigma, St Louis, MO, USA), acetylated tubulin (Sigma), GM130 (BD Biosciences, San Jose, CA, USA), Lamin A/C (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), β-actin (Abcam, Cambridge, UK), p115 (BD Biosciences), and MAP2 (Chemicon International, Temecula, CA, USA) were used according to vendor recommendations. Fluorescently labeled secondary antibodies were obtained from Molecular Probes (Carlsbad, CA, USA).

Protein isolation and biochemical fractionation

For total cell lysates (COS7, HEK293, and MDCK), cells were resuspended in Laemmli sample buffer. Ciliary axoneme preparations were a gift from Dr Larry Ostrowski (University of North Carolina at Chapel Hill) and were resuspended in Laemmli sample buffer. For precleared cell lysates (HeLa), cells were scraped up into NP40 buffer (53) and cleared by centrifugation. For biochemical fractionation, COS7 cells were extracted with 0.5% Triton X-100 in a microtubule-stabilizing buffer (61). Equal cell equivalents from detergent-soluble and detergent-insoluble fractions were analyzed by immunoblot.

GAS11 bacterial expression plasmids

A GAS11 expression plasmid was prepared by PCR amplification of mouse GAS11 cDNA (GenBank accession number AY692443) using primers containing nucleotides for direct cloning into a pET100/TOPO vector (Invitrogen, Carlsbad, CA, USA), which encodes a protein with a His tag (6× His) at the N-terminus: pET.GAS11^{FL}. Correct sequence was verified by the UCLA sequencing core facility using an ABI 3700 DNA Analyzer (Applied Biosystems, Foster City, CA, USA). The recombinant His-tagged GAS11 construct was transformed into BL21 bacterial cells (Invitrogen). His-tagged fusion proteins were purified by column chromatography using nickel-NTA His-bind® resin (Novagen, San Diego, CA, USA). Solubilized recombinant GAS11 was dialyzed overnight into buffer A (10 mM magnesium acetate, 15 mM EGTA, 20 mM β-glycerophosphate, 50 mM potassium acetate, 200 mM sucrose, 2 mM ATP, and 1 mM dithiothreitol, pH 7.5, at 4 °C) (41). Recombinant purified p115 was a gift of Dr Graham Warren (Yale University). Recombinant MAP2 protein was obtained from Cytoskeleton (Boulder, CO, USA). The protein samples were precleared by centrifugation at 100 000 × ***g*** for 5 min at 4 °C (TLA-100).

Preparation and treatment of Golgi membranes

Rat Golgi membranes were a gift of Dr Graham Warren (Yale University) and were prepared as described (40,62). Stacked rat liver Golgi membranes were prepared at 2 mg/mL protein (62). Membranes were salt-washed and stored at −80 °C in buffer A (41).

Recombinant GAS11-binding assay

GAS11–Golgi-binding assay was adapted from Levine *et al.* (41). Approximately 70 μL of buffer A containing varying amounts of recombinant His-tagged GAS11 (0, 0.1, 0.25, 0.5, 1, or 2 μg) or 0.5 μg of control protein was mixed with 5 μL of salt-washed Golgi membranes (derived from 10 mg of Golgi protein) or 5 μL of buffer A and incubated at 4 °C for 10 min. After incubation, the mixture was layered over 75 μL of buffer A containing 300 mM sucrose and centrifuged at 100 000 × ***g*** for 10 min (TLA-100). Membrane pellets were resuspended into buffer A, followed by addition of Laemmli sample buffer, and analyzed by immunoblot. Results are representative of three independent experiments using either rat or mouse Golgi membranes.

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