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The G alpha subunit Gα8 inhibits proliferation, promotes adhesion and regulates cell differentiation

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ABSTRACT

Heterotrimeric G protein-mediated signal transduction plays a pivotal role in both vegetative and developmental stages in the eukaryote *Dictyostelium discoideum*. Here we describe novel functions of the G protein alpha subunit Gα8 during vegetative and development stages. Gα8 is expressed at low levels during vegetative growth. Loss of Gα8 promotes cell proliferation, whereas excess Gα8 expression dramatically inhibits growth and induces aberrant cytokinesis on substrates in a Gβ-dependent manner. Overexpression of Gα8 also leads to increased cell–cell cohesion and cell–substrate adhesion. We demonstrate that the increased cell–cell cohesion is mainly caused by induced CadA expression, and the induced cell–substrate adhesion is responsible for the cytokinesis defects. However, the expression of several putative constitutively active mutants of Gα8 does not augment the phenotypes caused by intact Gα8. Gα8 is strongly induced after starvation, and loss of Gα8 results in decreased expression of certain adhesion molecules including CsA and tgrC1. Interestingly, Gα8 is preferentially distributed in the upper and lower cup of the fruiting body. Lack of Gα8 decreases the expression of the specific marker of the anterior-like cells, suggesting that Gα8 is required for anterior-like cell differentiation.

Introduction

Heterotrimeric G proteins are central mediators in signal transduction pathways, with cells utilizing them to respond to the environment and communicate with each other. Heterotrimeric G protein consists of an α subunit and an obligate βγ dimer, and localize to the cytosolic face of the plasma membrane. G proteins typically transduce extracellular stimuli from G protein-coupled receptors (GPCRs) to downstream effectors. Ligand binding to the GPCR activates the G protein heterotrimer by facilitating GDP/GTP exchange on the Gα subunit which leads to the dissociation of the Gα and Gβγ dimer (Oldham and Hamm, 2008). The activated GTP bound Gα and free Gβγ interact with their downstream effectors, respectively, including adenylyl cyclases (Pierre et al., 2009), phospholipases (Mizuno and Itoh, 2009) and ion channels (Padgett and Slesinger, 2010). GPCR-mediated signaling has been implicated in numerous physiological and pathological processes and represent 50–60% of current drug targets (Overington et al., 2006).

The social amoeba *Dictyostelium discoideum* has been employed as a model system to study G protein signaling. The amoeba has a relatively short life cycle, a haploid genome and is amenable to numerous biochemical and genetic techniques (Schaap, 2011b). The *D. discoideum* genome contains 14Gα subunits, 2Gβ subunits and a single Gγ subunit (Eichinger et al., 2005; Heidel et al., 2011). The Gα2-mediated cAMP chemotaxis pathway has been intensively studied in this organism. The amoeba usually lives in the soil feeding on bacteria. Once the food source is depleted, cells start a developmental process that leads to the secretion of propagating waves of cAMP (Schaap, 2011a). Gradients of cAMP are formed and can be sensed by other cells through the cAMP receptor cAR1 (Klein et al., 1988). Binding of cAMP to cAR1 in turn activates Gα2 and leads to the dissociation of Gα2 from the Gβγ subunit (Elzie et al., 2009; Janetopoulos et al., 2001; Kesbeke et al., 1988; Kumagai et al., 1989). The activated Gα2 and Gβγ elicit a plethora of cellular responses which allow thousands of cells to stream toward the aggregation center, undergo morphological changes and finally form environmental-resistant spores (Franca-Koh et al., 2006). Another Gα subunit, Gα9, has been suggested as an inhibitor of the cAMP pathway (Brzostowski et al., 2002, 2004).

Vegetative *D. discoideum* cells can sense the bacterial metabolite folic acid to help track down bacteria. This process has also been shown to be G protein-mediated. Cells lacking the Gβ subunit form tiny plagues on bacterial lawn (Wu et al., 1995), and Gα4 likely couples to the folic acid receptor (Hadwiger et al., 1994), although the folic acid receptor itself has remained elusive and is still not identified. A recent study shows that several elements

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thought to be required for cAMP chemotaxis are quite dispensable for folic acid chemotaxis (Srinivasan et al., 2012).

One of the Gα subunits, Gα8, has been investigated previously and no obvious function was revealed (Wu et al., 1994). Recently, Gα8 has been suggested to regulate the proliferation inhibition and chemorepellant activity of AprA (Bakthavatsalam et al., 2009; Phillips and Gomer, 2012). Here we generated *gα8*− cells in a new background and confirmed that the disruption of *gα8* leads to rapid proliferation. On the other hand, overexpression of *gα8* not only represses proliferation but also induces cytokinesis defects. We also found that overexpression of *gα8* promotes both cell–cell cohesion and cell–substrate adhesion, with the induced cell–substrate adhesion largely contributing to the cytokinesis deficiency. In addition, we present evidence showing that Gα8 modulates stalk cell fate determination and affects spore viability.

Materials and methods

Materials

Wild-type strains including Ax2, JH10, DH1, Ax3, KAx3, and the mutant strains summarized in Table 1 were obtained from dictyBase (*http://dictybase.org/*). Plasmids pLPBLP (Faix et al., 2004), pDM series (pDM304, pDM323, pDM326, and pDM358) (Veltman et al., 2009), GFP-Gβ (Jin et al., 2000), pDdGal-17 (Harwood and Drury, 1990), pVS (Zhang et al., 1999), pEcmAO-i-α-gal (Rafols et al., 2001), pEcmO-i-α-gal, pEcmB-i-α-gal, and pPsA-i-α-gal (Detterbeck et al., 1994) were also obtained from dictyBase. Polyclonal rabbit anti-Gα8 (Wu et al., 1994) and anti-Gα1 (Johnson et al., 1989) antisera were kindly provided by Dr. Peter Devreotes at John Hopkins University. Polyclonal rabbit anti-CadA antiserum (R851) (Knecht et al., 1987) and monoclonal mouse anti-CadA antibody (mLJ11) (Knecht et al., 1987) were kindly gifted by Dr. William Loomis at University of California San Diego. Rabbit anti-tgrC1 antiserum (Geltosky et al., 1979) was kindly provided by Dr. Charles Singleton at Vanderbilt University. Monoclonal mouse anti-CsA antibody (33-294-17) (Bertholdt et al., 1985) was obtained from the Developmental Studies Hybridoma Bank at the University of Iowa. Monoclonal mouse anti-Actin antibody (MAB1501R) was purchased from Millipore. Monoclonal mouse anti-c-myc antibody (46-0603) was purchased from Invitrogen. Monoclonal mouse anti-GFP antibody (11814460001) was purchased from Roche.

Cell culture, growth and development

Cells were axenically maintained in HL-5 medium or grown with *Klebsiella aerogenes* bacteria on SM plates at 22 °C. 100 μg/ml thymidine was supplemented in HL-5 medium for JH10 cells. Wild-type background used in each experiment was indicated in the figure legends. For proliferation measurements of suspension cultures, axenic cells were harvested from plastic petri-dishes, diluted in 50 ml HL-5 medium to 5×10^4 cells/ml, and shaken at 175 rpm, 22 °C. Cell density was measured by a hemacytometer. To measure adherent cell proliferation, cells were spread on 35 mm petri-dishes at a density of 1×10^4 cells/cm^2. At indicated time points, cells were removed thoroughly from the dish bottom by repeatedly pipetting, and the cell number was determined by a hemacytometer. The cell density was defined as cell number divided by petri-dish bottom area. To examine the developmental process, cells were collected from dishes or suspension culture, washed twice with developmental buffer (DB: 5 mM Na_2HPO_4, 5 mM KH_2PO_4, 0.2 mM $CaCl_2$, 2 mM $MgSO_4$, pH 6.5), and then plated on 1.5% non-nutrient DB agar at a density of 5×10^5 cells/cm^2.

Generation of mutant and overexpression strains

All primers used for molecular cloning are listed in Table S1. To disrupt *gα8* in wild-type Ax2 cells, a 677 bp 5′ homologous region and a 726 bp 3′ homologous region were amplified from genomic DNA and directionally cloned into the vector pLPBLP. The resulting construct replaced a small region on exon 2 of *gα8* (genomic DNA fragment bp 690–721, beginning with the start codon ATG) with the Bsr cassette. The knockout construct was linearized by NotI and 2 μg linear DNA was then electroporated into 5×10^6 Ax2 cells. 20 h after transformation, cells were selected with 10 μg/ml Blasticidin S for 10 days. The clones were isolated, diluted and then clonally spread on a *K. aerogenes* lawn for 5 days. Successful gene disruption in plaques was confirmed by PCR of genomic DNA using one primer inside the Bsr cassette and one primer outside the homologous region on the genome (Charette and Cosson, 2004).

The coding region of *gα8* was amplified from the first strand cDNA prepared from Ax2 cells starved for 5 h and cloned into the pDM304, pDM358 and pDM326 expression vectors, respectively. To generate the Gα8-GFP fusion, a SpeI restriction site was first introduced after the amino acid 110 of Gα8 by PCR and then *gα8* was inserted back into pDM304. *gfp* flanked by three glycine codons encoding "-GGG-GFP-GGG-" was amplified from the pEGFP-C1 vector and inserted into the SpeI site of *gα8*. For the inducible expression of Gα8–GFP fusion, the *gα8–gfp* fragment was amplified and cloned into the pVS vector. The point mutations G41V, S46C and Q203L of Gα8 were introduced by PCR and the resulting *gα8* mutants were cloned into the pDM304 vector. The truncated Gα8$^{\Delta Tail}$ was generated by removing the 51 amino acids at the COOH-terminus through PCR and cloning into the pDM304 vector. The DNA fragment "gaacaaaaactcatttcagaagaagattta" encoding the c-myc epitope "EQKLISEEDL" was fused to the NH_2-terminus of the Gγ gene, and the fusion protein was cloned into the pDM358 vector. The coding region of *gα1* was also amplified from the cDNA and cloned into the pDM304 vector. Cells transformed with these expression plasmids were selected with 20 μg/ml G418 or 50 μg/ml Hygromycin B or 10 μg/ml Blasticidin S as required until single colonies emerged. To reduce the expression level of Gα8-GFP, *gα8*−cells carrying *gα8-gfp* driven by the *discoidin I* promoter were either supplemented with 1 mM folate in HL-5 medium or co-cultured with *K. aerogenes* bacteria.

Immunocytochemistry

Cells were grown on coverslips in HL-5 medium overnight, and then washed with phosphate buffered saline (PBS: 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 2.7 mM KCl, 137 mM NaCl, pH 7.4) twice. Cells

Table 1
Summary of mutants used in this study.

Strain	DictyBase ID	Background	Phenotype mentioned in this study	References
gα8–	DBS0236107	JH10	Rapid proliferation	Bakthavatsalam et al. (2009)
gβ–	DBS0236530	JH10	Rapid proliferation	Bakthavatsalam et al. (2009)
paxB–	DBS0236728	Ax2	Reduced cell–substrate adhesion	Bukharova et al. (2005)
sadA–	DBS0236921	Ax3	Abolished cell–substrate adhesion	Fey et al. (2002)
cadA–	DBS0237013	KAx3	Loss of Ca^{2+}-dependent cell–cell cohesion	Wong et al. (2002)

were then fixed in 4% formaldehyde for 20 min, washed with PBS, and blocked in PBS containing 0.1% Triton X-100, 1% normal goat serum (NGS) and 1% bovine albumin serum (BSA) for 30 min. Primary rabbit anti-Gα8 was used at 1:200, and secondary FITC-labeled goat anti-rabbit IgG was used at 1:750. Images were taken on a Quorum WaveFX spinning disk confocal system running Metamorph software.

Microscopy

Images of developing structures on DB agarose were acquired with a Leica MZ16 stereomicroscope with a Q-Imaging Retiga 1300 camera and QCapture software. Live cells were photographed on coverslips or in Lab-Tek II chambers (Nalge Nunc International). Cells were imaged in DB for epifluorescence or confocal. For DAPI (4′,6-diamidino-2-phenylindole) stain, cells were first fixed with 4% formaldehyde in DB for 20 min, and then incubated with 1 μg/ml DAPI in DB for 5 min. To examine the cytokinesis process, cells were incubated with *K. aerogenes* bacteria overnight, collected and allowed to settle in Lab-Tek II chambers (Janetopoulos et al., 2005). The chambers were rinsed with DB three times to remove residual bacteria. Cells at the onset of cytokinesis were identified by their round shape, and imaged at 15 s intervals. Images were acquired on a Zeiss Axiovert Marianas Workstation from Intelligent Imaging and Innovations running Slidebook software. A 40 × PlanNeofluar (NA 1.3) wide-field lens was used. Confocal images were obtained by using a Quorum WaveFX spinning disk confocal system on a Nikon Eclipse Ti microscope with a PlanApo 60 × TIRF objective (NA 1.49) (Fig. 3B, F and Fig. S3C).

Cell–substrate adhesion and cell–cell cohesion assays

Cell–substrate adhesion assay was performed as described (Fey et al., 2002). 5×10^5 cells were plated in 35 mm petri-dishes in a total volume of 1 ml HL-5, and settled for 4 h to allow them to completely adhere to the substrate. The dishes were then set on a platform shaker and shaken at 125 rpm. At indicated time points, the number of floating cells in the medium was immediately scored by a hemacytometer. The total cell number was also counted in control dishes, which were not shaken. The number of floating cells divided by total cell number is the percentage of detached cells. For the cell–cell cohesion assay in vegetative cells, cells were shaken in suspension starting at 1×10^4 cells/ml. After 2 days, cell cultures were dropped on coverslips and immediately photographed using the 40 × PlanNeofluar (NA 1.3) wide-field lens. Cells in the field of view were then counted. Triplets and clumps containing more than three cells were defined as "cell clusters", while singlet and doublets were not. To confirm the accuracy of cell number counting in large cell clusters as seen in *gα8* overexpression cells, another photograph was taken after cells dissociated from each other, which usually happened a few minutes after the cell culture was plated. The dissociation process was also recorded to examine whether cells in the medium adhere to the coverslip and whether a cytoplasmic bridge between cells was formed during the separation, which indicates the existence of cytokinesis defects in the cluster. To test whether the cohesion was EDTA or EGTA sensitive, EDTA or EGTA was added to a final concentration of 10 mM and cells continued to shake for 3 h before they were counted. To test the effect of anti-CadA blocking, a final concentration of 20 μg/ml normal rabbit IgG or rabbit anti-CadA antiserum (R851) was added and continued to shake for 3 h before counting. The cell cohesion assay in starving cells was performed as described (Wong et al., 2002). Cells from petri-dishes were developed in DB suspension at 5×10^6 cells/ml, and cell aggregates were photographed after 3 h. Cell aggregates in 500 μl were dispersed by rigorously vortexing for 15 s and then examined under a microscope. The cells were then allowed to re-aggregate while shaking at 180 rpm. At indicated time intervals, only single cells were scored by a hemacytometer as non-aggregated cells. The percentage of cell–cell cohesion was defined as [(total number of cells minus non-aggregated cells)/total number of cells] × 100.

Western blots

To prepare crude membrane-enriched fractions, 5×10^7 vegetative cells were washed twice with cold DB and suspended in cold DB containing 1 × EDTA-free protease inhibitor cocktail (Roche). The cells were lysed by passing through an Acrodisc 5 μm pore size syringe filter (Pall). The crude membrane and cytosolic fraction were separated by centrifugation at 17,000 × *g* for 5 min at 4 °C. The membrane pellet was washed twice with DB containing protease inhibitor cocktail before dissolved in 1 × NuPAGE LDS Sample Buffer (Invitrogen) and 5% (v/v) β-mercaptoethanol (Sigma). For whole cells, 5×10^6 cells were washed twice with DB and lysed with 1 × LDS Sample Buffer and 5% (v/v) β-mercaptoethanol in a total volume of 20–50 μl. The cell lysate was incubated at 90 °C for 5 min and 3–10 μl was analyzed on 4–10% mini-protean TGX precast gel (Bio-Rad). After electrophoresis, proteins were transferred to a nitrocellulose membrane. The membrane was blocked with the Odyssey blocking buffer and incubated with the indicated antibodies. Unless otherwise mentioned, a 1:1000 dilution was used for primary antibodies and a 1:10,000 dilution was used for secondary antibodies. Secondary antibodies IRDye 680LT Donkey anti-Mouse IgG (LI-COR, 926-68022) and IRDye 800CW Goat anti-Rabbit IgG (LI-COR, 926-32211) were used for 2-color detection. The nitrocellulose membrane was developed using the Odyssey Infrared Imaging System (LI-COR, Lincoln, Nebraska).

β-Galactosidase stain and activity assay

The 1681 bp 5′ *gα8* region between position −1630 and position +51 was amplified from genomic DNA and inserted into the pDdGal-17, which resulted in a fusion of the first 17 codons of *gα8* in frame with *lacZ*. After transformation in wild-type Ax2 cells, β-galactosidase activity was stained with X-gal in developmental structures as described (Richardson et al., 1994). To visualize staining of structures, cells were starved on a 5 μm filter which was set on top of absorbent pads saturated with KK2 buffer (16.2 mM KH_2PO_4, 4.0 mM K_2HPO_4, pH 6.1). At different developmental stages, the filters were sprayed gently with 1% glutaraldehyde in Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, pH 7.0) to fix developing structures for 10 min, permeabilized with 0.1% NP-40 in Z buffer for 10 min. Filters were washed twice with Z buffer, and then incubated with X-gal stain solution (5 mM $K_3[Fe(CN)_6]$, 5 mM $K_4[Fe(CN)_6]$, 1 mM X-gal, 1 mM EGTA in Z buffer) for 5 min to 24 h. The developing of blue staining was examined under a stereomicroscope, and the filters were rinsed three times with Z buffer to remove X-gal stain solution before being photographed. Wild-type cells expressing *lacZ* driven by the endogenous *gα8* promoter were also cultured in suspension starting at 5×10^4 cells/ml, and cell density was measured by a hemacytometer. At indicated cell density, 5×10^7 cells were collected, washed twice with KK2 buffer, and lysed with 1 ml reporter lysis buffer. The β-galactosidase activity was measured using β-galactosidase enzyme assay system (Promega, Madison, WI). Specific enzyme activities are given as milliunit per mg total protein. One unit is defined as the enzymatic activity that hydrolyses 1 μM of *o*-nitrophenyl-β-D-galactopyranoside (ONPG) per minute at pH 7.5 and 37 °C.

Spore viability assay

The spore viability assay was performed as described (Brock and Gomer, 2005) with some modifications. All procedures were performed at room temperature. Cells were collected from dishes, washed twice with KK2 buffer, and suspended at 1×10^7 cells/ml. 1 ml of cells were then starved on KK2-saturated filters. After 4 days, the filter was put in a 50 ml tube and washed repeatedly with 2 ml KK2 buffer. 2 ml KK2 buffer with 0.8% NP-40 was then added to the tube. The tube was rocked gently for 10 min and then the filter was discarded. 11 ml KK2 buffer was added and thoroughly mixed to make a final 15 ml suspension. The density of ovoid spores was counted by a hemacytometer. 1 μl from the 15 ml suspension was diluted in 1 ml KK2 buffer, and a 100 μl dilution was plated with *K. aerogenes* bacteria on SM plates. The number of plaques was counted a week later.

Statistical analysis

The statistical significance of differences was determined by the two-tailed Student's *t* test or two-way ANOVA using software OriginPro 8.6.0 (OriginLab Corp., Northampton, MA, USA). $P < 0.05$ was considered to be statistically significant.

Results

Gα8 elicits aberrant cytokinesis and inhibits proliferation in a Gβ-dependent manner

The function of Gα8 was first investigated about twenty years ago and no evident phenotypes were observed for either *gα8* null mutant or *gα8* overexpression strains (Wu et al., 1994). Recently we revisited this study and explored Gα8 function further in development. Surprisingly, wild-type cells (Ax2 background) expressing *gα8* under the control of the *act*15 promoter were substantially larger than control cells when grown on solid substrates such as plastic or glass (Fig. 1A). When stained with DAPI, the large cells frequently harbored multiple nuclei. In control cells, the vast majority of them had a single nucleus, and only about 5% of them had two nuclei. No cells with three or more nuclei were identified (Fig. 1B). In contrast, only 40–50% of the cells overexpressing *gα8* had a single nucleus, about 30% of the cells have two nuclei, and more than 20% of the cells had four nuclei or more (Fig. 1B), suggesting cells overexpressing *gα8* divide abnormally on substrates. This phenotype was also confirmed in several other wild-type background strains including JH10, DH1, Ax3 and KAx3 cells (data not shown).

To test whether the deficient division of cells expressing Gα8 could be recapitulated when no adhesive force between the cell and substrate is present, cells were cultured in suspension and the nuclei number was quantified. Cells grown in axenic medium usually divide faster in suspension than on a substrate (Novak et al., 1995). This contributes to the increase in double-nucleated cells observed when cells are shifted from dishes to suspension cultures (Fig. 1C and D). When *gα8* overexpressing cells were grown in shaking culture, less than 2% of the cells with three or more nuclei were identified, which is comparable to control cells. This suggests that the adhesive force provided by the substrate contributes to the cytokinesis failure or that shaking shears the multinucleated cells. Interestingly, *gα8* overexpressing cells had a significantly higher percentage of single-nucleated cells and a significantly lower percentage of double-nucleated cells than untransformed cells (Fig. 1C and D). The expression level of Gα8 was revealed by western blots (Fig. 1E). Gα8 was expressed at a relatively low level in vegetative cells, and at least 20-fold higher in *gα8* overexpressing cells.

To test whether the Gβ subunit is required for the function of Gα8, *gα8* was overexpressed in *gβ*− cells (Lilly et al., 1993). *gβ*− cells overexpressing *gα8* were indistinguishable from wild-type cells in the number of nuclei when grown on substrates (Fig. 1A and B). When *gβ*− cells overexpressing *gα8* were grown in shaking culture, less single-nucleated cells and more double-nucleated cells were formed, as compared to control cells (Fig. 1C and D). The expression level of Gα8 was also examined in wild-type, *gβ*− cells and *gβ*− cells overexpressing *gα8* (Fig. 1F). The expression level of Gα8 was lower in *gβ*− cells than wild-type cells, consistent with previous studies reporting that loss of its binding partner Gβ results in a decreased amount of the Gα subunit (Marrari et al., 2007). Overexpression of *gα8* in *gβ*− cells led to at least a 20-fold increase in Gα8 expression level (Fig. 1F).

gα8− cells created in the JH10 background have been reported to proliferate rapidly (Bakthavatsalam et al., 2009). This phenotype was reproduced when *gα8* was disrupted in the Ax2 background. The successful disruption of *gα8* was confirmed by western blots (Fig. 1E). When grown in glass chambers, *gα8*− cells formed significantly less single-nucleated cells and more double-nucleated cells as compared to wild-type cells (Fig. 1A and B). A very small portion of cells (about 1.5%) had three nuclei and no cell had more than four nuclei. In suspension, about 54% *gα8*− cells had a single nucleus, comparing to about 79% in wild-type cells (Fig. 1C and D) and about 42% cells had double nuclei, twice as many as in wild-type cells, which is consistent with a previous report (Bakthavatsalam et al., 2009). In addition, about 4.5% cells had more than four nuclei, which was attributed to the rapid proliferation of *gα8*− cells previously (Bakthavatsalam et al., 2009). The increased proportion of cells with double nuclei suggests a higher proliferation rate in *gα8*− cells as compared to wild−type cells. To confirm this is the case, the proliferation rates of *gα8*− cells, as well as *gα8* overexpressing cells, were directly measured in suspension. *gα8*− cells proliferated faster than wild-type cells as previously described (Bakthavatsalam et al., 2009), whereas overexpression of *gα8* drastically inhibited proliferation (Fig. 1G). *gβ*− cells have been shown to proliferate faster than wild-type cells (Bakthavatsalam et al., 2009), and *gβ*− cells expressing *gα8* still exhibited a rapid proliferation rate (Fig. 1G) which is comparable to *gβ* − cells transformed with an empty pDM304 vector (data not shown), and suggests that the function of Gα8 is dependent on Gβ.

The overexpression of another Gα subunit, Gα1, has a similar multinucleated phenotype (Kumagai et al., 1989). Therefore we attempted to reproduce this data in a wild-type Ax2 background. Although overexpression of *gα1* in wild-type cells induced multinucleated cells on substrates (Fig. S1A), only about 2% of the cells had three or more nuclei, and the difference in the percentage of single-nucleus and double-nuclei cells between wild-type cells transformed with an empty vector and wild-type cells overexpressing *gα1* was insignificant (Fig. S1B). Gα1 was expressed at an extremely low level in axenic cells, and was strongly induced when overexpressed (Fig. S1C). Interestingly, overexpression of *gα1* did not significantly inhibit cell proliferation (Fig. S1D), suggesting that Gα1 functions differently than Gα8.

Gα8 promotes both cell–cell cohesion and cell–substrate adhesion

When grown in suspension, more than 80% of cells overexpressing *gα8* tended to form large clusters with other cells, whereas about 35% of control cells formed clusters (Fig. 2A and B). The natural dissociation of the clusters was carefully examined on a coverslip and with the addition of 10 mM EDTA or EGTA, which completely dissociated the clusters (Fig. 2A and B). This suggests that Ca^{2+}-dependent cell–cell interaction facilitates the formation of the clusters. In *D. discoideum*, CadA (Gp24) is the major cell adhesion molecule mediating Ca^{2+}-dependent cell–cell interaction

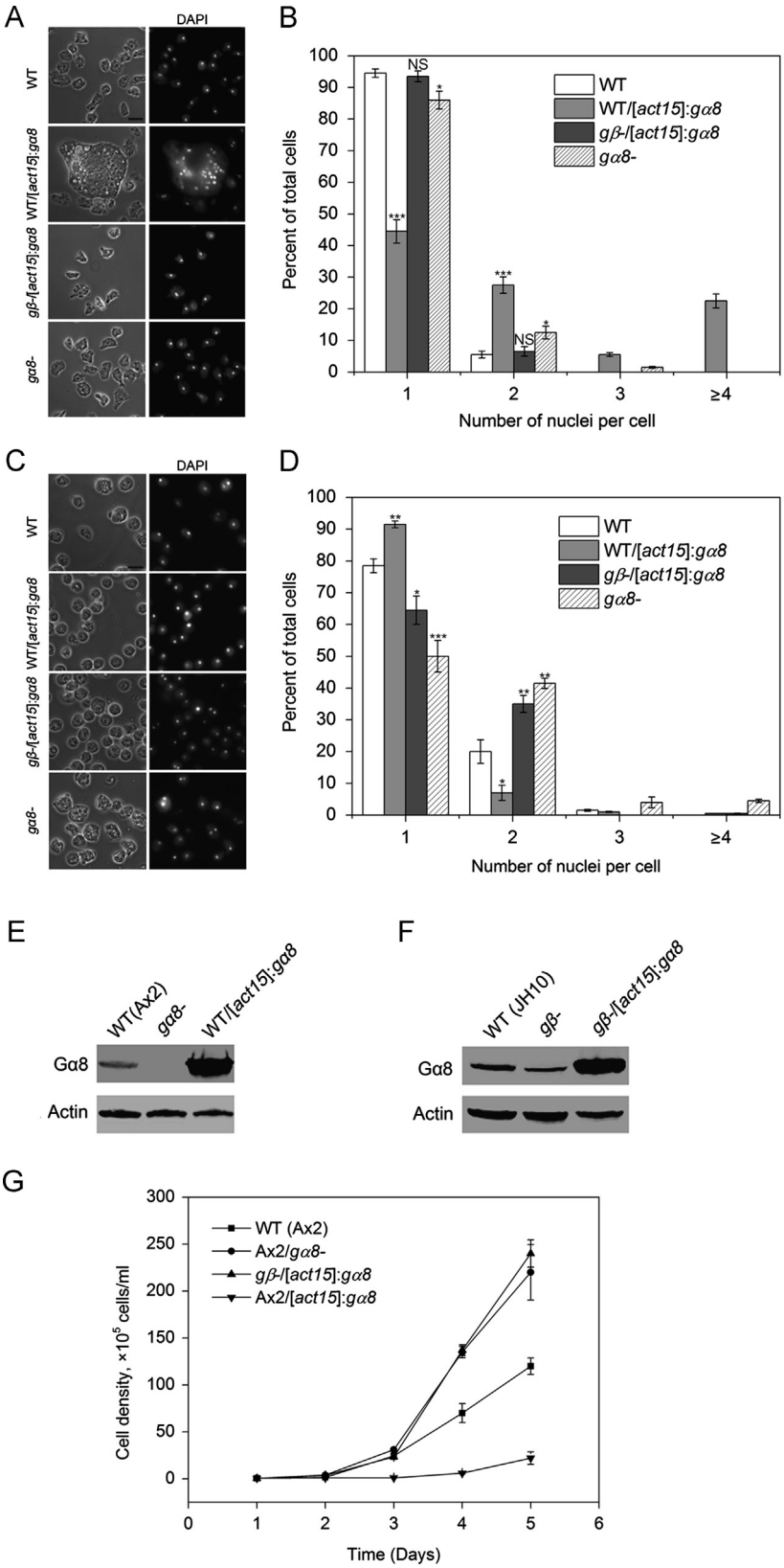

Fig. 1. Characterization of nuclei per cell and cell proliferation in cell lacking or overexpressing Gα8. (A) Cells were cultured in glass chambers, fixed in 4% formaldehyde and stained with DAPI. Ax2 cells were used as the wild-type (WT) cells. Images on the left panel were taken using phase microscopy. Bar, 10 μm. (B) Number of nuclei in cells from (A) was quantified. 200–300 cells were counted per sample in triplicates. Values are means ± s.e.m., and values are compared with WT values. $^{*}p < 0.05$; $^{***}p < 0.001$; NS, non-significant (two-tailed Student's *t* test). (C) Cells from suspension culture were fixed and stained with DAPI as in (A). Cells were then spread in glass chambers. WT cells and WT cells expressing *gα8* were pretreated with 10 mM EDTA to dissociate cell clusters before fixation. Bar, 10 μm. (D) Number of nuclei in cells from (C) was quantified as in (B). Values are means ± s.e.m. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ (two-tailed Student's *t* test). (E) Gα8 level was probed with anti-Gα8 serum in the parental WT cells, *gα8*– cells and WT cells expressing *gα8*. Actin was used as a loading control. (F) The Gα8 level was also probed in the parental JH10 cells, *gβ*– cells and *gβ*– cells expressing gα8. Actin was used as a loading control. (G) Cells were diluted to 5×10^4 cells/ml in HL-5 medium and the cell density was measured daily. The graph shows means ± s.e.m. from three independent experiments. The differences between each strain are as follows: *gα8*– cells versus WT cells, $p < 0.01$; *gβ*– cells expressing *gα8* versus WT cells, $p < 0.01$; WT cells expressing gα8 versus WT cells, $p < 0.001$ (Two-way ANOVA).

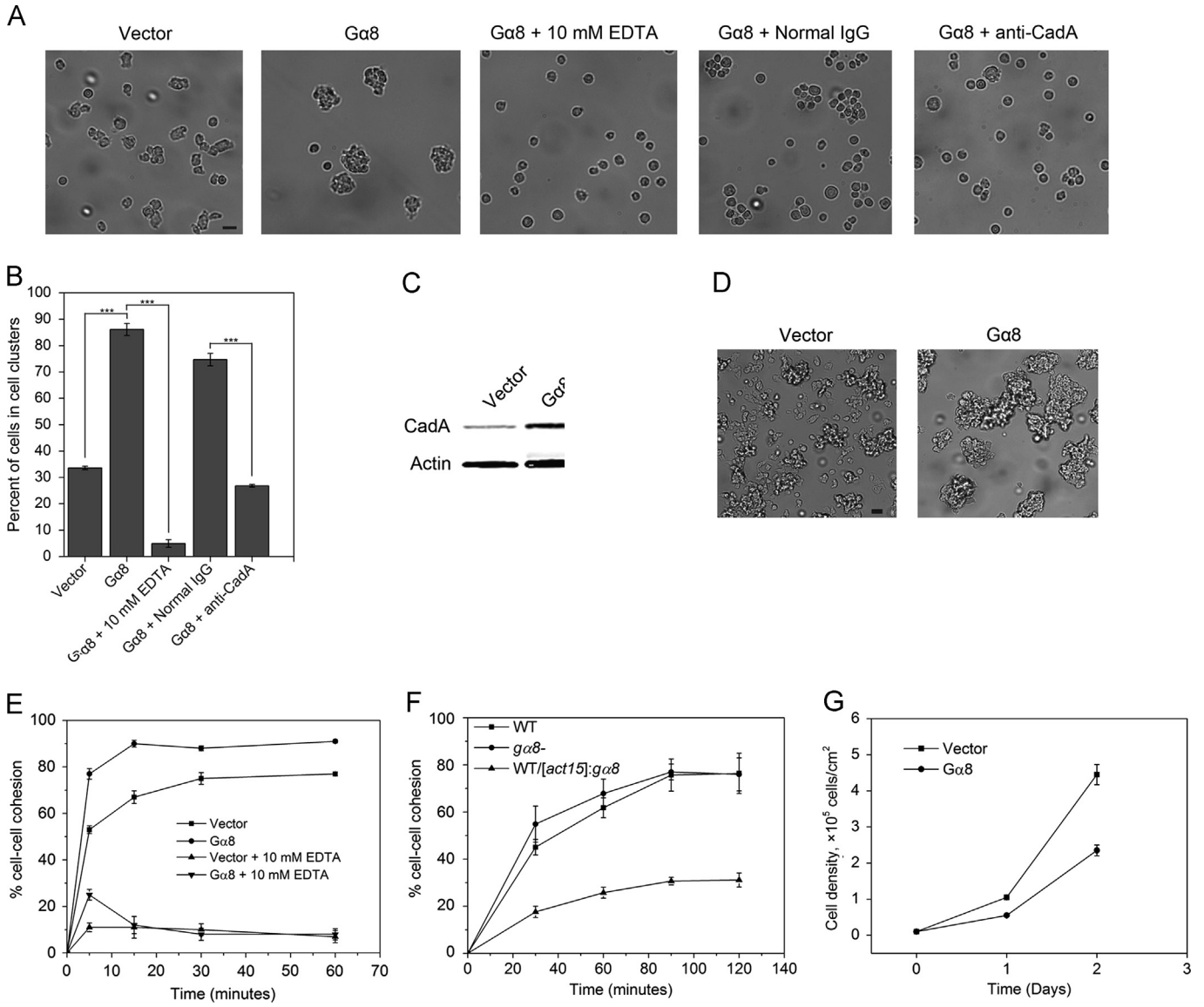

Fig. 2. Induction of adhesion in response to Gα8 overexpression. (A) WT cells expressing an empty vector or *gα8* were shaken in suspension starting at 5×10^4 cells/ml. After two days, cells were allowed to settle in glass chambers and photographed immediately. For EDTA and anti-serum treatment, 10 mM EDTA and 20 μg/ml normal IgG or anti-CadA serum were added to the suspension during 3 h continuous shaking. Bar, 10 μm. (B) The percentage of cells in clusters from (A) was quantified. 150–200 cells were counted per sample in triplicates. Values are means ± s.e.m. $^{***}p < 0.001$ (two-tailed Student's *t* test). (C) CadA levels were examined in cells expressing an empty vector or *gα8* from (A) using monoclonal anti-CadA antibody. (D) Cells expressing an empty vector or *gα8* were starved in DB while in shaking suspension for 3 h. Cells were then plated in chambers and photographed. Bar, 10 μm. (E) Cells from (D) were dissociated by rigorous vortexing and cell reassociation was monitored over time in the absence or presence of 10 mM EDTA. Data represent means ± s.e.m. of three independent experiments. WT cells expressing *gα8* versus WT cells expressing empty vector without EDTA, $p < 0.001$; with EDTA, $p = 0.17$ (two-way ANOVA). (F) 5×10^5 cells were plated in dishes and the dishes were set on a rotary shaker to detach cells. Cells floating in the medium were scored at indicated time. Data represent means ± s.e.m. of three independent experiments. *gα8*− cells versus WT cells, $p = 0.32$; WT cells expressing *gα8* versus WT cells, $p < 0.001$ (two-way ANOVA). (G) Cells were plated in dishes at 1×10^4 cells/cm^2, and cell density was measured daily. Data represent means ± s.e.m. of three independent experiments. WT cells expressing *gα8* versus WT cells harboring an empty vector, $p < 0.001$ (two-way ANOVA).

(Siu et al., 2011). Therefore, we tested whether anti-CadA serum blocks the formation of the clusters. A final concentration of 20 μg/ml anti-CadA serum successfully reduced clusters to 25% of total cells, which is in stark contrast to more than 70% in normal IgG treated cells (Fig. 2A and B). This suggests that CadA is responsible for the cluster formation. In addition, cells overexpressing *gα8* at a low density of 1.5×10^5 cells/ml (cells from Fig. 2A) showed an increased CadA expression level (Fig. 2C), indicating that overexpression of *gα8* promotes expression of *cadA* in suspensions with low densities. However, both *gα8*− cells and cells overexpressing *gα8* were indistinguishable in their CadA expression levels from wild-type cells collected from petri-dishes (Fig. S2A) or from high density suspension cultures (data not shown). Since the CadA levels are positively correlated with cell density and CadA is expressed at a high level in petri-dishes and in dense suspensions (Figs. S2A and S2B), overexpression of *gα8* may not significantly induce the amount of CadA at these stages. *gα8*− cells had a similar percentage of cells in clusters as wild-type cells, and comparable CadA levels at the low density of 1.5×10^5 cells/ml (data not shown). These results suggest that disruption of *gα8* is not sufficient to suppress the expression of CadA and inhibit adhesion in vegetatively growing cells.

CadA has been shown to initiate homophilic interactions between cells after starvation (Siu et al., 2011). Therefore, we tested whether overexpression of *gα8* could promote cell–cell aggregation after development. A large portion of control cells existed as small clumps or single cells after shaking for 3 h in DB.

By contrast, cells expressing *gα8* formed large clumps, and single cells were seldom observed (Fig. 2D). Examination of the re-aggregation of dispersed cells showed that cells expressing *gα8* rapidly aggregated, but could not aggregate in the presence of 10 mM EDTA (Fig. 2E). This suggests that overexpression of Gα8 promotes cell–cell aggregation after the onset of development, even though it does not substantially induce the expression of CadA when grown on petri-dishes (Fig. S2A).

Cells utilize cell–cell adhesion to communicate and form multicellular structures, and they require adhesive forces to move on a substrate. Different components are employed in cell–cell adhesion and cell–substrate adhesion in the social amoeba (Cornillon et al., 2006; Fey et al., 2002; Niewohner et al., 1997; Siu et al., 2011). Since overexpression of Gα8 induces cell–cell cohesion, the cell–substrate adhesion level was also examined. After 2 h in shaking suspension, only about 30% of cells expressing *gα8* were released from the dishes, whereas about 80% of wild-type cells were detached (Fig. 2F). No significant difference was observed in the percentage of detached cells between wild-type cells and *gα8*− cells (Fig. 2F). These results suggest that overexpression of Gα8 promotes cell–substrate adhesion. However, loss of Gα8 does not result in adhesion loss. Overexpression of Gα8 suppresses proliferation when grown in suspension (Fig. 1G), and it also substantially reduces proliferation when grown on substrates (Fig. 2G).

Previous studies have suggested that Gα8 is indispensable for the proliferation-inhibiting and chemorepellant activity of the autocrine signal AprA (Bakthavatsalam et al., 2009; Phillips and Gomer, 2012). AprA accumulation corresponds to cell density and reaches the highest level when density saturates (Choe et al., 2009). However, Gα8 was expressed at a very low level in suspension, and was only slightly induced when density increased (Fig. S2B). In addition, the reporter activity of *lacZ* driven by the *gα8* promoter did not change when grown in suspension (Fig. S2C).

GDP-bound Gα8 associated with Gβγ is essential for the aberrant cytokinesis induced by Gα8

The G protein heterotrimer is usually localized and functions at the plasma membrane, and efficient plasma membrane targeting requires the interaction of Gα with Gβγ (Fishburn et al., 2000; Hepler et al., 1993; Marrari et al., 2007). In *D. discoideum*, Gα2 failed to localize to the plasma membrane but instead was enriched in the cytoplasm in the absence of the Gβ subunit (unpublished data, Gus Wright). To verify the localization of Gα8 and examine whether loss of Gβ alters the localization of Gα8, crude membrane and cytosolic fractions were separated and examined by western blot analysis to determine Gα8 levels. Gα8 was enriched in the membrane fraction in both wild-type cells and in *gβ*− cells (Fig. 3A). In addition, the Gα8-GFP fusion was localized to the plasma membrane of both wild-type and *gβ*− cells (Fig. 3B). The fusion induced large multinucleated cells in wild-type cells (Fig. 3B), though the percentage of multinucleated cells was lower as compared to cells overexpressing untagged Gα8 (data not shown). It's worth noting that Gα8 was sometimes not uniformly enriched in the entire cell periphery of the extremely large multinucleated cells. Like untagged Gα8, the fusion did not induce cytokinesis failure in *gβ*− cells (Fig. 3B). Therefore, in addition to showing that an intact heterotrimer is involved in the cytokinesis failure, these results suggest that the plasma membrane localization of Gα8 is independent of Gβ.

We next tried to recapitulate the cytokinesis defect using putative constitutively active mutants of Gα8. Two different mutants were generated by replacing glycine with valine at position 41 ($G\alpha8^{G41V}$), or by replacing glutamate with leucine at position 203 ($G\alpha8^{Q203L}$) to theoretically ablate GTPase activity as described (Conklin and Bourne, 1993; Rens-Domiano and Hamm, 1995). If the function of Gα8 required activation, these mutations should augment the cytokinesis defect. To our surprise, none of these mutations induced any cytokinesis defects (Fig. 3C). We also generated a dominant negative Gα8 mutant by mutating the serine residue of the G1 motif ($G\alpha8^{S46C}$), which is essential for binding the phosphate moieties of guanine nucleotides and Mg^{2+} (Noel et al., 1993). This mutation leads to reduced affinity for GDP and Gβγ sequestration in Gα subunits (Natochin et al., 2006; Slepak et al., 1993), thereby inhibiting signaling by the wild-type G protein. The dominant negative $G\alpha8^{S46C}$ (Fig. 3C) did not lead to cytokinesis defects, consistent with *gα8*− cells, which also grow quite normally when grown on substrates (Fig. 1A and B). Interestingly, a truncated Gα8 with the removal of the last 51 amino acids ($G\alpha8^{\Delta Tail}$) led to cytokinesis failure (Fig. 3C), although the defect was less severe than that induced by intact Gα8. These data suggest that proper cycling of Gα8 between the GDP and GTP bound state is required to induce cytokinesis defects. With the exception of cells expressing $G\alpha8^{\Delta Tail}$, which lacks the antigen region where the anti-serum was raised, the levels of Gα8 in various strains shown in Fig. 3C were confirmed by western blot analysis (Fig. 3D).

In addition to displaying regular cytokinesis, cells expressing constitutively active $G\alpha8^{Q203L}$ proliferated normally in suspension (Fig. 3E). As expected, cells expressing dominant negative $G\alpha8^{S46C}$ grew significantly faster than control cells (Fig. 3E). One possible explanation for why constitutively active Gα8 mutants show no phenotypes when overexpressed is that these mutations modulate the G protein conformation and trigger the internalization of Gα8. To test this possibility, the Gα8 mutants were expressed in *gα8*− cells and stained with the anti-Gα8 serum. Intact Gα8, constitutively active $G\alpha8^{Q203L}$, and dominant negative $G\alpha8^{S46C}$ were all enriched on the plasma membrane, which demonstrate that these mutations do not alter the localization of Gα8 (Fig. 3F).

Another possibility is that Gα8 sequesters the Gβγ subunit. Overexpression of Gα8 might limit the amount of free Gβγ that could interact with other Gα subunits, therefore the phenotypes observed in cells overexpressing Gα8 might be from a lack of Gα signaling in general. To examine this possibility, Gβγ was co-overexpressed with Gα8 to see whether the cytokinesis defect could be rescued. Overexpression of Gβγ alone in wild-type cells did not induce any cytokinesis abnormalities (Fig. 3G). Overexpression of Gα8 in cells overexpressing Gβγ still exhibited a similar cytokinesis defect when compared to cells expressing Gα8 alone (Fig. 3G), indicating Gα8 does not function by sequestering Gβγ to induce cytokinesis defects. The induced level of Gβ and Gγ were confirmed by western blots (Fig. 3H), as were the amounts of Gα8 in cells coexpressing Gβγ (Fig. 3I).

We also examined the localization of Gα8 during cytokinesis. An inducible expression system of Gα8-GFP was generated using the *discoidin I* promoter. Expression of Gα8-GFP under the control of the *discoidin I* promoter in *gα8*− cells significantly increased the number of multinucleated cells, whereas repression of Gα8-GFP by adding 1 mM folate rescued the cytokinesis defect (Fig. S3A), suggesting that the cytokinesis defect depends on the amount of Gα8. The reduction of Gα8-GFP by addition of folate was confirmed by western blot (Fig. S3B). When cells with reduced Gα8-GFP level undergo cytokinesis, Gα8-GFP appears to be localized uniformly across the plasma membrane (Fig. S3C). GFP-Gβ is also localized uniformly across the plasma membrane during cytokinesis (Fig. S3D).

The cytokinesis defect induced by Gα8 is caused by increased cell–substrate adhesion

Since Gα8 induces CadA expression and promotes cell–cell cohesion, we examined whether loss of CadA might attenuate

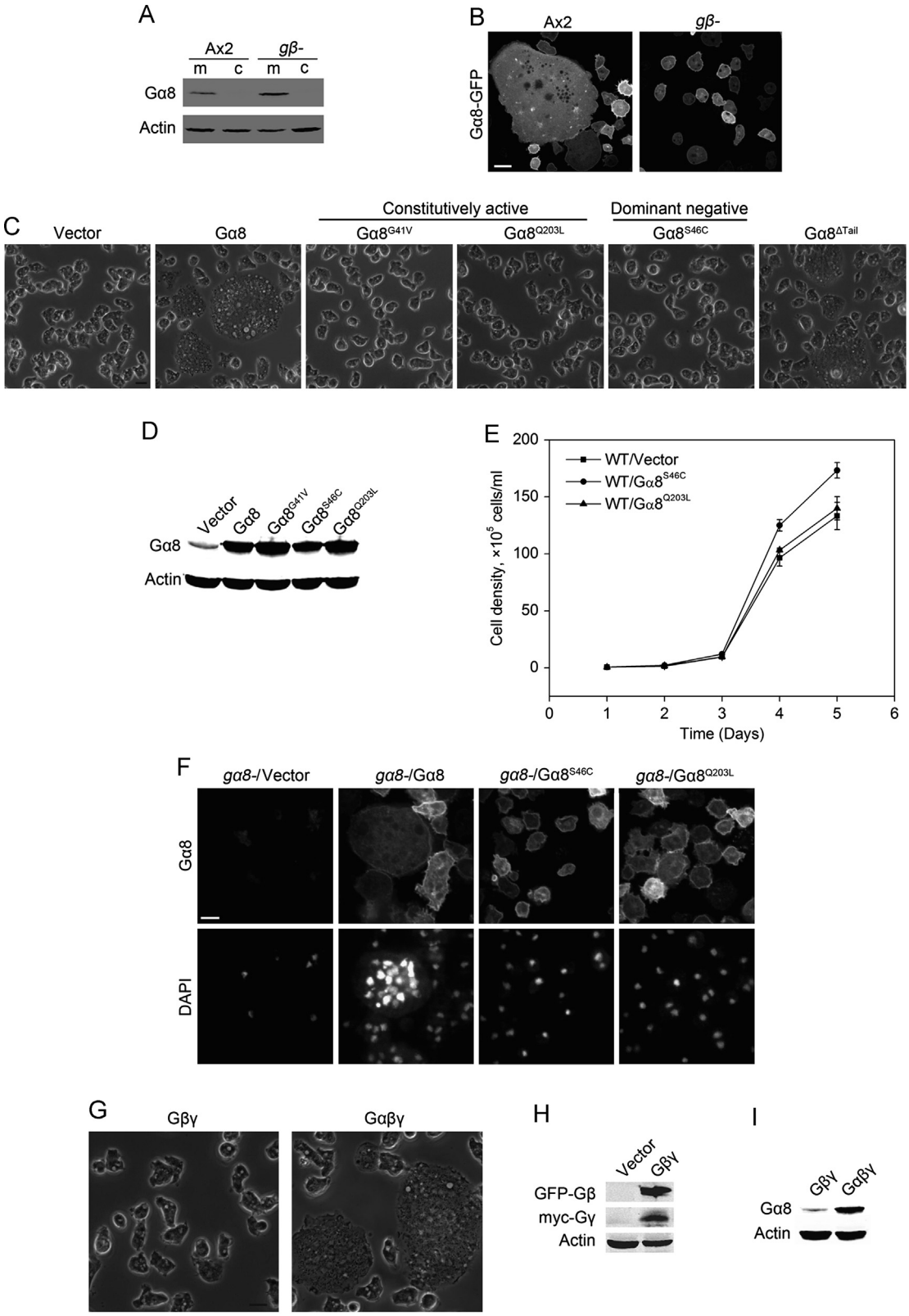

Fig. 3. Characterization of cell growth in wild-type and mutant forms of Gα8. (A) For western blot analysis, the crude membrane fraction and cytosol fraction were separated by filtering cells through 5 μm filters. Gα8 level in each fraction was examined using anti-Gα8 serum. m, membrane fraction; *c*, cytosol fraction. (B) The Gα8–GFP fusion was expressed in WT and *gβ*– cells. Images were taken on a spinning disk confocal microscope. Bar, 10 μm. (C) The empty vector pDM 304 or Gα8 forms with different acitvity were expressed in WT cells. Images were taken using phase microscopy. Bar, 10 μm. (D) Gα8 level in cells from (C) was confirmed using anti-Gα8 serum. (E) Cells were cultured in shaking suspension starting at 5×10^4 cells/ml and the cell density was measured daily. The graph shows means ± s.e.m. from three independent experiments. The differences between each strain are as follow: WT cells expressing dominant negative $G\alpha8^{S46C}$ versus WT cells expressing empty vector, $p < 0.01$; WT cells expressing constitutively active $G\alpha8^{Q203L}$ versus WT cells expressing empty vector, $p = 0.39$ (Two-way ANOVA). (F) Different forms of Gα8 were expressed in *gα8*– cells. Cells were then fixed and labeled with anti-Gα8 serum and DAPI and again images were taken by confocal microscopy. Bar, 10 μm. (G) myc-Gγ was cloned into pDM358 and co-transformed with GFP-Gβ into WT cells to generate cells expressing Gβγ. Gα8 was cloned into pDM328 and expressed in cells expressing Gβγ. Images were taken using phase microscopy. Bar, 10 μm. (H) Gβ and Gγ levels were examined in cells expressing Gβγ using anti-GFP antibody and anti-myc antibody. (I) Gα8 level was confirmed in cells expressing Gα8βγ using anti-Gα8 serum.

the cytokinesis defect caused by Gα8 overexpression. *cadA*− cells were previously generated and they exhibited very mild cytokinesis defects (Kim et al., 2011; Wong et al., 2002). Overexpression of *gα8* in *cadA*− cells resulted in a similar cytokinesis defect as seen for *gα8* overexpression in wild-type cells (Fig. 4A and B). This indicates that reducing CadA level could not rescue the cytokinesis defect induced by Gα8. Western blot showed that Gα8 was significantly induced by overexpression both in wild-type and *cadA*− background (Fig. 4B inset).

Since Gα8 also promotes cell–substrate adhesion, we next postulated that the increased cell–substrate adhesion may cause the cytokinesis defect. If this is true, low cell–substrate adhesion might rescue the defect, and the extent of rescue would depend on the level of cell–substrate adhesion. We used two mutants to test our hypothesis, *paxB*− and *sadA*− cells. It has been previously shown by two independent studies that loss of Paxillin results in decreased cell–substrate adhesion (Bukharova et al., 2005; Nagasaki et al., 2009). About 0.5% of *paxB*− cells transformed with an empty vector had four nuclei and had normal or slightly large morphology as (Fig. 4C arrow) and very few cells have five or more nuclei. Overexpression of Gα8 in *paxB*− cells still induced large multinucleated cells as shown in Fig. 4C (arrow head), however, only about 1.5% of the cells were evidently large and had more than four nuclei (Fig. 4D), significantly lower than *gα8* overexpression in wild-type background. Moreover, the percentage of cells with one or two nuclei was indistinguishable between control *paxB*− cells and *paxB*− cells expressing *gα8* (Fig. 4D). The induced Gα8 level in *paxB*− cells overexpressing *gα8* was confirmed by western blotting (Fig. 4D inset). Although overexpression of *gα8* in *paxB*− cells had no significant cytokinesis defect, it dramatically reduced the proliferation of *paxB*− cells in suspension (Fig. 4E). This suggests that the cytokinesis defect, but not the proliferation defect, as induced by *gα8* overexpression, is suppressed by loss of Paxillin.

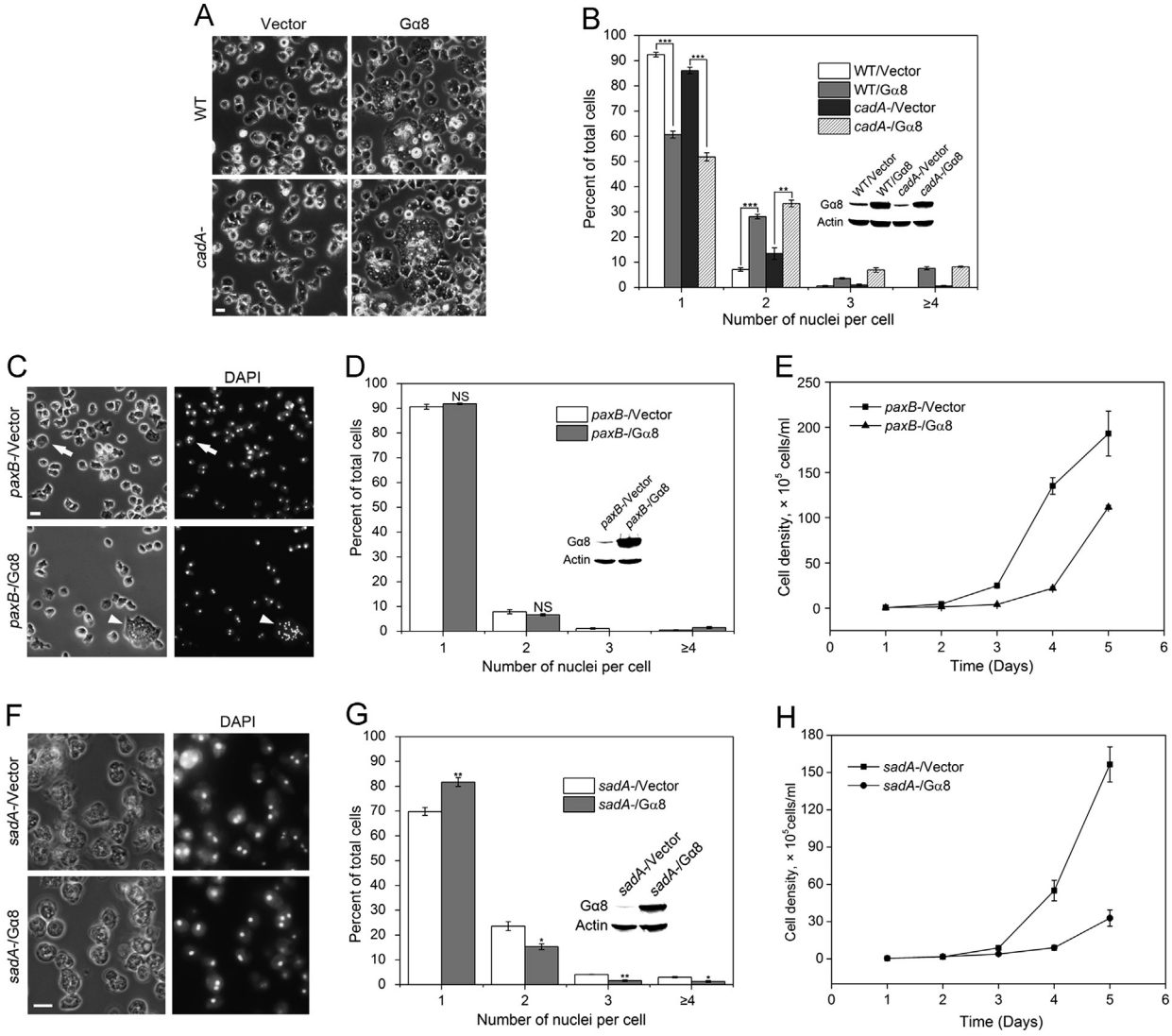

Fig. 4. Rescue of cytokinesis defects induced by Gα8 in adhesion mutants. (A) The empty vector pDM304 and Gα8 were expressed in the parental KAx3 cells (WT) and *cadA*− cells. Images were taken using phase microscopy. Bar, 10 μm. (B) Cells from (A) were fixed and stained with DAPI. 200–300 cells were counted per sample in at least triplicates. Values are means ± s.e.m. $^{**}p < 0.01$; $^{***}p < 0.001$ (two-tailed Student's *t* test). Inset, the Gα8 levels were examined in cells from (A) using anti-Gα8 serum. (C) The pDM304 vector and Gα8 were expressed in *paxB*− cells. Cells were fixed and stained with DAPI. Bar, 10 μm. (D) Number of nuclei in cells from (C) was quantified. 200–300 cells were counted per sample in at least triplicates. Values are means ± s.e.m. NS, non-significant (two-tailed Student's *t* test). Inset, the Gα8 levels were examined in cells from (C) using anti-Gα8 serum. (E) *paxB*− cells expressing pDM304 vector or Gα8 were diluted to 5×10^4 cells/ml, and cell density was measured daily. *paxB*− cells expressing *gα8* versus *paxB*− cells expressing pDM304 vector, $p < 0.001$ (two-way ANOVA). (F) The pDM304 vector and Gα8 were expressed in *sadA*− cells. Cells were fixed and stained with DAPI. Bar, 10 μm. (G) Number of nuclei in cells from (F) was quantified. 200–300 cells were counted per sample in at least triplicates. Values are means ± s.e.m. $^{*}p < 0.05$; $^{**}p < 0.01$ (two-tailed Student's *t* test). Inset, the Gα8 levels were examined in cells from (F) using anti-Gα8 serum. (H) Proliferation of *sadA*− expressing pDMA304 or Gα8 in suspension was measured as in (E). *paxB*− cells expressing *gα8* versus *paxB*− cells expressing pDM304 vector, $p < 0.01$ (two-way ANOVA).

The second mutant examined was *sadA* nulls, which have severely impaired cell–substrate adhesion, can barely attach to the substrate and have cytokinesis defects (Fey et al., 2002). *sadA*− cells exhibited strong cytokinesis defects with 7% having three or more nuclei and more than 20% having double-nuclei (Fig. 4F and G). Interestingly, overexpression of *gα8* in *sadA*− cells significantly increased the percentage of cells with a single nucleus, and reduced the percentage of cells with two or more nuclei (Fig. 4F and G). This indicates the cytokinesis defect caused by loss of adhesion in *sadA*− cells was partially rescued. The induced Gα8 level by overexpression was also confirmed by western blotting (Fig. 4G inset). Similar to *paxB*− cells expressing *gα8*, *sadA*− cells expressing *gα8* exhibited extremely slow proliferation (Fig. 4H). This suggests the proliferation-inhibiting activity of Gα8 is independent of induced cell–substrate adhesion. Taking these data together, the Gα8-induced cytokinesis defect can be rescued by reducing cell–substrate adhesion.

Gα8 is enriched in stalk cells and required for the differentiation of anterior-like cells

Gα8 levels rise after starvation (Wu et al., 1994), and disruption of *gα8* does not cause any dramatic phenotypes except rapid proliferation (Bakthavatsalam et al., 2009) (Fig. 1G). To address the functions of Gα8 during development, we examined the life cycle of *gα8*− cells and cells overexpressing *gα8*. Wild-type cells or those carrying the pDM304 vector exhibited a similar life cycle, with aggregation and mounds forming at 6 h after the onset of starvation (Fig. 5A). At 24 h, most of the cells form fruiting bodies (Fig. 5A). However, *gα8*− cells showed a slight delay (about 1–2 h) in aggregation, but were able to finish the life cycle in 24 h (Fig. 5A). In contrast, *gα8* overexpressing cells only formed small aggregates on the cell lawn (Fig. 5A). At 24 h, only a small portion of cells formed tiny fruit bodies, whereas most cells were culminating or still solitary (Fig. 5A). Cells expressing constitutively active $G\alpha8^{Q203L}$ developed normally (Fig. 5A). This is not surprising, as $G\alpha8^{Q203L}$ also did not display cytokinesis defects. We also confirmed the expression pattern of Gα8 after starvation. Consistent with a previous study (Wu et al., 1994), Gα8 protein levels gradually increase after starvation and peak at 4 h (Fig. 5B).

Overexpression of Gα8 promotes adhesion in vegetative cells. However, disruption of *gα8* barely reduces adhesion. When taken together with the temporal expression pattern of Gα8, we hypothesized that the adhesion loss of *gα8*− cells might happen in later development. To investigate this possibility, the expression of three adhesion molecules CadA, CsA (Gp80) and tgrC1 (Gp150) were examined during the development of *gα8*− cells. In wild-type cells, CadA expression decreased concomitantly with the increase of CsA. Interestingly, CsA was only weakly induced and CadA remained unchanged in *gα8*− cells (Fig. 5C). A previous study showed that the CsA level is increased to compensate the loss of CadA (Wong et al., 2002), therefore our data suggest that CadA compensates for low levels of CsA in *gα8*− cells. It has been reported that loss of CsA expression induces the precocious expression of TgrC1 (Wang et al., 2000). However, tgrC1 was still expressed at a relatively low level in *gα8*− cells when compared to wild-type cells (Fig. 5C). These data suggest that disruption of *gα8* inhibits expression of certain adhesion molecules.

To better understand the role Gα8 plays during development, we examined the spatial pattern of *gα8* expression using a *lacZ* reporter driven under the control of the endogenous *gα8* promoter. The gene fusion was transformed into wild-type cells and the developing structures were stained with X-gal and examined at different stages. Interestingly, *gα8* is strongly expressed throughout the slug and enriched in the anterior and rearguard of the slugs. Typical staining patterns are shown in Fig. 5D left panel, and the arrows indicate intensive staining. In fruiting bodies, Gα8 is specifically distributed in the upper cup, lower cup and basal disk but not in the stalk tube, similar to the distribution of anterior-like cells (ALCs) (Sternfeld and David, 1982) (Fig. 5D, right panel). The *ecmO* promoter is the distal portion of the full promoter for the *ecmA* gene, and it is the specific marker for ALCs (Jermyn et al., 1989). Therefore the *lacZ* reporter under the control of the *ecmO* promoter was examined in *gα8*− cells. The *ecmO* expression was significantly suppressed in *gα8*− cells (Fig. 5E), suggesting that Gα8 is required for the differentiation of ALCs.

Since Gα8 appears to be preferentially expressed in stalk cells, we examined whether Gα8 regulates cell fate. *gα8*− cells and cells overexpressing *gα8* were labeled with GFP and mixed with control wild-type cells to generate chimeras. At 6 h after the chimeras were starved on non-nutrient agarose, mounds were formed and wild-type cells were distributed throughout the mound (Fig. 6A and B). The *gα8*− cells exhibited a similar distribution pattern as compared to wild-type cells (Fig. 6C and D). However, cells overexpressing Gα8 were primarily distributed in the periphery of the mound (Fig. 6E and F), suggesting they were unable to populate the aggregation center. In early culminates, wild-type cells were distributed evenly in both the stalk and spore cells regions (Fig. 6G and H). The *gα8*− cells were predominantly localized in the spore cells region (Fig. 6I and J), whereas cells overexpressing Gα8 were primarily found in the tip, upper and lower cup regions (Fig. 6K and L). The above results suggest that loss of Gα8 biased cells towards the spore cell fate and excess Gα8 biased cells towards the stalk cell fate.

The percentage of spores was also examined in *gα8*− cells. Consistent with a previous report (Bakthavatsalam et al., 2009), *gα8*− cells have a reduced percentage of spores in contrast to wild-type cells, and these spores have significantly impaired viability when treated with the detergent NP-40 (Fig. S4A). In addition, overexpression of Gα8 also dramatically decreased the number and viability of spores (Fig. S4A). Since the Gα8 regulates cell fate, we further examined whether any particular cell type, other than the ALCs, would show a disproportionate localization in the *gα8*− cells. To examine this, the *lacZ* reporter gene driven by cell type-specific promoters (*ecmAO*, *ecmA*, *ecmB*, and *pspA*) was used to label different types of cells. The resulting structures were stained at 20 h of development. None of these specific markers showed significant changes in *gα8*− cells (Fig. S4B), suggesting that only the ALCs are affected by loss of Gα8.

Discussion

In this study, we characterized the functions of a G protein alpha subunit Gα8 during vegetative growth and development. Gα8 is induced after starvation, and distributed in the prestalk/stalk region during development.

Gα8 inhibits cells proliferation

Gα8 has been previously suggested to be part of the signal transduction pathway used by AprA to inhibit proliferation (Bakthavatsalam et al., 2009). Here we confirmed that loss of Gα8 leads to rapid proliferation. When over-expressed, excess Gα8 dramatically reduces the proliferation rate. Unlike the secreted chalone AprA, Gα8 remains at a low level during vegetative growth, and does not accumulate according to cell density. Upon starvation, Gα8 is promptly induced and probably functions through the accumulated AprA activated pathway to inhibit proliferation and facilitate development. Although excess Gα8 also promotes cell adhesion, it's likely that this effect of proliferation inhibition is independent of the high adhesion level since Gα8 still inhibits proliferation in several adhesion mutants. Cells without Gβ subunit

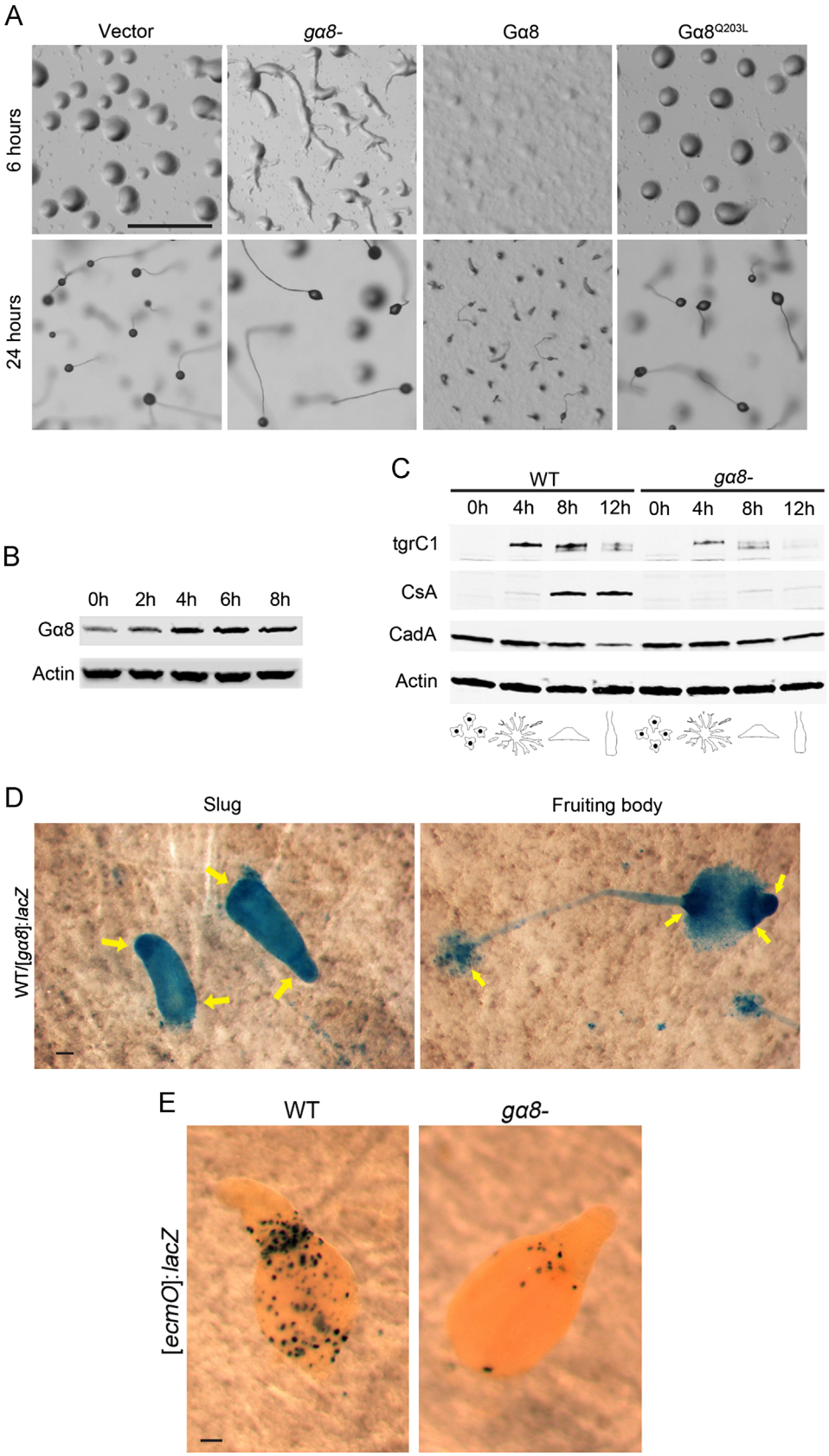

Fig. 5. Function of Gα8 in development. (A) 5×10^6 vegetative cells were washed with DB twice and plated on non-nutrient DB agarose. Images were taken at indicated hours after development. Bar, 1 mm. (B) 5×10^6 WT cells were starved on non-nutrient agarose. At indicated hours cells were collected, lysed and examined for Gα8 expression. (C) Same as in (B), WT cells and *gα8*– cells were starved on non-nutrient agarose and collected at indicated hours. The expression levels of adhesion molecules tgrC1, CsA and CadA were then examined. The cartoon at the bottom shows developmental stages at indicated hours. (D) WT cells expressing *lacZ* driven by the *gα8* promoter were developed on KK2 buffer-saturated filters. At 16 h (Slug stage) and 24 h (Fruiting body stage) after starvation, developing structures were fixed in 1% glutaraldehyde solution and stained with X-gal. Arrows indicates intensive blue staining. Bar, 50 μm. (E) β-galactosidase gene driven by the *ecmO* promoter was transformed in WT cells and *gα8*– cells. Cells were then developed on KK2-saturated filters. 20 h after development, developing structures were fixed and stained with X-gal. Typical stainings are shown. Bar, 50 μm.

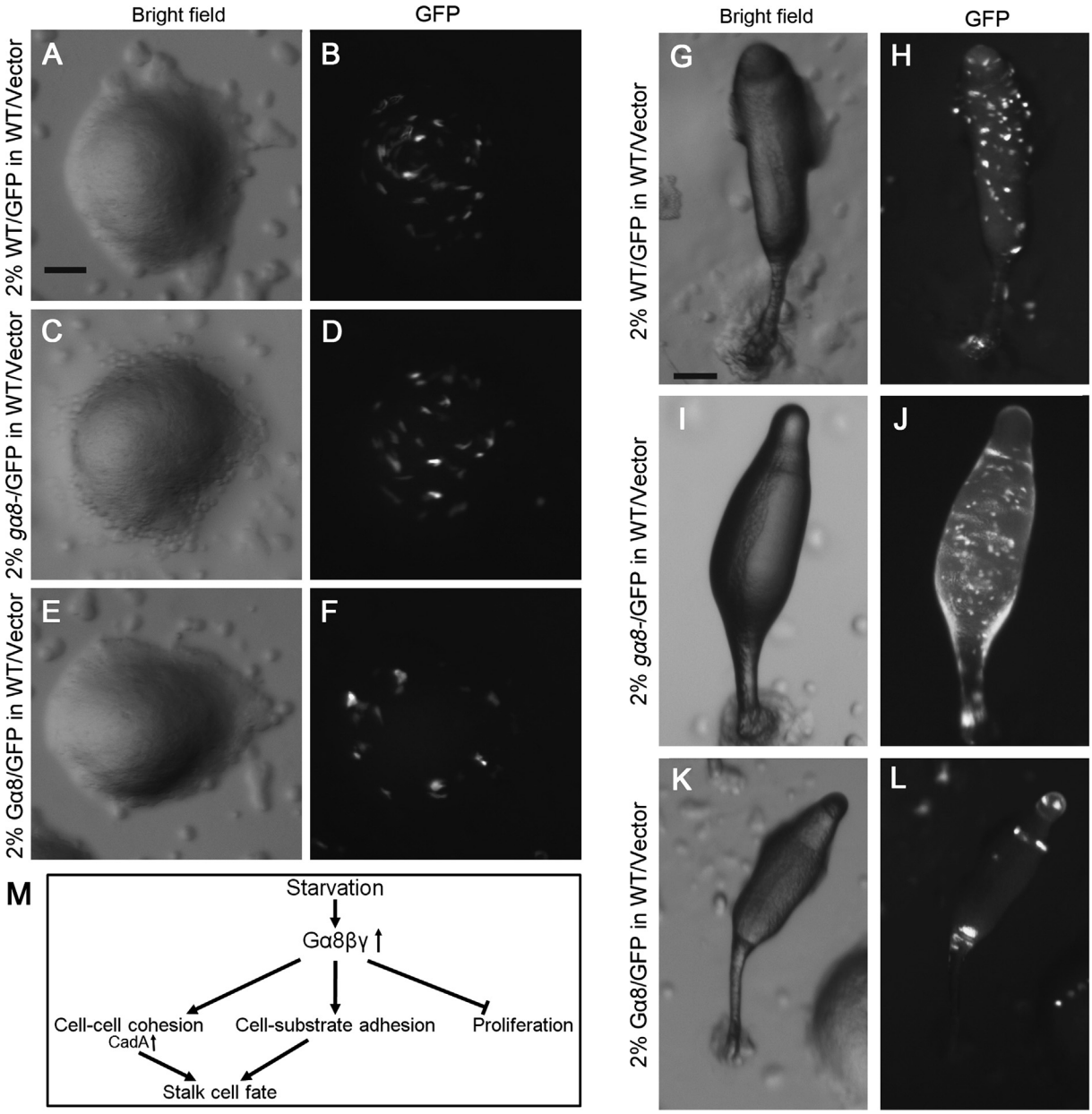

Fig. 6. Distribution of *gα8*– and *gα8* overexpression cells in chimeras. 2% wild-type Ax2 cells expressing GFP (Using pDM323 vector), *gα8*– cells expressing GFP (Using pDM323 vector), WT cells expressing *gα8* and GFP (Using *gα8* in pDM358 and pDM323 vector) were mixed with WT cells expressing pDM304 vector. 5×10^6 mixture cells were developed on non-nutrient agarose, and representative images were taken at mound stage (6 h after starvation, ((A)–(F)) and culmination stage (20 h after starvation, ((G)–(L)). Bar, 100 μm. Diagram showing proposed functions of the Gα8βγ heterotrimer is presented in panel M. The heterotrimer is increased in response to starvation. The heterotrimer thereby inhibits proliferation and promotes adhesion. The induced adhesion facilitates cell differentiation.

also exhibit rapid proliferation (Bakthavatsalam et al., 2009), and loss of Gβ suppresses Gα8-induced proliferation inhibition, suggesting Gβ is an indispensable component for the activity of Gα8. In mammalian systems, it was shown years ago that the inhibitory $G\alpha_i$ subunits likely regulate proliferation (Bloch et al., 1989; Hermouet et al., 1991). Gα8 is most similar to the $G\alpha_i$ subunit family of vertebrates (Brzostowski et al., 2002), suggesting this function of regulating proliferation in the inhibitory $G\alpha_i$ subunits is conserved.

Gα8 promotes cell adhesion

In addition to reduced proliferation, overexpression of Gα8 also causes cytokinesis deficiency on substrates. Subsequent results indicate excess Gα8 leads to both induced cell–cell cohesion and induced cell–substrate adhesion. This induced cell–cell cohesion was sensitive to the treatment of EDTA or EGTA, which indicates the cohesion is Ca^{2+}-dependent. Treatment with CadA antibody and analysis of CadA expression further suggest that the cell–cell cohesion is induced through up-regulating expression levels of CadA in cells overexpressing Gα8. Reducing cell–cell cohesion by removing CadA did not rescue the cytokinesis defect. However, reducing cell–substrate adhesion using different adhesion mutants including *paxB*– and *sadA*– successfully rescued the cytokinesis defect. These data also explained why no significant amount of multi-nucleated cells was observed when cells were grown in suspension since no cell–substrate adhesion is present in suspension. It is surprising that adhesion loss was not observed in *gα8*– cells. The low expression level of Gα8 in vegetative cells may explain this finding.

Although *paxB*– and *sadA*– rescued the cytokinesis defect, both Paxillin and SadA are not necessary components of the Gα8

signaling pathway. We showed that the cell-adhesion level governs the severity of the cytokinesis defect. Many proteins including Paxillin and SadA regulate cell–substrate adhesion in *D. discoideum* (Benghezal et al., 2003; Bukharova et al., 2005; Cornillon et al., 2006; Fey et al., 2002; Niewohner et al., 1997). The Gα8 signaling pathway might act through some of these proteins to induce cell–substrate adhesion, which causes more pulling force required for proper division. These data suggest that other adhesion mutants might also rescue the cytokinesis defect induced by excess Gα8. Interestingly, *sadA*− cells overexpressing Gα8 slightly increase adhesion toward substrates but are still severely impaired in cell–substrate adhesion. This suggests that SadA is the major substrate adhesion receptor and overexpression of Gα8 might promote expression of other minor substrate adhesion receptors in the absence of SadA.

The C terminus of Gα subunit has been recognized as a crucial receptor G protein interaction region (Bourne, 1997), and the truncated Gα8 lacking the C-terminal 51 amino acids causes similar cytokinesis failure as intact Gα8, suggesting Gα8 might induce the cytokinesis defect without the involvement of a GPCR, though the requirement for a GPCR is not absolutely ruled out. It is surprising to find that cells overexpressing two theoretically persistently activated Gα8 mutants failed to augment the overexpression phenotypes and instead grew and developed normally. The dominant negative Gα8 mutant theoretically binds to Gβγ but loses affinity for GDP, and overexpression of the heterotrimer with low affinity for guanine nucleotides does not induce cytokinesis defects. Since native Gα8 still induces cytokinesis defects when excess Gβγ exists, it is unlikely that overexpression Gα8 sequesters Gβγ from other Gα subunits. While it is possible that our Gα8 point mutations do not function as would be predicted and simply cause a loss of function, these data suggest that the activity of Gα8 requires the cycling of Gα8 between a GDP-bound and GTP-bound state.

Gα8 regulates cell differentiation

The expression pattern of Gα8 suggests that it functions after starvation. Moreover, the specified distribution of Gα8 in multicellular developing structures, the reduced *EcmO* marker and the decreased percentage of spores all suggest Gα8 regulates cell differentiation. We have shown that Gα8 inhibits proliferation, promotes cell–cell cohesion and cell–substrate adhesion. All three of these factors have been proposed to control cell fate determination when cells are shifted from the vegetative stage to starvation conditions. Cells that are starved at S phase or early or late G2 phase differentiate mostly into prestalk cells, whereas cells at middle G2 phase tend to differentiate into prespore cells (Gomer and Firtel, 1987; Weijer et al., 1984; Zimmerman and Weijer, 1993). Gα8 apparently regulates cell proliferation. However, it is still unclear whether the proportion of cell-cycle phase is altered with deficient or excess Gα8. *D. discoideum* cells have a prolonged G2 phase that accounts for over 90% of the cell cycle (Muramoto and Chubb, 2008). To explain the altered rate of proliferation, we speculate that *gα8*− cells have a shortened G2 phase and the proportion of G2 phase cells is lower, whereas cells overexpressing Gα8 have a prolonged G2 phase and the proportion of G2 phase cells is higher. However, our results showing differentiation of different cells in chimeras are contradictory to this cell cycle position theory. Cell-type specific alterations in adhesion have also been proposed in cell sorting. Cells with greater adhesiveness tend to differentiate into prestalk cells (Nicol et al., 1999; Sriskanthadevan et al., 2011). Our results are consistent with adhesion-dependent differentiation during development and suggest that cell adhesion likely precedes cell cycle position in determining cell differentiation.

In chimeras, cells expressing Gα8 are primarily located in the periphery of the mound. However, cells that show greater cohesiveness for one another usually stay in the center and the less cohesive ones typically sort to the periphery in chimeric mounds (Steinberg and Takeichi, 1994). One possible explanation is that cells overexpressing Gα8 have an impaired capacity for cAMP firing. This would explain why cells expressing Gα8 are seldom observed at aggregation centers. The strong cell–substrate adhesion of cells expressing Gα8 might also reduce cAMP chemotactic speed, which causes cells to reach the aggregates later than wild-type cells. Previous studies have suggested that spatial position in aggregates affects cell type differentiation (Krefft et al., 1984; Sriskanthadevan et al., 2011). Cells in the periphery differentiate mostly into prestalk cells and our results are consistent with position-dependent differentiation during development.

About 20% of prestalk cells populate the anterior region of the slug, and anterior-like cells with the prestalk features are scattered in the prespore zone (Sternfeld and David, 1982). Anterior-like cells could re-differentiate into prespore cells and form the upper cup and the lower cup which cradle the spore sorus (Jermyn et al., 1989), which possibly suggest that the decreased ratio of spores in *gα8*− cells is caused by the reduced number of anterior-like cells. A previous study revealed that the Gα4 subunit is distributed in anterior-like cells and disruption of *gα4* results in biased cell fate (Hadwiger and Firtel, 1992), which further suggests that the G protein is involved in anterior-like cell differentiation.

Our results provide evidence for novel functions of Gα8 during vegetative growth and development. These functions are summarized in Fig. 6M. Upon starvation, Gα8 is induced to negatively regulate proliferation and prepares cells for development. The induced levels of Gα8 promote cell adhesiveness, which is required for efficient cAMP chemotaxis and proper cell sorting. Further studies on the downstream targets of Gα8 will provide a better understanding of the mechanisms underlying this heterotrimeric G protein signaling cascade.

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Appendix A. Supplementary materials

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2013.05.001.

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