Clathrin adaptor GGA1 modulates myogenesis of C2C12 myoblasts

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Abstract

During myogenesis, myogenic stem cells undergo several sequential events, including cell division, migration, and cell-cell fusion, leading to the formation of multinucleated myotubes, which are the precursors of myofibers. To understand the molecular mechanisms underlying these complex processes, an RNA interference-based gene depletion approach was used. Golgi associated, gamma adaptin ear containing, ARF binding protein 1 (GGA1), a Golgi-resident monomeric clathrin adaptor, was found to be required for the process of myotube formation in C2C12 cells, a cultured murine myoblast cell line. Gga1 mRNA expression was upregulated during myogenesis, and Gga1 depletion prevented the formation of large multinucleated myotubes. Moreover, inhibition of lysosomal proteases in Gga1 knockdown myoblasts increased the amount of insulin receptor, suggesting that GGA1 is involved in the cell surface expression and sorting of the insulin receptor. These results suggested that GGA1 plays a significant role in the formation and maturation of myotubes by targeting the insulin receptor to the cell surface to establish functionally mature myofibers.

Introduction

Skeletal muscle tissue has essential roles within the body, such as movement, metabolism, glycopexis, and thermoregulation [1]. During muscle development, or muscle repair after damage, muscle satellite cells have crucial roles in the generation of muscle fibers. First, quiescent satellite cells are activated to become myoblasts and their number increase. Second, the differentiated myoblasts migrate into the damaged areas within the muscle. Third, multi-nucleated myotubes are formed through myoblast-to-myoblast or myoblast-to-myotube cell fusion [1]. The formation, maintenance, and growth of healthy skeletal muscle tissue are dependent upon these elementary steps.

During myogenic differentiation, myoblast cells undergo drastic changes in cell shape as a result of cell-to-cell fusion, becoming large, multi-nucleated myotubes that are the functional precursors of skeletal muscle cells. In the course of this differentiation, the secretion of several growth factors [2,3] and the cell surface expression of the fusion machinery are essential for proper muscle generation [4,5]. Therefore, the intracellular protein trafficking system is
thought to play a significant role in the stage-specific protein secretion and sorting of several plasma membrane proteins required for myogenesis.

Protein sorting at post-Golgi organelles requires the formation of carrier vesicles, such as clathrin-coated vesicles. A group of proteins termed clathrin adaptors is involved in the recognition of the cargo molecules and the physical formation of the membrane-bound clathrin-coated vesicles from the trans-Golgi network (TGN) and endosomes. Adaptor protein complex-1 (AP-1) and monomeric adaptor Golgi associated, gamma-adaptin ear containing, ARF binding proteins (GGA1, 2, and 3 in vertebrates) have been well studied in cultured cells and in animal models [6–10].

GGAs share some structural and functional features. All GGAs comprise three globular domains: VHS (Vps27/Hrs/STAM), GAT (GGA and Tom1), and GAE (Gamma-adaptin ear), with the last two domains being connected by a relatively unstructured hinge region. Although the VHS domains of all the GGAs possess similar binding capacity to the (D/E)xL-type sorting signal (where D is aspartate, E is glutamate, x is any amino acid, and L is leucine) of several membrane proteins in vitro [7], it is also believed that each GGA has its specific interactors. For example, the GAT domains of GGA1 and GGA3 have higher affinity for ubiquitin compared with that of GGA2 [11]. Recently, Uemura et al. showed that p56, an accessory protein of GGAs, is localized at the TGN in a GGA1-dependent manner [12,13]. In addition, while single knock-out (KO) of Gga1 or Gga3 caused no obvious phenotypes in mice, the Gga1/Gga3 double KO or single Gga2 KO mice were embryonic lethal [9,10]. These results strongly suggested that each GGA has specific physiological roles in vivo. However, the detailed physiological functions of GGA proteins remain to be determined.

GGAs have been suggested to be involved in the formation of insulin-responsible vesicles that contain glucose transporter type 4 (GLUT4), insulin-responsive aminopeptidase (IRAP), vesicle associated membrane protein 2 (VAMP2), and adiponectin in adipose tissues [14,15]. Although skeletal muscle is a major tissue responsible for the insulin-dependent uptake of blood glucose in vivo, the physiological function of GGA in skeletal muscle remains largely unknown.

In this study, using the cultured mouse myoblast cell line, C2C12, we found that GGA1 is specifically involved in the formation of mature large myotubes. Our data suggested that GGA1 has a pivotal role in the expression of the insulin receptor at the cell surface and in the downstream signaling required to establish mature myotubes.

Materials and methods

**Cell culture and short interfering RNA-mediated knockdown of Gga1 in C2C12 cells**

C2C12 cells were purchased from ATCC (#CRL-1772) and cultured in growth medium containing Dulbecco’s modified Eagle’s medium (DMEM) (Wako, Osaka, Japan) with 15% fetal bovine serum and 1% penicillin-streptomycin (growth medium). Muscle differentiation of C2C12 cells was induced by changing the medium to DMEM supplemented with 2% fetal bovine serum and 1% penicillin-streptomycin (differentiation medium) for 4 days. To knock down Gga gene expression, C2C12 cells were transfected with 20 nM of gene-specific small interfering RNA (siRNA, ON-TARGETplus SMART pool, Dharmacon) using the Lipofectamine RNAiMAX reagent (Thermo Fisher Scientific, Waltham, MA, USA). For control experiments, cells were transfected with non-targeted siRNA (Sigma, St Louis, MO, USA). To measure insulin-dependent glucose uptake, cells were shifted to a serum-free medium for 13 h and treated with Krebs-Ringer-Phosphate-HEPES buffer containing 2% (w/v) bovine serum
albumin (BSA) and 1 mM insulin for 5 min at 37 °C. All chemical reagents were purchased from Wako (Japan) unless described.

For the protease inhibition experiment, C2C12 cells were differentiated for 4 days and treated with lysosomal protease inhibitors (0.25 mg/ml leupeptin, 10 mg/ml pepstatin A, and 10 mg/ml E64d) or proteasome inhibitor MG132 at 5 mM in differentiation medium for 18 hours.

Cloning and expression of mouse GGAs and the preparation of anti-mGGA antibodies

Total RNA isolated from MEF cells was used for cDNA synthesis, and PCR amplification of mouse Gga1 (mGga1) and Gga2 (mGga2) and was carried out as described elsewhere [16]. The amplified full-length open reading frames were cloned into pCold-I vector (Takara, Kyoto, Japan) at the XhoI—XbaI sites. The expression of the recombinant His6-mGGAs was induced by cold shift at 15 °C and with 0.5 mM isopropyl β-D-1-thiogalactopyranoside for 24 h, following the manufacturer’s protocol. The His6-mGGAs were captured using a Talon metal affinity matrix (GE Healthcare, Chicago, IL, USA) and eluted with 250 mM imidazole, followed by dialysis. The purified recombinant mGGA proteins were used for immunization of rabbits (MBL, Nagoya, Japan) and the obtained antisera were further subjected to affinity purification using the recombinant protein coupled to N-hydroxysuccinimide-activated agarose beads (Thermo Fisher Scientific). The antibodies were checked for their specificity by immunoblotting using cell lysate from C2C12 cells treated with various siRNAs.

RNA isolation and quantitative real-time PCR

RNA was isolated from C2C12 cells using the RNAiso reagent (Takara) according to the manufacturer’s protocols. Synthesis of cDNA from the RNA samples was performed using PrimeScript 1st Strand cDNA Synthesis Kit (Takara). Real-time PCR amplification was carried out using Thunderbird SYBR qPCR Mix (TOYOBO, Osaka, Japan) on the StepOne Plus (Thermo Fisher Sciences) thermal cycler, according to the manufacturer’s instructions. In brief, real-time PCR was performed using the following cycles: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min for 40 cycles. At the conclusion of the 40 cycles, melting curve analysis was performed to confirm specific amplification. The StepOne software v2.2.2 (Thermo Fisher Sciences) was used for DDCT (cycle threshold) analysis of the data of the real-time PCR [17]. The PCR primer sets used for the real-time PCR amplification are following: Gga1 (F: CGTCCCCAAAGTCTAGAAGGT; R: TGGGGTTAGGGAAGGACAG), MyoD (myogenic differentiation 1) (F: CCCCGGGCCAGAATGCTAGC; R: GGTCTGGGTTC CCTTCTGTGTC), Myog (Myogenin) (F: CAACCAGGAGGCCGCTCCTCCG; R: AGG CGCTGGAAGTTGCAATCCTC), Myh3 (myosin heavy chain 3) (F: ATGAGTACCGACACC GAGATG; R: ACAAGCAGTAGGGTTGCGAT), Insr (insulin receptor) (F: CCCAGAA AAAACCTTCCAGCCA; R: TGGTGTCCACATCCCACCA), and Actb (beta actin) (F: AAA CATCCCCAAAGTTCTAC; R: GAGGACTTCTGTGACC).
with Alexa Fluor-conjugated secondary antibodies (Invitrogen, Carlsbad, CA, USA), diluted 1:400 in PBS, counterstained with Hoechst 33342, and mounted. The primary antibodies used were as follows: anti-GGA1 or 2 (this study), anti-Golgin subfamily A member 2 (GM130), anti-Ras-related protein Rab-4A (RAB4), anti-clathrin heavy chain (CHC), and anti-adaptor related protein complex 1 subunit gamma 1 (AP1G1) antibodies were from BD Biosciences (San Jose, CA, USA), whereas anti-lysosomal associated membrane protein 1 (LAMP1; 1D4B) rat monoclonal antibody, anti-MYH3, and anti-beta actin antibodies were purchased from Santa Cruz (Dallas, TX, USA). The immunofluorescent images were analyzed by BZ-9000 (Keyence, Osaka, Japan) and LSM5 Pascal laser scanning confocal microscopy (Zeiss, Jena, Germany).

**Assessment of cell fusion ability and myotube formation**

The fusion index was defined as the percentage of the number of nuclei contained in multinucleated myotubes to the total number of nuclei contained within the given field. For each experimental condition we selected six consecutive fields of view. Average fusion indices and standard errors were calculated, and the statistical analysis was conducted using Welch’s t-test. P-values of less than 0.01 were considered as statistically significant. To assess the level of MYH3, myotubes were stained with anti-MYH3 antibody followed by Alexa488-conjugated anti-Rabbit IgG (Thermo Fisher Scientific). The fluorescent images were captured by BZ-9000 (KEYENCE). The MYH3-positive, multinucleated myotubes were selected and the intensity of MYH3 was quantified using ImageJ software. To obtain the averaged width of myotubes, the area of MYH3-positive myotubes was divided by the longitudinal length.

**SDS-PAGE and immunoblotting analysis**

Cells were lysed in cell lysis buffer (PBS, 1% Triton X-100, and 1× Protease Inhibitor Cocktail (Roche)). The samples were then centrifuged at 20,600 × g for 20 min at 4 °C and the supernatant was recovered. After a protein assay was performed, samples were subjected to sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) (10 mg/lane). Proteins were transferred to polyvinylidene fluoride membranes (Millipore, Burlington, USA) in transfer buffer (25 mM Tris, 19.2 mM glycine, 0.1% SDS, and 20% methanol). After blocking with 10% non-fat dry milk or 0.1% (w/v) BSA in PBS-T (PBS with Tween-20), the membranes were incubated overnight at 4 °C with primary antibodies. The primary antibodies used were anti-GGA1, anti-GGA2, anti-MYH3 (Santa Cruz), anti-β-actin (Santa Cruz), anti-insulin receptor (Santa Cruz), anti-insulin like growth factor 1 receptor (IGFIR; Santa Cruz), anti-phospho-AKT serine/threonine kinase 1 (AKT1), and anti-AKT1 (Cell Signaling Technology, Danvers, MA, USA). Membranes were then washed with PBS-T and incubated with horseradish peroxidase-conjugated secondary antibodies (GE Healthcare). Lastly, membranes were washed with PBS-T and detected using the enhanced chemiluminescence system (GE Healthcare) and Ez-Capture ST (ATTO Corp., Tokyo, Japan).

**Measurement of glucose uptake**

Glucose uptake was assayed using the 2-deoxy-D-glucose (2-DG) Uptake Measurement kit (Cosmobio, Tokyo, Japan). In brief, differentiated (day 4) C2C12 cells were incubated in serum-free high glucose DMEM for 7 h at 37 °C. After incubation, cells were treated with 1 μM insulin for 18 min at 25 °C, and further incubated with 1 mM 2-DG (Peptide Institute, Osaka, Japan) for 20 min at 37 °C. Cells were recovered and the incorporated 2-DG was quantified by measuring the optical density at 405 nm.
Surface biotinylation
Growing or differentiated cells cultured in 6-well dishes were washed with ice-cold PBS three times on ice and then incubated with PBS containing 0.5 mM EZ-Link Sulfo-N-hydroxysuccinimid-SS-Biotin (Thermo Fisher Scientific) for 30 min on ice. After labeling, the labeling reagent was removed and replaced with ice-cold Tris-buffered saline (10 mM Tris-HCl (pH 7.5), 154 mM NaCl) for 15 min on ice, to quench the biotinylation reagent. The total lysate was generated by lysing the cells in lysis buffer (PBS containing 1% Triton-X-100 and 1× Protease Inhibitor Cocktail). The total lysate was then incubated with Streptavidin-beads (Sigma Aldrich) and the captured cell surface proteins were subjected to immunoblotting.

Statistical analysis
All data are presented as mean ± standard deviation and were compiled from at least two independent experiments performed in duplicate. P values were obtained using an unpaired Welch’s t-test or Student’s t-test analysis of variance. Values were considered statistically significant when P < 0.05.

Results
Preparation of specific antibodies recognizing mouse GGA1 and GGA2
To explore the physiological functions of GGAs in myogenesis, effective antibodies were required to detect endogenous GGAs. The commercially available antibodies for human GGAs did not work well to detect mouse GGAs in the mouse myoblast C2C12 cells; therefore, we generated anti-mouse GGA antibodies. The bacterially expressed full-length recombinant mGGA1 and mGGA2 (Fig 1A) were used for immunization of rabbits to raise specific antibodies. The affinity purified anti-mGGA antibodies were assessed for their specificity by immunoblotting using a C2C12 cell lysate (Fig 1B and 1C). The anti-mGGA1 and mGGA2 antibodies produced signals of approximately 81.8 kDa and 67.9 kDa, respectively, in the C2C12 lysate. Moreover, the signals were abrogated when the cells were treated with siRNAs targeting the corresponding gene. These results indicated that the antibodies could successfully detect the endogenous GGA proteins in C2C12 cells. Next, these antibodies were used for indirect immunofluorescence microscopy to check their utility in the morphological analysis. The C2C12 cells treated with siRNAs for Gga1 or Gga2 were stained with anti-GGA1 and GGA2 antibodies. In both cases, a perinuclear signal was predominantly observed and the immunoreactivity was reduced by treatment with the siRNAs targeting corresponding genes, indicating that the antibodies specifically recognize the endogenous gene products (Fig 1D).

GGA1 localizes to the Golgi apparatus and endosomes in differentiating C2C12 cells
GGA1 localizes to the TGN and endosomes in many cell types and contributes to the formation of clathrin-coated vesicles, which are required for intracellular transport of secretory and integral membrane proteins [7,18]. Thus, because its intracellular localization in differentiating myoblast cells was unknown, the intracellular localization of GGA1 and other organelle markers in growing or differentiating C2C12 cells was examined using indirect immunofluorescence microscopy with specific antibodies. Muscle differentiation was induced in C2C12 cells by shifting the culture medium to differentiation medium containing a low concentration of serum. Approximately four days after shifting to the differentiation medium, many large myotubes with multiple nuclei, which were positive for the developmental myosin heavy chain 3, MYH3, were observed in wild-type cells (Fig 2A).
GGA1 modulates myotube formation
GGA1 was localized at the perinuclear region and some peripheral puncta, in both the growing cells (day 0) and the multi-nucleated myotubes formed after four days of differentiation (day 4) (Fig 2B, a and a'). Co-staining with several organelle markers showed that the perinuclear localization was closely associated with GM130, a cis-Golgi marker (Fig 2B, a-c and a''-c'). The GM130-positive Golgi structure was also closely associated with, although it did not completely overlap with, the CHC signals (Fig 2B, j-l and j''-l'). GGA1 was also markedly colocalized with the gamma-subunit of the AP-1 complex at the Golgi area before and after differentiation (Fig 2B, d-f and d''-f'). Moreover, the GGA1-positive vesicular structures at the cell periphery also coincided with the signal for RAB4 (Fig 2B, g-i and g''-i'), which is localized at recycling endosomal compartments, whereas the GGA1 signal was completely segregated from that of LAMP-1, a marker for late endosomes and lysosomes (Fig 2B, m-o and m''-o'). These results indicated that GGA1 was distributed from the Golgi apparatus to the endosomes in C2C12 cells, throughout the differentiation period.

In addition to GGA1, the intracellular localization of GGA2 was also analyzed. GGA2 showed very similar staining patterns to those of GGA1 in C2C12 cells (Fig 2B, p-r). However, as the anti-GGA2 antibody gave less clear results compared with those of GGA1, we obtained only limited results from GGA2 staining of C2C12 cells.

GGA1 depletion causes a defect in myotube formation of C2C12 cells

To assess the physiological function of GGA1 in skeletal muscle differentiation, knockdown of Gga1 was performed in C2C12 cells. Morphological analysis showed that Gga1 knockdown (kd) cells showed fewer myotubes compared with control siRNA treated or Gga2-depleted cells (Fig 3A and S1 Fig). The results suggested that GGA1, but not GGA2, is possibly involved in myogenesis of C2C12 cells. To quantify the morphological data, the fusion index, which indicates the percentage of nuclei in myotubes as a proportion of the total number of nuclei in a given field, was calculated. The fusion indices of control, Gga1 kd and Gga2 kd cells were 63.3%, 46.7% and 56.9%, respectively. The result also indicated that the population of the unfused, mononuclear myoblasts in Gga1 kd cells was 16.7% higher than that of control cells, whereas no significant increase was observed by knockdown of Gga2 (Fig 3B). To further analyze which step of myotube formation is impaired by GGA1 depletion, a histogram analysis of the numbers of intracellular nuclei vs. the cell number of myotubes was constructed (S2 Fig and Fig 3C). The histogram showed a substantial decrease of 78.6% in the number of large myotubes containing over 20 nuclei in Gga1 kd cells, and the number of small myotubes with less than ten (2–9) nuclei in Gga1 kd cells significantly increased by 25.1% compared with that of control cells (Fig 3C). These results confirmed that GGA1 is partially involved in the fusion event of myoblasts throughout the myogenesis of C2C12 cells.

Induction of GGA1 expression during myogenesis of C2C12 cells

Silencing of Gga1 caused a reduction in the number of myotubes, and GGA1 has specific function(s) during myogenesis; therefore, to examine if the expression level of GGA1 is controlled...
GGA1 modulates myotube formation

A

Phase contrast  MYH3  Merge

a  b  c

a' b' c'

B

GGA1  GM130  Merge

da  e  f

d' e' f'

GGA1  AP1  Merge

g  h  i

g' h' i'

GGA1  Rab4  Merge

GGA1  Rab4  Merge

CHC  GM130  Merge

CHC  GM130  Merge

GGA1  Lamp1  Merge

GGA1  Lamp1  Merge

GGA2  AP1  Merge
under myogenesis, immunoblotting of GGA1 was performed. GGA1 protein expression was increased by approximately 3-fold during myogenic differentiation, whereas no significant change was observed in GGA2 protein expression. A decreased level of embryonic myosin heavy chain, MYH3, was observed in the Gga1kd cells, but not in the Gga2kd cells (Fig 4A), which was consistent with the results of the morphological analysis showing the reduced induction of myotubes (Fig 3).

GGA1 is not required for sequential induction of myogenic genes

During myogenesis, the sequential induction of myogenic transcription factors, including MyoD and Myogenin, leads to the expression of muscle specific genes [19]. The expression of MYH3 was affected by depletion of GGA1, which suggested that GGA1 might be involved in the induction of myogenic genes. To address this point, the expression levels of Myod and Myog were assessed using qPCR with Actb (encoding b-actin) as the control. The expression of Gga1 mRNA increased by 8-fold during myogenesis for 4 days. However, the transcriptional induction of myogenic genes was not affected by GGA1 depletion, except for a slight reduction of MyoD expression (Fig 4F–4H). These results suggested that the expression of Gga1 is transcriptionally controlled during myogenesis; however, its expression is not required for the sequential expression cascade of myogenic transcriptional factors.

Knockdown of Gga1 caused defects in the formation of mature myotubes

To gain more information about the physiological condition of the myotubes in the Gga1kd cells, morphological analysis was carried out. The intensity of MYH3 immunofluorescence in the GGA1-depleted myotubes was approximately 33.2% lower than that in the control cells (Fig 5A, b' and e', and 5B), and the average width of the myotubes in the Gga1kd cells was reduced by approximately 79.5% (Fig 5C). These data showed that knockdown of Gga1 resulted in the formation of smaller and thinner myotubes during myogenesis.

GGA1 deficiency affects insulin-dependent glucose uptake in C2C12 myotubes

Mature skeletal muscle has a large capacity to take up and store blood glucose upon insulin stimulation in vivo. During maturation of the skeletal muscle, the levels of the molecules that are involved in insulin-dependent glucose uptake increase to acquire the physiological role [20]. To assess whether the Gga1kd myotubes have the wild-type glucose uptake function, Gga1kd myotubes were examined for their capacity for insulin-dependent glucose uptake. Control and Gga1kd myotubes were prepared, and their glucose uptake ability was assayed using 2-DG, a non-metabolizable glucose derivative. As shown in Fig 6, 2-DG uptake was diminished by Gga1 knockdown under both basal and insulin-stimulated conditions, indicating that the Gga1kd cells failed to differentiate into physiologically mature myotubes.
GGA1 is involved in expression of the insulin receptor

Recently, it was shown that GGAs and the related clathrin adaptor AP-1 complex have functions not only in TGN-endosomal/lysosomal membrane trafficking, but also for the surface expression of a series of integral membrane proteins, including Notch and the epidermal growth factor receptor [13,21]. Moreover, Conejo et al. reported that insulin and insulin-like growth factors signaling play significant roles in the maturation of myotubes [22]. Thus, we
speculated that the immature Gga1kd myotubes with low MYH3 expression had a defect in the cell surface expression of the receptor proteins. To determine the possible changes in the expression levels of surface receptors, immunoblotting for the insulin receptor (IR) and the related receptor insulin-like growth factor receptor (IGF1R) was performed with total cell lysate and the cell surface protein fraction. The expression of the IR (both the b chain and ab precursor) was significantly induced by myogenic differentiation (Fig 7A) \[23\]. Immunofluorescent microscopy also confirmed that the induction of the IR was mainly observed in the multinucleated myotubes, and a low signal was observed in the surrounding unfused myoblasts (S3 Fig). In addition, the expression of the IR in both fractions was significantly decreased in Gga1kd cells, whereas little change was seen in IGF1R levels (Fig 7A). To examine the possibility that GGA1 affects the expression of the IR at the transcriptional level, the expression of Insr mRNA (encoding the IR) was quantified by qPCR. There was no significant change in the level of the Insr transcript in the Gga1kd cells (Fig 7B), suggesting that knockdown of Gga1 decreased the stability of the IR.

Next, to address the possibility that the IR was degraded in the absence of GGA1, Gga1kd myotube cells were treated with inhibitors of lysosomal proteases or the proteasome. The lysosomal inhibitors, but not the proteasomal inhibitor, successfully blocked the decrease in the cellular IR level in Gga1kd cells, indicating that the IR was degraded in the lysosomal compartment in the Gga1kd cells (Fig 7C–7E). Taken together, these results suggested that GGA1 is specifically required for the stable expression of the IR.

Insulin signaling is attenuated in GGA1kd myotubes

Gga1kd caused a reduction in IR surface expression as well as its total cellular level; therefore, we assessed IR downstream signaling to determine whether the signaling pathway was affected by Gga1kd at a physiological concentration of insulin. After serum depletion for 24 h, cells were stimulated by 0.1 and 1 nM insulin for 5 min and downstream signaling was examined by assessing AKT1 phosphorylation. Compared with the unstimulated cells, a significant reduction in AKT1 phosphorylation was observed in Gga1kd cells, indicating that GGA1 depletion affects insulin signaling (Fig 8A and 8B).

Insulin-dependent acceleration of myotube formation is affected in GGA1kd myoblasts

A previous study reported that modest insulin stimulation during myogenesis could enhance the formation of myotubes in C2C12 cells \[22\]. To determine if the insulin-dependent acceleration of myotube formation is also affected by Gga1 knockdown, C2C12 cells were subjected to myogenic differentiation in the presence of various concentrations of insulin. In the cells treated with the control siRNA, myotube formation increased under stimulation with 2 or 10 nM insulin. In contrast, the Gga1kd cells failed to respond to 2 nM insulin, suggesting that GGA1 is required for the enhancement of myotube formation at a physiological concentration of insulin (Fig 8C). At higher insulin concentrations, however, an increase in the fusion index
was observed in Gga1kd cells, consistent with the significant AKT1 phosphorylation in Gga1kd cells observed at higher concentrations of insulin (Fig 8A).

**Discussion**

In this study, we found that GGA1 has a crucial role in myogenesis of C2C12 cells. The GGA1-depleted cells showed small and thin myotubes with decreased expression of MYH3. The GGA family proteins are well known for their functions in recognition of cargo proteins that are sorted at the TGN; therefore, we initially speculated that GGA1 was involved in the sorting of fusion machinery to the plasma membrane. Indeed, a recent report showed that GGA2 has a crucial role in the cell surface expression of the epidermal growth factor receptor (EGFR), and the GGA-related clathrin adaptor AP-1 complex is also involved in targeting of the cell surface signaling molecule Notch and the secretion of certain soluble factors [13,21,24,25].

In the present study, morphological analysis indicated that GGA1 depletion caused a significant decrease in the population of myotubes with a large number (> 20) of nuclei, and increase in the number of myotubes with few nuclei. This result strongly suggested that GGA1 depletion affects the fusion process of myoblasts throughout the myogenesis of C2C12 cells. The immaturity of the myotubes in Gga1kd cells, however, also implied a significant physiological role of GGA1 in the metabolic maturation of myotubes. Thus, what is the physiological function of GGA1 during myotube formation? The regulation of the expression cascade [26] of myogenic genes Myod, Myog, and Myh3 was not significantly affected in Gga1kd cells, which suggested that GGA1 is not involved in the transcriptional control of these genes during the myogenic process.

During muscle differentiation of myoblasts, many extracellular factors, such as insulin, insulin-like growth factor I and II, and fibroblast growth factors, play important roles to support the growth of myotubes and myofibers [27,28]. Some of them are secreted from myoblasts and promote their own differentiation in an autocrine manner. In the current study, we found that IR expression was decreased in Gga1kd cells (Fig 7). Moreover, the GGA1-depleted cells also showed decreased sensitivity to the insulin stimulation that enhanced myotube formation (Fig 8C). These results suggested that the defect in the formation of large, mature myotubes in Gga1kd cells is, at least in part, caused by a reduction of cell surface receptor(s) and the subsequent impaired downstream signaling.

Insulin signaling is involved in skeletal muscle physiology [29]. Mice lacking mammalian target of rapamycin 1 (mTORC1) showed dystrophic skeletal muscle, mild glucose intolerance, and a shortened lifespan [30,31]. Mice lacking both insulin receptor substrate (IRS)1 and IRS2 in skeletal muscle also exhibited a much shorter lifespan than the control mice [32,33], suggesting that insulin action in skeletal muscle has a vital, but unrecognized, role in the control of lifespan, and that mTORC1 may contribute to these effects [34]. The decreased ability to respond to insulin stimulation observed in Gga1kd cells implied that GGA1 deficiency could cause diverse defects in insulin signaling in vivo.
GGA1 modulates myotube formation

**Graph: 2DG uptake (nmole/mg • min)**

- Insulin
  - Control
  - GGA1kd

*Significant difference (p < 0.05)
The reduction of IR levels in $Gga1$kd cells was suppressed by the inhibition of lysosomal proteases, which strongly suggested GGA1 depletion causes the IR to be missorted to the lysosome and degraded. One possible explanation for this result is that GGA1 functions in the recycling compartments that return the IR from the endocytic compartments to the cell surface. Alternatively, GGA1 could be involved in the sorting of IR at the Golgi compartments into the vesicle carriers destined for the plasma membrane. Currently, however, we have not addressed the precise functional site of action of GGA1.

More recently, Uemura and coworkers showed that GGA2 regulates the cell surface expression of EGFR via direct recognition and binding to the cytoplasmic region of EGFR [13].

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Fig 6. $Gga1$ silencing affects the insulin-dependent glucose uptake of myotubes. Control and Golgi associated, gamma adaptin ear containing, ARF binding protein 1 gene knockdown ($Gga1kd$) myotubes were prepared as in Fig 4, and the uptake of 2-deoxy-D-glucose (2-DG) in the absence or presence of 1 mM insulin was assayed as described in the Materials and methods. Error bars indicate the SD (n = 3). * $P < 0.05$ by a Student’s t-test.

Fig 7. $Gga1$ knockdown impaired the expression of the insulin receptor. (A) Total proteins (Total) and the cell surface proteins (Surface) purified using surface biotinylation from growing (day 0) and differentiated (day 4) cells were subjected to immunoblotting for golgi associated, gamma adaptin ear containing, ARF binding protein 1 (GGA1), the insulin receptor (IR), and the insulin-like growth factor 1 receptor (IGF1R). b-actin was used as the loading control. (B) Quantitative real-time PCR (qPCR) for $Insr$ (encoding IR) was carried out. (C) Lysosomal inhibitors suppressed the degradation of the IR in $Gga1kd$ cells. Myotubes differentiated for 4 days were treated with lysosomal inhibitors (L.I.) or proteasome inhibitor (P.I.) for 18 hours. The total lysates were subjected to immunoblotting for the IR. The signals for the ab precursor (D) and b-chain (E) of the IR were quantified and normalized by the b-actin signal. Error bars indicate the SD (n = 3). * $P < 0.05$ by Welch’s t-test.
Notably, the binding region of EGFR did not contain the (D/E)xxLL-type, conventional GGA binding motif. Although it remains unknown whether GGA1 could directly recognize the cytoplasmic domain of IR, which also does not contain a GGA binding motif, our preliminary results from the in vivo proximity labeling experiment suggested that GGA1 functions in proximity to the IR in vivo (S4 Fig)[35]. Thus, further investigation will clarify how GGA1 contributes to the trafficking of such surface receptors.

Previously, Pessin’s group proposed a model in which GGA proteins promote the loading of GLUT4 into GLUT4 storage vesicles (GSV) [36]. Another GSV resident membrane protein, IRAP, is also loaded into GSVs by GGA, through its di-leucine motif in the cytoplasmic region [37, 38, 39]. Thus GGA1 is likely to play a significant role in GLUT4 loading into GSVs; however, some of the data were obtained using the inhibitory effects of the VHS-GAT domains of GGA1. The VHS-GAT domain of GGAs selectively binds to the GTP form of Arf small GTPases and is presumed to block their actions broadly [40]. Arf1 functions in diverse steps throughout the secretory pathway; therefore, the inhibitory effects of VHS-GAT may not be specific to the GGA-dependent mechanisms at the TGN. We could not conduct an in vitro assay to show GSV formation from the TGN in Gga1kd C2C12 cells; therefore, it is currently unclear whether the defect in the insulin-dependent glucose uptake of Gga1kd is caused by a deficiency in the formation of functional GSVs.

According to previous biochemical approaches, GGA1 and GGA3, but not GGA2, were proposed to share certain functional and physiological features, such as their binding capacity to ubiquitin and their capability for self-inhibition [18]. Moreover, all three GGAs share similar structural features and binding affinity for the (D/E)xxLL-type signals of some membrane proteins [18]. Analysis of Gga KO mice also showed that single knock-out of Gga1 or Gga3 had no obvious phenotype, but that the Gga1/Gga3 double knock-out, or Gga2 single knock-out caused embryonic lethality, suggesting their diverse functional specificities in vivo [9,10]. In the current study, the result that GGA1, but not GGA2, was required for myotube formation in C2C12 cells, suggested that GGA1 might be responsible for the recognition of certain cargo molecules that are specifically required for myogenesis. Hence, the possible contribution of GGA3 to myogenesis is under investigation in our laboratory. In addition, our current approach using in vivo biotinylation for proximity labeling will provide clues to identify GGA1-specific interacting proteins that play important roles in myogenesis. Thus, further investigation into the specific cargo molecules associated with GGAs and their physiological analysis in skeletal muscle-specific Gga1 KO mice will shed light on the functions of GGA clathrin adaptors in muscle tissue.

Supporting information
S1 Fig. GGA1 knockdown caused a defect in myotube formation. (A) C2C12 cells treated with control siRNA (a-d) or siRNA targeting Gga1 (e-h) were differentiated for 0, 2, 4 and 7 days. Phase contrast images (gray) merged with the Hoechst33342 images (blue) are shown. (B) Fusion index in (A) was calculated as described in Materials and Methods. Bar indicates 10 mm. (TIIF)
S2 Fig. **GGA1 knockdown affected the myoblast fusion.** C2C12 cells treated with control siRNA (control) or siRNA targeting Gga1 (GGA1 kd) were differentiated for 4 days and the population of the myotubes with the indicated number of nuclei was plotted. (TIF)

S3 Fig. **Induction of IR expression in myotubes.** Wild-type C2C12 was subjected to differentiation for 4 days, then indirect immunofluorescent microscopy was carried out. Images for IR expression (green; a and a’, c and c’), nuclear staining images with Hoechst33342 (blue: b and b’, c and c’) and merged images are shown. Multinuclear myotube (arrow) showed IR staining whereas little or no staining was seen in the mononuclear myoblasts (arrow heads). (TIF)

S4 Fig. **In vivo proximity biotin labeling of IR by GGA1-BirA.** To perform the in vivo proximal biotinylation, the ORF of GGA1 was subcloned into pcDNA3.1 MCS-BirA(R118G), which was a gift from Dr. Kyle Roux (Addgene plasmid #336047)[35]. (A) pcDNA3.1 MCS-BirA (R118G) (BirA-HA) and pcDNA3.1-GGA1-BirA(R118G) (GGA1-BirA-HA) were transfected into C2C12 cells and intracellular localization of BirA-HA (a) and GGA1-BirA-HA (b) was examined by immunofluorescent microscopy with anti-HA antibody. The GGA1-BirA was localized at the perinuclear Gogi area (arrows) and at the peripheral puncta (arrow heads), whereas BirA alone did not localize at any intracellular compartments. (B) C2C12 cells were subjected to differentiation for 4 days and transfection of BirA-HA and GGA1-BirA-HA expression constructs was performed. Twenty-four hours after transfection, cells were incubated with 50 mM biotin for 6 hours and lysed. The biotinylated proteins were captured by streptavidin-sepharose (Wako). The input total lysate (5%) and the biotinylated proteins were subjected to immunoblotting with anti-IR antibody. The result indicated that GGA1-BirA-HA successfully biotinylated IR in vivo. (TIF)

**Acknowledgments**

We are grateful to Drs. Takefumi Uemura (Fukushima Medical University), Toyoshi Fujimoto and Yuki Ohsaki (Nagoya University), and Yasuo Uchiyama (Juntendo University) for extensive discussion and reagents. We also thank all the members of our laboratory. We would like to thank Editage (www.editage.jp) for English language editing.

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