G-protein coupled receptor BAI3 promotes myoblast fusion in vertebrates

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Muscle fibers form as a result of myoblast fusion, yet the cell surface receptors regulating this process are unknown in vertebrates. In *Drosophila*, myoblast fusion involves the activation of the Rac pathway by the guanine nucleotide exchange factor Myoblast City and its scaffolding protein ELMO, downstream of cell-surface cell-adhesion receptors. We previously showed that the mammalian ortholog of Myoblast City, DOCK1, functions in an evolutionarily conserved manner to promote myoblast fusion in mice. In search for regulators of myoblast fusion, we identified the G-protein coupled receptor brain-specific angiogenesis inhibitor (BAI3) as a cell surface protein that interacts with ELMO. In cultured cells, BAI3 or ELMO1/2 loss of function severely impaired myoblast fusion without affecting differentiation and cannot be rescued by reexpression of BAI3 mutants deficient in ELMO binding. The related BAI protein family member, BAI1, is functionally distinct from BAI3, because it cannot rescue the myoblast fusion defects caused by the loss of BAI3 function. Finally, embryonic muscle precursor expression of a BAI3 mutant unable to bind ELMO was sufficient to block myoblast fusion in vivo. Collectively, our findings provide a role for BAI3 in the relay of extracellular fusion signals to their intracellular effectors, identifying it as an essential transmembrane protein for embryonic vertebrate myoblast fusion.

myotube formation | myogenesis | model system | ced-12 | RhoGTP

The identity of molecular networks that control embryonic myogenesis and adult muscle regeneration is being unraveled (1). The intricacy of such networks reflects the complicated cascade of events underlying muscle development: myoblasts and satellite cells are first specified from precursors, then migrate to a precise location where they proliferate and fuse, and finally differentiate into postmitotic myofibers (2). Myoblast fusion is a critical step for the formation of embryonic muscle fibers, determines muscle size, and controls adult muscle regeneration (2), but its initiation remains poorly understood in vertebrates (3).

Genetic studies in *Drosophila* were instrumental in identifying the molecules that control myoblast fusion by relaying signals from the cell surface to the actin cytoskeleton (2). Cell adhesion receptors are expressed on two genetically distinguishable cell populations of founder and fusion-competent myoblasts and are critical to promote myoblast fusion (2). Dumbfounded/Kin-of-IrreC and Roughest/Irregular-optic-chiasma-C are expressed on the founder myoblasts and associate with Stick and Stones and Hibris found at the surface of fusion-competent myoblasts to trigger intracellular signaling cascades leading to fusion (4–7). The signaling intermediates include scaffold proteins (ELMO, Blown Fuse, Antisocial) (8–10), kinase (p21-activated kinase) (11), guanine nucleotide exchange factors (GEFs) [Myoblast City (MBC), Loner] (12, 13), GTPase (Rac), (14) and actin nucleation regulators (Kette, WAVE, WASP, WASP-interacting protein) (15–19). Some of these proteins ultimately promote the formation of actin-driven podosome-like structures in the fusion-competent cells that invade founder cells for membrane fusion (20, 21).

These findings raise the question of the identity of in vivo regulators of myoblast fusion in vertebrates. Previously, we demonstrated that the myoblast fusion function of DOCK1 (ortholog of MBC) is conserved from flies to mice (22). Extending this observation, conditional inactivation of Rac1 or *N*-WASP in developing mouse myoblasts was shown to impair fusion, further supporting an essential and evolutionarily conserved role for these genes in the fusion process (23, 24). However, mammalian orthologs of fly profusion receptors, Nephrin and Neph proteins, do not appear to be key regulators of primary myoblast fusion in mice (25, 26). Myomaker, or Transmembrane protein 8C, was recently shown to be essential for myoblast fusion, but its precise function remains to be defined (27). A number of cell adhesion proteins and promigratory receptors, including Integrin β1, Talin1/2, CDON/Brother of CDON and *M*-/*N*-Cadherins are implicated in myoblast fusion in cell culture models, and in vivo studies also support a contribution of Integrin β1 and Talin1/2 to fusion (28–32). Recently, activation of the brain-specific angiogenesis inhibitor 1 (BAI1) receptor by apoptotic cells residing in regenerating muscles was shown to promote myoblast fusion in muscle repair (33). Thus, the canonical cell surface receptors that control vertebrate primary myoblast fusion through the conserved DOCK1–Rac pathway have remained elusive.

BAI proteins belong to the cell adhesion subfamily of G-protein coupled receptors (GPCRs) and are characterized by long extracellular domains (ECDs) and intracellular domains (ICDs) (34). BAI3 regulates synapse density by binding to C1q-like proteins, a family of small secreted proteins highly expressed in the brain, via its Thrombospondin repeats (TSRs) (34). BAI1 recruits ELMO/DOCK1 and acts as an apoptotic cell clearance receptor by engaging phosphatidylserine exposed on the outer leaflet of dying cells via its TSRs (35). Recent data demonstrated

Significance

Myoblast fusion is essential for multinucleated muscle fiber formation. Regulators of fusion have been identified in *Drosophila*, but the molecular mechanisms controlling vertebrate myoblast fusion are poorly understood. We describe here previously unidentified in vivo functional evidence that the G-protein coupled receptor brain-specific angiogenesis inhibitor (BAI3) is a receptor controlling myoblast fusion in developing vertebrate muscles. We show that BAI3 binds the intracellular effector of myoblast fusion ELMO/DOCK1 and that this binding is obligatory for fusion. Using in vivo manipulations in the chicken embryo, we demonstrate that BAI3 binding to ELMO is essential for myoblast fusion. These experiments identify BAI3 as a long-sought-after receptor controlling vertebrate myoblast fusion.

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the presence of a GPCR–Autoproteolysis Inducing domain (GAIN) in ECDs of some cell adhesion GPCRs that is involved in autoproteolysis (36). In cancer cells, the BAI1 ECD is shed and cleaved into smaller fragments that display antiangiogenic properties (37).

Here, we present evidence for the identity of a transmembrane protein that orchestrates myoblast fusion by relaying cell surface signals to the underlying cytoskeleton. We identify the GPCR BAI3 as an interactor of ELMO/DOCK1 in myoblasts, whose down-regulation in cultured myoblasts abolishes fusion. Rescue experiments demonstrate that BAI3 engages the ELMO/DOCK1 pathway to mediate myoblast fusion. Finally, we show that interfering in vivo with BAI3's ability to interact with ELMO impaired primary myoblast fusion. Collectively, these results identify BAI3 as an indispensable protein for myoblast fusion in vivo.

Results

GPCR BAI3, an ELMO-Binding Receptor, Is Expressed in Myoblasts. We reasoned that a myoblast cell surface receptor that binds directly to the ELMO/DOCK1 GEF module might relay an extracellular signal to the cytoskeleton during fusion in vertebrates. We carried out a yeast two-hybrid screen to identify proteins that interact with ELMO1 and identified two potential candidate transmembrane receptors. One of them is the previously reported ELMO-interacting BAI1 GPCR (35), and the other was the related receptor BAI3 (Fig. 1*A*). Identity conservation between individual BAI protein family members is ∼40% and largely restricted to the TSRs, GAIN domain, seven transmembrane domains, and a short C-terminal region predicted to be α-helical (38). Notably, BAI1 is characterized by five TSRs, whereas BAI2/3 have only four and additionally lack an Arg-Gly-Asp (RGD) motif found exclusively on BAI1 (Fig. 1*A*). Our yeast two-hybrid screen suggests that ELMO1-interacting domains of BAI proteins are in the conserved predicted α-helical region at the C terminus (Fig. 1*A* and Fig. S1*A*).

Semiquantitative RT-PCR assays were performed to establish which DOCK, ELMO, and BAI members are expressed in C2C12, a myoblast differentiation and fusion model cell line. In these, we detected constant expression levels of DOCK1 and DOCK4 mRNAs, whereas DOCK5 mRNA levels increased upon differentiation (Fig. 1*B* and Fig. S1*B*). ELMO2, BAI2, and BAI3 transcripts were also readily detected in C2C12 cells (Fig. 1*B*). ELMO1 expression levels were apparently lower, whereas DOCK2-3, ELMO3, and BAI1 transcripts were below the limit of detection in this assay (Fig. 1*B* and Fig. S1*B*). These data suggest that BAI2 and BAI3 could be transmembrane proteins regulating myoblast fusion in vertebrates.

We next validated the interaction of ELMO1 with BAI3 in independent yeast two-hybrid assays. Full length ELMO1 and its N-terminal half (amino acids 1–495), but not the C terminus (amino acids 532–727), interacted with amino acids 1400–1522 of BAI3 (Fig. 1*C*), similar to the characterized ELMO1–BAI1 interaction (35). The C termini of BAI1, BAI2, and BAI3 precipitated Myc–ELMO1 in a GST pull-down assay suggesting that ELMO binding is a conserved feature of BAI receptors (Fig. 1*D*). Using a series of GST-BAI3 C terminus fusion proteins that contained BAI3 amino acids 1399–1522, 1399–1513, and 1399–1458, we identified a minimal BAI3 region required for ELMO binding (Fig. 1*E*). Furthermore, GST BAI3 was found to interact with ELMO1, ELMO2, and ELMO3, highlighting that binding to BAI receptors is a conserved feature of the ELMO family of proteins (Fig. 1*F*).

Next, a panel of ELMO1 and ELMO2 mutants was used to identify a minimal region responsible for binding to BAI3. In yeast two-hybrid interaction assays, specific N-terminal and C-terminal fragments of ELMO1 (amino acids 1–113, 1–212, 1–315, and 315–727) failed to bind BAI3 (Fig. S2*A*). Furthermore, internal deletions in full length ELMO1, namely those deleting

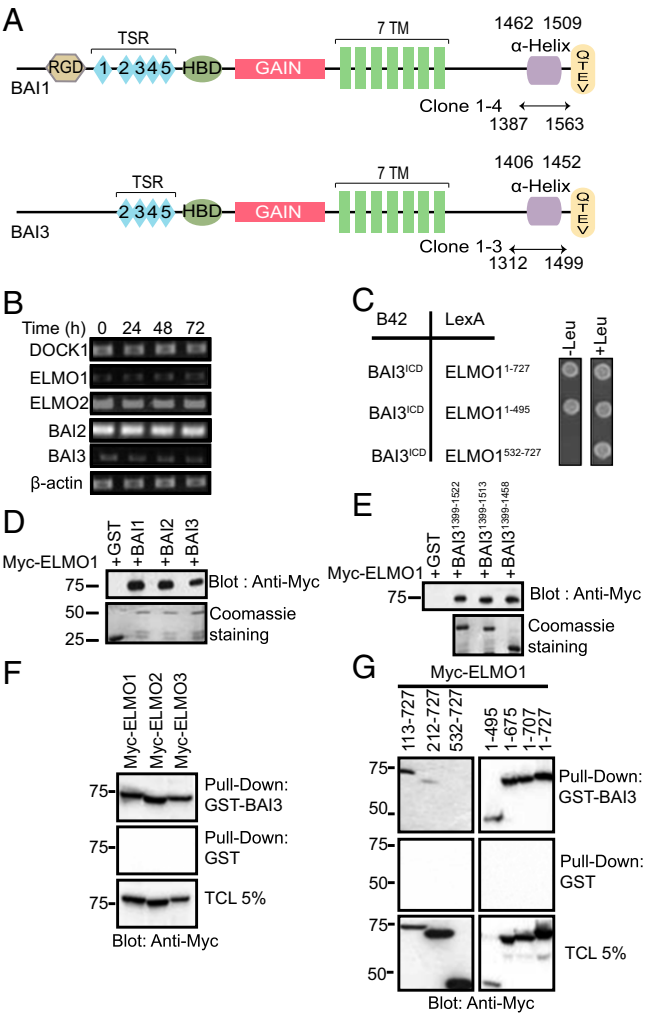

Fig. 1. The GPCR BAI3 is expressed in myoblasts and interacts with the ELMO family of proteins. (*A*) Schematic representation of mBAI1 and mBAI3 receptors isolated as ELMO-binding partners in a yeast two-hybrid screen. Four identical BAI1 clones and three identical BAI3 clones were recovered from the screen, and the binding regions are denoted in the schematics. Important structural domains are shown [hormone-binding domain (HBD); transmembrane domains (TM); predicted α-helix (α-Helix) (Fig. S1*A*); binding sequence for PDZ domains (Gln-Thr-Glu-Val, QTEV)]. (*B*) Expression of DOCK1, ELMO1, ELMO2, BAI2, and BAI3 mRNAs in differentiating C2C12 myoblasts (0–72 h) was measured by semiquantitative RT-PCR. (*C*) The N terminus of ELMO1 is required for BAI3 binding. Yeasts were cotransformed with the indicated LexA fusion ELMO constructs and a B42 fusion of BAI3 construct ICD and were grown on selective (−leu) and nonselective plates (+leu) to assay for protein–protein interaction. (*D*) The C termini of BAI1, BAI2, and BAI3 interact with ELMO1. HEK293 cells were transfected with Myc–ELMO1, and cleared lysate was subjected to GST pull-downs with the indicated GST–BAI fusion proteins or GST alone. Bound Myc–ELMO1 was detected by immunoblotting. (*E*) Identification of the minimal BAI3 region sufficient for interaction with ELMO1. Cleared lysates as in *D* were subjected to pull-down assays with the indicated GST–BAI3 fragments. Bound Myc–ELMO1 was detected by immunoblotting. (*F*) The BAI3 C terminus binds ELMO1–3. HEK293 cells were transfected with Myc–ELMO1, Myc–ELMO2, or Myc–ELMO3, and clarified lysates were subjected to GST–BAI3 pull downs. Bound Myc–ELMO1–3 were detected by immunoblotting. (*G*) Mapping the BAI3-binding site on ELMO1. HEK293 cells were transfected with a panel of Myc–ELMO1 constructs, and clarified lysates were subjected to GST–BAI3 pull downs. Bound Myc–ELMO1 fragments were detected by immunoblotting. TCL, Total Cell Lysate.

(Δ) amino acids 114–524, 213–524, or 310–492, also abolished the interaction between ELMO1 and BAI3 (Fig. S2*A*). Finally,

recombinant GST–BAI3 was able to precipitate Myc–ELMO1 N- and C-terminal deletion mutants encompassing amino acids 113–727, 212–727, 1–495, 1–675, and 1–707 and Myc–ELMO2 mutants 1–534 and 1–699 (Fig. 1*G* and Fig. S2*B*). Collectively, these data establish that BAI2/BAI3 are ELMO-binding transmembrane proteins expressed in myoblasts.

BAI3 and ELMO1/2 Are Essential for Myoblast Fusion. To directly test the requirement for BAI2, BAI3, and ELMO1/2 in myoblast fusion, we generated a loss of function of these proteins by expressing shRNAs from the pSIREN–RetroQ retroviral vector that also coexpresses the ZsGreen GFP protein. Stable C2C12 cell lines expressing ZsGreen GFP and shRNAs specifically targeting murine ELMO1, ELMO2, BAI1, BAI2, or BAI3 were generated by retroviral infections, and cells expressing high levels of GFP were selected via fluorescence-activated cell sorting and switched to differentiation conditions. Real-time quantitative (Q)-PCR analyses confirmed the decrease in BAI3 (95%), BAI2 (74%), ELMO1 (82%), and ELMO2 (92%) mRNAs in their respective knockdown conditions (Fig. 2 *B* and *C* and Figs. S3 and S4). Multiple and independent shRNAs targeting ELMO1, ELMO2, and BAI3 robustly blocked myoblast fusion, and quantification of fusion from multiple microscopic images revealed that most MHC-positive C2C12 cells in BAI3, ELMO1, and ELMO2 knockdown conditions were mononucleated (Fig. 2 *A–D* and Fig. S3). These knockdowns were specific such that shRNAs against ELMO1 or ELMO2 only affected their target mRNA and did not knockdown the other gene (Fig. 2 *G–L*). In contrast, expression of an empty vector or shRNAs targeting BAI1 or BAI2 had no impact on myoblast fusion (Fig. 2 *A–D* and Figs. S3 and S4). We found in some experiments that knockdown with one shRNA against BAI3 affected the number of MHC-positive cells appearing during differentiation in comparison with cells expressing shGFP (Fig. 2*A*). However, this was not observed with an independent shRNA sequence that targets BAI3 and blocks fusion (Fig. S3). To rule out a major contribution of BAI3 or ELMO2 to myoblast differentiation, we monitored the expression of Myogenin, Troponin-T, MHC, and MyoD during differentiation and found that this process was not affected by depletion of either BAI3 or ELMO2, excluding a central role for these proteins in myoblast differentiation (Fig. S5). In addition, myoblast fusion was also impaired when BAI3 or ELMO2, but not BAI1, were down-regulated by shRNAs in the Sol8 myoblast cell line (Fig. S6).

To confirm the specificity of the knockdown phenotypes, we carried out rescue experiments in stable C2C12 cell lines expressing BAI3, ELMO1, and ELMO2 shRNAs (Fig. 2 *E*, *G*, and *J*). The expression of murine Myc–ELMO1 in ELMO2 shRNA cells and of murine Myc–ELMO2 in ELMO1 shRNA cells restored myoblast fusion (Fig. 2 *G–L*). These data suggest that ELMO1/2 carry out similar functions during fusion and that a threshold level of their expression is critical for efficient fusion. Likewise, expression of Flag–hBAI3 C terminus fusion to mVenus (Flag–BAI3) rescued the myoblast fusion defect in cells in which endogenous BAI3 is depleted (Fig. 2 *E* and *F*). Collectively, these results establish BAI3 and ELMO1/2 as essential effectors of vertebrate myoblast fusion.

Lack of Myoblast Fusion Functional Redundancy of BAI Proteins. BAI3 and BAI2 proteins differ significantly from BAI1 in their extracellular domains (Fig. 1*A*). To address the significance of this divergence, we tested whether BAI1 can functionally substitute for BAI3 in myoblast fusion. To do this, we assayed myoblast fusion in C2C12 cells in which BAI3 protein levels were reduced but which also expressed a BAI1–eGFP fusion protein. In such cells, myoblast fusion remained severely reduced, indicating

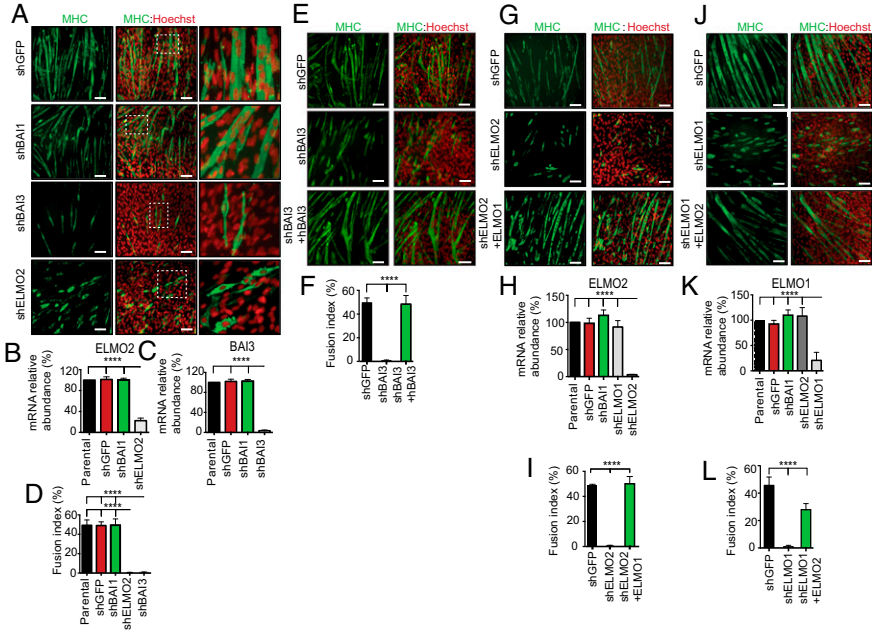

Fig. 2. BAI3, ELMO1, and ELMO2 are essential for myoblast fusion. (*A–L*) C2C12 cells expressing GFP (no hairpin) or shRNAs targeting BAI1, BAI3, ELMO1, or ELMO2 were generated by retroinfections. (*A*) Down-regulation of BAI3 and ELMO2 impairs myoblast fusion after 48 h in differentiation conditions. (*Right*) Dotted white boxes are shown at a higher magnification. (*B–C*) Real-time Q-RT-PCR amplifications against BAI3 or ELMO2 were performed to confirm specific knockdowns. (*D*) Quantification of experiments shown in *A*. (*E–L*) Expression of hBAI3 in BAI3–shRNA, Myc–ELMO1 in ELMO2–shRNA, and Myc–ELMO2 in ELMO1–shRNA C2C12 cells restored myoblast fusion. (*E*, *G*, *J*) Representative images of myoblast fusion for the indicated conditions. (*F*, *I*, *L*) Quantification of experiments shown in *E*, *G*, and *J*. (*H*, *K*) Real-time Q-RT-PCR amplifications against ELMO1 or ELMO2 were performed to confirm specific knockdowns. (*A*, *E*, *G*, and *J*) Myofibers were stained for Myosin Heavy Chain [MHC, MF20 antibody (red)] and nuclei revealed by Hoechst (blue). Error bars indicate SD. One-way ANOVA followed by a Bonferroni test calculated the *P* values; ****$P < 0.0001$. (Scale bar, 100 μm.)

that BAI1 cannot functionally substitute for BAI3 and suggesting that the structural basis for their functional divergence might reside in their extracellular domains (Fig. S7).

Expression of BAI3 Promotes Myoblast Fusion. Expression level or activity of BAI3 might be a limiting factor for myoblast fusion. To investigate this, we tested whether overexpression of Flag–BAI3 in the parental C2C12 cell line could promote myoblast fusion and found that expression of BAI3 potentiated myoblast fusion in comparison with control cells (Fig. S8). We also tested whether BAI3 could further potentiate myoblast fusion when coexpressed with Myc–ELMO2 and Flag–DOCK1 or if overexpression of ELMO2 and DOCK1 alone was sufficient to enhance fusion. Coexpression of BAI3 alongside either ELMO2 or ELMO2/DOCK1 did not further enhance fusion in comparison with cells expressing BAI3 alone (Fig. S8). Finally, expression of ELMO2/DOCK1 in C2C12 was not sufficient to enhance cell–cell fusion (Fig. S8). Although loss-of-function of BAI2 suggested that this receptor might not be involved in myoblast fusion, we noted that its knockdown was not as efficient as the one of BAI3 (Fig. S4 *A* and *B*). We therefore tested if the myoblast fusion promoting activity of BAI3 was also present in BAI2. However, overexpression of Flag–BAI2 failed to promote myoblast fusion (Fig. S4 *E* and *F*).

Binding of ELMO to BAI3 Is Essential for Myoblast Fusion. To investigate if physical coupling between BAI3 and ELMO is required for myoblast fusion, we next asked whether BAI proteins with mutations in the ELMO-interacting domain could functionally rescue the loss of BAI3 function. We derived a panel of GST–BAI3 proteins with mutations in the ELMO-binding site, which did not interact with ELMO1 in pull-down assays (Fig. 3*A* and Fig. S1*A*). We noted that similar mutations in BAI2 also abrogated ELMO binding (Fig. S4*D*). Two of these mutations were then introduced into the full length Flag–BAI3 fusion protein, and the ability of such mutants to localize to the cell surface was confirmed by staining nonpermeabilized myoblasts with an anti-Flag antibody (Fig. S9). Although expression of the wild-type Flag–BAI3 robustly rescued the fusion defects caused by the loss of endogenous BAI3, the ELMO1 binding-deficient Flag–BAI3$^{RKR/EEE}$ and Flag–BAI3$^{LDF/HHR}$ mutant proteins failed to restore myoblast fusion (Fig. 3 *B–D*). Such BAI3 mutants were also able to completely block myoblast fusion when expressed in the parental C2C12 cells with normal endogenous levels of BAI3, suggesting that they might be acting as dominant negative variants of BAI3 (Fig. 3 *E–F*). In addition, this result suggests that functional BAI3 is required on both fusion partners because cells expressing dominant-negative BAI3 constructs fail to fuse with neighboring wild-type cells (Fig. 3*E*). Together, these data demonstrate that BAI3–ELMO coupling is an essential signaling step for primary myoblast fusion.

BAI3 Is Essential in Vivo for Myoblast Fusion. Previous studies have taken advantage of the accessibility of the in ovo development of chicken embryos to manipulate in vivo the function of genes involved in myogenesis (39). Myoblasts actively populate limb buds at Hamburger–Hamilton (HH) stage 18 and undergo fusion at HH stage 20 (40, 41). Using in situ hybridization of antisense digoxigenin (DIG)-labeled riboprobes we found that during these stages, BAI3, but not BAI1 and BAI2, mRNA are coexpressed by muscle precursors that also express the myocyte differentiation marker MyoD (Fig. 4*A* and Fig. S10). In agreement with published data (42), we also found BAI1, BAI2, and BAI3 mRNAs in the developing spinal cord (Fig. 4*A* and Fig. S10).

To test the function of BAI3 in myoblast fusion in vivo, we used in ovo electroporation to express BAI3 proteins in developing myoblasts, as previously described (39). GFP-encoding expression plasmid or human Flag–BAI3–mVenus protein or

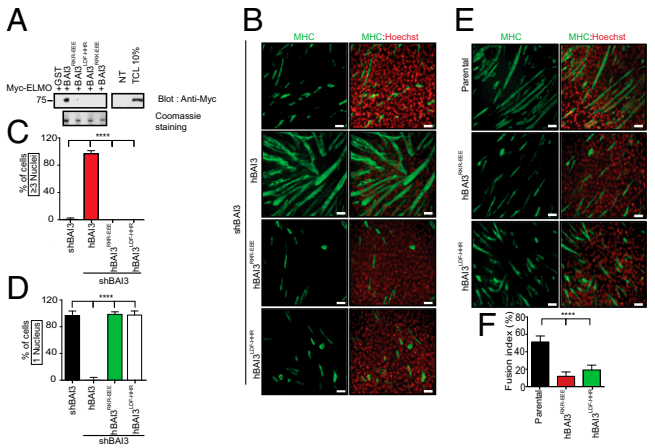

Fig. 3. BAI3 coupling to ELMO2 is necessary for myoblast fusion. (*A*) Identification of BAI3 residues important for interaction to ELMO1. HEK293 cells were transfected with Myc–ELMO1 and clarified lysates were subjected to pull-downs with GST alone, GST–BAI3, or the indicated mutants of BAI3. Bound Myc–ELMO1 was detected by immunoblotting. (*B*) Expression of BAI3 mutants unable to engage ELMO fails to rescue myoblast fusion in C2C12 cells depleted of BAI3. Experiments were carried out as in Fig. 2. (*C*) Quantification of fibers with three nuclei and more and (*D*) quantification of fibers with one nucleus. (*E*) Overexpression of BAI3 mutants lacking ELMO-binding activity blocks myoblast fusion in parental C2C12 cells. Cells were transfected with the indicated BAI3 mutants and differentiated for 48 h before analyzing fusion. (*F*) Quantification of the experiment shown in *E*. Error bars indicate SD. One-way ANOVA followed by a Bonferroni test calculated the *P* values; ****$P < 0.0001$. (Scale bar, 100 μm.)

human Flag–BAI3$^{RKR/EEE}$ mutant-encoding expression plasmids were microinjected into the somitocoele of developing interlimb somites of HH stage 12–17 embryos, and a current was applied (Fig. 4*B*). Seventy-two hours later, at a time when myoblast fusion produces many multinucleated fibers, embryos were fixed, sectioned, and stained with anti-Desmin and anti-MHC antibodies to assay for differentiation and myoblast fusion. This transfection approach was highly efficient, as most cells expressed GFP. None of the expression constructs impaired myoblast differentiation because GFP+ cells expressed apparently normal levels of Desmin and MHC under all conditions (Fig. 4 *C* and *D*). Expression of wild-type BAI3 had no impact on muscle development, and multinucleated muscle fibers could be detected with similar frequencies to that in GFP-expressing fibers (Fig. 4 *E* and *F*). In marked contrast, expression of the ELMO1 interaction domain mutant BAI3$^{RKR/EEE}$ blocked myoblast fusion such that only mononucleated MHC-expressing muscle fibers were observed (Fig. 4 *E* and *F*). To confirm that we were observing mononucleated cells and not an artifact of the sectioning plane, we performed 3D reconstructions of whole-mount developing limb muscles expressing either GFP or BAI3$^{RKR/EEE}$ (Movie S1). Although the global signal was weaker than on sections due imaging of native GFP fluorescence, we observed long GFP+ fibers in GFP control muscles, whereas only individual mVenus-positive cells were observed in muscles expressing BAI3$^{RKR/EEE}$ (Movie S1). These results indicate that BAI3 functions in vivo as a promoter of myoblast fusion by coupling the cell membrane to the ELMO/DOCK1 pathway.

Discussion

The nature of the transmembrane proteins initiating myoblast fusion in vertebrates has thus far remained elusive. Intracellular signaling molecules such as DOCK1 and Rac1 play a conserved role in primary myoblast fusion in both flies and mice, yet there is little evidence to suggest that *Drosophila* myoblast profusion

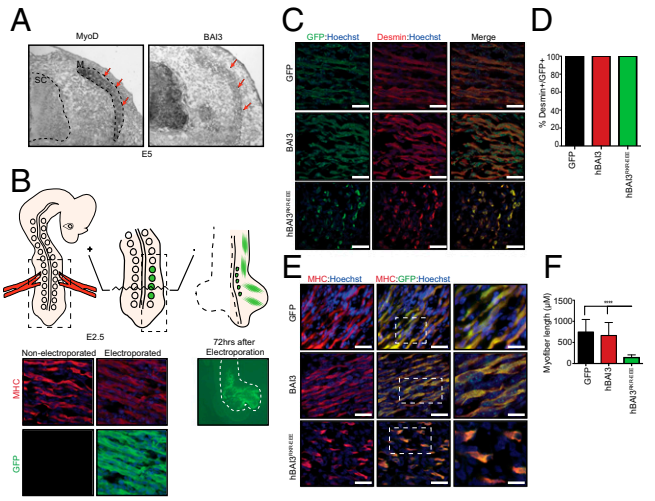

Fig. 4. The interaction of BAI3 with ELMO is essential in vivo for myoblast fusion. (*A*) In situ hybridization of antisense DIG-labeled riboprobes demonstrates that BAI3 (*Right*) is coexpressed by muscle precursors that also express the myocyte differentiation marker MyoD (*Left*) in the developing muscles of E5 chicken embryos. BAI3 is also expressed in the spinal cord. SC, spinal cord; M, muscle. (*B*) Schematic of the strategy to express constructs in muscle progenitors. Somitocoeles of interlimb somites of embryos between 13 and 18 HH (E2.5) were microinjected with plasmids and electroporated as indicated. Seventy-two hours after electroporation, embryos expressing GFP were collected and analyzed for myoblast fusion. Identical muscle development takes place in nonelectroporated and electroporated (GFP plasmid) sides of the embryo as demonstrated by staining for MHC and GFP. (*C*) Differentiation of myoblasts is not impaired by expression of GFP, BAI3, or the indicated mutant of BAI3 lacking ELMO-binding activity. Cryosections were stained with anti-Desmin and anti-GFP antibodies. (*D*) Quantification of cells double positive for Desmin and GFP. (*E*) Expression of BAI3 lacking ELMO-binding activity, but not GFP or wild-type BAI3, blocks myoblast fusion in vivo. Cryosections as in *C* were stained with anti-MHC and anti-GFP antibodies, and nuclei were revealed with Hoechst. (*F*) Length of myofibers was quantified (μM) in the indicated conditions. Error bars indicate SD. One-way ANOVA followed by a Bonferroni test calculated the *P* values; ****$P < 0.0001$.

receptors regulate primary fusion in vertebrates (22, 23). In this study, we aimed to identify such cell surface receptors and link them to key components of the myoblast fusion pathway, namely, ELMO–DOCK1–Rac. We describe here the finding that the GPCR BAI3 binds ELMO proteins in myoblasts and that this interaction is obligatory for myoblast fusion in cell lines. Using in ovo chicken embryo somite electroporation we expressed a loss-of-function mutant of BAI3 and observed a robust block in myoblast fusion in vivo. To our knowledge, this work is a previously unidentified documentation of a transmembrane protein that engages the conserved ELMO–DOCK1–Rac pathway to promote primary myoblast fusion.

Another BAI-family member, BAI1, was recently reported to promote myoblast fusion in the context of muscle cells undergoing apoptosis (33). Remarkably, the dying myoblasts are not engulfed but instead enhance myoblast fusion in a BAI1–ELMO-dependent manner. However, although BAI1-null animals exhibit a reduction in their ability to repair injured muscle, they do not display major defects in muscle development. These results argue for a specialized function of BAI1 in muscle regeneration and an indirect role in fusion (33, 43). Although our work does not rule out that BAI3 could also contribute to muscle regeneration, it highlights the unique ability of this GPCR to mediate primary myoblast fusion during embryonic development. Both BAI3 and BAI1 (33) depend on their common ability to bind ELMO–DOCK1 to play their respective functions during embryonic myogenesis and adult muscle regeneration. We were unable to rescue myoblast fusion defects in cells deficient in BAI3 via overexpression of BAI1. The numbers of TSRs in BAI1 (5) and BAI3 (4), in addition to an integrin-binding R-G-D motif found exclusively in BAI1, are distinctive features of these receptors (Fig. 1*A*). Our data therefore suggest that the extracellular domain of BAI3 contains a unique function in the context of myoblast fusion. Furthermore, our knockdown and overexpression experiments suggest that BAI2 is unessential for myoblast fusion and mouse null for BAI2 is viable and no defects in muscles were reported (44). In this study, we uncovered a missing link in the understanding of vertebrate myogenesis by defining a previously unidentified myoblast profusion receptor essential during primary embryonic myoblast fusion in vivo.

Our results suggest that functional BAI3 is required on both fusion partners. This is similar to additional vertebrate myoblast fusion components studied in vivo including DOCK1, Rac1, CDC42, and *N*-WASP (22–24). In contrast, the recently identified fusogenic transmembrane protein Myomaker is required only in one of the two fusion partners, and its forced expression in fibroblasts is sufficient to induce fibroblast–myoblast fusion (27). As Myomaker-induced myoblast fusion is also dependent on the actin cytoskeleton, it will be interesting to investigate whether the BAI3–ELMO–DOCK1–Rac1 pathway can crosstalk with Myomaker to promote fusion. We also found that ELMO1 and ELMO2 function similarly in myoblast fusion as loss-of-function of individual components revealed similar phenotypes. In addition, the loss-of-functions could be rescued with expression of the reciprocal ELMO proteins. Collectively, these data suggest that a threshold level of total ELMO1/2 is required for efficient myoblast fusion most likely to efficiently scaffold the recruitment of DOCK1 to BAI3 to orchestrate Rac signaling. Interestingly, ELMO1 mice are viable, and an up-regulation of ELMO2 in these animals was observed (45). Generating ELMO2-null mice will test if both genes must be inactivated in myoblasts to interfere with myoblast fusion.

Based on *Drosophila* and zebrafish findings (2, 25), we expected that the vertebrate transmembrane proteins coupling to ELMO–DOCK1–Rac would be similar in structure to canonical cell adhesion proteins. We were surprised to uncover such a profound role in primary myoblast fusion for a member of the large family of GPCRs. Recently, Pavlath and colleagues reported that several GPCRs are expressed in myoblasts (46). In particular, the mouse Odorant Receptor 23 is critical for myoblast migration in vitro and is important for muscle regeneration in vivo (46). These findings suggest that GPCRs could have multiple functions during myogenesis such as controlling migration, myoblast–myoblast or myoblast–myotube adhesion, and myoblast fusion. Our data suggest that BAI3–ELMO signaling is operating specifically at the step of myoblast fusion. In myoblast cell lines, depletion of BAI3 or ELMO1/2 did not interfere with cell differentiation. In vivo, we observed that myoblasts expressing BAI3 uncoupled from ELMO still migrated efficiently in the limb buds of chicken embryos. This is consistent with previous findings demonstrating that myoblasts lacking DOCK1 or Rac1 have normal migratory behaviors in vivo in mice (22, 23).

Material and Methods

Antibodies, plasmids, yeast two-hybrid assays, generation of shRNA-stable C2C12 cell lines, cell culture and transfections, mRNA isolation and Q-RT-PCR, protein-binding assays, in situ hybridization, immunofluorescence, immunohistochemistry, in ovo electroporation, and statistical analyses are described in the *SI Material and Methods*.

Differentiation of C2C12 and Sol8 myoblasts was induced by replacing the growth medium with differentiation medium [DMEM supplemented with 2% (vol/vol) horse serum and penicillin/streptomycin]. Cells were allowed to differentiate for 0–48 h, or up to 96 h for some experiments, before analyses including biochemical and immunostaining. To quantify myoblast fusion, MHC-positive cells with three nuclei or more were considered as multinucleated myofibers, and the fusion index was calculated by

dividing the numbers of nuclei in multinucleated fibers by the total number of nuclei for each described condition. In chicken embryos, the difference between the tested conditions was assessed by measurement of the fiber length (micrometers) using the Volocity image analysis software (Perkin–Elmer).

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1. Bentzinger CF, Wang YX, Rudnicki MA (2012) Building muscle: Molecular regulation of myogenesis. *Cold Spring Harb Perspect Biol* 4(2):1–16.
2. Abmayr SM, Pavlath GK (2012) Myoblast fusion: Lessons from flies and mice. *Development* 139(4):641–656.
3. Powell GT, Wright GJ (2012) Do muscle founder cells exist in vertebrates? *Trends Cell Biol* 22(8):391–396.
4. Ruiz-Gómez M, Coutts N, Price A, Taylor MV, Bate M (2000) Drosophila dumbfounded: A myoblast attractant essential for fusion. *Cell* 102(2):189–198.
5. Strünkelnberg M, et al. (2001) rst and its paralogue kirre act redundantly during embryonic muscle development in Drosophila. *Development* 128(21):4229–4239.
6. Bour BA, Chakravarti M, West JM, Abmayr SM (2000) Drosophila SNS, a member of the immunoglobulin superfamily that is essential for myoblast fusion. *Genes Dev* 14(12):1498–1511.
7. Artero RD, Castanon I, Baylies MK (2001) The immunoglobulin-like protein Hibris functions as a dose-dependent regulator of myoblast fusion and is differentially controlled by Ras and Notch signaling. *Development* 128(21):4251–4264.
8. Geisbrecht ER, et al. (2008) Drosophila ELMO/CED-12 interacts with Myoblast city to direct myoblast fusion and ommatidial organization. *Dev Biol* 314(1):137–149.
9. Doberstein SK, Fetter RD, Mehta AY, Goodman CS (1997) Genetic analysis of myoblast fusion: Blown fuse is required for progression beyond the prefusion complex. *J Cell Biol* 136(6):1249–1261.
10. Chen EH, Olson EN (2001) Antisocial, an intracellular adaptor protein, is required for myoblast fusion in Drosophila. *Dev Cell* 1(5):705–715.
11. Duan R, et al. (2012) Group I PAKs function downstream of Rac to promote podosome invasion during myoblast fusion in vivo. *J Cell Biol* 199(1):169–185.
12. Rushton E, Drysdale R, Abmayr SM, Michelson AM, Bate M (1995) Mutations in a novel gene, myoblast city, provide evidence in support of the founder cell hypothesis for Drosophila muscle development. *Development* 121(7):1979–1988.
13. Chen EH, Pryce BA, Tzeng JA, Gonzalez GA, Olson EN (2003) Control of myoblast fusion by a guanine nucleotide exchange factor, loner, and its effector ARF6. *Cell* 114(6):751–762.
14. Hakeda-Suzuki S, et al. (2002) Rac function and regulation during Drosophila development. *Nature* 416(6879):438–442.
15. Schröter RH, et al. (2004) kette and blown fuse interact genetically during the second fusion step of myogenesis in Drosophila. *Development* 131(18):4501–4509.
16. Richardson BE, Beckett K, Nowak SJ, Baylies MK (2007) SCAR/WAVE and Arp2/3 are crucial for cytoskeletal remodeling at the site of myoblast fusion. *Development* 134(24):4357–4367.
17. Schäfer G, et al. (2007) The Wiskott-Aldrich syndrome protein (WASP) is essential for myoblast fusion in Drosophila. *Dev Biol* 304(2):664–674.
18. Massarwa R, Carmon S, Shilo BZ, Schejter ED (2007) WIP/WASp-based actin-polymerization machinery is essential for myoblast fusion in Drosophila. *Dev Cell* 12(4):557–569.
19. Kim S, et al. (2007) A critical function for the actin cytoskeleton in targeted exocytosis of prefusion vesicles during myoblast fusion. *Dev Cell* 12(4):571–586.
20. Sens KL, et al. (2010) An invasive podosome-like structure promotes fusion pore formation during myoblast fusion. *J Cell Biol* 191(5):1013–1027.
21. Haralalka S, et al. (2011) Asymmetric Mbc, active Rac1 and F-actin foci in the fusion-competent myoblasts during myoblast fusion in Drosophila. *Development* 138(8):1551–1562.
22. Laurin M, et al. (2008) The atypical Rac activator Dock180 (Dock1) regulates myoblast fusion in vivo. *Proc Natl Acad Sci USA* 105(40):15446–15451.
23. Vasyutina E, Martarelli B, Brakebusch C, Wende H, Birchmeier C (2009) The small G-proteins Rac1 and Cdc42 are essential for myoblast fusion in the mouse. *Proc Natl Acad Sci USA* 106(22):8935–8940.
24. Gruenbaum-Cohen Y, et al. (2012) The actin regulator N-WASp is required for muscle-cell fusion in mice. *Proc Natl Acad Sci USA* 109(28):11211–11216.
25. Sohn RL, et al. (2009) A role for nephrin, a renal protein, in vertebrate skeletal muscle cell fusion. *Proc Natl Acad Sci USA* 106(23):9274–9279.
26. Donoviel DB, et al. (2001) Proteinuria and perinatal lethality in mice lacking NEPH1, a novel protein with homology to NEPHRIN. *Mol Cell Biol* 21(14):4829–4836.
27. Millay DP, et al. (2013) Myomaker is a membrane activator of myoblast fusion and muscle formation. *Nature* 499(7458):301–305.
28. Schwander M, et al. (2003) Beta1 integrins regulate myoblast fusion and sarcomere assembly. *Dev Cell* 4(5):673–685.
29. Hollnagel A, Grund C, Franke WW, Arnold HH (2002) The cell adhesion molecule M-cadherin is not essential for muscle development and regeneration. *Mol Cell Biol* 22(13):4760–4770.
30. Charlton CA, Mohler WA, Radice GL, Hynes RO, Blau HM (1997) Fusion competence of myoblasts rendered genetically null for N-cadherin in culture. *J Cell Biol* 138(2):331–336.
31. Cole F, Zhang W, Geyra A, Kang JS, Krauss RS (2004) Positive regulation of myogenic bHLH factors and skeletal muscle development by the cell surface receptor CDO. *Dev Cell* 7(6):843–854.
32. Conti FJ, Monkley SJ, Wood MR, Critchley DR, Müller U (2009) Talin 1 and 2 are required for myoblast fusion, sarcomere assembly and the maintenance of myotendinous junctions. *Development* 136(21):3597–3606.
33. Hochreiter-Hufford AE, et al. (2013) Phosphatidylserine receptor BAI1 and apoptotic cells as new promoters of myoblast fusion. *Nature* 497(7448):263–267.
34. Bolliger MF, Martinelli DC, Südhof TC (2011) The cell-adhesion G protein-coupled receptor BAI3 is a high-affinity receptor for C1q-like proteins. *Proc Natl Acad Sci USA* 108(6):2534–2539.
35. Park D, et al. (2007) BAI1 is an engulfment receptor for apoptotic cells upstream of the ELMO/Dock180/Rac module. *Nature* 450(7168):430–434.
36. Araç D, et al. (2012) A novel evolutionarily conserved domain of cell-adhesion GPCRs mediates autoproteolysis. *EMBO J* 31(6):1364–1378.
37. Kaur B, Brat DJ, Devi NS, Van Meir EG (2005) Vasculostatin, a proteolytic fragment of brain angiogenesis inhibitor 1, is an antiangiogenic and antitumorigenic factor. *Oncogene* 24(22):3632–3642.
38. Cork SM, Van Meir EG (2011) Emerging roles for the BAI1 protein family in the regulation of phagocytosis, synaptogenesis, neurovasculature, and tumor development. *J Mol Med (Berl)* 89(8):743–752.
39. Scaal M, Gros J, Lesbros C, Marcelle C (2004) In ovo electroporation of avian somites. *Dev Dyn* 229(3):643–650.
40. Hamburger V, Hamilton HL (1992) A series of normal stages in the development of the chick embryo. 1951. *Dev Dyn* 195(4):231–272.
41. Mok GF, Sweetman D (2011) Many routes to the same destination: Lessons from skeletal muscle development. *Reproduction* 141(3):301–312.
42. Kee HJ, et al. (2004) Expression of brain-specific angiogenesis inhibitor 3 (BAI3) in normal brain and implications for BAI3 in ischemia-induced brain angiogenesis and malignant glioma. *FEBS Lett* 569(1-3):307–316.
43. Yu SF, Baylies MK (2013) Cell biology: Death brings new life to muscle. *Nature* 497(7448):196–197.
44. Okajima D, Kudo G, Yokota H (2011) Antidepressant-like behavior in brain-specific angiogenesis inhibitor 2-deficient mice. *J Physiol Sci* 61(1):47–54.
45. Elliott MR, et al. (2010) Unexpected requirement for ELMO1 in clearance of apoptotic germ cells in vivo. *Nature* 467(7313):333–337.
46. Griffin CA, Kafadar KA, Pavlath GK (2009) MOR23 promotes muscle regeneration and regulates cell adhesion and migration. *Dev Cell* 17(5):649–661.