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N-CADHERIN/P120 CATENIN ASSOCIATION AT CELL-CELL CONTACTS OCCURS IN CHOLESTEROL-RICH MEMBRANE DOMAINS AND IS REQUIRED FOR RHOA ACTIVATION AND MYOGENESIS

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p120 catenin is a major regulator of cadherin stability at cell-cell contacts and a modulator of Rho GTPase activities. In C2C12 myoblasts, N-cadherin is stabilized at cell contacts through its association with cholesterol-rich membrane domains or lipid rafts (LR) and acts as an adhesion-activated receptor which activates RhoA, an event required for myogenesis induction. Here, we report that association of p120 catenin with N-cadherin at cell contacts occurs specifically in LR. We demonstrate that interaction of p120 catenin with N-cadherin is required for N-cadherin association with LR and for its stabilization at cell contacts. LR disruption inhibits myogenesis induction and N-cadherin-dependent RhoA activation as does the perturbation of the N-cadherin/p120 catenin complex after *p120 catenin* knockdown. Finally, we observe a N-cadherin-dependent accumulation of RhoA at PI(4,5)P₂-enriched cell contacts which is lost after LR disruption.

Thus, a functional N-cadherin/catenins complex occurs in cholesterol-rich membrane microdomains that allows the recruitment of RhoA and the regulation of its activity during myogenesis induction.

Skeletal myogenesis is a multistep process regulated by diffusible molecules and the interaction of muscle cell precursors with their neighbors and the extracellular matrix (1,2). Particularly, N-cadherin-dependent inter-cellular

adhesion has a major role in cell cycle exit and induction of skeletal muscle differentiation through activation of the Rho-family GTPases. RhoA positively regulates MyoD expression and skeletal muscle differentiation, as it is required for serum response factor-mediated activation of several muscle-specific gene promoters (3,4).

Dynamic association of cadherin complexes at the plasma membrane (PM) is crucial for cadherin-mediated signaling. Their extracellular domain mediates homophilic cell-cell adhesion, whereas the intracellular domain associates with catenins which produce attachment sites for the F-actin cytoskeleton (5-7). The juxtamembrane domain of cadherin cytoplasmic tail binds to p120 catenin which regulates cadherin stability at cell contacts and modulates Rho GTPase activities (8-11). Cadherin stability is directly dependent on p120 catenin and in its absence most cadherins are internalized and often degraded, suggesting that p120 catenin controls cadherin turnover at the cell surface (11,12). Moreover, mutations in the E-cadherin region that binds to p120 catenin dissociate the E-cadherin-p120 catenin complex and disrupt strong cell adhesion, although interaction with other catenins remains intact (13). Cadherin stability at cell-cell contacts is also regulated by homophilic binding between extracellular domains and association with the F-actin cytoskeleton (14,15). Association of N-cadherin with cholesterol-enriched microdomains, called lipid rafts (LR), at cell contacts, also stabilizes N-cadherin (16). Since p120 catenin interaction with cadherins and N-cadherin association with LR at cell contact sites are both involved in

cadherin stability at cell contact sites, we asked whether p120 catenin association with N-cadherin required LR. We observed that their association occurred specifically in these cholesterol-rich domains. Moreover, using an N-cadherin mutant unable to bind to p120 catenin, we showed that p120 catenin-N-cadherin interaction was required for N-cadherin association with LR and its stabilization at cell contacts. Since N-cadherin is implicated in the commitment to myogenesis through RhoA activation, we questioned whether its association with p120 catenin in LR was a prerequisite for RhoA activation. LR disruption inhibited myogenesis induction, association of p120 catenin with N-cadherin and N-cadherin-dependent RhoA activation as did the perturbation of the N-cadherin/p120 catenin complex after *p120 catenin* knockdown. Together, these data suggest a crucial role for N-cadherin/p120 catenin association in LR in the regulation of RhoA activity during myogenesis induction.

Experimental procedures

Cell lines- C2C12 myoblasts were cultured at 37°C and 5% CO₂ in DMEM/F-12 supplemented with 10% fetal calf serum; mouse L cells in DMEM supplemented with 10% fetal calf serum.

p120 catenin knockdown C2C12 cell lines were obtained after infection with pRS human *p120 catenin* siRNA or mouse *p120 catenin* siRNA retroviruses (11). Different clones were isolated by limited dilution and grown in 3 to 5 µg/ml puromycin.

Drug treatment, transfection and immunocytochemistry- Cells were treated with amphotericin B (ampho B) (25µg/ml for 4 hours; Sigma), methyl-β-cyclodextrin (MCD) (4 mM for 6 hours, Sigma) or Cholesterol Oxidase (1U/ml for 2 hours, Calbiochem) in culture medium containing 10% or 2% delipidized serum (Sigma-Aldrich). C2C12 myoblasts and L cells were transfected with N-cadherin/GFP, N-cadherin/RFP, N-cadherinAAA/YFP, PH-PLCδ/GFP and RhoAWT/RFP by jetPEI (Qbiogen) according to the manufacturer's instructions. Ganglioside GM1 patching was performed as described (16).

Cells were fixed for 5 min in 4% paraformaldehyde (in PBS) followed by a 2-min permeabilization in 0.1% Triton X-100 (in PBS)

and incubation in PBS containing 1% bovine serum albumin (BSA). Primary antibodies were: p120 catenin (1:100, Transduction Laboratories), N-cadherin (1:200, Transduction Laboratories), myogenin (1:30, Santa Cruz Biotechnology), troponin T (1:100, Sigma). Secondary antibodies were Alexa Fluor 350-, Alexa Fluor 546- or Alexa Fluor 488-conjugated goat anti-mouse antibody and Alexa Fluor 488-conjugated goat anti-rabbit antibody (Molecular Probes). Cells were stained for F-actin with tetramethylrhodamine B isothiocyanate (TRITC) or coumarin isothiocyanate (CPITC)-conjugated phalloidin (Sigma-Aldrich) and nuclei were stained with Hoechst (0.1 µg/ml, Sigma-Aldrich). Images were captured with a MicroMax 1300 CCD camera (RS-Princeton Instruments, Trenton, NJ, USA) driven by the MetaMorph Software (v.7, Universal Imaging Corp, Westchester, PA, USA). Images were processed using Adobe Photoshop and Adobe Illustrator.

Isolation of detergent-resistant membrane fractions (DRM) and immunoprecipitation- Cell lysates or plasma membrane-enriched fractions of C2C12 or L cells transfected or not with N-cadherin/GFP or N-cadherinAAA/YFP were fractionated through a 4 ml sucrose gradient as described (16). Fractions enriched in plasma membranes were prepared as described (16).

Pooled fractions 3-5 (LR fractions) and 8-10 (non lipid rafts NLR fractions) or fractions enriched in PM were analyzed by immunoblotting for the presence of N-cadherin, α-, β-, γ- and p120 catenins, caveolin (antibodies from Transduction Laboratories), actin (Sigma-Aldrich) or transferrin receptor (Zymed)). Alternatively, LR and NLR fractions were also used for immunoprecipitation with anti-N-cadherin antibodies. In this case, fractions 3 to 5 were pooled and diluted 5 folds in 25 mM MOPS, pH 6.5, 150 mM NaCl, 1% Triton X-100, and then centrifugated at 4°C at 100000g for 18 hours. Pellets were resuspended in 10 mM Pipes, pH 7, 100 mM NaCl, 300 mM sucrose, 3 mM MgCl₂, 0,5% NP-40, 1 mM EDTA, 1 mM Orthovanadate, 60 mM N-octylglucoside.

Protein concentration was determined with the BCA protein assay kit (Pierce).

C2C12 myoblasts expressing NcadAAA/YFP were lysed and extracts immunoprecipitated using an anti-GFP antibody (1 :100, Roche) and processed as described (17).

RhoA GTPase Activity Assay- Parental or *p120 catenin* shRNA C2C12 myoblasts were lysed

and processed to measure the total and GTP-bound RhoA levels as described previously (18). Alternatively, C2C12 myoblasts plated on Ncad-Fc chimera-coated dishes obtained as described (18) were used.

Fluorescence recovery after photobleaching (FRAP)- Lateral diffusion coefficients (D) and mobile fractions (M) of Ncad/GFP and NcadAAA/YFP expressed in mouse L cells were measured by FRAP using a Zeiss LSM Meta 510 confocal microscope as described (16).

RESULTS

p120 catenin association with N-cadherin occurs in cholesterol-rich membrane domains.

To dissect the mechanisms of catenins association with N-cadherin, we questioned whether the localization in cholesterol-rich membrane microdomains influenced cadherin/catenins complex formation. Therefore we co-immunoprecipitated similar amounts of proteins from either pooled fractions 3-5 (corresponding to LR fractions) or pooled fractions 8-10 (corresponding to Non Lipid Rafts fractions (NLR)) obtained from Triton X-100 lysates loaded onto sucrose density gradients (16). While α -, β - and γ -catenins associated with N-cadherin in both LR and NLR fractions, p120 catenin interacted with N-cadherin only in LR fractions (Figure 1A). In order to confirm that the interaction of p120 catenin with N-cadherin was impossible in NLR fractions, we repeated the experiment but using a ten fold higher amount of proteins from NLR fractions. Again, p120 catenin did not interact with N-cadherin, while α -, β - and γ -catenins strongly associated with N-cadherin under these conditions (Figure 1B, a). We confirmed that N-cadherin, β - and p120 catenin were present in both LR and NLR fractions (Figure 1B, b). To further confirm the co-distribution of N-cadherin and p120 catenin in LR, we used a technique based on lateral cross-linking of the raft-associated ganglioside GM1 (19). Patching of GM1 with the CTX-B subunit and antibodies against CTX-B resulted in co-patching of both N-cadherin and p120 catenin (Figure 1C). Moreover, LR disruption by cholesterol chelation through addition of amphotericin B (ampho B) impaired p120 catenin association with N-cadherin monitored by immunoprecipitation without modifying their concentration (Figure 1D, a and b). This result contrasts with previous data showing that cholesterol oxidase (CO) treatment did not affect

p120 catenin association with N-cadherin, although it perturbed N-cadherin accumulation and stabilization at cell-cell contacts (16). Thus, we carefully re-investigated the effect of three drugs (i.e., ampho B, CO and MCD) which affect cholesterol and confirmed that LR disruption by these compounds efficiently perturbed p120 catenin association with N-cadherin (data not shown). Nevertheless, data from cholesterol depletion experiments must be interpreted with caution since interactions between LR and F-actin cytoskeleton exist and association of N-cadherin with LR requires F-actin cytoskeleton (16). These data suggest that p120 catenin associates with N-cadherin specifically in LR and that LR are essential for p120 catenin/N-cadherin association.

Disruption of p120 catenin binding to N-cadherin impairs N-cadherin association with LR and destabilizes N-cadherin at cell junctions.

In order to analyze whether p120 catenin binding to N-cadherin was involved in the recruitment of N-cadherin in LR, we used a N-cadherin mutant with a triple alanine mutations in the juxtamembrane domain (NcadAAA) that abolishes binding to p120 catenin (20). Although NcadAAA did not bind to p120 catenin (data not shown), it surprisingly colocalized with p120 catenin in C2C12 myoblasts (Figure 2A). This unexpected observation could be explained by the likely formation of heterodimers between NcadAAA and endogenous wild type N-cadherin, this later being able to recruit p120 catenin. We thus checked this hypothesis by performing immunoprecipitation experiments using NcadAAA/YFP-transfected C2C12 myoblasts. Indeed, NcadAAA/YFP was co-immunoprecipitated with endogenous N-cadherin (Figure 2B). We then compared the distribution of Ncad/GFP and NcadAAA/YFP in C2C12 and mouse L cells which do not express endogenous cadherins (Figure 2C and D). Ncad/GFP accumulated at cell-cell contacts both in C2C12 myoblasts and L cells; conversely NcadAAA/YFP was present at cell contacts in C2C12 myoblasts (Figure 2C panel b) but rarely in L cells (Figure 2D panel c). This difference in Ncad/GFP and NcadAAA/YFP localization in L cells was not due to a variation in p120 catenin expression level (Figure 2D, right panel). We thus decided to use exclusively L cells to analyze p120 catenin contribution to N-cadherin association with LR. We analyzed the recruitment of Ncad/GFP and NcadAAA/YFP in LR by isolating LR from enriched plasma

membrane preparations on sucrose gradient. We observed a similar amount of Ncad/GFP and NcadAAA/YFP at the plasma membrane by Western blotting (Figure 2E, a) and by monitoring the level of plasma membrane-associated Ncad/GFP and NcadAAA/YFP by cell surface biotinylation (data not shown). Conversely, in pooled fractions 3-5 (corresponding to LR fractions) we detected Ncad/GFP but not NcadAAA/YFP (Figure 2E, b). Finally, we analyzed the distribution of N-cadherin/GFP and the LR marker ganglioside GM1 in parental and *p120 catenin shRNA* C2C12 myoblasts (see Figure 6A and B for the description of these cells). In parental cells, both N-cadherin and p120 catenin colocalized at cell-cell contacts with GM1, in agreement with their common distribution and association in biochemically isolated LR ((16), see also Figure 1) (Figure 2F). In *p120 catenin shRNA* myoblasts, N-cadherin neither accumulated nor colocalized with GM1 at cell-cell contacts. These data indicate that recruitment of N-cadherin in LR requires interaction with p120 catenin.

We previously showed that N-cadherin association with LR allows its stabilization at cell-cell contacts enabling the formation of a functional adhesive complex (16). To analyze the role of p120 catenin binding in N-cadherin stabilization at the cell contacts, we compared the assembly dynamics of Ncad/GFP and NcadAAA/YFP using fluorescent recovery after photobleaching (FRAP) experiments. We measured the diffusion coefficients (D) and mobile fractions (M) of Ncad/GFP and NcadAAA/YFP at cell-cell contacts of living L cells. We found a two-fold increase in the lateral diffusion of NcadAAA/YFP compared to Ncad/GFP (Figure 3A and B). Moreover, NcadAAA/YFP mobile fraction was bigger than that of Ncad/GFP indicating that NcadAAA/YFP was free to diffuse in the PM for the entire duration of the experiment. Typical images of FRAP experiments from Ncad/GFP and NcadAAA/YFP-expressing cells are shown in Figure 3C. Altogether, these data suggest that association of p120 catenin with N-cadherin participates in the recruitment of this cadherin to LR and in its immobilization during contact establishment.

LR disruption prevents myogenesis induction and RhoA activation. We previously showed that LR disruption leads to inhibition of cell-cell adhesion and disorganization of N-

cadherin-dependent cell-cell contacts (16). We now analyzed the effect of LR disruption on myogenesis. Treatment of C2C12 myoblasts with amphotericin B impaired N-cadherin association with LR (Figure 4A) as observed after CO or MCD addition (16). We next examined whether LR disruption could affect the expression of muscle-specific proteins by Western blot analysis and immunocytochemistry. Whereas parental C2C12 myoblasts expressed myogenin and troponin T after addition of differentiation medium (DM), C2C12 myoblasts treated with ampho B (Figure 4B and C, respectively) or with CO or MCD (data not shown) did not. We were also unable to detect myotube formation (data not shown). Addition of culture medium with cholesterol restored myogenesis (Figure 4D).

Treatment with ampho B (Figure 4E) or CO (data not shown) led also to a marked decrease in RhoA activity, known to be essential for myogenesis (18,21). We thus asked whether LR disruption specifically impaired the increase in RhoA activity mediated by N-cadherin adhesion. We measured RhoA activity by pull-down assays in C2C12 myoblasts plated onto dishes coated with either anti-Fc antibody or N-cad-Fc ligand, which allowed us to mimic N-cadherin-mediated adhesion (18). LR disruption by incubation with ampho B again decreased RhoA activity (Figure 4F), which was restored by addition of culture medium with cholesterol (data not shown). These experiments clearly show that LR are involved in the activation of RhoA downstream of N-cadherin.

PI(4,5)P₂ and RhoA are found at sites of N-cadherin adhesion in a p120 catenin-dependent manner. We next wanted to analyze at the cell-cell contacts, the content in lipid second messengers known to play a major role in protein recruitment and activation. Thus, we investigated whether phosphatidylinositol 4,5-bisphosphate (PI(4,5)P₂), a phosphoinositide known to be accumulated in LR (22,23), was present at the N-cadherin-mediated cell-cell contacts. C2C12 myoblasts were transfected with a construct expressing the PH domain of PLC δ fused to GFP (PH-PLC δ /GFP) that specifically binds to PI(4,5)P₂ (24). At cell contacts in C2C12 myoblasts, PI(4,5)P₂ accumulated and co-localized with N-cadherin (Figure 5A, panels a and b). This co-localization was lost after Ca²⁺ chelation with EGTA (panels c and d) and after LR disruption (data not shown). In L cells, PI(4,5)P₂ accumulated at cell contacts only when

N-cadherin was expressed (Figure 5C, compare panel a to panel d). In the absence of N-cadherin expression, PI(4,5)P₂ was found in F-actin-rich polymerization areas (panels a and b). LR disruption impaired the recruitment of PI(4,5)P₂ at N-cadherin-dependent cell-cell contacts (panels f-h). We also investigated whether PI(4,5)P₂ localization at cell-cell contacts correlated with RhoA accumulation in the same place. For this purpose, C2C12 myoblasts were co-transfected with PH-PLC δ /GFP and RhoAWT/RFP. In isolated C2C12 myoblasts, we observed no colocalization between RhoA and PI(4,5)P₂ (Figure 5B, panels a-d), whereas in contacting cells in which N-cadherin was engaged in cell-cell contacts, RhoA and PI(4,5)P₂ were colocalized (panels e-h). Treatment with amphotericin B impaired colocalization of RhoA and PI(4,5)P₂ in contacting C2C12 myoblasts (data not shown). In L cells, RhoA was not accumulated at cell-cell contacts (Figure 5D panel a), whereas it did so after N-cadherin expression (panels b-d). Again LR disruption impaired the recruitment of RhoA at N-cadherin-dependent cell-cell contacts (panels e-g). Conversely, NcadAAA did not recruit RhoA at cell contacts (Figure 5E). Altogether these data show that N-cadherin engagement and its subsequent recruitment in LR allows PI(4,5)P₂ localization at cell-cell contacts enabling RhoA accumulation at this place.

Perturbation of N-cadherin/catenins complexes inhibits myogenesis induction and RhoA activation. To investigate further the role of N-cadherin/p120 catenin complexes in RhoA activation and myogenesis induction we generated stable C2C12 cell lines in which the expression of *p120 catenin* was inactivated by RNA interference (*p120 catenin* shRNA) (Figure 6A and B). Parental and C2C12 myoblasts expressing human *p120 catenin* shRNA (*hp120 catenin* shRNA) were used as controls (data not shown). As previously reported (11,25), we observed that *p120 catenin* silencing led to a decrease in N-cadherin expression (Figure 6A and B). We next examined whether *p120 catenin* silencing affected the expression of myogenin and troponin T (Figure 6C). After four days in DM, parental C2C12 myoblasts expressed myogenin and troponin T, whereas *p120 catenin* shRNA myoblasts did not. In addition, we observed many myotubes in parental (Figure 6D, panel a) and control *hp120 catenin* shRNA cells (data not shown), but only few in the *p120 catenin* shRNA myoblasts (Figure 6D, panel b).

As N-cadherin-mediated adhesion activates RhoA which allows myogenesis induction (18,21), we analyzed the effect of *p120 catenin* silencing on RhoA activity in parental or *p120 catenin* shRNA C2C12 myoblasts. We first used the organization of the F-actin cytoskeleton as functional read-out. No modification was observed in both parental or *p120 catenin* shRNA C2C12 myoblasts (Figure 6E). We then analyzed RhoA activity using pull-down assays. We observed an increase in RhoA-GTP level after DM addition, in parental, but not in *p120 catenin* shRNA C2C12 myoblasts (Figure 6F). To confirm the involvement of RhoA in myogenesis inhibition after p120 catenin knock-down, we transfected *p120 catenin* shRNA C2C12 myoblasts with a RFP-tagged construct expressing RhoA wild-type (WT). Cells were cultured in DM, fixed and analyzed for expression of myogenin (Figure 6G). RhoA expression partially rescued the inhibition of myogenesis induction caused by *p120 catenin* silencing. This shows that perturbation of the N-cadherin/p120 catenin complex inhibits RhoA activation and subsequently myogenesis induction.

DISCUSSION

Skeletal myogenesis is a multistep process regulated by various molecules (1,2). N-cadherin-dependent adhesion controls induction of myogenesis through activation of RhoA which positively regulates MyoD expression and activation of several muscle-specific gene promoters (3,4).

Dynamic association of cadherin complexes at the plasma membrane is crucial for cadherin-mediated signaling. The juxtamembrane domain of the cadherin cytoplasmic tail binds p120 catenin which is a major regulator of cadherin stability at cell-cell contacts and a modulator of Rho GTPase activities (8-11). Mutations in the region of E-cadherin that binds to p120 uncoupled the E-cadherin-p120 catenin complex and disrupted strong adhesion, although the interaction with other catenins remains intact (13). Moreover, association of N-cadherin with cholesterol-enriched microdomains called lipid rafts (LR) at the cell-cell contacts, also stabilizes N-cadherin (16). Since p120 catenin association with cadherins and, N-cadherin association with LR at the cell-cell contact sites are both involved in cadherin stability, we have analyzed whether

p120 catenin association with N-cadherin requires LR. We observed that the association of p120 catenin with N-cadherin occurs specifically in these microdomains and reciprocally that LR are essential for p120 catenin/N-cadherin association. It has been suggested that p120 catenin is a relatively minor component of the cadherin complex as only a fraction of p120 catenin co-precipitates with cadherins in the presence of detergents (26,27). This fraction of p120 catenin might be the one associated with N-cadherin in cholesterol-rich membrane domains.

Although it is known that binding to p120 catenin promotes cadherin stabilization at cell-cell contacts, the underlying mechanisms are largely unknown (9,11,13,28). We demonstrate that p120 catenin associates with N-cadherin specifically and exclusively in LR and that, when this interaction is abolished, N-cadherin is not accumulated in LR and not stabilized at cell-cell contacts. This finding raises questions on how p120 catenin is recruited in LR and which mechanisms are involved in cadherin stabilization. Beside the function of the Arm domain of p120 catenin which binds to N-cadherin juxtamembrane domain, p120 catenin could also sense the lipid environment or interact with a protein located in these microdomains. Recently, p120 catenin C-terminal region was proposed to be involved in the recruitment of cytoplasmic E-cadherin to the plasma membrane (29). p120 catenin has been also involved in membrane stabilization of Desmoglein 3 through its binding to a juxtamembrane domain of this cadherin. This observation reinforces the potential contribution of the lipid environment in p120 catenin binding to this cadherin (30). Structural analysis of p120 catenin C-terminal region revealed the presence of potential CRAC (Cholesterol Recognition/Interaction Amino Acid Consensus) (personal observations) motifs which favor the interaction with cholesterol (31). In summary, binding to p120 catenin might stabilize N-cadherin through interaction with cholesterol-rich microdomains. This might contribute to N-cadherin clustering which enhances its adhesive strength. Moreover, binding to p120 catenin has also an indirect stabilizing function as it masks a conserved dileucine motif in N-cadherin juxtamembrane domain that is necessary for endocytosis (32). Indeed, unstabilized cadherin due to deficient binding to p120 catenin is endocytosed (11).

Moreover, we show that the association of p120 catenin with N-cadherin in LR is

involved in N-cadherin-mediated RhoA activation. This suggests that at cell contacts, N-cadherin/p120 catenin association with LR allows stabilization and formation of a functional adhesive complex able to organize signal transduction pathways in this specific area of the membrane (16). As RhoA is recruited at cell contacts after N-cadherin engagement (18), and its activity is required for N-cadherin stabilization at cell contacts in C2C12 myoblasts (33), we conclude that p120 catenin is required for N-cadherin-dependent RhoA activation which in turn allows N-cadherin stabilization at cell contacts. Interestingly *mammalian* RhoA and its *Drosophila* homolog, *Rho1*, directly interact with p120 catenin (34,35) and we have also detected a fraction of RhoA in LR (data not shown). Moreover, at cell contacts in C2C12 myoblasts, N-cadherin, RhoA and phosphatidylinositol 4,5-bisphosphate (PI(4,5)P₂), a phosphoinositide known to be accumulated in LR (22,23) and to play a critical role in the dynamic organization of the actin cytoskeleton (36,37), were co-localized (Figure 5A and B). This co-localization was lost after LR disruption. In L cells, PI(4,5)P₂ and RhoA accumulated at cell contacts only when N-cadherin was expressed. Again, LR disruption impaired the recruitment of PI(4,5)P₂ and RhoA at N-cadherin-dependent cell-cell contacts. Conversely, NcadAAA did not recruit RhoA at N-cadherin-mediated cell contacts. Recruitment and activation of PIP5K γ at sites of N-cadherin ligation resulting in PI(4,5)P₂ production has been reported (38). Segregation of PI(4,5)P₂ might allow the recruitment of pleckstrin homology domain-containing proteins, such as a guanine exchange factor (GEF) for RhoA that still remains to be identified. Altogether these data suggest that p120 catenin might be involved in recruiting RhoA in LR at cell contacts to allow its activation by a GEF and consequently its downstream function.

Finally, our results show that LR are required for the formation of the N-cadherin complex, downstream RhoA activation and subsequent myogenesis induction. Inhibition of myogenesis was already reported after perturbation of the N-cadherin/p120 catenin complex upon silencing of *p120 catenin* (39). In contrast to data obtained in fibroblasts (8), p120 knockdown in myoblasts does not increase RhoA activity as measured by Rhotekin pull-down assay and analysis of actin stress fibers, but the loss of p120 catenin impairs RhoA activation

induced by specific N-cadherin engagement. This was confirmed by the rescue of myogenesis inhibition in *p120 catenin* shRNA myoblasts after RhoA expression. These data obtained in myoblasts agree with the role of RhoA during myogenesis induction (18,21). Indeed, RhoA has been reported to positively regulate MyoD expression and skeletal muscle cell differentiation, as it has been demonstrated to be required for serum response factor-mediated activation of several muscle-specific gene promoters (3,4). These last years, roles for p120 catenin in controlling Rho GTPases activity emerged underlying the existence of cell type specific mechanisms. Indeed, in NIH3T3 fibroblasts, p120 catenin can target suppression of Rho to cadherin complexes via transient recruitment of p190RhoGAP (8) and in CHO

cells, p120 appears to be essential for cadherin-mediated activation of Rac1 (40).

p120 catenin is known to regulate cell-cell adhesion through its interaction with the cytoplasmic juxtamembrane domain of cadherins. Here we identified a novel mechanism by which p120 catenin acts as a direct stabilizer of N-cadherin at the cell contacts through its association with cholesterol-rich membrane microdomains. How p120 catenin senses this peculiar lipid membrane environment remains to be determined. LR serve as platforms for protein segregation and signaling and are often modified in cancer cells. Such modifications might thus participate to the perturbation of p120 catenin activity in tumors cells.

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FOOTNOTES

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The abbreviations used are: Ampho B, amphotericin B; CO, cholesterol oxydase; LR, lipid raft; MCD, methyl- β -cyclodextrin; NLR, non lipid raft; PM: plasma membrane; FRAP: Fluorescence recovery after photobleaching

FIGURE LEGENDS

Fig. 1. p120 catenin interacts with N-cadherin exclusively in LR. *A.* C2C12 myoblasts were lysed in 1% Triton X-100 and fractionated on a sucrose gradient. Fractions 3 to 5 (LR) and fractions 8 to 10 (NLR) were pooled. 30 μ g of each fraction were immunoprecipitated using an anti-N-cadherin antibody and probed for α -, β -, γ - and p120 catenin. *B.* a: NLR fractions (300 μ g) were immunoprecipitated using an anti-N-cadherin antibody and tested for α -, β -, γ - and p120 catenin. b: Cell lysates of C2C12 myoblasts (10 μ g), LR (2 μ g) and NLR fractions (10 μ g) were probed for N-cadherin (upper panel) and p120 catenin expression (lowest panel). Results are representative of three independent experiments. *C.* GM1 was labeled with rhodamine-conjugated cholera toxin B subunit and subsequently patched by the addition of a secondary antibody (a). Distributions of N-cadherin/GFP (b), p120 catenin (c) or merged image with the patched GM1 are shown (d). Arrows indicate some GM1 patches in which N-cadherin and p120 catenin are found. Bar: 5 μ m. *D.* a: Cell lysates of C2C12 myoblasts treated with ampho B, or not, were immunoprecipitated using an anti-N-

cadherin antibody and probed for the presence of p120 catenin. b: 20 μ g of cell lysates of C2C12 myoblasts treated with amphotericin B, or not, were probed for p120 catenin and N-cadherin.

Fig. 2. Disruption of p120 catenin binding to N-cadherin impairs its association with LR and increases its mobile pool and lateral mobility at cell contacts. *A.* C2C12 myoblasts transfected with a plasmid encoding NcadAAA/YFP were fixed 20 hours after transfection and monitored for YFP fluorescence and p120 catenin expression. Arrows illustrate the colocalization of NcadAAA and p120 catenin. Bar: 10 μ m. *B.* Lysates of parental (lane 2) or expressing NcadAAA/YFP C2C12 myoblasts (lane 3) were immunoprecipitated using an anti-GFP antibody and immunoblotted for N-cadherin. Lane 1: 20 μ g of C2C12 extracts. *C.* C2C12 myoblasts transfected with plasmids encoding Ncad/GFP or NcadAAA/YFP were fixed 20 hours after transfection and monitored for FP fluorescence. Bar: 10 μ m. *D.* Mouse L cells transfected with plasmids encoding Ncad/GFP or NcadAAA/YFP were fixed 20 hours after transfection and monitored for FP fluorescence and p120 catenin distribution. NcadAAA and p120 catenin do not accumulate at cell contacts (arrowheads in panels c and d) whereas Ncad and p120 catenin do (arrows in a and b). Bar: 10 μ m. The graph shows the percentage of cells with N-cadherin localization at cell contacts. Results are shown as mean \pm SED of three independent experiments (50 cells were counted for each condition). Cell lysates (20 μ g) of L cells expressing Ncad/GFP or NcadAAA/YFP were probed for p120 catenin and α -tubulin expression. *E.* a: 30 μ l of fractions enriched in PM obtained from L cells expressing Ncad/GFP or NcadAAA/YFP were probed for GFP and transferrin receptor (TfR) expression. b: PM fractions from L cells expressing Ncad/GFP or NcadAAA/YFP were fractionated through sucrose gradients. Fractions 3-5 (i.e. LR) were pooled and probed for Ncad/GFP and NcadAAA/YFP expression with an anti-GFP antibody. Actin is a loading control. Results are representative of three independent experiments. *F.* Analysis of the distribution of the LR marker ganglioside GM1 by using Rhodamin-conjugated Cholera toxin B (CTX-B) (panels a and e) and of p120 catenin (panels c and g) in control and *p120 catenin shRNA* C2C12 myoblasts expressing N-cadherin/GFP (panels b and f). Panels d and f: merge of N-cadherin/GFP and GM1. Arrows indicate cell-cell contacts displaying GM1 and N-cadherin colocalization. Scale bar: 10 μ m.

Fig. 3. Disruption of p120 catenin binding to N-cadherin increases its mobile pool and lateral mobility at cell-cell contact sites. *A.* Shown are the first 95 s of photobleach recovery curves of Ncad/GFP and NcadAAA/YFP. The solid lines represent the best fit to the lateral diffusion equation as described in (16). *B.* D and M coefficients of Ncad/GFP and NcadAAA/YFP in membranes involved in cell contacts. Significance (by paired Student's *t* test) is shown on the graph. *C.* Shown are typical images of a FRAP experiment. Fluorescence of selected area (circles) of N-cadherin or N-cadherinAAA-dependent cell-cell contacts (visualized in the left panels) were photobleached (the first image recorded after bleaching is marked by an asterisk), and fluorescence recovery was measured with time.

Fig. 4. Effect of LR disruption on myogenesis and RhoA activation. *A.* C2C12 myoblasts treated with amphotericin B (right) or mock-treated (left) were fractionated on sucrose gradients. 30 μ l of each fraction were analyzed for N-cadherin and caveolin distribution by immunoblotting. *B.* Cell lysates (20 μ g/well) of C2C12 myoblasts treated with amphotericin B, or not, cultured in GM or DM as indicated (D=day) were assessed by Western blot analysis for myogenin, troponin T and α -tubulin expression. *C.* C2C12 myoblasts treated with amphotericin B, or not, cultured in DM for 3 days were analyzed by immunocytochemistry for myogenin and troponin T. Bar: 10 μ m. *D.* C2C12 myoblasts cultured in DM containing amphotericin B alone or amphotericin B and cholesterol for 3 days were analyzed by Western blot for myogenin, troponin T and α -tubulin expression. *E.* GTP-bound RhoA level was measured using GST fused to the Rho-binding domain of the RhoA effector Rhotekin (GST-RBD) in lysates obtained from C2C12 myoblasts treated with amphotericin B or not. RhoA was detected by immunoblotting. The histogram shows a quantification of the results of three independent experiments. *F.* GTP-bound RhoA was measured in lysates obtained from C2C12 myoblasts plated on surfaces coated with either anti-Fc antibody or N-cad-Fc and treated with amphotericin B or not. The histogram represents GTP-bound RhoA after normalization to the amount of total RhoA protein. Results are the mean of three independent experiments.

Fig. 5. PI(4,5)P₂ and RhoA are found at sites of N-cadherin adhesion. *A.* C2C12 myoblasts transfected with PH-PLCδ/GFP (panels b and d) were treated with EGTA (panels c and d). Cells were stained for N-cadherin (panels a and c). Arrows show N-cadherin and PH-PLCδ/GFP enrichments at cell contacts. Bar: 10μm. *B.* C2C12 myoblasts transfected with PH-PLCδ/GFP and RhoAWT/RFP. In confluent conditions (panels e-h) PH-PLCδ/GFP and RhoAWT/RFP colocalize at cell contact sites (arrows in panel g), whereas they do not in isolated myoblasts (panels a-d). Bar: 10μm. *C.* L cells transfected with PH-PLCδ/GFP alone (panel a) or together with N-cadherin/RFP (panels c and d) were stained for F-actin (panels b and e). PH-PLCδ/GFP is located in F-actin-rich structures (lamellipodia) in L cells (panel a) and is recruited at the cell contacts when N-cadherin/RFP is expressed (panel d, arrows). Recruitment at cell contacts is lost after LR disruption (panels f-h). Bar: 10μm. *D.* L cells were transfected with RhoAWT/RFP alone (panel a) or together with N-cadherin/GFP (panels b and c). RhoAWT/RFP is recruited at cell contacts when N-cadherin/GFP is expressed and they colocalize (arrows in panel d). This is lost after LR disruption (panels e-g). Bar: 10μm. *E.* L cells were co-transfected with N-cadherinAAA/YFP (panel a) and RhoAWT/RFP (panel b). Bar: 10μm.

Fig. 6. Effect of p120 catenin silencing on myogenic differentiation and RhoA activation. *A.* The expression of p120 catenin, N-cadherin and α-tubulin is shown in one representative clone and parental C2C12 myoblasts. *B.* p120 catenin (panels b and d) and N-cadherin (panels a and c) expression in parental myoblasts (panels a and b) and in one representative *p120 catenin* shRNA clone (panels c and d). *C.* Cell lysates (20μg/well) of parental, and *p120 catenin* shRNA myoblasts were cultured in DM for 4 days and probed for myogenin, troponin T and α-tubulin expression. *D.* Phase-contrast images of parental (panel a) and *p120 catenin* shRNA myoblasts (panel b) after 4 days in DM. *E.* F-actin expression in parental (panel a) and *p120 catenin* shRNA myoblasts (panel b). Bar: 10μm. *F.* GTP-bound RhoA was measured using GST-RBD in lysates from parental (left panels) and *p120 catenin* shRNA (right panels) C2C12 cells grown in GM or DM for 6 hours. RhoA was detected by immunoblotting. Data are representative of three independent experiments. *G.* Parental, *p120 catenin* shRNA and *p120 catenin* shRNA myoblasts expressing RFP-tagged RhoAWT were cultured in DM for 2 days and probed for myogenin expression by immunohistochemistry. The histogram represents the percentage of myogenin-positive cells and summarizes the data from three independent sets of experiments; 60-70 cells were analyzed in each experiment.

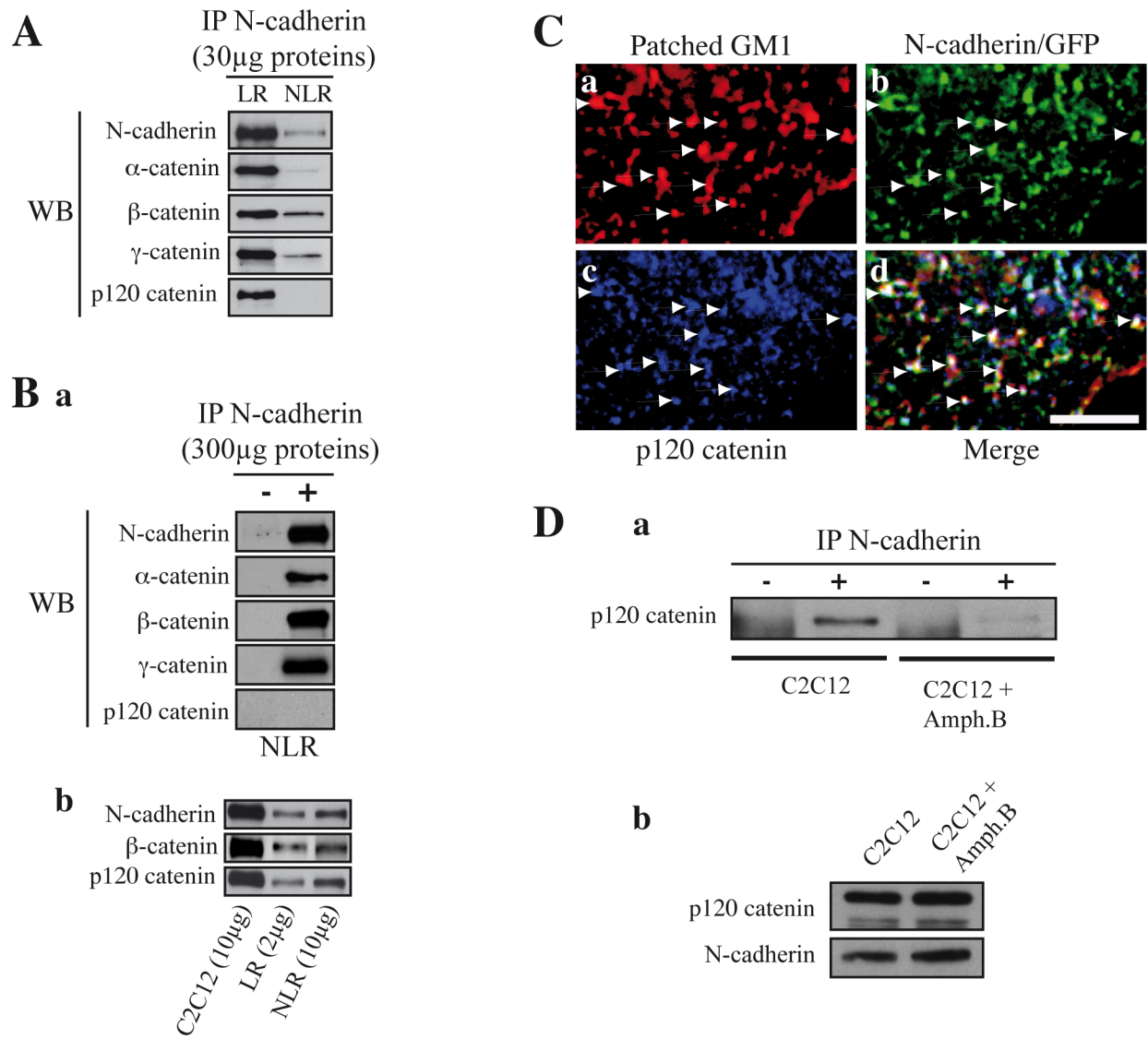


Figure 1

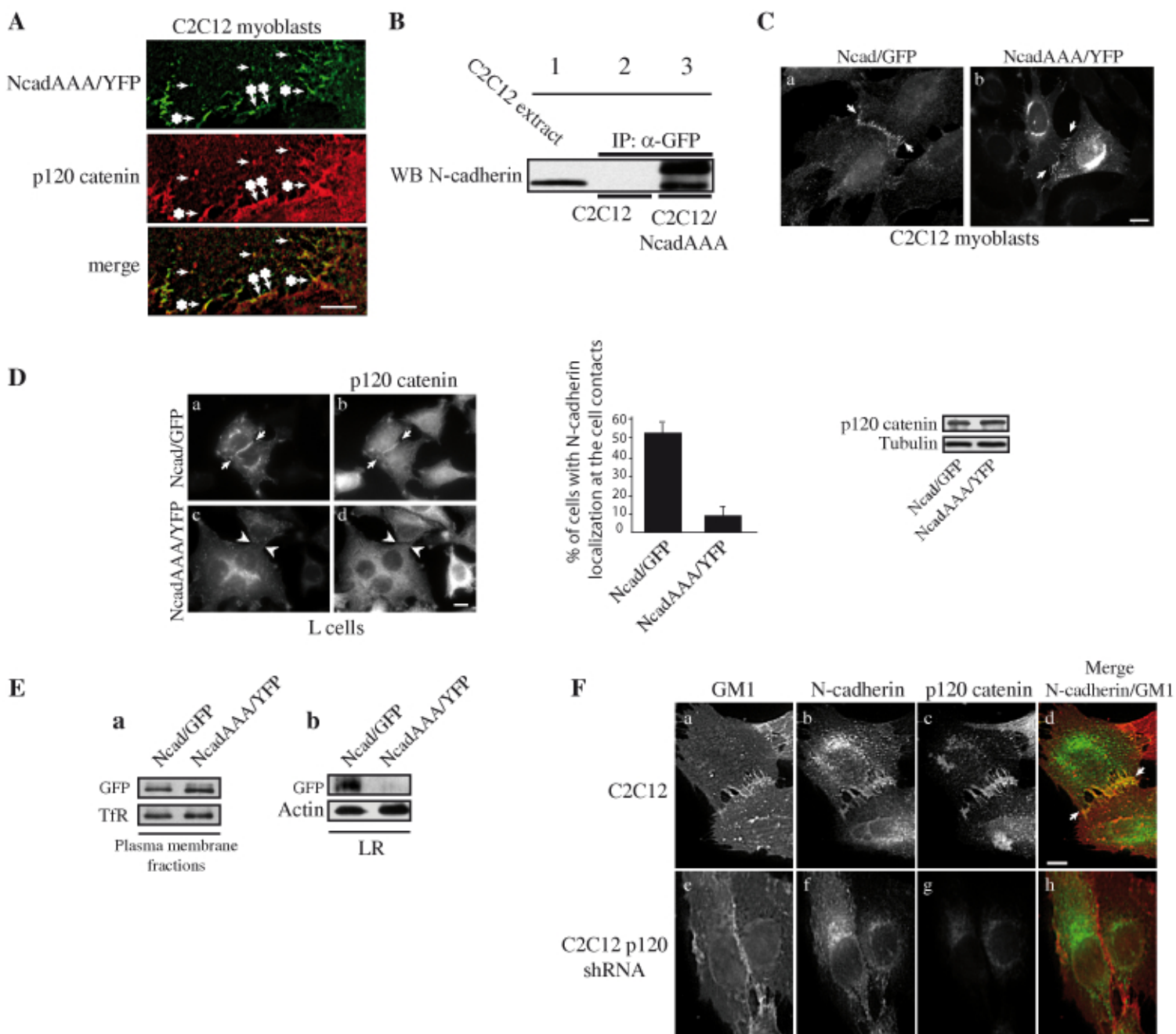


Figure 2

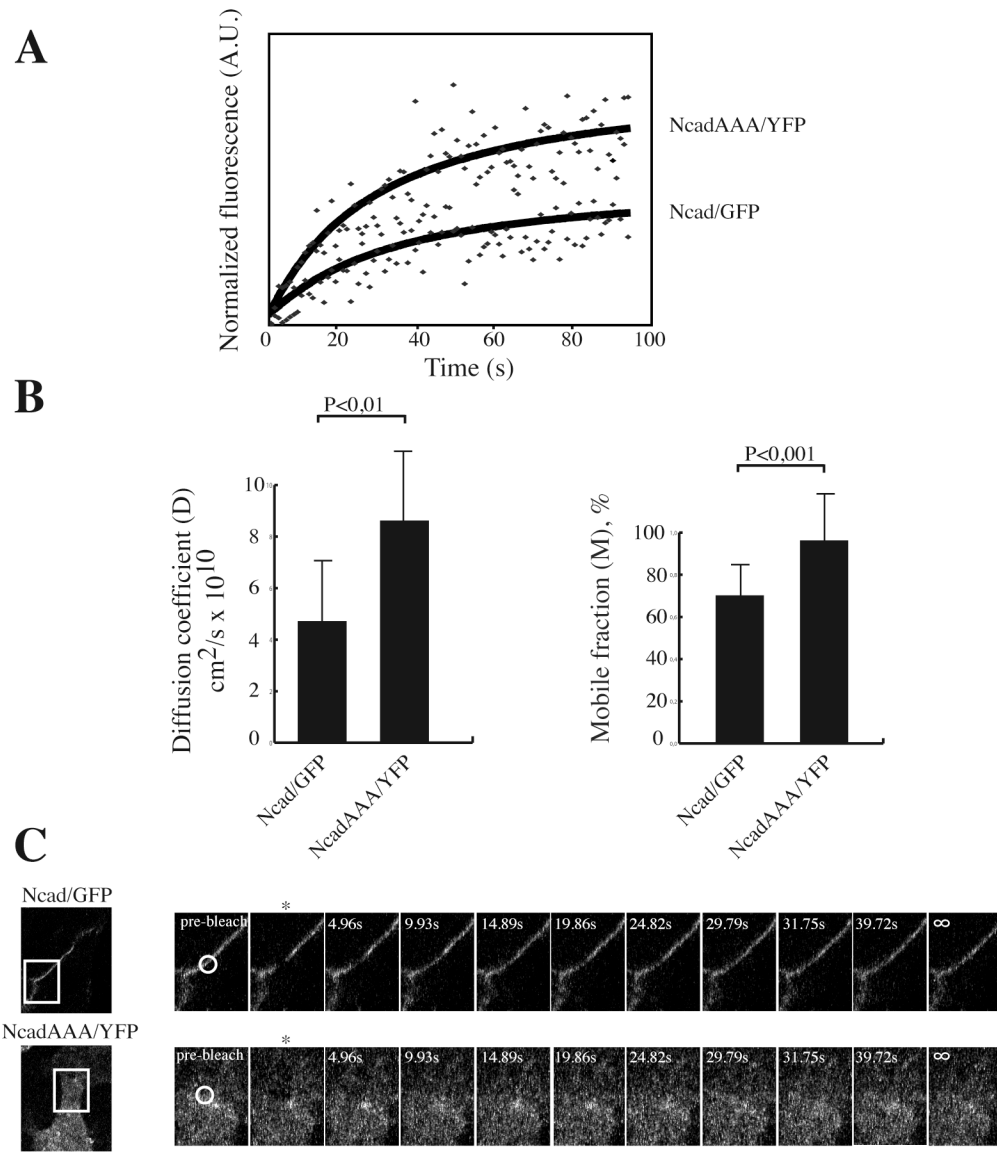


Figure 3

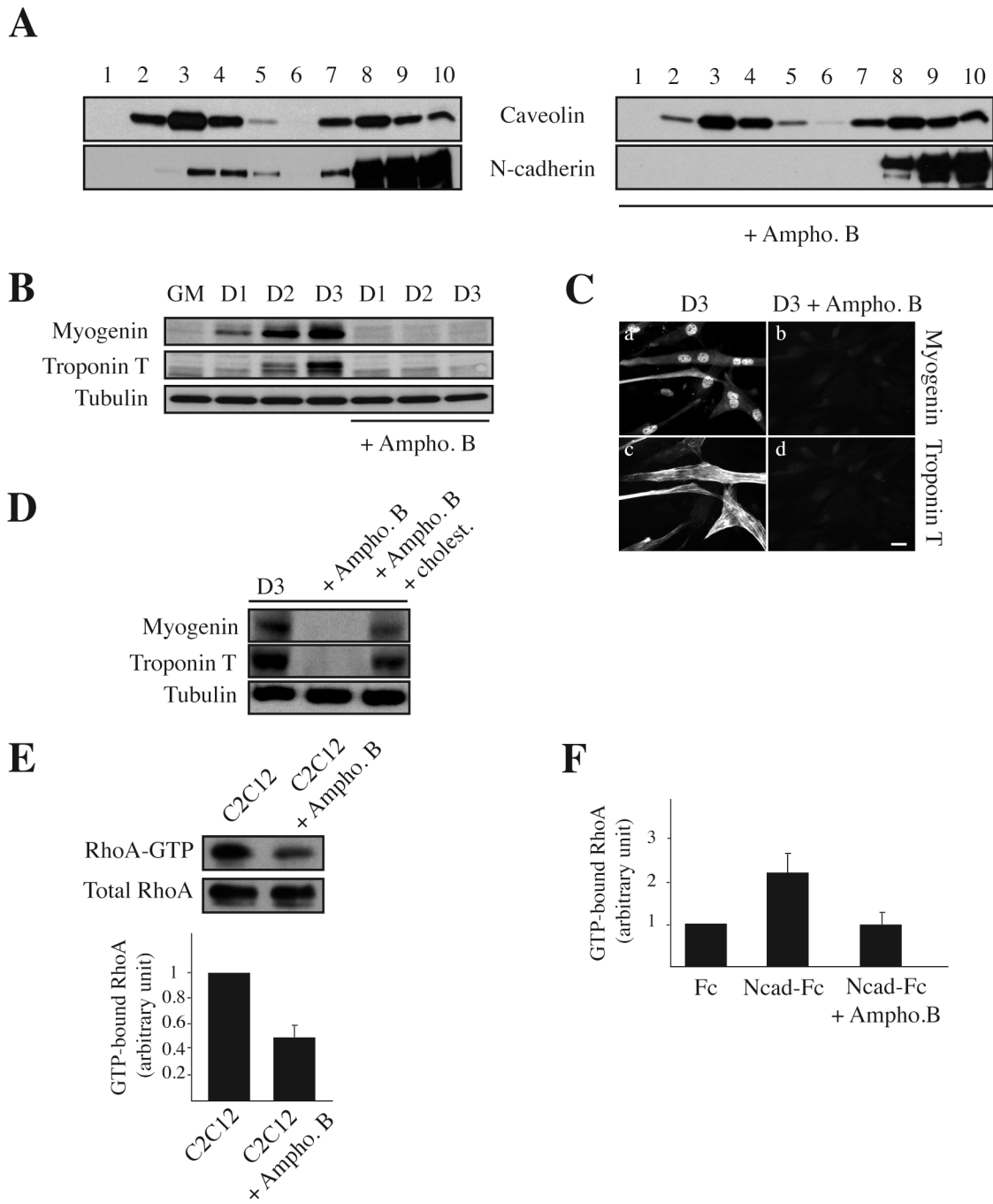


Figure 4

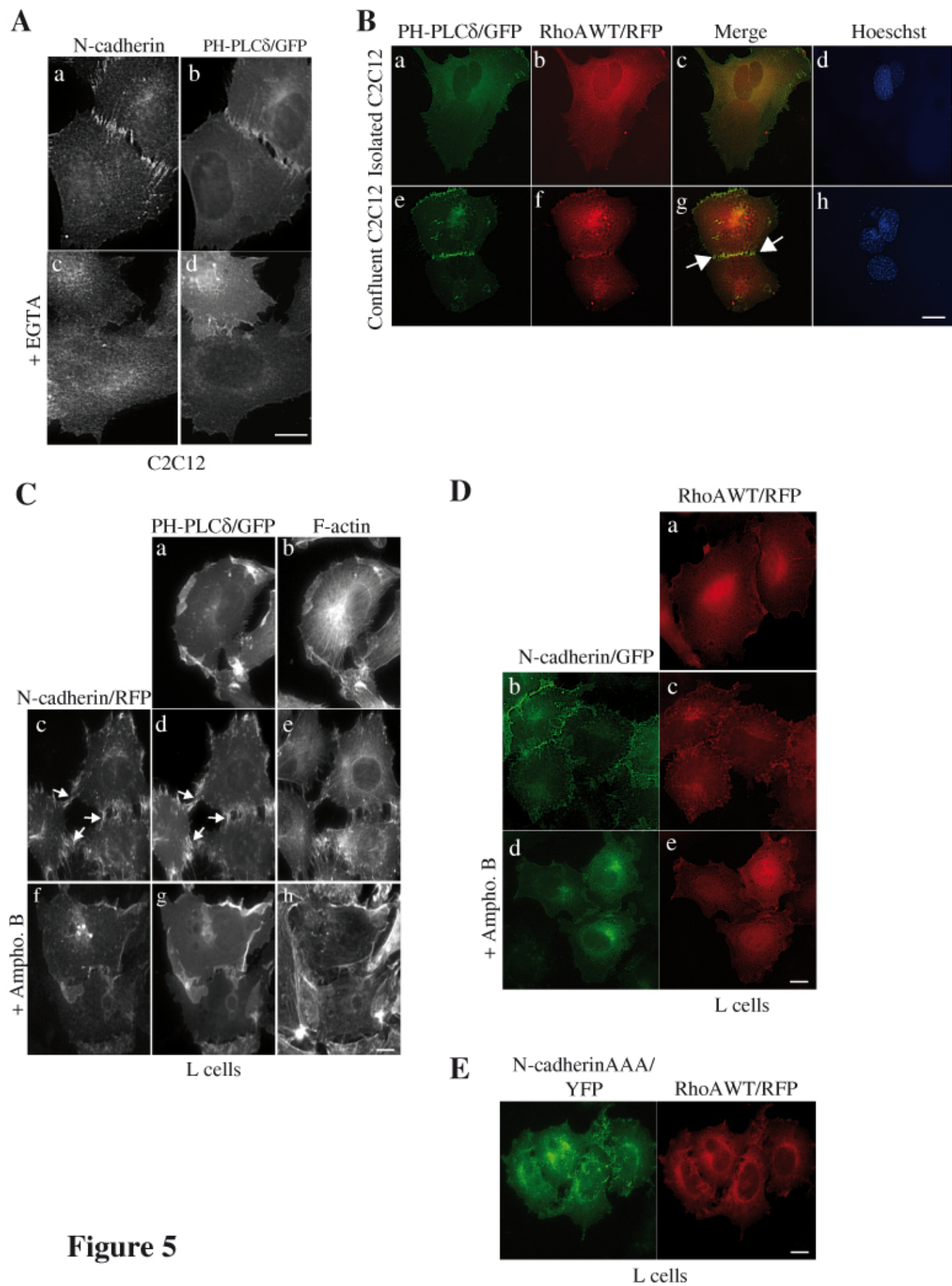


Figure 5

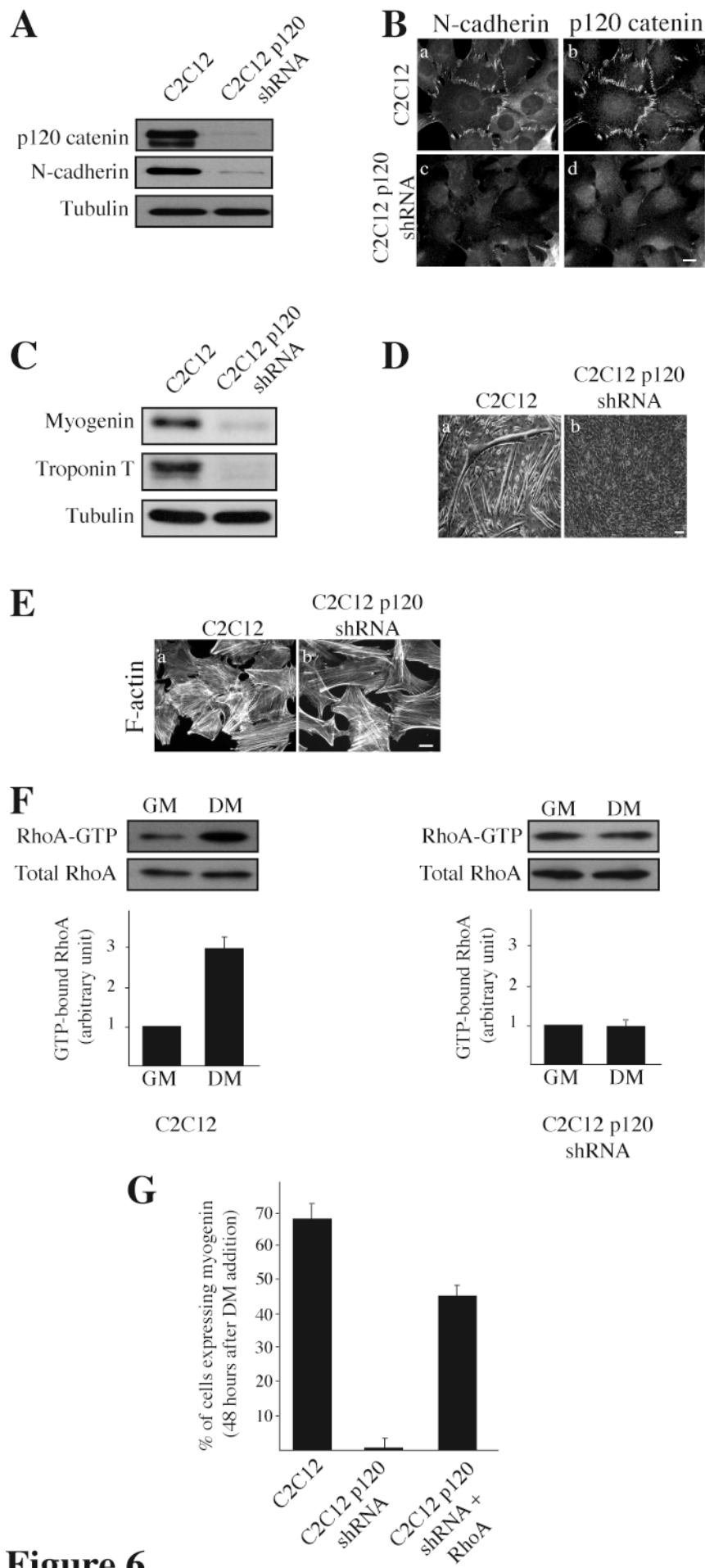


Figure 6