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**R-cadherin expression inhibits myogenesis and induces myoblast
transformation via Rac1 GTPase**

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Abstract

Cadherins are transmembrane glycoproteins that mediate Ca^{2+} -dependent homophilic cell-cell adhesion and play a crucial role in proliferation, differentiation and cell transformation. The goal of this study was to understand why R-cadherin is found in rhabdomyosarcomas, tumors of skeletal muscle origin, while it is absent in normal myoblasts. We show that R-cadherin expression in C2C12 myoblasts causes inhibition of myogenesis induction and impairment of cell cycle exit, when cells are cultured in differentiation medium. Furthermore, R-cadherin expression elicits myoblast transformation, as shown by anchorage-independent growth in soft agar, *in vivo* tumor formation assays and increased cell motility. In contrast, inhibition of R-cadherin expression using RNA interference hinders growth of RD cell line in soft agar and its tumorigenicity in mice. The analysis of the nature of R-cadherin-mediated signals shows that R-cadherin-dependent adhesion increases Rac1 activity. Dominant negative forms of Rac1 inhibit R-cadherin-mediated signaling and transformation. In addition, expression of R-cadherin results in perturbed function of endogenous N- and M-cadherin. Together, these data suggest that R-cadherin expression inhibits myogenesis and induces myoblast transformation through Rac1 activation.

Therefore, the properties of R-cadherin make it an attractive target for therapeutic intervention in rhabdomyosarcomas.

Introduction

Cadherins are homophilic cell-cell adhesion molecules essential for the organization of cells into tissues during embryonic development. They are also involved in cell growth, migration and differentiation. Cadherins are highly conserved transmembrane glycoproteins that mediate homotypic cell-cell adhesion through their extracellular domain. The cadherin cytoplasmic domains provide F-actin cytoskeleton attachment points via association of catenins and other cytoskeletal associated proteins (1). These adhesive receptors regulate diverse functions, beyond the basic adhesive process, including intracellular signaling events (2). In particular, cadherins activate Rho-family GTPases and participate in receptor tyrosine kinase signaling (3). Developing skeletal muscles express N-, M-, R-cadherins and cadherin-dependent adhesion is required for a variety of cellular events. N-cadherin-dependent inter-cellular adhesion has a major role in the cell cycle exit and in the induction of the skeletal muscle differentiation program (4-7). N-cadherin regulates RhoA and Rac1, two Rho GTPases whose activity needs to be tightly controlled to allow myogenesis induction (8). Indeed, RhoA is activated in a N-cadherin-dependent manner which allows Serum Response Factor-dependent genes expression and myogenesis induction. In contrast, N-cadherin activation decreases Rac1 which is known to inhibit myogenesis by preventing the withdrawal of myoblasts from the cell cycle (9, 10). During embryonic development M-cadherin is expressed later than N-cadherin. Its expression increases at the onset of secondary myogenesis, and it has been implicated in terminal myoblast differentiation, mainly during myoblast fusion (11). R-cadherin is detected in somites and limbs, and it is localized at the cell-cell contacts of primary myoblasts during primary myogenesis, as well as at the primary myotube/secondary myoblast boundaries during secondary myogenesis (12). R-cadherin expression is lost in adult muscles and is found expressed in rhabdomyosarcoma (RMS), a

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highly malignant soft-tissue tumor committed to the myogenic lineage, but arrested prior to terminal differentiation (13). RMS are divided into two major histological subtypes, alveolar and embryonal (14); both express R-cadherin. R-cadherin expression in RMS is accompanied by a decrease in the expression of N- and M-cadherin which is reminiscent of the cadherin switching observed in tumorigenesis, particularly in metastasis (15) (16). Indeed, cadherin switching is often observed in the epithelium-to-mesenchyme transition (EMT) during normal embryonic development and when cancer cells invade adjacent tissues (17-19). R-cadherin was first found to be expressed in the chick embryonic retina and, later, was also detected, besides skeletal muscle, in heart, kidney, thymus and lung during development (12, 20-22). R-cadherin mRNA has been detected in bladder and prostate carcinomas and its expression in various cell lines downregulates expression of other cadherins (23-25). Conversely, R-cadherin is silenced by promoter methylation in gastrointestinal tumors (26). This suggests that the cellular context is important for R-cadherin-induced downstream effects.

Here we show that R-cadherin expression in C2C12 cell line results in myogenesis inhibition and myoblast transformation. On the contrary, inhibition of R-cadherin expression by siRNA decreases the transformation potential of RMS cells. Furthermore, we demonstrate that R-cadherin-mediated adhesion increases Rac1 activity and that R-cadherin-induced myoblast transformation is dependent upon Rac1 activation. Finally, we evidence a cadherin switch induced by R-cadherin with down-regulation of the endogenous expression of M-cadherin and delocalization of M and N-cadherin, processes mediated by Rac1.

Materials and methods

Cell Culture and Immunocytochemistry

C2C12 mouse myoblasts and the embryonic rhabdomyosarcoma cell line (ERMS) RD were grown as described (13).

Cells growing onto 35-mm dishes were fixed in 3.7% formaldehyde in PBS followed by a 5 min permeabilization in 0.1% Triton X-100 in PBS and then incubated in PBS containing 0.1% BSA. Myogenin, troponin T and N-cadherin expression were detected as described (8, 10). Monoclonal anti-myc (Invitrogen) and anti cyclin D1 (BD Pharmingen) antibodies were used respectively at 1:1000 and 1:500. Primary antibodies were revealed with either an Alexa Fluor 546- or an Alexa Fluor 488-conjugated goat anti-mouse or goat anti-rabbit antibodies (Molecular Probes, Interchim). Cells were stained for F-actin using TRITC conjugated phalloidin (Sigma, France). Cells were analyzed as described (8).

The Rac1 inhibitor NSC23766 (Calbiochem, La Jolla, CA) was used at 100 μ M and was added 30 h before cell harvesting, in proliferation medium.

Establishment of stable cell lines

C2C12 were transfected with either pEGFPN1 plasmid (Clontech), in which the R-cadherin cDNA was subcloned, or infected by recombinant retroviruses produced by Phoenix cells transfected with the LZRS-MS-Rcad-2Xmyc plasmid or the LZRS-MS-R(AAA)cad-2Xmyc plasmid (kindly provided by Dr. MJ. Weelock). Stably transfected cells were selected in 1 mg/ml G418 and sorted by fluorescence-activated cell sorting for GFP expression.

To produce stable RD cell lines in which R-cadherin expression is inhibited, short interfering RNA constructs were made by cloning a double strand DNA corresponding to the R-cadherin sequence (13) in the retroviral vector pSIREN-RetroQ according to the manufacturer's protocol (BD Biosciences). A second R-cadherin small hairpin RNA was also used. For this,

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annealed double strand oligonucleotides
GATCCGTCAGAACGTGAAATGCAAAGTTCAAGAGAGACTGTGTACTGCTGAAC
TCTTTTTTACGCGTG (top) and
AATTCACGCGTAAAAAAGAGTTCAGCAGTACACAGTCTCTCTTGAACTTTGCAT
TTCACGTTCTGACG (bottom) were inserted into the RNAi-Ready pSIREN-RetroQ and the
RNAi-Ready pSIREN-RetroQ-ZsGreen vectors (Clontech, BD Biosciences). As a control, we
made RD cell lines stably expressing a siRNA for the unrelated gene luciferase. Stable
transfectants were selected in medium containing puromycin (2.5µg/ml) and different clones
were isolated by limited dilution.

Immunoblotting and Immunoprecipitation

Cell extracts were prepared as described (8). Equal amounts of protein (20 µg to 40 µg) were
resolved on SDS-PAGE and transferred to PVDF membranes. Membranes were blocked with
5% milk protein or 1% BSA when an anti-phosphorylation antibody was used. Membranes
were incubated with monoclonal anti-N-cadherin (1:250), -β-catenin (1:500), -γ-catenin
(1:200), -p120 catenin (1:200), anti-cyclin D1 (1:500) (all from BD Transduction
Laboratories, Lexington, KY), -α-tubulin (1:100, P. Mangeat, France), -α-actin (1:500,
Sigma), and anti-phospho-cJun (Ser61) (1:1000, Cell Signaling) antibodies, rat monoclonal
anti-R-cadherin antibody MRCD5 (1:100, a gift from M. Takeichi, Japan), polyclonal anti-M-
cadherin (13) and -α-catenin (1:200, Sigma) antibodies. Membranes were processed as
described (8).

For immunoprecipitation, C2C12 Rcad/GFP or C2C12 Rcad/2myc cells were lysed as
described previously (8). Lysates were immunoprecipitated using a polyclonal anti-GFP
(1:250, Torrey Pines) or a monoclonal anti-myc antibody (Invitrogen, 1:1000) followed by

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sequential incubation with protein A-Sepharose or protein G-sepharose (Amersham). The immunoprecipitates were analyzed by immunoblotting.

BrdU incorporation

When appropriate density was reached, cells were incubated with bromodeoxyuridine (BrdU, Boehringer) and processed as described (27).

Luciferase assay

C2C12 cells plated in 35-mm dishes (20000 cells for subconfluent condition; 50000 cells for confluent conditions) were co-transfected using the JetPEI method with 0.6 µg of construct containing the cyclin D1 promoter fused to the luciferase gene (28) and 40 ng of pRL cytomegalovirus CMV vector (renilla luciferase cytomegalovirus). For DM conditions, 250000 cells were co-transfected as soon as they were attached to the plate. Cells were induced to differentiate four hours after transfection. Forty eight hours after transfection, luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega).

Plasmid construction and production of the chimeric R-cad-Fc protein

To construct the cDNA coding for the entire mouse R-cadherin ectodomain linked to the human Fc fragment (Fc), a fragment from nucleotides 1 to 2156 was amplified by PCR reaction using oligonucleotides flanked by BamHI sites and subcloned into the TOPO vector (Invitrogen). The resulting plasmid was digested with BamHI restriction enzyme and the full length ectodomain was subcloned into the BamHI site of the pREP10-Fc plasmid (kindly provided by RM Mège, Paris, France). The production of the chimeric protein corresponding

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to the ectodomain of R-cadherin fused to Fc fragment (R-cad-Fc) was performed as previously described (8).

Time lapse imaging

C2C12 Rcad/GFP and C2C12 cells were analyzed to calculate their migration (29).

Statistical analysis

For experiments with $n \geq 30$, t test was used to assess the statistical differences between two experimental conditions (Figure 3B, 5B). For experiments where $n < 30$ (Figures 2A, 2C, 2D, 3A, 3C and 5B), the non-parametric Mann–Whitney U test was used to assess the statistical differences between two experimental conditions. A linear regression was used to analyse the repeated data (Figure 3A, 3D, 5C). The tumor progress is significantly different with regard to the two groups ($p=0.001$).

GTPase activity assays

C2C12 myoblasts or RD cells were processed as described to measure Rac1 activity (9).

Mice and xenografting

Female C3H HEN/HSD mice and athymic mice were purchased from Harlan Laboratories and used at 6-8 weeks of age. All mice were maintained at the animal facility of Centre de Recherche en Cancérologie de Montpellier, CRLC Val d'Aurelle-Paul Lamarque, Montpellier. Experimental procedures and handling were performed in a laminar flow hood for nude mice. For xenografts, 2×10^6 C2C12 cells or 5×10^6 RD cells were injected subcutaneously into the leg or in the thigh muscle. Tumors were detected by palpation and measured periodically with callipers and tumor volume deduced using the formula

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$D_1 \times D_2 \times D_3 / 2$, where D_1 is the length, D_2 the width and D_3 the depth of the tumor.

Results

R-cadherin expression inhibits myogenesis induction

To establish the role of R-cadherin in skeletal muscle cells, we made stable mouse C2C12 myoblasts expressing either R-cadherin fused to a GFP-tag (Rcad/GFP) or to a 2myc tag (Rcad/2myc). C2C12 myoblasts expressing GFP alone were used as control. The characterization of these clones is shown in supplementary Figure 1.

To gain further insight into the role of R-cadherin in myogenesis, we quantified the differentiation rate of parental, GFP- and Rcad/GFP-expressing myoblasts. Confluent C2C12 myoblasts were induced to differentiate by replacing the growth medium (GM) with differentiation medium (DM) and analyzed for myotube formation and expression of muscle-specific proteins. Two days after serum withdrawal (D2), myotubes were present both in parental (data not shown) and GFP-expressing C2C12 cells (Figure 1A, panel c). Numerous myotubes were visible 3-4 days after DM addition (panels d and e). In a sharp contrast, no myotubes were visible in Rcad/GFP expressing myoblasts (Figure 1A, bottom panels) even when differentiation conditions were maintained up to 7 days (data not shown), indicating that myoblast to myotube transition is efficiently blocked and not simply delayed. Similar data were observed in a separate Rcad/GFP-expressing clone and in 3 independent Rcad/2myc-expressing clones demonstrating that these effects were not due to clonal variations or influence of the tag (data not shown).

We next examined, whether R-cadherin could affect the expression of myogenin and troponin-T, two muscle-specific proteins. As expected, myogenin and troponin T were detected after 1 day and 2 days in DM, respectively, in parental and GFP-expressing C2C12 myoblasts, while C2C12 Rcad/GFP cells faintly expressed these proteins (Figure 1B and 1C). Taken together, these data suggest that R-cadherin expression blocks myogenesis induction.

R-cadherin expression inhibits myoblast cell cycle exit

Since myogenic differentiation and cell cycle progression are mutually exclusive events (30), we asked whether the failure of R-cadherin expressing C2C12 myoblasts to differentiate could be ascribed to a high proliferative rate retained in the differentiation conditions. No obvious differences in the proliferation rate of control and Rcad/GFP-expressing cells were observed in sub-confluent cells maintained in GM. In contrast, confluent Rcad/GFP-expressing C2C12 myoblasts proliferated significantly more than control cells cultured at similar density (Figure 2A). This effect was even sharper when cells were induced to differentiate demonstrating that R-cadherin acts positively on cell cycle progression, bypassing not only cell contact inhibition but also cell cycle exit induced by growth factor deprivation. Cyclins are key factors for cell cycle progression. Cyclin D1 plays a critical role by regulating the transit through the G1 phase of the cell cycle and its expression markedly decreases during the C2C12 myoblast differentiation process (31). Cyclin D1 was normally expressed both in proliferating control and Rcad/GFP-expressing C2C12 myoblasts and was reduced in control cells induced to differentiate (Figure 2B). In contrast, cyclin D1 expression was maintained in C2C12 Rcad/GFP cells cultured in DM. The maintenance of cyclin D1 expression in Rcad/GFP-expressing cells after DM addition was confirmed also by immunocytochemistry (Figure 2C). To test whether sustained cyclin D1 expression was due to transcriptional regulation of the promoter, we performed luciferase assays in which parental and Rcad/GFP-expressing C2C12 were transfected with the cyclin D1 promoter fused to the luciferase reporter gene. In proliferative conditions the level of cyclin D1 activation was comparable between control and C2C12 Rcad/GFP cells (Figure 2D). However, upon differentiation this level of activity was maintained only in R-cadherin-expressing C2C12 myoblasts, whereas in control cells the activity of the promoter diminished.

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Next, we examined the level of cyclin A expression, a cyclin that acts during S phase, which is rather linked to proliferation (32). Cyclin A was similarly maintained at high level in C2C12 Rcad/GFP-expressing myoblasts upon differentiation conditions, while was down-regulated in control cells (Figure 2B).

R-cadherin expression induces myoblast transformation and increases cell motility

Since R-cadherin induces the loss of cell contact inhibition and of the expression of cyclin D1, two characteristics of tumor cells (33), we asked whether R-cadherin might be sufficient to induce myoblast transformation. Therefore, we performed soft agar assay to analyze whether R-cadherin expression might affect anchorage-independent cell growth (Figure 3A, left panel). C2C12 Rcad/GFP cells were able to form colonies in soft agar, while parental C2C12 did not. To estimate the ability of R-cadherin to induce myoblast transformation *in vivo*, we transplanted either subcutaneously or in thigh muscles GFP- and Rcad/GFP-expressing C2C12 cells in mice. We monitored the appearance of tumors by palpation and we measured the size of the tumors formed. Two months after injection, the mice that received two different C2C12 Rcad/GFP clones had measurable tumors, while mice injected with control C212 GFP cells did not (Figure 3A, right panel). To assessed if R-cadherin expression on C2C12 cells could influence their migration as it was already reported for breast tumor cells (23), C2C12 cells that stably expressed GFP alone or R-cadherin-GFP were seeded at a low density and random cell movement was recorded by phase-contrast microscopy for 4 h (Figure 3B). Rcad/GFP-expressing cells showed a faster migrating rate compared with GFP-expressing cells indicating that R-cadherin increases C2C12 myoblast migration.

To gain further insight into the role of R-cadherin in muscle cell transformation in the pathological context of rhabdomyosarcoma, we generated a stable embryonal RMS cell line

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RD, in which the expression of R-cadherin was inactivated by RNA interference (RDsiRcad) (Figure 3C, left panel). Transformation assays showed that the number and the size of colonies were reduced in RDsiRcad cells in comparison to control RDsiLuc cells (Figure 3C, right panel). Similar data were obtained using a second R-cadherin shRNA (data not shown). Importantly *in vivo* experiments showed that RDsiRcad cells grew slower than control RD cells in nude mice as demonstrated by monitoring the size of the tumors (Figure 3D). Taken together these data show that i) R-cadherin expression induces myoblast cell transformation *ex vivo* and *in vivo* and that ii) inhibition of the R-cadherin expression in the RD cell line significantly decreases its transforming activity.

R-cadherin expression activates Rac1 GTPase

To examine whether the R-cadherin-dependent cell-cell contacts might control the Rho GTPase activity, we used the organization of the F-actin cytoskeleton as a functional read-out (Figure 4A). An increase of lamellipodia was detected in C2C12 Rcad/GFP cells (panel d) when compared to control C2C12 GFP myoblasts (panel b). In addition, lamellipodia were detected in C2C12 Rcad/GFP cells plated onto dishes coated with R-cad-Fc ligand when compared to C2C12 Rcad/GFP cells plated onto dishes coated with anti-Fc antibody (Fc) (compare panels f and e and see Supplementary Online Videos). This result suggests that Rac1 could be activated by the forced R-cadherin expression in C2C12 myoblasts and, thus, led us to assess Rac1 activity using pull-down assays. Rcad/GFP-expressing myoblasts showed an increase in the level of activated Rac1 relative to control GFP-expressing cells (Figure 4B, left panel). To further confirm that R-cadherin engagement activates Rac1, isolated Rcad/GFP-expressing C2C12 cells were plated on dishes coated either with an Fc fragment (Fc) or the R-cad-Fc ligand, which allow us to mimic R-cadherin-mediated adhesion. Two hours after plating on R-cad-Fc, we observed a marked Rac1 activation

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(Figure 4B, right panel). We then wanted to know whether the Jun N-terminal kinase pathway (JNK), a downstream target of Rac1, had been activated in the R-cadherin-expressing C2C12 cells. The phosphorylation status of the c-Jun transcription factor was analyzed by immunoblotting parental C2C12 and C2C12 Rcad/GFP cellular extracts at different times of differentiation. Both parental and R-cadherin-expressing C2C12 myoblasts cultured in GM showed a marked phosphorylation of c-Jun (Figure 4C). Upon DM addition, c-Jun phosphorylation decreased in control cells whilst it was noticeably maintained in C2C12 Rcad/GFP myoblasts, demonstrating that R-cadherin activates the JNK pathway in myoblasts. In parallel, we used extracts from the RMS cell line RD in which R-cadherin expression was inactivated by RNA interference. Significantly, the down-regulation of R-cadherin expression in the RMS cell line RD was accompanied by a lower Rac1 activity level, indicating that R-cadherin is partly accountable for the Rac1 activity in RD (Figure 4D).

Rac1 inhibition decreases R-cadherin-dependent signaling

We next investigated the effect of Rac1 inhibition on R-cadherin-dependent pathways. First, we analyzed the effect of dominant negative Rac1 (Rac1N17) on R-cadherin-induced cyclin D1 expression. C2C12 Rcad/GFP myoblasts were transfected with empty pRFP or RFP-tagged Rac1N17 and induced to differentiate by addition of DM. Cells were fixed 2 days thereafter, and analyzed for cyclin D1 expression. Cyclin D1 was detected in less than 20% of cells expressing Rac1N17 (Figure 5A, panels d-f and Figure 5B) whereas it was expressed in almost 50% of the parental cells (data not shown) and of the cells transfected with empty pRFP (Figure 5A, panels a-c and Figure 5B). A comparable inhibition of R-cadherin-induced cyclin D1 expression was obtained using the specific Rac inhibitor NSC23766 ((34), data not shown). Moreover, mice injected with Rac1N17-infected C2C12 Rcad/GFP myoblasts

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developed significantly smaller tumors than mice injected with C2C12 Rcad/GFP cells (Figure 5C).

Taken together, these findings emphasize that the R-cadherin-induced Rac1 activation is critical for R-cadherin-induced cyclin D1 expression and for myoblast transformation *in vivo*.

R-cadherin expression down-regulates N- and M-cadherin

Cadherin switching has been implicated in tumorigenesis (15, 16). We thus analyzed whether R-cadherin influences expression of the two main cadherins, i.e. N- and M-cadherin, present in C2C12 myoblasts. C2C12 Rcad/GFP and C2C12 Rcad/2myc cells expressed significantly less M-cadherin (Figure 6A). Both N- and M-cadherin accumulated less at cell contacts in R-cadherin-expressing myoblasts (Figure 6B, compare panel a to panel d, and panel b to panel f). Taken together, these data show that i) R-cadherin decreases the expression of M-cadherin, and that ii) N- and M-cadherin only slightly accumulate at the cell contacts in R-cadherin-expressing myoblasts.

Cadherin switching induced by R-cadherin has been linked to competition for p120^{ctn}, a mechanism in which p120^{ctn} plays the role of a rheostat to regulate the levels of cadherin in the cell (35). To test this hypothesis in our system we generated stable mouse C2C12 myoblasts expressing the R-cadherin mutant (Rcad-AAA) that do not interact with p120^{ctn} (35). Ten clones were established that behave similarly, shown are the data obtained with one of them. Figure 6C shows that expression of Rcad-AAA in C2C12 cells did not downregulate M-cadherin in C2C12 cells. Moreover C2C12 expressing Rcad-AAA cells expressed normal levels of troponin T and myogenin upon addition of differentiation medium, and fused normally compared to parental myoblasts (data not shown). In contrast, M-cadherin as well as myogenin and troponin T levels were significantly decreased in C2C12 cells expressing R-cadherin. We show that p120^{ctn} level did not change when the cells expressed wild-type or

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mutant R-cadherin. Finally, expression of Rcad-AAA did not increase Rac1 activity in contrast to wild-type R-cadherin (right panel).

Since Rac1 is activated by R-cadherin, we assessed if Rac1 could also be implicated in cadherin switching mediated by R-cadherin in C2C12 cells. As shown in Figure 6D, Rac1 inhibition, using the specific Rac inhibitor NSC23766 (34), led to an increase of M-cadherin expression and to a relocalization at the membrane junction of N- and M-cadherin. No modification of M- and N-cadherin expression was observed in parental C2C12 myoblasts treated with NSC23766 (Supplementary Figure 2). This suggests that Rac1 activation contributes to the mechanism of cadherin switching mediated by the expression of R-cadherin in C2C12 cells.

Discussion

The data presented here provide evidence that R-cadherin is a key player in myogenesis inhibition and myoblast transformation. R-cadherin has been detected in developing skeletal muscles and it might be implicated in the formation of this tissue since its expression can rescue striated muscle in embryonic stem cells that lack E-cadherin (12). On the other hand, its expression in C2C12 myoblasts inhibits myogenesis induction and causes their transformation. Moreover, we have previously observed R-cadherin expression in RMS cell lines and biopsies, while this protein is absent from normal adult tissues (13). This suggests that R-cadherin has to be tightly controlled during skeletal muscle development. In this study, we further demonstrate that R-cadherin is a major actor of tumorigenesis and/or tumor progression in muscle cells because i) its expression is able to drive myoblast transformation *ex vivo* and *in vivo* ii) R-cadherin inhibition in the RMS RD cells decreases their tumorigenic potential *in vivo*. Our results show that R-cadherin expression in C2C12 myoblast is sufficient to induce their transformation and to increase their motility, as it was recently reported in various epithelial tumor cell lines (23). The present study is the first demonstration of such an « oncogenic potential » in normal cells. This suggests that whereas R-cadherin might be important during embryonic development for the proliferation and/or the migration of muscle precursor cells, its deregulation can participate in developmental abnormalities and also in pathological processes such as RMS formation.

In addition, we deciphered a novel pathway, in which R-cadherin activates Rac1, an event that induces muscle cell transformation through the expression of cyclin D1. Johnson et al. (2004) previously reported that forced expression of R-cadherin in breast tumor cells acts positively on cell migration through a Rac1-dependent pathway (23). Thus, by activating Rac1 and consequently both the growth and the migration of tumor cells, R-cadherin is likely to function as a potent oncogene in muscle cells. The implication of Rac1 in myoblast

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transformation has been previously described, since a constitutively active form of Rac1 was able to induce rat L6 myoblast cells transformation and constitutive Rac1 activation was found in RMS cells (9). Here we show that i) inactivation of R-cadherin expression in RMS cells decreases Rac1 activation and, as a consequence, their transformation ability *in vivo* is reduced ii) inhibition of Rac1 in R-cadherin-expressing cells decreases their tumorigenic potential *in vivo*, validating the notion that R-cadherin in the pathological context of RMS might play a critical role in tumor initiation and progression through a Rac1-dependent mechanism. In contrast, RhoA activity is decreased by R-cadherin expression (data not shown) supporting the existence of a balance between Rac1 and RhoA activities downstream of R-cadherin.

Tumor cells often show a decrease in cell-cell and/or cell-matrix adhesion. An increasing body of evidence now indicates that aberrant cell adhesion is causally involved in tumor progression and metastasis, rather than merely being a consequence of it (36). Amongst these adhesion defects, cadherin switching plays a critical role in the progression of some tumors (15). In particular, the switch from E- to N-cadherins has been shown to enhance the motility, invasiveness and metastatic potential of cancer cells (37). Moreover, P-cadherin overexpression promotes N-cadherin downregulation and consequently facilitates the motility of pancreatic cancer cells (38). These changes in cadherin expression not only modulate tumor cell adhesion, but also affect signal transduction and hence tumor malignancy. Such a cadherin switching was observed in RMS cell lines and biopsies since we detected R-cadherin expression concomitant with a perturbation in N- and M-cadherin function (13). Here we demonstrate that this cadherin switch is induced by R-cadherin expression in myoblasts, suggesting that R-cadherin deregulation during muscle development might be sufficient to induce the loss of N- and M-cadherin-mediated cell-cell adhesion and to support tumour transition towards malignancy. In this regard, beside the control of myogenesis induction, N-

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cadherin-dependent signaling mediates contact inhibition of cell growth through the cyclin-dependent kinase inhibitor p27Kip1 (8, 39, 40). p120^{ctn} and Rac1 play an important role in the molecular mechanisms by which R-cadherin induces N- and M-cadherin down-regulation. Use of an R-cadherin mutant that could not bind p120^{ctn} does not perturb the distribution of N- and M-cadherin at the membrane of C2C12 cells as well as their ability to undergo differentiation. Since p120^{ctn} seems to be limiting in the cell, its binding to exogenously expressed R-cadherin could lead to the destabilization and degradation of N- and M-cadherin. A similar mechanism has been described recently in epithelial cells where ectopic expression of R-cadherin induces down regulation of E- and P-cadherin by competition for p120^{ctn} (35). We also show that Rac1, which is activated by R-cadherin expression, is implicated in this process since Rac1 inhibition clearly restores the levels of M-cadherin in R-cadherin expressing C2C12 cells. Additional unidentified pathways might be activated downstream of R-cadherin since Rac1 activation alone is not sufficient to induce M-cadherin degradation whereas it leads to M and N-cadherin delocalization from the cell-cell contacts (Supplementary Figure 3).

R-cadherin has recently emerged as a marker of RMS (13, 41). It remains to be determined whether R-cadherin has been maintained due to the development stage of RMS, or whether it has been re-expressed in these cells. Interestingly, mice that express, in postnatal terminally differentiated Myf6-positive myofibers, the fusion protein *Pax3/Fkhr*, issued from a chromosomal translocation found in alveolar RMS, later develop such tumors (42). Therefore, altered expression or activity of transcription factors, which regulate the R-cadherin promoter, might influence the expression of R-cadherin either in muscle precursor cells or in cells already engaged in the differentiation program. The R-cadherin promoter has recently been characterized and contains at least three Pax DNA-binding consensus sites (26). Moreover, the paired box gene *Pax-6* appears to be an important regulator of R-cadherin

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expression in neurons (43). Interestingly, Pax3 and Pax7 are required for the proper migration and specification of myogenic precursor cells and to prevent terminal differentiation (44). In addition, Pax7 is a key regulator of satellite-cell self-renewal as it promotes their proliferation and inhibits their differentiation (45). Pax3 and Pax7 expression has also been reported in RMS and seems involved in the proliferation and survival of these tumor cells (review in (46)). It will be interesting to analyze the potential cross-talk between *Pax* genes and R-cadherin in skeletal muscle cells or to see whether Pax3 and/or Pax7 might control R-cadherin expression by using the various RMS mouse models available.

Another crucial issue is to explore the molecular mechanisms of the functional differences of cell-cell adhesion induced by N- and R-cadherins in myoblastic cells. Recent data which demonstrate an interaction between cadherins and tyrosine kinase receptors suggest that changes in cadherin expression may not only modulate tumor cell adhesion but also affect signal transduction and, hence, the malignant phenotype. Analyzing the interaction of these cadherins with various tyrosine kinase receptors and in particular c-met, due to its role in fetal myoblasts proliferation and tumorigenesis (47) might help in the understanding the processus of R-cadherin-mediated rhabdomyosarcogenesis.

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Figure legends

Figure 1: R-cadherin expression blocks C2C12 myoblasts differentiation.

A- B) GFP- or Rcad/GFP-expressing C2C12 myoblasts were maintained in growth medium containing 10% FBS up to confluency (P) and then shifted to differentiation medium (DM).

A) Pictures were taken during 4 days (D1-D4) using an inverted microscope to follow the number of myotubes formed.

B) Cells were also fixed and stained for Myogenin and Troponin T expression. Bar: 10µm.

C) 50 µg of cell lysates were used in Western blot analysis to probe for expression of Myogenin and Troponin T.

Figure 2: R-cadherin induces C2C12 hyperproliferation in differentiation conditions.

A) GFP- or Rcad/GFP-expressing C2C12 were seeded at different stages of confluency. For the D1 conditions, cells were shifted to differentiation medium (DM) for 24 hours when 100% confluency was reached. Cells were incubated for 8 hours (overgrown and D1 conditions) or 2 hours (proliferative conditions, GM) with BrdU, a thymidine analog. ** $p < 0.05$, *** $p < 0.001$.

B) C2C12 GFP or C2C12 Rcad/GFP myoblasts were grown in DM for up to 4 days. At the indicated times, lysates were prepared and Western blot analysis was performed to probe for expression of cyclin D1 and cyclin A. P: proliferative condition; D: day in DM.

C) Immunofluorescence micro-photographs of Hoescht (panels a and c) and cyclin D1 (panels b and d) staining on control and Rcad/GFP expressing C2C12 myoblasts grown in DM for 2 days. The histogram represents the % of cyclin D1-positive cells in proliferative conditions and two days after DM addition and summarizes the data from three independent

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sets of experiments; 100-200 cells were analyzed in each experiment. ** $p < 0.05$, *** $p < 0.001$. Bar: 10 μ m.

D) The luciferase reporter gene driven by the full-length wild-type (-944) cyclin D1 promoter was transfected in parental and Rcad/GFP-expressing C2C12 cells at different stage of confluency. Firefly luciferase activity was analyzed in cells cultured in GM or in DM for 24 hours as described in Material and Methods. The results are representative of at least three independent experiments. *** $p < 0.001$.

Figure 3: R-cadherin expression is sufficient to induce myoblast transformation *in vivo*

A) Left: The ability to form colonies in soft agar of control or Rcad/GFP-expressing myoblasts was analyzed. Shown are typical phase-contrast microscopy images. The histogram represents the quantification of the colonies obtained from three independent experiments. *** $p < 0.001$.

Right: Control GFP- or Rcad/GFP-expressing C2C12 clones were inoculated either subcutaneously or by injection into the thigh of C3H mice ($2 \cdot 10^6$ cells/mouse) and tumor size was measured with calipers at the indicated times. Results represent the kinetic of tumor growth (in mm³) as the mean \pm SEM in either 6 mice for subcutaneous injection of C2C12 GFP and for thigh muscle injection of C2C12-Rcad/GFP or 9 mice for subcutaneous injection of C2C12-Rcad/GFP. Images show a mouse with a tumor 65 days after injection of Rcad/GFP-expressing cells (arrow) and a tumor after its removal.

B) Box-and-whisker plot showing the distance covered by control GFP- or Rcad/GFP-expressing C2C12 myoblasts during the recording (left) and their migrating speed (right). $N \geq 80$ for each point. ** $p < 0.01$.

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C) Left: R-cadherin siRNA (RDsiRcad) and control luciferase siRNA (RDsiLuc) were delivered in RD cell lines by retroviral infection. Cells were selected and pools of resistant cells were analyzed for the expression of R-cadherin and α -tubulin by immunoblot analysis. Right: Phase-contrast microscopy images of colony growth in soft agar from RDsiLuc or RDsiRcad cells. The histogram represents the quantification of the colonies obtained from three independent experiments. *** $p < 0.001$.

D) Pools of control RDsiLuc or RDsiRcad cells were inoculated subcutaneously into nude mice and tumor size was measured with calipers at the indicated days. Tumor volume is expressed as the mean \pm SEM of 5 mice.

Figure 4: Overexpression of R-cadherin induces Rac1 activation in C2C12 myoblasts.

A) Control GFP- (panels a and b) and Rcad/GFP-expressing C2C12 myoblasts (panels c and d) were stained for F-actin distribution with rhodamine-labeled phalloidin (panels b and d). Rcad/GFP-expressing C2C12 myoblasts, which have been transfected with mRFP-actin, were plated onto anti-Fc-antibody (panel e) or R-cad-Fc (panel f) coated dishes. Bar: 10 μ m.

B) The level of GTP-bound Rac1 was measured using GST-Pak CRIB in lysates obtained from C2C12 GFP or Rcad/GFP myoblasts (left panel) or 2 h after plating the C2C12 Rcad/GFP cells on culture plates whose surface has been coated with either a human Fc fragment or a chimeric Rcadec-hFc, a protein obtained by fusing the R-cadherin ectodomain with a human Fc fragment (right panel). Rac1 and α -tubulin were detected by immunoblotting. The data are representative of three independent experiments.

C) Parental C2C12 cells or Rcad/GFP C2C12 cells were maintained in a proliferative state or induced to differentiate and immunoblotting was performed to detect c-Jun phosphorylation using an antibody against the phosphorylated fraction of c-Jun. As a positive control, anisomycin-treated C2C12 myoblasts were analyzed in parallel.

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D) The level of GTP-bound Rac1 in RDsiLuc or RDsiRcad cells was measured as described in B.

Figure 5: Inhibition of Rac1 decreases R-cadherin-induced transformation

A) C2C12 Rcad/GFP cells transfected with empty pRFP (panels a-c) or with RFP-Rac1N17 (panels d-f) were grown in DM for 48 hours. Cells were stained for cyclin D1 expression (panels c and f) and for DNA with Hoechst (panels a and d). Bar: 10 μ m. Arrows indicate cyclin D1 positive nuclei and the arrowhead a cyclin D1 negative nucleus.

B) Quantification for cyclin D1 expression in C2C12 Rcad/GFP-expressing myoblasts transfected with pRFP or with RFP-Rac1N17 as shown in A. Around 100 cells were analyzed for each condition. The data are representative of at least three independent experiments. *** p < 0.001.

C) C2C12 Rcad/GFP cells alone or C2C12 Rcad/GFP cells infected with retroviral vectors encoding for Rac1N17 were inoculated subcutaneously into nude mice and tumor size was measured with calipers at the indicated days. Tumor volume is expressed as the mean \pm SEM of 3 mice. The tumor progress is significantly weaker for the mice injected with C2C12 Rcad/GFP+Rac1N17 with regard to those injected with C2C12 Rcad/GFP (p=0.001).

Figure 6: Ectopic expression of R-cadherin downregulates N- and M-cadherin in C2C12 myoblasts.

A) Protein extracts (20 μ g/well) from parental, Rcad/GFP- and Rcad/2myc-expressing C2C12 myoblasts were immunoblotted to check for the expression levels of R-cadherin, M-cadherin and α -tubulin. The data are representative of at least three independent experiments.

B) N- and M-cadherin localization was analyzed by indirect immunofluorescence in control C2C12 (a and c) and C2C12 Rcad/2myc (c-f). Bar: 10 μ m.

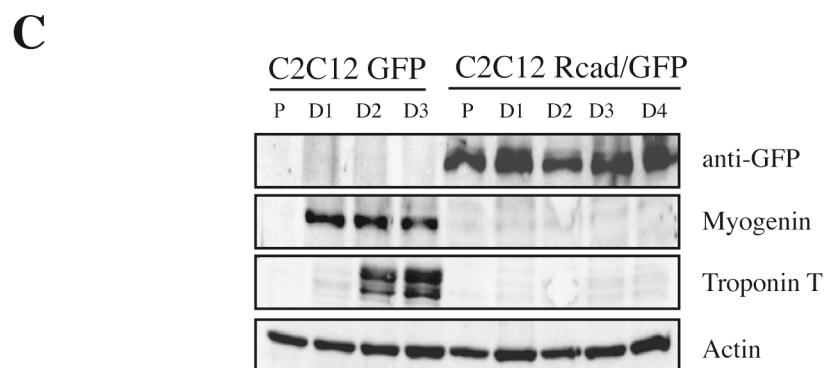
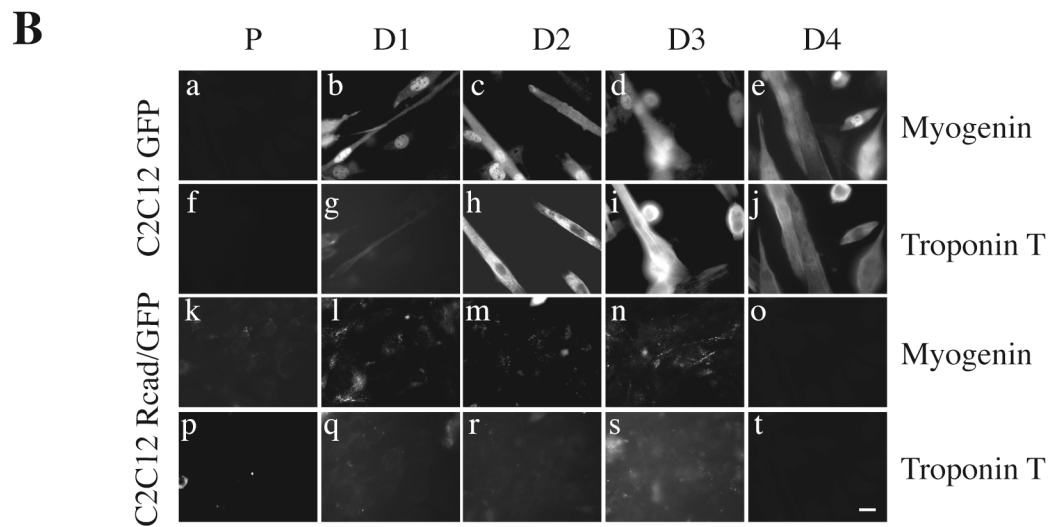
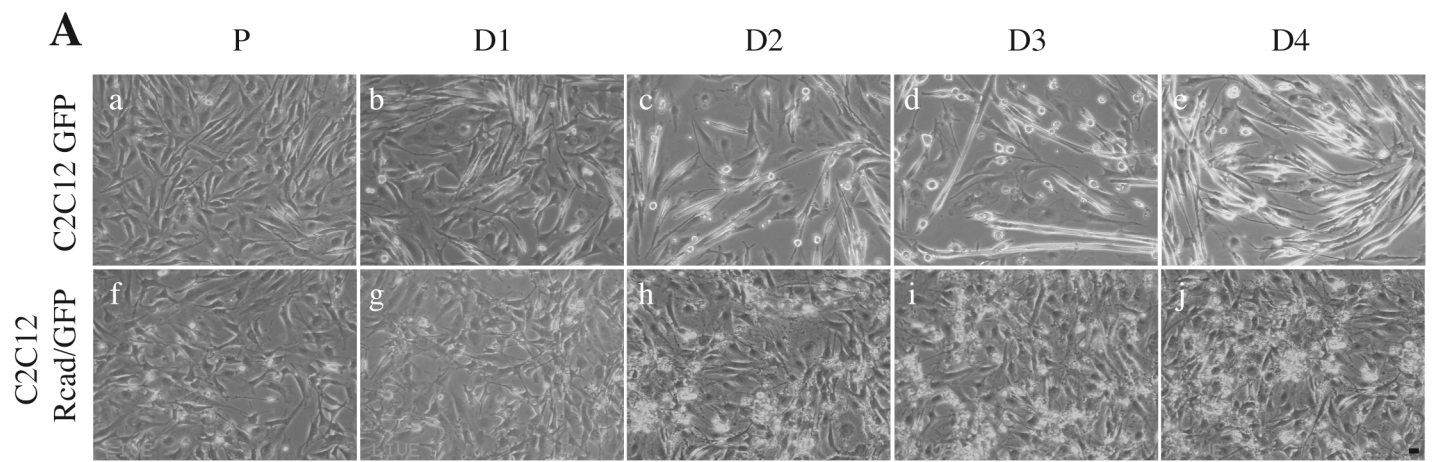
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C) Left: Protein extracts (20µg/well) from parental, Rcad/2myc and RcadAAA/2myc-expressing C2C12 myoblasts grown in DM for 3 days were immunoblotted to check for the expression levels of myc, M-cadherin, myogenin, troponin T, p120^{ctn} and Actin.

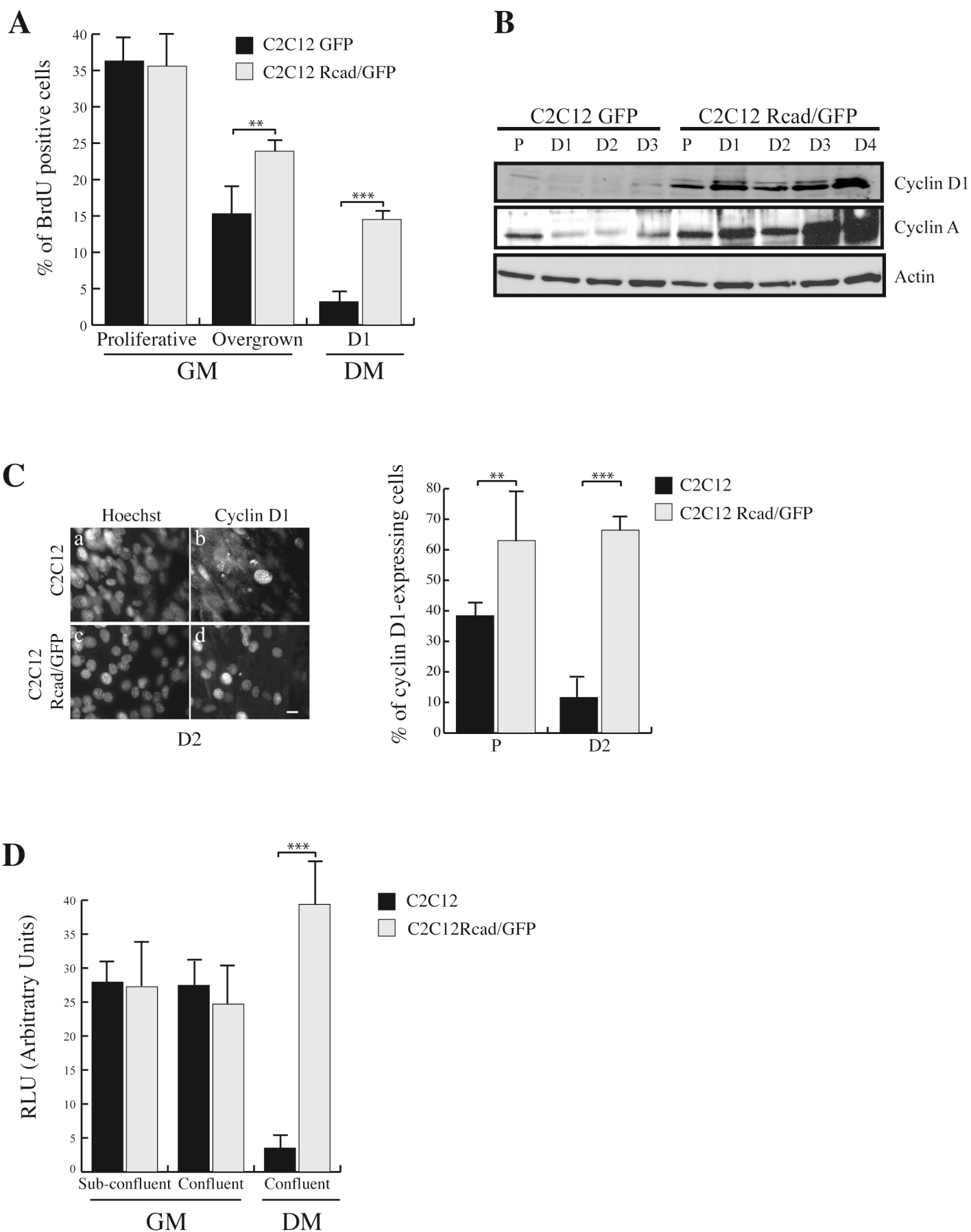
Right: The level of GTP-bound Rac1 was measured in lysates obtained from parental, Rcad/2myc and RcadAAA/2myc-expressing C2C12 myoblasts. Rac1 was detected by immunoblotting.

D) Left: Protein extracts (20µg/well) from parental and Rcad/2myc-expressing C2C12 myoblasts treated with the Rac inhibitor NSC23766 as indicated were immunoblotted to check for the expression levels of M-cadherin and α -tubulin. The data are representative of at least three independent experiments.

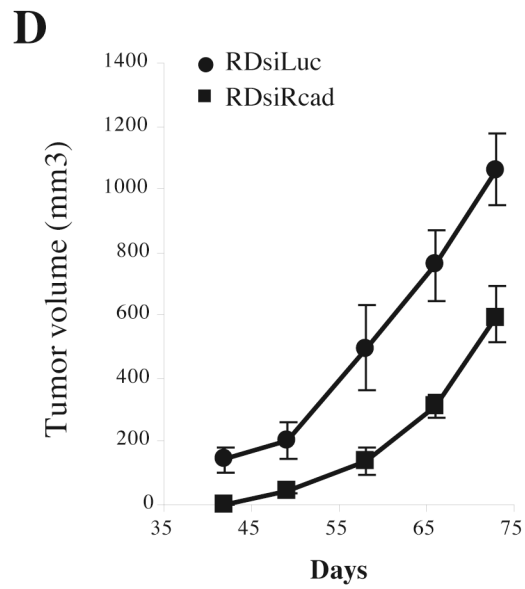
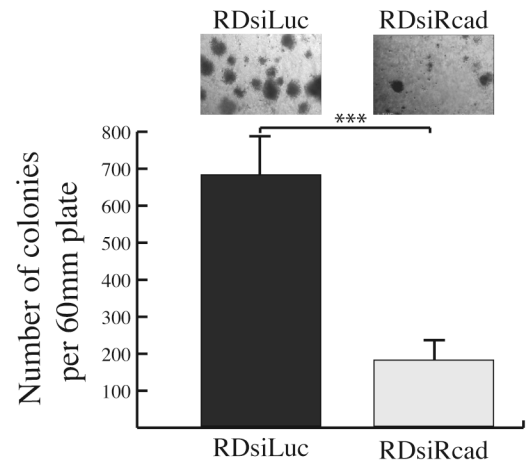
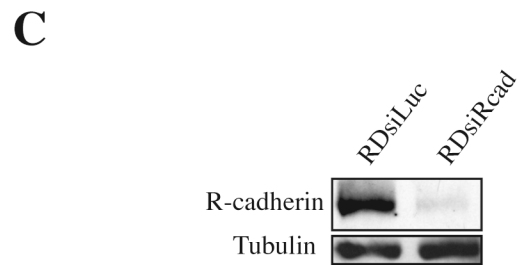
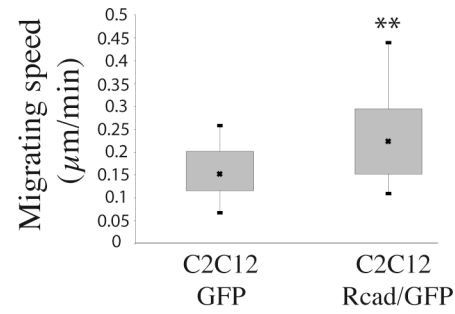
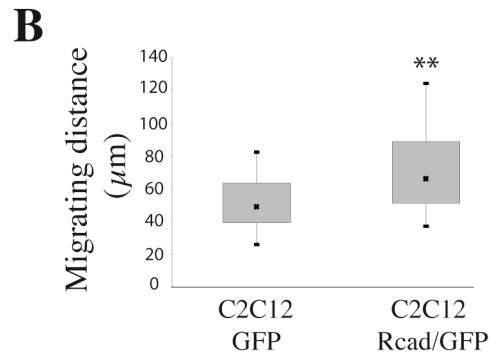
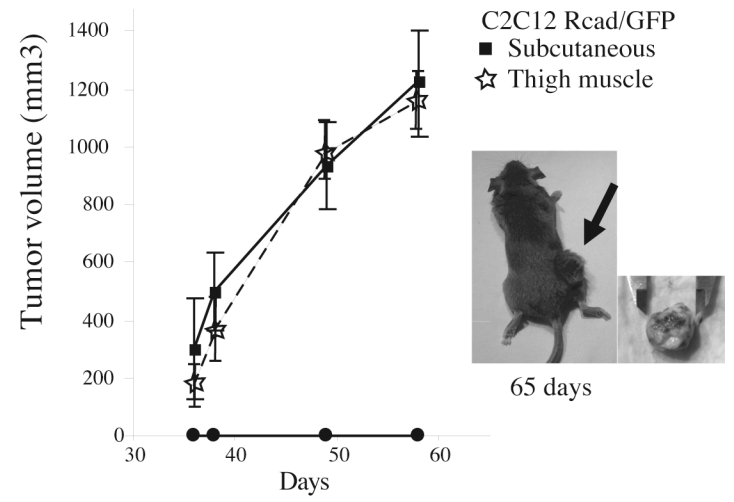
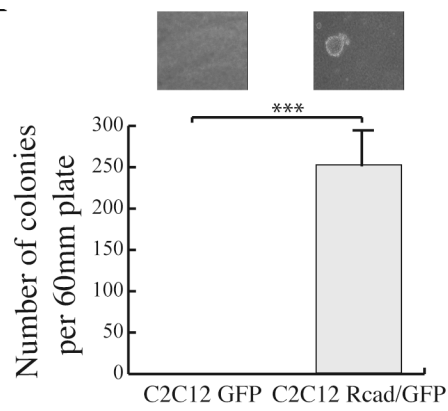
Right: Rcad/2myc-expressing C2C12 myoblasts either untreated (panels a, c) or treated (panels b, d) with the Rac inhibitor NSC23766 for 24 hours were analyzed for N- and M-cadherin distribution. Bar: 10µm.

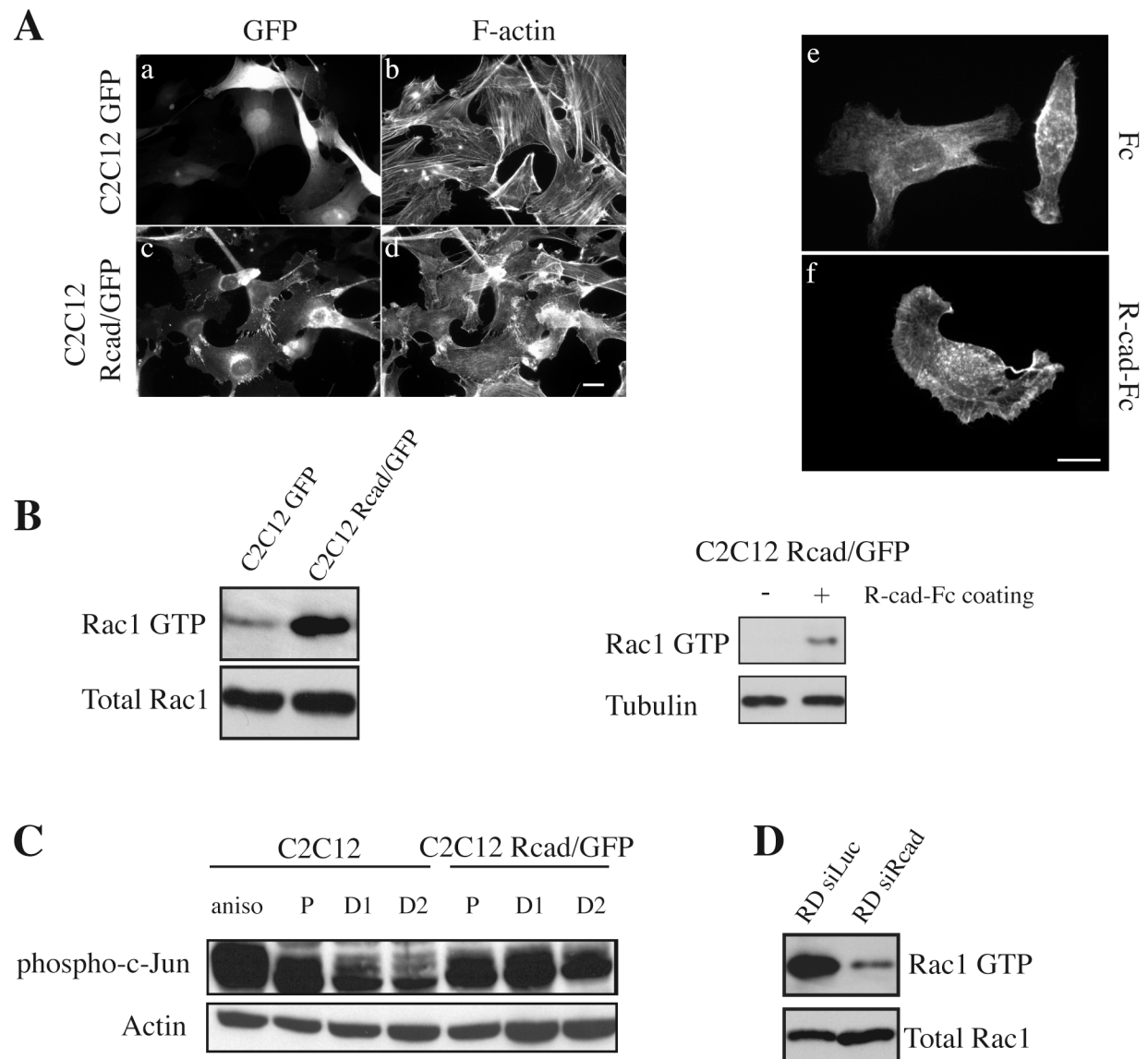


Kucharczak, Figure 1

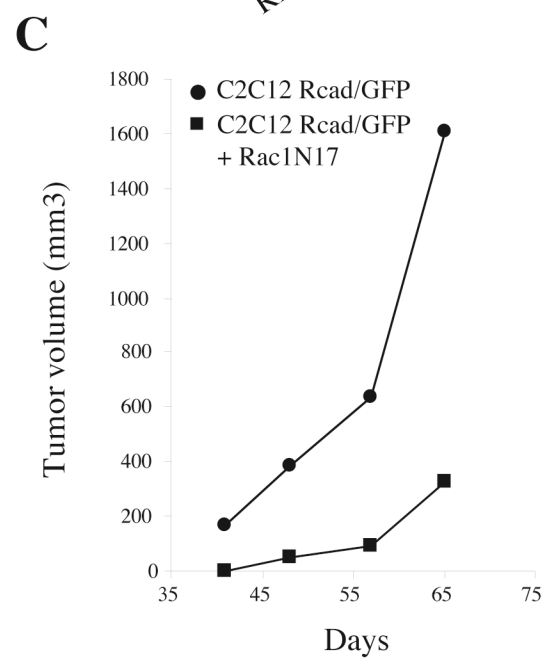
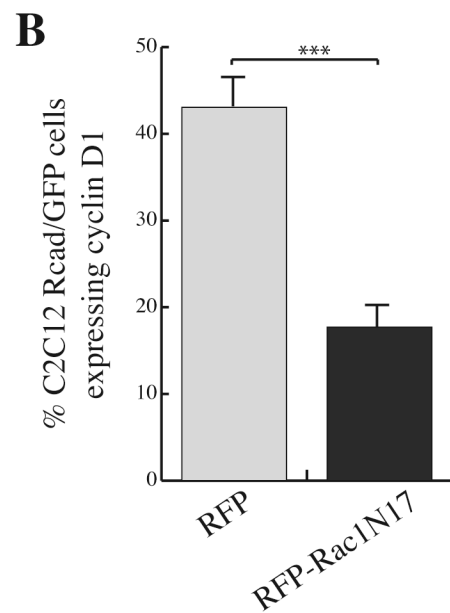
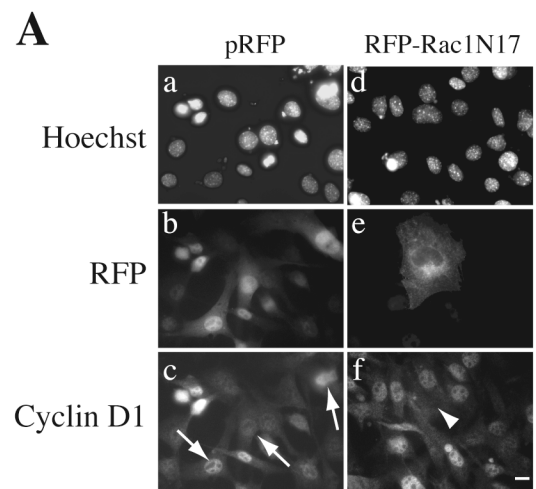


Kucharczak, Figure 2

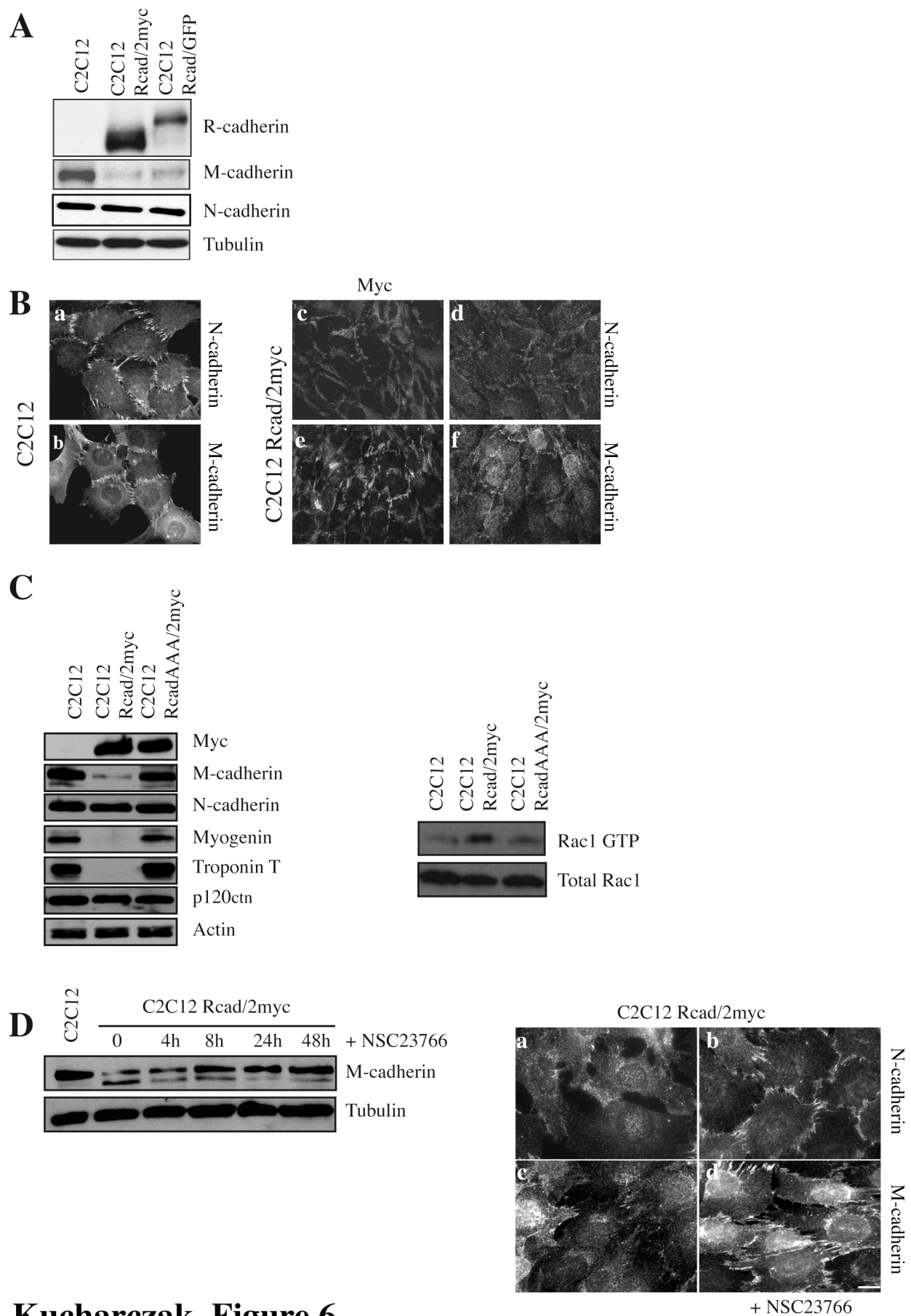




Kucharczak, Figure 4



Kucharczak, Figure 5



Kucharczak, Figure 6