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Running title: Erythroblast maturation and constitutive ROCK-1

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ABSTRACT

Stem cell factor (SCF) and erythropoietin (EPO) are strictly required for preventing apoptosis and stimulating proliferation, allowing the differentiation of erythroid precursors from CFU-E to the polychromatophilic stage. In contrast, terminal maturation to generate reticulocytes occurs independently of cytokine signaling by a mechanism not fully understood. Terminal differentiation is characterized by a sequence of morphological changes including a progressive decrease in cell size, chromatin condensation in the nucleus and disappearance of organelles, which requires transient caspase activation. These events are followed by nucleus extrusion as a consequence of plasma membrane and cytoskeleton reorganization. Here we show that in early step, SCF stimulates the Rho/Rock pathway until the basophilic stage. Thereafter, ROCK-1 is activated independently of Rho signaling by caspase-3-mediated cleavage, allowing terminal maturation at least in part through phosphorylation of the light chain of myosin II (MLC2). Therefore, in this differentiation system, final maturation occurs independently of SCF signaling through caspase-induced ROCK-1 kinase activation.

Keywords

Erythropoiesis, ROCK-1, caspases

Introduction

Erythropoiesis is a multistep process that occurs in the bone marrow and involves the sequential formation of proerythroblasts - basophilic, polychromatic and orthochromatic - erythroblasts and enucleated reticulocytes. Terminal erythroid differentiation is characterized by hemoglobin synthesis and major morphological changes, including chromatin condensation, cell size reduction and finally, nucleus extrusion to generate reticulocytes that enter in the blood circulation [1]. This differentiation process involves the transcription factor GATA-1 [2 3], which positively regulates promoters of erythroid genes such as glycophorin A, the erythropoietin receptor (EPO-R) and hemoglobin chains.

Some features of terminal differentiation of erythroid cells are similar to features associated with apoptosis, such as chromatin condensation, organelle loss and membrane remodeling. We and others have previously demonstrated that transient activation of effector caspases at the basophilic stage is required for terminal erythroid maturation [4 5 6]. Caspases induce cleavage of several proteins involved in the nuclear structural changes observed during terminal erythroid differentiation, such as lamin B, acinus or PARP [7]. In contrast, GATA-1, a target of caspases during apoptosis, is not cleaved during differentiation, allowing expression of erythroid genes and survival. This protection of GATA-1 from cleavage by caspases is explained by the translocation into the nucleus at the onset of caspase activation of the chaperone protein HSP70. At this stage, HSP70 interacts physically with GATA-1 resulting in protection against caspase cleavage [8].

Identification of each caspase substrate might be helpful for understanding the mechanisms that determine the fate (differentiation versus apoptosis) of erythroid cells in different physiological or pathological conditions.

Erythropoiesis is regulated by a number of growth factors. Two of these growth factors are

critical and have distinct and non-redundant functions, namely the c-KIT ligand or stem cell factor (SCF) [9] and erythropoietin (EPO) [10]. SCF induces an increase in cell survival and expansion of BFU-E and CFU-E, and slows down the differentiation of these cells towards the basophilic stage [11] [10]. Thereafter, cell-surface c-KIT expression is down-modulated [12]. EPO, which acts synergistically with SCF from late BFU-E until the basophilic stage is strictly required for preventing apoptosis and stimulating proliferation, allowing differentiation of erythroid progenitors and precursors, from CFU-E until the polychromatophilic stage [13]. In contrast, terminal maturation from polychromatophilic erythroblasts to reticulocytes occurs independently of signaling by these two cytokines.

This cytokine independence of terminal maturation of erythroid precursors is a peculiar phenomenon that may depend *in vivo* on integrins signaling for erythroid progenitors proliferation. However, it remains to be understood by which mechanisms orthochromatic erythroblasts undergo cytoskeleton reorganization, specifically contractile actin ring formation for extrusion of pycnotic nucleus, a process highly dependent on kinases signaling.

Small GTPases of the Rho family (RhoA, Rac, Cdc42) transduce signals from receptors at the plasma membrane to the actin and microtubule cytoskeleton as well as to kinase cascades that regulate cell morphology and many different processes such as motility and cell cycle progression. Upon activation, Rho proteins exchange GDP for GTP, transduce signals to downstream effector proteins and finally return to the inactive GDP-bound form by hydrolyzing the bound GTP [14] [15] [16]. Whereas RhoA, Rac1 or Cdc42 are ubiquitously expressed, Rac2 expression is confined to the hematopoietic compartment [17]. Rac GTPases are dynamic regulators of erythrocyte cytoskeleton organization [18] and are required in mice for early erythropoietic expansion in the bone marrow but not in the spleen. Rac1 and Rac2 deficiency may disrupt signaling downstream of SCF and IL3 to affect bone marrow erythropoiesis. By contrast, the splenic microenvironment can circumvent this deficiency [19].

Rac GTPases are required for the formation of the contractile actin ring and enucleation of mouse fetal erythroblasts [20]. In contrast, the role of RhoA has not been investigated in erythroblasts.

Two isoforms of a serine/threonine kinase (Rho-associated kinases ROCK-1 and ROCK-2) have been identified as effectors of Rho. In non-muscle cells, ROCK proteins regulate several cellular functions downstream of Rho and control actin cytoskeleton assembly and cell contractility [21]. Thereby, they contribute to several physiological processes by phosphorylation of various substrates such as MLC2 (myosin light chain 2) phosphatase, LIM kinase, and ERM (ezrin–radixin–moesin) proteins. Phosphorylation of the regulatory MLC of myosin II induces its interaction with actin, which thereby activates myosin ATPase and results in enhanced cell contractility [22]. Both ROCK-1 and ROCK-2 regulate MLC2 phosphorylation either directly by phosphorylating MLC2 on Ser19 or indirectly by inhibiting MLC phosphatase [23]. Cleavage of the C-terminal domain of ROCK proteins results in the generation of a constitutively active kinase. This occurs *in vivo* during the execution phase of apoptosis, when ROCK-1 but not ROCK-2 is cleaved by caspase-3, inducing membrane blebbing [24 25].

Because terminal erythroid differentiation is associated with actin filaments contractility and caspase activation, we wondered (i) whether the Rho/ROCK signaling pathway could be involved in differentiation of erythroid progenitors (ii) whether ROCK-1 could be a caspase target. In the present study, we show that inhibition of ROCK-1 but not Rho activity impairs erythroblasts terminal maturation. In addition, our data reveal that the Rho pathway is primarily activated by SCF/c-KIT during the early stage of erythroid precursors maturation. Caspase-3-mediated cleavage of ROCK-1 allows constitutive activation from the basophilic stage to mature cells, at the time of extinction of the c-KIT/Rho pathway. Therefore, we identified an additional role of caspase activation during erythroid differentiation, allowing

cytokine independency.

Materials and methods

Reagents and antibodies

Y-27632 and blebbistatin were from Calbiochem. Z-VAD-fmk was from Alexis Biochemicals. The cell permeable Rho inhibitor C3 transferase was from Cytoskeleton. The following antibodies were used for western blots: anti-MLC2 (FL-172) from Santa Cruz, anti-phospho-MLC2 monoclonal antibody from Cell signaling, anti-ROCK-1 monoclonal antibody from BD Biosciences, anti-Rho monoclonal antibody from Cytoskeleton, anti-HSC70 from Stressgen, anti-caspase 3 from Neomarker, c-KIT and phospho-c-KIT from Cell Signaling.

In vitro generation of erythroid cells

Umbilical cord blood units from normal full-term deliveries were obtained after informed consent of the mothers from the Obstetrics Unit of Hôpital Necker-Enfants Malades. Erythroid cells were generated with a two–step amplification culture system as previously described [4] with the following modification: Because 20 to 35% of the cells were GPA+ after the first step of CD34+ expansion, to better synchronize the culture, we isolated CD36+GPA$^-$ progenitors at the end of the first step. For this purpose, cells were stained with anti-CD36-APC monoclonal antibody (mAb) (BD Pharmingen) and anti-glycophorin A-PE mAb (BD Pharmingen) and the CD36+ GPA- fraction was purified by cell sorting (Facs ARIA Becton Dickinson) (day 0). These cells were subsequently cultured for 10 to 12 additional days in the presence of IL-3, SCF and EPO. To investigate the role of ROCK during terminal differentiation, the ROCK inhibitor Y-27632 was added at a concentration of 10 μM in the second step of the culture and the cells were diluted every day with fresh medium without or

with Y-27632.

Cell proliferation, differentiation and apoptosis assay

Cell proliferation was assessed by counting cells every day after trypan blue dye exclusion staining. Apoptosis was assessed by annexin V binding and propidium iodide (PI) or 7AAD staining.

Differentiation was assessed by morphological analysis after May-Grünwald- Giemsa coloration. Cells were examined under a Leica DMRB microscope with a PLFluotar 40X oil objective. The number of mature cells (polychromatic and orthochromatic erythroblasts) was assessed in each experiment by counting 300 cells in consecutive oil immersion fields and is expressed as a percentage of total cells.

Differentiation was also evaluated after dead cell and debris exclusion by flow cytometric analysis, which allowed quantitative assessment of the maturation stage of differentiating erythroblasts. Terminal maturation is characterized by morphological changes including progressive cell size reduction, chromatin condensation and nucleus extrusion. Forward light scatter and side scatter FACS analysis indirectly measures changes in cell volume and structure. After dead cells exclusion, we could define three populations A, B and C in differentiating CD36 cells **(see results Figure 3A)**. The relative number of cells in each population varied between the onset of caspases activation at day 8 and day 12. Cells from each population were sorted and analyzed morphologically. Proerythroblasts were identified in gate C, polychromatic and orthochromatic erythroblasts in gate B and a mix of orthochromatic erythroblasts, reticulocytes and nuclei were present in gate A. Erythroid differentiation was also evaluated by flow cytometry after double labeling with phycoerythrin (PE)-conjugate anti-GPA (BD Pharmingen) antibody and APC-conjugate anti-c-KIT (BD Pharmingen) antibody.

Western blot analysis

For detection of ROCK, cells were lysed buffer in Triton buffer (50mM Tris pH7.5, 1% Triton X-100, 100mM NaCl, 10mM tetrasodium pyrophosphate) and 10mM NaF, 1mM EDTA, 1mM NaV, 1mM EGTA, 1mM phenylmethylsulphonyl fluoride (PMSF) and 1µg/ml each of aprotinin, leupeptin and pepstatin. For detection of Rho, cells were lysed according to the protocol of the Rho activation assay kit. For detection of caspases, MLC2 and c-KIT, cells were lysed in Laëmmli buffer (60mM Tris pH6.8, 2% SDS). Protein concentration was assessed using the BCA kit (Pierce). Proteins were loaded at 50µg per well and were separated by SDS–PAGE. After transfer to a polyvinylidene difluoride (PVDF) membrane (GE Healthcare) and blocking for at least 1h in PBS, 5% non-fat milk and 0.1% Tween 20, blots were hybridized and proteins were visualized by enhanced chemiluminescence (ECL, Pierce).

To investigate the role of ROCK or Rho in MLC2 phosphorylation, Y-27632 (10 µM) or C3 (5 and 10 µg/ml) respectively, was added to the culture for 24 hours. At the end of the culture (day10), cells were stained with annexin V-APC and PI, and dead cells were excluded by cell sorting before lysis.

Rho pull-down assay

Pull-down assays were performed with a Rho activation assay kit (Cytoskeleton). To analyze Rho activity, CD36+ GPA- cells isolated by cell sorting were grown in serum-free medium with IL3, SCF and EPO. At day 4 and 10 of the culture (before and after the onset of caspases activation), cells were washed with serum-free medium and cytokine-deprived for eight hours. Cells were then stimulated with EPO (10 U/ml), SCF (100 ng/ml) or SCF+EPO for 5 to

30 minutes. Cells were collected, washed in PBS at 4°C and manipulated according to the protocol of the Rho activation assay kit. Pull-down assays were performed with 300 µg of protein.

shRNA ROCK-1 lentiviral production

Four shRNAmir constructs targeting the human ROCK-1 sequence, cloned in the pGIPZ lentiviral vector (Open Biosystems) were tested and the two that knockdown ROCK-1 protein in western blot, (nucleotides 2428 -2450 Open Biosystems V2LHS_70607 and nucleotides 2358-2380 Open Biosystems V2LHS_70609) were used in this study. pGIPZ lentiviruses were produced by 293T cells cotransfected by pCMV-Gag-Pol and pCMV-VSV-G plasmids (Open Biosystems). Supernatants were collected 48 hours and 72 hours after transfection, and were pooled and concentrated by ultracentrifugation. Virus stocked were kept frozen at -70°C.

Retroviral production

Retrovirus MIGR-GFP and the retrovirus expressing the dominant negative form of Rho MIGR RhoA-N19, were produced as described [26].

Erythroid cell infection.

CD34+ cells isolated from cord blood were cultured for five days as described then infected by shRNA GIPZ lentiviruses or MIGR-GFP retroviruses. A second round of infection was performed 24 hours later upon changing to fresh medium with cytokines. After an additional 24 hours, cells were extensively washed in PBS and stained with the anti-CD36-APC mAb and anti-GPA-PE mAb. The CD36+ GPA- GFP+ cell population was purified by cell sorting and cultured in serum-free medium in the presence of IL3+SCF+EPO.

Erythoid cells transient transfection

Transient transfections were performed with $3x10^6$ CD36 cells cultured for five days in the presence of IL3, SCF and EPO by nucleoporation in 100 µl of nucleofector solution (Amaxa biosystems) with 2.5 µg of pcDNA-GFP or with 2.5 µg of a mix of pcDNA-GFP and constitutively truncated ROCK-1 [24] (pCAG-Myc–ROCK1 G1114*opa* kindly provided by Dr Michael Olson) at a 1:4 ratio. After transfection, CD36 cells were cultured without SCF to reduce proliferation. To determine the percentage of GFP positive cells exhibiting Myc-associated fluorescence, cells were fixed, permeabilized, and labeled for Myc expression using the 9E10-PE mAb (Santa Cruz sc-40). Differentiation was assessed 24 and 48 hours after nucleoporation by analyzing FSC-SSC cell distribution of GFP positive cells. Differentiation was also analyzed by cell-surface GPA expression of cells exhibiting Myc-associated fluorescence. For this purpose, day 5 CD36 cells were transfected as described with 2 µg of the pCAG-Myc–ROCK1 G1114*opa* plasmid or 2 µg of the empty vector. Twenty four and forty eight hours after nucleoporation, the transfected cells were cell-surface labeled with APC-conjugate anti-GPA (BD Pharmingen), then fixed, permeabilized, and labeled for Myc expression with the 9E10-PE mAb and analyzed with flow cytometry.

Statistical analyses

Analysis was performed using Prism software and paired *t*-tests were used for the statistical analyses. Asterisks indicate significant differences : * *p value <0.05*), ** *p value <0.01*, *** *p value <0.00,*

Results

Rho is primarily activated through the SCF/c-KIT pathway

Erythroid cells were generated with a two–step amplification culture system as described in the Methods. In this culture system, differentiation was asynchronous and most of the cells remained immature until day 8, after which polychromatic and orthochromatic erythroblasts were gradually generated. In these conditions, the onset of caspase-3 activation occurred at day 7- 8.

Because ROCK proteins are classically activated by GTP-bound Rho, we analyzed Rho expression during erythroid differentiation. As compared to HSC70, used as a control for loading, the level of Rho protein decreased at the onset of caspase-3 activation **(Figure 1A)**. Pull-down analyses were performed at day 4 to determine Rho activity before the onset of caspase-3 activation. GST-RBD (Rhotekin Rho-binding domain) was used to pull down the active Rho-GTP form. For this purpose, CD36 cells were cytokine-deprived for eight hours then stimulated by SCF, EPO or SCF+EPO for 5 to 30 minutes. Rho activity peaked at five minutes (data not shown) and was strongly activated by SCF and to a lesser extent by EPO **(Figure 1B)**. EPO and SCF had an additive effect indicating that SCF/EPO signaling is involved in Rho activation at this stage of erythroid differentiation. In contrast, when pull-down analyses were performed at day 10 (after the onset of caspase-3 activation), Rho activity was equivalent to residual activity after EPO and SCF starvation for eight hours. Therefore, we could conclude that Rho is primarily activated through SCF stimulation as long as c-KIT is expressed. Thus, after the onset of caspase activation, Rho can no longer be activated because c-KIT is down modulated **(Figure 1C)** [12]**.**

Effect of ROCK-1 inhibition on cell proliferation

The requirement for ROCK function for proliferation in numerous cell types has previously been demonstrated [27]. We therefore monitored the proliferation of erythroid progenitors in which ROCK-1 activity was inhibited using RNA interference. For this purpose, $CD34^+$ cells

isolated from cord blood were infected as described with lentiviruses encoding short hairpin RNA (shRNA) targeting ROCK-1 mRNA. A scrambled shRNA sequence was used as a control. Western blots performed at day 6 of the CD36 lysates from selected GFP^+ cells showed a 47% reduction in ROCK-1 protein level **(Figure 2A)** demonstrating the efficacy of the shRNA.

The culture condition that we used allowed massive proliferation of the erythroid progenitors with a 300- to 400-fold amplification at day 10. Analyses using annexin V labeling along with PI staining showed that ROCK-1 inhibition did not significantly increase apoptosis in the culture except at the onset of caspase activation (supplementary information 1). ROCK-1 shRNA infected cells had a similar proliferation rate to cells in the control culture (**Figure 2B)**. Therefore, we could conclude that ROCK-1 is not involved in erythroblast proliferation.

ROCK-1 inhibition impairs terminal maturation of erythroblasts

To investigate the role of ROCK-1 in differentiation of erythroid progenitors, we first used Y-27632, a cell-permeable compound that is specific and efficient for inhibition of the activity of both ROCK-1 and ROCK-2. ROCK inhibitor Y-27632 was added at a concentration of 10 μM from day 0 to day 12 of the CD36 culture. Terminal differentiation was analyzed by examining the morphologic features of the erythroid cells using May-Grünwald-Giemsa staining at serial time intervals in the presence or absence of Y-27632. Erythroid maturation was also evaluated by flow cytometric analysis of FSC-SSC cell distribution as described in the Methods (**Figure 3A)** and by cell-surface c-KIT/GPA expression that allowed quantitative assessment of the maturation stage of differentiating erythroblasts. By day 6, the majority of erythroid cells are $KIT^{pos}GPA^{int}$. At the onset of caspases activation, c-KIT is down-modulated and the ratio of $KIT^{neg}GPA^{high}$ is progressively increased (**Figure 3B and 3C)**.

At day 12 of the control culture, a mean of 25.8% ± 1.3 of the cells exhibited a mature phenotype (polychromatic and orthochromatic erythroblasts) as identified by morphological analyses. In the Y-27632 treated culture, multinucleate cells appeared at day 3 (0.5 to 2%). However, the proportion of these cells was low compared to 25-30% observed with blebbistatin, a selective inhibitor of myosin II ATPase activity (data not shown), suggesting that ROCK does not play a critical role in cytokinesis of early erythroblasts. At day 12, the proportion of mature cells was dramatically decreased in the presence of the ROCK inhibitor with a mean of 12.5% ± 0.9 **(Figure 4Ai).** FSC-SSC cell distribution in flow cytometric analysis, as described Figure 3, also indicated that the Y-27632 treatment significantly increased the proportion of immature cells (39.9% ± 1.6 versus 20.3% ± 4.2 in controls within gate C) and decreased the proportion of mature cells (11.9% ± 1.1 versus 22.8% ± 3.6 in controls within gate A and 4.3% ± 1 versus 6.3% ± 0.8 in controls within gate B) **(Figure 4Aii)**.

Similar results were observed when the ROCK inhibitor was added at the onset of caspase activation from day 7 to day 12 as assessed by morphological analyses (22.5% ± 0.8 of mature cells, versus 29.7% ± 1.1 in controls) **(Figure 4Bi)**, and FSC-SSC analyses (36.3% ± 3.6 versus 24.9% ± 2.9 in controls within gate C, 4.2% ± 1.5 versus 6.2% ± 1.2 in controls within gate B, and 10.9% ± 1.4 versus 17.2% ± 1.9 in controls within gate A) **(Figure 4Bii)**.

Erythroid maturation was also evaluated by GPA and c-KIT cell-surface expression. Two populations with specific staining characteristics were defined using flow cytometry. The populations c-KIT^{neg} GPA^{high} and c-KIT^{pos} GPA^{int} corresponded respectively to mature and immature cells (**Figure 3C).** The ROCK inhibitor added from day 0 to day 10 or from day 7 to day 10 of the CD36 culture significantly decreased the proportion of c-KIT^{neg} GPA^{high} cells (44.65 ± 1.52 in controls, versus 25.50 ± 3.03 in the Y-27632 treated culture, versus 32.20 ± 3.03 when the ROCK inhibitor was added from day 7) and increased the proportion of c-

KIT^{pos} GPA^{int} cells (26.35 ± 0.45 in controls, versus 37.40 ± 1.44 in the Y-27632 treated culture, versus 31.50 ± 3.46 when the ROCK inhibitor was added from day 7) **(Figure 4Biii).** Then, to discriminate between ROCK-1 and ROCK-2 function, we examined the effect of ROCK-1 knock down by RNA interference. Although shRNA partially inhibited ROCK-1 expression at day 6 **(Figure 2A),** a significant decrease of cell maturation was observed in ROCK-1shRNA infected culture. At day 12, a mean of 9.5% ± 2.1 of the cells exhibited a mature phenotype versus 22.4% ± 2.5 in controls **(Figure 4Ci)**. FSC-SSC cell distribution analysis indicated that ROCK-1 knock-down significantly increased the proportion of immature cells (44.2% ± 3.4 versus 38.9% ± 2.9 in controls within gate C) and decreased the proportion of mature cells (8.8% ± 1.5 versus 14.4% ± 0.2 in controls within gate A, and 2.2% ± 0.5 versus 7.9% ± 2.4 in controls within gate B) **(Figure 4Cii)**. The ratio of GPA^{high} cells was also decreased in ROCK-1 shRNA infected cells (27.45 ± 2.39 versus 38.30 ± 2.20 in scrambled) **(Figure 4Ciii)**. Taken together, these results suggest that ROCK-1 is involved in terminal maturation, which occurs after the onset of caspase activation.

ROCK-1 is activated independently of Rho

We next investigated the mechanism of ROCK-1 activation. To determine whether ROCK-1 is dependent on Rho activity during the late stage of maturation, we over-expressed a dominant-negative form of RhoA by infecting CD36 cells with the MIGR-Rho-N19 retrovirus. To evaluate the functionality of the construct, pull down analyses were performed with EPO-stimulated CD36 cells overexpressing the MIGR-Rho-N19 construct, which inhibited Rho activity (**Figure 5A).** Terminal differentiation was not significantly impaired when the CD36 cells were infected with the MIGR-Rho-N19 retrovirus compared with the MIGR-GFP control retrovirus as shown by morphological (**Figure 5B)** and FACS analyses

(Figure 5C). This is consistent with our results showing that Rho was not activated at this stage of maturation **(Figure 1B).**

ROCK-1 is activated by caspase-3 cleavage

Because caspase-3 was shown to constitutively activate ROCK-1 during apoptosis in many cells, whereas ROCK-2 cannot be cleaved by caspase-3, western blotting of whole cell lysates was performed at several time intervals to evaluate the possible activation of ROCK-1 through caspase-3 cleavage during erythroid differentiation. In this *in vitro* culture system, the percentage of cells undergoing apoptosis could reach 15% to 20% at the end of differentiation. To discriminate differentiation from apoptotic ROCK-1 cleavage, day 10 annexin V- and propidium iodide (PI)-positive cells were excluded by cell sorting before cell lysis. By probing with a ROCK-1 specific antibody, in addition to the full-length 160-kDa protein, a 130-kDa fragment was detected in non apoptotic cells from the onset of caspase-3 activation until the end of differentiation **(Figure 6A)**. Likewise, treatment of the culture during the second step of differentiation with z-VAD-fmk at a dose of 150 μM, which inhibited terminal erythroid differentiation [4], abolished the ROCK-1 cleavage. As a control, the same 130-kDa-cleavage product was present after overnight EPO starvation, strongly suggesting that this truncated ROCK-1 corresponds to the fragment generated by caspase-3 cleavage. Treatment of the CD36 culture by QVD-OPH, another broad-spectrum inhibitor of caspases, at a dose of 50 or 200 μM also abolished the Rock-1 cleavage and induced a dose-dependent decrease of terminal erythroid differentiation (supplementary information 2). This finding demonstrated that ROCK-1 is caspase-3 cleaved during the late step of erythropoiesis in non-apoptotic cells.

To determine whether a constitutively truncated form of ROCK-1 expressed before the onset of caspase activation could affect terminal differentiation, we transiently transfected CD36

cells at day five of the culture with a constitutively active mutant of ROCK-1: pCAG-Myc–ROCK1 G1114*opa* (ROCK-1DC) [24] that contains a truncated auto-inhibitory domain. This experiment was performed without SCF to rule out any activation of ROCK-1 through Rho induced phosphorylation.

For this purpose, cells were co-transfected with a mix of ROCK-1ΔC and a plasmid encoding GFP protein at a 4:1 ratio.

The percentage of cells expressing the ROCK-1 mutant was determined after permeabilization by measuring Myc-associated fluorescence using flow cytometry. For this purpose, a gate was established above the level of autofluorescence seen in mock-transfected cells. The cells exhibiting Myc-associated fluorescence also exhibited GFP fluorescence **(Figure 6Bi)**. Therefore, differentiation was assessed 24 and 48 hours post-transfection by SSC-FSC FACS analyses on live GFP-positive cells. Cell distribution analysis indicated that the truncated ROCK-1ΔC construct significantly decreased cell size distribution (7.4% ± 0.8 gating with mature cells versus 4.7% ± 0.9 in controls) **(Figure 6Bii)**. Differentiation was also assessed by cell-surface GPA expression. For this purpose, day 5 CD36 cells were transfected with the pCAG-Myc–ROCK-1ΔC plasmid or with the empty vector. Twenty four and forty eight hours post-transfect ion, cells were labeled for GPA then MYC after permeabilization and analyzed by flow cytometry. By 24 hours post-transfection, the ratio of GPA^{high} erythroblasts was 2.2-fold increased in Myc–labeled ROCK-1ΔC transfected cells (8.35 ± 2.17 versus 3.79 ± 1.60 in controls) and 2.7-fold increased by 48 hours (33.35 ± 2.83 versus 12.33 ± 0.62 in controls) **(Figure 6C)**. These results indicated that the truncated ROCK-1ΔC construct accelerated maturation. Taken together, our data demonstrate that ROCK-1 is activated through an SCF-independent mechanism, through caspase-induced cleavage, to allow erythroblast terminal maturation.

ROCK-1 is the main MLC2-regulating kinase

One major role of the Rho/ROCK pathway is to regulate actomyosin-based contractility by modifying the MLC2 phosphorylation status. We analyzed the expression of total MLC2 and p-MLC2 (Ser 19) on CD36+ cells at serial time intervals. The level of MLC2 decreased at the end of the CD36 culture **(Figure 6A)**. To investigate whether or not ROCK was involved in MLC2 phosphorylation, western blot analyses were performed on whole cell lysates of the Y-27632-treated and control cultures at various times. At day 10, to discriminate differentiation-induced from apoptotic MLC2 phosphorylation, annexin V- and PI-positive cells were excluded by cell sorting before cell lysis. MLC2 phosphorylation was strongly inhibited by the Y-27632 inhibitor throughout the differentiation process **(Figure 7B)**. Similar results were obtained after selective inhibition of ROCK-1 **(Figure 7C)** showing that ROCK-1 is the main MLC2-regulating kinase in erythroid cells. To determine whether MLC2 activation is Rho-independent in the late stage of differentiation, MLC2 phosphorylation was assessed at day 10 of the CD36 culture in which Rho activity was inhibited. For this purpose, the level of MLC2 phosphorylation was determined after annexin V- and PI-positive cell exclusion by cell sorting following a 24 hours treatment with 5 µg/ml and 10 µg/ml of a cell penetrating form of the *Clostridium Botulinum* toxin C3, which induced a dose-dependent inhibition of Rho GTPase activity (data not shown). As expected in our culture system in which some immature cells, which are Rho dependent, are still present at the end of the culture, a weak inhibition of MLC2 phosphorylation was observed with C3 transferase treatment, but this inhibition was weak compared to Y-27632 treatment as shown by analysis of the p-MLC2:HSC70 ratio **(Figure 7D)**. Therefore, we could conclude that MLC2 phosphorylation occurrs independently of Rho via caspase-activated ROCK-1.

Discussion

Terminal erythroid differentiation is characterized by a sequence of morphological changes that require transient caspase activation. These events are followed by nuclei extrusion, which involves a profound reorganization of the cytoskeleton. We demonstrate here that the Rho effector protein ROCK-1 is required for terminal maturation occurring after the onset of caspase activation. At this stage, we show that Rho is not required, which is in agreement with reduced Rho expression at the end of differentiation. Late erythropoiesis can be characterized in successive stages with respect of cytokine dependency. First, early colony-forming units (CFU-E) express both c-KIT and EPO-R, the ligands of which are required for survival and proliferation. Then, basophilic erythroblasts down-regulate c-KIT, after which caspases are transiently activated, inducing cleavage of selected substrates. c-KIT expression, which is critical for activation of the Rho/ROCK pathway, is lost at the end of erythroid differentiation, but this could be overcome by caspase-3-induced ROCK-1 cleavage. This phenomenon allows terminal maturation of erythroblasts independently of SCF signaling. This is confirmed by our results showing that a constitutively truncated construct of ROCK-1 expressed before the onset of caspase activation accelerated terminal maturation. This truncated form exhibits a higher intrinsic kinase activity [24 25] that could be required for terminal maturation. However, terminal differentiation, which is inhibited in the presence of a caspase inhibitor [4], could not be rescued by expressing the constitutively cleaved form of ROCK-1 (data not shown) suggesting that other caspase targets are involved in terminal maturation.

Rock-1-/- newborn mice exhibit developmental defects in the eyelid and the ventral body wall closure [28]. No obvious anemia was described; nevertheless, erythroblast development has not been investigated in these mice [29] and compensatory changes in other kinases including MLCK, a caspase-3 target during apoptosis [30], cannot be excluded.

ROCK-1 could be involved in erythroid terminal maturation at least in part through contractility control by regulating MLC2 phosphorylation. In the erythroblast, myosin II could function in an actomyosin contractile apparatus responsible for maintaining a normal cell shape during cell condensation. Rho/ROCK-1 pathway has been shown to be involved in other differentiation systems, including proplatelet formation through MLC2 phosphorylation [26], and keratinocyte differentiation through stratification and enlargement of cell size [31]. Recent findings also define a cytoskeleton-independent function for ROCK in regulating morphogenesis and cell size through insulin/IGF signaling [32]. Interestingly, ROCK activity was also shown to be a component of the switch in the adipogenesis/myogenesis fate [33] [34]. Thus, we cannot exclude that in addition to its role in mediating MLC2 phosphorylation, ROCK-1 may have another function required for terminal erythroid differentiation. Our data provide another evidence that ROCK is a critical mediator of cell differentiation.

Recent analyses have provided substantial evidence that apoptotic caspases have important functions in the differentiation of various cell types. Keratinocyte cornification involves caspase-14-mediated cleavage of profilaggrin [35] [36], and lens fiber epithelial cells are characterized by complete removal of the nucleus [37]. Another type of anucleate cell, platelets, is produced by megakaryocytes in which selected substrates are cleaved by compartmentalized activation of caspase-3 [38]. Moreover, myoblast to myotube differentiation, which involves caspase-3-mediated cleavage of the kinase MST1, shares other features with apoptosis such as disassembly and reorganization of the actin fiber [39]. Nevertheless, little is known concerning the identity of caspases substrates involved in differentiation and the exact requirement for caspase activity in these systems. We show here that caspase-3 activation in basophilic erythroblasts, cells in which c-KIT receptor expression is lost, allows constitutive activation of ROCK-1, one of their substrates. We could therefore propose a model in which ROCK is Rho-dependent until the basophilic stage, whereas in the later steps of

differentiation, when Rho is no longer activated by SCF signaling, caspase-3-activated ROCK-1 allows terminal maturation. Further exploration of caspase targets in the future will shed light on the mechanism of erythroid differentiation as well as on other systems in which caspases are activated for cell differentiation

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Disclosure of conflicts of interest

The authors declare no conflict of interest.

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Titles and legends to figures

Figure 1. Rho is primarily activated through SCF stimulation

Immunoblots were performed from CD36 lysates at indicated times in the presence of cytokines, or with eight-hours deprivation of growth factors. HSC70 or actin are shown as loading control. (A) Rho expression decreased after the onset of caspases activation as shown by western blot quantification showing the Rho/HSC70 ratio. (B) Pull-down RBD-bound Rho indicates that Rho is primarily activated by SCF before the onset of caspase activation (day 4) but not after the onset of caspase activation (day 10). (C) Immunoblots indicate that 120-kDa c-KIT is down modulated at the onset of caspase-3 activation.

Figure 2. ROCK-1 inhibition is not involved in erythroblasts proliferation

CD36 cells infected by scrambled or shRNA lentiviruses targeting ROCK-1 were cultured as described in the Methods. (A) Immunoblot analysis performed at day 6 of the CD36 culture shows a 47% reduction in ROCK-1 protein level in ROCK-1 shRNA infected cells. (B) Viable cells were assessed daily by trypan blue staining at indicated times. The graph indicates the cumulative cell number. Means of cumulative cell number of five representative experiments are shown.

Figure 3. Flow cytometric analysis of differentiating erythroblasts

(A) CD36 cells cultured as described were analyzed from day 9 to day 12 of the culture by forward light scatter and side scatter flow cytometry density plots after dead cells (PI-positive) and debris (low forward scatter) exclusion as described in the Methods. Regions A, B and C were defined and cells from each population were sorted and analyzed

morphologically with PLFluotar 40X and 100X oil objectives after May-Grünwald-Giemsa staining. Proerythroblasts were identified in gate C, polychromatic and orthochromatic erythroblasts in gate B and a mix of orthochromatic erythroblasts, reticulocytes and nuclei were present in gate A.

(B) Flow cytometric dot plots from CD36 cells cultured as described, representative of 3 experiments and analyzed from day 1 to day 10 of the culture. Cells were double-labeled for c-KIT-APC and GPA-PE as described in the Methods. The relative number of cells in each region is indicated.

(C) Left panel: Flow cytometric dot plots from day10 CD36. Regions A, B and C were selected as indicated. The right-panels show the flow cytometric dot plots for c-KIT and GPA for cells derived from each region as described in B. These results show that the majority of the cells from regions A and B are c-KIT^{neg}GPA^{high} and the majority of the cells from region C are c-KIT^{pos}GPA^{int} .

Figure 4. ROCK-1 inhibition impairs terminal differentiation

(A) CD36 cells were generated with a two–step amplification culture system as described in the continue presence of the ROCK inhibitor Y-27632. Data representatives of several experiments show that ROCK inhibition impairs erythroid terminal differentiation.. Differentiation was evaluated at day 12 (i) by morphological analyses after May-Grünwald-Giemsa staining. The scale bar represents 20 μm. The percentage of matures cells (polychromatic and orthochromatic erythroblasts) was assessed by counting 300 cells in consecutive oil immersion fields (N=12) (ii) by flow cytometric dot plots as described in Figure 3A showing the relative number of cells in each region and FSC distribution histograms of a representative experiment. The graph indicates the mean percentage of cells in gates A, B and C.

(B) The ROCK inhibitor Y-27632 was added from day 7 to day 12. (i) (ii) Differentiation was assessed as described in (A) (N=6) (iii) Flow cytometric dot plots of CD36 cells at day 10 of the culture in the presence or not of the Y-27632 inhibitor added from day 0 to 10 or from day 7 to 10 in one representative experiment out of 4. Cells were labeled for c-KIT and GPA as described in the Methods. Two populations were defined: c-KIT^{neg} GPA^{high} and c-KIT^{pos} GPA^{int} corresponding respectively to mature and immature cells. The relative number of cells in each region is indicated. The graph on the right-panel indicates the mean percentage of cells in each region.

(C) CD36 cells were infected with scrambled or ROCK-1 shRNA lentiviruses as described. (i)(ii) Differentiation was assessed as described in (A) (N=5) (iii) Flow cytometric GPA distribution histogram at day 12 of the culture from a representative experiment of scrambled and ROCK-1 shRNA infected cultures. The gate corresponding to GPA^{high} cells is indicated. The graph on the right-panel indicates the mean percentage of GPA^{high} cells in both cultures (N=3).

Figure 5. Rho is not involved in terminal differentiation

CD36 cells were infected with MIGR-GFP or MIGR-Rho-N19-GFP retroviruses as described (N=3). (A) A pull-down Rho-GTP assay was performed at day 4 of the MIGR-GFP control or MIGR-Rho-N19-GFP infected CD36 culture after stimulation with EPO for five minutes or after cytokine deprivation as described. It shows that the GTPase activity is inhibited in MIGR-Rho-N19-GFP+ infected cells. (B) Morphological analyses after May-Grünwald-Giemsa staining and (C) FSC-SSC density plots analyses as described in Figure 3A and FSC distribution histograms of a representative experiment both indicate that the dominant negative Rho-N19 overexpression does not significantly affect terminal differentiation.

Figure 6. ROCK-1 is activated by caspase-3 cleavage

(A) On the left-panel: Immunoblots of a 12% acrylamide gel were performed from whole cell lysates from CD36 cells cultured as described for the indicated times in the presence of cytokines or in the presence of the caspase inhibitor z-VAD-fmk at 150 μM, or overnight deprived of growth factors (-EPO 16H) at day 9. The blots detect the 32-kDa procaspase-3 and its 17-kDa cleavage product (p17), showing the onset of caspase activation at day 7-8. The same lysates were loaded on an 8% acrylamide gel to detect ROCK-1 (160-kDa) and its 130-kDa cleavage product. HSC70 is shown as a loading control. At day 9, to discriminate apoptotic from differentiating cells, lysates were performed before (9 total cells) or after annexin V and PI-positive cell exclusion by cell sorting (9 alive cells). On the right-panel, western blot quantification showing the ROCK-1 p160+p130/HSC70 ratio.

(B) (i) CD36 cells were mock-transfected (control) or transfected with a mix of pCAG-Myc–ROCK1 G1114*opa* (ROCK-1ΔC) and pGFP at a 4:1 ratio. Twenty-four hours post-transfection, cells were fixed, permeabilized, and Myc-associated or GFP fluorescence was analyzed by fow cytometry using the 9E10-PE mAb (Santa Cruz sc-40). Gates were established to analyze Myc-associated fluorescence, which was negative in mock-transfected cells (left-panel) and positive in pGFP+ROCK-1ΔC transfected cells (middle-panel). Histogram (right-panel) shows that cells exhibiting Myc-associated fluorescence also exhibited GFP fluorescence. One representative out of six experiments is shown. (ii) CD36 cells were transiently transfected as described in Bi. Forward scatter distribution histograms overlays of ROCK-1ΔC+pGFP and pGFP (control) transfected cells, assessed 48 hours post-transfection on GFP positive cells. The gate corresponded to mature cells (regions A and B as defined in Figure 3A). Results are the average of five experiments performed in triplicate.

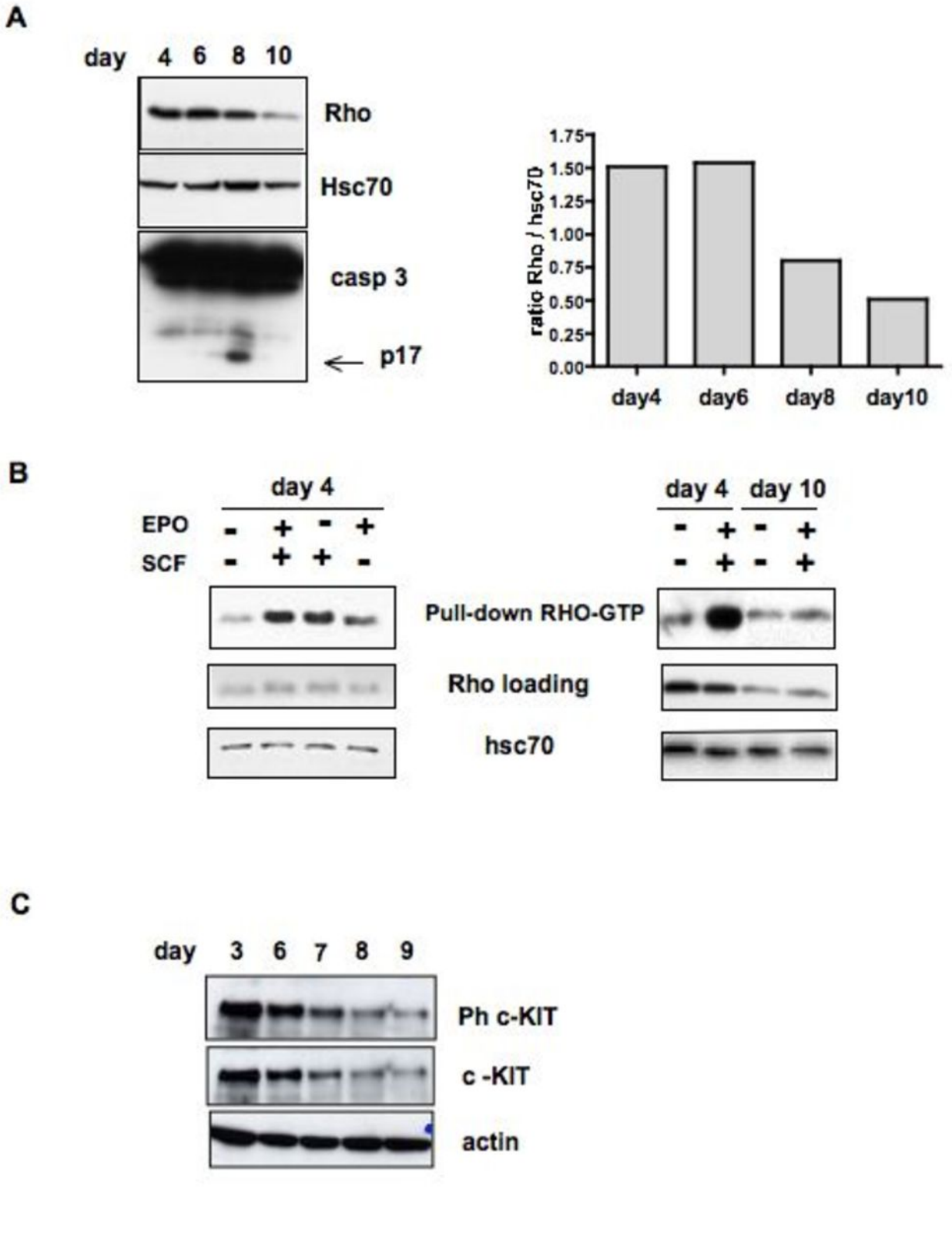
(C) Day 5 CD36 cells were transfected with the pCAG-Myc–ROCK-1ΔC plasmid or with the empty vector as described in the Methods. Differentiation was assessed 24 hours and 48 hours

post-transfection by cell-surface GPA expression from the MYC positive population selected from pCAG-Myc–ROCK-1ΔC transfected cells and compared to controls. Representative flow cytometric analysis of one experiment assessed 24 and 48 hours post-transfection of MYC positive cells from pCAG-Myc–ROCK-1ΔC transfected cells in respect to GPA and size (FSC), compared to controls (N=4). A region corresponding to GPA^{high} cells was defined and the relative number of cells in this region is indicated. The graph on the right-panel indicating the mean percentage of GPA^{high} cells in this region shows that the truncated ROCK-1ΔC construct accelerated maturation.

Figure 7. ROCK-1 is the main MLC2-regulating kinase

Erythroid cells were generated with a two–step amplification culture system as described. (A) Immunoblots were performed from whole cell lysates for indicated times to detect MLC2. Control shows HSC70 levels. (B) To investigate the role of ROCK in MLC2 phosphorylation, the Y-27632 inhibitor was added to the culture for 24 hours before cell lysis. At day 10, to discriminate apoptotic from differentiating cells, lysates were made after annexin V- and PI-positive cell exclusion by cell sorting (alive cells). Immunoblots detecting p-MLC2, MLC2 and HSC70 as loading control. (C) A CD34 culture was infected by ROCK-1 shRNA or scrambeled lentiviruses and cultured as described. Immunoblots were performed at day 9 of the culture to detect p-MLC2, MLC2 and HSC70 as loading control**.** (D) At day 9, C3 transferase at a dose of 5 or 10 μg/ml was added to the culture for 24 hours and annexin V-, PI-positive cells (alive cells) were excluded before cell lysis. Immunoblots detecting p-MLC2 and HSC70 as loading control are shown. Western blot quantification shows the P-MLC2/HSC70 ratio.

Figure 1.


A
day 4 6 8 10
Rho
Hsc70
casp 3
p17
ratio Rho / hsc70
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1.50
1.25
1.00
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0.50
0.25
0.00
day4
day6
day8
day10
B
day 4
EPO
SCF
day 4 day 10
Pull-down RHO-GTP
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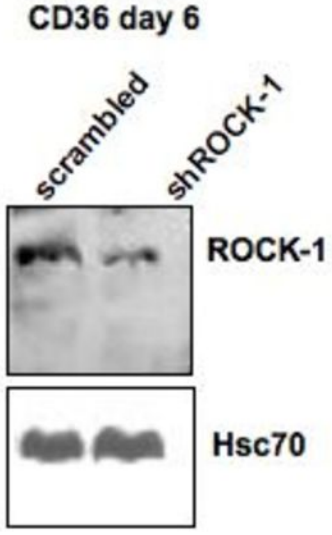
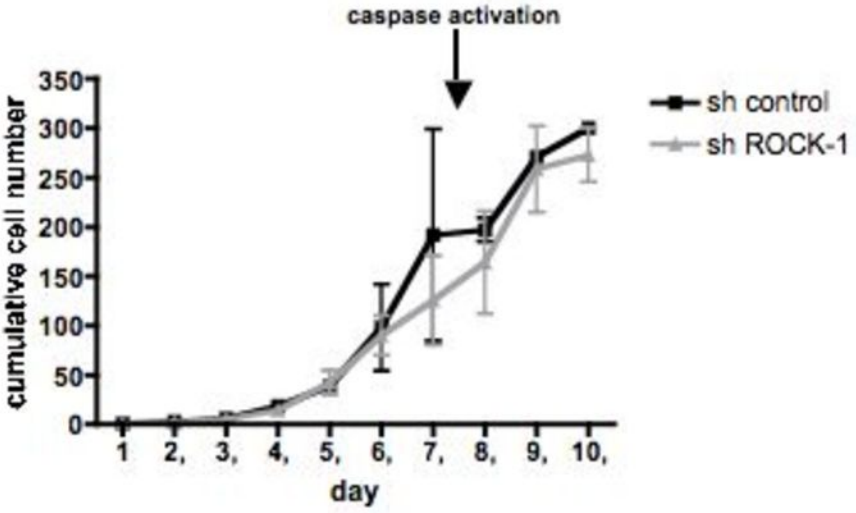
hsc70
C
day 3 6 7 8 9
Ph c-KIT
c -KIT
actin

Figure 2.

A


CD36 day 6
scrambled
shROCK-1
ROCK-1
Hsc70

B


caspase activation
cumulative cell number
350
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50
0
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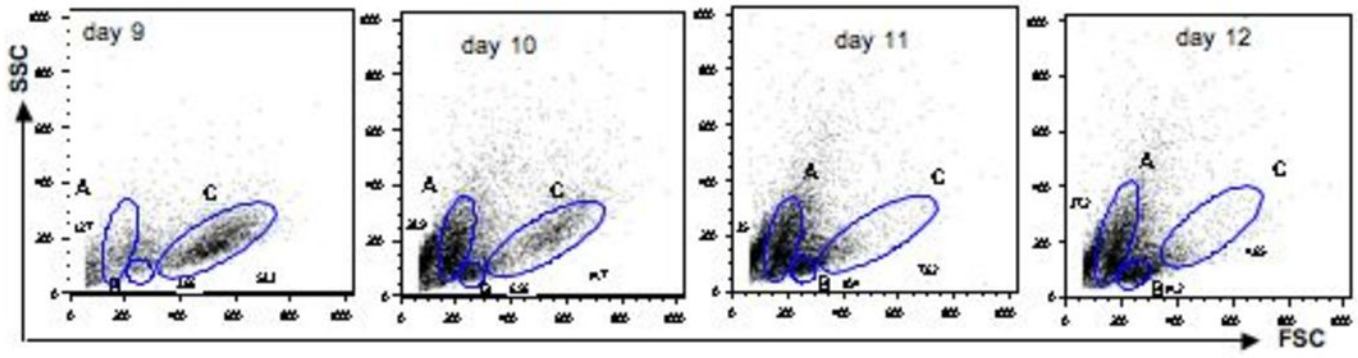
Figure 3 A.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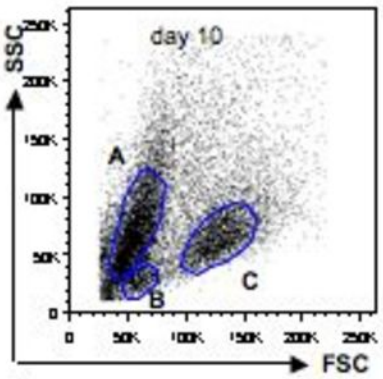
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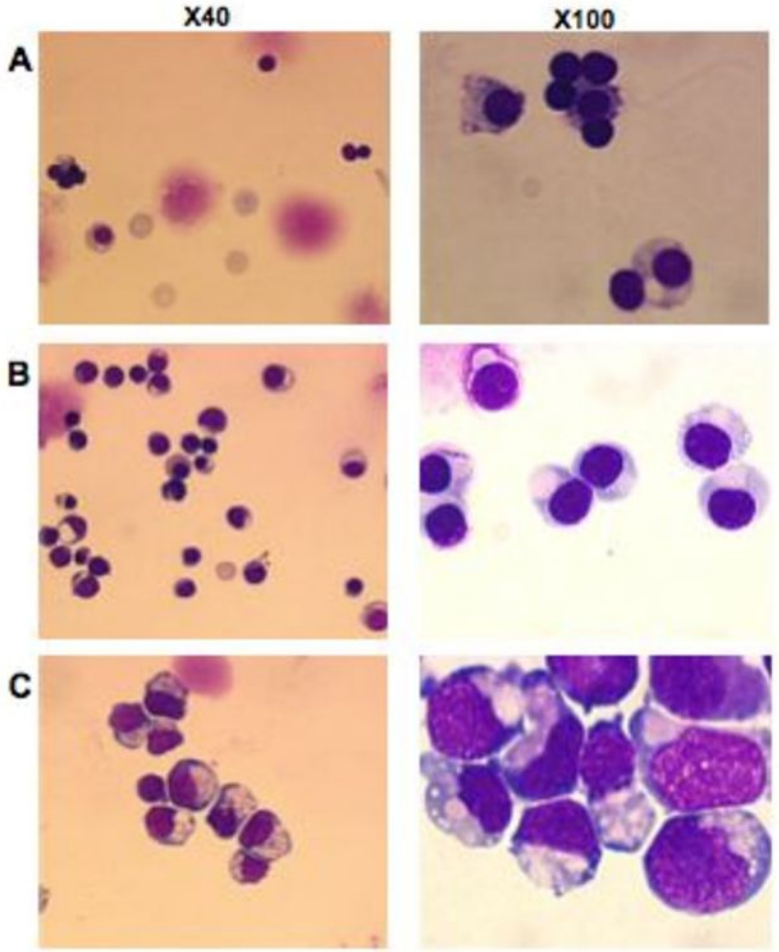
X40
X100
A
B
C

Figure 3 B.

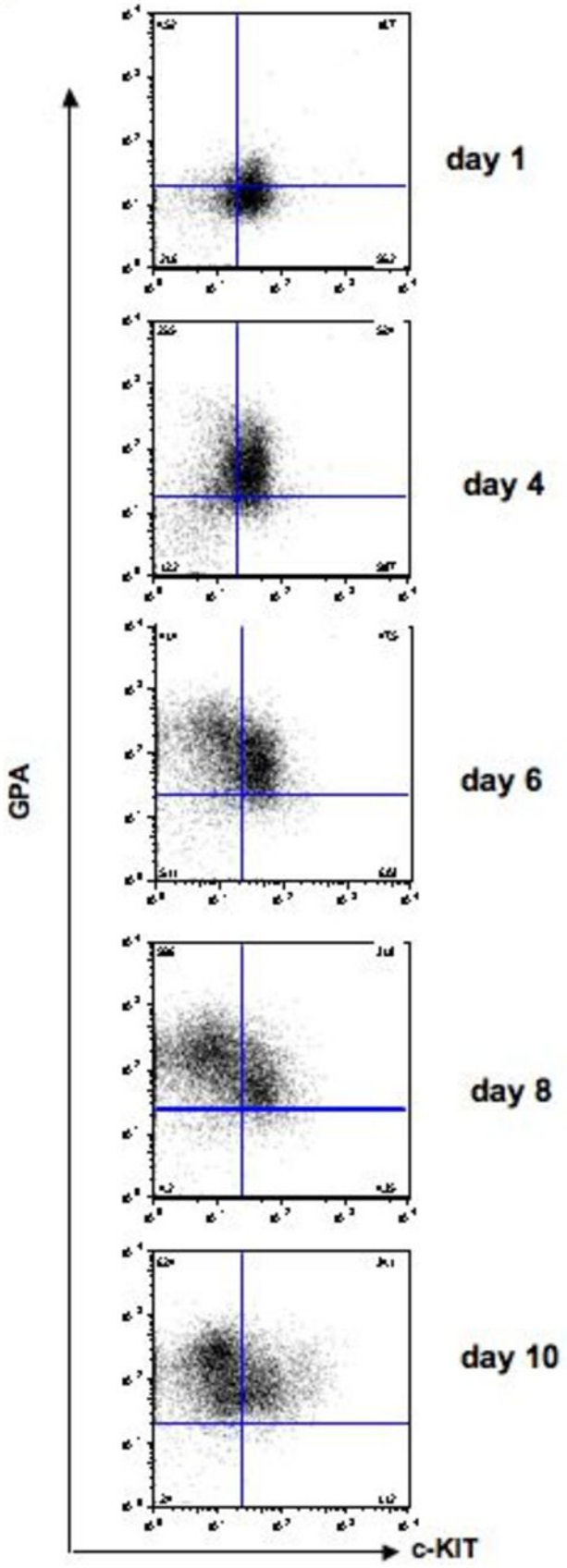

Figure 3 C.

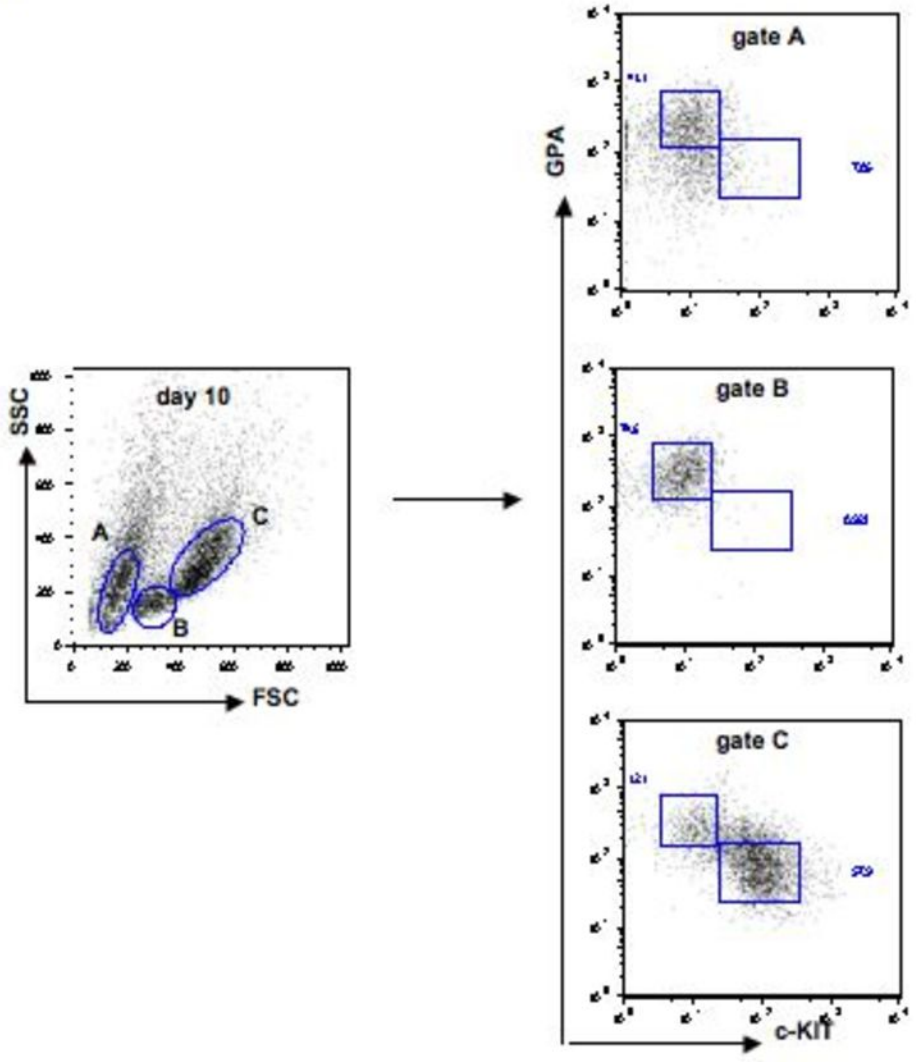

Figure 4A.

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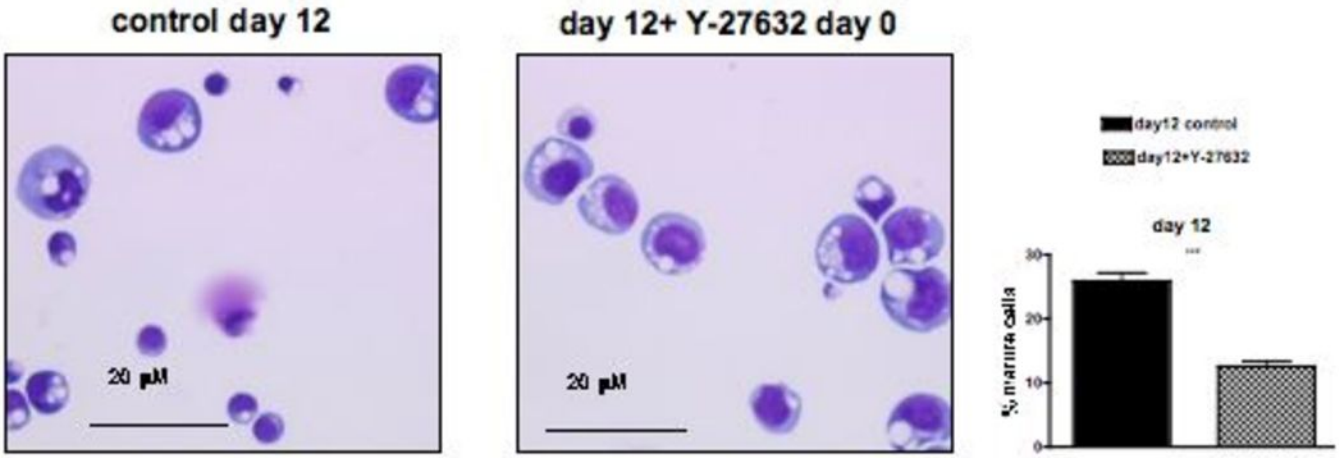

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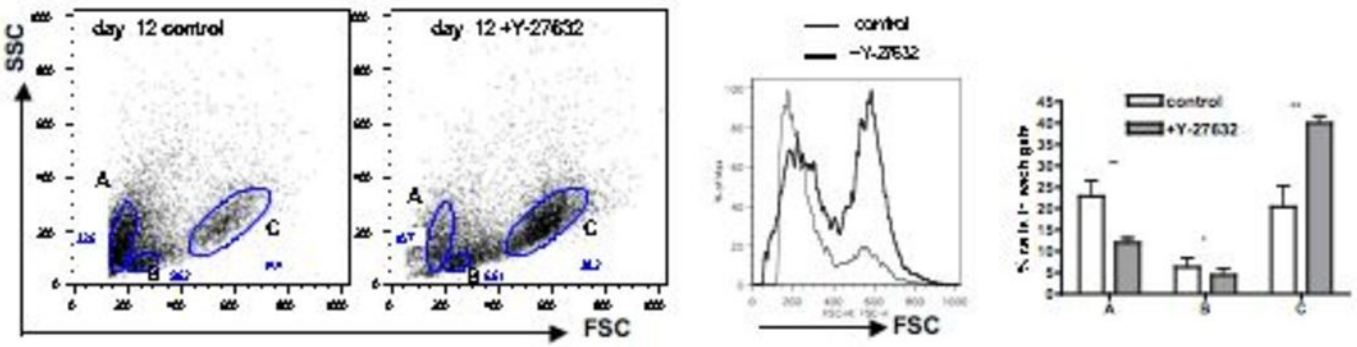

Figure 4B.

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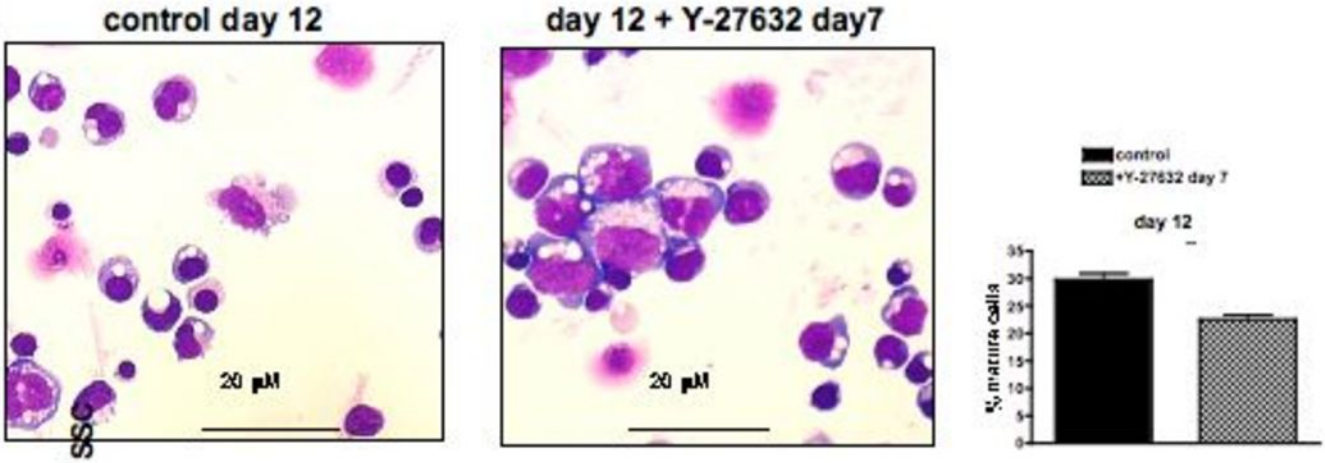

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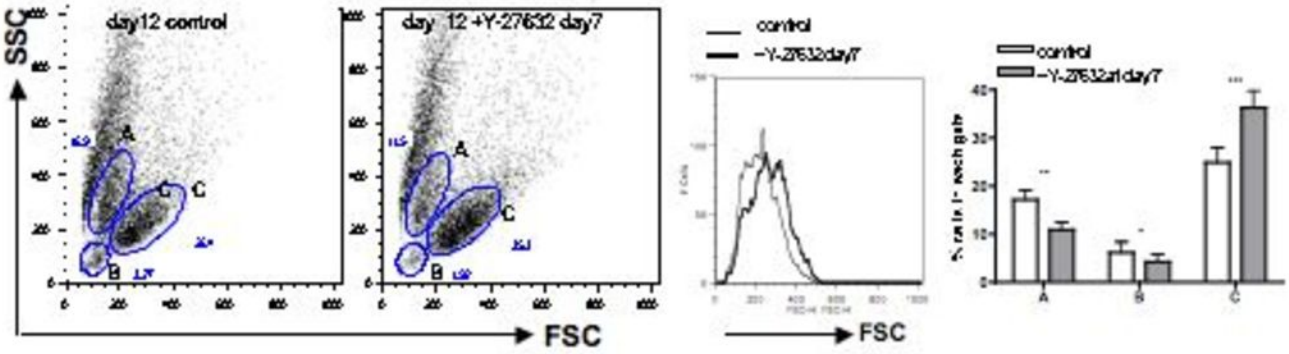

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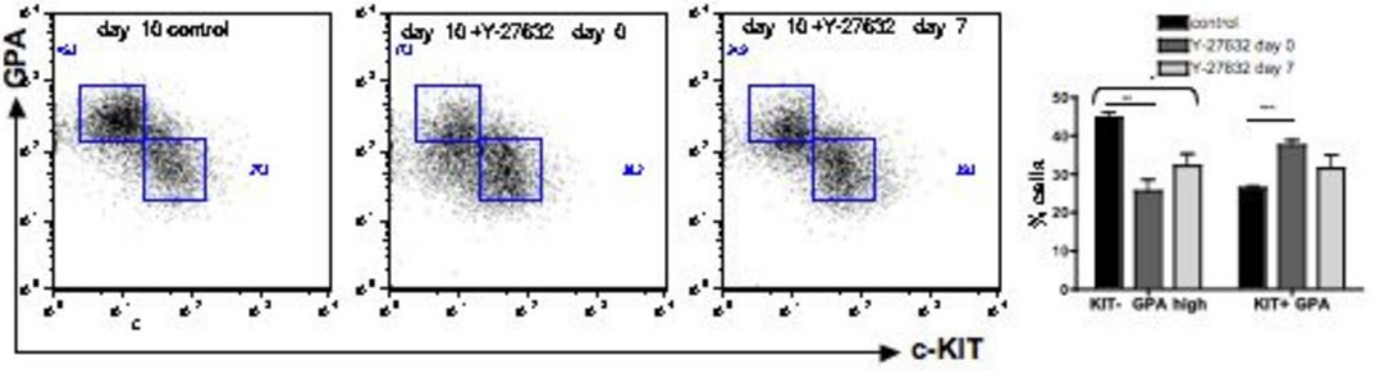
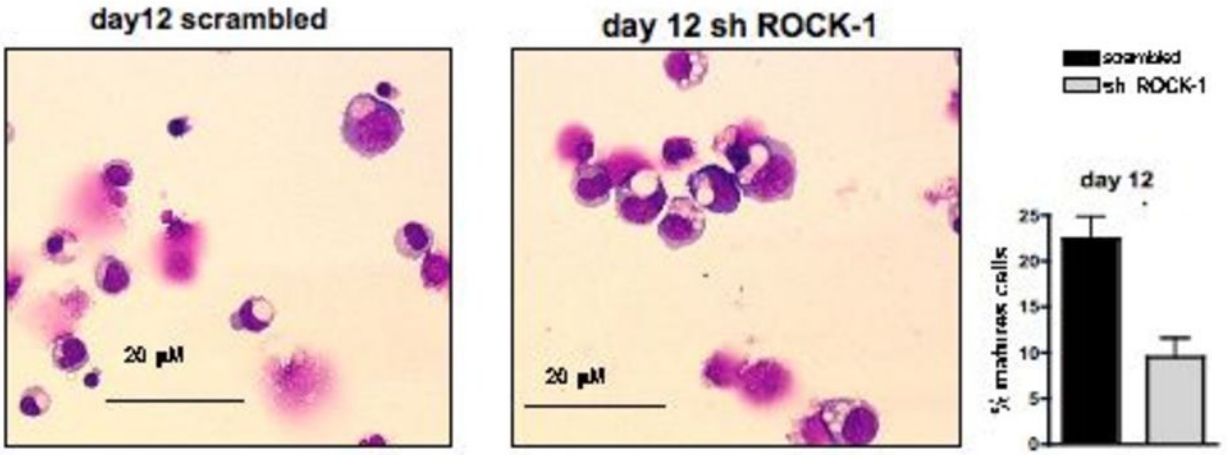


Figure 4C.

i



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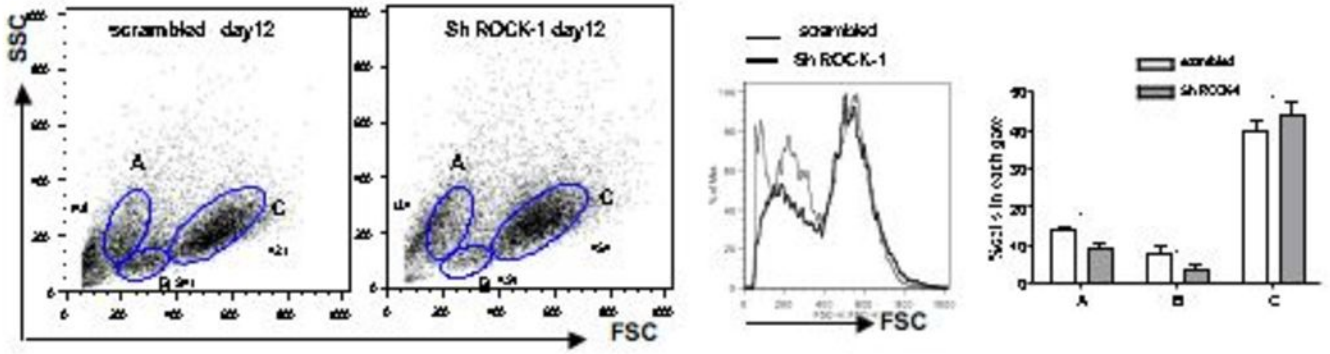

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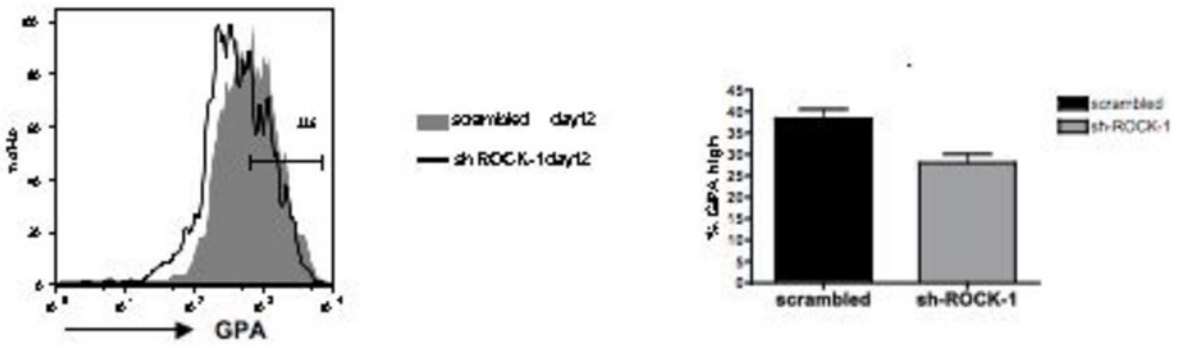


Figure 5.

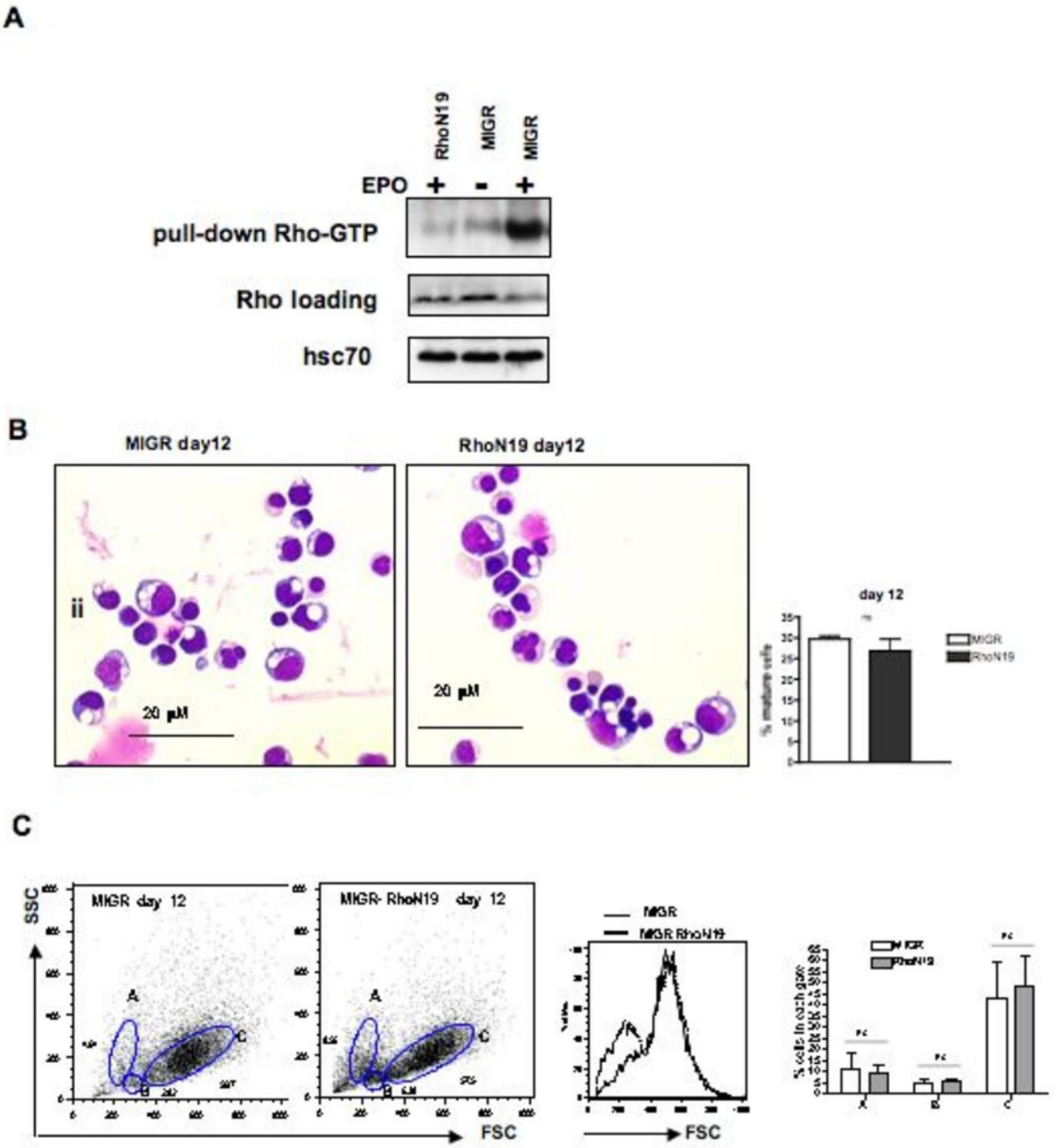

A
RhoN19
MIGR
MIGR
EPO + - +
pull-down Rho-GTP
Rho loading
hsc70
B
MIGR day12
RhoN19 day12
ii
20 µM
20 µM
day 12
MIGR
RhoN19
C
MIGR day 12
MIGR- RhoN19 day 12
SSC
FSC
A
B
C
MIGR
MIGR RhoN19
FSC

Figure 6

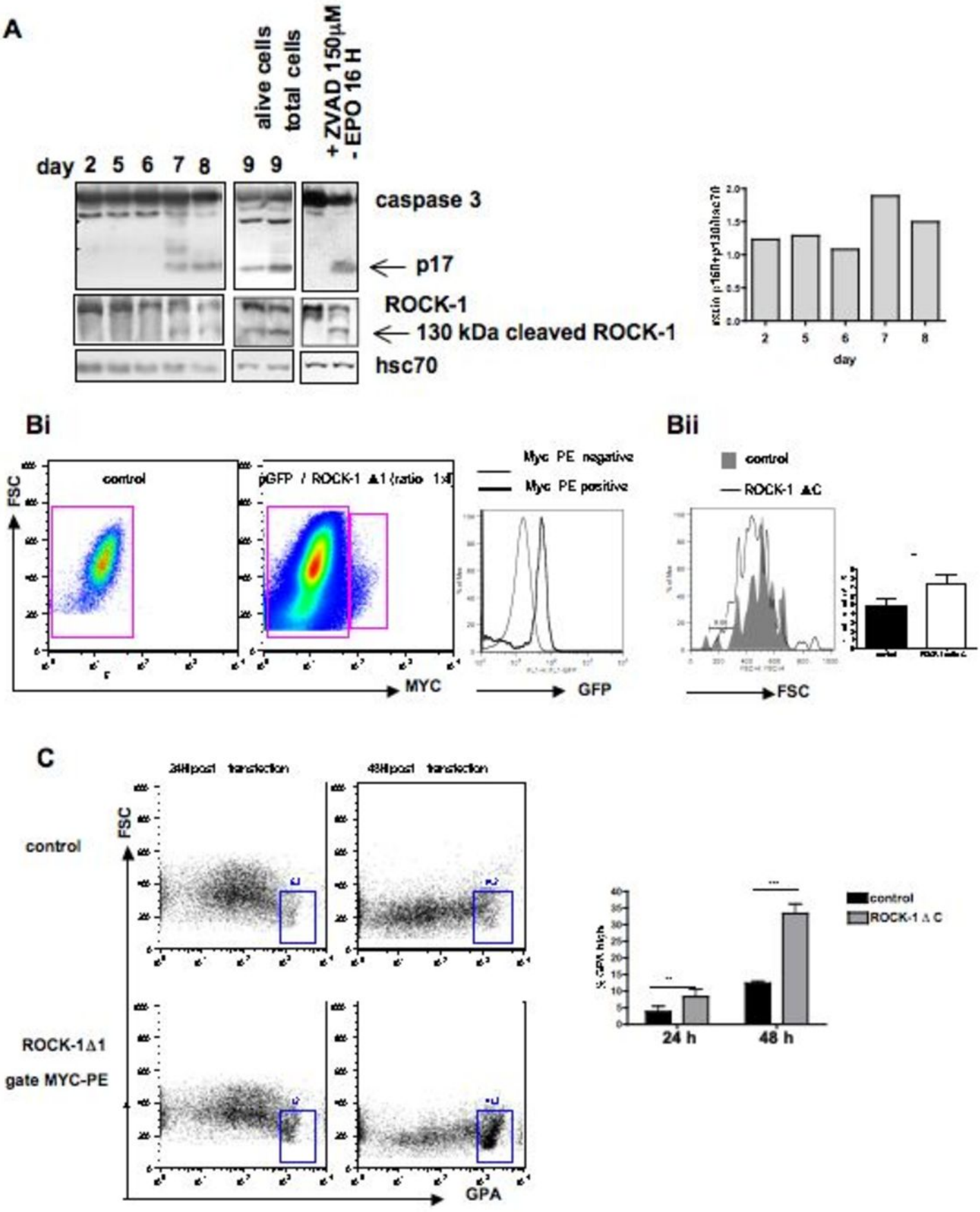
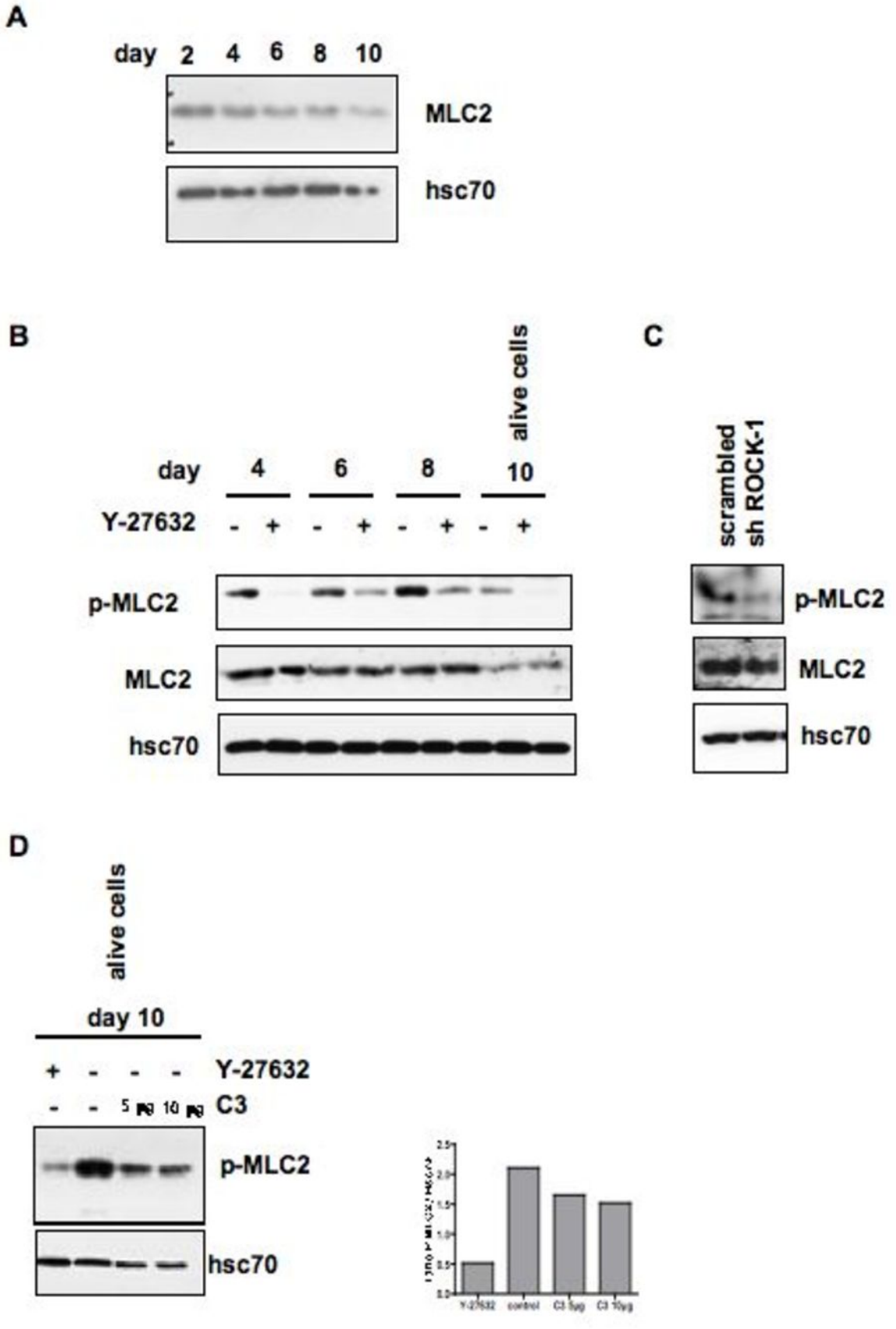

A
alive cells
total cells
+ ZVAD 150µM
- EPO 16 H
day 2 5 6 7 8 9 9
caspase 3
p17
ROCK-1
130 kDa cleaved ROCK-1
hsc70
day
Bi
control
pGFP / ROCK-1 Δ1 (ratio 1:4)
FSC
MYC
Myc PE negative
Myc PE positive
GFP
Bii
control
ROCK-1 ΔC
FSC
C
24H post transfection
48H post transfection
control
ROCK-1Δ1
gate MYC-PE
FSC
GPA
24 h
48 h
control
ROCK-1 Δ C

Figure 7


A
day 2 4 6 8 10
MLC2
hsc70
B
alive cells
day 4 6 8 10
Y-27632 - + - + - + - +
p-MLC2
MLC2
hsc70
C
scrambled
sh ROCK-1
p-MLC2
MLC2
hsc70
D
alive cells
day 10
+ - - - Y-27632
- - 5 10 C3
p-MLC2
hsc70