A TRANSCRIPTIONAL CROSS–TALK BETWEEN RhoA AND c–Myc
INHIBITS THE RhoA/Rock–DEPENDENT CYTOSKELETON

by

Vincent Sauzeau, Inmaculada M. Berenjeno, Carmen Citterio, and Xosé R. Bustelo

SUPPLEMENTARY INFORMATION*‡

*This file contains:
(1) Supplementary Text (pages 2–5)
(2) Supplementary Materials and Methods (pages 6–12)
(3) References used in the Supplementary Information (pages 13–14)
(4) Legends to Supplementary Figures (page 15–16)
(5) Supplementary Figures 1–3 (pages 17–end)

‡NOTE: Two Excel documents containing the microarray data have been included in two additional supplemental files.
(1) SUPPLEMENTARY TEXT

Analysis of the transcriptome present in RhoA<sup>Q63L</sup>/c–Myc–expressing NIH3T3 cells

As indicated in the Main Text, we found a number of differentially expressed genes that could modify the signaling output derived from the RhoA pathway. Those genes encoded:

(i) Direct upstream regulators of the RhoA pathway. (ii) GTPases that antagonize RhoA signaling routes. (iii) Structural elements of the cell cytoskeleton. In the first class, we found that c–Myc repressed three RhoA–specific GDP/GTP exchange factors (ArhGEF2, ArhGEF5, ArhGEF10) (Fig. 5B). We surmised that those GEFs were unlikely to contribute to the downmodulation of the F–actin cytoskeleton in RhoA<sup>Q63L</sup>–transformed NIH3T3 cells, because the activity of the constitutively active form of RhoA is totally independent on the action of upstream GEFs. In this category, however, we found that c–Myc induced the upregulation of Ect2 (Fig. 5B), a RhoA–specific GEF that plays crucial roles in cytokinesis (Tatsumoto et al., 1999). This GEF can also promote activation of Cdc42, Rac1 and Pak1 indirectly via the engagement of the Par6/Par3/atypical protein kinase C (aPKC) complex (Justilien & Fields, 2009; Liu et al., 2004) (see below in this section). In the second class, we found that c–Myc upregulates the expression of genes encoding the GTPase Cdc42 and three of its signaling elements, Pak1, Nck1, and Par6 (Fig. 5B). Pak1 is a serine/threonine kinase activated by both Cdc42 and Rac1 that counteracts RhoA–dependent pathways by phosphorylating and inhibiting MLC kinase (Sanders et al., 1999; Wirth et al., 2003) and the Net1 RhoA GEF (Alberts et al., 2005) (Fig. 5B). Pak1 activity also contributes to the destabilization of focal adhesions (Bokoch, 2003; Frost et al., 1998; Manser et al., 1997) (Fig. 5B). Nck1 is a SH3/SH2–containing adaptor protein that binds to Pak1 and facilitates
the proper subcellular localization of that kinase (Bokoch, 2003; Lu et al., 1997). Par6 is a protein that contributes to Cdc42–dependent polarity events by forming a heteromolecular complex with Par3 and aPKCs (Etienne–Manneville & Hall, 2001). This protein can contribute to the downmodulation of RhoA–dependent pathways by favoring the activation of the Rac1/Pak1 route (Liu et al., 2004; Regala et al., 2005) and by promoting RhoA inhibition via the aPKC–dependent stimulation of p190RhoGAP (Zhang & Macara, 2008) (Fig. 5B). This latter pathway is unlikely to be relevant for the downmodulation of the cell cytoskeleton by c–Myc in RhoAQ63L–transformed cells, because this RhoA mutant is GTPase deficient and, therefore, immune to the action of RhoA GAPs. In contrast to the above gene upregulations, c–Myc promoted the repression of the Borg3/septin signaling branch that works downstream of Cdc42 (Fig. 5B) (Joberty et al., 2001). It is possible that this is part of the proliferative program of c–Myc, since Septins have been associated to tumor–suppressor activities in some biological contexts (Kremer et al., 2007). However, the knockdown of Septin mRNAs has been shown to induce disassembly of stress fibers in HeLa cells (Fig. 5B) (Kremer et al., 2007). Finally, the third class of genes included the downmodulation of the expression of several integrin subunits (Itgβ1l, Itgβ5 and Itgα5), integrin–associated proteins (CD9, Zdhhc2), integrin regulatory kinases (Fyn), caveolin2, and a number of cytoskeletal regulatory molecules (actinin α1, transgelin 2, the Rock target profilin 2, F–actin capping protein, tropomyosin α) (Fig. 5B). We did not find genes encoding previously described RhoA or Rock regulators such as Shp2 (Lee & Chang, 2008), Smurf1 (Sahai et al., 2007; Wang et al., 2003), Gem (Hatzoglou et al., 2007; Ward et al., 2002), Rad (Hatzoglou et al., 2007; Ward et al., 2002) or RhoE (Riento et al., 2003)
(Supplementary Table I). These results indicate that the overexpression of c–Myc with RhoA\textsuperscript{Q63L} leads to alterations in a relatively low number of genes (4.28% out of 12,500 genes interrogated in the arrays), some of which can be associated to the regulation and/or stability of stress fibers and focal adhesions.

**The genes differentially expressed in RhoA\textsuperscript{Q63L}/c–Myc–expressing NIH3T3 cells result from synergistic transcriptional interactions between the RhoA and the c–Myc route**

Consistent with the synergistic effects inferred from our limited RT–PCR data, we observed that Cdc42, Pak1, Itgb1, Itbl1, Itgb5 and Itga5 genes were not upregulated in RhoA\textsuperscript{Q63L}–expressing cells when compared to the parental NIH3T3 cells. We also observed that the overexpression of c–Myc did not affect the expression of integrin–encoding genes (Itgα6, Itgβ7) previously seen upregulated in RhoA\textsuperscript{Q63L}–transformed cells, indicating that the effect of c–Myc in integrin–encoding genes is highly selective. The dependency of RhoA\textsuperscript{Q63L}/c–Myc synergistic interactions for the regulation of gene expression can be extrapolated to other gene targets identified in the present work, because the genes encoding the proteins AhrGEF2, ArhGEF5, ArhGEF10, Par6, RockII, caveolin 2 and profiling 2 were not detected deregulated when the transcriptomes of the parental and RhoA\textsuperscript{Q63L}–expressing NIH3T3 cells (Berenjeno et al., 2007) were compared side–by–side. In fact, 65% and 94% of the 100 genes showing highest upregulation and downmodulation in RhoA\textsuperscript{Q63L}/c–Myc–expressing cells could be included in this category, respectively. Another genes do appear to be already regulated, but at lower levels, by RhoA\textsuperscript{Q63L}–dependent signals. For example, the expression of *Ect2* mRNA was seen already increased
in RhoA^{Q63L}–expressing cells (Berenjeno et al., 2007) and was further enhanced in RhoA^{Q63L}/c–Myc–expressing cells (this work). 25% and 5% of the 100 genes showing highest upregulation and repression in RhoA^{Q63L}/c–Myc–expressing cells could be assigned to this category, respectively. The regulation of other genes seems to follow opposite trends in RhoA^{Q63L}– and RhoA^{Q63L}/c–Myc–expressing cells. For example, we described before that the expression of the genes encoding actinin α1 and transgelin 2, two loci that are downmodulated in RhoA^{Q63L}/c–Myc–expressing cells, was upregulated in RhoA^{Q63L}–expressing cells when compared with the parental cell line (Berenjeno et al., 2007). 10% and 1% of the 100 genes showing highest upregulation and downmodulation in RhoA^{Q63L}/c–Myc–expressing cells belonged to this category, respectively. These observations indicate that the overexpression of c–Myc leads to synergistic interactions with RhoA–dependent signals that result in new gene expression patterns rather than in the modification of the expression levels of the transcriptome present in RhoA^{Q63L}.
(2) MATERIALS AND METHODS

**Antibodies.** Mouse monoclonal antibodies to actin and vinculin were obtained from Sigma and Merk/Calbiochem, respectively. Rabbit polyclonal antibodies to β–tubulin and p21WAF1 were obtained from Merk/Calbiochem. Mouse monoclonal antibodies to MYPT1, RockI, RockII, and Itgβ1 were purchased in BD Biosciences. Rabbit polyclonal antibodies to RockI were obtained from Santa Cruz Biotechnology. Rabbit polyclonal antibodies to phosho–MLC and phosho–MYPT1 were sold by Cell Signaling and Upstate Biotechnology, respectively. Rabbit polyclonal antibodies to Pak1 were purchased in Zymed Laboratories. Rabbit polyclonal antibodies to phosho–Pak1 were from Cell Signaling. Horseradish peroxidase–conjugated secondary antibodies to rabbit and mouse IgGs were obtained from GE Healthcare. The Alexa 594–labeled secondary antibodies to either rabbit or mouse IgGs were from Invitrogen.

**Plasmids.** The mammalian expression vector encoding EGFP–RhoAQ63L (pNM41) was generated by subcloning the RhoAQ63L cDNA into pEGFP–C1 vector (Clontech). Retroviral vectors encoding the wild type and catalytically–inactive (L121G mutant) versions of the EGFP–RockIIKD–ER protein were obtained from Dr. M. Olson (Beatson Institute for Cancer Research, Glasgow, UK) and reported previously (Croft et al., 2004). A retrovirus expressing bicistronically wild type Pak1 and EGFP (pVS7) was generated by subcloning the Pak1 cDNA into the pMIEG3 vector (Williams et al., 2000) according to standard procedures. The retroviral vector (pACC30) encoding the catalytically inactive Pak1\textsuperscript{K298R} mutant was generated by subcloning the Pak1\textsuperscript{K298R} cDNA into the pCMV6–XL4 vector...
(Origene). pMIEG3 vectors encoding the Rac1$^{F28L}$ and Cdc42$^{F28L}$ mutants were obtained from Dr. Y. Zheng (Cincinnati Children’s Hospital Medical Center, Cincinnati, OH, USA) (Guo & Zheng, 2004). The plasmids encoding the Rac1$^{F28L+F37A}$ (pVS5) and Rac1$^{F28L+Y40C}$ (pVS6) mutants were generated by site–directed mutagenesis using the QuikChange mutagenesis kit (Stratagene) and the pMIEG3–Rac1$^{F28L}$ vector described above as template. All vectors were verified by DNA sequencing analysis.

**Immunofluorescence studies**

Cells were fixed with 4% formaldehyde in phosphate buffered saline solution (PBS) for 20 min and then permeabilized for 10 min in PBS containing 0.5% Triton X–100 (Sigma). For F–actin staining, cells were incubated with 2 units/ml of rhodamine–conjugated phalloidin (Molecular Probes/Invitrogen) at room temperature for 30 min to visualize the F–actin cytoskeleton (Berenjeno et al., 2007). For staining of nuclei, cells were incubated with 4’,6–diamidino–2–phenylindole (Sigma) (Berenjeno et al., 2007). When appropriate, cells were stained with antibodies to vinculin or p21$^{WAF1}$ following previously described protocols (Couceiro et al., 2005). In the case of immunofluorescence studies to detect microtubules, cells were fixed with ice–cold methanol and processed as above. Cells were then analyzed using either confocal (LSM 510, Zeiss) or standard immunofluorescence (Axiovert 2000, Zeiss) microscopy.
Quantitation of F–actin levels

In the case of immunofluorescence images, images of phalloidin–decorated cells were captured using a fluorescence Axiovert 2000 microscope. To make it possible side–by–side comparisons, we captured fluorescent signals keeping always constant the time of image capturing and the image intensity gain. Quantitation of fluorescence signals was done using the Metamorph/Metaview software (Universal Imaging). To this end, we first subtracted electronically the background fluorescence signal present in cell–free areas of the captured images and, subsequently, measured the rhodamine–derived fluorescence signals of selected cells. F–actin levels are given in arbitrary units considering that the signals obtained in the control samples were assigned a value of 1. In all cases, we utilized a minimum of 50 single, randomly picked cells (in the case of experiments involving non–transfected cells) or 50 EGFP–positive cells (in the case of retrovirally–infected cells) in each experimental condition. In the case of flow cytometry determinations, the indicated cell lines were grown on two 10–cm plates until they reached approximately 80% confluency. At that time, cells were harvested, washed with PBS and fixed using an 8% paraformaldehyde solution in PBS for 10 min at room temperature. After three additional washes with PBS, cells were permeabilized with 0.3% Triton X–100 in PBS for 1 min at room temperature, washed thrice in PBS, and stained with 0.6 mM rhodamine–phalloidin in PBS for 10 min at room temperature. Finally, cells were washed thrice with PBS and analyzed by flow cytometry using a FACSCalibur system (BD Biosciences).
Immunoblot experiments

To detect proteins in total cellular lysates, cells were lysed in 10 mM Tris–HCl (pH 8.0), 1% Triton X–100, 150 mM NaCl, 1 mM NaF, 1 mM orthovanadate and a protease inhibitor cocktail (Complete, Roche Molecular Biochemicals). Extracts were cleared by low–speed centrifugation and supplemented with an equal volume of 2x SDS–polyacrylamide gel electrophoresis (PAGE) sample buffer. After boiling for 10 min, proteins were separated electrophoretically, transferred to nitrocellulose filters, and subjected to immunoblot analysis with the appropriate primary antibody. Protein/antibody complexes were detected with horseradish peroxidase–linked goat secondary antibodies to either anti–mouse or rabbit immunoglobulins using a chemiluminiscent method (Pierce Biotechnology).

Migration during wound healing

These experiments were performed as indicated before (Couceiro et al., 2005).

Cell transfections

In the case of retroviral infections, recombinant retroviruses were generated using the packaging Phoenix cell line. To this end, cells were transfected with 4 µg of the appropriate retroviral vector, 7 µg of a plasmid encoding the viral Gag–Pol protein, and 3.5 µg of plasmid encoding the viral Env protein. 48 h after transfection, the culture medium was removed and used to infect NIH3T3 cells for 24 h. 48–72 h postinfection, infected NIH3T3 were subjected to subsequent analyses. In the case of standard transfections, 1 µg of the indicated plasmids was introduced into NIH3T3 cells using Lipofectamine (Invitrogen) and
analyzed 24–48 h later. When appropriate, cells were treated with 4–hydroxytamoxifen (1 µM, Sigma) for 16 hr before cell staining.

**Determination of mRNA expression levels**

Microarray experiments were performed exactly as indicated before (Berenjeno & Bustelo, 2008; Berenjeno et al., 2007), using the Genomics and Proteomics Unit of our Center. Normalization, filtering and analysis of the raw data obtained from microarrays were carried out with the Bioconductor software (www.bioconductor.com) using the ReadAffy package and the RMA application. Statistical analyses were performed using F–statistics. For the graphical presentation of microarray data, we performed hierarchical clustering analysis using the WPGA average–linkage and the standard correlation similarity metric method with the J–Express application. Functional annotation of gene functions was performed using the Ingenuity Pathways Analysis program (www.ingenuity.com) (Calvano et al., 2005). To allow statistical analysis, we used RNAs obtained from three independent cultures of each cell line. To identify genes of interest, we used two main criteria: (i) That the variations in gene expression relative to the control RhoA$^{Q63L}$–expressing cell line were statistically significant. (ii) That the changes in gene expression were not identical between RhoA$^{Q63L}$/c–Myc– and RhoA$^{Q63L}$/MadMyc–expressing cells. The latter selection criteria was based on the prior observation that the disruption of stress fibers and focal adhesions were observed in cells co–expressing RhoA$^{Q63L}$ and c–Myc but not in those co–expressing the GTPase with the MadMyc protein (see Results Section in main text, Fig. 1).
The comparison of the transcriptome of RhoA\textsuperscript{Q63L/c–Myc–expressing} cells with those from parental cells and RhoA\textsuperscript{Q61L–transformed} NIH3T3 cells treated with the Y27632 Rock inhibitor was done using the previously published data on the RhoA\textsuperscript{Q61L–dependent} transcriptome (Berenjeno et al., 2007). The side–by–side comparison of these gene networks was facilitated by the use of the same type of Affymetrix chips in both analysis (Berenjeno et al., 2007). In the case of quantitative reverse transcription PCR, total RNAs were extracted using the RNeasy mini kit (Qiagen), reverse transcribed using the iScript one–step RT–PCR kit (Bio–Rad) and amplified by PCR in an iCycler apparatus (Bio–Rad). The expression levels of mouse \textit{Rplp0} mRNA in each RT–PCR sample were used as internal control.

\textbf{siRNA knockdown}

A scrambled control siRNA and siRNAs targeting the \textit{Itgb1} and \textit{Itgb5} mRNAs were purchased from Dharmacon RNAi Technologies/Thermo Scientific. siRNAs were transfected using Lipofectamine according to the manufacturer’s instructions. The efficacy of the knockdown and the biological effects of the siRNA expression in NIH3T3 cells were studied 48 h after each transfection using RT–PCR experiments.

\textbf{Invasion and adhesion assays}

A CytoSelect 24–well cell invasion assay kit (CBA–100, Cell Biolabs) was used for the cell invasion experiments. Cells were trypsinized and resuspended in serum–free medium at a density of 1 × 10\textsuperscript{6} cells/ml. Cells were added into the cell culture inserts (300 µl/well),
which were then placed into a 24–well plate containing complete growth medium and incubated for 16 h. Cells which migrated to the other sides of the inserts were stained and measured following the manufacturer’s instructions. Adhesion experiments were carried out using the CytoSelect 48–well cell adhesion assay, ECM array (Cell Biolabs). In this case, cells (1 x 10^5) were seeded onto the wells and incubated at 37°C for 90 min before washing and staining according to the manufacture’s protocol.
(3) REFERENCES

Alberts AS, Qin H, Carr HS and Frost JA. (2005). *J Biol Chem*, **280**, 12152–61.

Berenjeno IM and Bustelo XR. (2008). *Clin Transl Oncol*, **10**, 726–38.

Berenjeno IM, Nunez F and Bustelo XR. (2007). *Oncogene*, **26**, 4295–305.

Bokoch GM. (2003). *Annu Rev Biochem*, **72**, 743–81.

Calvano SE, Xiao W, Richards DR, Felciano RM, Baker HV, Cho RJ, Chen RO, Brownstein BH, Cobb JP, Tschoeke SK, Miller–Graziano C, Moldawer LL, Mindrinos MN, Davis RW, Tompkins RG and Lowry SF. (2005). *Nature*, **437**, 1032–7.

Couceiro JR, Martin–Bermudo MD and Bustelo XR. (2005). *Exp Cell Res*, **308**, 364–80.

Croft DR, Sahai E, Mavria G, Li S, Tsai J, Lee WM, Marshall CJ and Olson MF. (2004). *Cancer Res*, **64**, 8994–9001.

Etienne–Manneville S and Hall A. (2001). *Cell*, **106**, 489–98.

Frost JA, Khokhlatchev A, Stippec S, White MA and Cobb MH. (1998). *J Biol Chem*, **273**, 28191–8.

Guo F and Zheng Y. (2004). *Oncogene*, **23**, 5577–85.

Hatzoglou A, Ader I, Splingard A, Flanders J, Saade E, Leroy I, Traver S, Aresta S and de Gunzburg J. (2007). *Mol Biol Cell*, **18**, 1242–52.

Joberty G, Perlungher RR, Sheffield PJ, Kinoshita M, Noda M, Haystead T and Macara IG. (2001). *Nat Cell Biol*, **3**, 861–6.

Justilien V and Fields AP. (2009). *Oncogene*.

Kremer BE, Adang LA and Macara IG. (2007). *Cell*, **130**, 837–50.

Lee HH and Chang ZF. (2008). *J Cell Biol*, **181**, 999–1012.
Liu XF, Ishida H, Raziuddin R and Miki T. (2004). *Mol Cell Biol*, 24, 6665–75.

Lu W, Katz S, Gupta R and Mayer BJ. (1997). *Curr Biol*, 7, 85–94.

Manser E, Huang HY, Loo TH, Chen XQ, Dong JM, Leung T and Lim L. (1997). *Mol Cell Biol*, 17, 1129–43.

Regala RP, Weems C, Jamieson L, Copland JA, Thompson EA and Fields AP. (2005). *J Biol Chem*, 280, 31109–15.

Riento K, Guasch RM, Garg R, Jin B and Ridley AJ. (2003). *Mol Cell Biol*, 23, 4219–29.

Sahai E, Garcia–Medina R, Pouyssegur J and Vial E. (2007). *J Cell Biol*, 176, 35–42.

Sanders LC, Matsumura F, Bokoch GM and de Lanerolle P. (1999). *Science*, 283, 2083–5.

Tatsumoto T, Xie X, Blumenthal R, Okamoto I and Miki T. (1999). *J Cell Biol*, 147, 921–8.

Wang HR, Zhang Y, Ozdamar B, Ogunjimi AA, Alexandrova E, Thomsen GH and Wrana JL. (2003). *Science*, 302, 1775–9.

Ward Y, Yap SF, Ravichandran V, Matsumura F, Ito M, Spinelli B and Kelly K. (2002). *J Cell Biol*, 157, 291–302.

Williams DA, Tao W, Yang F, Kim C, Gu Y, Mansfield P, Levine JE, Petryniak B, Derrow CW, Harris C, Jia B, Zheng Y, Ambruso DR, Lowe JB, Atkinson SJ, Dinauer MC and Boxer L. (2000). *Blood*, 96, 1646–54.

Wirth A, Schroeter M, Kock–Hauser C, Manser E, Chalovich JM, De Lanerolle P and Pfitzer G. (2003). *J Physiol*, 549, 489–500.

Zhang H and Macara IG. (2008). *Dev Cell*, 14, 216–26.
(4) LEGENDS TO SUPPLEMENTARY FIGURES

SUPPLEMENTAL FIGURE 1. Status of the microtubule system in the cell lines under study in this work. NIH3T3 cells expressing the indicated proteins were fixed and stained with anti–β–tubulin antibodies. After staining, microtubules (green color) were analyzed by microscopy. Scale bar, 20 µm.

SUPPLEMENTAL FIGURE 2. c–Myc overexpression does not rescue the loss of cell polarity of RhoA<sup>Q63L</sup>–transformed NIH3T3 cells. NIH3T3 cells expressing the indicated ectopic proteins (left) were subjected to a wound–healing assay for the indicated period of time (top).

SUPPLEMENTAL FIGURE 3. Deregulated expression of Pak1 and Itgβ1 in RhoA<sup>Q63L</sup>/c–Myc–expressing NIH3T3 cells. (A,B) Immunoblot analysis showing the protein expression of Pak1 (A) and Itgβ1 (B) in total cellular lysates obtained from the cell lines used in this study (top) that had been cultured under low (A,B) and high density (B) conditions. In A, RhoA<sup>Q63L</sup>+c–Myc #1, #2 and #3 refer to the IMB11–1–P, IMB11–2P and IMB11–3P cell lines, respectively. Samples used in panel A are aliquots of the same extracts used in Fig. 3B.

SUPPLEMENTAL FIGURE 4. Cell density affects the total protein levels of Pak1 (A) Immunoblot analysis showing the levels of phospho–Pak1 (upper panel), total Pak1 (middle panel), and tubulin (bottom panel) in total cellular lysates obtained from the indicated cell
lines (top) that had been cultured under the described conditions (bottom). (B) Quantification of the Pak1/tubulin and phospho–Pak1/Pak1 rations in the indicated cells lines. Bands corresponding to proteins under study were scanned and subjected to densitrometric analysis \((n = 3)\). *, \(P \leq 0.01\) compared to either sparsely cultured RhoA\(^{Q63L}\)–expressing cells or the indicated experimental pairs (brackets).

SUPPLEMENTAL FIGURE 5. Pak1 and integrin β subunits are involved in the disruption of stress fibers in RhoA\(^{Q63L}/c–Myc\)–expressing fibroblasts. (A) RhoA\(^{Q63L}\)–transformed NIH3T3 cells were infected with retroviral particles encoding the indicated proteins (top) and F–actin fibers detected by immunofluorescence techniques after staining with rhodamine–labeled phalloidin (upper panels, red color). Infected cells were identified by the expression of EGFP (bottom panels, green color). (B,C) Effect of the overexpression of wild type Pak1 (B) and a dominant negative mutant (DNM) of Pak1 (C) in the F–actin cytoskeleton (red color) in the indicated cell types (left). Infected cells were identified as in A. (D) NIH3T3 stably expressing the indicated proteins (left) were infected with retrovirus encoding bicistronically wild type Pak1 and EGFP (labeled as Pak1) or, alternatively, with retroviruses containing only EGFP (labeled as Mock). Cells were fixed and F–actin stress fibers visualized by staining with rhodamine–labeled phalloidin (red color). Infected cells were identified as in A. (E) RhoA\(^{Q63L}\)–transformed NIH3T3 cells were transfected with the indicated siRNAs and, 48 h later, stained with rhodamine–labeled phalloidin and analyzed by immunofluorescence microscopy. Scale bars in panels A–E, 20 μm.
(5) SUPPLEMENTARY FIGURES
Supplementary Figure 1
Sauzeau et al.
Supplementary Figure 2
Sauzeau et al.

| Time (hours) | 0       | 6       |
|--------------|---------|---------|
| **NIH3T3**   | ![Image](image1) | ![Image](image2) |
| **RhoA<sub>Q63L</sub>** | ![Image](image3) | ![Image](image4) |
| **RhoA<sub>Q63L</sub> + c-Myc** | ![Image](image5) | ![Image](image6) |
| **RhoA<sub>Q63L</sub> + c-Myc** | ![Image](image7) | ![Image](image8) |
| **RhoA<sub>Q63L</sub> + MadMyc** | ![Image](image9) | ![Image](image10) |
Supplementary Figure 3
Sauzeau et al.

A

B

Pak1

ltgβ1

 Sparse culture

 Dense culture

NIH3T3

 c-Myc

 RhoA G02L

 RhoA G02L +

 #1

 #2

 #3

 RhoA G02L +

 MaxMyc

RhoA G02L + c-Myc

 RhoA G02L + c-Myc siRNA

 RhoA G02L + MedMyc

 RhoA G02L + c-Myc

 RhoA G02L + c-Myc siRNA

 RhoA G02L + MedMyc
