Myosin IIA drives membrane bleb retraction

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ABSTRACT Membrane blebs are specialized cellular protrusions that play diverse roles in processes such as cell division and cell migration. Blebbing can be divided into three distinct phases: bleb nucleation, bleb growth, and bleb retraction. Following nucleation and bleb growth, the actin cortex, comprising actin, cross-linking proteins, and nonmuscle myosin II (MII), begins to reassemble on the membrane. MII then drives the final phase, bleb retraction, which results in reintegration of the bleb into the cellular cortex. There are three MII paralogues with distinct biophysical properties expressed in mammalian cells: MIIA, MIIB, and MIIC. Here we show that MIIA specifically drives bleb retraction during cytokinesis. The motor domain and regulation of the nonhelical tailpiece of MIIA both contribute to its ability to drive bleb retraction. These experiments have also revealed a relationship between faster turnover of MIIA at the cortex and its ability to drive bleb retraction.

INTRODUCTION

The actin cortex is a thin network of actin filaments underneath the plasma membrane that allows a cell to maintain and change shape in response to internal and external stimuli (Salbreux et al., 2012; Fritzsche et al., 2016; Sezgin et al., 2017). Membrane blebs are created upon a local detachment of the cortex from the membrane, which leads to an influx of cytosol, thus creating a spherical protrusion of the membrane (Salbreux et al., 2012). These specialized protrusions play multiple roles, such as releasing cytoplasmic pressure at the polar cortex during cytokinesis, as well as driving pressure-driven cell migration (Sedzinski et al., 2011; Bergert et al., 2015). As such, the mechanisms driving bleb growth and retraction remain active areas of interest.

The newly formed membrane bleb lacks the majority of cortical components. New cortex assembly occurs on this membrane, with ezrin appearing nearly instantaneously, followed by actin appearing ~2 s after bleb formation, and with nonmuscle myosin II (MII) following ~8 s later (Charras et al., 2006). Ezrin was recently shown to recruit MYOGEF to the bleb, which activates RhoA signaling (Jiao et al., 2018). This results in recruitment and activation of MII, which then drives retraction of the bleb. Previous work has suggested that turnover of actin, MII, and actin cross-linkers is critical for bleb retraction (Fritzsche et al., 2013). Specifically, the MII regulatory light chain was shown to turn over at rates intermediate between actin and actin cross-linking protein alpha actinin (Fritzsche et al., 2013). It was proposed that this allows myosin II to reorganize actin network architecture, even in the presence of passive cross-links (Fritzsche et al., 2013). Because the regulatory light chain binds multiple MII paralogues, the specific parologue responsible for bleb retraction is unknown. Furthermore, the relationship between turnover of that specific parologue and its correlation with bleb retraction has not been established.

There are three MII paralogues, MIIA, MIIB, and MIIC, with mammalian cells commonly expressing MIIA and MIIB (Vicente-Manzanares et al., 2009). Distinctive roles for MII paralogues have been proposed in multiple contexts, such as stress fiber formation in migrating cells (Beach et al., 2017), cell–cell junction formation in epithelial cells (Smutny et al., 2010), growth cone advance in neurons (Brown and Bridgman, 2003), and proplatelet formation in mice (Lordier et al., 2008; Bluteau et al., 2012). Here we show evidence for MIIA in specifically driving bleb retraction during cytokinesis. We further show that both motor activity at the N-terminus and regulation of the nonhelical tailpiece at the C-terminus control this activity. These experiments have also revealed a correlation between the rate of MII turnover and bleb retraction.

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Abbreviations used: CRISPR, clustered regularly interspaced short palindromic repeats; DIC, differential interference contrast; FRAP, fluorescence recovery after photobleaching; KO, knockout; MII, myosin II; MIIA, myosin IIA; MIIB, myosin IIB; MIIC, myosin IIC; Scr, scrambled siRNA; UT, untransfected.

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N.T. and D.T.B. conceived the study, designed the experiments, and wrote the manuscript. N.T. performed the experiments and analyzed the data.

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RESULTS AND DISCUSSION

To create a bleb in a controlled manner, we utilized laser ablation as previously described (Tinevez et al., 2009; Sedzinski et al., 2011; Goudarzi et al., 2012). Ablation of the polar cortex in a control cell undergoing cytokinesis in the formation of a bleb followed by retraction over a time period of ~2 min as previously reported (Figure 1A; Charras et al., 2008). We depleted either MIIA or MIIB in HeLa cells, which express only these two paralogues (Supplemental Figure S1) and created blebs. We found that knockdown of MIIA resulted in failure of bleb retraction, whereas MIIB knockdown did not (Figure 1, A and B). We observed a similar trend in spontaneously occurring blebs at the polar cortex (Figure 1B). The lack of an effect on bleb retraction upon MIIB knockdown could result from an inability of MIIB to get recruited to the bleb. To test this, we monitored MIIA and MIIB recruitment to newly formed blebs in live cells coexpressing MIIA mApple and MIIB mEGFP (Figure 1C). We found that both paralogues were recruited to blebs after their formation (Figure 1, C and D). Given that both MII paralogues are recruited to blebs, other mechanisms likely drive the differences we observed in bleb retraction upon knockdown.

We next wanted to confirm that MIIA was required to drive bleb retraction. To that end, we used a myh9 (MIIA) knockout HAP1 cell line we previously generated using CRISPR (Fenix et al., 2016), which expresses only MIIB (Supplemental Figure S1). We compared bleb retraction rates in this cell line versus the parental HAP1 cells. Knockout of MIIA resulted in failure of bleb retraction in HAP1 myh9 KO cells (Figure 2, A and B). Expression of full-length MIIA at 72.6 ± 33% of parental levels restored bleb retraction rates comparable to the parental cell line (Figure 2, B and C, and

**FIGURE 1:** MIIA but not MIIB is necessary for bleb retraction. (A) Laser-induced polar cortex ablation in control, MIIA- or MIIB-depleted HeLa cells. Representative control DIC montage shows the ablation ROI (magenta circle) used to create a membrane bleb (yellow arrow). Dotted yellow line represents ROI used to create kymographs. Representative kymographs for each condition are shown below. White arrows show the measurement method for calculating retraction rates. (B) Tukey plots comparing bleb retraction rates for controlled and spontaneous blebs in control vs. MIIAlo or MIIBlo cells. Controlled blebs: n = 25 control, 15 MIIAlo and 25 MIIBlo cells from three independent experiments. Spontaneous blebs: n = 18 control blebs from 9 cells, 15 MIIAlo blebs from 10 cells, 15 MIIBlo blebs from 10 cells over three independent experiments. (C) Representative time montage of HeLa cell coexpressing MIIA mApple and MIIB mEmerald showing the ablation ROI (magenta circle). Representative kymographs created using the solid white line show MIIA and MIIB recruitment to the bleb. Yellow ROI shows the region of the kymograph compared for recruitment (first 60 s). (D) Comparison of IIA and IIB recruitment to blebs in HeLa and HAP1 fibroblasts. n = 10 cells for each cell line over three independent experiments. Exact p values stated over respective bars. Solid black circles represent outliers. Scale bar: 10 µm.
FIGURE 2: The motor domain and non-helical tailpiece of MIIA are sufficient to drive bleb retraction. (A) Representative kymographs from HAP1 parental and myh9 KO cells following cortex ablation. n = 21 parental cells and 12 KO cells over three independent experiments. (B) Representative DIC and fluorescence images showing the localization of MII paralogues and mutants in HAP1 KO cells. (C) Representative kymographs from MIIA, MIIB, and MIIC expressing HAP1 KO cells following cortex ablation, as in Figure 1. Tukey plots comparing retraction rates in HAP1 KO cells expressing MIIA, MIIB, or MIIC, and Cos7 cells expressing MIIA, MIIB, MIIC, or untransfected (UT). For HAP1 KO cells, n = 27 MIIA, 10 MIIB, and 15 MIIC expressing cells over more than three independent experiments. For Cos7 cells, n = 16 untransfected, 16 MIIA, 11 MIIB, and 10 MIIC expressing cells over three independent experiments. (D) Representative kymographs showing MIIA N93K, MIIA/B, MIIB/A, and MIIA/B/A expressing HAP1 KO cells following cortex ablation. (E) Retraction rates comparing mutants shown in D. n = 21 N93K, 18 MIIA/B, 8 MIIB/A, and 21 MIIA/B/A expressing cells over more than three independent experiments. MIIA bar is from the same data set as C and is displayed only for comparison. Exact p values stated over respective bars. Solid circles in Tukey plots represent outliers. Scale bar: 10 µm.
Supplemental Table S1). Similar levels of MIIB or MIIC expression did not rescue bleb retraction (Figure 2, B and C, and Supplemental Table S1). We next wanted to further test the potential roles of MIIB and MIIC in driving bleb retraction. Therefore, we turned to Cos7 cells, which express only MIIB and MIIC (Even-Ram et al., 2007). While cortex ablation resulted in bleb formation, wild-type Cos7 cells failed to retract these blebs (Figure 2C, UT bar). Overexpression of either MIIB or MIIC did not result in bleb retraction (Figure 2C). However, Cos7 cells expressing exogenous MIIA did retract their blebs (Figure 2C). Taken together, our data show that MIIA is required to drive bleb retraction.

MIIA and MIIB primarily differ in their N-terminal motor domain as well as in their C-terminal nonhelical tailpiece (Vicente-Manzanares et al., 2009). We first hypothesized that motor activity of MIIA could play a role in bleb retraction. To test this, we expressed MIIA containing a N93K mutation in the motor domain, which results in reduced ATPase activity of MIIA (Figure 2B; Hu et al., 2002). Expression of MIIA N93K in HAP1 myh9 KO cells resulted in significantly slower bleb retraction, suggesting the motor domain of MIIA plays a role in bleb retraction (Figure 2, D and E). To test whether the motor domain of MIIA is sufficient to drive bleb retraction, we used chimeric motors, where the motor domains of the MIIA and MIIB were swapped (see schematics, Figure 2D; Vicente-Manzanares et al., 2008). Expression of MIIB/A, bearing the motor domain of MIIB, and the helical rod and nonhelical tailpiece of MIIA, did not rescue bleb retraction (Figure 2, D and E). On the other hand, expression of MIIA/B, bearing the motor domain of MIIA, and the helical rod and nonhelical tailpiece of MIIB significantly rescued bleb retraction (Figure 2, D and E). Interestingly, the MIIA/B chimera did not rescue bleb retraction rates to the same extent as full-length MIIA (p = 0.0009). This suggests that in addition to the motor domain of MIIA, other factors also contribute to drive bleb retraction.

Because MIIA and MIIB also differ in their nonhelical tailpiece, we hypothesized that the tailpiece of MIIA may also contribute to bleb retraction. Therefore, we created a chimeric motor, bearing the motor domain and nonhelical tailpiece of MIIB, and the helical rod domain of MIIA (MIIA/B/A). Expression of this construct at levels similar to MIIB/A (55 ± 21% for MIIB/A vs. 48 ± 10% for MIIB/A) resulted in statistically indistinguishable rates of bleb retraction compared with full-length MIIA (Figure 2, D and E). Taken together, these data show that the motor domain and nonhelical tailpiece of MIIA, with the rod domain of either MIIA or MIIB, are sufficient to drive bleb retraction.

Current models of bleb retraction propose that myosin II turnover plays a critical role in bleb retraction (Charras et al., 2008; Fritzsche et al., 2013). We therefore measured the turnover of MIIA and MIIB at the cortex using fluorescence recovery after photo-bleaching (FRAP), as previously performed for the regulatory light chain (Figure 3A; Fritzsche et al., 2013). In HeLa cells, we found that MIIA recovered twice as fast as MIIB (Figure 3B). We confirmed this finding in HAP1 myh9 KO cells expressing full-length MIIA or MIIB (Figure 3, C and E). Interestingly, MIIA recovered over markedly more slowly than MIIA and MIIB in HAP1 myh9 KO cells (Figure 3, D and E). Similar trends were observed in Cos7 cells (Figure 3F). Taken together, these data suggest that the relatively fast turnover of MIIA correlates with its ability to drive bleb retraction.

To further test whether changes in bleb retraction correlated with changes in turnover of MIIA at the cortex, we measured the turnover of the MIIA mutants and chimeras in HAP1 KO cells. MIIA N93K showed a slower recovery than full-length MIIA, indicating that lower motor activity correlates with slower turnover (Figure 4, A and B). Interestingly, the MIIA/B chimera recovered significantly more slowly than MIIA, but not MIIB (Figure 4, B and C). This suggests that the nonhelical tailpiece of MIIA plays a role in turnover, and its absence in the MIIA/B chimera results in its slower turnover compared with full-length MIIA. To test this, we expressed the MIIA/B/A chimera, comprising the motor domain and nonhelical tailpiece of MIIA and the helical rod domain of MIIB. The MIIA/B/A chimera rescued turnover rates to levels comparable to full-length MIIA, in agreement with our observation that this chimera also rescued bleb retraction rates (Figure 4, B and C). Taken together, these data suggest that both the motor domain and the nonhelical tailpiece of MIIA contribute to its fast turnover relative to MIIB or MIIC. We next wanted to determine the basis for regulation of MIIA turnover by the nonhelical tailpiece.

Previous studies have proposed phosphorylation of the myosin heavy chain at the nonhelical tailpiece results in MIIA filament disassembly during interphase (Dulyaninova et al., 2007; Dulyaninova and Bresnick, 2013; Breckenridge et al., 2008). To test whether phosphorylation of the nonhelical tailpiece regulates MIIA turnover at the polar cortex during cytokinesis, we expressed an MIIA mutant lacking the nonhelical tailpiece (Figure 5A). Deletion of the nonhelical tailpiece significantly slowed MIIA turnover (Figure 5B). The nonhelical tailpiece contains a single phosphorylation site that could regulate turnover (Ser 1943). We therefore created a phospho-null mutant of MIIA at this site (S1943A) and measured turnover using FRAP. We found that MIIA S1943A also recovered more slowly than wild-type MIIA (Figure 5B). Given the slower turnover of these MIIA tail mutants, we hypothesized that bleb retraction should be slower in these mutants. Indeed, both the MIIA Δtailpiece and MIIA S1943A mutants showed significantly slower bleb retraction (Figure 5, C and D).

Finally, we wanted to test the distinct role of MIIA in driving bleb retraction during interphase. To that end, we used filamin-deficient M2 cells that constitutively bleed during interphase, a classic model system used to study membrane blebbing (Figure 5E; Charras et al., 2006; Bovellan et al., 2014). We probed the expression of the three MII paralogues in M2 cells, and found they express all three paralogues (Supplemental Figure S1). To test their potential roles, we depleted each of the MII paralogues using small interfering RNA (siRNA) knockdown, and measured bleb retraction rates. Knockdown of MIIA, but not MIIB or MIIC, significantly impaired bleb retraction, suggesting that MIIA is the paralogue that drives bleb retraction even during interphase (Figure 5, F and G).

Here we show that MIIA is the specific paralogue that is necessary and sufficient to drive bleb retraction during interphase and cytokinesis. We confirmed this finding using three independent approaches: knocking down MIIA, knocking out MIIA, and expressing IIA in a cell line that does not normally express it. We also showed that the motor domain and the nonhelical tailpiece both regulate bleb retraction. The motor domain of MIIA has distinct biophysical properties compared with MIIB and MIIC, namely, having higher ATPase activity, as well as spending a smaller proportion of its mechanochemical cycle bound to actin in the force-generating state (i.e., lower duty ratio; Kovács et al., 2003; Wang et al., 2003). Interestingly, we found these biophysical properties correlated with faster turnover at the cortex compared with MIIB and MIIC.

Reducing MIIAs motor activity using the N93K mutation slowed the turnover of MIIA at the cortex, which correlated with slower bleb retraction. Replacing the motor domain of MIIA with the motor domain of MIIB also slowed turnover (p = 0.0257); this construct also did not support bleb retraction. These findings thus establish a link between the turnover rate and motor activity of MIIA. The positive correlation between MIIA turnover and bleb retraction supports previous experimental and theoretical studies suggesting that
turnover is critical for bleb retraction (Charras et al., 2008; Fritzsche et al., 2013). A limitation of our current study, as well as previous studies that have measured MII turnover, is that we correlated measurements made at the cortex with bleb retraction rates (Fritzsche et al., 2013). We were unable to combine photoablation to create a bleb and photobleaching to measure MII turnover within the same bleb.

We further showed that the nonhelical tailpiece of MIIA also regulates its turnover. Deletion of this segment resulted in both slower turnover and slower bleb retraction. Of note, this effect was FIGURE 3: MIIA shows fast turnover compared with MIIB and MIIC at the cortex. (A) Representative time montages from two separate cells showing FRAP of MIIA and MIIB mEGFP expressed in HeLa cells. Inset shows an enlarged view of the yellow box. Dotted white box represents the bleaching region. (B) Averaged FRAP curves for MIIA and MIIB in HeLa cells. n = 15 cells each for MIIA and MIIB over three independent experiments. See Materials and Methods for the curve fitting method. (C, D) Averaged FRAP curves for MIIA and MIIB mEGFP (C), and MIIC mEGFP (D) expressed in HAP1 KO cells. (E) Tukey plots showing time for half-maximal recovery for MIIA, MIIB, and MIIC in HAP1 KO cells. n = 11 MIIA, 13 MIIB, and 11 MIIC expressing cells over three independent experiments. (F) Tukey plots showing time for half-maximal recovery for MIIA, MIIB, and MIIC in Cos7 cells. n = 11 MIIA, 9 MIIB, and 10 MIIC expressing cells over three independent experiments. Exact p values stated over respective bars.
not specific to MIIA, because deletion of the nonhelical tailpiece of MIIB also resulted in slower turnover of MIIB (Supplemental Figure S2). Not surprisingly, this did not change MIIB’s inability to drive bleb retraction (Supplemental Figure S2). While the motor domain of MIIA is absolutely required for bleb retraction, the nonhelical tailpiece may add an additional layer of regulation. This finding is in agreement with previous studies that have proposed that heavy chain phosphorylation controls MIIA disassembly from filaments (Dulyaninova et al., 2007; Dulyaninova and Bresnick, 2013; Brekenridge et al., 2008). Our finding that Ser1943 regulates bleb retraction also suggests an important role for casein kinase II in regulating MIIA heavy chain phosphorylation, which has been shown to phosphorylate Ser1943 in vitro (Kelley and Adelstein, 1990; Dulyaninova et al., 2005). The development of biosensors as well as specific, fast acting inhibitors will likely be necessary to better understand the mechanisms regulating MIIA assembly state during the cell cycle and at the polar cortex.

Membrane blebs play diverse roles in cellular physiology. During cytokinesis, blebs serve as pressure release valves to regulate intracellular pressure and when deregulated, result in dramatic cell shape instabilities and cytokinetic failure (Sedzinski et al., 2011). We have established that MIIA is the major parologue that drives bleb retraction during cytokinesis. Therefore, a high level of MIIA expression may amplify genome instability through deregulation of cell shape during cytokinesis in cancer cells. Although we did not find a role for MIIB or MIIC in bleb retraction, it is still possible that they may have other roles at the cortex during cytokinesis. The slower turnover of MIIB and MIIC, combined with their lower ATPase activity may make these paralogues better suited to act as cross-linkers at the cellular cortex, thus maintaining cortex stability.

Cells also employ blebbing as a mode of migration, both during development and cancer progression (Trinkaus, 1973; Kageyama, 1977; Friedl and Wolf, 2003; Sahai and Marshall, 2003; Blaser et al., 2006). Interestingly, certain types of cancers up- or down-regulate MII paralogues (Maeda et al., 2008; Derycke et al., 2011). Our finding that MII paralogues have distinct roles in bleb retraction may thus have interesting implications for bleb-based cell migration in both developmental and pathological contexts.

**MATERIALS AND METHODS**

**Cell lines, growth conditions, and chemicals**

HeLa (American Type Culture Collection [ATCC], CCL-2) and Cos7 (ATCC CRL-1651) cells were cultured in growth media composed of DMEM (Mediatech, Manassas, VA; #10-013-CV) containing 4.5 g/l L-glutamine, L-glucose, and sodium pyruvate, and supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO; #F2442). HAP1 myh9 (MIIA) KO and parental cells were purchased from Haplogen Genomics as previously described (Fenix et al., 2016), and cultured in Iscove’s minimal essential medium (IMDM) supplemented with 10% FBS. M2 melanoma cells were cultured in MEM supplemented with Earle’s salts, 10 mM HEPES, and 10% FBS. Growth substrates were prepared by coating #1.5 glass cover slips (In Vitro Scientific; #D35-20-1.5N) with 10 μg/ml fibronectin (Corning, NY; #354008) in phosphate-buffered saline (Mediatech; #46-013-CM) at 37°C for 1 h.

For protein expression, cells were transiently transfected using Fugene 6 (Promega, Madison, WI; #E2691) as per the manufacturer’s instructions overnight in a 24-well tissue culture plate (Corning) before plating on a growth substrate.

Alexa Fluor 488–goat anti-rabbit (#A11034) and Alexa Fluor 568–goat anti-rabbit (#A11036) were purchased from Thermo Fisher Scientific (Waltham, MA). Rabbit anti-myosin IIA (#909801) was purchased from BioLegends (San Diego, CA). Rabbit anti-myosin IIB (#88245) and myosin IIC (#8189) were purchased from Cell Signaling Technology (Danvers, MA). Mouse anti-tubulin (#T6199) was purchased from Millipore Sigma (Darmstadt, Germany).
Plasmids
MIIA mApple (Addgene; #54929) and MIIB mEmerald (Addgene; #54192) were gifts from Michael Davidson (National High Magnetic Field Laboratory). MIIA mEGFP (Addgene, Cambridge, MA; #11347) was a gift from Robert Adelstein (National Institutes of Health). MIIB mEGFP (Addgene; #35691) was a gift from Venkiah Betapudi (Case Western Reserve University). MIIA Δtail (Addgene; #35689) was a gift from Thomas Egelhoff (Case Western Reserve University). The mEGFP tagged MIIB/A and MIIA/B chimeras were generously provided by Miguel Vincente-Manzanares (Universidad Autonoma de Madrid, Spain). MIIA S1943A and MIIB Δtail were created by mutagenizing MIIA mEGFP and MIIB mEmerald, respectively, using a site-directed PCR mutagenesis protocol as described previously (Liu and Naismith, 2008). The A/B/A chimera was created using Gibson Assembly. The fragments corresponding to the rod region of MIIB and corresponding to the motor and nonhelical tailpiece of MIIA were amplified using PCR, following which they were PCR purified and assembled using the NEB HiFi Gibson Assembly Kit according to the instructions provided by the manufacturer.

Live imaging and FRAP
Live imaging and FRAP experiments were performed on a Nikon Eclipse Ti-E inverted microscope equipped with a Yokogawa CSU-X1 spinning-disk head, 1.4 NA 60x oil objective, Andor DU-897 EMCCD, and a dedicated 100-mW 405-diode ablation laser, generously provided by the Nikon Center of Excellence at Vanderbilt University. The instrument was controlled using Nikon Elements AR software. Bleaching was performed for 500 ms using a pixel dwell time of 500 μs and 15% laser power with the same 2.7 μm × 2.7 μm region of interest (ROI) for all FRAP experiments. Samples were maintained at 37°C with 5% CO₂ in a Tokai Hit Stage incubator.

To image endogenous MIIA in fixed HAP1 cells, single optical sections through the middle of the cell were acquired using the ablation and FRAP experiments. To image M2 cells, we used a Nikon Eclipse Ti wide-field system equipped with a 1.45 NA 100x objective with a Tokai Hit heated stage.

Cortical ablation
Laser damage–induced ablation of the polar cortex was performed on a Nikon Eclipse Ti-E inverted microscope equipped with a Yokogawa CSU-X1 spinning-disk head, 1.4 NA 60x oil objective, an Andor DU-897 EMCCD, and a dedicated 100-mW 405-diode ablation laser, generously provided by the Nikon Center of Excellence at Vanderbilt University. A 1.4 μm × 1.4 μm ROI was used for all experiments. A differential interference contrast (DIC) and/or fluorescence image was acquired before ablation, followed by ablation using a miniscanner. A pixel dwell time of 500 μs, 50% laser power was used for a duration of 1 s, followed by acquiring DIC or fluorescence images at 2 s intervals.

Knockdown experiments
Smart Pool Accell siRNA against MIIA (myh9 gene, #E-007668, #1-CCGUUGACUCAGUAUUAGUU, #2-UCCACAUCUCAGUAUUAGGU, #3-CCGUUGACUCAGUAUUAGCU, #4-UCCACAUCUCAGUAUUAGCU, and #5-UCCACAUCUCAGUAUUAGCU) were provided by the SMARTpool company and were transfected into M2 cells using Metafectene Pro (Biontex Laboratories, Berlin, Germany). The MIIA knockdown experiments were repeated three times. A representative experiment is shown in Figure 5E.
For quantification of FRAP data, confocal time montages acquired at 2 s intervals were first aligned using the StackReg plug-in in Fiji, followed by drawing ROIs around the bleached region, an unbleached region, and background. Mean intensity over time was calculated for each of the three ROIs using the multimeasure function in the ROI manager. Subsequent analysis was performed using the EasyFRAP algorithm in MATLAB as previously described (Rapsomanski et al., 2015). Briefly, a double normalization was performed to account for background correction and photobleaching, followed by fitting the normalized curves to a second-degree exponential to obtain the half-maximal recovery time and mobile fraction (see Rapsomanski et al., 2015, for details on fitting equations and normalization). Curves with poor fits (with $R^2 < 0.9$) were not included for analysis. We noted negligible bleaching (<5% for GFP). FRAP curves averaged using entire data sets for display (Figures 3–5) were generated in Excel.

**Statistics**

Statistical significance was determined using Mann–Whitney’s U test using GraphPad Prism. All graphs are represented as Tukey plots showing boxes (with median, Q1, Q3 percentiles), whiskers (minimum and maximum values within 1.5 times interquartile range), and outliers (solid circles). No outliers were removed from the analysis.

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