Resolving Subcellular miRNA Trafficking and Turnover at Single-Molecule Resolution

Graphical Abstract

Highlights

- diSHiRLoC and CCA quantify miRNA unwinding, turnover, and activity inside cells
- miRNA stability and nuclear retention is dependent on Argonaute and targets
- miRNA unwinding, strand selection, and cytoplasmic retention are Ago2 dependent
- Nuclear miRNAs do not repress cognate targets

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In Brief

Pitchiaya et al. describe tools to interrogate gene-regulatory microRNAs inside living cells at single-molecule resolution. They find that the RNA silencing machinery and RNA targets mediate gene silencing surveillance by modulating the abundance and subcellular location of microRNAs. These findings and tools promise to facilitate single-cell screening of microRNA activity.

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Resolving Subcellular miRNA Trafficking and Turnover at Single-Molecule Resolution

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SUMMARY

Regulation of microRNA (miRNA) localization and stability is critical for their extensive cytoplasmic RNA silencing activity and emerging nuclear functions. Here, we have developed single-molecule fluorescence-based tools to assess the subcellular trafficking, integrity, and activity of miRNAs. We find that seed-matched RNA targets protect miRNAs against degradation and enhance their nuclear retention. While target-stabilized, functional, cytoplasmic miRNAs reside in high-molecular-weight complexes, nuclear miRNAs, as well as cytoplasmic miRNAs targeted by complementary anti-miRNAs, are sequestered stably within significantly lower-molecular-weight complexes and rendered repression incompetent. miRNA stability and activity depend on Argonaute protein abundance, whereas miRNA strand selection, unwinding, and nuclear retention depend on Argonaute identity. Taken together, our results show that miRNA degradation competes with Argonaute loading and target binding to control subcellular miRNA abundance for gene silencing surveillance. Probing single cells for miRNA activity, trafficking, and metabolism promises to facilitate screening for effective miRNA mimics and anti-miRNA drugs.

INTRODUCTION

MicroRNA(miRNA)-mediated gene silencing is a pervasive, evolutionarily conserved cellular pathway wherein endogenous small non-coding RNAs (ncRNAs) guide the RNA-induced silencing complex (RISC) to translationally repress and eventually degrade complementary sequence-matched mRNA targets (Pasquinelli, 2012). Biosynthesis of functional miRNAs follows a tightly regulated multi-step pathway, encompassing both the nuclear and cytoplasmic compartments of the cell (Ha and Kim, 2014; Lin and Gregory, 2015). Following the transcription of a miRNA gene, a primary miRNA transcript (pri-miRNA) is processed in the nucleus to generate a corresponding precursor-miRNA (pre-miRNA), which is then exported to the cytoplasm and further processed to yield mature, ~22-bp miRNA duplexes. One strand of the miRNA duplex (the guide strand [G]) is preferentially retained within the Argonaute (Ago) protein containing RISC, whereas the other strand (passenger [P], or ‘*’*) is released and possibly degraded. Activated microRNA-induced silencing complex (miRISC) then engages mRNAs by hybridizing the G strand with complementary seed sequences, leading to RNA silencing (Pasquinelli, 2012). Recent reports suggest that many RISC factors and mature miRNAs are also present in the nucleus, allowing for potentially widespread nuclear functions (Gagnon et al., 2014; Khudayberdiev et al., 2013; Liao et al., 2010; Weinmann et al., 2009; Zisoulis et al., 2012). Collectively, miRNAs regulate many developmental and physiological processes, and their dysregulation is known to lead to pathologies, including cancer (Lin and Gregory, 2015). The cellular abundance and subcellular localization of miRNAs is thus central to maintaining physiological homeostasis.

miRNA levels are regulated via both transcriptional and post-transcriptional mechanisms. Post-transcriptional regulation of miRNAs can occur via protein- or target RNA-mediated pathways. One protein-mediated pathway involves the regulation of the levels or activities of key role players in the miRNA biosynthesis pathway (Ha and Kim, 2014; Pasquinelli, 2012). Another pathway encompasses proteins, including the XRN1/2 5’-to-3’ (Chatterjee and Grosshans, 2000) and SDN 3’-to-5’ exonuclease complexes (Ramachandran and Chen, 2008), that directly mediate miRNA turnover (Kai and Pasquinelli, 2010). There is conflicting evidence, however, whether targets of miRNAs promote or rather inhibit turnover of mature miRNA. Brown and coworkers (Baccarini et al., 2011) reported that extensive complementarity between miRNA targets and miRNAs promotes miRNA degradation in human cells, suggesting that miRNA decay through 3’ uridylation is promoted by targets. Moreover, Zamore and coworkers have found evidence that targets promote 3’ adenylation and/or trimming of cognate miRNA 3’ ends to initiate degradation in Drosophila (Ameres et al., 2010). In contrast, mRNA targets have been shown to protect miRNAs in C. elegans (Chatterjee et al., 2011).

Recent reports suggest that sub-cellular localization is critical to miRNA function (Leung, 2015). In particular, the discoveries of mature miRNAs in the nucleus (Gagnon et al., 2014; Khudayberdiev et al., 2013; Liao et al., 2010) and of the ability of small RNAs to guide RNA target cleavage in the nucleus (Gagnon et al., 2014) were unexpected. Several groups have suggested
that engineered and exogenously added small RNAs and Ago proteins can mediate nuclear gene regulation in the forms of inhibition (Castanotto et al., 2005; Janowski et al., 2005, 2006; Kim et al., 2006; Morris et al., 2004; Napoli et al., 2009; Ting et al., 2005) or activation of transcription (Janowski et al., 2007; Li et al., 2006; Matsui et al., 2013) and of control over alternative splicing (Alió et al., 2008; Liu et al., 2012), but mechanisms mediating these processes largely remain unresolved. While import and export factors mediating the nucleo-cytoplasmic trafficking of mature miRNAs have been identified (Castanotto et al., 2009; Ohrt et al., 2006; Yi et al., 2003), factors that retain mammalian miRNAs in the nucleus and the kinetics associated with trafficking are largely unknown.

Consequently, there is an urgent need for approaches that can dissect miRNA localization, function, and turnover within the cellular environment. To this end, we previously reported the ability of intracellular single-molecule, high-resolution localization and counting (iSHiRLoC) to dissect two kinetically sequential miRNA assembly pathways in single HeLa cervical cancer cells (Pitchiya et al., 2012, 2013). Here, we have extended iSHiRLoC to exploit single, microinjected, mature miRNA reporters for a comprehensive survey of the subcellular localization, maturation, turnover, and function of miRNAs. Our current technology interrogates RISC loading and subsequent steps of the miRNA-mediated RNA silencing pathway but is potentially extendable to investigate upstream pathway steps, simultaneously visualize small RNAs and RISC complexes, and probe target search mechanisms of RISC inside cells. Introducing a corelative counting analysis (CCA) of live- and fixed-cell images, we find that Ago proteins and seed-matched targets play a critical role in mediating nuclear retention and stabilization of mature miRNAs. In cells lacking Ago2, P strand discard is diminished and nuclear localization enhanced compared to cells either over-expressing Ago2 or lacking Ago1, suggesting additional non-redundant functions of nuclear Ago proteins. We further find that nuclear miRNAs are not as effective in silencing their cognate targets as their cytoplasmic counterparts. Our work reveals underappreciated roles of both cognate RNA targets and Ago2 in controlling subcellular miRNA abundance for surveillance of gene regulation.

RESULTS

iSHiRLoC Resolves miRNA Localization, Unwinding, and P Strand Removal

To dissect the RNA silencing pathway in mammalian cells, we expanded our previously established iSHiRLoC assay (Figure 1A). We used the highly conserved tumor suppressor miRNA let-7-a1 (henceforth l7/l7*, where l7 refers to the guide or G strand and l7* refers to the P strand, known to have ~1,200 mRNA targets in human cells per targetscan 7.1) (Rough and Slack, 2008) as a reporter and the flat, microscopically amenable human U2OS osteosarcoma cells as our model system. Based on prior reports that fluorophore labels on miRNAs do not affect the activity and binding kinetics of eukaryotic Ago in vitro (Chandra-doss et al., 2015), we further confirmed that the conjugation of a fluorescent dye to the 3’ end of individual or both strands of a mature, double-stranded miRNA (ds-miRNA) does not affect their RNA silencing activity in U2OS cells (Figure S1). To avoid photobleaching of the miRNA-conjugated Cy3 or Cy5 dye during initial visualization of cells, microinjection samples were supplemented with a spectrally distinct, fluorescein- or Alexa-Fluor-405-labeled dextran. Hydrophilic dextrans are biologically inert and stable and cannot permeate through the cell membrane, so they are retained within cells (Ludtke et al., 2002). Moreover, high-molecular-weight (>70 kDa) dextrans are refractory to nucleo-cytoplasmic transport so that they remain within the intracellular compartment of microinjection (Kee et al., 2012). In addition to serving as a visual aid to identify cellular and nuclear boundaries, the co-injected dextrans were used henceforth as a loading control to normalize for the amount of material microinjected (Figures 1B, 1C, and 1D).

Upon microinjection of just ~10,000 miRNA molecules, or less than 5% of the total cellular miRNA count, l7-Cy5/l7* assembled into spatially resolved, diffusing particles in living U2OS cells 2 hr after microinjection (Figure 1B), akin to our previous observation in HeLa cells (Pitchiya et al., 2012, 2013). Using an optimized counting algorithm (Figure S2; Supplemental Experimental Procedures), we quantified the normalized subcellular abundance of diffusing particles and found that l7 predominantly remained in the cytoplasm, with a small yet significant fraction of 9% ± 3% found in the nucleus (Figure 1C). Consistent with our observations in HeLa cells (Pitchiya et al., 2012, 2013), diffusion coefficients of cytosolic l7 in U2OS cells distributed within two Gaussian populations that resembled MS2-MCP-labeled miRNAs and GFP labeled P-bodies respectively (Figure 1D), as expected for a functional miRNA (Pitchiya et al., 2012). To assess subcellular miRNA abundance more quantitatively, we performed stepwise photobleaching counting of similarly microinjected cells after fixation (Figure 1E). We found that >85% of all labeled particles contained only a single miRNA reporter molecule (Figure S2) and that 17% ± 4% of these molecules localized to the nucleus (Figures 1E and F). Considering that U2OS cells are only ~2.5–5 μm deep (Macdonald et al., 2013) and the depth of our highly inclined laminar optical sheet (HILO) illumination is ~3 μm (Liu et al., 2015), miRNAs present within the focal plane of illumination represent ~50% of all miRNAs in the cell (Figure S2).

In the cytosol, ds-miRNAs are unwound to retain the single-stranded G strand within RISC and to eject and possibly degrade the P strand. To further test for full functionality of our miRNA reporters, we performed two-color iSHiRLoC on fixed cells with the G and P strands of l7 miRNA labeled with spectrally distinct Cy5 and Cy3 dyes (l7-Cy5/l7*-Cy3), respectively (Figures 1A and 1G). Strikingly, we found that cells contained a large (5-fold) excess of l7 over l7* in both cytoplasm and nucleus (Figure 1H,I), independent of dye identity (Figure S3), ruling out differential fluorescence detection sensitivity as a cause. These observations are consistent with the known asymmetric loading of l7 over l7* into RISC (Chatterjee et al., 2011), combined with loss of the ejected P strand due to RNA degradation and the expected expulsion of the resulting free dye from the cell (Homoya et al., 1993) or other forms of exocytic pathways. 2 hr after microinjection, less than 5% of G molecules still co-localized with P strand (Figure 1J), indicating that very few miRNA molecules are still double stranded and attesting to efficient loading of the guide. Taken together, our data establish iSHiRLoC as a quantitative
single-molecule tool for detecting the subcellular localization, unwinding, and degradation of miRNAs in individual cells. Conversely, each cell in effect becomes an isolated reaction vessel for probing miRNA processing pathways.

**Correlative Live- and Fixed-Cell Counting Probes miRNA Integrity and Activity**

To verify that our microinjected ds-miRNAs functionally engage mRNA targets in the form of guide-strand loaded RISC, as opposed to merely hybridizing to complementary targets in the absence of a protein complex, we performed a series of iSHiRLoC assays with several RNA and DNA variants of I7/I7* miRNA. 2 hr after microinjection, I7-Cy5/I7* in live cells had again assembled into slowly diffusing RNPs (Figures 2A and 2B), which together with corresponding reporter gene assays (Figure 2C) indicated that I7 loaded RISC binds to and actively represses target mRNAs (Pitchiaya et al., 2012; Pitchiaya et al., 2013; Shankar et al., 2016). Strikingly, microinjecting an equivalent
quantity of single-stranded l7 (l7-Cy5) resulted in similarly diffusing complexes; however, the (normalized) number of such particles was significantly (~3-fold) less than for l7-Cy5/l7* (Figures 2A and 2B). This observation suggests that a majority of l7-Cy5 either resides in rapidly diffusing particles (with a diffusion constant >10 μm²/s and a molecular weight <0.5 MDa) that blur out at 100-ms time resolution (Liu et al., 2015; Pitchiaya et al., 2012) or is degraded and/or lost from the cell. To distinguish these possibilities, we performed iSHiRLoC’s stepwise photobleaching counting in fixed cell (Pitchiaya et al., 2013) and found that the number of l7-Cy5 molecules was diminished by ~3-fold compared to l7-Cy5/l7*, very similar to our observations in live cells (Figures 2A–2D). These results suggest that single-stranded RNAs (ssRNAs) are actively degraded and the dye then expelled from the cell, similar to our observation for the unused P strand (Figure 1). Accordingly, the ssRNA does not downregulate a potential target in a gene reporter assay, unlike the corresponding dsRNA (Figure 2C). Further supporting our interpretation, guide-strand-labeled double-stranded DNA (dsDNA) or single-stranded DNA (ssDNA) versions of l7/l7*, expected to

**Figure 2. Validation of the CCA**

(A) Representative images of live and fixed U2OS cells injected with various Cy5-labeled oligonucleotides (red) bearing the sequence of let-7 miRNA. Scale bar, 10 μm. Images were acquired 2 hr after microinjection.

(B) Scatterplot depicting the total number of particles for each “live” sample in (A). Error bars represent SEM (n ≥ 2, number of cells ≥ 20).

(C) Luciferase reporter assays of U2OS cells transfected with the pmG-mH3U plasmid and the appropriate oligonucleotide. Error bars represent SEM (n = 3, with four technical replicates per trail).

(D) Scatterplot depicting the total number of molecules for each “fixed” sample in (A). Error bars represent SEM (n ≥ 2, number of cells ≥ 20).

(E) Plot correlating the abundance of particles in live and fixed cells. Dotted line represents a perfect correlation. Each data point depicts the mean value, and error bars depict SEM. Shaded regions were arbitrarily chosen.

(F–H) Luciferase reporter assays of U2OS cells transfected with the pmG-mH3U plasmid and either a scrambled double-stranded oligonucleotide (Scr) or l7-Cy5/l7* under various treatment conditions. Error bars represent SEM (n = 3, with four technical replicates per trail). For inhibiting RISC loading (F), cells were treated with Geld and compared to control cells treated with DMSO. For competitive inhibition experiments (G), cells were treated with a 5-fold excess of unlabeled miR21 (m21/m21*) or an equivalent amount of dl7/dl7*. For blocking target binding (H), cells were treated with a 3-fold excess of anti-let7 anti-miR (LNA) or control anti-miR (ctrl LNA) bearing no complementarity to let7.

(I) Plot correlating the abundance of l7-Cy5/l7* particles in live and fixed cells, as plotted in (E).

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be more stable intracellularly but incapable of RNA silencing, failed entirely to assemble into slowly diffusing RNP (Figures 2A and 2B), even though their intracellular abundance in fixed cells remained high (Figures 2A and 2D), yet without regulatory potential (Figure 2C).

Since live-cell particle counting provided complementary information to our fixed-cell analysis, we correlated the two datasets within a comprehensive plot that we refer to here as CCA (Figure 2E). Plotting the live-cell imaging data on the x axis with the fixed-cell data on the y axis, we used the standard error of our data to empirically determine 35:65 and 30:70 splits of the x and y axes, respectively, to delineate the plot into four practically useful quadrants. Samples such as l7-Cy5/l7* that efficiently assemble into complexes diffusing like mRNPs in live cells and are stably retained as observed in fixed cells, will occupy the top right quadrant (Figure 2E, “I”). Samples prone to degradation, such as l7-Cy5, lead to low live- and fixed-cell counts and will occupy the bottom-left quadrant (Figure 2E, “III”). By contrast, stable samples that fail to assemble into slowly diffusing complexes but are retained in the cell, such as single-stranded or double-stranded let-7 DNA (dI7-Cy5 or dI7-Cy5/MI7*), will occupy the top-left quadrant of the plot (Figure 2E, “II”). Quadrant IV is populated when the particles identified in live cells exceed those identified in fixed cells, a scenario that typically manifests due to fixation induced dye photobleaching or loss from cell permeation. We find that quadrant IV is never populated in our assays. This correlative plot thus delineates the differences in intracellular behavior between species and further supports the notion that microinjected miRNAs act functionally through RNP assembly and not simple hybridization to mRNA targets.

To further test the robustness of our interpretation, we blocked two distinct steps of the RNA silencing pathway and performed iSHiRLoC-based correlative live/fixed-cell counting (Figures 2F–2I). First, we inhibited the loading of miRNA into RISC by treating cells with geldanamycin (Geld), an inhibitor of the Hsp90/Hsc70 chaperone, a known RISC loading factor (Wasaki et al., 2010). Alternatively, we co-microinjected a 5-fold excess of unlabeled miR21 (m21/m21*) miRNA as a direct competitor for RISC loading. Finally, we co-microinjected an anti-miR complementary to I7 strand (anti-let7) to block target binding by the miRNA. All three strategies relieved miRNA-mediated silencing in our luciferase gene reporter assay (Figures 2F–2H), in contrast to controls in which cells were treated with only DMSO (the geldanamycin solvent), with dark dI7/dI7*, or with a scrambled anti-miR (Figures 2F–2H), as expected. Concordantly, all three controls occupied quadrant I of the CCA plot, suggesting that the microinjected I7-Cy5/l7* stably assembled into functional RNPs (Figure 2I). In contrast, both strategies for blocking RISC loading shifted I7-Cy5 into quadrant III (Figure 2I), consistent with the miRNA no longer efficiently loading into RISC and consequently being degraded. Strikingly, anti-let7-treated cells occupied quadrant II of the plot (Figure 2I), indicative instead of intracellular stabilization of the miRNA within low molecular weight complexes of limited efficacy in engaging mRNAs. Our data thus suggest that 2 hr after microinjection, ~50%–80% of all observed miRNA molecules are stably retained inside cells as functional RNPs, while the rest are still undergoing RISC assembly or are involved in other cellular pathways. Our results also establish iSHiRLoC as a quantitative approach to dissecting miRNA integrity and activity.

**Seed-Matched Targets Enhance Intracellular miRNA Stability**

Based on the calibration of our microinjector (Pitchiya et al., 2013), an intra-needle concentration of 1 μM should introduce ~10,000 molecules of miRNA per cell. 2 hr after microinjection, we typically observed only ~200–600 miRNA molecules per cell, suggesting that signal from a large fraction of molecules is lost due to degradation, expulsion from the cell, and/or other processes. To test for miRNA turnover and fluorophore expulsion from U2OS cells, we performed time courses of fixed- and live-cell counting of microinjected I7-Cy5/l7*-Cy3 and I7-Cy5/ I7*, respectively. Observing fixed cells, loss of I7-Cy5 signal occurred in two distinct phases, fitted well with a double-exponential decay function (Figure 3A; Table S1). Signal loss was rapid during the first phase, i.e., within 1 hr after microinjection (t1 ~20 min), with a second miRNA population observed to be lost 7-fold more slowly over the next several hours (t2 ~8–24 hr) so that at 16 hr, no miRNAs were left (Figure 3A; Table S1). The half-life of I7-Cy5 measured using iSHiRLoC is similar to that of endogenous I7 in other cell lines (Rieger and Großhans, 2012). Strikingly, at zero time, the fit intersects the y axis at ~9,700 molecules, consistent with the microinjection of ~10,000 molecules per cell (Table S1). Importantly, loss of I7 P strand was also biphasic and considerably more rapid throughout the time course (Figure 3A; Table S1), wherein a significant fraction of the molecules disappeared with a t1 of ~7 min with the remaining molecules disappearing from the cells with a t2 of 84 min. These data further corroborate our finding of asymmetric RISC loading of the G strand (Figure 1) and suggest that although a large fraction of the microinjected miRNA G strand is degraded, a significant fraction is stabilized over the P strand. Further supporting this view, in live cells, the appearance of slowly diffusing I7-Cy5 particles also occurred in two phases (Figure 3A), with the number of observable particles rapidly increasing within the first hour (t1 ~6 min) and slowly disappearing from view (t2 ~6 h) until no observable signal was detected 16 hr after microinjection. Taken together, these results suggest that a large fraction of I7/I7* molecules assemble as guide-strand-enriched miRISC (Figures 1, 2, and 3A), with a small-yet-significant fraction rapidly assembling into quite stable miRISC-mRNP complexes.

We next asked how the intracellular assembly and turnover of other miRNAs compares to I7/I7* by performing correlative live- and fixed-cell counting 2 hr after microinjection, when stable assembly of I7 was observed (Figure 3A). We found that a guide-strand-labeled let-7 seed mutant (m7-Cy5/m7*), which cannot repress targets with I7 binding sites (Figure S3C) and consequently has few intracellular targets, was rapidly turned over and unable to assemble into active miRISC-mRNP complexes, as indicated by a shift into quadrant III (Figure 3B). Similarly, the artificial cxcr4 miRNA (cx) and a negative control dsRNA (Scr), both of which have limited intracellular targets, were unstable and inefficiently assembled into diffusing complexes, also confining them to quadrant III (Figure 3B).
guide-strand-labeled miR-21 (m21-Cy5/m21*), which has ~300 distinct seed-matched targets in human cells (predicted by targetscan 7.1), stably assembled into miRISC-mRNP complexes and was found in quadrant I, next to l7 (Figure 3B). These data suggest that miRNA stability is correlated with the abundance of intracellular targets. To directly test whether seed-matched targets enhance miRNA stabilization and assembly into miRISC-mRNP complexes, we co-microinjected random, seed-mismatched, or seed-matched mRNA targets with the ml7-Cy5/ml7* or cx-Cy5/cx* miRNAs. As predicted, seed-matched targets mediated miRNA protection and miRISC-mRNP assembly, whereas all mismatches do not (Figure 3C).

Based on our previous observation that l7 is stabilized by anti-l7 in the form of small-molecular-weight complexes (Figure 2I), we reasoned that anti-miRs might act as "minimal" seed-matched targets. If so, seed-matched anti-miRs should generally mediate miRNA protection, just as the mRNA targets they mimic. We therefore co-microinjected various combinations of anti-miRs with either matched or mismatched miRNAs, confirming that only little anti-miR hybridized with the ds-miRNA in the injection needle (Figure S4A). As predicted, correlative live- and fixed-cell analysis showed that anti-cxcr4 miRNA robustly stabilized matched cx miRNA while not forming the slowly diffusing particles resembling miRISC-mRNP complexes, as evident from this combination appearing in quadrant II (Figure 3D). Indeed, an anti-miR generally shifts a matched or nearly matched miRNA into quadrant II (Figure 3D). Notably, anti-l-let7 shifted the nearly matched mutant ml7-Cy5 into quadrant II but stabilized it less than the matched l7-Cy5, as evident from its lower fixed-cell count (Figure 3D). This observation is consistent with the only partial relief of ml7-mediated reporter gene repression by anti-l-let7 (Figure S4B). We conclude that target-mediated miRNA protection (Chatterjee et al., 2011) can be observed in human cells, involves both mRNA and anti-miR targets, and competes with miRNA degradation to sustain intracellular gene silencing.

Mature miRNAs with mRNA Targets Localize to the Nucleus in Rapidly Diffusing Complexes

To understand the subcellular distribution and fate of mature miRNAs, we performed iSHiRLoC and CCA upon microinjecting l7-Cy5/l7* miRNA into the cytoplasm or nucleus of U2OS cells, as confirmed by imaging of the co-injected Alexa-Fluor-405-labeled high-molecular-weight dextran. Regardless of the compartment of injection, 2 hr after microinjection, a large majority of miRNAs had assembled into miRISC-mRNP complexes in the cytosol (quadrant I), with only a few remaining in the nucleus as low-molecular-weight complexes (quadrants II and III; Figures 4A and S5A). Notably, the retention of miRNAs in the nucleus was ~2-fold higher when first introduced there (Figures 4A and S5A). Consistent with previous reports for small interfering RNAs (siRNAs) (Ohrt et al., 2008), our data suggest that

Figure 3. Seed-Matched Targets Protect miRNAs In Cellulo

(A) Time course analysis of microinjected let-7 abundance in live (blue) and fixed cells (green and red). Assays were done over a time course of 0.3–16 hr. The number of l7-Cy5 and l7*-Cy3 molecules in two-color iSHiRLoC assays are depicted in red (squares) and green (triangles), respectively, and their corresponding double-exponential fits are represented as appropriately colored dotted lines. iSHiRLoC live-cell assays using l7-Cy5/l7* miRNA were done on a complementary set of samples. Particle abundances are depicted in blue (circles), and the corresponding double-exponential fit is represented as a blue dotted line.

(B) CCA plot of various miRNAs 2 hr after microinjection.

(C) CCA plot of various miRNAs co-injected with target mRNAs 2 hr after microinjection. All miRNAs were labeled with Cy5 on the G and were co-injected with the appropriate target. RL-ml7 and RL-cx6x are seed-matched targets of ml7 and cx, respectively. RL-ml7 has two miRNA recognition elements (MREs) for ml7, whereas the RL-cx6x has six MREs for cx. RL-l7 bears a seed mismatch for ml7 at each of the MREs and is a target of l7, whereas RL mRNA does not have any 3' UTR.

(D) CCA plot of various miRNAs co-injected with anti-miRs 2 hr after microinjection.
nuclear miRNAs assemble into low-molecular-weight complexes compositionally different from cytoplasmic high-molecular-weight miRISC-mRNP complexes.

To test the broader validity of our observations, we performed iSHiRLoC fixed-cell counting with a panel of miRNAs 2 hr after microinjection. While l7-Cy5/l7* and m21-Cy5/m21* localized in the nucleus of U2OS cells to similar extents, miRNAs without miRNA targets (m21-Cy5/m21*, cx-Cy5/cx*, and Scr-Cy5/Scr*), as well as the P strand of let-7, i.e., l7/17*-Cy5, generally displayed lower nuclear localization, independent of the compartment of injection (Figures 4B and S5B). Similar observations were made when l7-Cy5/l7* or cx-Cy5/cx* was instead microinjected into HeLa cells (Figure S5C). Analysis of l7 and cx miRNAs over a time course showed that significantly higher percentages of signal in each subcellular compartment retained the nucleus at any given time than cx, irrespective of the compartment of injection (Figures 4B and S5D). We further found that an initial rapid influx of l7 miRNAs into the nucleus was followed by a slow efflux, whereas cx showed very little influx to begin with (Figures S4C and S5D). These data suggest that nuclear localization appears to be specific to miRNAs with potential mRNA targets and that a G strand is preferentially retained in the nucleus over a P strand.

A previous report used oligofectamine-based transfection followed 24 hr later by subcellular fractionation and northern blotting to suggest that miR-29b accumulates in the nucleus of HeLa cells based on a hexanucleotide nuclear localization signal (Hwang et al., 2007). Using this experimental approach, U2OS cells transfected with fluorophore-labeled miRNAs indicated a more pronounced retention of l7 and m21 over cx in the nucleus (Figure 4D), consistent with our iSHiRLoC results. Notably, we found that transfection with lipofectamine generally retained miRNAs in the nucleus at 24 hr (Figure S5E), suggesting that this reagent is not ideal for localization studies. As a control, we also probed for endogenous l7 in U2OS cells, whose subcellular distribution closely matches that of our microinjected l7-Cy5/l7* but was different from transfected miRNAs, further supporting the notion that transfection, in contrast to microinjection, is not an ideal method of tracking miRNA localization. Next, we performed subcellular fractionation and northern blotting of HeLa cells transfected using oligofectamine and one of three different miR-29b variants to reproduce the previous work (Hwang et al., 2007). Surprisingly, we found that si-miR29a, a miRNA that does not contain the hexanucleotide signal and was previously suggested to predominantly localize to the cytosol, displayed similar nuclear abundance as si-miR29b and fluorophore-labeled miR-29b that both contain the signal (Figure S6A). Similarly, the extent of miR-29b nuclear localization after microinjection as measured by iSHiRLoC was similar to those of l7 and m21 in both U2OS and HeLa cells (Figures S6B and S6C). This suggests that miR-29b localizes significantly to the nucleus of human cells, but not more than other miRNAs with cellular miRNA targets that lack the previously described nuclear localization signal of miR-29b (Hwang et al., 2007). That is, nuclear miRNA localization appears to be primarily target driven.

Intracellular miRNA Unwinding and Nuclear Localization Are Dependent on Ago Identity

Small double-stranded oligonucleotides, such as miRNAs, siRNAs, and dsDNAs, used in our assays, are <40 kDa and therefore have the potential to passively shuttle across the nuclear membrane. To test whether target-dependent retention of miRNAs in the cell nucleus requires a saturable protein factor, we used our previous strategy (Figure 2) of blocking RISC loading with competing dsRNAs. We performed iSHiRLoC...
fixed-cell counting 2 hr after microinjecting mixtures of l7-Cy5/l7* with a 5-fold excess of unlabeled l7/l7*, m21/m21*, cx/cx*, or dl7/dl7* either into the cytoplasm or nucleus of U2OS cells. Nuclear localization of l7-Cy5 was diminished 10-fold upon co-injection of competing dsRNAs into the cytosol compared to the control and 3-fold when the RNA mixture was delivered directly into the nucleus (Figure 5A). These data suggest that a saturable protein factor and perhaps Ago loading are critical for nuclear localization of miRNAs. Next, we used two-color iSHiRLoC to test whether miRNAs are unwound when microinjected directly into the nucleus. To capture the rapidly degrading P strand (Figure 1), we performed these assays at the earliest possible time point just 20 min after microinjection. Strikingly, only 8%-15% of all l7 strands were still co-localized with l7*, although both strands were still found in the nucleus (Figures S7A–S7C). Additionally, l7 abundance was at least 2-fold higher than l7* levels (Figure S7C), very similar to our observations on miRNA unwinding upon cytoplasmic injection (Figures 1G–1J). Our data are consistent with the notion that miRNAs can be potentially unwound in the nucleus, in addition to the cytoplasm, and selectively retained in the nucleus.

Mammalian genomes encode four Ago isoforms, the relative expression of which depends on the cell type (Meister, 2013). The isoforms are thought to be functionally redundant in mediating canonical RNA silencing, but only Ago2 possesses endonucleolytic RNA cleavage ("slicer") activity. We therefore tested whether miRNA function and nuclear localization are dependent on Ago identity by employing a panel of three isogenic mouse embryonic fibroblast (MEF) cells that largely lack Ago1 (MEF-Ago1<sup>−/−</sup>), lack Ago2 (MEF-Ago2<sup>−/−</sup>), or overexpress Ago2 40-fold over the other two MEFs (MEF-Ago2<sup>+/+</sup>+Ago2) (Broderick et al., 2011). Consequently, unlabeled ml7/ml7* repressed a cognate reporter with at least 10-fold less potency in MEF-Ago1<sup>−/−</sup> or MEF-Ago2<sup>−/−</sup> cells than in MEF-Ago2<sup>+/+</sup>+Ago2 cells (Figure S7D; Table S3). Fluoro-phore labeling of ml7 made these distinctions even more pronounced, as the miRNA was not functional in MEF-Ago1<sup>−/−</sup> or MEF-Ago2<sup>−/−</sup> cells even at high doses, whereas it robustly repressed its target in MEF-Ago2<sup>+/+</sup>+Ago2 cells (Figure S7E). Consistent with these observations, CCA conducted 2 hr after microinjecting l7/l7* showed that the miRNA was highly destabilized in MEF-Ago1<sup>−/−</sup> and MEF-Ago2<sup>−/−</sup> cells (quadrant III; Figure 5B), whereas stable miRNA assembly into miRISC-mRNA complexes was observed in MEF-Ago2<sup>+/+</sup>+Ago2 cells (quadrant I; Figure 5B). These data support the notions that Argonaute proteins can be a limiting silencing factor in cells (Broderick et al., 2011; Janas et al., 2012) and that, consequently, miRNA stability and
repression activity are enhanced by increasing intracellular Ago levels, at the very least Ago2. To further test whether the lack of specific Ago proteins affects miRNA maturation and nuclear localization, we performed two-color iSHiRLoC fixed-cell counting on l7-Cy5/l7*-Cy3 at the earliest possible time point after microinjection (20 min). We observed, first, that the relative abundances of l7 and l7* were almost identical in MEF-Ago2/C0/C0 cells, as expected if both strands are degraded to similar extents; in contrast, there was asymmetric retention of l7 over l7* in both MEF-Ago1/C0/C0 and MEF-Ago2/C0/C0+Ago2 cells (Figures 5C and 5D). Consistent with our observation of limited stability upon depletion of either Ago1 or Ago2, however, the overall levels of I7 and I7* were almost identical in MEF-Ago2−/− cells, as expected if both strands are degraded to similar extents; in contrast, there was asymmetric retention of I7 over I7* in both MEF-Ago1−/− and MEF-Ago2−/−+Ago2 cells (Figures 5C and 5D). Consistent with our observation of limited stability upon depletion of either Ago1 or Ago2, however, the overall levels of I7 and I7* were almost identical in MEF-Ago2−/− cells, as expected if both strands are degraded to similar extents; in contrast, there was asymmetric retention of I7 over I7* in both MEF-Ago1−/− and MEF-Ago2−/−+Ago2 cells (Figures 5C and 5D). 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Seed-Matched RNA Targets Enhance Nuclear Retention of Mature miRNAs

To probe the role of RNA targets in nuclear retention of miRNAs directly, we performed iSHiRLoC fixed-cell counting on I7-Cy5/* or cx-Cy5/cx* miRNAs in U2OS cells treated for 4 hr with 5 μg/mL actinomycin D (ActD), a transcription inhibitor that drastically reduces levels of nascent transcripts (Bensaude, 2011; Figure S7F). As a result, the relative nuclear localization of I7-Cy5/*, 2 hr post-injection and incubation in ActD-containing media, was significantly reduced (~3- to 4-fold for cytoplasmic injections and 5- to 6-fold for nuclear injections), whereas that of cx-Cy5/cx* remained almost unaltered (Figure 6A). To verify
that targets are directly involved in nuclear retention of miRNAs, we co-microinjected either ml7-Cy5/ml7* or cx-Cy5/cx* miRNAs with random or seed-matched mRNA targets into the cytoplasm or nucleus of U2OS cells. We found that miRNAs are retained more strongly in their compartment of injection upon co-microinjection with cognate target (Figures 6B and 6C). Conversely, co-microinjection of mRNA with seed-matched miRNA allows for a particularly robust cytoplasmic mRNA expression (Figures 6D–6F), suggesting that a significant fraction of the mature mRNA is exported from the nucleus and its expression then not affected by the nucleus-retained miRNA. Cytoplasmic co-microinjection resulted in robust RNA silencing, confirming that microinjection does not affect canonical RNA silencing (Figures 6D–6F).

**DISCUSSION**

Small ncRNAs have been found to perform widespread structural, catalytic, and regulatory roles in the eukaryotic cell. Our human cell data unveil a fundamental competition between incorporation of mature miRNAs into the intracellular RNA silencing and degradation pathways (Figure 7), which is shifted toward silencing by the availability of Ago proteins and RNA targets. These observations are consistent with reports that Ago is typically a limiting factor in enabling miRNA activity (Broderick et al., 2011; Janas et al., 2012) and that the introduction of high levels of siRNAs in knockdown experiments can have side effects by competing with intracellular miRNAs (Khan et al., 2009) (Figures 2G and 5A). We additionally find that threshold target abundance is potentially crucial for miRNA protection (Figure 3). Of note, it is possible that the 3’ fluorophore on our miRNA probes suppresses 3’ tailing and trimming, which in turn decouples 3’ end remodeling from target-mediated effects, allowing us to corroborate target-mediated miRNA protection as a viable pathway for controlling miRNA levels, as previously described to occur during developmental processes (Chatterjee et al., 2011).

Strikingly, we find that MEF cells lacking Ago2 exhibit miRNA unwinding and localization profiles (Figure 5) distinct from those lacking Ago1. More specifically, miRNA unwinding was reduced in cells containing Ago1 but lacking Ago2, leading to both high-molecular-weight miRISC-mRNP complexes primarily in the cytoplasm.

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strands of the miRNA localizing to the nucleus. This suggests that Ago2 mediates miRNA unwinding and promotes retention of miRNAs in the cytosol for canonical RNA silencing (Figure 7), expanding on the notion that mammalian Ago isoforms exhibit distinct miRNA binding/processing profiles and thus non-redundant functions (Meister, 2013).

Ago-bound small ncRNAs have been reported to perform important functions in the cellular nuclei of several lower organisms. For instance, small ncRNAs mediate RNA-induced transcriptional silencing (RITS) and epigenetic regulation in yeast, C. elegans, and plants, as well as transposon silencing in fruit flies (Castel and Martienssen, 2013). Moreover, the mammalian let-7 miRNA has been shown to regulate its own biogenesis, hypothetically via nuclear processing of its primary miRNA transcript (Zisoulis et al., 2012). The rapid uptake of our miRNA probes into the nucleus and their RNA target-dependent retention suggest that similar pathways may exist in mammals. Our data indicate that mammalian miRNAs play a role in nuclear-RNA-dependent processes such as transcription or splicing. In support of this hypothesis, miRNAs that were co-microinjected with cognate targets into the nucleus of mammalian cells were retained in the nucleus most strongly, even though the co-microinjected mature miRNAs were, as expected, efficiently exported into the cytoplasm, where they faced little RNA silencing from the largely nucleus-retained miRNAs (Figure 6).

Very few methods currently enable detection of single functional miRNAs in live or fixed cells. Tsourkas and coworkers have developed methods to count endogenous miRNAs in situ (Lu and Tsourkas, 2009), but only in formaldehyde-fixed cells that lose spatiotemporal features critical to living cells. Schwille and coworkers developed techniques based on microinjection and fluorescence correlation spectroscopy (FCS) to characterize diffusion properties of nuclear and cytoplasmic RISC (Ohrt et al., 2006, 2008) in live cells. However, the confocal illumination required by FCS only yield data from selected subsections of a mammalian cell at any given time, and deconvoluting more than two molecular species of distinct mobility is challenging. Moreover, Schwille and coworkers typically investigated Ago2-bound miRNAs, with limited access to aspects of miRNA binding and turnover not requiring Ago2. We demonstrate here that our iSHiRLoC technique can account for all miRNA molecules introduced into a single cell at a defined zero time point and provides an unbiased, quantitative survey of intracellular pathways involving miRNAs at single-molecule resolution. In live-cell experiments, our typical 50–100 ms time resolution elegantly filters out “free” miRNAs (with a diffusion constant of 10–20 μm²/s) and minimal RISC complexes (5–6 μm²/s), both of which can be detected complementarily by FCS (Ohrt et al., 2008). Complementarily, fixed-cell iSHiRLoC experiments account for these complexes, as exemplified by the ability to stabilize yet non-functional miRNA-anti-miR complexes (Figure 3).

RNA therapeutics have exhibited remarkable promise in treating various pathologies (Kole et al., 2012), and a significant subset of these drugs target the miRNA pathway. For instance, anti-miR drugs that target miR-21 and miR-122 are currently in clinical trials for the treatment of Alport syndrome (Gomez et al., 2015) and pancreatic cancer (Sicard et al., 2013), respectively, as well as for hepatic pathologies (Haussecker and Kay, 2010). Moreover, miRNAs that mimic miR-34 activity (MRX34) have shown promise in targeting previously untreatable forms of cancer (Bouchie, 2013). iSHiRLoC uses individual cells as reaction vessels in ways that promise to facilitate screening for effective oligonucleotide-based drugs such as anti-miRs, especially in preclinical cell culture models, which has the potential to propel single-cell, single-molecule analysis into the realm of therapeutics.

### EXPERIMENTAL PROCEDURES

#### Plasmids

Description of plasmids pmG-mH3U, pmG-mH3UM, pmG-cx6x, pEF6-mCh-cr6x, pEF6-mH, and pEF6-mHM can be found in Supplemental Experimental Procedures.

#### DNA and RNA Oligonucleotides

All DNA and RNA oligonucleotides used for iSHiRLoC experiments were obtained from IDT with a 5’ phosphate and, in the case of fluorophore-labeled oligonucleotides, a Cy3 or Cy5 dye at the 3’ end. G and P strands were heat- annealed in a 1:1.1 or 1:1 ratio, resulting in duplex miRNAs, and were frozen for further use. Negative control siRNA and siluc2 siRNA were purchased as ready-to-use duplex samples from Ambion and Dharmacon, respectively. Oligonucleotide sequences are listed in Tables S4 and S5.

#### mRNA Synthesis

In vitro transcriptions and mRNA purifications were performed as previously described (Pitchiaya et al., 2012).

#### Cell Culture

HeLa (CCL-2, ATCC) and U2OS (HTB-96, ATCC) cells were propagated as per elsewhere (Pitchiaya et al., 2012, 2013). Cells grown on DeltaT dishes (Bioptechs) were microinjected as described previously (Gagnon et al., 2014; Hwang et al., 2007). Northern blotting and northern blotting were performed as described previously (Pitchiaya et al., 2012, 2013).

#### Luciferase Reporter Assays

100 μL of 10,000–20,000 cells were seeded per well of a 96-well plate. Transfection conditions and luminescence readouts are as described previously (Pitchiaya et al., 2012, 2013).

#### EMSA

Appropriate RNA samples were mixed with non-denaturing gel-loading buffer (10 mM Tris-HCl, 100 mM NaCl, 10 mM EDTA, 0.1% SDS, 0.02% NP40, and 10% glycerol) on ice, and 15 μL of each sample was loaded onto each well of a pre-cast 20% TBE gel (Life Technologies). Gels were run at 200 V for ~2.5 hr at 4°C.

#### Real-Time qPCR

U2OS cells were treated with DMSO or ActD (5 μg/mL) in regular medium for 0 hr or 4 hr in six-well plates and harvested, and total RNA was extracted and quantified by real-time qPCR. Primer sequences are listed in Table S6.

#### Biochemical Fractionation and Northern Blotting

Nucleo-cytoplasmic fractionation and northern blotting were performed as described previously (Gagnon et al., 2014; Hwang et al., 2007). Northern blotting probe sequences are listed in Table S5.

#### Microinjection

Cells grown on DeltaT dishes (Bioptechs) were microinjected as described elsewhere (Pitchiaya et al., 2012, 2013).

#### Microscopy and Image Analysis

Imaging and analysis was performed as described previously (Pitchiaya et al., 2012, 2013), with some minor modifications. Briefly, particle tracking analysis was performed using tracks that spanned at least four video frames. Stepwise
photobleaching analysis in fixed cells was done using custom written Lab-view codes and ImageJ.

Statistical Analysis
GraphPad Prism and Origin were used for statistical analysis and plotting. For pairwise comparisons, p values were calculated based on non-parametric unpaired t tests with Kolmogorov-Smirnov test. For comparisons involving more than two samples, one-way-ANOVA tests were used with Geisser-Greenhouse correction.

SUPPLEMENTAL INFORMATION
Supplemental Information includes Supplemental Experimental Procedures, seven figures, and six tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2017.03.075.

AUTHOR CONTRIBUTIONS
S.P. designed and performed all assays. L.A.H. performed the northern blotting analysis. E.J.C. assisted with microinjection experiments. J.I.P. assisted with reporter assays and EMSA. S.P. and N.G.W. conceived the study, and all authors wrote the manuscript.

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Supplemental Information

Resolving Subcellular miRNA Trafficking and Turnover at Single-Molecule Resolution

Sethuramasundaram Pitchiaya, Laurie A. Heinicke, Jun I. Park, Elizabeth L. Cameron, and Nils G. Walter
**Figure S1. Effect of fluorophore modification on miRNA function.** Related to Figures 1 and 6. (A) Plasmid maps of the various luciferase reporter systems we have used in our assays. Asterisks ("**") represents locations of miRNA binding sites, whose sequences are
depicted below each construct. All reporters were constructed in the pmiR-GLO (pmG) plasmid backbone, wherein the appropriate 3`UTRs were cloned downstream of the firefly luciferase (FL) gene. (B) Luciferase reporter assays of U2OS cells transfected with the appropriate reporter plasmids. Zoomed-in view of the dotted red box is shown on the right. Renilla luciferase (RL) activity was used for internal normalization of FL activity within each sample. Error bars, SEM (n = 4). (C-E) Luciferase reporter assays of U2OS cells co-transfected with the appropriate reporter plasmids and various labeled and unlabeled miRNAs. A siluc2 siRNA (siR/siR*) that is designed to target the FL gene was used as a positive control for repression. All samples were normalized with respect to a scrambled, negative control RNA (Scr/Scr*). Grey dotted line represents average repression of the pmG-mH3U reporter with the l7/l7* miRNA. *** represents statistical significance of data points, p < 0.001.
Figure S2. Representative example of the single-molecule counting analysis routine.

Related to Figure 1. (A) Representative image of formaldehyde-fixed U2OS cells containing Alexa-405-labeled, 500 kDa dextran (cyan). Distribution of Alexa-405 signal was used to identify cellular (dashed) and nuclear (dotted) boundaries. Five random 25 x 25 pixel regions outside of the cell which were used for background subtraction are depicted as white boxes. Total, corrected fluorescence intensity \((89.3 \times 10^7 \text{ A.U.})\) was calculated by subtracting the average background intensity from the cellular intensity (Supplementary experimental procedure). Scale bar, 10 µm. (B) Cellular and nuclear boundaries as outlined by the dextran were applied on the Cy5 channel to identify miRNAs within the cell. Inset, magnified view of
the white box, 5.32 x 5.32 µm² area. (C) A Laplacian of Gaussian (LoG) filter was then applied to the Cy5 channel to aid particle identification. Inset, magnified view of the white box, 5.32 x 5.32 µm² area. (D) The number of particles detected by setting discrete intensity thresholds over the entire intensity range was plotted. The resulting plot typically distributed into two populations, depicting “background” or “false-positive” spots (grey) and “true” spots (red). The black dotted line depicts the maxima of the red population, which was used as the threshold to count miRNA spots in the image. The total (T), nuclear (N) and cytoplasmic (C) spots are depicted in the plot. (E and G) Representative intensity trajectories of individual miRNA particles within the cell shown in B. For some traces, the number of photobleaching steps could be accurately determined (E), whereas this was not always the case (F). These latter set of traces (F) were classified as non-determinable (ND) and the number of photobleaching steps was calculated by scaling the average intensity of the first three frames of the trajectory (~4800 A.U. in this example) by that of a single photobleaching step (~1250 A.U.) to deduce the number of molecules per particle (~3 in this example). A few particles bleached within 3 frames of image acquisition (G). These particles constituted anywhere between 2-5% of the total population and were discarded from further analysis. (H) Top panel depicts the intensity distribution of all particles that photobleached as a single step (blue regime), the lowest intensity value of such particles in this cells was 325 A.U. The grey region depicts intensity values from 0 – 325 A.U., for which we do not have data points. Bottom panel depicts the intensity distribution of all particles. The blue regime in the top panel was applied here to demarcate single miRNA molecules. The green and yellow regime includes particles that contain two and more miRNA molecules respectively. The total (T), nuclear (N) and cytoplasmic (C) molecules are depicted in the plot. (I) Distribution of miRNA photobleaching steps, before (top) and after (bottom) after scaling all ND particles with the average intensity of a single photobleaching step. (J) the number of particles and molecules before (top) and after
(bottom) normalizing the counts with intensity of Alexa-405 dextran (100 x 10^7 A.U.). (K) 3D image of two representative microinjected cells. Scale bar (x-y axis), 7 µm. (L) 3D image of two representative microinjected cells with software (imaris) rendered particles. Scale bar (x-y axis), 7 µm. Z-scale range, 0 – 5 µm. As a vast majority of miRNAs are distributed within the z-scale range, a HILO beam width of ~3 µm will account for ~ 50% of all miRNAs in the cell.
Figure S3. Chemical composition and photophysical properties of dye molecules do not affect miRNA unwinding. Related to Figure 1. (A) Representative image of formaldehyde-fixed U2OS cells containing Alexa-405 (A405) labeled, 500 kDa dextran (blue) and individual particles of (top) Cy3-labeled miRNA G strands (green), Cy5-labeled miRNA P strands (red) or (bottom and as in Figure 1G) Cy5-labeled miRNA G strands (red), Cy3-labeled miRNA P strands (green). Schematics of miRNAs used in each case are depicted to the left of images. Black represents G strands and grey represents P strands. Scale bar, 10 µm. (B) Scatter plot depicting the number of G and P strand molecules for each sample.
Figure S4. Seed-matched targets stabilize intracellular miRNAs. Related to Figure 3. (A) Double stranded miRNAs do not pre-anneal with their cognate antimiRs during sample preparation. Top and bottom gels depict the mobility of samples containing I7-Cy5 and cx- Cy5 respectively. “ss” corresponds to single-stranded, Cy5-labeled, G strand, whereas “ds"
corresponds to G-labeled double stranded miRNA. The "ss : antimiR’ sample was prepared by annealing the single-stranded G strand with the appropriate animiR. AntimiRs supplemented with 0.3x, 1x and 3x the amount of double-stranded miRNAs were also loaded. (B) Luciferase reporter assays of U2OS cells transfected with the appropriate reporter plasmids and miRNAs. Samples were also treated with anti-let7 antimiR at the appropriate ratio.
Figure S5. Nuclear localization of mature miRNAs. Related to Figure 4. (A) Representative pseudocolored images of fixed or live U2OS cells injected in the cytoplasm (Cyt Injection) or directly into the nucleus (Nuc Injection) with I7-Cy5/I7* or cx-Cy5/cx*. 500 kDa FITC Dextran and the appropriate miRNA are represented in green and red respectively. Scale bar, 10 µm. White dotted lines represent nuclear and cytoplasmic boundaries. (B) Scatter plot representing the number of appropriate miRNA molecules in the nucleus (n ≥ 2, ≥ 15 cells) when injected in the nucleus of U2OS cells. (C) Scatter plot representing the number of appropriate miRNA molecules in the nucleus (n = 3, ≥ 10 cells) when injected in the cytoplasm or nucleus of HeLa cells. (D) Time course analysis of nuclear I7-Cy5/I7* or cx-Cy5/cx*, upon nuclear injections. The molecular abundance in the nucleus (left y-axis, # I7-Cy5/I7* and # cx-Cy5/cx*) and the nuclear fraction (right Y-axis, Fr. I7-Cy5/I7* and Fr. cx-Cy5/cx*) of each miRNA is represented.
(n ≥ 2, ≥ 15 cells). The red hexagon at t = 0, is an assumed data-point, considering that all miRNAs are delivered directly into the nucleus during injection. (E) Northern blot analysis of subcellular fractionated U2OS cells to detect miRNAs transfected with lipofectamine. Nuclear and cytoplasmic subcellular fractions are annotated as Nuc and Cyt. U6 small nuclear RNA (U6) and cytoplasmic tRNA-Lys serve as subcellular loading controls. Percent of signal in each subcellular compartment has been corrected for leakage of U6 and tRNA-Lys and is mentioned below each blot (n = 2).
Figure S6. miR-29b does not accumulate in the nucleus. Related to Figure 4. (A) Northern blot analysis of subcellular fractionated HeLa cells to detect miRNAs transfected with oligofectamine. Nuclear and cytoplasmic subcellular fractions are annotated as Nuc and Cyt. U6 small nuclear RNA (U6) and cytoplasmic tRNA-Lys serve as subcellular loading controls. Percent of signal in each subcellular compartment has been corrected for leakage of U6 and tRNA-Lys and is mentioned below each blot (n = 2). (B) Representative pseudocolored images of fixed U2OS cells injected in the cytoplasm (Cyt Inj) or directly into the nucleus (Nuc Inj) with m29b-Cy5/m29b*. 500 kDa FITC Dextran and the appropriate miRNA are represented in blue and red respectively and samples were imaged 2 h post injection. Scale bar, 10 µm. (C) Scatter plot representing the number of miR-29b molecules in the nucleus (n ≥ 2, ≥ 15 cells) when injected in the cytosol or nucleus of U2OS or HeLa cells, 2 h post injection. The nuclear
abundance of microinjected let-7 in U2OS cells (reproduced from Figures 4B and S5B) is depicted for comparison.
Figure S7. Ago proteins and seed-matched targets enhance nuclear localization of miRNAs. Related to Figures 5 and 6. (A) Representative, pseudocolored image of a U2OS cell injected in the nucleus with Alexa-405 labeled 500 kDa dextran (blue) and I7-Cy5/I7*-Cy3 (Cy5 – red, Cy3 - green). Scale bar, 10 µm. Zoomed-in view of particles within grey box is depicted.
beside the image. Scale bar, 5 µm. Images were acquired 20 min post injection. (B) Scatter plot representing the percentage of colocalized l7-Cy5 and l7*-Cy3 molecules, as normalized to the more abundant G strand molecules in U2OS cells as in (A). (C) Scatter plot representing the ratio of G:P strands in U2OS cells as in (A). Grey line represents a 1:1 ratio of G:P. (D) Luciferase reporter assays of MEF cells transfected with the pmG-mH3UM plasmid and various concentrations of mI7/mI7*. Renilla luciferase (RL) activity was used for internal normalization of FL activity within each sample. Error bars, SEM (n = 3, with 4 technical replicates per trial). IC₅₀ values (in nM) are depicted for each cell line. (E) Luciferase reporter assays of MEF cells transfected with the pmG-mH3UM plasmid and 10nM fluorophore labeled (red) or unlabeled (black) mI7/mI7* miRNA. Normalization, as in (D). (F) qPCR analysis of GAPDH or Myc transcript levels after ActD treatment. Error bars, SEM (n = 3). (G) Double stranded miRNAs do not pre-anneal with their cognate mRNA targets during sample preparation. Top and bottom gels depict the mobility of samples containing l7-Cy5/l7* and cx-Cy5/cx* respectively. “ss” corresponds to single-stranded, Cy5-labeled, G strand, whereas “ds” corresponds to G-labeled double stranded miRNA. The “ss : mRNA’ sample was prepared by annealing the single-stranded G strand with the appropriate mRNA. mRNAs supplemented with 0.3x, 1x and 3x the amount of double-stranded miRNAs were also loaded.
**SUPPLEMENTARY TABLES**

Table S1. Details on double exponential fit. Related to Figure 3A.

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</tr>
<tr>
<td>$A_2$</td>
<td>480 ± 57</td>
<td>846 ± 363</td>
<td>333 ± 39</td>
</tr>
<tr>
<td>$t_1$ (h)</td>
<td>0.08 ± 0.01</td>
<td>0.30 ± 0.01</td>
<td>0.13 ± 0.01</td>
</tr>
<tr>
<td>$t_2$ (h)</td>
<td>5.40 ± 1.30</td>
<td>21.40 ± 15.10</td>
<td>1.40 ± 0.20</td>
</tr>
</tbody>
</table>

Fit equation: \( y = y_0 + A_1 \cdot e^{-x/t_1} + A_2 \cdot e^{-x/t_2} \); At \( x = 0 \), \( y = y_0 + A_1 + A_2 \).

Table S2. Details on double exponential fit. Related to Figures 4C and S5E.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Injection site</th>
<th># Mols / Fr.</th>
<th>$t_1$ (h)</th>
<th>$t_2$ (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>let-7</td>
<td>Cyt</td>
<td># Mols</td>
<td>0.46 ± 0.06</td>
<td>4.76 ± 8.61</td>
</tr>
<tr>
<td>cxcr4</td>
<td>Cyt</td>
<td># Mols</td>
<td>0.26 ± 0.01</td>
<td>3.33 ± 0.67</td>
</tr>
<tr>
<td>let-7</td>
<td>Cyt</td>
<td>Fr.</td>
<td>0.47 ± 0.21</td>
<td>0.30 ± 0.12</td>
</tr>
<tr>
<td>cxcr4</td>
<td>Cyt</td>
<td>Fr.</td>
<td>0.13 ± 0.04</td>
<td>1.47 ± 0.28</td>
</tr>
<tr>
<td>let-7</td>
<td>Nuc</td>
<td># Mols</td>
<td>0.21 ± 0.01</td>
<td>3.13 ± 0.09</td>
</tr>
<tr>
<td>cxcr4</td>
<td>Nuc</td>
<td># Mols</td>
<td>0.12 ± 0.01</td>
<td>2.27 ± 0.21</td>
</tr>
<tr>
<td>let-7</td>
<td>Nuc</td>
<td>Fr.</td>
<td>0.69 ± 0.04</td>
<td>4.55 ± 0.83</td>
</tr>
<tr>
<td>cxcr4</td>
<td>Nuc</td>
<td>Fr.</td>
<td>0.08 ± 0.03</td>
<td>0.90 ± 0.51</td>
</tr>
</tbody>
</table>

Fit equation: \( y = y_0 + A_1 \cdot e^{-x/t_1} + A_2 \cdot e^{-x/t_2} \); At \( x = 0 \), \( y = y_0 + A_1 + A_2 \).
Table S3. Details on hill plot. Related to Figure S7D.

<table>
<thead>
<tr>
<th>Cell</th>
<th>IC$_{50}$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEF-Ago1$^{+/}$</td>
<td>13.34 ± 4.30</td>
</tr>
<tr>
<td>MEF-Ago2$^{+/}$</td>
<td>11.81 ± 0.86</td>
</tr>
<tr>
<td>MEF-Ago2$^{+/}$ + Ago2</td>
<td>0.21 ± 0.20</td>
</tr>
</tbody>
</table>

Fit equation: $y = \frac{[x]^n}{([x]^n + \text{IC}_{50})}$
Table S4. Sequences of oligonucleotides used for microinjection and reporter assays. Related to Figures 1-7.

<table>
<thead>
<tr>
<th>Name</th>
<th>DNA / RNA</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>I7</td>
<td>RNA</td>
<td>P-UGAGGUAGUAGGUUGAUAGUU-X</td>
</tr>
<tr>
<td>I7*</td>
<td>RNA</td>
<td>P-CUAUACAAUCUACUGUCUUCC-X</td>
</tr>
<tr>
<td>dl7</td>
<td>DNA</td>
<td>P-TGAGGTAAGTAGGGTTGTATAGTT-X</td>
</tr>
<tr>
<td>dl7*</td>
<td>DNA</td>
<td>P-CTATACAATCTACTGTCTTTCC-X</td>
</tr>
<tr>
<td>ml7</td>
<td>RNA</td>
<td>P-UGCGUUAGUAGGUUGAUAGUU-X</td>
</tr>
<tr>
<td>ml7*</td>
<td>RNA</td>
<td>P-CUAUACAAUCUACUGUCUUCC-X</td>
</tr>
<tr>
<td>cx</td>
<td>RNA</td>
<td>P-UGUUAGCUGGAGUGAAAACUU-X</td>
</tr>
<tr>
<td>cx*</td>
<td>RNA</td>
<td>P-GUUUCACAAGCUACACA-X</td>
</tr>
<tr>
<td>m21</td>
<td>RNA</td>
<td>P-UAGCUUAUCAGACUGAUUGUUGA-X</td>
</tr>
<tr>
<td>m21*</td>
<td>RNA</td>
<td>P-CAACACCAGUGCAUUGGCUGU-X</td>
</tr>
<tr>
<td>m29b</td>
<td>RNA</td>
<td>P-UAGCACCAUUUGAAAUCAGUGUU-X</td>
</tr>
<tr>
<td>m29b*</td>
<td>RNA</td>
<td>P-GCUGGUUUCAUAUGGUGGUUUGA-X</td>
</tr>
<tr>
<td>si-miR-29a</td>
<td>RNA</td>
<td>P-UAGCACCAUCAGAAUCGUGU-X</td>
</tr>
<tr>
<td>si-miR-29a*</td>
<td>RNA</td>
<td>P-CCGAUUUCAGAUGGUGCUAUU-X</td>
</tr>
<tr>
<td>si-miR-29b</td>
<td>RNA</td>
<td>P-UAGCACCAUUUGAAAUCAGUGUU-X</td>
</tr>
<tr>
<td>si-miR-29b*</td>
<td>RNA</td>
<td>P-CACUGAUUUCAAAUGGUGCUAUU-X</td>
</tr>
<tr>
<td>Scr</td>
<td>RNA</td>
<td>P-CCGUAUUCGUAGCAUGCUUU-X</td>
</tr>
<tr>
<td>Scr*</td>
<td>RNA</td>
<td>P-AGUACUGCUUACGUAACGGU-X</td>
</tr>
<tr>
<td>siluc2</td>
<td>RNA</td>
<td>GAAGUGCUUCGUCCUCGUCCUU</td>
</tr>
<tr>
<td>siluc2*</td>
<td>RNA</td>
<td>GGACGAGGACGAGACUUCUU</td>
</tr>
</tbody>
</table>

*P: 5` Phosphate, X: 3` hydroxyl or C6 linker + Cy3 or C6 linker + Cy5*
Table S5. Sequences of oligonucleotide probes used for northern blotting. Related to Figures 4, S5E and S6A.

<table>
<thead>
<tr>
<th>Probe against</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>I7 (LNA)</td>
<td>AACTATAACACCTACTACCTCA</td>
</tr>
<tr>
<td>U6</td>
<td>GAATTTGCGTGTCATCCTTTGCGCAGGGGCCATGCTAA</td>
</tr>
<tr>
<td>Lys-tRNA</td>
<td>CTGATGCTCTACCCGACTGAGCTATCCCGGTC</td>
</tr>
<tr>
<td>si-miR-29a</td>
<td>AACCGATTTTCAGATGGCTA</td>
</tr>
<tr>
<td>m29b / si-miR-29b</td>
<td>AACACTGATTTCAATGGCTA</td>
</tr>
<tr>
<td>cx</td>
<td>AAGTTTTCACTCCAGCTAACA</td>
</tr>
</tbody>
</table>

Table S6. Sequences of qPCR primers. Related to Figures 6 and S7F.

<table>
<thead>
<tr>
<th>Target</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>F: CCATCACCATCTTCCAGGAGCGA</td>
</tr>
<tr>
<td></td>
<td>R: GGTGGTGAAAAGACGCCAGTGGA</td>
</tr>
<tr>
<td>Myc</td>
<td>F: GCTCGTCTCAGAGAAGCTGG</td>
</tr>
<tr>
<td></td>
<td>R: GCTCAGATCCTGCAGGTACAA</td>
</tr>
</tbody>
</table>
SUPPLEMENTARY EXPERIMENTAL PROCEDURES

Plasmids. pmG-mH3U and pmG-mH3UM were described previously (Pitchiaya et al., 2012; Pitchiaya et al., 2013). Plasmid pmG-cx6x was constructed by restriction enzyme digesting (XhoI and NotI) the cxcr4 3`UTR from pRL-TK-cxcr4-6x (a gift from Phil Sharp, Addgene plasmid # 11308) and ligating it into the pmiR-GLO (Promega) plasmid. pEF6-mCh-cx6x was constructed in 2 steps. First the mCherry ORF was restriction enzyme digested from the pRSET-mCherry vector (a gift from Roger Tsien, UCSD), using BamHI and EcoRI and ligated into the multiple cloning site of the pEF6-myc-His-B (Invitrogen) vector. The cxcr4 3`UTR was then PCR amplified and ligated into the NotI and XbaI sites of the above plasmid. pRL-TK-let7-A and pRL-TK-let7-B plasmids were obtained as gifts from Phil Sharp (Addgene plasmids # 11324 and #11325).

DNA and RNA oligonucleotides. All DNA and RNA oligonucleotides used for iSHiRLoC experiments were obtained from IDT. Oligonucleotides contained a 5` Phosphate (P) and, in the case of fluorophore labeled oligonucleotides, a Cy3 or Cy5 dye at the 3` end. Dyes were attached after oligonucleotide synthesis to a 3` amino group on a C6 carbon linker and were HPLC purified by the vendor. Guide and passenger strands were heat-annealed in a 1:1.1 ratio or 1:1 ratio in 1x PBS for single-color or two-color iSHiRLoC assays respectively, to achieve 10 µM stock solutions and were frozen until further use. Negative control siRNA and siluc2 siRNA, designed towards the coding sequence of the firefly luciferase gene (luc2), were purchased as ready-to-use duplex samples from Ambion and Dhharmacon respectively. Oligonucleotide sequences are listed in Table S1.

mRNA synthesis. pRL-TK-cx6x was linearized with XhoI or NotI to generate RL or RL-cx6x mRNAs respectively. pRL-TK-let7-A and pRL-TK-let7-B were both linearized with NotI used to
generate RL-l7 and RL-ml7 respectively. pEF6-mCh-cx6x was linearized with XbaI to generate mCh-cx6x mRNA. The pCFE-GFP plasmid (Thermo Scientific) was directly used in the in vitro transcription reactions to generate the GFP mRNA. The linearized plasmids were extracted with phenol and chloroform and subsequently ethanol precipitated. In vitro transcriptions were performed using the MegaScript T7 kit (Ambion) according to manufacturer's protocol. Transcription reactions were then DNase treated (turbo DNase, Ambion) and the respective RNAs were purified by sequential gel-filtration chromatography (Nap-5 followed by Nap-10, GE healthcare) and ethanol precipitation. The RNAs were 5’capped and polyadenylated and were further purified by sequential gel-filtration chromatography and ethanol precipitation. The length of the polyA tails was estimated based on electrophoretic mobility on a 1.2% formaldehyde agarose gel.

**Cell culture.** HeLa (CCL-2, ATCC) cells were propagated in DMEM (GIBCO, # 11995) supplemented with 10% FBS (GIBCO, # 16000) and U2OS (HTB-96, ATCC) cells were propagated in McCoy's 5A (GIBCO, # 16600) supplemented with 10% FBS (GIBCO, # 16000). MEF cells, namely MEF-Ago1-/-, MEF-Ago2-/- and MEF-Ago2-/- + Ago2 were obtained from Phil Zamore’s lab and were propagated as described (Broderick et al., 2011) in DMEM (GIBCO, # 11995) supplemented with 15% FBS (GIBCO, # 16000). Medium typically contained 1x Penicillin-Streptomycin (GIBCO, #15140). For experiments involving actinomycin D (ActD), cells were treated with the drug (5 µg/µL) in regular medium for 4 h prior to microinjection.

**Luciferase reporter assays.** 100 µL of 0, 000 -20, 000 cells were seeded per well of a 96 well plate in antibiotics-free medium containing DMEM (GIBCO, # 11995) supplemented with 10% FBS (GIBCO, # 16000) for HeLa and MEF cells or in medium containing McCoy’s 5A
(GIBCO, # 16600) supplemented with 10% FBS (GIBCO, # 16000) for U2OS cells. Transfection conditions and luminescence readouts are as described previously (Pitchiaya et al., 2012; Pitchiaya et al., 2013).

**EMSA.** Appropriate RNA samples were mixed with non-denaturing gel-loading buffer (10 mM Tris-HCl, 100 mM NaCl, 10 mM EDTA, 0.1% SDS, 0.02% NP40, 10% glycerol) on ice and 15 µL of each sample was loaded onto each well of a pre-cast 20% TBE gel (Life Technologies). Gels were run at 200 V for ~ 2.5 h at 4 °C.

**Quantitative Real-Time RT-PCR (qRT-PCR).** U2OS cells were treated with DMSO or ActD (5 µg/µL) in regular medium for 0 h or 4 h in 6-well plates. Treated cells were then harvested and total RNA was extracted using the QIAzol Lysis reagent and RNeasy kit (QIAGEN). Relative RNA levels determined by qRT-PCR were measured on an Applied Biosystems 7900HT Real-Time PCR System, using Power SYBR Green MasterMix (Applied Biosystems). All of the primers were obtained from Integrated DNA Technologies (IDT), and gene-specific sequences are listed in Supplementary Table 6. The fraction of RNA left after ActD treatment was calculated as a ratio of DMSO:ActD treatment at each time point and further normalized to the 0 h time point.

**Biochemical fractionation and northern blotting.** Nucleo-cytoplasmic fractionation and northern blotting were performed as described (Gagnon et al., 2014; Hwang et al., 2007). Briefly, nuclear and cytoplasmic RNA was isolated from untransfected cells (endogenous miRNAs) or cells transfected for 24 hours (Cy5-labeled miRNA). Synthetic RNA duplexes were transfected using Oligofectamine or Lipofectamine 2000 (Life Technologies) according to the manufacturer’s protocol. For 10 and 15 cm plates, cells were transfected with 250 and 575...
pmol RNA, respectively. RNA was harvested at 24 h post-transfection. Cells growing in 10 cm or 15 cm dishes were rinsed twice with ice-cold 1x PBS, harvested in 1 mL (or 2 mL) ice-cold 1x PBS by scraping, and centrifuged at 100 x g for 10 min at 4 °C. Cell pellets were resuspended by gentle pipetting in 200 µL lysis buffer (10 mM Tris.HCl, pH 8.0, 140 mM NaCl, 1.5 mM MgCl₂, 0.5% NP-40), incubated on ice for 5 min, and then centrifuged at 1,000 x g for 3 min at 4 °C. The supernatant, containing the cytoplasmic fraction was added to 1 mL Trizol for RNA purification. Nuclear pellets were washed 2X with 500 µL lysis buffer and finally with 1x 500 µL lysis buffer containing 1% Tween-40 and 0.5% deoxycholic acid. Between washes, cells were pelleted at 1,000 x g for 1 min at 4 °C. Purified nuclear pellets were then resuspended in 1 mL Trizol. For northern blotting, equal microgram amounts of nuclear and cytoplasmic RNA was added to each lane.

Total RNA was collected using Trizol (Invitrogen) following the manufacturer’s protocol. 5-20 µg of total RNA was separated on denaturing 15% polyacrylamide gels and transferred to BrightStar-Plus Positively Charged Nylon Membranes (Life Technologies) using Trans-Blot SD Semi-Dry Transfer Cell (BIO-RAD). Transferred RNA was then crosslinked to the membrane using 254 nm irradiation for 2 min. DNA oligonucleotide probes (IDT) were 5’ end-labeled with [γ- 32P] ATP using T4 polynucleotide kinase (Invitrogen). To enhance probe sensitivity, a miRCURY LNA probe (Exiqon) was used to detect endogenous and transfected let-7a-1. Probe sequences are provided in Table S1. RNA-crosslinked membranes were pre-hybridized for 30 min with hybridization buffer (50% Formamide, 0.5% SDS, 5x SSPE - 750 mM NaCl, 50 mM NaH₂PO₄ and 5 mM EDTA, 5X Denhard’s solution - 0.1% Ficoll 400, 0.1% Polyvinylpyrrolidone, Bovine serum albumin (Invitrogen), and 10 µg/µL denatured Salmon Sperm DNA (Invitrogen). After pre-hybridization, 32P-labeled probes were added at 1,000,000-5,000,000 counts per million to hybridization buffer. Membranes were incubated with DNA probes overnight at 42 °C or LNA probe for 3 h at 37 °C. After probe hybridization, membranes
were washed 3 times for 5 min with Membrane Wash Buffer (0.1% SDS, 300 mM NaCl and 30 mM Sodium citrate, pH 7.0). Membranes were exposed to a phosphor screen for 2 h to 2 weeks and radioactive signals were visualized using a PhosphorImager. Band quantifications were done either in Image quant (GE healthcare) or Image J (NIH).

**Microinjection.** Cells grown on DeltaT dishes (Bioptechs) were microinjected as described (Pitchiaya et al., 2012; Pitchiaya et al., 2013). Briefly, injection solutions contained the appropriate miRNA at 0.1 or 1 µM concentration, 1x PBS and 0.5 mg/mL of 10 kDa or 500 kDa dextran. Competing miRNAs or double-stranded DNA were present at a 5-fold excess to the labeled miRNA. mRNAs were added at a stoichiometric amount based on the number of miRNA binding sites, for instance, 0.16 µM of RL-cx6x mRNA, bearing 6 cxcr4 binding sites, was added along with 1 µM cxcr4 miRNA. Solutions were filtered through a 0.45 µm Ultrafree-MC filter (Millipore) and then centrifuged at 16,000 x g for 15min at 4 °C immediately before injection. The solution was loaded into a femtotip (Eppendorf). Injections were performed using a Femtojet pump (Eppendorf) and an Injectman (Eppendorf) mounted to the microscope. Microinjections were performed at 100 hPa injection pressure for 0.5 s with 20 hPa compensation pressure. This pressure translates to a volume of 0.02 pL and 10,000-20,000 miRNA molecules. Cells were incubated in basal medium containing 2% FBS after microinjection for the duration of the assay. For experiments involving ActD, cells were treated with the abovementioned medium with the drug (5 µg/µL) for 2 h after microinjection.

**Microscopy and Image Analysis.** Imaging was performed as described (Pitchiaya et al., 2012; Pitchiaya et al., 2013) using a cell-TIRF system based on an Olympus IX81 microscope equipped with a 60x 1.49 NA oil-immersion objective (Olympus), as well as 405 nm (Coherent ©, 100 mW at source, ~65 µW for imaging Alexa-405), 488 nm (Coherent ©, 100 mW at
source, ~38 µW for imaging fluorescein), 532 nm (Coherent ©, 100 mW at source, ~8.5 mW for imaging Cy3) and 640 nm (Coherent ©, 100 mW at source, 13.5 mW for imaging Cy5) solid-state lasers. Quad-band filter cubes consisting of z405/488/532/640rpc or z405/488/561/640rpc dichroic filters (Chroma) and z405/488/532/640m or z405/488/561/640m emission filters (Chroma) were used to filter fluorescence of the appropriate fluorophores from incident light. Emission from individual fluorophores was detected sequentially on an EMCCD camera (Andor iXon Ultra). Image analysis was performed as described (Pitchiaya et al, EMBO report, 2012) with some minor modifications. Briefly, particle tracking analysis was performed by using tracks that spanned at least four video frames. Step-wise photobleaching analysis in fixed cells was done using custom written Lab-view codes and ImageJ macros that can be shared upon request. Notably, imaging immediately after microinjection (within 20 min) of 1 µM (in the femtotip) labeled miRNAs resulted in extremely high intracellular miRNA density which was unresolvable by our analysis routines. In these cases, 0.1 µM miRNA (in the femtotip) were injected, which resulted in a particle density of ~0.1 molecules per µm², easily resolvable by our spot-detection algorithm.

Correlative counting analysis (CCA) was performed as follows. Live cell and fixed cell imaging of single-stranded l7 and double stranded l7/l7* were first performed. As expected and observed, l7 was rapidly degraded while l7/l7* was comparably stably retained. We calculated the mean ± 3 x SEM of the number of particles observed in live cells (x-axis) and the number of molecules observed in fixed cells (y-axis) for both l7 and l7/l7* and then averaged those values to set boundaries in the CCA plot. This incidentally split the x- and y-axes in ratios of 35:65 and 30:70, respectively, irrespective of the range of the plot.

Statistical analysis. Graphpad-Prizm and Origin were used for statistical analysis and plotting. For pairwise comparisons, p-values were calculated based on non-parametric
unpaired t-tests with Kolmogorov-Smirnov test. For comparisons involving more than 2 samples, one-way-ANOVA tests were used with Geisser-Greenhouse correction.