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MicroRNA-122 Regulates Polyploidization in the Murine Liver

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Abstract

A defining feature of the mammalian liver is polyploidy, a numerical change in the entire complement of chromosomes. The first step of polyploidization involves cell division with failed cytokinesis. Although polyploidy is common, affecting ~90% of hepatocytes in mice and 50% in humans, the specialized role played by polyploid cells in liver homeostasis and disease remains poorly understood. The goal of this study was to identify novel signals that regulate polyploidization, and we focused on microRNAs (miRNAs). First, to test whether miRNAs could regulate hepatic polyploidy we examined livers from *Dicer1* liver-specific knockout mice, which are devoid of mature miRNAs. Loss of miRNAs resulted in a 3-fold reduction in binucleate hepatocytes, indicating that miRNAs regulate polyploidization. Secondly, we surveyed age-dependent expression of miRNAs in wild-type mice and identified a subset of miRNAs, including miR-122, that is differentially expressed at 2–3 weeks, a period when extensive polyploidization occurs. Next, we examined *Mir122* knockout mice and observed profound, life-long depletion of polyploid hepatocytes, proving that miR-122 is required for complete hepatic polyploidization. Moreover, the polyploidy defect in *Mir122* knockout mice was ameliorated by adenovirus-mediated over-expression of miR-122, underscoring the critical role miR-122 plays in polyploidization. Finally, we identified direct targets of miR-122 (*Cux1*, *Rhoa*, *Iqgap1*, *Mapre1*,

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Nedd41 and *Slc25a34*) that regulate cytokinesis. Inhibition of each target induced cytokinesis failure and promoted hepatic binucleation.

Conclusion—Our data demonstrate that miR-122 is both necessary and sufficient in liver polyploidization. Among the different signals that have been associated with hepatic polyploidy, miR-122 is the first liver-specific signal identified. These studies will serve as the foundation for future work investigating miR-122 in liver maturation, homeostasis and disease.

Keywords

hepatocyte; polyploidy; binucleation; miR-122 microRNA-122; postnatal development

Introduction

Polyploidy, a numerical change in the complement of chromosomes, is a defining feature of the liver. Most easily observed as binucleate hepatocytes in liver sections, polyploidy is determined by the number of nuclei per cell and the DNA content of each nucleus. Diploid hepatocytes are exclusively mononucleate and polyploid hepatocytes (e.g., tetraploid, octaploid) are mono- or binucleate. Polyploidization begins early in life when a subset of proliferating diploid hepatocytes fails to undergo cytokinesis, resulting in binucleate tetraploids with two diploid nuclei (1, 2). A binucleate tetraploid then enters the cell cycle and, following successful cytokinesis, generates a pair of mononucleate tetraploid hepatocytes. A proliferating mononucleate tetraploid then undergoes cytokinesis failure and generates a binucleate octaploid, which, in turn, proliferates to yield a pair of mononucleate octaploids after successful cytokinesis. The process repeats, generating hepatocytes with even greater ploidy values. In this way, the liver develops with a combination of mononucleate (diploid) and binucleate (tetraploid) hepatocytes. Approximately half of hepatocytes in adult human livers are polyploid (3) and as many as 90% of adult mouse hepatocytes are polyploid (4). It is unclear whether polyploid hepatocytes play a specialized role in liver homeostasis (reviewed in (5)). Nearly a dozen factors have been shown to regulate hepatic polyploidy, including p53 (6), E2F1/7/8 (7–9), cMyc (10), insulin (11) and Rb (12). How all of these diverse signals are integrated is unknown, and it remains to be seen if there is a liver-specific regulator that drives polyploidization.

MicroRNAs (miRNA) are ~22 nucleotide-long, single-stranded, non-coding RNAs that typically negatively regulate expression of protein coding genes. When associated with Argonaute in the miRNA-induced silencing complex, mature miRNAs bind to miRNA-specific “seed sequences” in the 3' untranslated region (3'UTR) of target genes and facilitate mRNA degradation or impair translation (reviewed in (13)). A single miRNA can attenuate expression of numerous target genes/proteins. In the liver, miRNAs contribute to diverse cellular functions, including hepatic differentiation, regeneration from injury and metabolism. miRNAs are involved in progression of liver diseases, including viral hepatitis, steatosis, cirrhosis and liver cancers. miRNA-122 (miR-122) is a liver-abundant miRNA that accounts for over 70% of total miRNAs in hepatocytes (14, 15) and is also expressed at low levels in the heart (16). During liver development, expression of miR-122 initiates around embryonic day 12.5 (E12.5) and increases continuously until adulthood (17, 18); thus, miR-122 expression closely associates with hepatic maturation. In diet-induced obesity

animal models, transient depletion of miR-122 in adults by antisense oligonucleotides significantly inhibits hepatic lipogenesis and improves hepatic steatosis (19). In contrast, long-term deletion of *Mir122* in knockout mice promotes early onset of steatohepatitis, fibrosis and eventually leads to spontaneous hepatocarcinogenesis (20, 21), which is facilitated after exposure to the hepatocarcinogen diethylnitrosamine (22). Although the duration and timing of miR-122 deletion leads to differences in hepatic phenotype for reasons that are not fully understood, these reports highlight a critical role for miR-122 in liver homeostasis and disease.

We set out to identify signals that control polyploidization in the liver, and we focused on cytokinesis regulators (reviewed in (23)). No individual signal functions as the dominant driver of hepatic polyploidization, which suggests that initiation/promotion of binucleation is coordinated by the combined activity of multiple effectors. Since miRNAs can coordinate the expression of sets of genes/proteins, we hypothesized that one or more miRNAs could alter expression of key cytokinesis effectors, induce cytokinesis failure and initiate/promote hepatic binucleation. Consistent with this hypothesis, miR-15 family members have been shown to regulate binucleation of cardiac myocytes (24). Here, we identified miRNAs differentially expressed in livers of mice at 2 and 3 weeks of life, ages that correspond to rapid hepatic binucleation. We demonstrate that miR-122 is necessary and sufficient for hepatic polyploidization. *Mir122* knockout livers are depleted of polyploid hepatocytes, and this defect is rescued by miR-122 over-expression. Furthermore, we identified the mechanism of miR-122-regulated polyploidy: miR-122 antagonizes the expression of the pro-cytokinesis effectors *Cux1*, *Rhoa*, *Mapre1*, *Iqgap1*, *Nedd4l* and *Slc25a34*, which leads to cytokinesis failure and expansion of binucleate hepatocytes.

Materials and Methods

Mouse strains

The Institutional Care and Use Committees of the University of Pittsburgh, The Ohio State University and Children's Hospital of Philadelphia approved all mouse experiments. The following inbred strains were used: wild-type (WT) C57BL/6 (Jackson Labs, Bar Harbor, ME), *Mir122* germline knockout ("Mir122-KO") (20), *Mir122* liver-specific knockout ("Mir122-LKO;" *Mir122^{loxP/loxP}* Albumin-Cre⁺) (20) and *Dicer1* liver-specific knockout ("Dicer1-LKO;" *Dicer1^{loxP/loxP}* Alfp-Cre⁺) (25). Control mice for *Mir122* deletion studies were *Mir122^{+/+}* littermates or *Mir122^{loxP/loxP}* Albumin-Cre⁻. Control mice for *Dicer1* deletion studies were *Dicer1^{loxP/+or loxP/loxP}* Alfp-Cre⁻. Unless noted, male mice were used for experiments.

Quantification of mono- and binucleate hepatocytes in liver sections

Mono- and binucleate hepatocytes were quantified in frozen liver sections stained with Phalloidin/Alexa Fluor 488 (Life Technologies, Carlsbad, CA) + Hoechst 33342 or in paraffin sections stained with anti- β -catenin antibody (Santa Cruz, Dallas, TX) + hematoxylin or Hoechst 33342. The binucleation curve in Fig. 1A was derived from C57BL/6 livers ages 8, 10, 12, 14, 18, 20, 29, 42 and 56d (mixed gender, 8–12d; males, 14–56d). Livers from mice 8–20d-old were imaged with an Olympus Fluoview 1000 confocal

microscope using 0.2 μm Z-stacks, and livers >20d-old were imaged with Nikon TiU by manually focusing from the top to bottom of each cell. A minimum of 500 hepatocytes/sample was scored.

Microscopy

Unless otherwise noted, fluorescent images were captured with a Nikon TiU fluorescent microscope equipped with a monochrome camera; non-fluorescent images were captured with a Nikon TS100 microscope equipped with a color camera. Images were processed with Nikon NIS Elements Basic Research software and Adobe Photoshop CS6.

Statistical analysis

Statistical significance was calculated using 2-tailed Student's *t* test unless otherwise noted. *P* values < 0.05 were considered significant.

Additional methods

Please refer to Supporting Information.

Results

miRNAs are necessary for hepatic binucleation

Binucleate hepatocytes were previously shown to emerge at the time of weaning in rats (11), but the timing of hepatic binucleation differs in mice. As early as 20–21d (prior to weaning), 30–40% of hepatocytes in WT C57BL/6 mice are binucleate and tetraploid (4), indicating that binucleation initiates before 3 weeks (wk) of life. To determine the kinetics of hepatocyte binucleation in mice, we counted the number of mono- and binucleate hepatocytes in liver sections (Fig. 1A). Between 8 and 14 days (d) the percentage of binucleate hepatocytes was <3% and then from 14–18d binucleate hepatocytes rapidly expanded to ~35%. Binucleate cells continued to increase slowly after 21d to comprise ~70% of hepatocytes by 8wk, consistent with the number of binucleate cells seen in suspensions of hepatocytes isolated by collagenase perfusion (4). The data indicate that a specialized mitotic program between 14–18d promotes rapid polyploidization of the liver. Postnatal liver development is characterized by robust hepatocyte proliferation, as measured by the cell cycle marker Ki-67 (Fig. 1B) and reported by others (26), so the burst in binucleate hepatocytes is likely a consequence of enhanced proliferation by diploid hepatocytes predisposed for cytokinesis failure.

To test whether miRNAs affect liver binucleation, we examined livers in *Dicer1* liver-specific knockout mice (*Dicer1*-LKO) (25). Cells lacking *Dicer1*, an RNase III-type enzyme that cleaves miRNA precursors, are devoid of mature miRNAs. Livers from 22–28d-old control and *Dicer1*-LKO mice contained the same number of cells per section, but loss of *Dicer1* led to 60–70% reduction in binucleate hepatocytes (Fig. 1C). Loss of *Dicer1* leads to a 10–20-fold increase in the percentage of Ki-67+ hepatocytes at 28d of age, compared to controls, indicating that *Dicer1*-LKO livers are highly proliferative during postnatal development (25); thus, the loss of binucleate hepatocytes in *Dicer1*-LKO livers is not simply explained by failure to proliferate. We were unable to track binucleation in adults

because *Dicer1*-LKO livers are spontaneously repopulated by *Dicer1*⁺ hepatocytes as early as 6 weeks (27). Together, the data support the idea that functional miRNAs are required for polyploidization in the liver.

A subset of miRNAs is differentially expressed during postnatal development

We performed a screen to identify miRNAs differentially expressed in 2–3wk-old livers, ages when rapid binucleation takes place. We compared expression of ~600 miRNAs using the NanoString nCounter Mouse miRNA Expression Assay in embryonic livers (embryonic day 15.5, E15.5), postnatal livers (2 and 3wk) and adult livers (7wk). A total of 181 miRNAs were differentially expressed in embryonic and postnatal livers compared to the adult (Fig. 2A). As expected, miRNA expression was age-dependent (Fig. 2B and Supporting Table 1): 73 miRNAs were highly expressed in embryonic liver and decreased into adulthood; in contrast, 23 miRNAs were lowly expressed in embryos and increased with age. Additionally, nearly half of the miRNAs were differentially expressed during postnatal development: 25 miRNAs were down-regulated at 2 and/or 3wk, whereas 60 miRNAs were up-regulated.

To validate the NanoString data, we interrogated expression of a subset of miRNAs that are enriched in the liver: miR-23, -30d, -122, -148 and -194 (28). Consistent with the NanoString observations, these miRNAs were specifically up-regulated during the postnatal time period (Fig. 2C and Supporting Fig. 1). For example, miR-122 was previously shown to be ~50-fold up-regulated in adult livers compared to E15.5 (17, 18). We also observed robust expression of miR-122 in adults, but peak miR-122 expression occurred specifically at 2wk. Although *RNU6b* is frequently used for normalization, we found that miR-16 was more consistently expressed at all ages (Supporting Fig. 1A). Regardless of the normalization method used, however, similar expression patterns were seen for each miRNA (Fig. 2C and Supporting Fig. 1B).

We speculated that one or more of the miRNAs could antagonize pro-cytokinesis genes, thereby promoting cytokinesis failure and the emergence of binucleate hepatocytes. To determine which miRNAs were predicted to target cytokinesis genes, we screened all 60 of the miRNAs with elevated expression at 2 and/or 3 weeks. The 3'UTRs of cytokinesis-associated genes (defined as the 323 genes classified by Ingenuity Pathway Analysis, IPA, as “affecting” or “activating” cytokinesis) were screened using TargetScan 6.2 for seed matching sequences to each of the miRNAs. As seen in Supporting Table 2, most of the miRNAs are predicted to target multiple genes. Together, these data indicate that mouse liver miRNAs are expressed in an age-dependent manner, a subset is differentially expressed during postnatal development and numerous miRNAs are predicted to antagonize cytokinesis-associated genes.

miR-122 is required for complete hepatic polyploidization

We decided to focus on miR-122, which is predicted to target 38 of 323 cytokinesis-associated genes (Supporting Table 2). miR-122 is a compelling candidate because *Mir122*-deficient livers were previously noted to have fewer binucleate hepatocytes compared to controls (Hsu and Ghoshal, unpublished observations 2012), which suggested that miR-122 could contribute to polyploidization. Moreover, as a liver-specific miRNA, we hypothesized

that miR-122 could serve as a tissue-specific regulator of polyploidy in the liver. To determine how loss of miR-122 affects nuclearity, ploidy and aneuploidy we utilized liver-specific *Mir122* knockout mice (*Mir122*-LKO) and germline *Mir122* knockout (*Mir122*-KO) mice (20). First, we counted the number of binucleate hepatocytes in *Mir122*-LKO and -KO livers (Supporting Fig. 2A). At 28d, following the period of rapid binucleation, *Mir122*-LKO and -KO livers displayed 60–70% reduction in the number of binucleate hepatocytes (Fig. 3A). Consistent with enrichment for mononucleate hepatocytes that are smaller than binucleate polyploids, *Mir122*-LKO and -KO livers contained 20–25% more hepatocytes per liver section than controls (Fig. 3B). Livers from 2.5- and 6.0-month (mo)-old *Mir122*-KO mice also contained 60–70% fewer binucleate hepatocytes (Fig. 3A), indicating that the binucleation defect caused by miR-122 loss persists throughout life. Aside from the binucleation defect, *Mir122*-KO livers were nearly identical to controls in terms of proliferation, cell cycle arrest and apoptosis (Supporting Fig. 2B–D).

Secondly, we measured hepatocyte ploidy. In adult mice that are predominantly quiescent (4, 29), hepatic ploidy is accurately determined by flow cytometric quantification of nuclear content. We isolated hepatocytes from 2.5mo-old mice and determined ploidy of live hepatocytes (Supporting Fig. 3). As expected, only a small fraction of control hepatocytes were 2c (3%) and the majority were 4c (59%) or 8c (35%) (Fig. 3C,D). In contrast, the ploidy spectrum of *Mir122*-KO hepatocytes was significantly altered. Most *Mir122*-KO hepatocytes were 2c (60%) and many fewer cells were 4c (33%) or 8c (6%).

Finally, we measured aneuploidy by metaphase cytogenetic analysis. Chromosome counts for control hepatocytes clustered at ~40 (diploid) or ~80 (tetraploid), but there were also numerous cells with hypo-diploid, hypo-tetraploid and hyper-tetraploid chromosome counts (Fig. 3E and Supporting Fig. 4). Similar to previous studies (4, 30), 60% of control hepatocytes contained random whole chromosome gains and/or losses, chromosome losses outnumbered chromosome gains and structural rearrangements were uncommon (Fig. 3F and Supporting Fig. 4). Loss of miR-122 dramatically altered hepatic aneuploidy. *Mir122*-KO hepatocytes were enriched for diploid chromosome counts, and, for each sample analyzed, only 2–3 aneuploid karyotypes were identified per 20 cells (12.5%), a level that, in our experience, is slightly above background. Collectively, the data show that *Mir122*-deficient livers are predominantly diploid and euploid, which indicates that miR-122 is necessary for complete hepatic polyploidization.

miR-122 promotes hepatic binucleation

We next hypothesized that miR-122 activity is sufficient to induce hepatic binucleation. To test this idea, we over-expressed miR-122 in WT diploid hepatocytes and tracked binucleation *in vitro* (Fig. 4A). Although miR-122 is robustly expressed by WT hepatocytes, expression decreases 75–80% after 1d in culture and remains depressed for additional days (Fig. 4B). We isolated hepatocytes from 14d-old WT livers that are 97% diploid, transfected with synthetic miR-122 mimic or control mimic (25 nM or 200 nM) and monitored proliferation. On day 1, prior to transfection, hepatocytes appeared spread-out and were predominantly mononucleate (Fig. 4C). Proliferation kinetics were similar between control- and miR-122-transfected cells, but there was a trend toward delayed S-phase at days

2 and 3 in hepatocytes over-expressing miR-122 (Supporting Fig. 5A,B). Cell death was minimal (Supporting Fig. 5C). Hepatocytes migrated together during the culture period, became smaller and formed tightly packed clusters. By day 4, miR-122 was induced 10–20-fold in miR-122-transfected cells (Fig. 4D). Hepatocyte cultures were comprised of a heterogeneous mixture of mono- and binucleate cells, and multinucleate cells were rarely observed (<1%) (Fig. 4C). It was difficult to identify individual cells in the tightly packed clusters; therefore, we harvested samples by trypsinization and prepared cytopspins, which allowed identification of single cells and quantification of the number of nuclei per cell (Supporting Fig. 6). Binucleate hepatocytes were seen in 30–35% of miR-122-transfected hepatocytes compared to 10% of control-transfected hepatocytes (Fig. 4C). Thus, the data show that miR-122 promotes hepatic binucleation.

To complement the *in vitro* approach, we next tested whether exogenous miR-122 could promote hepatic binucleation in *Mir122*-KO mice. We generated an adenovirus encoding four tandem copies of miR-122 (Ad-miR122), which robustly induces miR-122 expression *in vitro* (Supporting Fig. 7). *Mir122*-KO mice were injected with Ad-miR122 or control adenovirus (Ad-control) on postnatal day 12 and livers harvested at postnatal day 22 (Fig. 4E). We rationalized that exogenous miR-122 activity would promote cytokinesis failure and the emergence of binucleate hepatocytes. At postnatal day 22, we failed to detect elevated levels of miR-122 in Ad-miR122-injected mice (data not shown). This is not surprising because expression by non-integrating viruses is diluted and frequently lost in proliferating cells (31), and hepatocytes proliferate extensively between postnatal days 12 and 22 (Fig. 1B). However, we observed a significant change in phenotype on postnatal day 22. *Mir122*-KO mice infected with Ad-miR122 had nearly twice as many binucleate cells compared to *Mir122*-KO livers infected by Ad-control (Fig. 4F). There was also a trend toward increased proliferation, as measured by Ki-67 staining, but the difference was not significant (Fig. 4F). Together with the *in vitro* observations, these data show that exogenous miR-122 rescues the binucleation defect in *Mir122*-KO mice, indicating that miR-122 is sufficient to induce binucleation during postnatal liver development.

miR-122 directly regulates genes involved in cytokinesis

Control and *Mir122*-KO livers have similar cell cycle kinetics (Supporting Fig. 2B–D), so differences in proliferation, arrest or apoptosis cannot explain the ploidy defect caused by miR-122 loss. Based on these observations, we hypothesized that miR-122 regulates hepatic binucleation by antagonizing genes involved in cytokinesis. To identify candidate cytokinesis target genes with miR-122 seed matching sequences we used bioinformatic and literature-based screening approaches (Fig. 5A). First, using published expression profiles of *Mir122*-LKO and control livers (20), IPA revealed 63 cytokinesis-associated genes that were differentially expressed in *Mir122*-LKO livers (Supporting Table 3). We screened 3'UTRs and identified 4 genes with conserved seed matching sequences that were up-regulated 1.3-fold: *Mapre1*, *Nedd4l*, *Rhoa* and *Vps4a* (Table 1 and Supporting Table 4). Secondly, in an unbiased screen for genes involved in cell division, Neumann et al. identified 427 genes involved in binucleation (32). Comparison of these genes with the *Mir122*-LKO/control microarray data revealed 31 genes that were up-regulated in *Mir122*-LKO livers (Supporting Table 5). We screened 3'UTRs and narrowed the list to 3 genes with conserved miR-122

seed matching sequences that were most highly up-regulated (1.3-fold): *Galc*, *Nedd4l* and *Slc25a34* (Table 1). Finally, we searched the literature for genes involved in cytokinesis that were also validated miR-122 targets. We identified *Cux1* (17, 33), *Iqgap1* (20, 34, 35), *Mapre1* (36, 37) and *Rhoa* (2, 20, 38, 39), genes previously implicated in cytokinesis and demonstrated up-regulated in *Mir122*-deficient livers (Table 1). Three of the targets, *Rhoa*, *Mapre1* and *Nedd4l*, were identified by multiple methods, which supports our screening approach. Thus, among the cytokinesis-associated genes identified, we decided to focus on the most promising candidates for further analysis: *Cux1*, *Rhoa*, *Mapre1*, *Iqgap1*, *Nedd4l* and *Slc25a34*.

- *Cux1* is a transcription factor that promotes expression of centralspindlin components *Racgap1*, *Ect2* and *Kif23/Mklp1* (33). The centralspindlin complex regulates cleavage furrow ingression and abscission (reviewed in (40)). Inhibition of *Racgap1*, *Ect2* and *Kif23/Mklp1* blocks cytokinesis in a variety of cell types, and these proteins have been associated with liver binucleation (2, 9). We found 10-fold up-regulation in *Cux1* protein in *Mir122*-KO livers but not at the mRNA level (Fig. 5B,C), indicating that miR-122 controls *Cux1* translation and not mRNA stability (41). Although *Racgap1*, *Ect2* and *Kif23/Mklp1* lack miR-122 seed matching sequences in their 3'UTR (data not shown), these genes were up-regulated 2–5-fold in *Mir122*-KO livers (Fig. 5D). Thus, the data indicate that miR-122 regulates the centralspindlin complex via the transcription factor *Cux1*.
- *Rhoa* is a small GTPase that promotes formation of the actomyosin ring and facilitates myosin II constriction to induce cleavage furrow ingression (38). Cytokinesis failure in hepatocytes was previously associated with loss and/or mislocalization of active *Rhoa* (2). In agreement with earlier work (20), we found 2.4-fold up-regulation of *Rhoa* in *Mir122*-KO livers (Fig. 5B,C).
- *Mapre1* is a microtubule-tip associated protein that performs multiple roles, including cell division. During cytokinesis, *Mapre1* binds and stabilizes Aurora B kinase (36, 37), which phosphorylates centralspindlin complex member *Kif23/Mklp1* and promotes cytokinesis completion (42). Similar to previous results (20, 43), we found that *Mapre1* was up-regulated 3–5-fold in *Mir122*-KO livers (Fig. 5B,C).
- *Iqgap1* is a Ras GTPase-activating-like protein that localizes to the spindle midbody during cytokinesis where it promotes abscission and completion of cytokinesis (35). Depletion of *Iqgap1* leads to a dramatic increase in the number of bi- and multinucleate cells (35). Consistent with earlier work (20, 34), *Iqgap1* was up-regulated 6–7-fold in *Mir122*-KO livers (Fig. 5B,C).
- *Nedd4l* is an E3 ubiquitin ligase that facilitates proteasome-mediated degradation (reviewed (44)). Identified by Neumann et al. (32), *Nedd4l* inhibition blocked cytokinesis and induced formation of binucleate cells; however, it is unknown how *Nedd4l* affects cytokinesis. *Nedd4l* has two

isoforms (a “short” 110 kDa form and a “long” 130 kDa form) that are encoded as distinct transcripts from the same gene (45). Both isoforms were significantly elevated in 2–4-fold in *Mir122*-KO livers (Fig. 5B,C).

- *Slc25a34* is a member of the *Slc25* family and is believed to facilitate transport over the mitochondrial membrane (46). *Slc25a34* was also identified in the Neumann et al screen, and it is unclear how this gene affects cytokinesis. We found that *Slc25a34* was up-regulated ~6-fold in *Mir122*-KO livers (Fig. 5B,C).

Cux1, *Rhoa* and *Mapre1* were previously shown to function as direct miR-122 targets (17, 20, 39). They contain miR-122 seed sequences in their 3'UTR that facilitate miR-122-induced degradation. To confirm whether *Iqgap1*, *Nedd4l* and *Slc25a34* were also direct targets of miR-122 we performed luciferase reporter assays. The 3'UTR of each gene (either containing the predicted seed sequence(s) or with the seed sequence deleted) was cloned downstream of *Renilla* luciferase in psiCHECK2 reporter vector (Supporting Fig. 8A). NIH 3T3 cells with undetectable levels of miR-122 were then co-transfected with “full length” (FL) or deleted (DEL) reporter constructs plus synthetic miR-122 mimic or control mimic. Luciferase activity is expected to be high in all conditions except when exogenous miR-122 interacts with a bona fide seed sequence and represses *Renilla* luciferase expression. As a positive control we started with *Mapre1*, which has a single predicted miR-122 seed sequence (Supporting Fig. 8B). Indeed, miR-122 repressed luciferase activity ~50% in the FL reporter but had minimal effect on the DEL reporter, confirming that *Mapre1* is a direct miR-122 target (Fig. 5E). We identified 1–5 putative miR-122 seed sequences in the 3'UTRs of *Iqgap1*, *Nedd4l* and *Slc25a34* (Supporting Fig. 8B) and generated FL and DEL reporter constructs for each gene. *Renilla* luciferase activity was repressed 25–50% in a miR-122-dependent manner for each FL reporter but not when the seed sequences were deleted (Fig. 5E). The data show that *Cux1*, *Mapre1*, *Iqgap1*, *Nedd4l* and *Slc25a34* are direct targets of miR-122.

Depletion of miR-122 targets promotes formation of binucleate hepatocytes

Cux1, *Rhoa*, *Mapre1*, *Iqgap1*, *Nedd4l* and *Slc25a34* are all direct targets of miR-122 that are up-regulated in *Mir122*-KO livers (Fig. 5). To determine how these genes affect cell division in hepatocytes we tracked proliferation *in vitro*. Hepatocytes from 14–15d-old WT livers that are 97% diploid were transfected with siRNAs to each of the target genes (Fig. 6A). In addition to the target genes, we also depleted diploid hepatocytes of *Racgap1*, a member of the centralspindlin complex that is essential for successful cytokinesis (47). Approximately 75% of the cells were successfully transfected, and targets were down-regulated 70–90% by day 4 (Fig. 6B and Supporting Fig. 9). We tracked proliferation on days 2–4 using BrdU incorporation and phospho-histone H3 staining (Supporting Fig. 10A,B). Proliferation by target-depleted hepatocytes varied day-by-day, but there was a trend toward decreased proliferation by cells depleted of *Cux1*, *Mapre1* and *Slc25a34*. Cell death was < 2% in all of the cultures (Supporting Fig. 10C). By day 4, cultures contained mono- and binucleate hepatocytes, and multinucleate cells were rare (<1%) (Fig. 6C and Supporting Fig. 11). Cultures transfected with scrambled siRNA contained 3% binucleate hepatocytes. As expected, *Racgap1*-depleted hepatocytes were highly enriched (30%) for binucleate cells.

Depletion of the other targets also resulted in a significant increase in binucleate hepatocytes: *Cux1* (7%), *Rhoa* (17%), *Mapre1* (13%), *Iqgap1* (11%), *Nedd4l* (10%) and *Slc25a34* (15%) (Fig. 6D). Taken together, the data indicate that *Cux1*, *Rhoa*, *Mapre1*, *Iqgap1*, *Nedd4l* and *Slc25a34* are necessary for complete cytokinesis in hepatocytes.

Discussion

In this work, we investigated the role of miRNAs in hepatic polyploidy. Compared to livers with a full complement of miRNAs, *Dicer1* knockout livers had 60–70% fewer binucleate hepatocytes at 22–28d. Loss of *Dicer1* is also accompanied with increased hepatic proliferation (25); thus, our data suggest that miRNAs affect cytokinesis fidelity as well as proliferation. To determine which miRNAs were involved, we identified 85 miRNAs differentially expressed at 2 or 3 weeks, ages associated with extensive binucleation. miR-122 and miR-194, for instance, were expressed at low levels during embryonic development and then increased 50–100-fold in adults. Intriguingly, miR-122 and miR-194 expression spiked around 2wk at a level 2–3 times higher than is typically seen in adults. The transient differential expression of numerous miRNAs is not surprising because livers undergo profound changes during postnatal development, including acquisition of “adult” drug-metabolizing and metabolic processes, expansion of hepatocytes and bile duct epithelial cells and loss fetal hematopoietic cells (26, 48). Hence, the changes in miRNA expression we observed could be related to many different processes. We focused on miR-122, a pleiotropic miRNA among the most abundantly expressed miRNAs in the liver (14, 15). Loss of miR-122 led to 60–70% reduction in binucleate hepatocytes at 28d, and this defect persisted throughout life. The ploidy spectrum was also dramatically altered in *Mir122*-KO livers – diploid hepatocytes were enriched nearly 20-fold. Proliferating polyploid hepatocytes can undergo chromosome segregation errors and generate aneuploid daughters. Consistent with increased numbers of diploid hepatocytes and corresponding decrease in polyploids, the degree of aneuploidy in *Mir122*-KO livers was minimal. In addition to being required for polyploidy, miR-122 actively promotes polyploidy. Adenovirus-mediated expression of miR-122 in *Mir122*-KO livers promoted hepatic binucleation and rescued the binucleation defect; similarly, miR-122 over-expression induced binucleation in cultured WT diploid hepatocytes. Taken together, the data demonstrate that miR-122 is necessary and sufficient for polyploidization of the liver.

Proliferating diploid hepatocytes can go through mitosis, generate a pair of diploid nuclei and then experience cytokinesis failure, which leads to the formation of a binucleate tetraploid hepatocyte with two diploid nuclei (1, 2). We reasoned that miR-122 could control hepatic binucleation by directly targeting genes involved in cytokinesis. We identified and characterized miR-122 targets that influence cytokinesis progression: *Cux1*, *Rhoa*, *Mapre1*, *Iqgap1*, *Nedd4l* and *Slc25a34* (Fig. 7A). Among these targets, *Cux1* (17), *Rhoa* (39) and *Mapre1* (20) were previously characterized as direct targets of miR-122, and we showed that *Iqgap1*, *Nedd4l* and *Slc25a34* were also direct miR-122 targets via seed sequences within their 3'UTRs. As expected, all of the targets were notably up-regulated in *Mir122*-KO livers. We finally tested how deletion of each target affects hepatocyte proliferation. Consistent with the targets promoting successful cytokinesis, cultures of primary diploid hepatocytes depleted of each target generated populations of binucleate hepatocytes.

We propose a model in which miR-122 collaborates with other signals to regulate hepatic binucleation and polyploidy (Fig. 7B). During postnatal liver development, miR-122 is expressed at exceptionally high levels (2–3-fold greater than in adults), allowing miR-122 to directly down-regulate targets involved in cytokinesis, including *Cux1*, *Rhoa*, *Mapre1*, *Iqgap1*, *Nedd41* and *Slc25a34*. Down-regulation of *Cux1*, in turn, reduces centralspindlin complex members *Racgap1*, *Ect2* and *Kif23/Mklp1*. Although miR-122 is an important regulator of hepatic polyploidy, loss of miR-122 does not completely block binucleation, which suggests that other signals/networks are involved. For example, transcription factors E2F7 and E2F8 are robustly expressed during postnatal development, and they also antagonize *Racgap1*, *Ect2* and *Kif23/Mklp1* (the same set of centralspindlin targets indirectly regulated by miR-122) (9). Other signals may also contribute, including insulin/AKT signaling (11), unknown genes and even other miRNAs differentially expressed during postnatal development (e.g., miR-148, miR-194). Thus, the combined activity of all the signals in the network leads to inhibition of cytokinesis machinery, failure of cytokinesis and expansion of binucleate hepatocytes. The opposite situation occurs in adults where hepatocyte proliferation following partial hepatectomy involves successful cytokinesis (49). In adult livers, miR-122, E2F7, E2F8 and possibly other genes/miRNAs have reduced expression (compared to postnatal livers). Consequently, pro-cytokinesis signals are expressed appropriately, cytokinesis machinery remains functional and proliferating hepatocytes generate mononucleate daughter cells.

Despite our understanding of the myriad signals that have been shown to affect hepatic polyploidy, a major unanswered question is why polyploidy is so common, affecting 50% and 90% of adult hepatocytes in humans and mice, respectively (3, 4). Is there an evolutionarily conserved, liver-specific driver of polyploidy? We speculate miR-122 is a likely candidate. miR-122 is expressed nearly exclusively in the liver (16), and we hypothesize that it functions as a *tissue-specific* signal, which provides an explanation for the high percentage of polyploid cells in the liver. Consistent with this idea, it is intriguing to consider that cardiac myocytes become binucleate via a failed cytokinesis mechanism (50) and miR-122 is expressed (albeit at low levels) in the heart (16). Future studies should examine a role for miR-122 in cardiac myocyte polyploidy.

In summary, we identified a novel function for miR-122 and showed for the first time that miR-122 is required for complete hepatic polyploidization. Up-regulation of miR-122 during postnatal liver development, in collaboration with other signals, promotes a cell division program associated with failed cytokinesis. We propose that miR-122 regulates polyploidy in two major ways: first, by tissue-specific expression in the liver and, secondly, by inducing cytokinesis failure in an age-dependent manner.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

miRNA	microRNA
3'UTR	3' untranslated region
miR-122	microRNA-122
WT	wild-type
<i>Dicer1</i>-LKO	<i>Dicer1</i> liver-specific knockout
<i>Mir122</i>-LKO	<i>Mir122</i> liver-specific knockout
<i>Mir122</i>-KO	<i>Mir122</i> germline knockout
mo	month
wk	week
d	day
h	hour
min	minute
IPA	Ingenuity Pathway Analysis
LKO	liver-specific knockout
KO	knockout
2c	diploid cellular content
4c	tetraploid cellular content
8c	octaploid cellular content
FL	Full-length 3'UTR reporter
DEL	deleted 3'UTR reporter
kDa	kilodalton
SEM	standard error of mean
qRT-PCR	quantitative reverse transcriptase polymerase chain reaction

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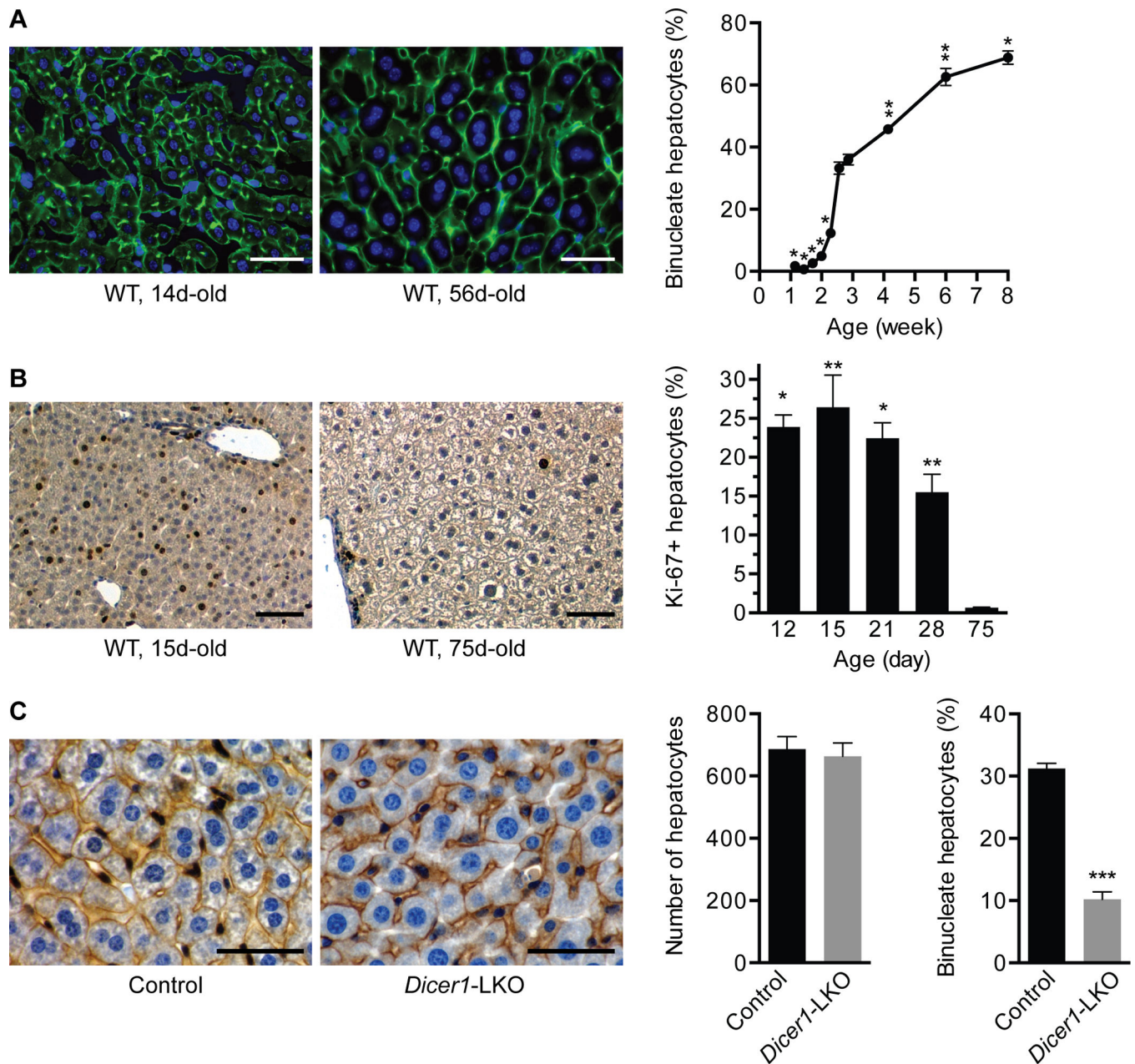


Fig. 1. Hepatic binucleation begins at 14d and is impaired in *Dicer1*-deleted livers

(A) Livers from WT C57BL/6 mice ages 8–56d ($n = 3$) were stained with phalloidin (green) to mark cell boundaries and Hoechst 33342 (blue) to mark nuclei and the number of mono- and binucleate hepatocytes counted. Representative images show a 14d-old liver with small hepatocytes that are predominantly mononucleate and a 56d-old liver with large hepatocytes that are mono- or binucleate (left). The percentage of binucleate hepatocytes at each age is indicated (right). * $P < 0.003$, ** $P < 0.02$, compared to 18 and 20d. (B) Livers from WT C57BL/6 mice ages 12–75d ($n = 3$) were stained with the proliferation marker Ki-67 (brown) and hematoxylin (blue) for nuclei. Representative images show Ki-67+ hepatocytes in 15 and 75d-old livers (left). The percentage of Ki-67+ hepatocytes is indicated (right). P

values at 12–28d are compared to 75d. (C) Livers from control or *Dicer1*-LKO mice ages 22–28d ($n = 5$) were stained for β -catenin (brown) to mark cell membranes and hematoxylin (blue) for nuclei. Representative images are shown (left). Control and *Dicer1*-LKO livers had equivalent numbers of cells per 200 \times field-of-view (middle), and binucleate hepatocytes were reduced 3-fold in *Dicer1*-LKO livers (right). *** $P < 0.0001$. Graphs show mean \pm SEM. Scale bars are 50 μ m.

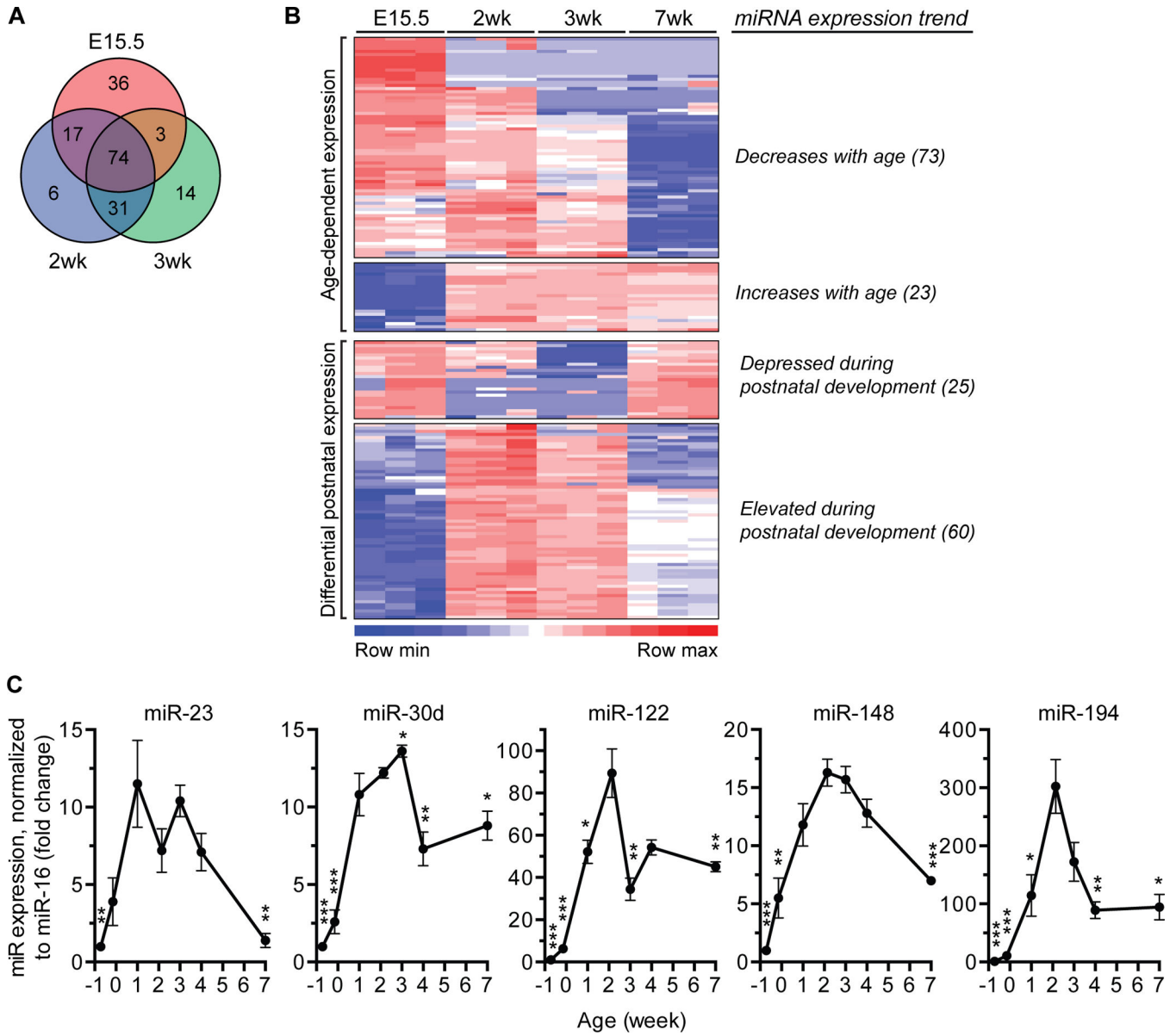


Fig. 2. miRNAs are temporally expressed in the mouse liver

(A,B) miRNA expression was assessed in livers from WT C57BL/6 mice at ages embryonic day 15.5 (E15.5), 2-, 3- and 7wk ($n = 3$) using the nCounter Mouse miRNA Expression Assay from NanoString Technologies. Among the ~600 miRNAs examined, 181 were differentially expressed at E15.5, 2- and 3wk compared to 7wk-old livers ($P < 0.05$), as indicated in the Venn diagram (A). The heat map illustrates 4 major expression patterns (B). The number of miRNAs in each category is indicated in parentheses. Detailed expression profiles are in Supporting Table 1. (C) Expression of select miRNAs (elevated during postnatal development) was tracked in WT C57BL/6 livers at ages E15.5, E19.5, 1-, 2-, 3-, 4- and 7wk ($n = 3-4$) by quantitative reverse transcriptase PCR (qRT-PCR). Peak expression of miR-23, -30d, -122, -148 and -194 occurred at 1–3wks. Expression values were

normalized to miR-16. * $P < 0.04$, ** $P = 0.009$, *** $P < 0.0008$, compared to 2wk. Graphs show mean \pm SEM.

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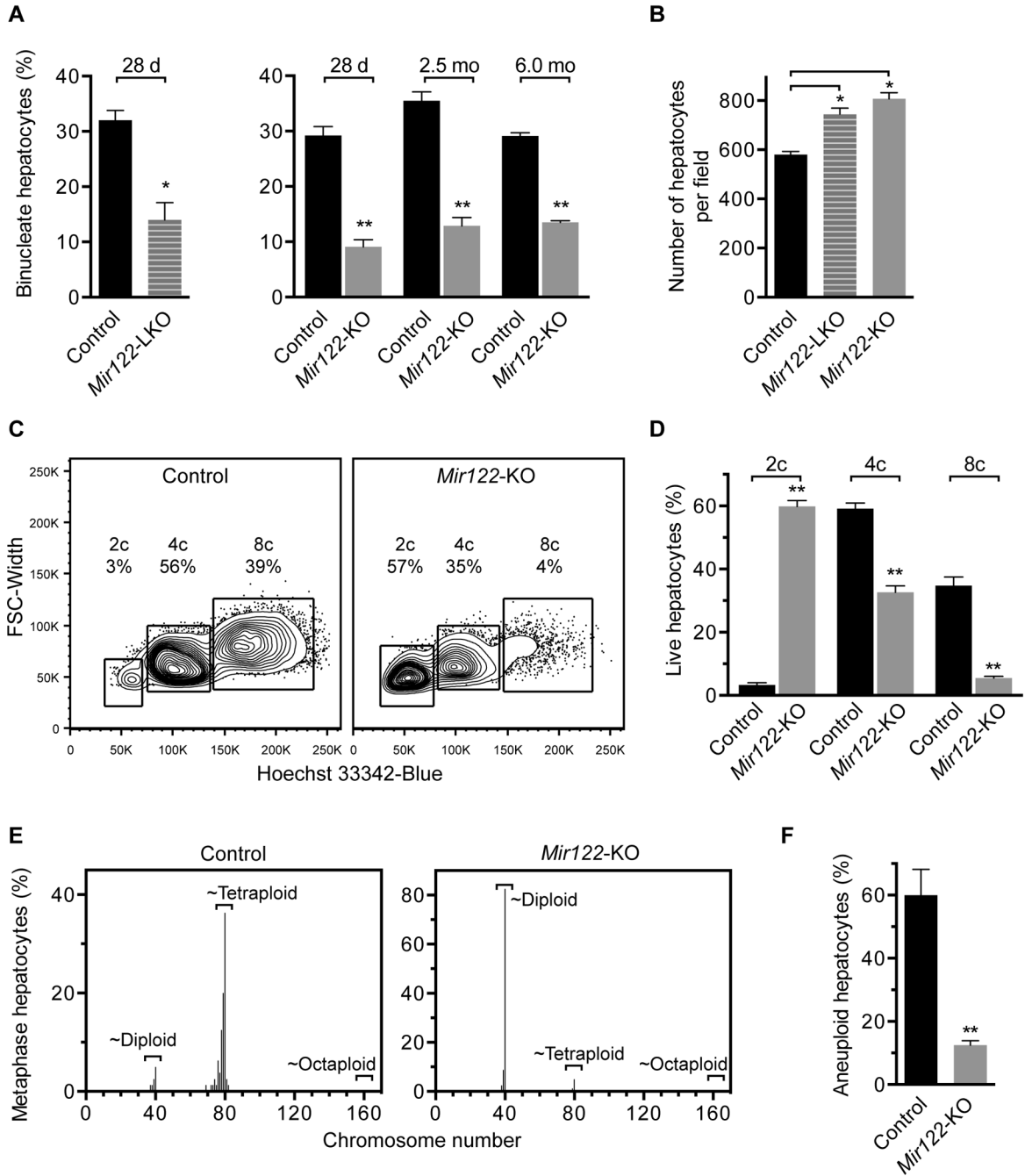


Fig. 3. *Mir122*-deficient livers are enriched for diploid, euploid hepatocytes

(A,B) Livers from control, *Mir122*-LKO and *Mir122*-KO livers were stained with phalloidin and Hoechst 33342, as shown in Supporting Fig. 2A ($n = 4-5$ mice/age/genotype). The percentage of binucleate hepatocytes was reduced 2–3-fold at 28d (LKO and KO), 2.5mo (KO) and 6.0mo (KO) (A). 28d-old *Mir122*-LKO and *Mir122*-KO livers had 20–25% more hepatocytes per 150 \times field-of-view (B). (C,D) Single cell suspensions of hepatocytes were stained with a viability marker and Hoechst 33342, allowing identification of live cells with 2c, 4c and 8c nuclear content, corresponding to diploid, tetraploid and octaploid

hepatocytes, respectively. Representative ploidy profiles are shown (C) and the percentage of each population quantified ($n = 4$; 2.5mo) (D). (E,F) Metaphase cytogenetic analysis revealed skewed chromosome counts (hypo-diploid, hypo-tetraploid and hyper-tetraploid) in control hepatocytes but not *Mir122*-KO hepatocytes ($n = 4$) (E). The overall degree of aneuploidy in *Mir122*-KO hepatocytes was reduced 4–5-fold compared to controls (F). See Supporting Fig. 4 individual karyotypes. * $P = 0.03$, ** $P = 0.0001$. Graphs show mean \pm SEM.

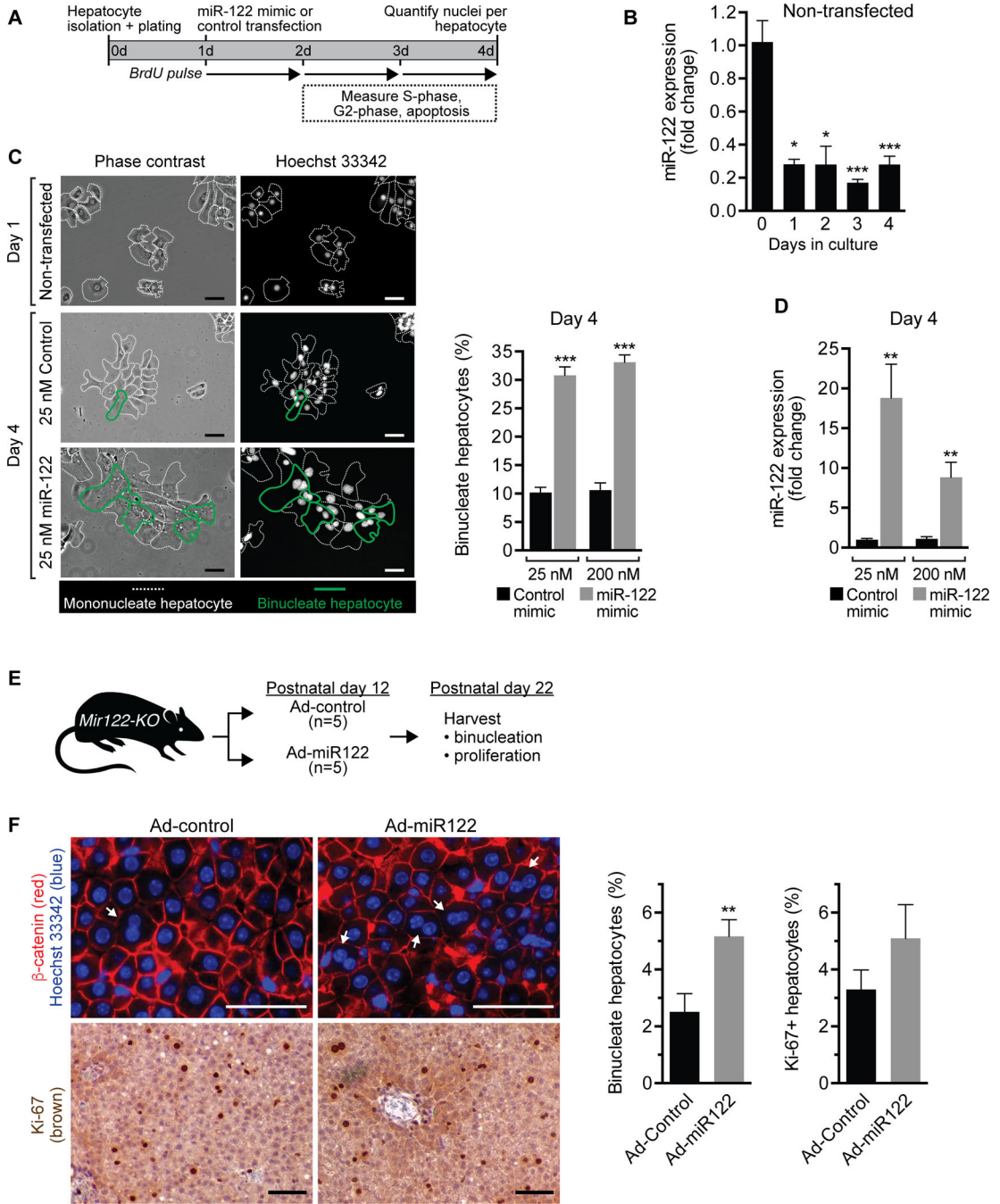


Fig. 4. miR-122 induces binucleation *in vitro* and *in vivo*.

(A–D) Diploid hepatocytes isolated from 14d-old WT C57BL/6 mice were plated on day 0, transfected with miR-122 mimic or control mimic (25 nM or 200 nM) on day 1 and proliferation/nucleation tracked until day 4. (A) Experimental design. (B) Primary non-transfected hepatocytes rapidly lost miR-122 expression in culture, as measured by qRT-PCR and normalized to RNU6b ($n = 3$). (C) Cultured non-transfected hepatocytes were predominantly mononucleate on day 1, and by day 4 hepatocytes transfected with 25 nM or 200 nM miR-122 mimic contained 3-fold more binucleate hepatocytes compared to controls.

Representative images are shown (left). Dashed white lines indicate mononucleate hepatocytes, and bold green lines mark binucleate hepatocytes. Graph shows the percentage of binucleate hepatocytes observed at day 4 ($n = 3-6$) (right). (D) Hepatocytes transfected with miR-122 mimic contained 10–20-fold more miR-122 at day 4. miR-122 expression was normalized to RNU6b ($n = 3$ from 2 independent experiments). (E,F) *Mir122*-KO mice were injected with Ad-miR122 or Ad-control (1×10^9 viral particles) on postnatal day 12 ($n = 5$) and livers harvested on postnatal day 22. (E) Cartoon showing the experimental design is shown. (F) Livers were stained with β -catenin (red) to mark membranes and Hoechst 33342 (blue) to mark nuclei. Arrows indicate binucleate hepatocytes. Additionally, livers were stained with Ki-67 (brown) to identify proliferating cells. Representative images are shown (left). The percentage of binucleate hepatocytes and Ki-67+ hepatocytes is shown in the graphs (right). * $P < 0.04$, ** $P < 0.02$, *** $P < 0.007$. Graphs show mean \pm SEM. Scale bars are 50 μm .

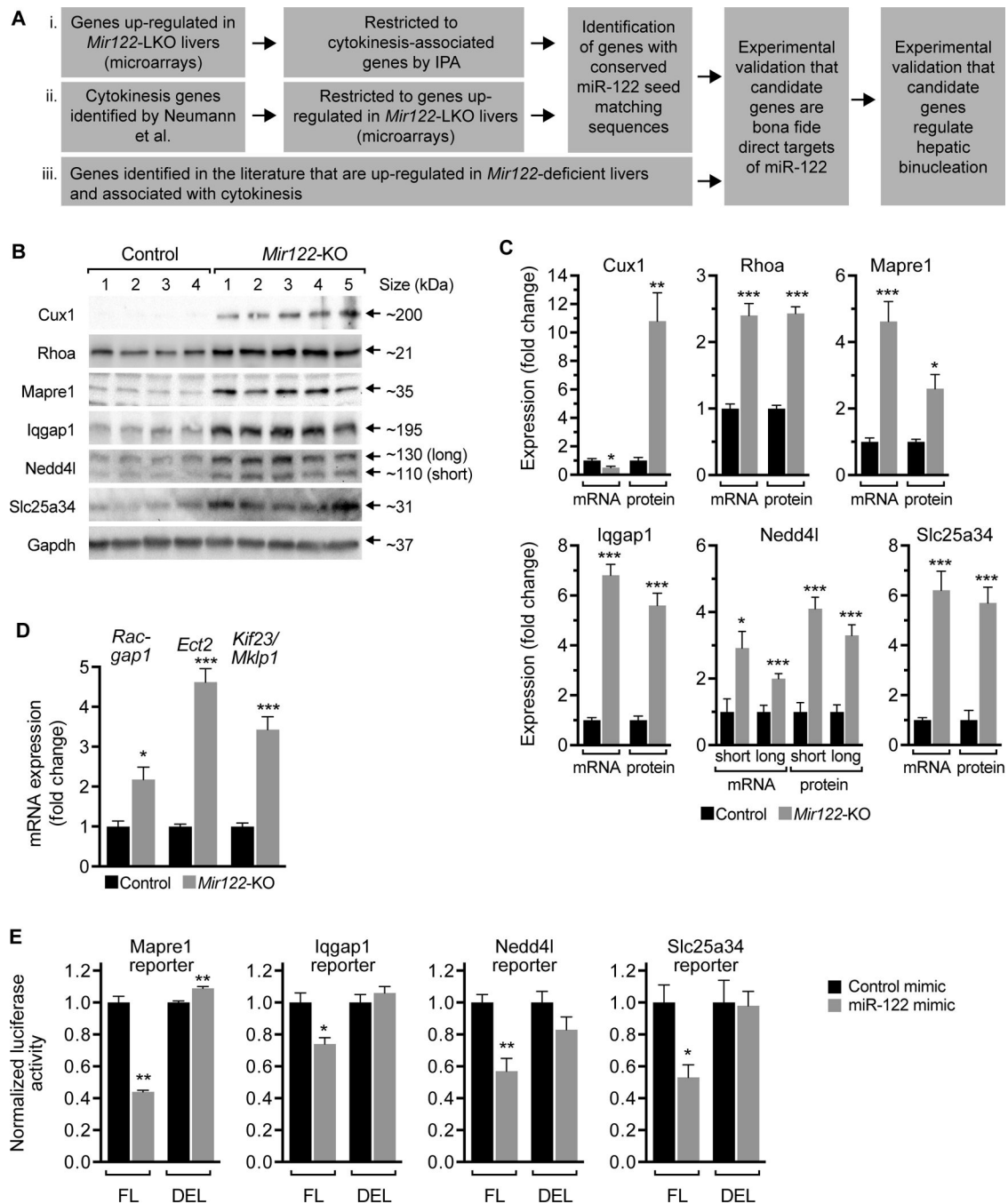


Fig. 5. miR-122 regulates expression of cytokinesis-related targets

(A) Strategy for identifying miR-122 target genes that regulate cytokinesis. (B,C) Cux1, Rhoa, Mapre1, Iqgap1, Nedd4l and Slc25a34 were up-regulated in 28d-old *Mir122*-KO livers compared to the controls at the protein and/or mRNA levels ($n = 4-5$). Western blots are shown for 4 control and 5 *Mir122*-KO livers; Gapdh was used as a loading control (B). Graphs show relative mRNA and protein expression levels normalized to Gapdh (C). (D) Centralspindlin complex members *Racgap1*, *Ect2* and *Kif23/Mklp1* were also up-regulated in 28d-old *Mir122*-KO livers ($n = 5$). The graph shows mRNA expression normalized to

Gapdh. (E) NIH 3T3 cells were transfected with *Mapre1*, *Iqgap1*, *Nedd4l* or *Slc25a34* reporter plasmids containing predicted miR-122 3'UTR seed sequences (full length, FL) or with the seed sequences deleted (DEL). Reporter constructs are detailed in Supporting Fig. 8. Cells were co-transfected with control or miR-122 mimic (25 nM) and luciferase activity measured after 48h. For each set of reporters, miR-122 expression reduced reporter activity 26–47% in the presence of FL reporters but not DEL reporters. Graphs show normalized luciferase activity (*Renilla* luciferase activity controlled by FL or DEL normalized to firefly luciferase activity expressed by the same plasmid) ($n = 3$ replicates from 2–3 independent experiments). * $P < 0.03$, ** $P < 0.008$, *** $P < 0.0008$. Graphs show mean \pm SEM.

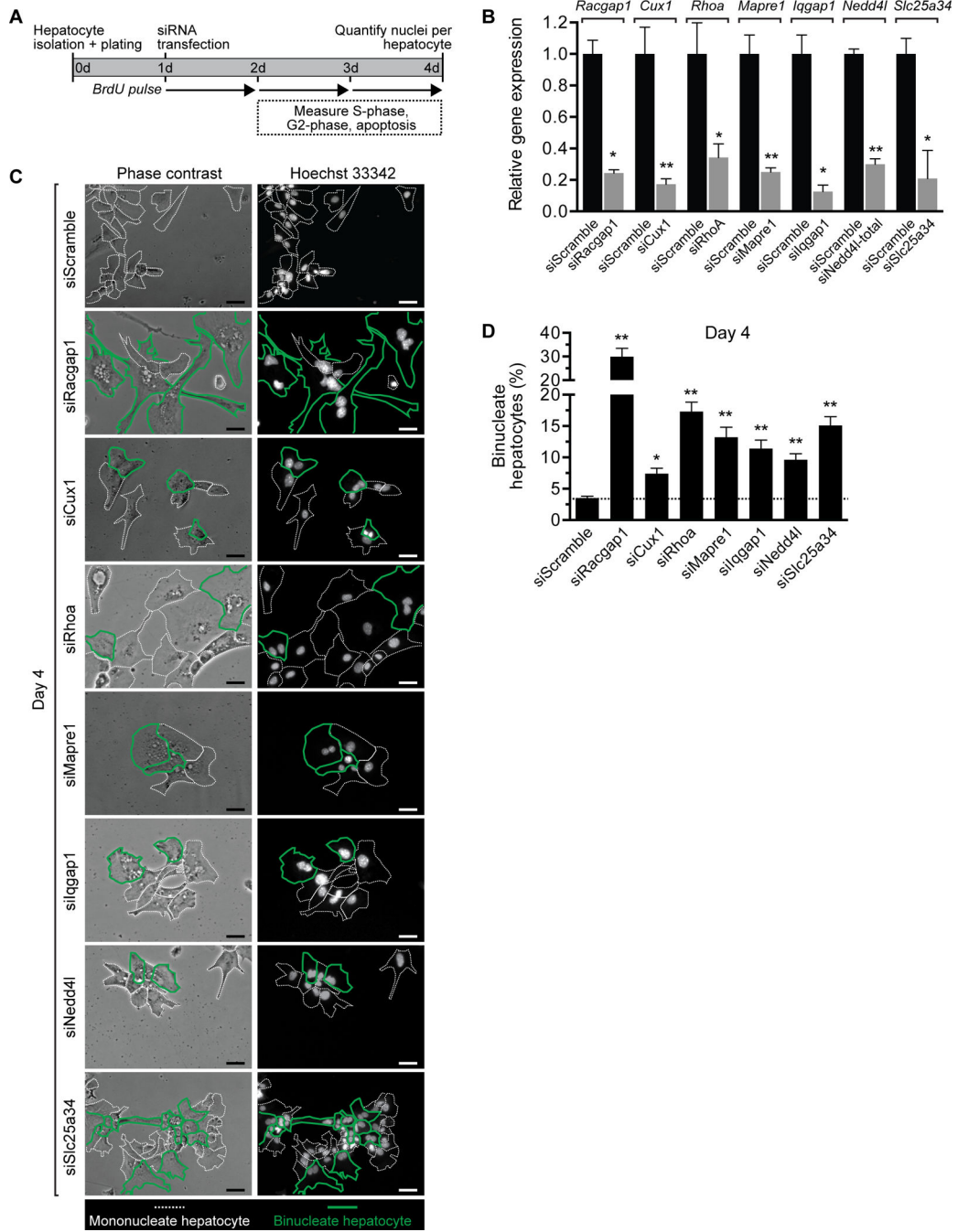


Fig. 6. Inhibition of miR-122 target genes promotes formation of binucleate hepatocytes (A) Experimental design for tracking diploid hepatocytes depleted of target genes. Diploid hepatocytes isolated from 14–15d-old WT C57BL/6 mice were plated on day 0, transfected with 20–40 nM siRNAs on day 1 and proliferation/nucleation tracked until day 4. (B) Hepatocytes transfected with scrambled siRNA or siRNAs to *Racgap1*, *Cux1*, *Rhoa*, *Mapre1*, *Iqgap1*, *Nedd4l* or *Slc25a34* showed reduced target expression on day 4 ($n = 2–4$ replicates from 2 independent experiments). The graph shows relative gene expression measured by qRT-PCR normalized with *Gapdh*. Total *Nedd4l* was measured using primers

detecting both the short and long forms. Protein levels are shown in Supporting Fig. 9. (C,D) The number of nuclei/hepatocyte was determined on day 4. Representative images illustrate a mixture of mono- and binucleate hepatocytes (C), and the percentage of binucleate hepatocytes calculated ($n = 3-6$) (D). Dashed white lines indicate mononucleate hepatocytes, and bold green lines mark binucleate hepatocytes. The dashed line on the graph indicates the percentage of binucleate cells in cultures of hepatocytes transfected with scrambled siRNA. * $P < 0.04$, ** $P < 0.009$. Graphs show mean \pm SEM. Scale bars are 50 μm .

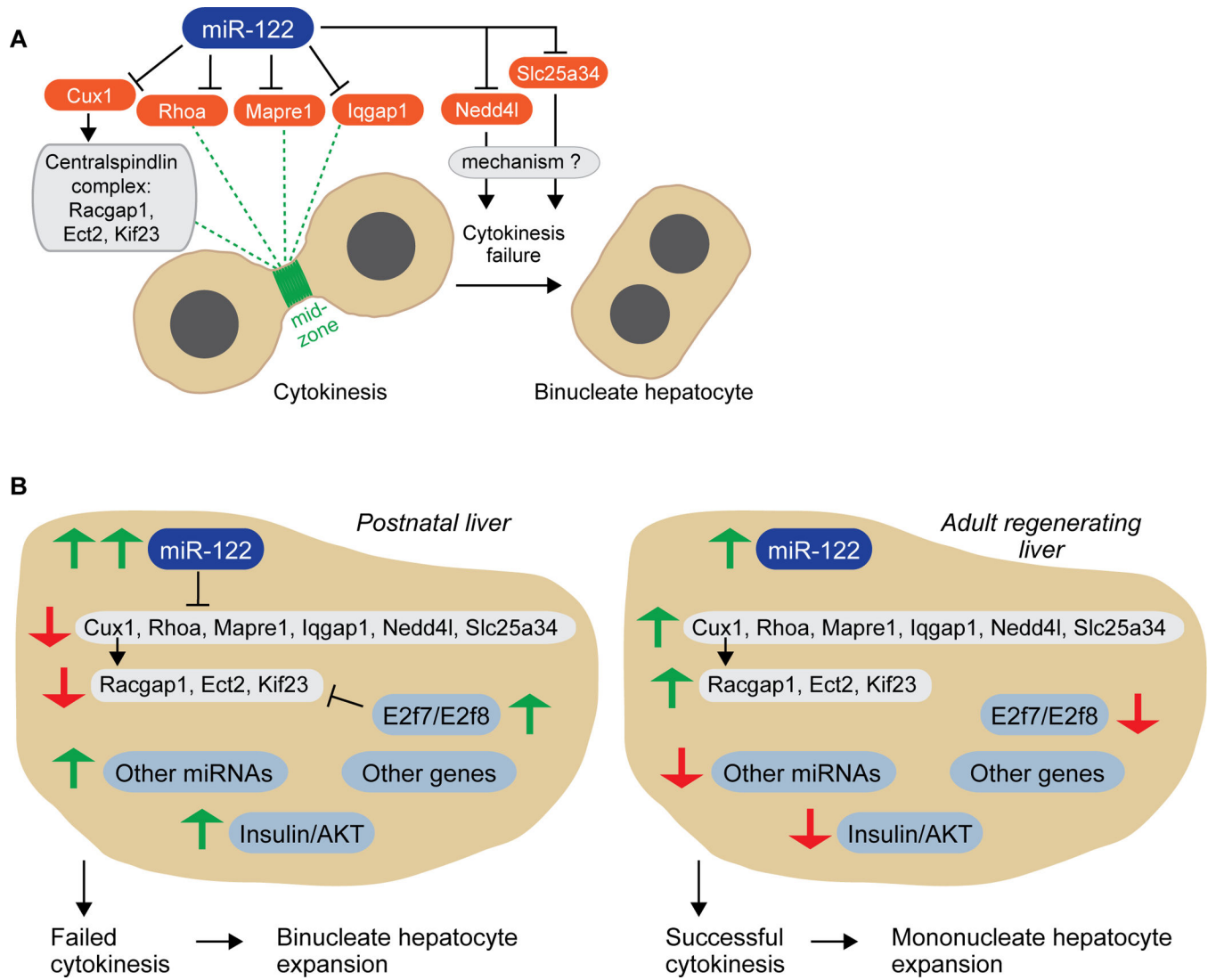


Fig. 7. A network of signals regulates hepatic binucleation
 (A) The model details a mechanism of miR-122-regulated cytokinesis. miR-122 directly antagonizes pro-cytokinesis targets (shown in orange), induces cytokinesis failure and emergence of binucleate hepatocytes. (B) The cartoon illustrates signaling networks involved in cytokinesis failure in postnatal developing livers and successful cytokinesis in adult regenerating livers. Green arrows indicate elevated expression; red arrows indicate reduced expression.

Table 1

Cytokinesis-associated genes with conserved miR-122 seedmatching sequences in the 3'UTR

Accession no.	Symbol	Gene name	Alias	Identification Method
NM_009986	Cux1	Cut-like homeobox 1	Cutl1	c
NM_008079	Galc	Galactosylceramidase		b
NM_016721	Iqgap1	IQ domain-containing GTPase-activating protein 1		c
NM_007896	Mapre1	Microtubule-associated protein, RP/EB family, member 1	Eb1, Bim1p	a, c
NM_031881	Nedd4l	Neural precursor cell expressed, developmentally down-regulated gene 4-like	Nedd4b, Nedd4-2	a, b
NM_016802	Rhoa	Ras homolog gene family, member A	Arha, Arha1, Arha2	a, c
NM_001013780	Slc25a34	Solute carrier 25 family, member a34	Gm1369	b
NM_126165	Vps4a	Vacuolar protein sorting 4a (yeast)		a

^aIPA analysis (JCI 2012;122:2871) revealed 63 cytokinesis-associated genes ($P < 0.05$) differentially expressed in *Mir122*-LKO livers. Among these, 4 genes up-regulated 1.3-fold are indicated with conserved miR-122 seed matching sequences as determined by TargetScan and/or MiRanda algorithms: Mapre1, Nedd4l, Rhoa and Vps4a. See Supporting Tables 3 and 4 for additional information.

^bNeumann et al. (Nature 2010;464:721) characterized 427 genes involved in binucleation, and we showed that 31 genes were up-regulated in *Mir122*-LKO livers ($P < 0.05$) (JCI 2012;122:2871). Among these, 3 genes up-regulated 1.3-fold are listed with conserved miR-122 seed matching sequences as determined by TargetScan and/or MiRanda algorithms: Galc, Nedd4l and Slc25a34. See Supporting Table 5 for additional information.

^cGenes identified in the literature that were implicated in cytokinesis and up-regulated in *Mir122*-deficient livers: Cux1, Iqgap1, Mapre1 and Rhoa.