## Supplementary data

**Table S1** – miRNA expression array

**Table S2** – primer sequences

**Table S3** – siRNA and miRNA sequences

**Figure S1. Differential expression of mature miRNA and pri/pre-miRNA of the miR17~92 cluster**

Primary human melanocytes were stimulated with forskolin for the indicated times and then harvested for qRT-PCR analysis. qRT-PCR results of mature miRNA (upper) and pri/pre miRNA gene expression (lower) at different time points after stimulation are shown. Mature miRNAs were normalized to RNU48 and pri/pre-miRNAs to Actin and relative to non stimulated (-) cells. Error bars indicate the standard deviation of 5 replicates from different primary melanocyte donors.

**Figure S2. Differential expression of mature miRNA and pri/pre-miRNAs**

Primary human melanocytes were stimulated with forskolin for the indicated times and then harvested for qRT-PCR analysis. qRT-PCR results of mature miRNAs (upper) and the pre miRNA genes (lower) at different time points upon stimulation are shown. Mature miRNAs were normalized to RNU48 and pre-miRNAs to Actin and relative to non stimulated (-) cells. A table summarizing the expression pattern of those miRNAs is present in Figure 2B. Error bars indicate the standard deviation of 5 replicates from different primary melanocyte donors.

**Figure S3. Gene expression upon melanocyte stimulation**

(A) Both isoforms of the DICER gene are upregulated upon melanocyte stimulation

Mammalian genomes encode one DICER gene with two alternative 5' UTRs. Primary human melanocytes were stimulated with forskolin for the indicated times and total RNA was then subjected to qRT-PCR analysis of both DICER isoforms. mRNA values were normalized to actin and relative to non-stimulated cells. Error bars indicate the standard deviation of 5 replicates from different primary melanocyte donors.

(B) miR17 and 92 upregulation is MITF dependant

Primary human melanocytes were stimulated with forskolin and total RNA was then subjected to qRT-PCR analysis of the indicated miRNAs. miRNA values were normalized to RNU48 and relative to non-stimulated cells. Error bars indicate the standard deviation of 5 replicates from different primary melanocyte donors.

(C) Primary human melanocytes were stimulated with forskolin for the indicated times and total RNA was subject to qRT-PCR analysis. BIM mRNA expression is presented at each indicated time point with results normalized to Actin and to non-stimulated cells. Error bars indicate the standard deviation of 3 replicates.

(D) Primary human melanocytes were stimulated with forskolin for the indicated times and total RNA was subject to qRT-PCR analysis. CT values of 4 different endogenous controls are shown.

**Figure S4. MITF and DICER expression levels correlate in-vivo**

(A) Three-color immunofluorescence staining of MITF (*mitf*$^{wh/+}$) heterozygous skin compared to MITF (*mitf*$^{+/+}$) wild-type skin sections permits comparison of expression of Dicer (green, cytoplasmic) in wildtype vs. MITF-mutant melanocytes and wildtype vs. MITF-mutant keratinocytes, normalized to DAPI staining of the same cells (400x magnification). MITF-stain is red and is used to identify melanocytes; DAPI-stained nuclei appear blue. Merged images show co-localization of Dicer and MITF in melanocytes ("merge"). Dicer and DAPI staining intensities were quantitatively measured in melanocytes (identified by MITF-positivity) and keratinocytes (identified by location and MITF-negativity). Dicer signal was normalized to DAPI staining of the identical cells, and plotted in Figure 6B. Please see Supplemental Experimental Procedures for details of the Microscopy and quantitative methodology.
(B) Control immunofluorescence stain without primary antibodies. Outlines represent the hair follicle structures.

**Figure S5. Post-natal conditional knockout of *Dicer* in the melanocyte lineage leads to age-dependent depigmentation with melanocyte loss.**

(A) Dorsal and ventral views of p19 Dicer(fl/fl); Dct(Cre/Cre); Dct-lacZ mice compared to Dicer(fl/+); Dct(Cre/Cre); Dct-lacZ littermates. The Dct-CRE allele leads to gradual, age-dependent deletion of Dicer (based upon staining for Dicer within melanocytes in skin of different aged mice, data not shown).
(B) Dorsal views of P35 Dicer (fl/fl); Dct(Cre/Cre); Dct-lacZ; K14-SCF transgenic mice compared to Dicer(fl/+); Dct(Cre/Cre); Dct-lacZ; K14-SCF transgenic littermates. The

images show dorsal views before hair depilation (left) or after (right) and reveal significant loss of epidermal pigmentation in the homozygous Dicer(fl/fl) mice.

(C) Dorsal and ventral views of P420 and P502 mice exhibit significant and progressive depigmentation on the Dicer(fl/fl); Dct(Cre/Cre); Dct-lacZ background.

(D) Hematoxylin/eosin staining of skin sections from Dicer(fl/fl); Dct(Cre/Cre); Dct-lacZ, Dicer(fl/+); Dct(Cre/Cre); Dct-lacZ and Dicer(+/+); Dct(Cre/Cre); Dct-lacZ mice, revealing grossly intact skin architecture.

(E) Whole-mount X-gal staining of whiskers generated from Dicer(fl/fl); Dct(Cre/Cre); Dct-lacZ revealed partial loss of *Dct-lacZ* labeled melanoblasts in whiskers of Dicer(fl/fl);Dct(Cre/Cre) mice. The Dct-CRE allele leads to gradual, age-dependent deletion of Dicer (based upon staining for Dicer within melanocytes in skin of different aged mice, data not shown). Thus depigmentation correlates with diminished melanocyte numbers, rather than diminished pigment within viable melanocytes.

(F) Immunofluorescence staining of skin from Dicer(fl/fl); Dct(Cre/Cre) and control (Dicer(fl/+); Dct(Cre/Cre)) mice reveals absence of melanocytes (S100 positive cells) in the Dicer(fl/fl); Dct(cre/Cre) unpigmented skin, compared to littermate control. Cytoplasmic staining for Dicer is seen in S100 negative cells (keratinocytes).

**Figure S6. Post natal Dicer depletion leads to melanocyte loss**

(A) Dorsal P32 mouse depigmentation in Dicer(fl/fl); Tyr::CreER mutants compared to Dicer(fl/+); Tyr::CreER littermate control. Please see Supplemental Experimental Procedures for details of the topical OH-Tamoxifen treatment regimen.

(B) Immunofluorescence staining of skin from Dicer(fl/fl); Tyr::CreER and control (Dicer(fl/+); Tyr::CreER) mice reveals absence of melanocytes (S100 positive cells) in the Dicer(fl/fl); Tyr::CreER unpigmented skin, after OH-Tamoxifen topical treatment. Cytoplasmic staining for Dicer is seen in S100 negative cells (keratinocytes).

(C) Mouse primary melanocytes generated from Dicer (fl/fl) mice were infected with either adenovirus Cre or vector control (GFP) adenovirus (MOI400). Total protein was extract at the indicated time post infection. Protein levels of Cre and Tubulin were assessed by Western blotting.

(D) Mouse primary melanocytes generated from Dicer(fl/fl) mice treated as in (A). Genomic DNA was extracted at the indicated time post infection and subjected to genotyping. Dicer genotype was assessed by PCR and XPC genotyping was used as a control.

(E) Mouse primary melanocytes generated from Dicer(fl/fl) mice were treated as in (A) and total RNA was subjected to qRT-PCR analysis of mature miRNAs at indicated time points post Cre infection. Mature miRNAs were normalized to RNU48 and relative to control empty vector (GFP). Error bars indicate the standard deviation of 5 replicates from different primary melanocyte donors.

(F) Growth/survival curve of infected melanocytes are presented. Cre or GFP infected cells were fixed and crystal violet stained, at the indicated time points post infection. Results are triplicates from one experiment (mean ± s.d.) and representative of three independent experiments.

(G) Typical bright light picture of melanocyte 5, 15 and 25 days post Cre infection.

**Figure S7. DICER depletion leads to human melanocyte loss in-vitro.**

(A) Human primary melanocytes were transfected with 2 different siDICER (Dic1, Dic2) or siScramble (con); total protein was extracted at the indicated time post transfection. Protein levels of DICER and Tubulin were assessed by Western blotting.

(B) Human primary melanocytes were transfected as in (A) and total RNA was subjected to qRT-PCR analysis of mature miRNAs at indicated time points post siDICER transfection. Mature miRNAs were normalized to RNU48 and relative to siScramble. Error bars indicate the standard deviation of 5 replicates from different primary melanocyte donors.

(C) Growth/survival curve of transfected melanocytes are presented. siDICER (Dicer si1, si2) or siScramble (siScrm) cells were fixed and crystal violet stained, at the indicated time points after indicated siRNA transfections. Results are triplicates from one experiment (mean ±s.d.) and representative of three independent experiments.

(D) Bright field image of melanocytes 3 and 7 days after the indicated siRNA transfections.

## Supplementary Experimental Procedures

### Cells, oligonucleotide transfection and growth assay

Primary human melanocytes were isolated from neonatal foreskin as described (McGill et al., 2002). Primary melanocytes between passages 2 and 5 were stimulated with 20 uM forskolin (Sigma Aldrich) or with DMSO (as a vehicle control), for indicated times after

an overnight starvation in F10 media supplemented with penicillin/streptomycin/glutamine (PSQ). Serum starvation conditions did not affect cell viability (data not shown). For microarrays primary melanocyte were stimulated for 16 hours with forskolin. 501mel human melanoma cells (gift from Dr. Ruth Halaban, Yale University) were maintained in Ham's F-10 media (Cellgro) supplemented with 10% fetal bovine serum (FBS) (Sigma Aldrich) and 1% PSQ. The UACC62 human melanoma cell line was obtained from NCI and maintained in RPMI-1640 media (Mediatech) supplemented with 10% fetal bovine serum (FBS) (Sigma Aldrich) and 1% PSQ .Primary human fibroblasts were isolated from abdominal or breast skin that was rinsed with 70% ethanol, maintained in PBS while the fat attached at the dermis was removed. Fat free skin was cut in small ($2cm^2$) pieces and incubated with Dispase II (Koche) over night at 4ºC. Forceps were used to separate the epidermis from the dermis. In a flask 8 dermis pieces were mixed with 12mL of Hank's Balanced Salt Solution (Cellgro) and 0.5mL of collagenase (2mg/mL H2O) (Gibco). The mixture above was shaken at maximum speed for 30min at room temperature. The solution was filtered using a 70um cell strainer (Falcon). 3-4mL of the filtered dermis suspension was cultured in 20mL of DMEM media (Mediatech) + 10% calf serum (Sigma Aldrich) + 1% Penicillin-Streptomycin-Glutamine (PSQ). Cells were incubated at 37ºC, 5.0% CO2 until confluent. Primary mouse melanocytes were established from Dicer fl/fl mice as described before (Sviderskaya et al., 1997) with slight modification. Trunk skin from neonatal mice was harvested, chopped and maintained in RPMI 1640 medium containing 10% fetal bovine serum, 200nM 12-O-tetradecanoyl phorbol 13-acetate and 200 pM cholera toxin, gassed with CO2 (10% vol/vol). MiRNA mimics, antagomirs or siRNAs were transfected into

primary human melanocytes using HiPerFect according to manufacturer's protocols (Qiagen). The sequences and manufacturers of the miR-17-3p, miR-92, miR-107 mimics, scrambled control miRNA mimic, DICER siRNA, and scrambled control siRNA are listed in Supplementary material, Table S3. Cells were transfected twice with 100 pmol of oligonucleotide per well ($0.5 \times 10^6$ cells) at 24h intervals. 48 h after the second siDICER transfection, primary human melanocytes were transfected with miRNA mimics. Transfected primary human melanocytes were assayed 48 h after the last transfection. For cell growth assays, cells ($10^4$/well) were plated in multiple 24 well plates. Cells were treated as indicated and the following day was considered day 0. For analysis of cell growth/number, cells were fixed using 10% ethanol/10% acetic acid, and stained with 0.2% crystal violet:fixative. Following washes with water, the plates were dried. Each 24 well plate was quantified by crystal violet staining. The crystal violet dye was re-dissolved in fixation solution. One hundred μl of the re-dissolved dye was used for color measurement, in duplicate Color intensity was measured at $OD_{595}$ in a 96-well plate reader, and normalized to day 0 as 100%.

**Gel electrophoresis and immunoblotting**

Primary human melanocytes were harvested in 50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 10 μM leupeptin, 1 mM AEBSF, 100 units/ml aprotinin, 10 mM NaF, and 1 mM $Na_3VO_4$. Samples (30 μg) were resolved by electrophoresis through a 10% SDS-PAGE gel, transferred to nitrocellulose membranes, and then exposed to the appropriate antibodies: 1:5 diluted mouse anti-MITF (C5) (tissue culture supernatant), 1:500 dilution anti-DICER (Abcam), 1:1000 dilution anti-phospho-CREB (Cell Signaling Technology), 1:1000 dilution anti-BIM (MBL International), 1:1000 dilution anti-Caspase3 (Cell

Signaling Technology), 1:2000 dilution anti-tubulin (Sigma Aldrich). Proteins were visualized with the ECL system (Perkin Elmer Life Sciences) using horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibody (GE Healthcare) according to manufacturer's protocols. Western blot assays are representative of at least three experiments.

**Adenovirus production and infection**

Adenoviruses encoding wild type MITF, dominant negative MITF or vector control encoding a short inert polypeptide, were cloned and purified as previously described (Garraway et al., 2005). Briefly, viruses were propagated in HEK293 cells, which were collected upon detection of viral cytopathic effect. Cell pellets were re-suspended in PBS and lysed by freeze-thaw cycles. Concentration and purification of virus was performed using a cesium chloride gradient, followed by intensive dialysis. Multiplicity of infection (MOI) was estimated by $OD_{260/280}$, using a multiplier of $2x10^6$ cfu/μl per OD unit. For infection, $5x10^5$ cells were plated in 6-well dishes, and on the next day overlaid with 0.5 ml serum-free media containing 10 mM $MgCl_2$ and concentrated adenovirus at the indicated titer. Following incubation at 37°C for 30 min, 2ml of fresh full media was added and the cells were grown until harvest. Adenoviruses encoding Cre or GFP, were purchased from the Gene Transfer Vector Core of Iowa University. For infections, primary mouse melanocytes were plated in culture dishes and the next day overlaid with serum free media and 400 MOI viruse. Following incubation at 37°C for 30 min, fresh

2% serum mouse melanocyte media was added, and 72h post infection, media was changed to full media.

**Chromatin Immunoprecipitation**

ChIP assays were performed with primary melanocytes grown to logarithmic phase. Cells were subjected to 1% formaldehyde in PBS for 20 min at room temperature with gentle shaking. Cells were then harvested by scraping and homogenized in hypotonic buffer (Du et al., 2004) on ice using a Dounce homogenizer. The nuclei were isolated by centrifugation over a 10% sucrose pad. Nuclei were then spun down, resuspended in ChIPs buffer (Du et al., 2004) and sonicated. Antibodies, RNA polymerase II CTD4H8 (Covance) or Placental Protein 4 (Assay Designs) as a nonspecific control antibody, were added to a 10-fold ChIPs buffer diluted sample and incubated on a rotator for 10 h at $4^{o}$C. Ultralink protein-A/G-beads (Pierce) were added to the sample and a control sample and incubated for an additional hour at room temperature. Immunoprecipitates were then washed twice with ChIPs buffer, twice with 500 mM NaCl ChIPs buffer, and once with TE (pH 8). The immunoprecipitates were released from the beads by incubating at 65°C for 20 min in 1% SDS/TE and treated with proteinase K side by side with an unprecipitated sample as input control. Crosslinks were released by heating at 70°C for 10 h, and DNA was recovered by extraction with phenol and chloroform at high-salt (0.6 M Na Acetate [pH 8]) and then ethanol precipitated. qRT- PCR was then performed on samples to amplify a fragment occupied by MITF. For additional information on buffers, please see Supplementary reference (Du et al., 2004).

**Northern blot analysis**

5-10 micrograms of total RNA were resolved on a 12.5% Urea–Polyacrylamide gel (BioRad) and transferred to a Hybond-N+ membrane (Amersham). The membrane was dried, UV crosslinked, preincubated with ULTRAhyb-Oligo Hybridization Buffer (Ambion) for 1 h and incubated overnight at 42°C with 107 cpm of an antisense RNA probe directed against mature miR211 miR17-3p or miR92a (IDT); miR211 probe: 5' rArGrGrCrGrArArGrGrArUrGrArCrArArArGrGrGrArA 3'; miR92a-1 probe: 5' rArGrC rArUrU rGrCrA rArCrC rGrArU rCrCrC rArArC rCrU 3'; miR17-3p probe: 5' rCrUrA rCrArA rGrUrG rCrCrU rUrCrA rCrUrG rCrArG rU 3'. tRNA-Met probe: 5' GGT AGC AGA GGA TGG TTT CGA TCC 3'. Probes were 5' end labeled using mirVana Probe and Marker kit (Ambion) in the presence of [$\gamma$-$^{32}$P] ATP 4000-7000 Ci/mmol, according to manufacturer's protocols. The membrane was washed for 15 min at 42°C in 2× SSC, 0.1% SDS, exposed and scanned using a Storm PhosphorImaging system (Molecular Dynamics). Sizes of pre and mature miRNAs were confirmed using a labeled small RNA ladder (Ambion). The membrane was re-probed with DNA probe directed against tRNA for loading controls. pre-miRNA, mature miRNA and tRNA bands intensities were quantified using Imagequant software (GE Life Sciences).

## References

Du, J., Widlund, H.R., Horstmann, M.A., Ramaswamy, S., Ross, K., Huber, W.E., Nishimura, E.K., Golub, T.R., and Fisher, D.E. (2004). Critical role of CDK2 for melanoma growth linked to its melanocyte-specific transcriptional regulation by MITF. Cancer cell *6*, 565-576.

Garraway, L.A., Widlund, H.R., Rubin, M.A., Getz, G., Berger, A.J., Ramaswamy, S., Beroukhim, R., Milner, D.A., Granter, S.R., Du, J., *et al.* (2005). Integrative genomic analyses identify MITF as a lineage survival oncogene amplified in malignant melanoma. Nature *436*, 117-122.

McGill, G.G., Horstmann, M., Widlund, H.R., Du, J., Motyckova, G., Nishimura, E.K., Lin, Y.L., Ramaswamy, S., Avery, W., Ding, H.F., *et al.* (2002). Bcl2 regulation by the melanocyte master regulator Mitf modulates lineage survival and melanoma cell viability. Cell *109*, 707-718.

Sviderskaya, E.V., Bennett, D.C., Ho, L., Bailin, T., Lee, S.T., and Spritz, R.A. (1997). Complementation of hypopigmentation in p-mutant (pink-eyed dilution) mouse melanocytes by normal human P cDNA, and defective complementation by OCA2 mutant sequences. The Journal of investigative dermatology *108*, 30-34.

**Supplemental Figure**

Figure S1_levy et al

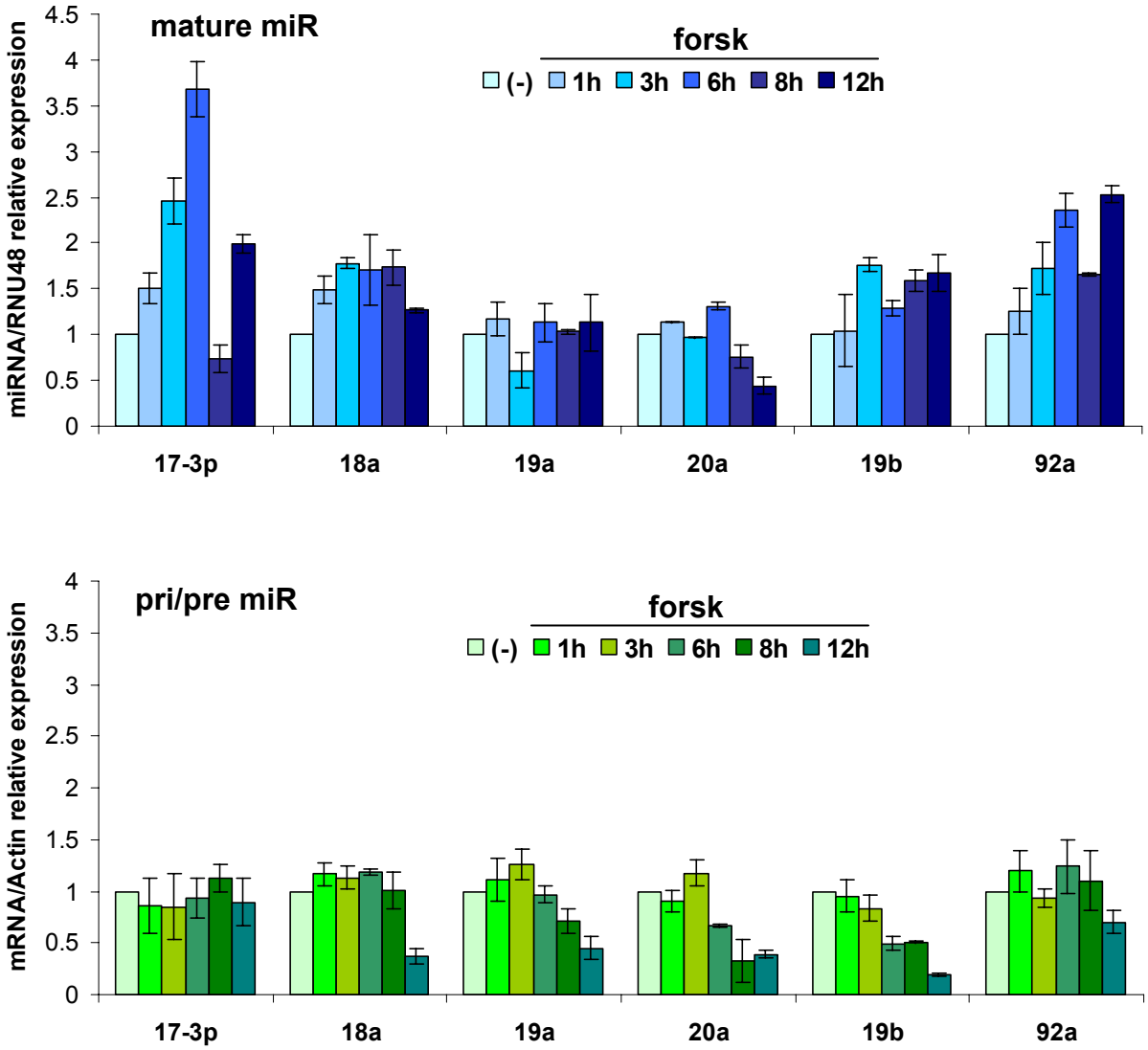
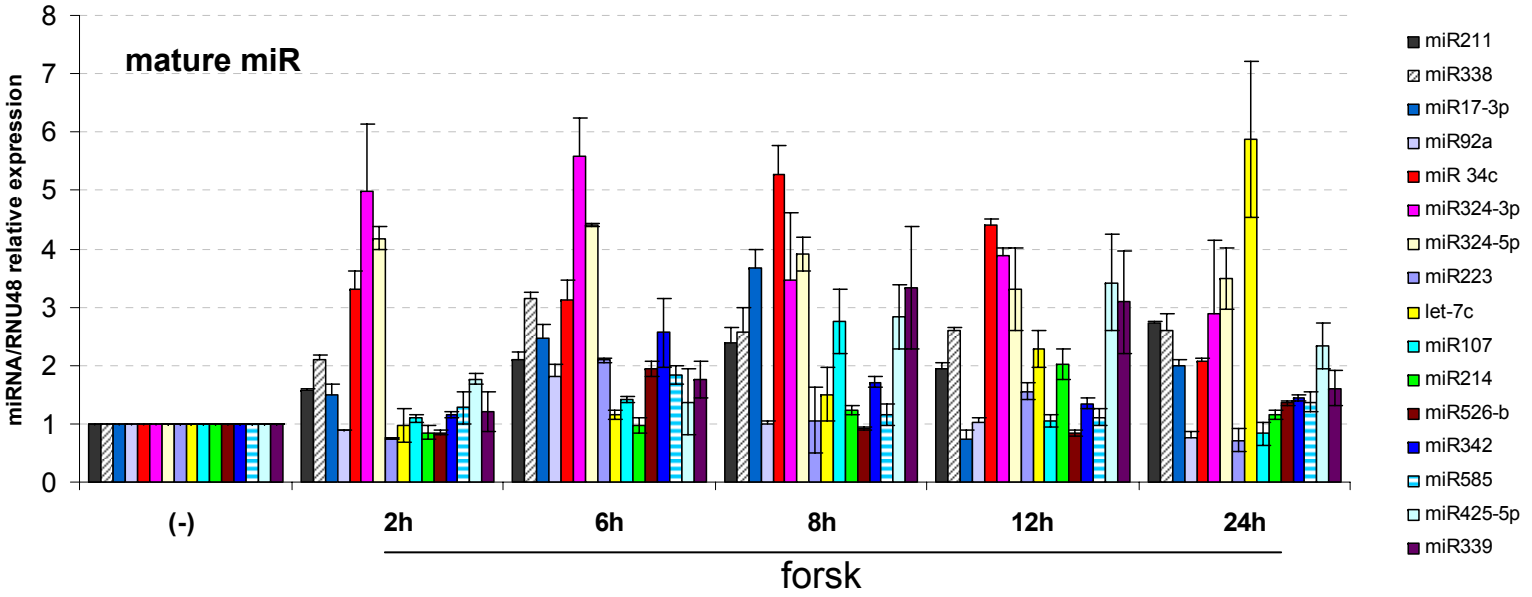
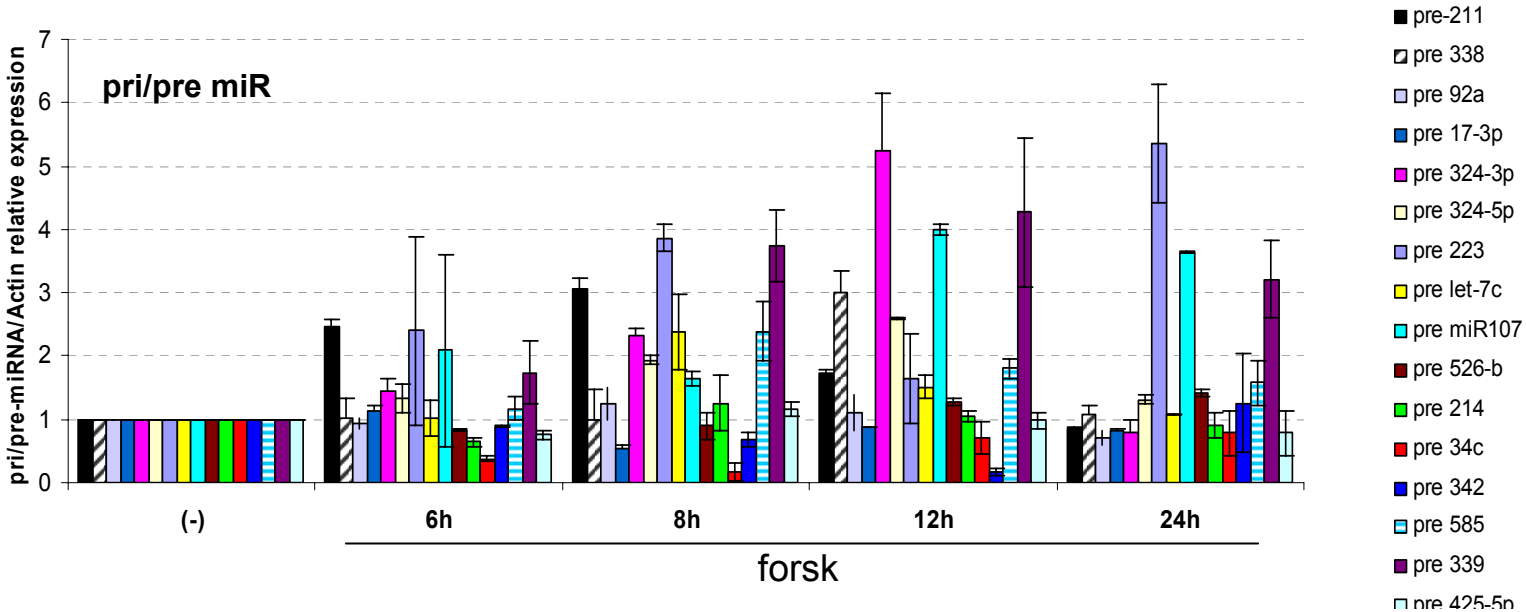


Figure S2_levy et al



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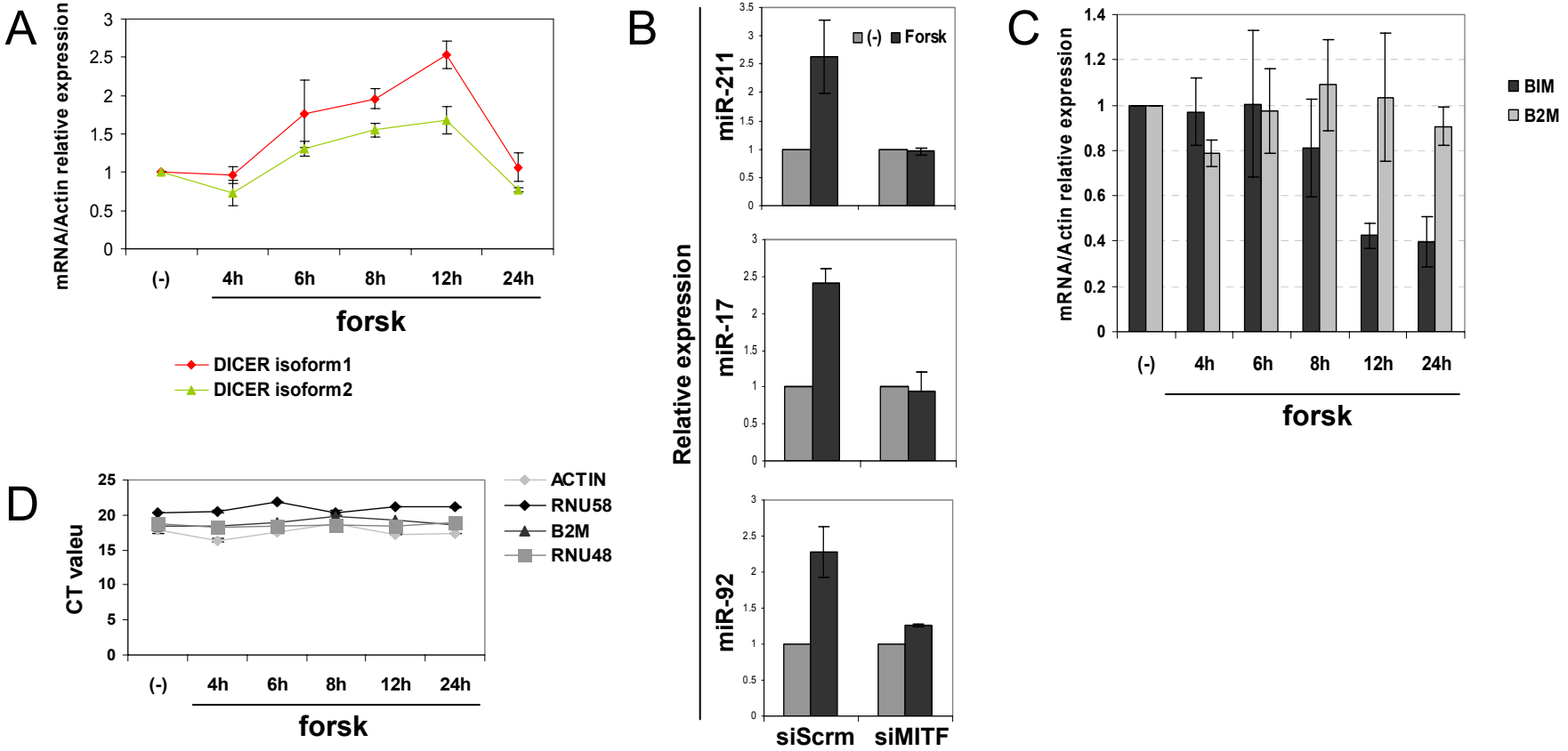

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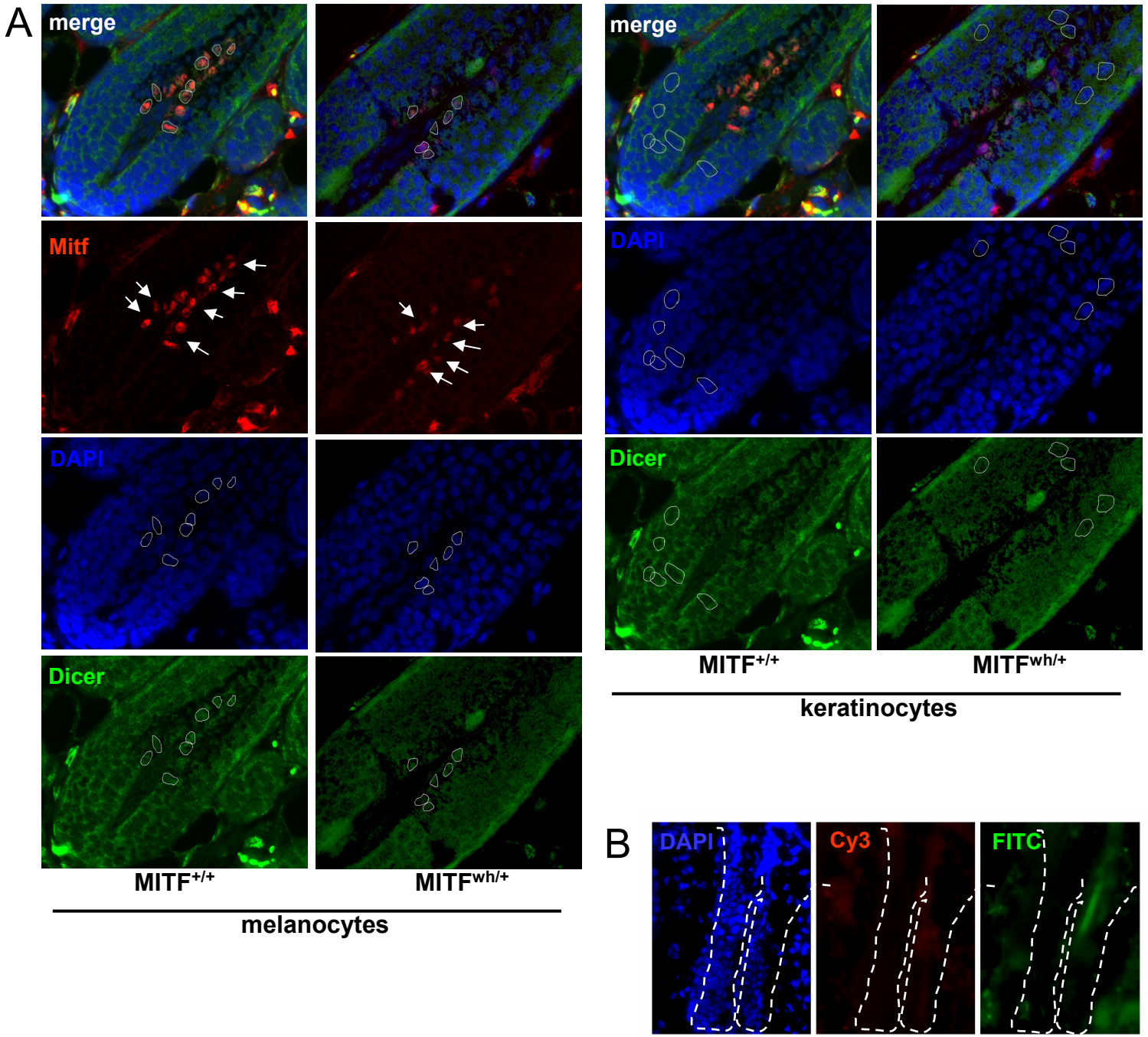

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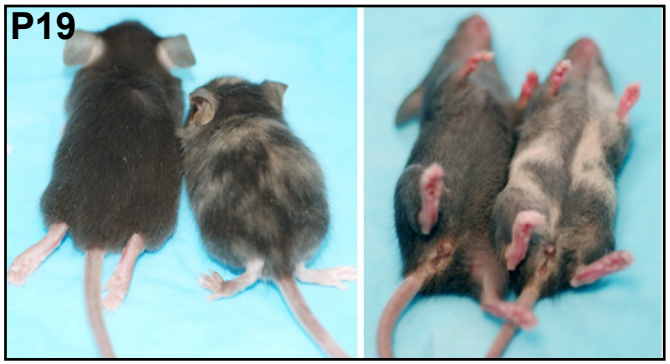


Dicer fl/+ Dicer fl/fl Dicer fl/+ Dicer fl/fl

DCT-LacZ, DCT-Cre

B


Dicer fl/+ Dicer fl/fl Dicer fl/+ Dicer fl/fl

DCT-LacZ, DCT-Cre, K14-SCF

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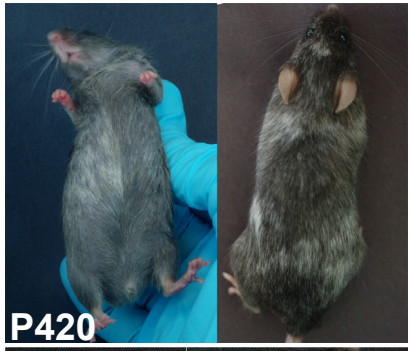


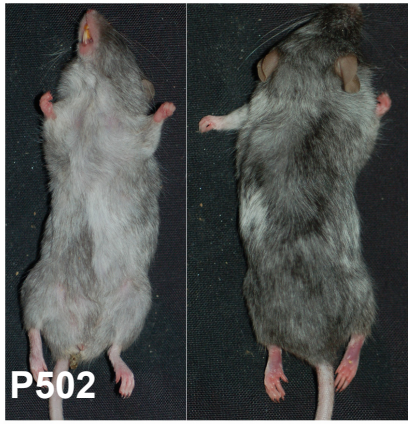


Dicer fl/fl, DCT-LacZ, DCT-Cre

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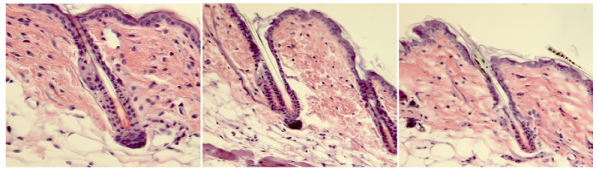


Dicer +/+ Dicer fl/+ Dicer fl/fl

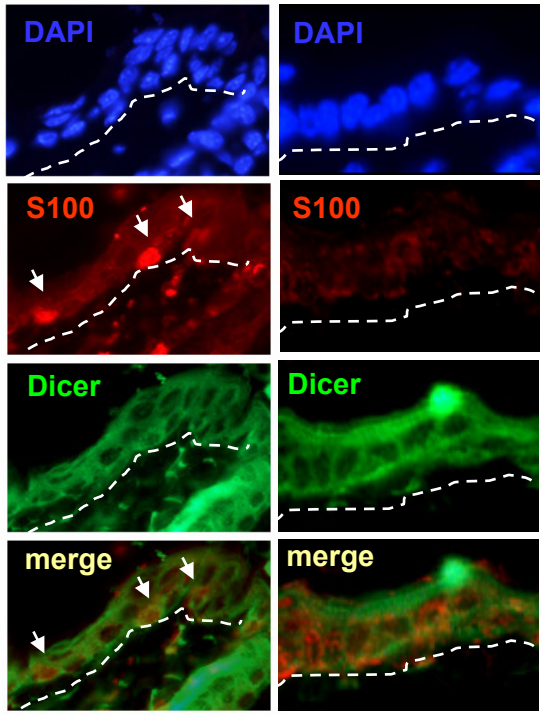
DCT-LacZ, DCT-Cre

E

pigmented unpigmented

Dicer fl/fl, DCT-LacZ, DCT-Cre

F




Dicer fl/+ Dicer fl/fl

DCT-LacZ, DCT-Cre, K14-SCF

Figure S6_levy et al

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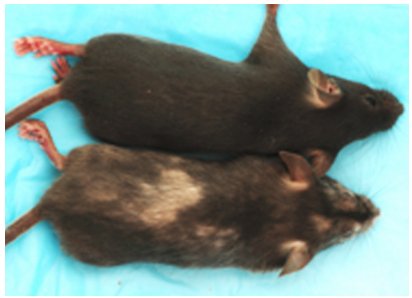


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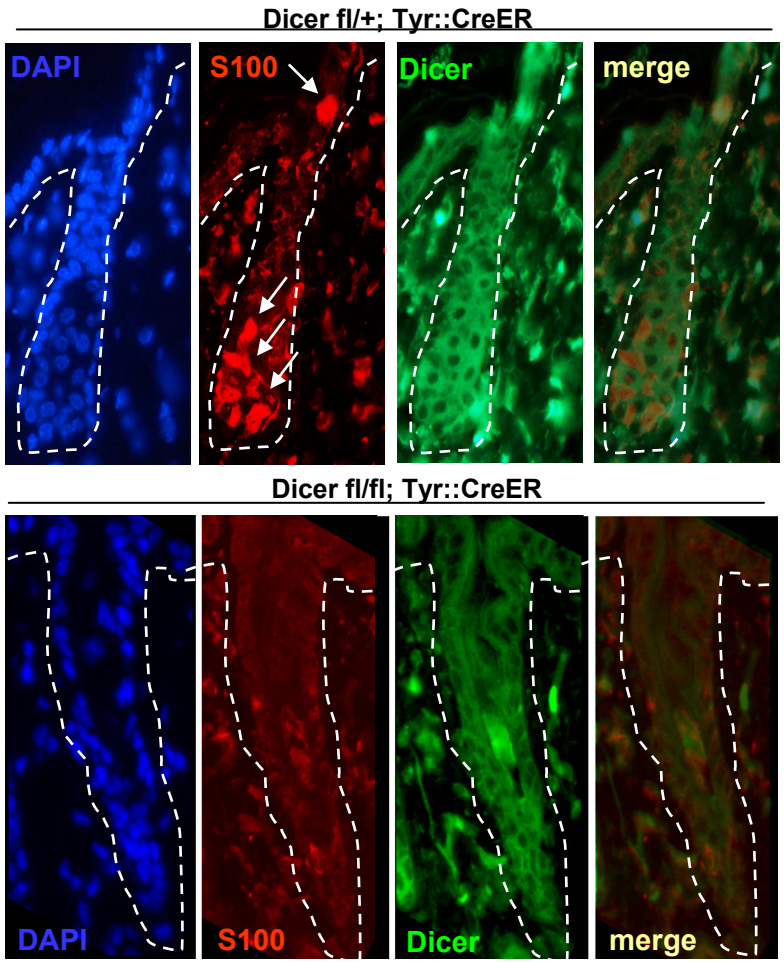


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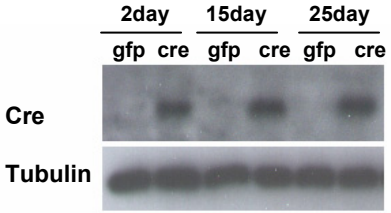


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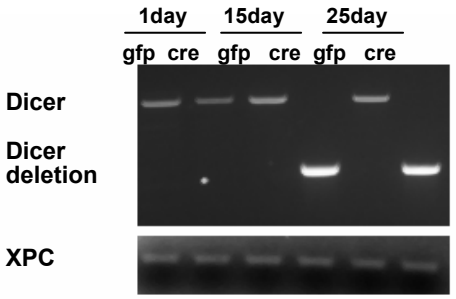


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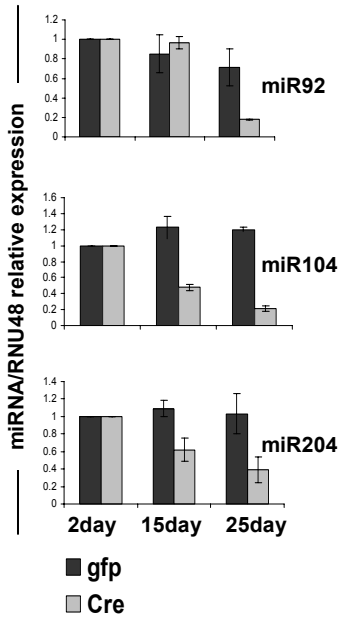


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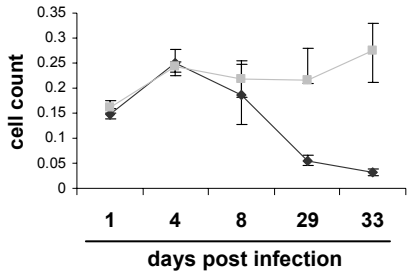


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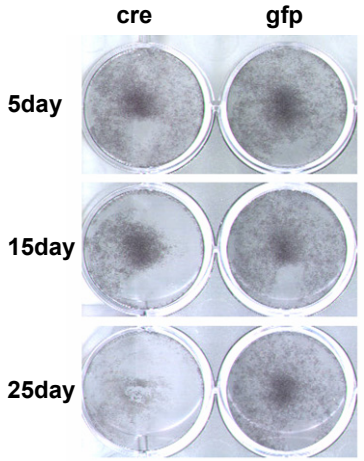

Figure S7_levy et al

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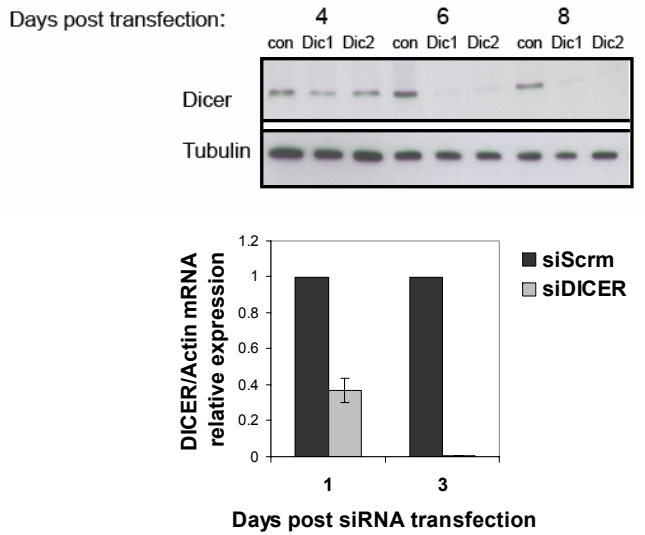


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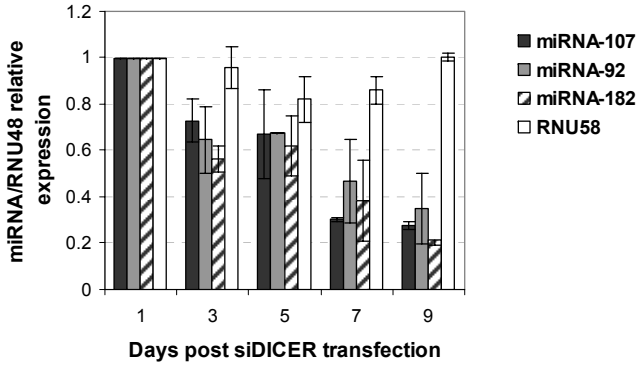


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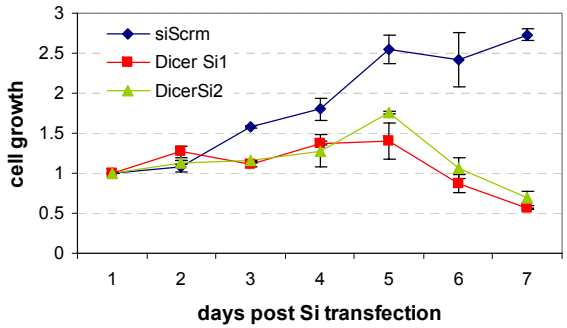


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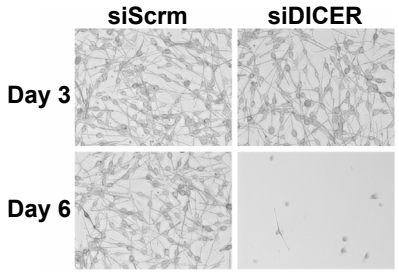

## ETOC paragraph

DICER is a central regulator of microRNA maturation. However little is known about mechanisms regulating its expression. Now, Levy et al. demonstrate that in differentiating melanocytes, a significant population of fully processed miRNAs is dependent upon stimulation of DICER expression—an event which occurs via direct transcriptional targeting of DICER by the melanocyte master transcriptional regulator MITF. Targeted knockout of DICER is lethal to melanocytes, at least partly via MITF-DICER-dependent processing of the pre-miRNA-17~92 cluster which targets the pro-apoptotic factor BIM. These observations highlight a central mechanism underlying miRNA regulation during lineage development.