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Loss of Dicer in newborn melanocytes leads to premature hair greying and changes in integrin expression.

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Abstract

Premature hair greying occurs due to the depletion of melanocyte stem cells (McSCs) in the hair follicle, which can be accelerated by stress caused by genetic or environmental factors. However, the connection between stress and McSC loss is not fully understood. MiRNAs are molecules that control gene expression by regulating mRNA stability and translation, and are produced by the enzyme Dicer, which is repressed under stress. Here, using two mouse genetic models and human and mouse cell lines, we found that the inactivation of Dicer in melanocytes leads to a misplacement of these cells within the hair follicle, resulting in a lack of melanin transfer to keratinocytes in the growing hair and the exhaustion of the McSC pool. We also show that miR-92b, which regulates *ItgaV* mRNA and protein levels, plays a role in altering melanocyte migration. Overall, our findings suggest that the Dicer-miR92b-ItgaV pathway serves as a major signalling pathway linking stress to premature hair greying.

INTRODUCTION

The cycle of hair follicles consists of three phases: growth (=anagen), destruction (=catagen), and rest (=telogen) (Fuchs 2009). For hair to be properly pigmented, there must be coordination between the proliferation and differentiation of keratinocytes and melanocytes during the anagen phase (Yardman-Frank and Fisher 2021). During each anagen phase, melanocyte stem cells (McSCs) located in the bulge region become active and multiply into transient amplifying cells (=TACs) (Sun et al. 2023). These TACs move along the growing hair follicle and develop into pigmented dendritic melanocytes in the bulb. The pigmentation process depends on the synthesis, transport, and transfer of melanin from melanocytes to keratinocytes that are committed to differentiating into hair-producing cells (Luciani et al. 2011). This intricate process is carefully regulated by both cell autonomous and non-cell autonomous signals such as Wnt, Edn, Tgf- β , and Notch, which control the timing of differentiation and quiescence (Chang et al. 2013; Moriyama et al. 2006; Nishimura et al. 2010; Rabbani et al. 2011). There is currently limited knowledge about how TACs migrate towards the new hair bulb. It is possible that TACs may simply follow the growth of the hair follicle passively, or they may actively migrate towards the hair bulb in a directed manner, or perhaps a combination of both mechanisms occurs.

The cycle of pigmented hair is carefully controlled by a combination of genetic factors and external signals, such as stress from the environment (Fialkowski et al. 2019; Harris et al. 2015). Dicer is an enzyme of the RNase III family that plays a critical role in the precise regulation of gene expression through its involvement in the maturation of microRNAs (= miRNAs) (Torrez et al. 2023). Both the activity and expression of Dicer are regulated by different types of stress, both *in vitro* and *in vivo*, across a variety of cell types, including trophoblasts, pre-adipocytes, and breast cells. For instance, *in vitro*, exposure to chemical and physical stressors like hydrogen peroxide, hypoxia, interferon α , phorbol ester, trichostatin A,

and UV radiation has been shown to reduce Dicer levels (van den Beucken et al. 2014; Mori et al. 2012; Wiesen and Tomasi 2009). *In vivo*, Dicer plays a role in mediating resilience to psychological stress by facilitating the pro-resilient effect of β -catenin in a mouse model of depression (Dias et al. 2014). Different types of stress can modulate Dicer levels and subsequently impact miRNA expression. For example, UV radiation controls the cellular stress response by regulating Dicer and various miRNAs (Dynoodt et al. 2013; Emde and Hornstein 2014).

miRNAs are generated in the nucleus by RNA polymerase II/III as stem-loop RNAs known as primary microRNAs (pri-miRs). The pri-miRs are then converted into precursor miRs and exported to the cytoplasm by Exportin-5. In the cytoplasm, Dicer, which belongs to the RNA-induced silencing loading complex (RISC RLC) complex, processes the precursor miRs into duplex miRs (Torrez et al. 2023). These duplex miRs are then processed by RISC, and mature miRNAs are loaded onto Argonaut proteins that recognize and bind to target mRNAs, leading to the inhibition of their expression by either blocking translation or causing mRNA degradation (Ha and Kim 2014). As a result, Dicer and miRNAs can regulate a vast number of genes. Dicer is crucial for mammalian embryonic development, and mice with germline Dicer inactivation die before E7.5 (Bernstein et al. 2003).

The loss of Dicer in the developing melanocyte lineage during embryonic development resulted in a white adult mouse lacking melanocytes in hair follicles (Levy et al. 2010). Similarly, the knockout of *Dicer1* in zebrafish also results in abnormal pigmentation with a reduced number of melanocytes in the head and ventral trunk (Weiner et al. 2019). The miRNA-17 cluster and BIM were identified as partially responsible for the lack of viability of primary human melanocytes in the absence of Dicer (Levy et al. 2010). Several miRNAs have been found to play a role in the regulation of pigmentation and melanin transport, while others have been associated with melanoma cell migration, EMT, progression, and invasion *in vitro*

(Ozsolak et al. 2008; Rambow et al. 2016; Rambow et al. 2015; Rambow et al. 2014). In this study, after evaluating the consequence of the lack of Dicer in mouse melanoblasts during embryonic development, we investigated the impact of *Dicer* inactivation on mouse melanocytes by specifically targeting *Dicer* at birth in the melanocyte lineage. Our results revealed that the absence of Dicer resulted in abnormal melanocyte localization in hair follicles and, premature greying of hair, due to the reduction of miR-92b and the increase of one of its targets, *ItgaV*.

RESULTS

Dicer conditional knockout during embryonic development reduces the number of melanoblasts.

To assess the effects of Dicer inactivation during the establishment of the melanocyte lineage, we conducted crosses between $Dicer^{Flox22-23}$ mice, which carry loxP sequences in introns 21 and 23, and $Tyr::Cre$ mice, and monitored the phenotype of the mutant mice as they aged (Supplementary Figure S1a). At birth, homozygous Dicer knockout mice ($Dicer$ -cKO, "c" for constitutive) were pigmented, but notably less than control littermates (WT), indicating that Dicer may affect the growth of melanoblasts or affect its differentiation (Supplementary Figure S1b-d). Heterozygous mutant mice had no coat color phenotype (not shown). At 2 months of age, $Dicer$ -cKO mice showed salt-and-pepper coloring on their back but were white on the belly (Supplementary Figure S1e1). By 6 months of age, $Dicer$ -cKO mice were completely white (Supplementary Figure S1f). Hair greying appears to be a gradual process that depends on the number of original melanocytes at birth, as there are more melanocytes on the back than on the belly (Luciani et al. 2011).

Previously, the inactivation of Dicer was performed using the same $Tyr::Cre$ mice and another Dicer allele: $Dicer^{Flox23}$ (Levy et al. 2010). For both Dicer alleles ($Dicer^{Flox23}$ and $Dicer^{Flox22-23}$), the RNase III domain of this enzyme is inactive (Harfe et al. 2005; Murchison et al. 2005). These studies demonstrated that mice gradually became fully white as they aged. However, it is important to note that there are some differences in the coat color phenotype between $Tyr::Cre; Dicer^{Flox23/Flox23}$ (Levy et al. 2010) and the $Tyr::Cre; Dicer^{Flox22-23/Flox22-23}$ mice used in this study. The $Dicer^{Flox23}$ mice “were virtually white from birth, except for the tip of the initial hairs on the dorsal trunk”. However, the $Dicer^{Flox22-23}$ mice were slightly pigmented at birth and then had salt-and-pepper hairs before becoming fully white. We investigated the

impact of Dicer inactivation on melanogenesis and melanin production by studying a mouse melanocyte cell line (Melan-a) and a human melanoma cell line (MNT-1). The knock-down of Dicer in both cell lines, resulted in a minor, yet significant increase in melanin production per cell, a slight reduction in the growth of the cells, but did not affect the mRNA levels of *Tyrp1*, *Dct*, and *Tyr* (Figure 1a-h). Further analysis revealed a significant decrease in the number of melanoblasts at E14.5 in the entire body of mutant embryos compared to wild-type (Figure 1g-j). Therefore, the overall reduction in pigmentation in the Dicer-cKO mutant pups could be attributed to a decrease in the number of melanocytes rather than a decrease in melanogenesis.

Dicer plays a crucial role in pigmentation renewal

To further investigate the role of Dicer in regulating pigmentation, we created spatio-temporal inducible *Tyr::CreER^{T2}; Dicer^{Flox22-23/Flox22-23}* mutant (Dicer-iKO) mice. Dicer was defloxed in the melanocyte lineage, including McSCs, TACs, and differentiated melanocytes (Mc), by Tamoxifen (=Tam) treatment at p1, p2, and p3. Defloxing was confirmed by PCR analysis of hair bulb DNA (Supplementary Figure S2a-c). There was no significant difference in coat color until p30 after Tam treatment of both wild-type (WT) and Dicer-iKO mice (Supplementary Figure S2d-f, Figure 2a). At p30, after the first hair cycle, the belly of Dicer-iKO mice was lighter than that of WT mice, but the color of the back was similar. At p60, after the second hair cycle, the belly of Dicer-iKO mice was even lighter, displaying salt-and-pepper hairs (Figure 2b-e). By p180, after the fifth hair cycle, the whitening of Dicer-iKO mice had significantly increased, with a higher number of white hairs than at p60. These results suggest that Dicer plays a direct role in pigmentation renewal and that its inactivation leads to gradual whitening of the hair coat.

The absence of Dicer in melanocytes leads to their improper localization in the hair follicle

We determined the location of WT and Dicer-iKO melanocytes in hair follicles at p60 by Immuno-histochemistry (IHC) using as a Dct marker. In WT mice, Dct-positive cells were found at the level of the bulge and bulb (Figure 3a). In Dicer-iKO mice, most hairs were black and Dct-positive cells were found at the bulge and bulb but can be mislocated as shown in Figure 3b. The majority of Dicer-iKO ventral hairs were white at this age; either the Dct-positive cells were poorly mislocated (Figure 3c-e) or they were absent (Figure 3f). These observations suggest that in the absence of Dicer, melanocytes may undergo cell death as previously shown by Levy and colleagues (Levy et al. 2010) and/or melanocytes have a migratory defect.

Dicer knock down affects the level of integrins

To gain a better understanding of the effects caused by a reduction of Dicer in melanocytes, we knocked-down Dicer using specific siRNAs in normal C57BL/6 mouse melanocyte Melan-a cells. This resulted in a decrease of Dicer protein levels to approximately 40% compared to the control siScramble (siScr) after 24 hours of transfection (Figure 4a,b). Microarray analysis was used to examine gene expression, revealing that 280 genes were deregulated in siDicer cells compared to siScr (Figure 4c,d and Supplementary Table S1). Of these genes, 200 were upregulated and 80 were downregulated ($p\text{-value} \leq 0.05$ and $FC \geq 1.5$), which is consistent with decreased miR maturation and increased levels of their target RNAs. The top 20 most significantly enriched GO terms showed that seven were associated with genes related to "membrane", "cell surface", "cell-matrix adhesion", "cell migration", "focal adhesion", "integrin mediated signaling pathway", and "integrin complex", suggesting that Dicer regulates Melan-a cell adhesion and migration through integrins (Figure 4e). KEGG and REACTOME pathways of the deregulated genes revealed enrichment in functions associated with integrins, "ECM receptor interaction", "Focal adhesion", "Regulation of actin cytoskeleton", "Cell

adhesion molecules", "Integrin", and "ECM proteoglycans" (Figure 4f,g). The volcano plot displayed upregulation of several mRNA integrins, including *Itgav*, *Itga8*, *Itga9*, *Itgb5* along with the expected downregulation of Dicer (Figure 4d). We confirmed the increased levels of *Itgav*, *Itga8*, *Itga9*, and *Itgb5* after transfection of Melan-a cells with siDicer (Figure 4h,i, and Supplementary Figure S3a,b). *In vivo*, the amount of integrin α V and β 5 in Dct-positive cells appeared to be higher in Dicer-iKO melanocytes compared to WT melanocytes (Figure 4j-m).

Dicer regulates the level of *ItgaV* through miR-92b

To dissect the molecular mechanisms by which Dicer regulates integrin expression and melanocyte migration *in vitro*, we performed a miRNome analysis by microarray on Melan-a cells transiently transfected with siDicer or siScr (Figure 5a, Supplementary Table S2). Twenty-four and 48 hours after transfection, 151 and 189 miRs were differentially expressed with $p \leq 0.05$, respectively. After pooling the 24- and 48-hour data, a total of 327 miRs were differentially expressed. Amongst these, five are putative regulators of *ItgaV*, according to MirTarBase and miRnome: miR-92b-3p, miR-16-5p, miR-195a-5p, miR-322-5p and miR-761 (Figure 5b).

Transient transfection of Melan-a cells with miR-92b-3p mimics allowed the validation of the decreased of *ItgaV* mRNA and protein levels by RT qPCR and western blot analyses 48 hours after transfection (Figure 5c,d). A miRglo experiment confirmed that miR-92b-3p reduced the stability of luciferase mRNA when fused to the *ItgaV* 3'-UTR sequence, but not when the putative targeted sequence was mutated (Figure 5e). Thus, miR-92b-3p specifically targets *ItgaV* in mouse melanocytes.

Dicer regulates melanocyte migration through miR-92b/*ItgaV*

We investigated the role of ItgaV, Dicer and miR-92b in regulating melanocyte migration by analysing single-cell migration on non-coated plastic dishes. In Melan-a cells, the reduction of ItgaV with siRNA reduced cell velocity (Figure 5f,g) and the reduction of Dicer, with siRNA, or the antagomir for miR-92b-3p increased cell velocity and induced ItgaV level (Figure 5h-j). Altogether, these results identify the Dicer-miR92b-ItgaV axis as a signalling pathway affecting melanocyte migration.

DISCUSSION

This study used conditional and inducible mouse mutants to demonstrate that Dicer is essential for hair pigmentation in adult mice by regulating melanocyte positioning and renewal, leading to the maintenance of melanocyte stem cells (McSC) in hair bulges. In particular, reduced Dicer levels altered miR-92b and α V integrin levels, which regulate melanocyte migration. Integrin α V β 5 contributes to proper melanocyte migration along hair follicles and that melanocytes may actively migrate along the growing hair follicle using their integrins repertoire. Dicer inactivation also induced McSC loss, possibly due to premature differentiation, death, and/or exhaustion.

During the hair cycle, keratinocyte and melanocyte stem cells exit quiescence together, in response to Wnt, Bmp/Tgf- β , and Edn (Chang et al. 2013). As keratinocytes form hair follicles, TACs migrate to the hair bulb and differentiate into melanocytes. While proteins involved in cell adhesion, cytoskeleton remodeling, and cellular traction have been studied for melanocyte migration (Hamidi and Ivaska 2018; Petit and Larue 2016; Pinon and Wehrle-Haller 2011), little is known about TAC migration during the hair cycle. We propose that the expression of integrin α V β 5, which mediates cell adhesion to the extracellular matrix, affects TAC migration *in vivo* due to the diverse ECM components in various parts of the hair follicle (Couchman and Gibson 1985; Fujiwara et al. 2011; Jahoda et al. 1992; Tanimura et al. 2011).

The role of integrins is of a particular importance because we know that the mobility and plasticity of McSCs is a key element of melanocytes renewal (Sun et al. 2023). TAC migration differs between mice and humans, with mice showing migration primarily to the bulb and humans to both the apex and bulb. This suggests that TACs undergo two directed migrations, which may be cell-autonomous or non-autonomous. In mice lacking Dicer, integrin levels are altered, resulting in increased melanocytes at the hair's apex. It is possible that

differences in integrin expression between humans and mice contribute to the bidirectional movement of TACs observed in humans.

The salt-and-pepper phenotype observed in all mutant mice progressed over time, with an increase in the proportion of white hairs, which is unlikely due to inefficient Dicer inactivation, as we demonstrated the high efficiency of TAM induction at birth using Tyr::CreER^{T2} mice (Le Coz et al. 2021; Yajima et al. 2006). Premature hair greying in mice can be attributed to three potential causes: premature differentiation, cell death, or exhaustion of melanocyte stem cells (McSCs). The premature differentiation of McSC in the bulge was reported in mouse mutants lacking Notch1/2, Rbp-j, TgfbRII, β -catenin, or Col17a1 (Aubin-Houzelstein et al. 2008; Moriyama et al. 2006; Nishimura et al. 2010; Rabbani et al. 2011; Tanimura et al. 2011). McSCs cell death was observed in the absence of Bcl2 and Dicer (Levy et al. 2010; Nishimura et al. 2005), while McSC exhaustion was observed in the absence of TgfbRII (Koludrovic et al. 2015; Nishimura et al. 2010). The absence of Dicer resulted in mislocated pigmented Dct-positive cells and premature differentiation (Figure 3). It is possible that, in the absence of Dicer, McSC die in a cell autonomous way and the combination of mislocation and premature differentiation/death exhausts McSCs. Abnormal localization of melanocytes and loss of McSCs may or may not be linked. On one hand, if the two events occur independently, Dicer/miR/Itg-dependent melanocyte migration explains their abnormal localization, and death is only associated with apoptosis as it was shown by Levy et al. 2010. On the other hand, both events may occur in a coordinated fashion. As the number of Mc localized in the hair bulb is lower than normal, we can imagine the existence of a feedback mechanism by which TAC/Mc signal McSCs to regulate their proliferation. In the Dicer mutant, where the number of TAC/Mc is lower, this feedback signaling is diminished, and the McSCs undergo excessive proliferation to compensate, and are therefore prematurely depleted. This second possibility does not mean that apoptosis does not occur. In all cKO-Dicer and iKO-

Dicer mice, the whitening of the fur is occurring faster on the ventral side than on the dorsal side (Supplementary Figure S1 and Figure 2). This occurrence can be attributed to a lower presence of melanoblasts in the ventral region of the embryo, resulting in fewer melanocytes and McSC in that area. This suggests that the exhaustion of McSC happens more swiftly on the ventral side due to its initially lower abundance.

The transcriptome analysis of Dicer knockdown Melan-a cells demonstrated deregulation of multiple genes involved in cell adhesion and migration, consistent with *in vivo* observations. Integrins αV , $\alpha 8$, $\alpha 9$, and $\beta 5$ (*Itgav*, *Itga8*, *Itga9*, and *Itgb5*) were among the upregulated genes at the RNA level, and their induction was confirmed in Dicer-iKO melanocytes *in vivo* and Dicer knockdown Melan-a cells *in vitro*. MiR-92b-3p was identified as a strong repressor of *ItgaV*. In humans MiR-92b-3p is known to decrease *ITGAV* and repress migration and invasion of esophageal squamous cell carcinoma cells *in vitro* in another context (Ma et al. 2011). However, the role of MiR-92b in proliferation is not clear, it promotes proliferation during pre-eclampsia (Li et al. 2020) but paradoxically inhibits proliferation and promotes apoptosis in glioma cells (Song et al. 2016). Moreover, performing similar analysis, we could show that (i) miR-155-5p decreased *Itgb5* mRNA level, but not those of *Itga8*, *Itga9*, or *ItgaV*, (ii) miR-466i-5p decreased *Itga9* mRNA levels 48 and 72 hours after transfection, but not those of *ItgaV*, *Itga8*, or *Itgb5*, and (iii) *let7b-5p* decreased *Itga8* mRNA level.

Knocking down Dicer in Melan-a cells led to changes in the expression of several genes involved in melanocyte migration, including integrins and ECM components such as *Npnt* and *Col6a1* (Supplementary Table S1). *Kit* is also one of the most up-regulated genes. *Kit* is well known to be involved in melanoblast migration during embryogenesis (Jordan and Jackson 2000; Nocka et al. 1990), is a prerequisite for melanoblast migration in hair at birth (Peters et al. 2002), and is involved in melanoma cell migration (Bernex et al. 1996). Furthermore, while

Kit is described as participating in the regulation of McSC differentiation (Chang et al. 2013), its involvement in the migration of normal adult melanocytes has never been demonstrated."

Previous studies have demonstrated the expression of several integrins in the melanocyte lineage but not Itga8, Itga9 and Itgb5. In the future, the role of Itga8 and Itga9 in the melanocyte lineage should be looked at more carefully, and especially after stress in various diseases that includes vitiligo. However, the level of these two integrins remain low compared to ItgaV and Itgb5 in melan-A cells (see supplementary Figure S3c). Melanoblasts and melanoma cells rely on integrins for their migration. Melanoblasts migrate along the dorso-lateral axis and tightly regulate the usage of various integrins, including ItgaV, to establish the melanocyte lineage (Beauvais-Jouneau et al. 1999; Haage and Tanentzapf 2023). We were expecting that the migration of melanoblasts during embryogenesis would be enhanced since integrins and Kit, to the least, were induced in the absence of Dicer in Melan-a cells. The analysis of the location of melanoblasts in WT and cKO embryos at E14.5 revealed that proportionally there was slightly more melanoblasts in the ventral part in cKO embryos, but it was not significant.

In vivo, it has been shown that melanocytes and melanoblasts express $\alpha 3\beta 1$, $\alpha 6\beta 1$, and $\alpha \text{v}\beta 3$ (Beauvais-Jouneau et al. 1999; Haass et al. 2005). In culture, melanocytes produce $\alpha 3\beta 1$, $\alpha 5\beta 1$, $\alpha 6\beta 1$, and $\alpha \text{v}\beta 3$ (Hara et al. 1994; Scott et al. 1994). Additionally, melanoma cell lines and melanoma exhibit the expression of $\alpha 5\beta 1$, $\alpha 6\beta 1$, $\alpha 5\beta 1$, $\alpha 7\beta 1$, and $\alpha \text{v}\beta 3$ (Haass et al. 2005; Pinon and Wehrle-Haller 2011). Melanoma is the most aggressive form of skin cancer and is known for its rapid metastasis. Integrins play a direct role in various cellular processes involved in melanoma progression (Buommino et al. 2009; Voura et al. 2001). The regulation of integrin activity depends on factors such as mRNA and protein levels, as well as the abundance of specific α - β dimers. In cancer, miRs have been shown to regulate integrin levels, but it remains unknown whether miR-92 can regulate ITGAV (Verhoeff et al. 2023). Our analysis of TCGA

data revealed significantly higher *ITGAV* mRNA levels in melanoma metastasis compared to primary melanoma (Supplementary Figure S4a). Additionally, we observed an inverse relationship between *ITGAV* mRNA and miR-92 in various human melanoma cells (Supplementary Figure S4b). These findings suggest that miR-92 may play a crucial role in the regulation of *ITGAV* during homeostasis and the proper movement of TAC/melanocytes in the hair follicle.

In summary, our study reveals to our knowledge previously unreported pathway and mechanism for hair greying that involves stress-induced reduction in Dicer levels and dysregulation of integrins, which are involved in the directed migration of TACs through their interaction with the extracellular matrix. This suggests a retro-control mechanism for the bulge cycle, and highlights the link between stress, Dicer, integrins and hair greying.

Materials and Methods

Detailed materials and methods can be found in the supplemental information.

Animal Models.

Transgenic mice used in this study were previously described (Murchison et al. 2005)(MacKenzie et al. 1997)(Delmas et al. 2003; Yajima et al. 2006).

All animals were housed in a specific pathogen-free mouse colony at the Institut Curie. Animal care, use, and experimental procedures were conducted in accordance with recommendations of the European Community (86/609/EEC) and Union (2010/63/UE) and the French National Committee (87/848). Animal care and use were approved by the ethics committee of the Curie Institute in compliance with the institutional guidelines.

Embryo X-gal staining. E14.5 embryos were X-gal stained and imaged as previously described (Berlin et al. 2012).

Tamoxifen administration. Tamoxifen treatment was performed following a previously published protocol (Pitulescu et al. 2010).

Verification of defloxing by PCR. Primers and programs are described in Supplementary Tables S3,S4.

Quantification of melanin levels. Melanin was quantified in mouse hairs following a previously published protocol (Berlin et al. 2012)(Gallagher et al. 2011).

Tissue processing, immunostaining, and microscopic imaging. Tissues were fixed, stained and visualized as previously described (Berlin et al. 2012).

Cell culture, transfection, and treatments. Plasmids and sRNA (siRNA, mimics, and hairpin inhibitors) used for transfection in Melan-a cells line (Bennett et al. 1987) are described in Supplementary Table S5).

Western blotting. Cells were lysed into RIPA buffer. Twenty μ g total protein was separated by SDS-PAGE, transferred to nitrocellulose membranes, and probed with various antibodies.

Single-cell migration assay. Forty-eight hours after transfection, cells were transfected 48 hours prior, trypsinization and seeded for 5h at low concentration prior imaging cells every 4 min for 12 h. Tracking of the cells and path analysis was performed using iTrack4U software (Cordelieres et al. 2013).

Quantitative real-time PCR. After preparing and quantification of RNA, real-time PCR was performed using primers listed in Supplementary Table S3. Mouse Gapdh housekeeping gene and the internal control using $\Delta\Delta C_t$ methods.

mRNA microarray. Sample preparation and hybridization were performed by the Genomic platform of the Institut Curie. Affymetrix Mouse ClariomD arrays were hybridized according to the recommendations of Affymetrix (Santa Clara, CA, United States) using the WT PLUS protocol and labelling and hybridization kits from Affymetrix.

microRNA microarray. Sample preparation and hybridization were performed by the Genomic platform of the Institut Curie. The flashTag Biotin HSR RNA labelling kit (Affymetrix) was used to label 100 ng total RNA according to the manufacturer's protocol.

Volcano plot methods. RNA-seq counts and phenotypic traits of the cohort: GDC TCGA Melanoma (SKCM), were downloaded from the UCSC's Xena Browser at <https://xenabrowser.net>. Analysis was performed as previously described (Petit et al. 2019)

pmirGLO luciferase assay. Melan-a cells were transfected with 50 nM miR-92b-3p mimic (Dharmacon), 250 ng various pmirGLO Dual-Luciferase miRNA target expression vector constructs (Promega), and 250 ng TK::Renilla (Promega). The negative control, without insert, did not contain any matching miR-92b-3p sequence. Details can be found in supplementary information.

Statistics and reproducibility. Quantitative data were performed at least in triplicate for analysis and expressed as mean \pm SEM or mean \pm SD as indicated in the figure legends. The exact number of replicates, statistical test and significance are reported in the figure legends. Differences between two groups were assayed using unpaired Student's t-test in Prism (Graphpad software).

Data availability. Datasets related to this article can be found at <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE128757>. The accession number for the mRNA and miRNA data reported in this paper is GEO: GSE128757.

Journal Pre-proof

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CRedit

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Conflict of interest statement

We declare that we have no conflict of interest

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Figure Legends

Figure 1. Absence of Dicer reduces melanoblast numbers without altering melanogenesis gene levels.

Western blot analysis (a,e) showed siRNA-mediated Dicer knockdown in Melan-a (a-d) and MNT-1 (e-h) cells. The melanin content (b,f) melanogenesis gene RNA level (c,g) and cell number evaluation (d,h) analyses were performed as previously described (Gallagher et al. 2011). Scr = scramble. The number of melanoblasts was decreased in E14.5 Dicer-cKO ($=Tyr::Cre^{flox}$; $Dicer^{flox22-23/flox22-23}$) mice compared to WT controls (i-l). The number of Dct::LacZ^o WT and mutant head and truncal melanoblasts was determined after beta-galactosidase staining (Berlin et al. 2012). Each experiment was repeated at least three times, and statistical analysis was performed using t-test with ns indicating not significant, * $p < 0.05$, and ** $p < 0.01$.

Figure 2. The knockout of Dicer at birth induces a salt-and-pepper phenotype.

(a) Ventral and dorsal views of Dicer-iKO ($=Tyr::CreER^{T2}$; $Dicer^{flox22-23/flox22-23}$) and littermate WT controls. Melanin amount in Dicer-iKO and WT hairs from the dorsal (b) and ventral (d) parts at p60. No difference was observed between females (pink) and males (blue). $n = 5$ to 7 . t-test analysis: *** $p < 10^{-3}$, ** $p < 10^{-2}$. Black and white hairs on the back (c) and belly (e) of Dicer-iKO and WT mice from the same litter. Relative abundance of black and white hairs on a representative pair of mice is given. Scale bar: $20 \mu\text{m}$.

Figure 3. Abnormal location of melanocytes in Dicer-iKO hairs.

Following hair growth synchronization, $10\text{-}\mu\text{m}$ skin sections of p60 Dicer-iKO and WT were stained for Dct (red, highlighted by white arrows) and DAPI (blue). Twenty-six hairs from three

mutants and 25 hairs from three wild-type mice were followed. Among the wild-type hairs, all 25 were pigmented, and 24 exhibited a normal distribution pattern with the profile a; Dct+ cells are located in the dermal papilla of the bulb (Bb) and in the bulge (Bg). From the mutant hairs, six were pigmented and twenty were not pigmented. The pigmented hairs had two profiles: two had the profile a, and four had the profile b. The profile b corresponds to hairs with Dct+ cells in the bulb and some with abnormal localization. The not pigmented had four profiles: four hairs had the profile c (without Dct+ cells in the bulb but with Dct+ in the apex of the hair), six the profile d (below and on the side of the bulb), four the profile e (below the bulb only), and six the profile f (without any Dct+ cells were observed). Hair follicles were schematically represented by black and white rectangles for pigmented and non-pigmented hairs, respectively, and a black diamond for melanocytes (Mc). Scale bar: 25 μm .

Figure 4. ItgaV is induced in cells knocked down or knockout for Dicer

Melan-a cells were subjected to Dicer knockdown (n=3), and subsequent analyses were performed, including Western blot (a), RT-qPCR (b), and transcriptomic (c-g). A heatmap (c) and a volcano plot (d) show genes differentially expressed between siDicer and siScr conditions with $p \leq 0.05$ and fold-change ≥ 1.5 . In (d) upregulated and downregulated genes are shown as red and green dots. Gene ontology (e), KEGG (f), and REACTOME-pathway (g) analyses reveal an enrichment of pathways involved in cell adhesion and cell-matrix interactions (red). Validation of the upregulation of integrin mRNA and protein levels by RT-qPCR (h) and western blot (i) analyses, respectively, 24 and 48 h after transfection with siDicer or siScr. n = 4. t-test analysis: **** $p \leq 10^{-4}$, ** $p \leq 10^{-2}$, * $p \leq 5 \cdot 10^{-2}$. au: arbitrary unit. (j-m) Ten-week-old Dicer-iKO and WT mice from the same litter were depilated on the left side of the back, and a 0.25cm² biopsy sampled 10 days later from the depilated area. Dct (red) and ItgaV (green) co-staining (j,k) and Dct (red) and Itgb5 (green) co-staining (l,m) were performed on 10- μm skin

sections. In Dicer-iKO, all Dct-positive cells displayed an increase of ItgaV or Itgb5. Scale bars: 10 μ m.

Figure 5. ItgaV is targeted by miR-92b-3p.

Melan-a cells were transfected with siRNA Dicer (si-Dicer) or control siRNA (si-Scr) prior miRnome analysis (a,b), with mouse mimic of miR-92b-3p (92b-3p) (+) or a mimic (-) control in the absence prior RT-qPCR (c) and western blot analysis (d) or presence of a miRgluciferase construct (e), with si-ItgaV or siScr prior migration analysis (f) and western blot analysis (g), and with si-Dicer, si-Scr (-), mimic of miR-92b or a mimic control (-) prior migration analysis (h) and western blot (i) and mRNA (j) analyses. (a) Heatmap presenting the differentially expressed miR between both conditions at 24 and 48h with $p \leq 0.05$ for a total of 327 miR. b) Venn diagram comparing the miRNA from miRnome to the mirTarBase list of putative miRNA that target ItgaV. Vinc =Vinculin. Three biological replicates were used for each condition. t-test analysis: **** $p \leq 10^{-4}$, *** $p \leq 10^{-3}$, ** $p \leq 10^{-2}$, * $p \leq 5.10^{-2}$, ns: non-significant. r.u. = relative unit.

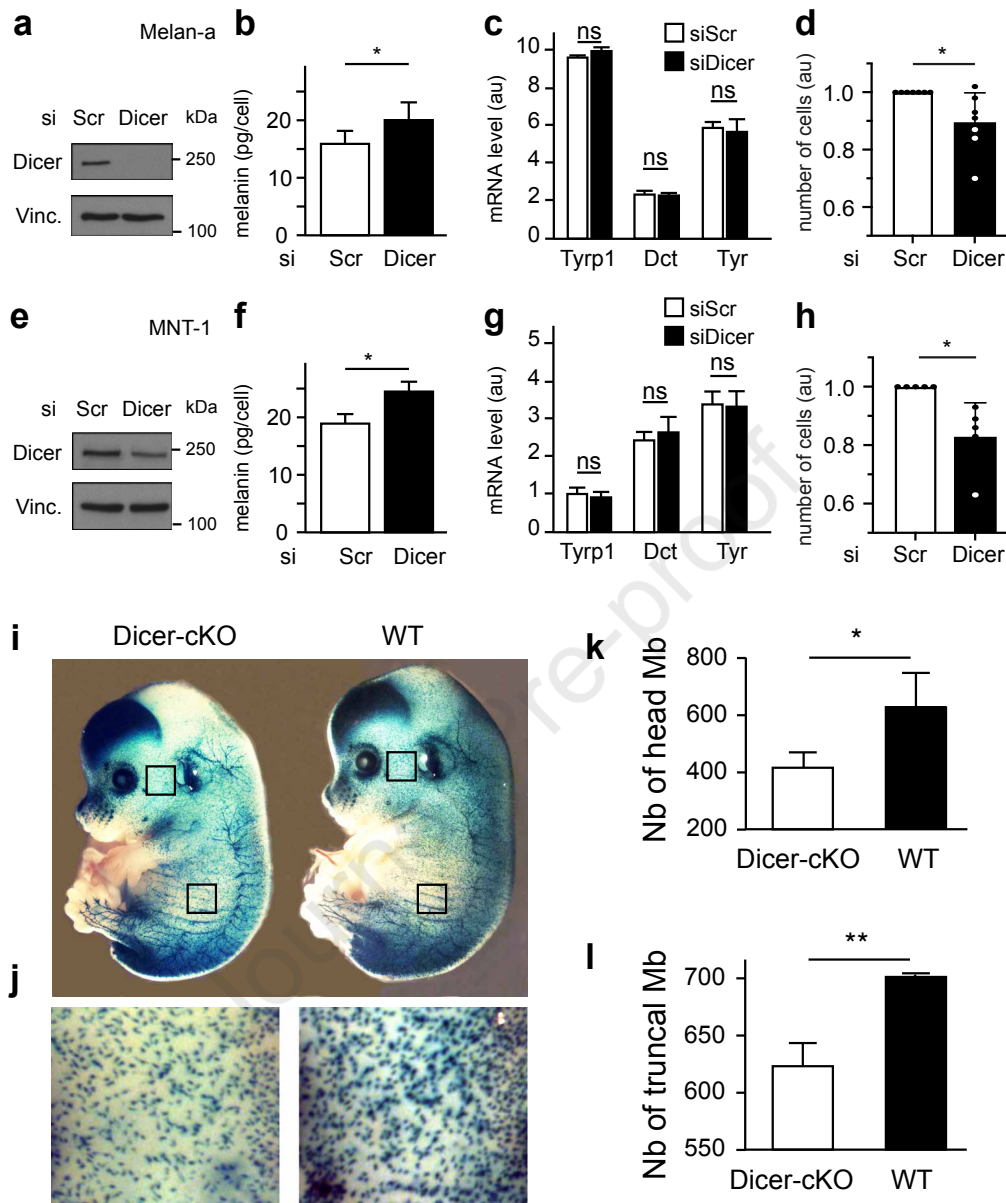


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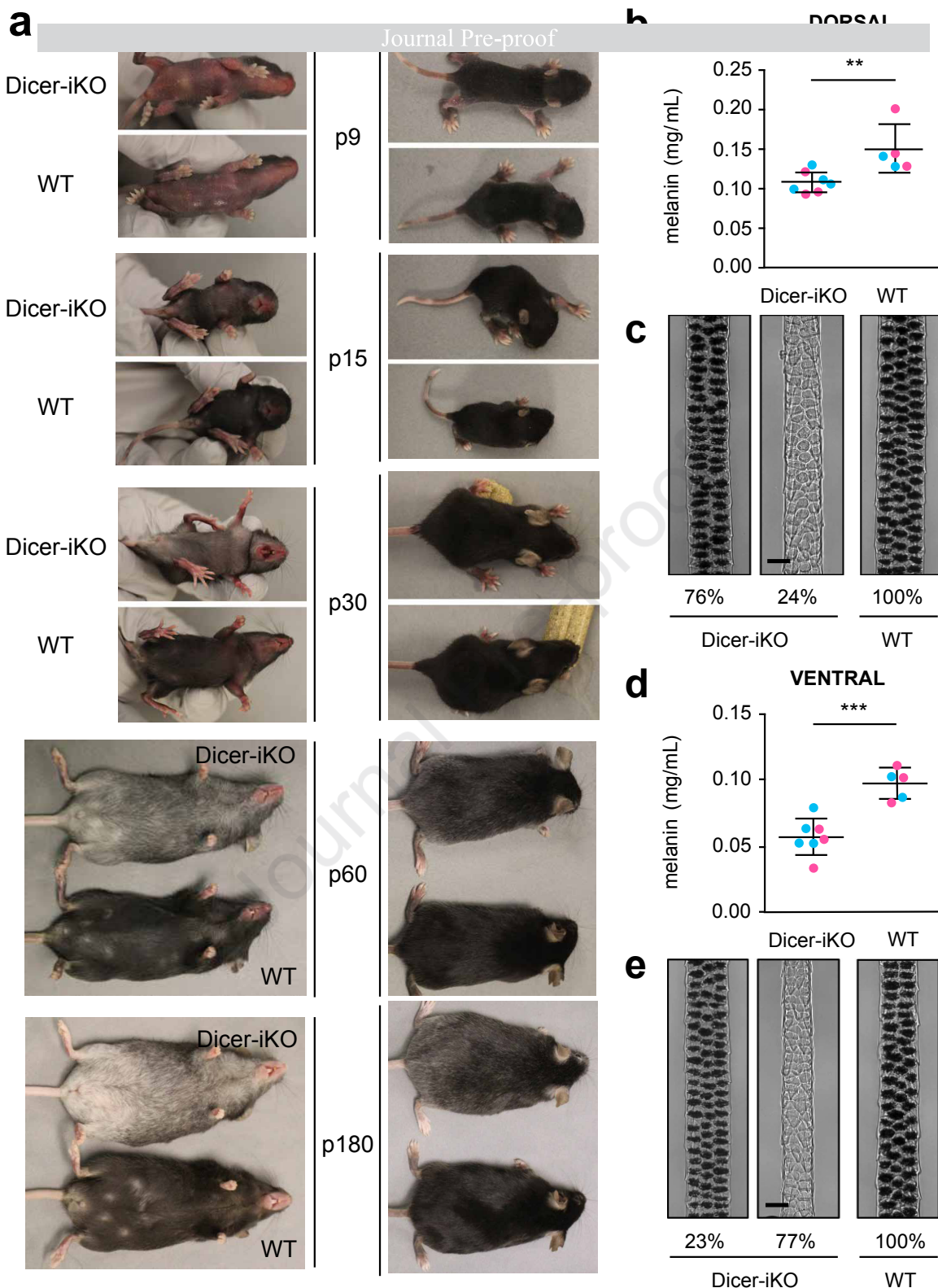


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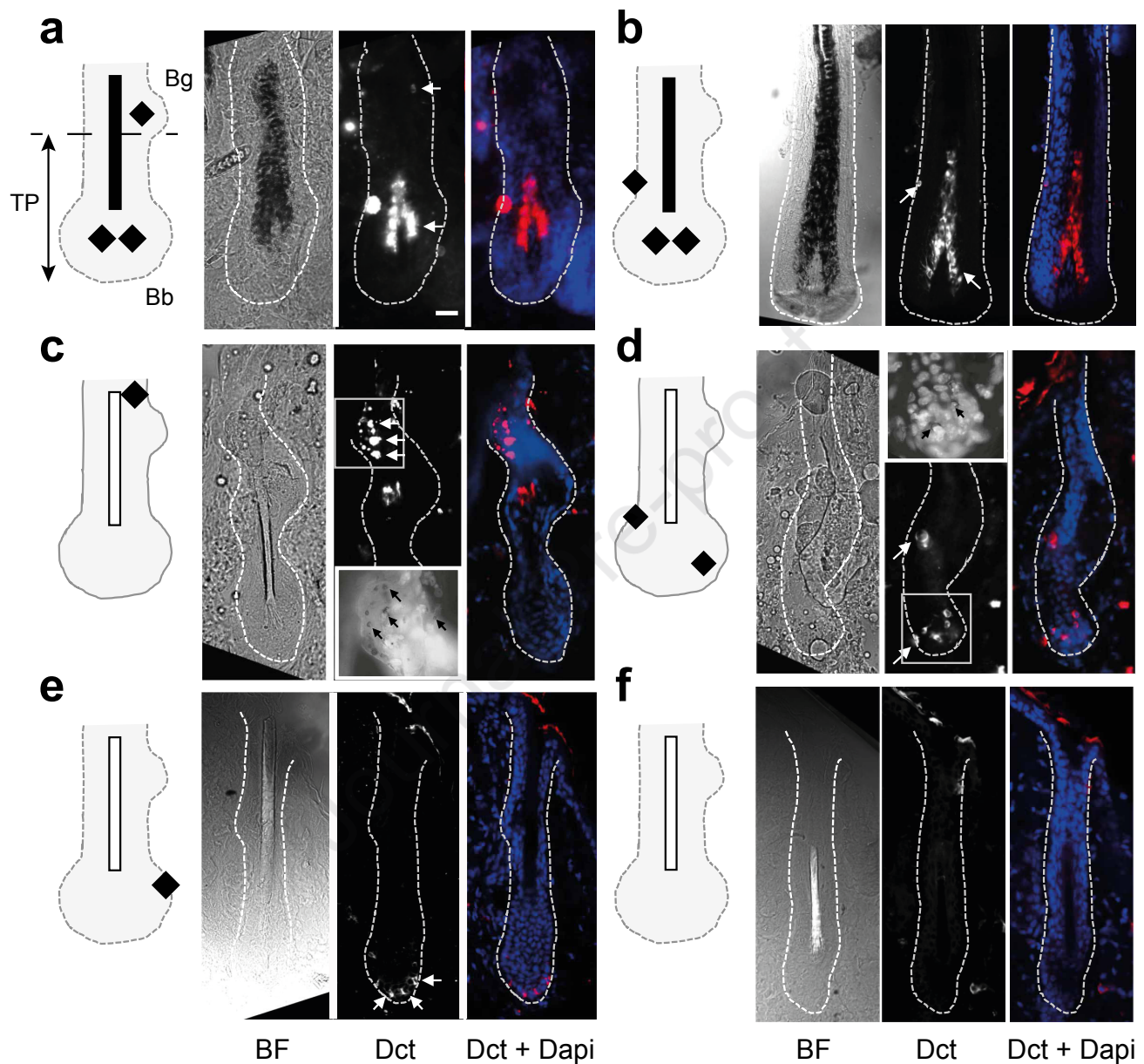


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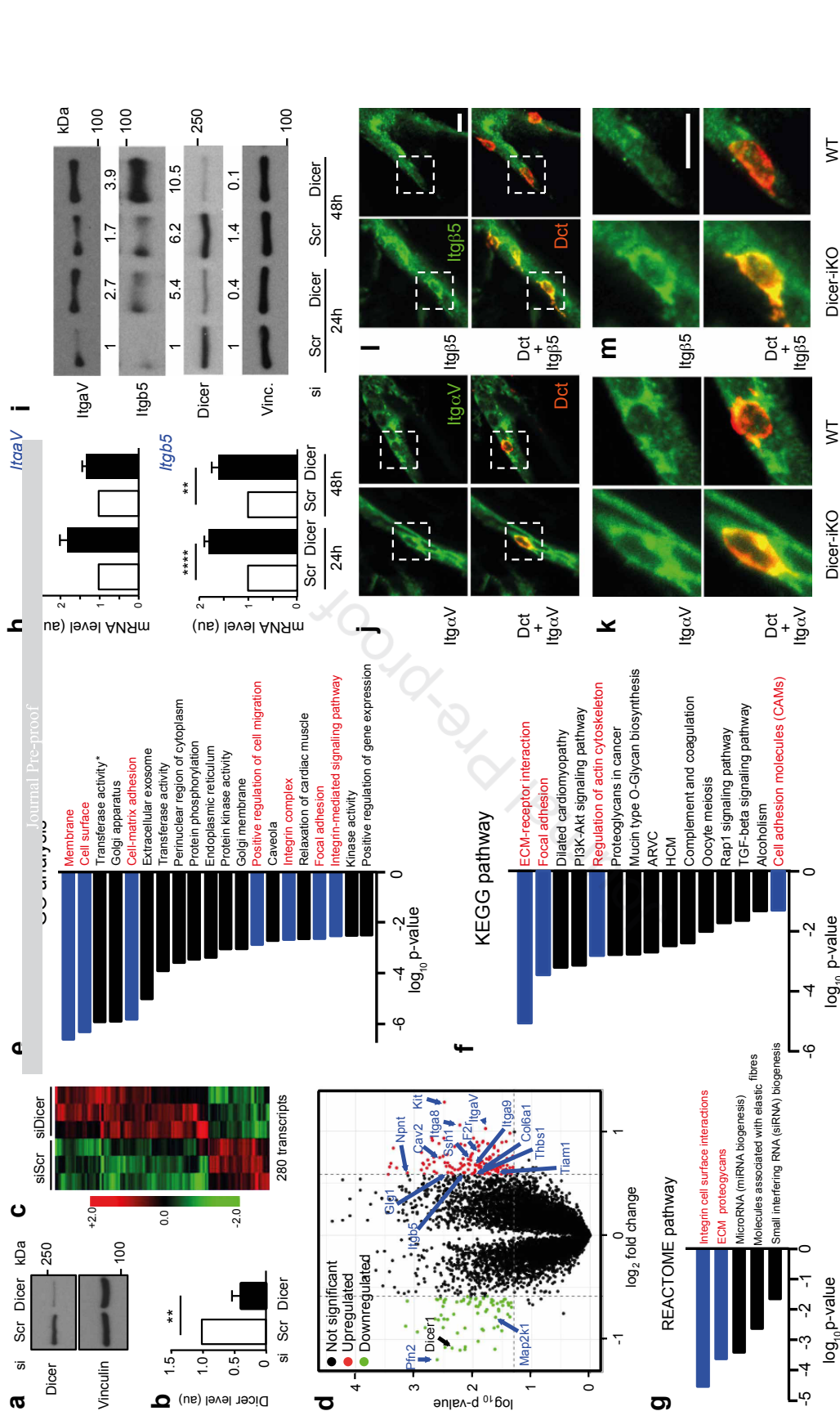


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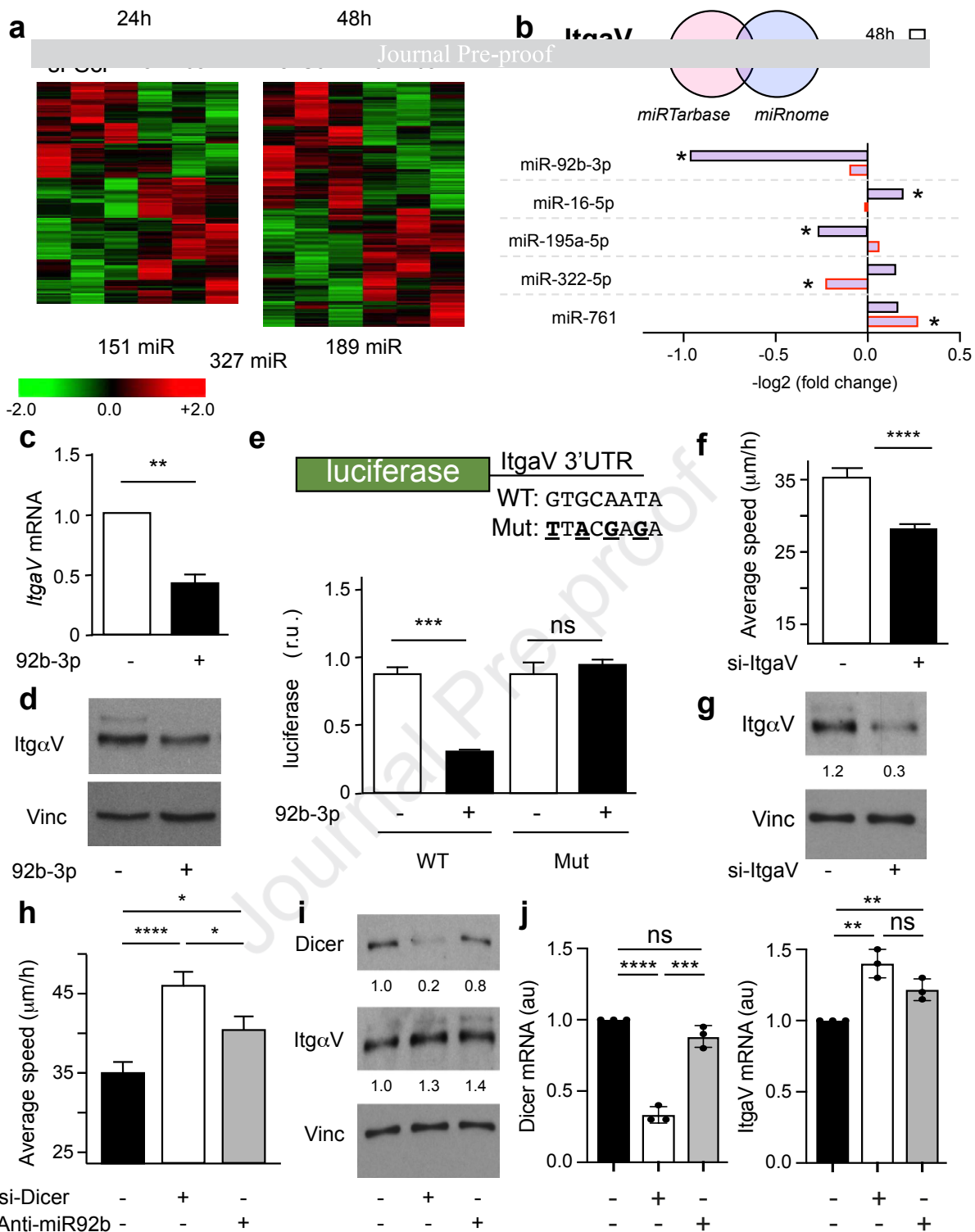


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