**An imbalance between proliferation and differentiation underlies the development of microRNA-defective pineoblastoma**

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**ABSTRACT**

Mutations in the microRNA processing genes *DICER1* and *DROSHA* drive several cancers that resemble embryonic progenitors. To understand how microRNAs regulate tumorigenesis, we ablated *Drosha* or *Dicer1* in the developing pineal gland to emulate the pathogenesis of pineoblastoma, a brain tumor that resembles undifferentiated precursors of the pineal gland. Accordingly, these mice develop pineal tumors marked by loss of microRNAs, including the let-7/miR-98-5p family, and de-repression of microRNA target genes. Pineal tumors driven by loss of *Drosha* or *Dicer1* mimic tumors driven by *Rb1* loss, as they exhibit upregulation of S-phase genes and homeobox transcription factors that regulate pineal development. Blocking proliferation of these tumors facilitates expression of pinealocyte maturation markers, with a concomitant reduction in embryonic markers. Select embryonic markers remain elevated, however, as the microRNAs that normally repress these target genes remain absent. One such microRNA target gene is the oncofetal transcription factor *Plagl2*, which regulates expression of pro-growth genes, and inhibiting their signaling impairs tumor growth. Thus, we demonstrate that tumors driven by loss of microRNA processing may be therapeutically targeted by inhibiting downstream drivers of proliferation.

## INTRODUCTION

Mutations in microRNA processing genes, such as *DROSHA*, *DICER1*, and *DGCR8*, drive ~2% of childhood cancers [1, 2]. These mutations arise in a family of cancers that resemble embryonic progenitors, including Wilms tumor, pleuropulmonary blastoma, rhabdomyosarcoma, and the brain tumor pineoblastoma [3-11]. It is thought that loss of microRNA production leads to de-repression of the genes that are normally repressed by these microRNAs, leading to tumor formation [12, 13]. However, microRNA loss leads to transcriptome-wide changes, with different effects in each cell type, and it is challenging to separate genes upregulated through loss of direct microRNA targeting from secondary effects. In addition, little is known about the specific microRNAs or microRNA target genes that regulate the balance between proliferation and differentiation in each cancer type. As a result, there is currently no way to therapeutically target these mutations.

Pineoblastoma is a rare brain tumor that resembles embryonic neurogenic progenitors of the pineal gland, and it is treated with chemotherapy, surgery, and radiation [14]. However, the morbidities associated with each of these treatment modalities limits their utility; cranial radiation therapy, for instance, is generally avoided in young children. Most other *DICER1/DROSHA*-related tumors, which exhibit point mutations in these genes that incompletely impair microRNA production, but pineoblastoma can uniquely exhibit complete loss of *DROSHA* or *DICER1*, leading to complete loss of microRNA production [11, 15]. Specifically, recent molecular studies have identified three mutational patterns in pineoblastoma: (1) bi-allelic loss of *DROSHA*, *DICER1*, or *DGCR8*; (2) bi-allelic loss of *RB1*; or (3) activation of *MYC* or *FOXR2* [16-19]. The RB1 tumor suppressor normally regulates E2F transcription factors to allow progression through the G1/S cell cycle checkpoint [20], and MYC orchestrates tumorigenesis by binding “E-box” motifs in promoters or enhancers of tumor-promoting genes [21]. However, the microRNAs and microRNA target genes that mediate tumorigenesis in tumors driven by loss of *DROSHA* or *DICER1* remain poorly understood.

Previous studies have used *Rb1* loss, cyclin D1 upregulation, and *Dicer1* loss to model the development of pineoblastoma in mice [22-27]. To dissect how microRNA loss drives formation of pineoblastoma, we generated mice with loss of microRNAs in the developing pineal gland. Here we show that loss of *Drosha* or *Dicer1* mimics loss of the *Rb1* tumor suppressor by driving cell cycle progression through de-repression of cyclin D2 (*Ccnd2*). Uncontrolled proliferation itself restrains tumor cells in an undifferentiated state, and restraining proliferation

leads them to upregulate differentiation markers. In addition to being directly targeted by microRNAs, we find that *Ccnd2* is also indirectly regulated by microRNAs through the transcription factor *Plagl2,* and blocking other Plagl2 target genes also slows tumor growth. Lastly, we show that human pineoblastoma tumors exhibit parallel expression patterns.

## RESULTS

*Pineal tumors driven by loss of Drosha, Dicer1, or Rb1 resemble embryonic pineal progenitors*

Prior studies had demonstrated that mice develop pineoblastoma upon pineal-directed ablation of *Rb1* and *p53* (*IrbpCre*;*p53*$^{flox/flox}$;*Rb1*$^{flox/flox}$, hereafter abbreviated as IPRb1) [27]. These studies used a Cre recombinase line driven by the promoter of interphotoreceptor retinoid binding protein (*Irbp*, also known as *Rbp3*), which is expressed in retinal and pineal progenitors [28, 29]. To understand how microRNAs regulate pineal tumor formation, we developed mice with analogous loss of *Drosha* and *p53* (*IrbpCre*;*p53*$^{flox/flox}$;*Drosha*$^{flox/flox}$, abbreviated as IPDrosha) (**Fig. 1A,B**). These mice were healthy at birth, but at 5-10 months of age, they began to exhibit signs of brain tumor formation. Within days of developing an enlarged skull, they become moribund (**Suppl. Fig. S1A**). Similarly, pineal tumors also arise upon conditionally deleting *Dicer1* and *p53* (*IrbpCre*;*p53*$^{flox/flox}$;*Dicer1*$^{flox/flox}$, or IPDicer1) (**Fig. 1A,B**). By microscopic evaluation, IPDrosha, IPDicer1, and IPRb1 tumors contain large, pleomorphic, primitive cells with a high nucleus-to-cytoplasm ratio and rare inconspicuous nucleoli that resemble embryonic pineal progenitors. These cells are arranged as sheets, in a vaguely nodular pattern, with nuclear molding, frequent mitosis, and areas of necrosis. These cells stain positive for markers of proliferation and neuronal lineage (Ki-67 and synaptophysin, respectively) and negative for a glial lineage marker (glial fibrillary acidic protein, or GFAP) (**Fig. 1C,D**). Furthermore, just as *DROSHA/DICER1*-related pineoblastomas arise later in childhood than *RB1*-driven pineoblastoma, IPDrosha and IPDicer1 tumors appeared to arise at later ages than IPRb1 tumors (median age of onset 269 days, 239 days, and 182 days, respectively), though we observed tumors as early as ~5 months of age in all three groups. We did not observe any histological abnormalities in the retina, where *IrbpCre* is also active (not shown).

To understand how these tumors arise, we next profiled gene expression and histone modification patterns, focusing on acetylation of histone H3 lysine 27 (H3K27ac), which marks active promoters and enhancers, and trimethylation of histone H3 lysine 4 (H3K4me3), which marks active promoters. We found that IPDrosha and IPRb1 tumors retain the gene expression patterns of embryonic pineal progenitors, with enrichment for embryonic pineal markers and depletion for adult markers (**Fig. 1E-F**, **Suppl. Fig. S1B-E**). Specifically, the pineal homeobox transcription factors *Otx2, Crx, Pax6*, and *Rax* are expressed at high levels in IPDrosha, IPDicer1, and IPRb1 tumors, reflecting their shared pineal progenitor identity (**Fig. 1G**) [30]. Accordingly, the genomic regions surrounding *Otx2, Crx, Rax*, and *Pax6* are marked by broad

H3K27ac signal, sometimes termed “super-enhancers” [31] (**Suppl. Fig. S1F**). Broad H3K27ac signal at these homeobox transcription factors indicates the importance of these genes to maintain embryonic identity in these pineal tumors. Together, these results indicate that IPDrosha, IPDicer1, and IPRb1 tumors all arise from a similar pineal gland progenitor.

*Drosha-driven tumors lack microRNAs and overexpress microRNA target genes*

To understand how IPRb1 tumors form with intact microRNA processing and, conversely, how IPDrosha/IPDicer1 tumors form with intact Rb1 function, we next investigated differences between IPRb1 and IPDrosha/IPDicer1 tumors. We first performed small RNA sequencing in IPDrosha tumors, IPRb1 tumors, matched normal brain, and age-matched normal adult pineal glands to identify the microRNAs regulating normal pineal gland development. As expected, IPDrosha tumors are devoid of canonical microRNAs (**Fig. 2A,C**, **Suppl. Fig. S2A**). Some microRNAs can be produced without *Drosha*, through a non-canonical pathway, if they are processed by the splicing machinery (called “miRtrons”) or if they are transcribed as a capped microRNA [32, 33]. These “Drosha-independent” microRNAs remain expressed in IPDrosha tumors at levels similar to matched normal brain, age-matched adult pineal glands, or IPRb1 tumors (**Suppl. Fig. 2B-D**). These findings are consistent with *Drosha* loss.

We identified the most abundant microRNAs in age-matched adult pineal glands and in IPRb1 tumors, which resemble embryonic pineal progenitors. Members of the let-7/miR-98-5p, miR-99/100-5p, and miR-125/351-5p families are among the most abundant microRNA families in both IPRb1 tumors and adult pineal glands (**Fig. 2A,C, Suppl. Fig. S2E-F**). These microRNAs are co-transcribed from the same genomic loci in an evolutionarily conserved manner [34]. They are not expressed in neural progenitors but are induced late in neural or retinal development to downregulate progenitor genes [35-37]. In IPRb1 tumors and adult pineal glands, we detected nearly all let-7/miR-98-5p family members (**Fig. 2B,D**). As expected, IPDicer1 tumors similarly expressed undetectable levels of microRNAs (**Fig. 2A,C,E**). The depletion of mature let-7/miR-98-5p microRNAs was seen in IPDrosha tumors despite broad H3K27ac and H3K4me3 at the host genes of let-7/miR-98-5p transcripts (**Suppl. Fig. S2G**).

To understand which microRNAs have the greatest effect on gene expression in IPDrosha tumors, we next examined the expression of genes predicted to be targeted by the 50 microRNAs expressed in IPRb1 tumors but not in IPDrosha tumors. Based on TargetScan

calculations of sequence context and conservation, we found 781 genes predicted to be targeted by these 50 microRNAs [38]. These predicted microRNA targets are expressed at higher levels in IPDrosha tumors than IPRb1 tumors, consistent with a loss of microRNA repression (**Fig. 2F**). To understand which microRNAs are the key contributors, we examined enrichment for the genes targeted by each individual microRNA family. Predicted gene targets of the let-7/miR-98-5p and miR-125/351-5p families were the most significantly enriched sets of microRNA targets in IPDrosha compared to IPRb1 tumors or adult pineal glands, consistent with the loss of their expression in IPDrosha tumors (**Fig. 2G**, **Suppl. Fig. S2H-J**).

However, observing loss of a microRNA and upregulation of a predicted target transcript cannot fully distinguish between the direct and indirect effects of microRNA targeting. Some genes may be upregulated as a secondary effect of another de-repressed microRNA target gene. Thus, we next used microRNA enhanced cross-linking immunoprecipitation (miR-eCLIP) sequencing to confirm which genes are directly targeted by microRNAs in IPRb1 tumors [39]. This technique directly identifies microRNA-mRNA interactions through immunoprecipitation of Argonaute 2 (AGO2) and ligation of microRNA-mRNA “chimeras”. We performed miR-eCLIP sequencing in two independent IPRb1 tumors, and we examined the non-chimeric peaks and chimeric peaks that were reproduced between the two samples (**Suppl. Fig. S3A**). As expected, reproduced reads clustered primarily in the 3’ untranslated regions (3’UTRs) of coding genes, confirming enrichment for regions captured by microRNA targeting [12] (**Suppl. Fig. S3B**). In non-chimeric transcripts immunoprecipitated with AGO2, two of the three most significantly enriched motifs were let-7/miR-98-5p recognition sequences (**Suppl. Fig. S3C**). Furthermore, let-7/miR-98-5p family members were commonly identified in the microRNA side of chimeric reads (**Suppl. Fig. S3D**). Altogether, these results highlight the let-7/miR-98-5p family as key regulators of gene expression in IPRb1 tumors.

Next, we examined whether the genes identified as direct microRNA targets in miR-eCLIP sequencing of IPRb1 tumors are repressed compared to IPDrosha tumors. Genes represented in microRNA-mRNA chimeric reads were slightly upregulated in IPDrosha tumors, and they were more likely to be upregulated in IPDrosha tumors than downregulated (**Suppl. Fig. S3E,F**). Furthermore, while transcripts represented in any chimeric reads were upregulated in IPDrosha tumors, chimeras which corresponded to a true 7- or 8-mer perfect seed match were upregulated even further (**Suppl. Fig. S3E**). We thus considered genes with true seed matches to be “confirmed top microRNA targets”. These 1,454 confirmed top microRNA genes were even more upregulated than the microRNA targets predicted by TargetScan (**Fig. 2F,H**).

Similarly, the confirmed top targets of the let-7/miR-98-5p family were more enriched than the set of predicted let-7/miR-98-5p targets (**Fig. 2G,I**).

Together, these results demonstrate that de-repression of direct microRNA targets explain many of the differences between IPDrosha and IPRb1 tumors. Nevertheless, genes represented in microRNA-mRNA chimeras only constituted 25.1% of all genes overexpressed in IPDrosha tumors (**Suppl. Fig. S3F**). In other words, while microRNAs directly drive many of the transcriptomic differences between IPDrosha and IPRb1 tumors, up to 75% of the genes overexpressed in IPDrosha tumors may be attributable to other effects.

*Proliferation restrains differentiation in Drosha-driven tumors*

Thus, we next examined other gene sets upregulated by loss of *Drosha*, whether directly or indirectly. Specifically, we identified “hallmark” gene sets differentially enriched in IPDrosha tumors, IPRb1 tumors, and normal adult pineal glands. We noted that four of the top five most enriched hallmark gene sets overlapped between IPDrosha and IPRb1 tumors, demonstrating their overlapping paths to tumor formation (**Fig. 3A**). These similarities in overall transcriptome, despite independent molecular mechanisms, suggests that *Drosha* loss and *Rb1* loss converge to drive cancer formation through overlapping downstream effects.

As expected, since Rb1 normally regulates E2F transcription factors, the most enriched “hallmark” gene set in IPRb1 tumors is “E2F target genes” (**Fig. 3A**, **Suppl. Fig. S4B**). However, E2F target genes were also the most enriched gene set in IPDrosha tumors (**Fig. 3A**, **Suppl. Fig. S4A**). In cells with intact Rb1, D-type cyclins (cyclin D1, D2, or D3) partner with cyclin-dependent kinases 4 and 6 (CDK4/6) to phosphorylate Rb1 and release it from inhibiting E2Fs [20, 40]. It had previously been shown that *Ccnd1* overexpression was sufficient to replace Rb1 loss and drive pineal tumor formation [23, 41]. Thus, we examined the expression of D-type cyclins in IPDrosha and IPDicer1 tumors and found that cyclin D2 (*Ccnd2*) was the only one significantly overexpressed (**Fig. 3B**, **Suppl. Fig. S4C**). *Ccnd2* is a target of several microRNAs; for example, it is thought to drive proliferation of leukemias driven by loss of miR-16 [42, 43]. We confirmed that it is regulated by several microRNAs in our miR-eCLIP sequencing (it was also the most overexpressed predicted let-7/miR-98-5p target gene in IPDrosha tumors, though we did not detect any chimeras demonstrating let-7/miR-98-5p microRNAs interacting *Ccnd2* transcript in miR-eCLIP, **Suppl. Fig. S4D**). Using immunohistochemistry, we also

confirmed that IPDrosha and IPDicer1 tumors exhibit high levels of CDK4/6 activity, as nearly every cell stained positive for phosphorylated Rb1 (**Fig. 3C**). Thus, *Drosha* loss and *Rb1* loss converge on the same proliferation-driving pathway to drive cancer cells through the G1/S cell cycle checkpoint.

In the developing brain, proliferation maintains progenitors in an undifferentiated state, and differentiation is allowed to occur once proliferation slows down [44]. Accordingly, IPRb1 tumors remain undifferentiated solely because of unrestrained proliferation of these embryonic progenitors. Thus, to understand whether IPDrosha/IPDicer1 tumors with high levels of *Ccnd2* are dependent on CDK4/6 activity to remain undifferentiated, we treated them with the clinically approved CDK4/6 inhibitor palbociclib [45]. Specifically, we implanted tumor cells as subcutaneous allografts and administered either vehicle or palbociclib to recipient mice. Over two weeks, palbociclib suppressed proliferation and extended survival in IPDrosha and IPDicer1 tumors (**Fig. 3D** and **Suppl. Fig. S4E**). Palbociclib-treated tumors exhibited loss of Rb1 phosphorylation, consistent with a loss of CDK4/6 activity (**Fig. 3E**). Histologically, the palbociclib-treated IPDrosha and IPDicer1 tumors exhibit increased karyorrhexis and increased background neuropil, a sign of differentiation.

Next, we used RNA-seq to understand how palbociclib affected IPDrosha tumor cells. Consistent with inhibition of CDK4/6, E2F target genes were the most downregulated hallmark gene set in palbociclib-treated tumors (**Fig. 3F**). In contrast, interferon alpha and gamma response pathways were enriched in palbociclib-treated tumors, consistent with reports that CDK4/6 inhibitors can induce antitumor immunity [46-49] (**Suppl. Fig. S4F**). When we examined pineal differentiation markers, we found that palbociclib-treated tumors were enriched for adult pineal markers such as *Chrna3* and *Nefh* (**Fig. 3G,H, Suppl. Fig. S4G**). On the other hand, embryonic markers were mildly depleted as a whole (**Fig. 3G**, **Suppl. Fig. S4H**). Many embryonic genes remain highly expressed upon palbociclib treatment, such as the embryonic transcription factor *Plagl2* (**Fig. 3H**). In other words, blocking the effect of *Ccnd2* partially restores differentiation in IPDrosha tumors, but microRNAs remain absent, allowing some embryonic pineal markers to remain highly expressed.

*The microRNA target gene Plagl2 regulates proliferation drivers Igf2 and Ccnd2*

We next identified the proliferation-independent microRNA target genes that contribute to maintaining an embryonic expression program. Specifically, we next identified embryonic pineal markers that were overexpressed in IPDrosha tumors, confirmed “top” let-7/miR-98-5p target genes by miR-eCLIP, and independent of palbociclib. Three genes met these criteria: *Bach1*, *Tgfbr1*, and *Plagl2*. We focused on *Plagl2* (*PLAG1*-like gene 2) because of previous evidence that it is an oncogenic, developmental transcription factor, and because its most well-characterized target gene, *Igf2*, was the single most overexpressed gene in IPDrosha tumors [50-52] (**Fig. 4A-B**). Our miR-eCLIP sequencing demonstrated that the 3’ UTR of *Plagl2* interacted with several microRNAs (**Fig. 4C**). In fact, let-7/miR-98-5p microRNAs interacted with *Plagl2* at three sites, including one highly conserved site with a 7-mer perfect match.

Histone marks at the *Plagl2* locus were similar in IPDrosha and IPRb1 tumors, consistent with a gene whose expression is primarily regulated by microRNAs post-transcriptionally (**Suppl. Fig. S5A**). At the *Ccnd2* locus, however, IPDrosha tumors had much higher levels of H3K27ac and H3K4me3, suggesting that *Ccnd2* upregulation is due to a combination of transcriptional and post-transcriptional regulation (**Fig. 4D**). Since the *Ccnd2* promoter also contains two clusters of predicted Plagl2 binding sites, we examined whether Plagl2 resides at the *Ccnd2* promoter in IPDrosha tumors (**Fig. 4D**). Using ChIP-qPCR, we verified Plagl2 binding at a region of the *Ccnd2* promoter with four Plagl2 binding motifs clustered in a 300bp range (“region 2” in **Fig. 4D,E**). Lastly, we noted that levels of *Plagl2*, *Igf2,* and *Ccnd2* are high in embryonic pineal glands and fall in parallel during pineal development, suggesting coordinated regulation (**Suppl. Fig. S5B**). Together, these results suggest that *Ccnd2* is upregulated by both increased transcription and decreased microRNA repression, and that the microRNA target gene *Plagl2* contributes to the maintenance of the embryonic expression program, in part by regulating *Igf2* and *Ccnd2*.

*Igf2*, whose overexpression drives many other childhood cancers, signals through the insulin signaling pathway primarily by binding the insulin-like growth factor 1 receptor (Igf1r), leading to activation of mTORC1 and other kinase pathways [53]. Accordingly, we observed that the “insulin signaling” and “mTORC1 signaling” gene sets were significantly enriched in IPDrosha tumors (**Fig. 5A** and **Fig. 3A**). Thus, to study the importance of Igf2 signaling, we treated tumor-bearing mice with ceritinib, a small-molecule Igf1r inhibitor [54]. In both IPDrosha and IPDicer1 tumors, we found that ceritinib significantly impaired tumor growth (**Fig. 5B**). Vehicle- and ceritinib-treated IPDrosha and IPDicer1 tumors are histologically similar, though ceritinib-treated tumors appeared to have slightly more background neuropil (**Fig. 5C**). By

immunostains, ceritinib-treated tumors exhibited decreased phosphorylation of S6 (a measure of Igf1r/mTORC1 activity) and Rb1 (**Fig. 5C**). Similarly, RNA sequencing revealed depletion of the insulin signaling genes in ceritinib-treated IPDrosha tumors (**Fig. 5D**).

*E2F targets are enriched in human pineoblastoma tumors with low DROSHA*

Lastly, we examined RNA-seq data from human pineoblastoma tumors. Among these tumors, we noted that *DROSHA* expression was essentially undetectable in two of nine tumors. We compared expression patterns in these two "*DROSHA*-low" tumors to the other seven "*DROSHA*-high" tumors. Consistent with impairment in microRNA processing, we found that *DROSHA*-low tumors are enriched for genes predicted to be targeted by let-7/miR-98-5p and other microRNAs (**Suppl. Fig. S6A,B**). Furthermore, consistent with our findings in IPDrosha tumors, the most enriched hallmark gene set in *DROSHA*-low tumors is "E2F targets" (**Suppl. Fig. 6C,D**). Lastly, although they did not reach statistical significance, the two *DROSHA*-low tumors expressed slightly higher levels of *CCND1, CCND2*, *PLAG1*, and *PLAGL2* (**Suppl. Fig. S6E**). (*IGF2* was not consistently detected in these tumors). In sum, these data in human tumors are consistent with findings from our mouse model that impaired microRNA processing dysregulates the G1/S checkpoint.

## DISCUSSION

*Drosha* loss leads to de-repression of microRNA target genes, which has cascading effects on the expression of other genes. In this study, we dissect *Drosha*-dependent gene networks with a combination of microRNA sequencing, RNA-sequencing, miR-eCLIP sequencing and histone profiling. Our study shows that loss of *Drosha* drives pineal tumors by mimicking *RB1* loss. Together, these studies revealed *Plagl2* and *Ccnd2* as microRNA target genes driving IPDrosha tumors and small-molecule inhibitors of these effectors that can block tumor growth (**Suppl. Fig. S6F**). In other words, while loss of microRNAs cannot be therapeutically targeted currently, the microRNA target genes that drive tumorigenesis could be blocked with available drugs.

Our results shed light on the cells from which pineoblastoma arise. IPDrosha, IPDicer1, and IPRb1 tumors express high levels of *Pax6*, *Otx2*, *Crx*, and *Rax* – the homeobox transcription factors that cooperate to regulate pinealocyte development. In particular, *Pax6* and *Otx2* are expressed in rapidly dividing cells at the earliest stages of pineal development [30, 55]. *Otx2* regulates the expression of *Crx*, a specific marker for retina and the pineal gland that may even be used as a diagnostic marker for retinoblastoma and pineoblastoma [19, 56-58]. *Otx2* and *Crx* recognize the same binding motif and thus activate the same target genes, including *Irbp* and *Rax* [30, 59, 60]. In fact, *OTX2* and *CRX* are key oncogenic drivers in group 3 medulloblastoma and are likely to play similar roles in pineoblastoma [61, 62]. Together, these results suggest that pineoblastomas driven by loss of *Drosha*, *Dicer1*, or *Rb1* arise from an embryonic pineal progenitor marked by *Pax6*, *Otx2*, *Crx*, *Rax*, and *Irbp*.

Using a Cre driver initially developed for the mammary gland, Chung et al. previously showed that *Dicer1* ablation also drives development of pineal tumors; to our knowledge, our study is the first report of pineal tumors arising in mice through loss of *Drosha* [26, 63]. Our findings recapitulate many of their findings, including incomplete penetrance and delayed onset compared with *Rb1*-driven tumors, suggesting that tumors from the two Cre drivers represent the same cell of origin. Nevertheless, the incomplete penetrance and delayed onset of *Drosha/Dicer1*-driven tumors suggest that other factors might also contribute to or restrain tumor development. For instance, in both studies, loss of *Rb1*, *Dicer1,* or *Drosha* alone was not sufficient to drive tumor development. In other words, while these genetic events are critical drivers for tumor development, other tumor-suppressive mechanisms, such as p53 and $p18^{Ink4c}$, may compensate to restrain cell proliferation, as previously reported in a cyclin D1-driven

mouse model of pineal tumors [23]. The role of such mechanisms is unclear in human pineoblastoma, which rarely exhibits loss or mutation of p53. Future studies are thus required to characterize the genetic status of these genes in human pineoblastoma and understand the effect of p53 loss on the proliferative potential of pineal cells losing *Rb1*, *Dicer1,* or Drosha.

Previous studies showed that pineal tumors arise through dysregulation of the G1/S checkpoint, either by ablating *Rb1* or upregulating *Ccnd1* [23, 41]. By demonstrating that IPDrosha/IPDicer1 tumors drive proliferation by upregulating D-type cyclins, we build upon these studies and show similarities between loss of *Drosha/Dicer1* and loss of *Rb1*. CDK4/6 inhibitors are clinically approved for use in hormone receptor-positive breast cancer and under investigation for treatment of other undifferentiated pediatric and adult tumors that retain intact RB1-E2F responses [20, 40, 64, 65]. Specifically, CDK4/6 inhibitors have been found to induce differentiation in neuroblastoma [66], rhabdomyosarcoma [22], medulloblastoma [67], and prostate cancer [68]. To date, the only known genomic predictors of sensitivity to CDK4/6 inhibition directly drive overexpression of *CCND1*, *CCND2*, or *CCND3* [69]. These may arise through translocation, amplification, or variants that remove or alter microRNA binding sites in the 3' UTR. Our results suggest that removal of the microRNAs themselves could also contribute to overexpression of D-type cyclins and thus sensitivity to CDK4/6 inhibitors.

We have also previously reported overlapping results in Wilms tumor, a pediatric cancer of embryonic renal progenitors frequently driven by mutations in microRNA processing genes [3-7]. Unlike the complete loss of microRNA production seen in pineoblastoma, Wilms tumors most commonly exhibit point mutations in *DROSHA* that lead to incomplete impairment of microRNA processing. In two separate studies, we previously found that *DROSHA* regulates *CCND2* and *PLAG1/IGF2* in Wilms tumor; it remains to be seen whether *PLAG1* or *PLAGL2* interacts with *CCND2* in Wilms tumor [70, 71]. Regardless, together, our observations suggest that despite arising from different cell types and through different effects on microRNA processing, *DROSHA*-mutant Wilms tumor and pineoblastoma harness similar pathways to drive proliferation of embryonic progenitors.

Another family of tumors, including pleuropulmonary blastoma, Sertoli-Leydig cell tumor, and rhabdomyosarcoma, arises through point mutations in the RNase IIIB domain of *DICER1,* which cleaves the 5p arm of pre-microRNA hairpins [9, 10]. This results in loss of 5p-derived microRNAs but retention of 3p-derived microRNAs. Many of these *DICER1*-related tumors also resemble highly proliferative, undifferentiated progenitors. In fact, in a recently published mouse

model of *Dicer1* RNase IIIb mutation, the gynecologic tumors that arose exhibited loss of 5p microRNAs and upregulation of E2F and Myc target genes, similar to our findings [72]. Furthermore, many of the microRNAs we identified as key regulators of gene expression in pineoblastoma are 5p-derived and thus lost in tumors with *DICER1* missense variants, including members of the let-7/miR-98-5p, miR-99/100-5p, and miR-125/352-5p families. It remains to be seen whether D-type cyclins and *PLAG1* family members are also important in these cancer types.

Recently, a subset of embryonal brain tumors was found to be defined by amplification and overexpression of *PLAGL2*, and our study expands on its oncogenic importance [73]. *PLAGL2* was initially described as a homolog of the *PLAG1* transcription factor, whose translocation-driven overexpression defines pleomorphic adenoma of the salivary glands [74]. In the embryonic brain and in brain tumors, *Plagl2* regulates the balance between differentiation and proliferation, and its overexpression shifts neural progenitors towards proliferation [75, 76]. Outside the brain, *PLAGL2* also contributes to oncogenesis in lung cancer [77, 78], colorectal cancer [79-82], gastric cancer [83], breast cancer [84, 85], and acute myeloid leukemia [86]. In these cancers, *PLAGL2* has been shown to contribute to proliferation, epithelial-mesenchymal transition, and “stemness” by regulating target genes such as *Igf2* and regulators of Wnt signaling [73, 76, 79, 80, 82, 85]. To our knowledge, however, this is the first description of *PLAGL2* upregulation in *DROSHA*- or *DICER1*-related pediatric cancers.

In this study, we identify two small molecules that impaired tumor proliferation by blocking signaling pathways normally regulated by microRNAs. Although they are both clinically approved, it remains to be seen whether they will be effective in children with these cancers. In our models, neither drug was cytotoxic, and they may need to be combined with other therapies to harness clinical benefit. For instance, previous studies have found synergy between CDK4/6 inhibitors and immune checkpoint blockade, and we also found that palbociclib upregulated immune gene signatures [46-49] (**Suppl. Fig. S4E**). Alternatively, human tumors may harness other oncogenic signaling pathways or growth factors, such as RAS/MAP kinase; indeed, RAS pathway genes are the most frequently mutated genes in *DICER1*-related tumors after *TP53* [87-89]. Future studies will be needed to identify the mitogenic signaling pathways used in human pineoblastomas. Nevertheless, our results demonstrate the potential for the signals upregulated by microRNA loss to be therapeutic targets in cancers driven by impairment of *DROSHA* or *DICER1*.

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## AUTHOR CONTRIBUTIONS

C.R.F., K.D., L.K.M., U.O., T.S., K.O., and K.S.C. conceived and performed experiments. C.R.F., Y.W., and K.S.C. wrote the manuscript. V.R. provided expertise, feedback, and analysis. J.L. and K.S.C. performed data analysis and secured funding.

## FIGURE LEGENDS

### Figure 1. Pineal tumors driven by loss of *Drosha*, *Dicer1*, or *Rb1* resemble embryonic pineal progenitors.

**(A)** Kaplan-Meier plots showing tumor-free survival of IPDrosha, IPDicer1, and IPRb1 tumors. IrbpCre;Drosha$^{f/f}$;p53$^{f/f}$ (IPDrosha, dark green) mice have a tumor incidence of 36% at 1 year; IrbpCre;Dicer1$^{f/f}$;p53$^{f/f}$ (IPDicer1, light green) mice have a tumor incidence of 34% at 1 year; IrbpCre;Rb1$^{f/f}$;p53$^{f/f}$ (IPRb1, purple) mice have a tumor incidence of 90% at 1 year (***p<0.001 and ****p<0.0001; vs. control genotypes; by log-rank test). **(B)** Photomicrographs of brains from control (Drosha$^{f/f}$;p53$^{f/f}$), IPDrosha, IPDicer1, and IPRb1 mice show expansile pineal tumors expanding into the skull above and normal brain below (scale bar 1000 µm). **(C)** Photomicrographs of IPDrosha, IPDicer1, and IPRb1 tumors (scale bar 50 µm). Top row shows H&E, and bottom row shows immunostaining for Ki-67. **(D)** Photomicrographs of IPDrosha, IPDicer1, and IPRb1 tumors (scale bar 50 µm), with immunostaining for synaptophysin (top) and GFAP (bottom). **(E)** Gene set enrichment analysis (GSEA) of enrichment for pineal development markers comparing IPDrosha tumor with normal adult pineal gland. **(F)** GSEA of enrichment for pineal development markers comparing IPRb1 tumor with normal adult pineal gland. **(G)** qPCR for pineal homeobox genes (*Otx2, Crx, Pax6*, and *Rax*) in brain, IPDrosha, IPDicer1, and IPRb1 tumors. Values shown are mean ± SD (**p < 0.01 and **p < 0.001; vs. normal brain; two-sided t-test). See also Supplementary Figure S1.

### Figure 2. *Drosha*-driven tumors lack microRNAs and overexpress microRNA target genes.

**(A)** Small RNA sequencing displaying the most abundant microRNA families in IPRb1 tumors and IPDrosha tumors. **(B)** Expression profile of individual let-7/miR-98-5p family microRNAs in IPRb1 and IPDrosha tumors. **(C)** Small RNA sequencing displaying the most abundant microRNA families in adult pineal glands and IPDrosha tumors. **(D)** Expression profile of let-7/miR-98-5p family microRNAs in IPDrosha tumors and adult pineal glands. **(E)** TaqMan miRNA expression of let-7a-5p, let-7d-5p, let-7i-5p, miR-100-5p, and miR-183-5p in normal adult brain, IPDrosha, IPDicer1, and IPRb1 tumors. **(F,G)** GSEA of enrichment for predicted targets of all microRNAs expressed in IPRb1 tumors **(F)** or predicted let-7/miR-98-5p targets **(G)** in IPDrosha

vs. IPRb1 tumors. **(H,I)** GSEA for enrichment of confirmed top targets of microRNAs **(H)** or let-7/miR-98-5p **(I)** in IPDrosha vs. IPRb1 tumors. See also Supplementary Figures S2 and S3.

**Figure 3. Proliferation restrains differentiation in *Drosha*-driven tumors.**

**(A)** Hallmark gene sets most enriched in IPDrosha tumors vs. age-matched adult pineal gland, IPRb1 tumors vs. age-matched adult pineal gland, and IPDrosha tumors vs. IPRb1 tumors. **(B)** qPCR for *Ccnd2* in normal adult brain, IPDrosha, IPDicer1, and IPRb1 tumors. Values shown are mean ± SD (*p< 0.05; vs. normal brain; two-sided t-test). **(C)** Photomicrographs of IPDrosha, IPDicer1, and IPRb1 tumors stained for phospho-Rb1 (Ser807/811) (scale bar 50 µm). **(D)** Tumor volumes for IPDrosha or IPDicer1 tumors in mice dosed with vehicle or palbociclib. Values shown are mean ± SD (*p<0.05 and *p<0.01; vs. vehicle-treated; two-sided t-test). **(E)** Photomicrographs of vehicle- or palbociclib-treated IPDrosha and IPDicer1 tumors (scale bar 50 µm). Top row shows H&E, and bottom row shows immunostaining for phospho-Rb1 (Ser807/811). **(F)** GSEA for E2F target genes in RNA sequencing of IPDrosha tumor allografts treated with palbociclib vs. vehicle. **(G)** GSEA of enrichment for pineal development genes in IPDrosha tumors treated with palbociclib vs. vehicle. **(H)** qPCR for adult pineal marker genes (*Chrna3* and *Nefh*) and an embryonic pineal marker gene (*Plagl2*) in IPDrosha and IPDicer1 tumors treated with palbociclib vs. vehicle. Values shown are mean ±SD (*p<0.05; vs. vehicle-treated; two-sided t-test). See also Supplementary Figure S4.

**Figure 4. The microRNA target gene *Plagl2* regulates proliferation drivers *Igf2* and *Ccnd2*.**

**(A)** qPCR for *Igf2* and *Plagl2* in normal adult brain, IPDrosha, IPDicer1, and IPRb1 tumors. Values shown are mean ± SD (*p< 0.05 and ***p<0.001; vs. normal adult brain; two-sided t-tests). **(B)** Heat map from total RNA sequencing of IPDrosha and IPRb1 tumors highlighting *Igf2*. **(C)** miR-eCLIP chimeric reads from IPRb1 tumors of the 3' UTR of *Plagl2,* with microRNA chimeric read peaks denoted below, including three peaks containing let-7/miR-98-5p family members. The conservation of the second let-7/miR-98-5p binding site (marked with asterisk) is featured on the right. **(D)** H3K27ac and H3K4me3 signal at the *Ccnd2* promoter in IPDrosha and IPRb1 tumors, with predicted Plagl2 binding sites and regions targeted by qPCR marked below. **(E)** ChIP-qPCR detecting Plagl2 occupancy in IPDrosha and IPRb1 tumors at the mouse *Ccnd2*

promoter, *Igf2* promoter, and a negative-control intergenic region. Values shown are mean ± SD (*p<0.05 and *p<0.01; vs. IgG; two-sided t-test). See also Supplementary Figure S5.

**Figure 5. The Igf inhibitor ceritinib suppresses tumor growth.**

**(A)** GSEA of enrichment for insulin signaling in IPDrosha vs. IPRb1 tumors (Wikipathway WP481). **(B)** Tumor volumes for IPDrosha and IPDicer1 tumors in mice dosed with vehicle or ceritinib. Values shown are mean ± SD (*p< 0.05; vs. vehicle treated; two-sided t-test). **(C)** Photomicrographs of vehicle- or ceritinib-treated IPDrosha and IPDicer1 tumors (scale 50 µm). Top row shows H&E, the middle row shows phospho-S6 (Ser235/236), and the bottom row shows phospho-Rb1 (Ser807/811). **(D)** GSEA of enrichment for insulin signaling gene set in IPDrosha tumors treated with ceritinib vs. vehicle.

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# Fig 1. Pineal tumors driven by loss of *Drosha*, *Dicer1*, or *Rb1* resemble embryonic pineal progenitors.

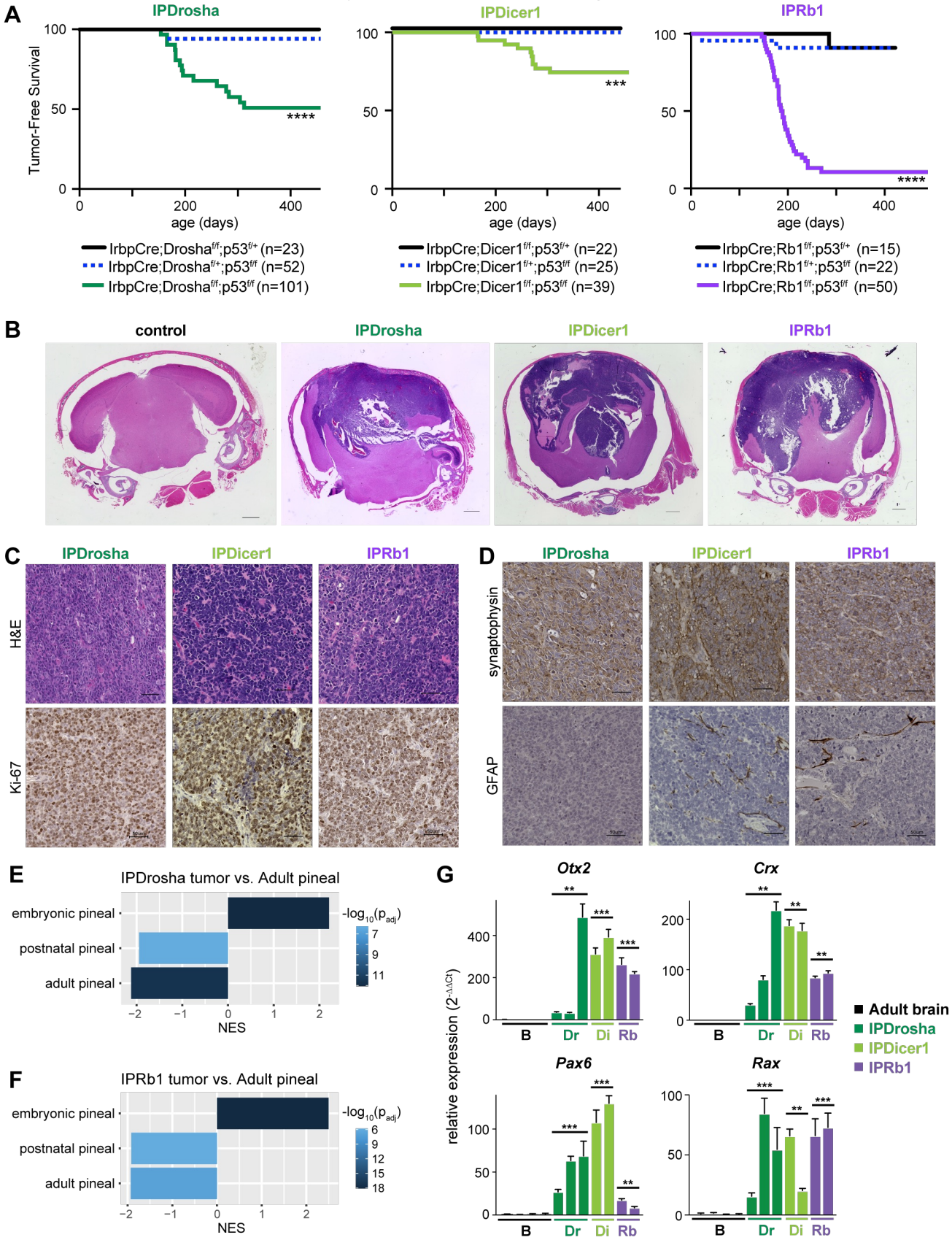
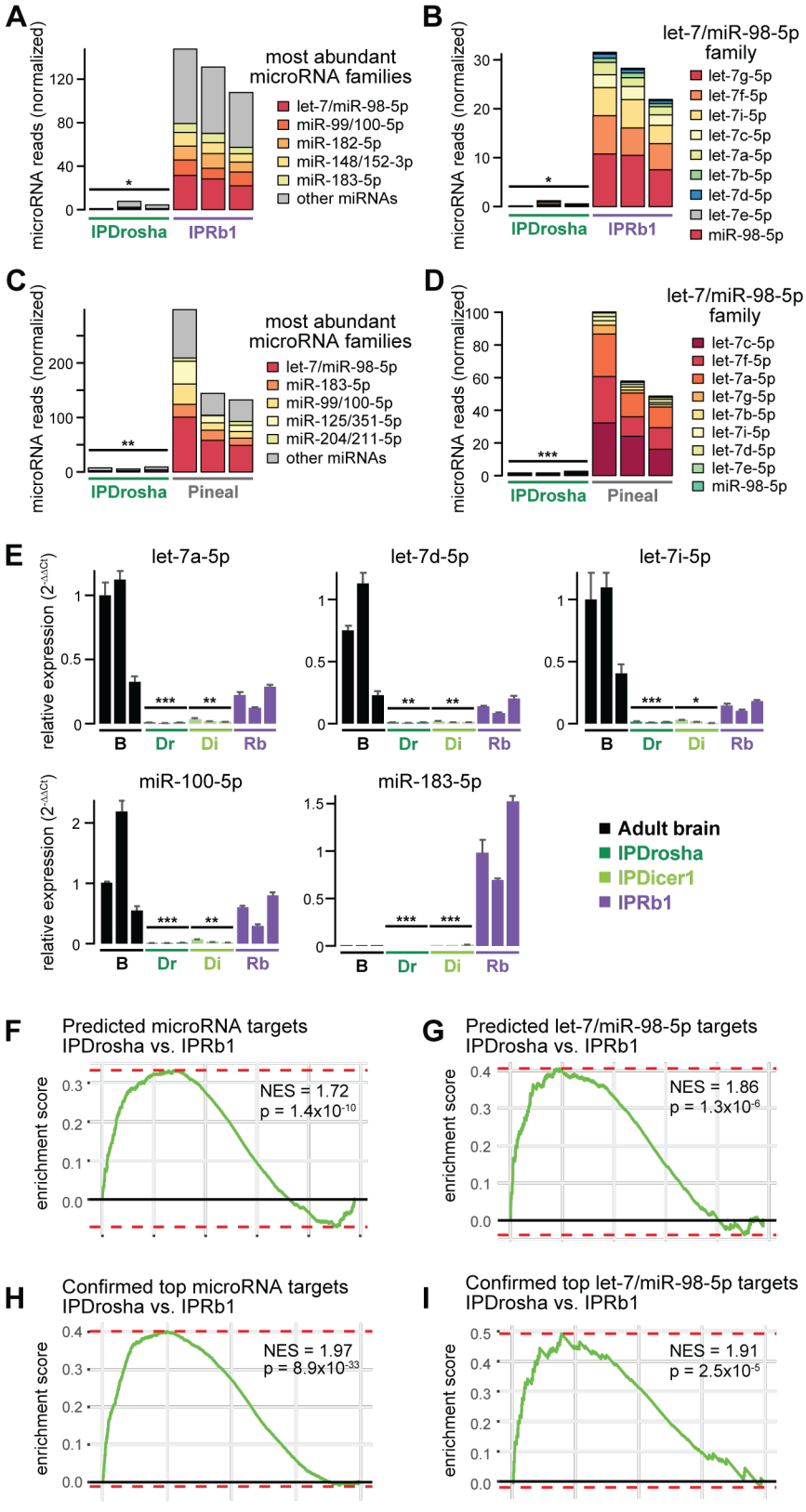
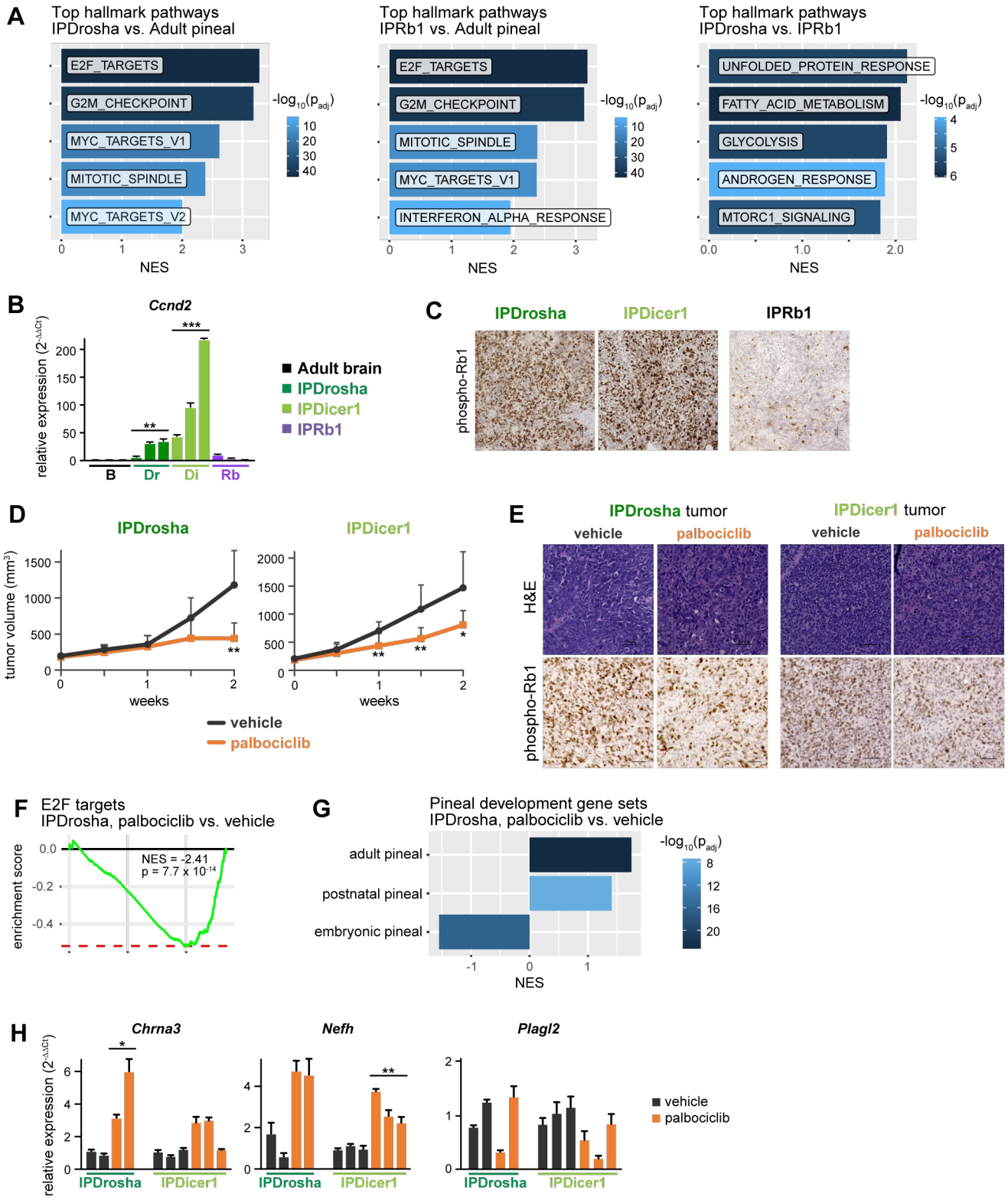


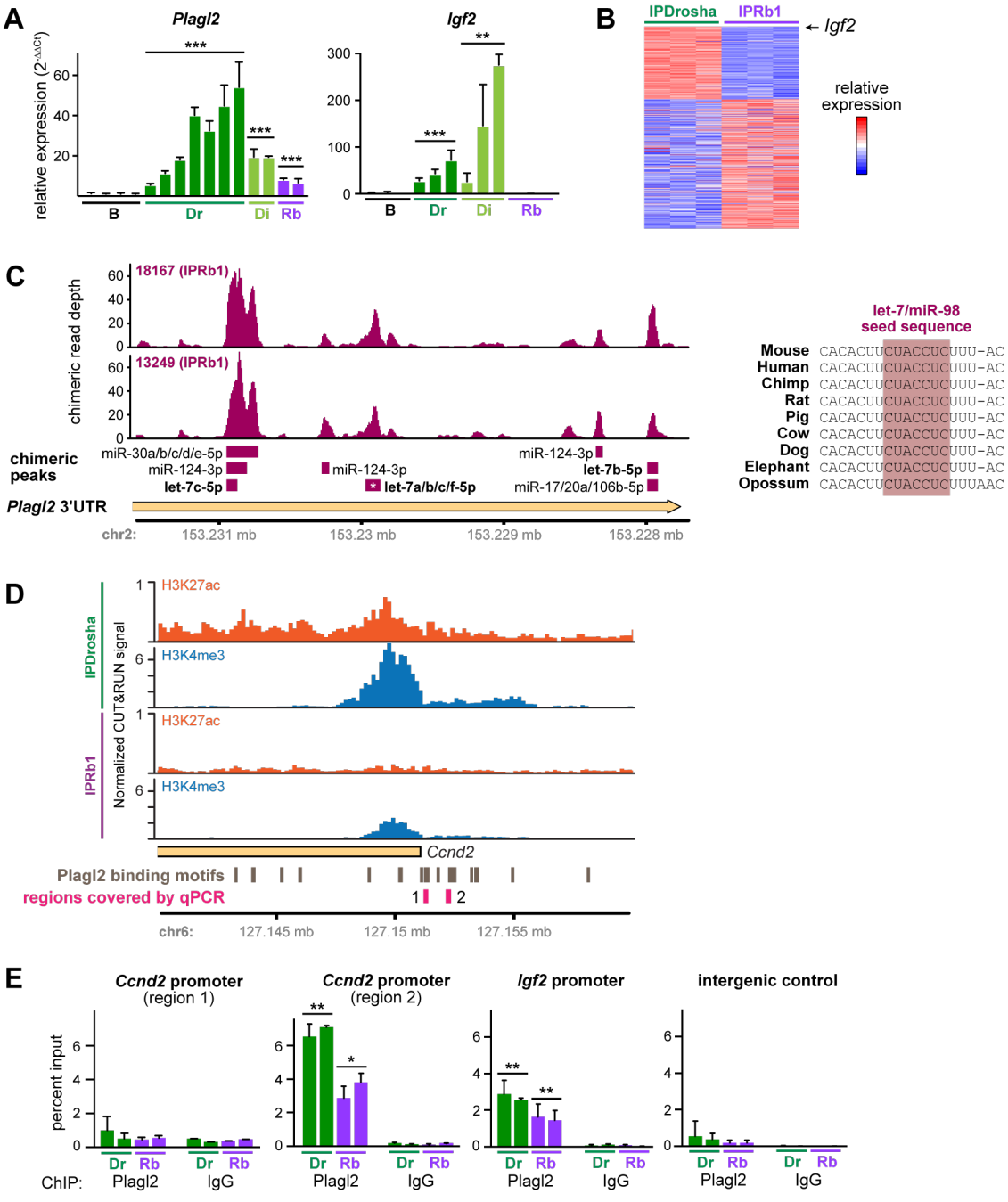
Fig 2. *Drosha*-driven tumors lack microRNAs and overexpress microRNA target genes.



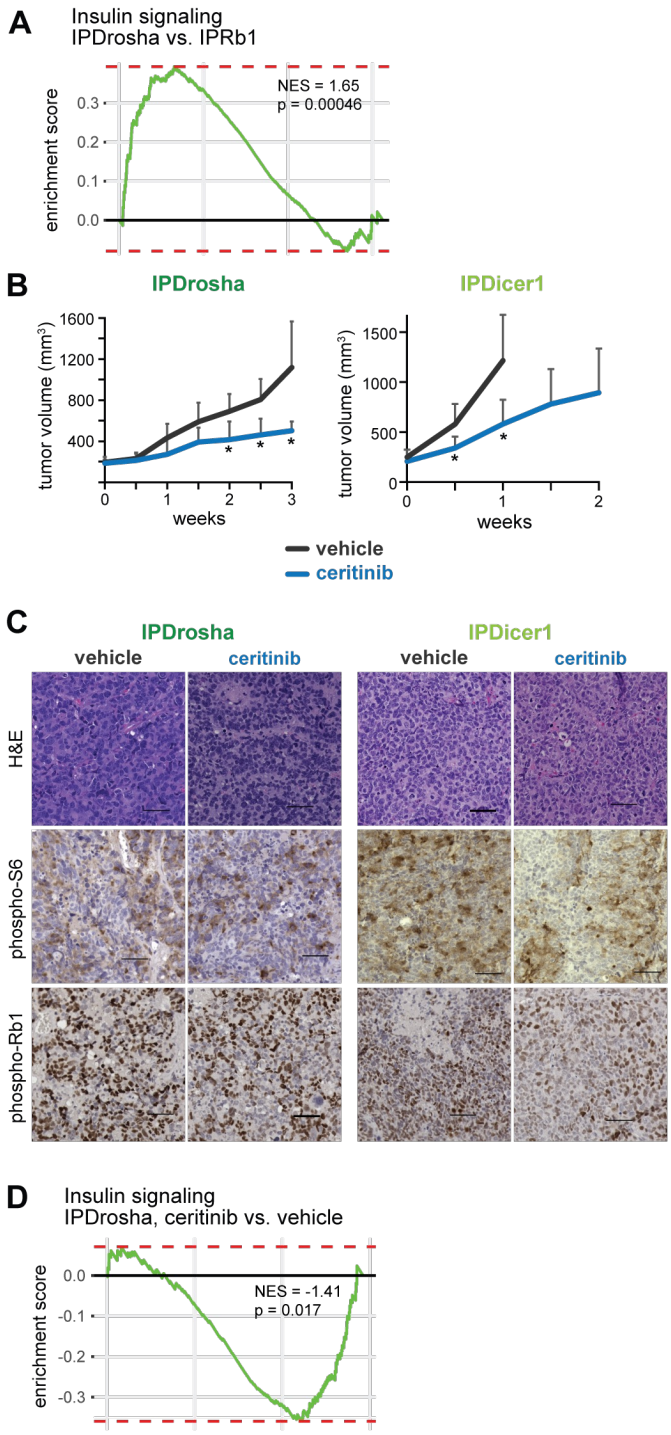
# Fig 3. Proliferation restrains differentiation in *Drosha*-driven tumors.



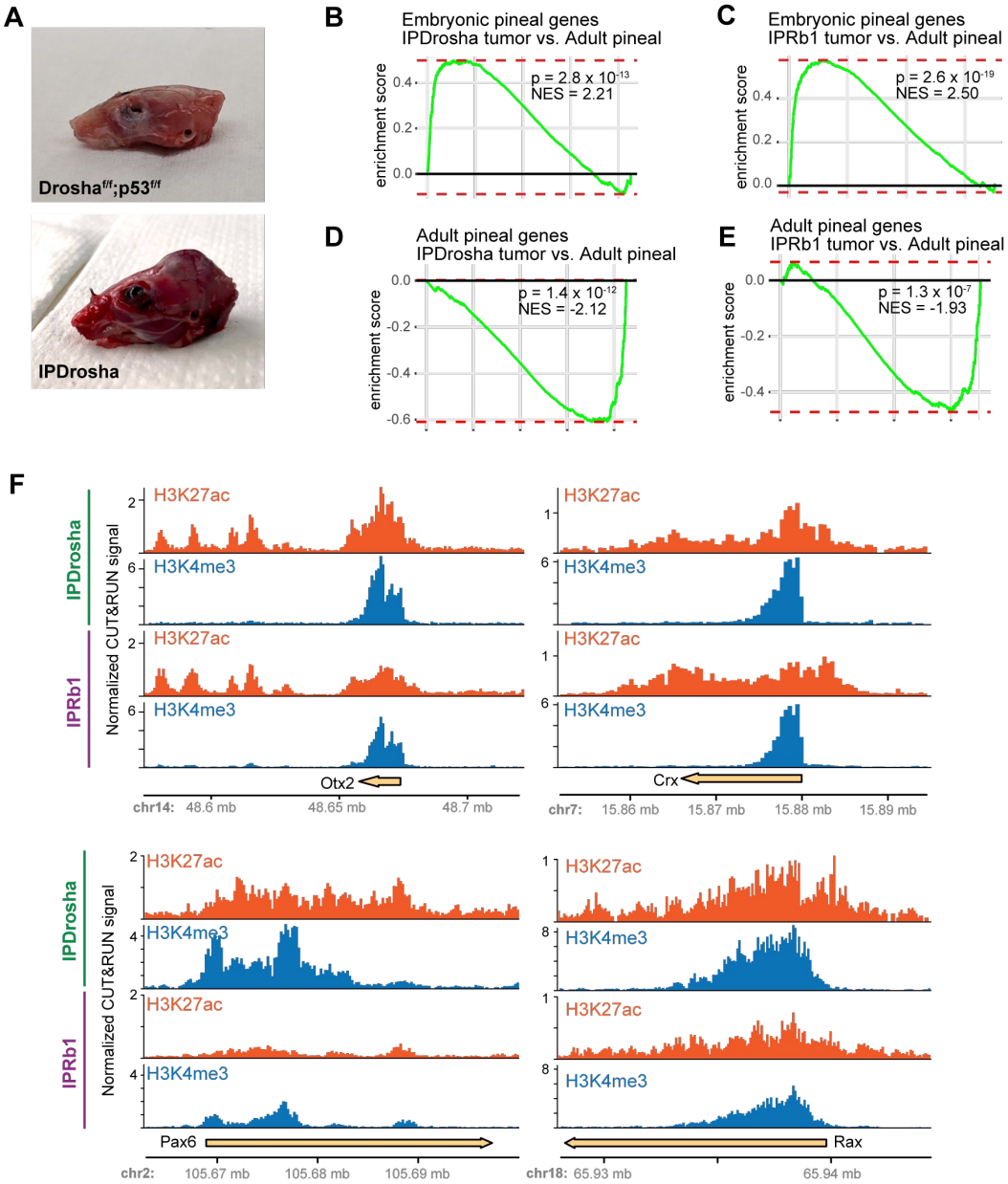
# Fig 4. The microRNA target gene *Plagl2* regulates proliferation drivers *Igf2* and *Ccnd2*.



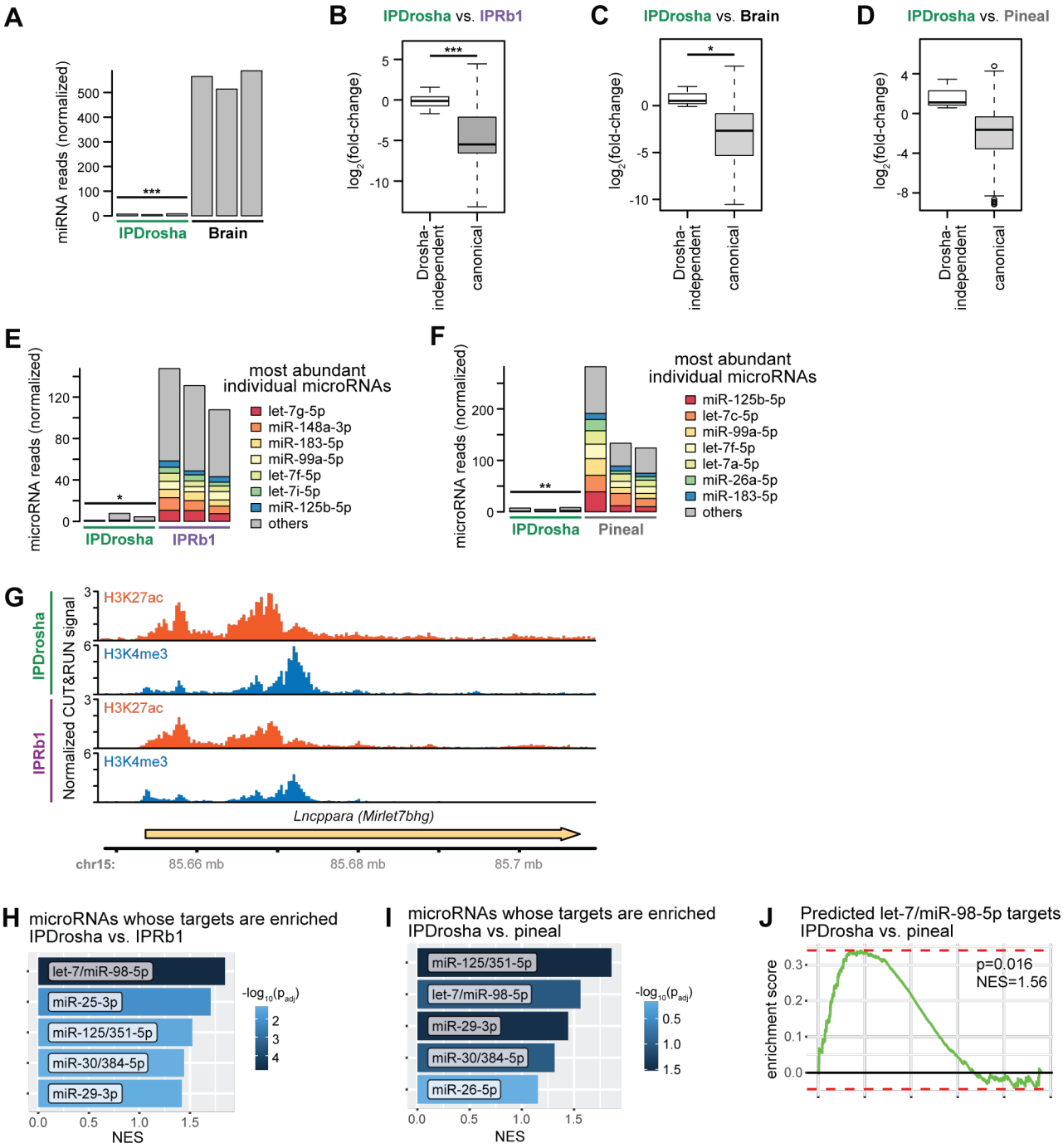
# Fig 5. The Igf inhibitor ceritinib suppresses tumor growth.



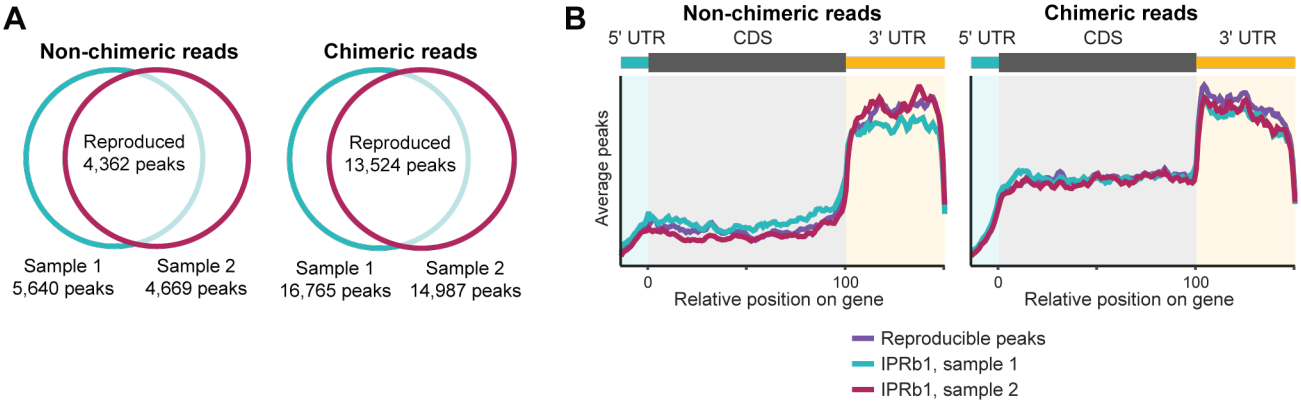
# Fig S1. IPDrosha and IPRb1 pineal tumors retain embryonic pineal expression pathways.



# Fig S2. Drosha tumors exhibit de-repression of let-7/miR-98-5p target genes.

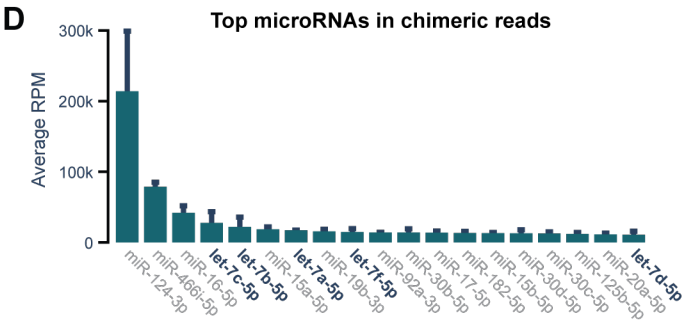


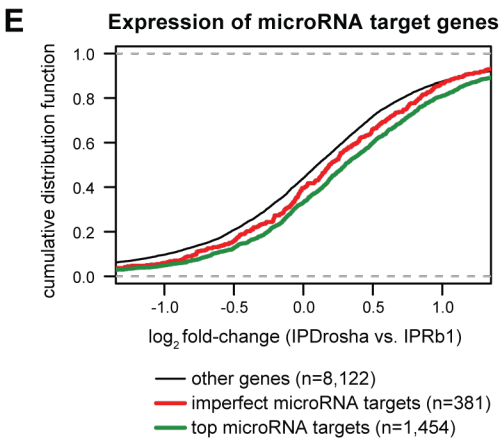
# Fig S3. Patterns of microRNA regulation in miR-eCLIP sequencing of IPRb1 tumors.

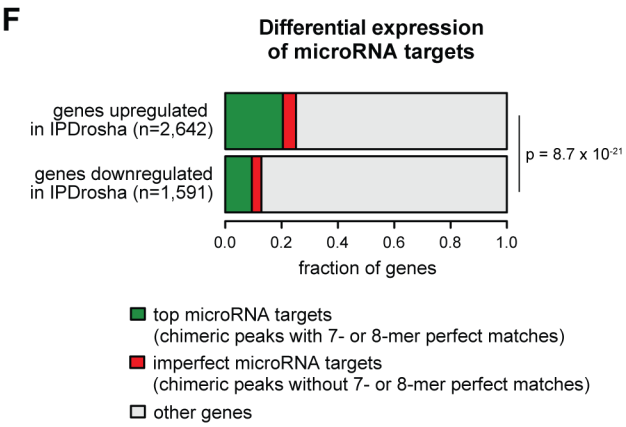



**C** Top motifs in non-chimeric reads

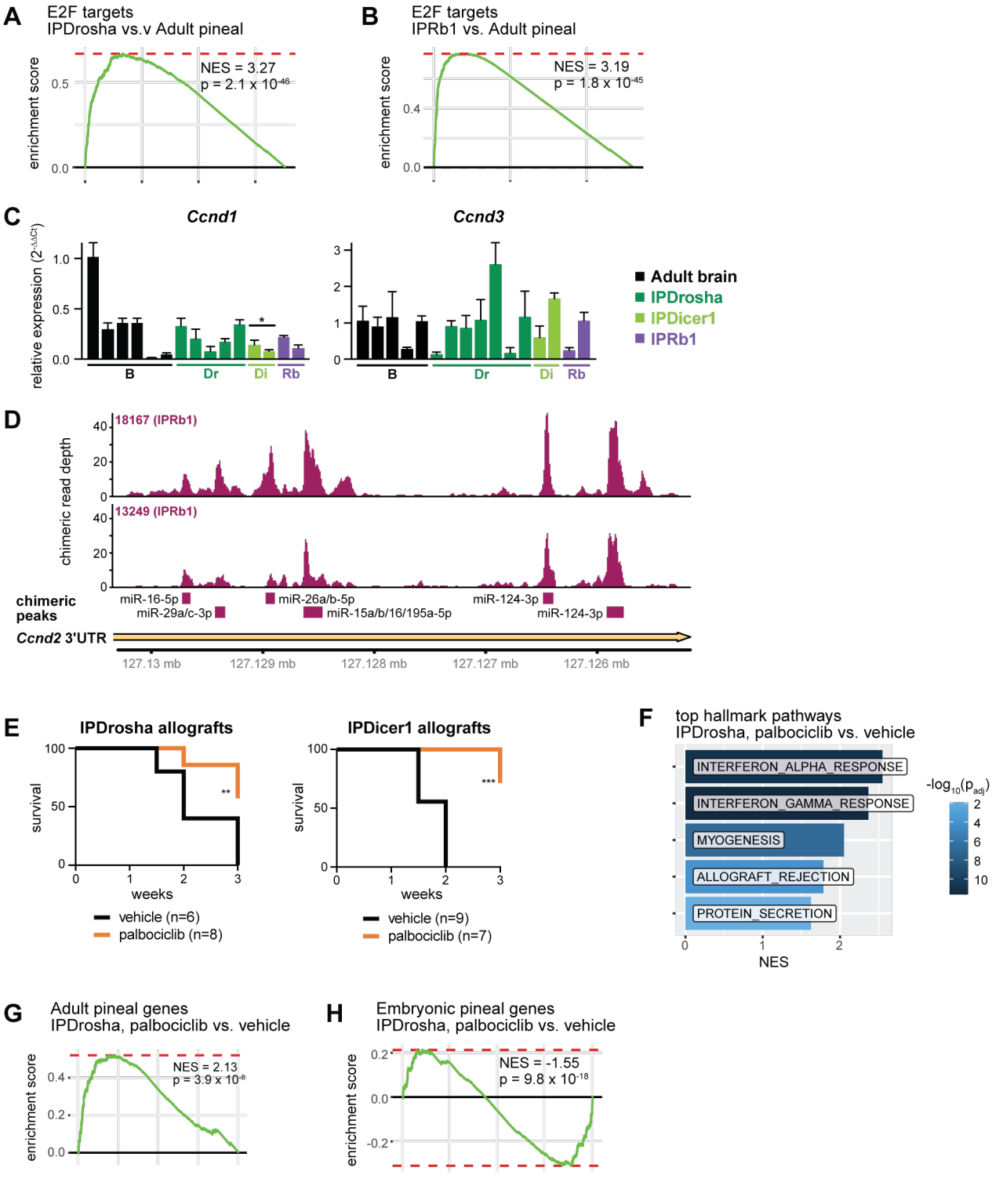
| Motif | Seed match | % of peaks with motif | $-\log_{10}(p)$ |
|---|---|---|---|
| HWGTGCCTA | miR-124-3p | 8.85% | 748.9 |
| NHHCTACCTCAD | let-7/miR-98-5p | 8.57% | 745.6 |
| ATCTACCTCA | let-7/miR-98-5p | 6.54% | 715.9 |
| CTAAGTGCCTA | miR-124-3p | 8.47% | 709.2 |



# Fig S4. IPDrosha tumors are highly proliferative.



# Fig S5. Epigenetic regulation of *Ccnd2.*

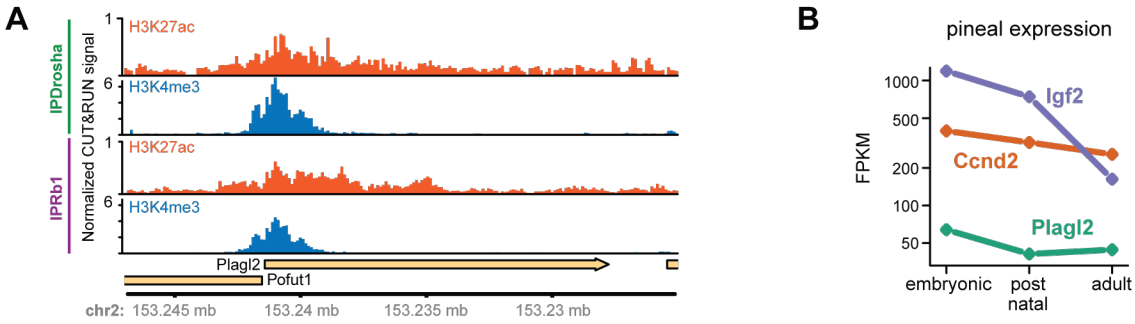
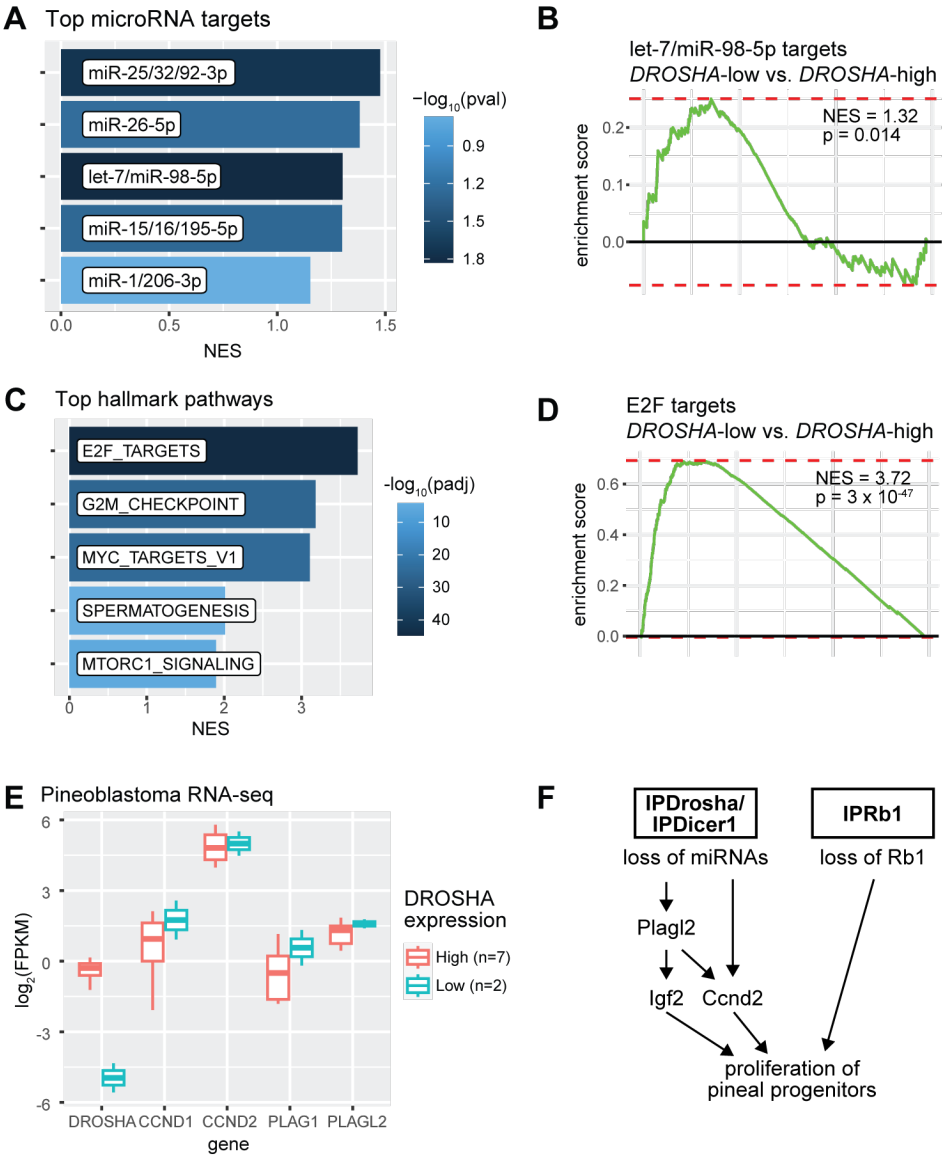



Fig S6. *DROSHA* expression is negatively correlated with let-7/miR-98-5p targets and E2F targets in human tumors.