Title Page

Title:

MYCN-regulated miRNAs Inhibit Secretion of the Tumor Suppressor *DICKKOPF-3* (*DKK3*) in Neuroblastoma

Running Title:

Dickkopf-3 Regulating miRNAs in Neuroblastoma

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Abstract

The *MYCN* oncogene is frequently amplified in neuroblastoma. It is one of the most consistent markers of a bad prognosis for this disease. Dickkopf-3 (DKK3) is a secreted protein of the Dickkopf family of Wnt regulators. It functions as a tumor suppressor in a range of cancers, including neuroblastoma. *MYCN* was recently found to downregulate *DKK3* mRNA. In this study, we show that *MYCN* knockdown in *MYCN*-amplified (MNA) neuroblastoma cell lines increases secretion of endogenous DKK3 to the culture media.

MiRNAs are ~20-nt-long RNAs encoded by the genome that downregulate mRNAs by targeting the 3`untranslated region (3'UTR). Many miRNAs regulate genes involved in the pathogenesis of cancer and are extensively deregulated in different tumors. Using miRNA target prediction software, we found several *MYCN*-regulated miRNAs that could target the 3'UTR sequence of *DKK3*, including *mir-92a*, *mir-92b* and *let-7e*. Luciferase expression from a reporter vector containing the *DKK3*-3`UTR was decreased when this construct was cotransfected with *mir-92a*, *mir-92b* and *let-7e* in HEK293 cells. Mutation of the *mir-92* seed sequence in the 3'UTR completely rescued the observed decrease in reporter expression when cotransfected with *mir-92a* and *mir-92b*. Antagomir and miRNA-mimic transfections in neuroblastoma cell lines confirmed that DKK3 secretion to the culture media is regulated by these miRNAs.

Consistent with reports from other cancers, we found DKK3 to be expressed in the endothelium of primary neuroblastoma samples and to be absent in tumors with *MYCN* amplification. These data demonstrate a previously unknown tumor promoting mechanism for *MYCN*-regulated miRNAs.

Keywords: *MYCN*, neuroblastoma, miRNA, *DKK3*

Introduction

Neuroblastoma accounts for 7% of childhood malignancies and 15% of pediatric cancer-related deaths. It is a heterogeneous disease, ranging from spontaneous regression to metastatic tumors resistant to multimodal treatment, as reviewed in (Maris *et al.*, 2007). The clinical outcome depends on several prognostic factors, with gene copy amplification of the *MYCN* oncogene being the most consistent marker of aggressive disease (Brodeur and Seeger, 1986). *MYCN* is a member of the MYC oncogene family, which also consists of *c-myc* and *MYCL* (Schwab, 2004; Schwab *et al.*, 1983), and functions by up and downregulating genes directly by transcriptional binding or through indirect pathways or mediators. It is known to regulate a wide range of microRNAs (Buechner *et al.*, 2010; Loven *et al.*, 2010; Mestdagh *et al.*, 2010; Schulte *et al.*, 2008) and is responsible for the methylation of target genes by activating methyltransferases, as well as affecting the chromatine structure (Liu *et al.*, 2007; Marshall *et al.*, 2010).

MicroRNAs (miRNAs) are small non-coding RNAs which participate in diverse biological processes including tumorigenesis by sequence-specific targeting of particular mRNAs, primarily in the 3' untranslated region (UTR) (Bartel, 2009; Kwak *et al.*, 2010). MiRNAs negatively regulate protein production by translational repression, mRNA destabilization or a combination of both (Filipowicz *et al.*, 2008). In mammals, miRNAs were recently shown to act predominantly through mRNA degradation (Guo *et al.*, 2010).

DKK3 is a member of the Dickkopf family of secreted Wnt antagonists, including DKK1-4 and Soggy, a member resembling DKK3. The functions of other members of this family have been well elucidated, although the role of DKK3 still remains unclear

(Barrantes Idel *et al.*, 2006; Niehrs, 2006). DKK3 is an N-glycosylated, secreted, tumor suppressor protein which inhibits proliferation, is of prognostic significance in neuroblastoma (Koppen *et al.*, 2008; Revet *et al.*, 2009) and functions as a tumor suppressor in a range of other tumors (Fong *et al.*, 2009; Hoang *et al.*, 2004; Hsieh *et al.*, 2004; Lee *et al.*, 2009; Maehata *et al.*, 2008; Mizobuchi *et al.*, 2008; Saeb-Parsy *et al.*, 2008; Veeck *et al.*, 2009; Yue *et al.*, 2008). The tumor suppressor mechanisms of DKK3 include inhibition of the canonical Wnt signaling by blocking β-catenin translocation to the nucleus (Caricasole *et al.*, 2003; Hoang *et al.*, 2004; Lee *et al.*, 2009; Yue *et al.*, 2008). However, DKK3 does not seem to affect Wnt/β-catenin signaling in neuroblastoma, thereby suggesting other mechanisms to suppress tumorigenicity in this tumor (Koppen *et al.*, 2008). This indicates that DKK3 has functions beyond mere canonical Wnt inhibition.

Previous reports have demonstrated that *MYCN* downregulates *DKK3* at the mRNA level in neuroblastoma by inducing *MYCN* expression in non-amplified cell lines (Bell *et al.*, 2007; Koppen *et al.*, 2008). The expression of *DKK3* mRNA in cell lines and tumor material was inversely correlated with *MYCN*, and variations in *DKK3* were solely attributed to changes in *MYCN* expression. No direct promoter binding was identified, which suggests an indirect regulatory mechanism (Koppen *et al.*, 2008). We therefore hypothesized that *MYCN* suppresses *DKK3* through miRNAs.

Results

MYCN Knockdown Increases Secretion of DKK3 Proteins in MYCN-amplified Neuroblastoma Cell Lines

The expression of *Dickkopf-3* (*DKK3*) mRNA has previously been shown to be inversely correlated with *MYCN* mRNA expression in neuroblastic tumors and neuroblastoma cell lines (Bell *et al.*, 2007; Koppen *et al.*, 2008).

Using a tetracycline-inducible retroviral *MYCN*-shRNA expression system, we observed that *MYCN* knockdown increased the expression of *DKK3* mRNA in the *MYCN*-amplified (MNA) neuroblastoma cell lines SK-N-BE(2) and Kelly (**Figure 1a**). Since DKK3 is a secreted glycoprotein, we further investigated DKK3 protein levels in culture supernatants from SK-N-BE(2) and Kelly cells upon induced *MYCN* knockdown. As shown in **Figures 1b and 1c,** increased levels of secreted DKK3 protein were detected. Similarly, SHEP Tet21N, a derivative of the *MYCN* single-copy SHEP neuroblastoma cell line containing a constitutively expressed doxycycline-repressible *MYCN* gene, revealed increased DKK3 secretion into the culture media when induced to suppress *MYCN* expression (**Figure 1d**).

These data clearly show an inverse correlation between *MYCN* expression and secretion of DKK3 proteins in neuroblastoma cell lines.

Methylation Status of the DKK3 Promoter in Neuroblastoma

Inactivation of *DKK3* expression by promoter methylation has been shown in several cancers (Ding *et al.*, 2009; Lee *et al.*, 2009; Veeck *et al.*, 2008). Because of this, we analyzed the methylation status of the *DKK3* promoter in ten primary neuroblastoma (five MNA, five non-MNA) samples and five neuroblastoma cell lines (three MNA, two non-MNA) using methylation-specific PCR (MSP). The breast cancer cell lines MDA-MB231 and HS578T were used as methylated and non-methylated *DKK3*

promoter controls, respectively (Veeck *et al.*, 2008). As shown in **Figure 2**, all neuroblastoma tumor samples and cell lines, irrespective of their *MYCN* status, showed specific amplification of the 150 bp band generated by the non-methylated primers.

These data demonstrate that the expression of *DKK3* is not regulated by promoter methylation in neuroblastoma.

MYCN-regulated miRNAs are Potential Regulators of DKK3 Expression

In order to investigate whether *MYCN* regulates *DKK3* expression indirectly through miRNAs, we used three independent miRNA target prediction programs (TargetScan v5.1, Diana-microT 3.0 and MicroCosm) to identify miRNAs that could target the 3'UTR of *DKK3* transcripts. The analyses revealed that *mir-92a*, *mir-92b* and *mir-32* were predicted to target the *DKK3*-3'UTR at position 25-31 (site-1) by all three programs used. In addition, *let-7* and *mir-98* were predicted to target a seed sequence at position 550-556 (site-2) by Targetscan 5.1 and Diana-microT 3.0, while *mir-363* was predicted to target site-1 by Targetscan 5.1 and MicroCosm (**Supplementary Figure 1a)**. We have recently shown that *mir-92a*, *mir-92b* and *let-7* expression levels in MNA neuroblastoma cell lines are regulated by *MYCN* (Buechner *et al.*, 2010).

Mir-92a, mir-92b and let-7e Target the 3'UTR Sequence of DKK3

Several reports have shown a correlation between *MYCN* and *mir-92a* expression in neuroblastoma. *Mir-92a* is a member of the oncogenic *mir*-17-92 cluster that has been

documented to be transcriptionally activated by *MYCN* (Fontana *et al.*, 2008; Loven *et al.*, 2010; Schulte *et al.*, 2008). We recently reported that both *mir-92a* and *mir-92b* are downregulated when *MYCN* expression is repressed by anti-*MYCN* shRNAs in MNA neuroblastoma cell lines (Buchner *et al.*, 2010). Since *mir-92a* and *mir-92b* are both inversely correlated with *MYCN* expression and predicted to target *DKK3*, we decided to investigate these miRNA:*DKK3*-3'UTR interactions experimentally. In addition, we also investigated the binding of *let-7e* to the target sequence at site-2 of the *DKK3*-3'UTR.

The full-length 3'UTR sequence of *DKK3* was cloned downstream of the *Firefly* luciferase gene in the pMIR-REPORT vector (pMIR-DKK3). HEK-293 cells were cotransfected with pMIR-DKK3 and microRNA mimics of *mir-92a, mir-92b, let-7e* or a negative control miRNA (mir-NC). Luciferase assays revealed that an overexpression of *mir-92a, mir-92b* and *let-7e* significantly reduced luciferase activity from the reporter vector when compared to the negative control miRNA (**Figure 3**). We observed a 38%, 45% and 30% decrease in luciferase activity with *mir-92a, mir-92b* and *let-7*e, respectively. These results indicate efficient repression of the luciferase *DKK3*-3'UTR reporter since large-scale proteomics studies have revealed that most miRNA:mRNA interactions result in the silencing of 30% or less (Baek *et al.*, 2008).

To test whether *mir-92a, mir-92b* and *let-7e* downregulated the luciferase activity of pMIR-DKK3 through binding to the predicted target sites, we mutated the putative seed sequences at site-1 and site-2. When the site-1 mutated version of pMIR-DKK3 (mut mir-92 seed) was coexpressed with *mir-92a* or *mir-92b*, the luciferase activity was rescued to levels similar to those observed for the negative control miRNA

(**Figures 3a and 3b**). Only a slight rescue of luciferase activity was observed when the site-2 mutated reporter vector (mut let-7 seed1) was cotransfected with *let-7e* (**Figure 3c**). In searching for alternative *let-7e* seed sequences in the *DKK3*-3'UTR sequence, the RNA22 miRNA software detected a new candidate seed sequence at position 180-185 (site-3) (Miranda *et al.*, 2006) (**Supplementary Figures 1b and 1c**). The mutated version of site-3 alone (mut let-7 seed2), or in combination with the mutated site-2 (mut let-7 seed1+2), did not result in the major rescue of luciferase activity (**Figure 3c**).

Since *mir-92a* is coexpressed with the other members of the mir-17-92 cluster, we also examined the effect of these miRNAs on the 3'UTR sequence of *DKK3*. Cotransfection of pMIR-DKK3 and the individual mir-17-92 miRNA mimics resulted in a slight decrease in luciferase activity by *mir-19a* and *-19b*, which was predicted to target *DKK3* by Targetscan. Cotransfections with *mir-17* or *mir-20a* had no effect when compared to the negative control. We unexpectedly observed an increase in luciferase activity when the reporter vector was cotransfected with *mir-18a* (**Supplementary Figure 2**).

DKK3 Secretion is Regulated by mir-92a, mir-92b and let-7e in Neuroblastoma Cell Lines

To investigate how changes in the expression of *MYCN*-regulated miRNAs influence secretion of DKK3 in neuroblastoma cell lines, we transfected the MNA neuroblastoma cell lines SK-N-BE(2) and Kelly with antimir-92a and -92b (antagomirs) to reduce the endogenous expression of these miRNAs. A stem-loop qRT-PCR assay revealed an approximate 50% decrease in *mir-92b* expression

compared to a negative control antagomir (antimir-NC) (**Supplementary Figure 3a**). After 72 h of antagomir treatment, secretion of DKK3 into the culture media was measured. As shown in **Figure 4a**, suppression of both *mir-92a* and *mir-92b* resulted in a 2-2.5 fold increase in DKK3 secretion.

Next, we transfected the non-MNA neuroblastoma cell lines SH-SY-5Y and SK-N-AS with the *mir-92a*, *mir-92b* and *let-7e* miRNA mimics. Transfection of SH-SY-5Y with the *mir-92b* mimic increased the cellular *mir-92b* levels approximately 100 fold (**Supplementary Figure 3b**). The overexpression of *mir-92a* and *mir-92b* resulted in a major reduction of secreted DKK3 proteins from SH-SY-5Y and SK-N-AS cells, while *let-7e* overexpression revealed a minor, but significant decrease (**Figure 4b**). Consistent with these results, *DKK3* mRNA levels were reduced when SH-SY-5Y cells were transfected with miRNA mimics of *mir-92a*, *mir-92b* and *let-7e* (**Supplementary Figure 4**).

In summary, these data show that the *MYCN*-regulated miRNAs *mir-92a*, *mir-92b* and *let-7e* reduce secretion of DKK3 proteins from human neuroblastoma cell lines.

DKK3 is Expressed in Neuroblastoma Vasculature

We investigated 25 primary neuroblastoma tissue samples from various biological subsets and clinical stages for the expression of DKK3 (**Supplementary Table 2)**. Twenty of 26 samples revealed specific DKK3 staining of the endothelial cells of the tumor, though no staining was seen in the tumor cells themselves (**Figure 5a**). Co-staining with CD31 (an endothelial marker) demonstrated a co-localization to the endothelium. The immunopositivity for DKK3 in the endotehelial cells was of a

different intensity between different tumors. The unfavorable MNA tumors demonstrated a very low or absent staining (**Supplementary Table 2**). Four ganglioneuromas were investigated and all were immunopositive for DKK3 in the tumor-derived ganglion cells, but not in the surrounding stroma (**Figure 5b, Supplementary Table 2**).

Discussion

MYCN-regulated miRNAs Decrease DKK3 Secretion from Neuroblastoma Cell Lines

DKK3 is an established tumor suppressor gene that inhibits the proliferation of several cancers, including neuroblastoma (Abarzua *et al.*, 2005; Chen *et al.*, 2009; Koppen *et al.*, 2008; Kurose *et al.*, 2004; Mizobuchi *et al.*, 2008; Sakaguchi *et al.*, 2009). It has previously been shown that *DKK3* mRNA levels are inversely correlated to *MYCN* mRNA expression in neuroblastic tumors and neuroblastoma cell lines (Bell *et al.*, 2007; Koppen *et al.*, 2008). In our study, we used an inducible retrovirally delivered anti-*MYCN* shRNA to downregulate *MYCN* expression in two MNA neuroblastoma cell lines, and observed increased secretion of the endogenous DKK3 protein into the cell culture media. We also documented that the *MYCN*-regulated microRNAs, *miR-92a*, *mir-92b* and *let-7e*, efficiently decreased expression of a luciferase reporter containing the 3'UTR sequence from *DKK3*. The predicted target seed sequence for *mir-92a* and *mir-92b* in the *DKK3*-3'UTR sequence was validated by mutagenesis. Two candidate seed sequences were found for *let-7e*. Mutation of the putative let-7e seed sequences, whether alone or in combination, could not rescue the *let-7e* repression of luciferase reporter expression similar to that observed for the *mir-92*

seed sequence. This indicates that other as yet unidentified *let-7e* seed sequences and/or *let-7e* targets are responsible for the majority of the observed *DKK3* repression by *let-7e*. By the use of miRNA mimics and antagomirs treatment, we further demonstrated that both *DKK3* mRNA expression and protein secretion into the media were inversely correlated to *mir-92a*, *mir-92b* and *let-7e* expression in neuroblastoma.

Mir-92a is a member of the oncogenic mir-17-92 cluster, which consists of six coexpressed miRNAs: *mir-17*, *mir-18a*, *mir-19a, mir-19b*, *mir-20 and mir92a* (Mendell, 2008). Members of this cluster have been shown to be aberrantly expressed and to promote tumorigenicity in neuroblastoma (Beveridge *et al.*, 2009; Fontana *et al.*, 2008), as well as in other types of cancer (Diosdado *et al.*, 2009; Hayashita *et al.*, 2005). Several reports have documented a correlation between the expression of *MYCN* and the mir-17-92 cluster (Fontana *et al.*, 2008; Loven *et al.*, 2010; Schulte *et al.*, 2008). With the exception of *mir-92a,* and to some extent *mir-19a* and *-19b*, none of the other mir-17-92 members reduced expression of the *DKK3* 3'UTR luciferase reporter. On the contrary, we observed an unexpected increase in luciferase activity when *mir-18a* was overexpressed. A similar observation has previously been reported and was proposed to be due to interference between *mir-18a* and regulation of the CMV promoter (Vreugdenhil *et al.*, 2009).

The DKK3 Promoter is Not Inactivated by Hypermethylation in Neuroblastoma

Hypermethylation of CpG islands in the promoter regions of genes is a powerful mechanism for transcriptional repression (reviewed in Illingworth and Bird, 2009). Nonetheless, the differential methylation of promoters is not a general mechanism for regulating gene expression since most inactive promoters remain unmethylated

(Weber *et al.*, 2007). *DKK3* is frequently inactivated by promoter methylation in cancers of the gastrointestinal tract, lung, cervix and breast (Ding *et al.*, 2009; Kobayashi *et al.*, 2002; Lee *et al.*, 2009; Maehata *et al.*, 2008; Sato *et al.*, 2007; Veeck *et al.*, 2009; Yue *et al.*, 2008). For the neuroblastic tumors arising from the sympathetic adrenal lineage of the neural crest, increased *DKK3* mRNA levels have been reported as a strong marker of differentiation. The well-differentiated ganglioneuromas showed a high *DKK3* mRNA expression, while the undifferentiated neuroblastomas showed a low *DKK3* expression, which also correlated with a bad prognosis (Koppen *et al.*, 2008)

The c-myc protein has previously been shown to repress gene expression through promoter hypermethylation by interaction with Miz-1 and Dnmt3a (Brenner *et al.*, 2005). Furthermore, global *MYCN* transcription factor binding analysis in neuroblastoma has revealed an association of the MYCN protein to regions of DNA hypermethylation (Murphy *et al.*, 2009).

We analyzed the methylation status of the *DKK3* promoter in 10 neuroblastoma primary tumors and five neuroblastoma cell lines using methylation-specific PCR (MSP). The results revealed that neither the primary tumors nor the cell lines were hypermethylated at the investigated CpG island of the *DKK3* promoter. In addition, a chromatin immunoprecipitation (ChIP) analysis performed by others failed to reveal a direct interaction between the MYCN protein and regulatory elements in the *DKK3* promoter (Koppen *et al.*, 2008). Low levels of DKK3 have also previously been reported in the absence of promoter methylation in malignant melanoma cell lines (Kuphal *et al.*, 2006).

These findings indicate that DKK3 levels in neuroblastoma are regulated by mechanisms other than promoter hypermethylation. Specifically, we have documented that *mir-92a*, *mir-92b* and *let-7e* target the 3'UTR sequence of *DKK3* to repress its expression.

DKK3 Expression in Tumor Endothelium is Inversely Correlated with *MYCN* Levels

Recently, several papers have shown *DKK3* to be involved in tumor vessel biology and to be highly expressed in tumor endothelium (Fong *et al.*, 2009; Muhlmann *et al.*, 2010; Pei *et al.*, 2009; Untergasser *et al.*, 2008; You *et al.*, 2010; Zenzmaier *et al.*, 2008). Consistent with these findings, we demonstrate an increased expression of DKK3 in neuroblastoma vasculature. It has been suggested that this vascular expression of DKK3 antagonizes the inactivation that frequently takes place in tumor cells during malignant transformation (Zenzmaier *et al.*, 2008). DKK3 has also been reported to stimulate vascular growth and increase vascular density in tumors (Untergasser *et al.*, 2008). The underlying mechanisms are not known, but bioinformatic analyses have indicated that DKK3 contains a cysteine-rich prokineticine domain also present in the potent angiogenic endocrine gland-derived vascular endothelial growth factor (EG-VEGF).

We observed that the expression of the DKK3 protein in neuroblastoma vasculature was significantly higher in non-MNA tumors and more benign ganglioneuroma in comparison to MNA tumors. This is in line with a previous report by Valentijn and co-workers, who demonstrated higher levels of *DKK3* mRNA expression in both non-MNA neuroblastoma tumors and ganglioneuromas (Koppen *et al.*, 2008).

The mir-17-92 cluster has been related to angiogenesis (Dews *et al.*, 2006; Doebele *et al.*). A recent work demonstrated that the *in vivo* inhibition of *mir-92a* enhanced blood vessel formation (Bonauer *et al.*, 2009). Here, we provide one possible explanation for this observation since DKK3 has been shown to stimulate angiogenesis (Untergasser *et al.*, 2008).

An increased serum level of *mir-92a* has also been reported to be of diagnostic value in colorectal cancer (Huang *et al.*, 2009), although *mir-92a* has been shown to be downregulated in the serum of patients with acute lymphatic leukemia (Tanaka *et al.*, 2009). The *mir-92a* homologue, *mir-92b*, is a marker for primary brain tumors and regulates cell cycle control (Nass *et al.*, 2009; Sengupta *et al.*, 2009). DKK3 is present in high levels in the adult brain and central nervous system (Krupnik *et al.*, 1999), thereby making it plausible that the link between *mir-92b* and *DKK3* may play a contributing role in these cancers and deserves further investigation. *Mir-92b* is also a negative regulator of PRMT5, which is an epigenetic regulator of several tumor suppressors in vivo (Pal *et al.*, 2007).

In some cellular settings, DKK3 has been shown to stimulate growth and inhibit apoptosis. Additionally, DKK3 could also stimulate or inhibit the canonical wnt pathway, depending on the cellular context (Nakamura and Hackam, 2010; Nakamura *et al.*, 2007). These contradictory findings illustrate the complex and cell specific nature of DKK3, so it therefore not surprising to find that *DKK3* is regulated by miRNAs with postulated oncogenic as well as tumor suppressor functions.

Material and Methods

Cell Cultures and miRNA Overexpression

The human SH-SY-5Y neuroblastoma cell line was cultivated in a DMEM/Ham'sF12 medium (1:1) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine and 1% NEAA (non-essential amino acid). The HEK-293 cell line was grown in DMEM supplemented with 10% FBS. The SK-N-AS, SK-N-BE(2), Kelly and SHEP Tet21N cell lines were grown in RPMI-1640 medium supplemented with 10% FBS. SHEP Tet21N cells express high *MYCN* in the absence of doxycycline (Lutz *et al.*, 1996). To switch off *MYCN* expression, 1 µg/ml doxycycline was added to the growth media at least 24 hours prior to the experiments. All cells were split before confluence and incubated at 37˚C in 5% humidity and 4.5% CO_2.

MicroRNA mimics (*let-7e*, *mir-92a*, *-92b*, *-17*, *-18a*, *-19a*, *-19b* and *-20*a) and antagomirs (antimir-92a and -92b), with corresponding negative controls, were from Shanghai GenePharma, (Shanghai, China). Transfections of miRNA mimics and antagomirs were carried out using Lipofectamine-2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

MYCN Knockdown

SK-N-BE(2) and Kelly cells stably transfected with pcDNA6/TR (constitutively expressing the Tetracycline Repressor) were transduced with a retrovirus (RV-aMN1658) containing a tetracycline-inducible anti-*MYCN* shRNA (aMN1658) expression module (Henriksen *et al.*, 2010). Retroviruses containing an inducible scrambled shRNA (SCR) were used as a negative control, and cells were cultivated as previously described (Henriksen *et al.*, 2007). Expression of the shRNAs was induced by adding 1 µg/ml doxocycline to the culture medium.

Human Tissue Samples

Tissue samples from tumors were obtained from neuroblastoma patients during surgery, snap-frozen in liquid nitrogen and transferred to storage at -80°C for future analysis. Twenty-six neuroblastoma samples derived from children of different ages and all clinical stages, including different biological subsets, were analyzed (**Supplementary Table 2**). Five childhood ganglioneuromas from children aged 12-25 months were also included. Genomic DNA was extracted from frozen (-70°C) tumor tissue according to standard procedures, and ethical approval was obtained by the Karolinska University Hospital Research Ethics Committee (approval 03-308).

Methylation-specific PCR (MSP)

DNA (100 ng) from neuroblastoma tumors or cell lines was bisulfite-treated using the EZ methylation gold kit (Zymo Research, CA, USA), and further purified using the DNA Clean and Concentrate Kit (Zymo). Previously published primers, specific to the methylated (ON-479/ON-480) and unmethylated sequence (ON-481/ON-482), were used to perform a methylation-specific PCR (MSP) on the promoter region of *DKK3* (Sato *et al.*, 2007). The primer sequences are listed in **Supplementary Table 1.** The Zymotaq premix polymerase (Zymo) was used in a 25 µl reaction, including 50 ng of treated template. Products were visualized on ethidium bromide-stained 3% agarose gels. We included DNA from the MDA-MB231 and HS578t as methylated and unmethylated controls, respectively (Veeck *et al.*, 2008).

In Silico Target Prediction

Diana microT v3.0 (www.microrna.gr/microT), EMBLs MicroCosm Targets v5 (www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/) and TargetScan v5.1 (www.targetscan.org/) target prediction software were used to identify miRNAs that could potentially target the human *DKK3* 3'UTR.

Luciferase Reporter Assay

The *DKK3*-3`UTR region was amplified from human total DNA (neuroblastoma LAN5 cell line) using primers ON-361 and ON-327 that contain in-fusion recombinase overhangs. The insert was cloned into the pMIR-REPORT vector (Ambion, Austin, TX, USA) using the In-Fusion Cloning Kit (Clontech, CA, USA) to generate pMIR-DKK3(wt). Mutations were introduced in the miRNA seed sequences at positions 25-31 (site-1), 550-556 (site-2) and 180-185 (site-3) using the Site-Directed Mutagenesis Kit (Stratagene) with ON-447, ON-505 and ON-507 as primers to generate pMIR-DKK3(mut mir-92 seed), pMIR-DKK3(mut let-7 seed1) and pMIR-DKK3(mut let-7 seed2), respectively. A double mutant, pMIR-DKK3 (mut let-7 seed1 + seed2), was also generated using ON-505 and ON-507 as primers. All vectors were verified by DNA sequencing using ON-005 and ON-363. The primer sequences are listed in **Supplementary Table 1.**

Hek-293 cells grown in 12 well plates were Lipofectamin-2000-transfected with 25 pmoles synthetic miRNA or negative control, 0.05 µg pGL-renilla vector (Promega, WI, USA) and 0.1 µg luciferase-UTR reporter vector (pMIR-DKK3(wt) and mutated versions). Luciferase was measured two days after transfection using the dual

luciferase reporter kit according to the manufacturer's instructions (Promega). Firefly luciferase was normalized against renilla luciferase. All experiments were done in two parallels and at least three times.

Quantitative Polymerase Chain Reaction (qPCR)

SHSY5Y cells were seeded in 6-well plates and transfected with 100 pmoles of synthetic miRNAs. Cells were harvested 48 hours after transfection before total RNA was extracted and DNase-treated using the miRNeasy mini kit (Quiagen Inc., Valencia, CA, USA).

cDNA was created using the miScript reverse transcriptase kit (Qiagen) and 1 ug RNA. Quantitative PCR was performed using the *Power* SYBR Green PCR Master Mix (Applied Biosystems Inc., Carlsbad, CA, USA). *DKK3* mRNA levels were quantified using HPRT1 and UBC as housekeeping genes. 2.5 µl of a 20x diluted RT mix was loaded in a 25 µl reaction. The primer sequences are listed in **Supplementary Table 1.**

Quantification of *mir-92b* was performed on the same cDNA using a miScript SYBR GREEN detection kit with *mir-92b* primer assay (Quiagen). HPRT1 and UBC were used for normalization.

The ABI 7300 (Applied Biosystems) was used as a detection system.

DKK3 Enzyme Linked Immunosorbent Assay (ELISA)

SHSY5Y and SKNAS cells were seeded in 6-well plates, and transfected in three parallels with 100 pmol of synthetic miRNAs or a negative control miRNA. The cell

medium was changed 48 hours after transfection, and the cells were then incubated for another 24 hours before the conditioned medium was harvested for analysis using a DKK3 duoset Elisa assay (R&D Systems, Cambridge, UK). A high standard of 2000 pmoles/ml was chosen and diluted to a low standard of 15 pmoles/ml.

The transfection of antagomirs and the measuring of the DKK3 levels secreted by the SK-N-BE(2) and Kelly cell line were performed in the same manner as the overexpression of synthetic miRNAs (Shanghai GenePharma, China). The cell medium was changed 24 hours after transfection, while the harvesting of total cell protein and culture medium for ELISA was done after another 24 hours.

The human neuroblastoma cell lines Kelly and SK-N-BE(2), containing RV-aMN1658 and RV-SCR constructs, were incubated in the presence of 1 µg/ml doxycycline for six days before analyzing the DKK3 content in the medium (Henriksen *et al.*, 2007). The medium was changed 24 hours before analysis.

Levels of secreted DKK3 were normalized against total protein, measured at the time of harvesting, using the Biorad protein assay (Biorad Laboratories, Hercules, CA, USA).

Immunohistochemistry

Formalin-fixed and paraffin-embedded tissue sections were deparaffinized in xylene and graded alcohols, hydrated and washed in PBS. After antigen retrieval on sodium citrate buffer (pH 6) in a microwave oven, the endogenous peroxidase was blocked by 0.3% H2O2 for 15 min. Sections were incubated overnight at 4°C with a biotinylated goat anti-human DKK3 antibody (R&D Systems). Thereafter, sections were incubated with streptavidin-HRP (Invitrogen) for 30 min at room temperature. A matched

isotype control was used as a control for nonspecific background staining. For co-localization studies of DKK3 and CD31, tumor tissue sections were simultaneously stained with biotinylated anti-human DKK3 and mouse-anti-human CD31 (Dako, Glostrup, Denmark). For fluorescence visualization, streptavidin-Alexa Fluor 594 and anti-mouse Alexa Fluor 488 were used, respectively.

Statistical Analysis

All tests were performed two-sided. Differences between the two groups were studied using the two-sided Student`s *t* test. When more than two treatments were compared, we performed the one-way analysis of variance (ANOVA).

Conflict of Interest:

The authors declare no conflict of interests.

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Supplementary information is available at the Oncogenes website.

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Figure Legends:

Figure 1: ***MYCN*** **knockdown increase** ***Dickkopf-3*** **(*DKK3*) mRNA and secreted protein in neuroblastoma cells - (A)** Real-time qRT-PCR measurements of *DKK3* mRNA levels in SK-N-BE(2) and Kelly cells induced to knock down *MYCN* expression. Secreted endogenous DKK3 proteins from **(B)** SK-N-BE(2) and **(C)** Kelly cells induced to knock down *MYCN* expression, and **(D)** SHEP Tet21N cells induced to repress exogenous *MYCN* overexpression were measured with ELISA.

Figure 2: ***DKK3*** **promoter methylation status in neuroblastoma tumors and cell lines -** Methylation-Specific PCR (MSP) was performed on bisulfite-treated DNA from neuroblastoma tumors and cell lines. Lanes labeled U and M contain PCR products amplified from primers recognizing unmethylated and methylated *DKK3* promoters, respectively. All neuroblastoma samples investigated were unmethylated. DNA from the breast cancer cell lines MDA-MB231 and HS578t were used as methylated and unmethylated controls, respectively (Veeck *et al.*, 2008). NTC represents the no template control. *MYCN*-amplified samples are marked with ▲.

Figure 3: **Luciferase assays for** ***mir-92a, mir-92b*** **and** ***let-7e*** **-** Luciferase activity of HEK293 cells cotransfected with the wild type (pMIR-DKK3 wt) or mutated *DKK3*-3'UTR luciferase vector and miRNA mimics of *mir-92a* **(A)**, *mir-92b* **(B)** or *let-7e* **(C)**. Mut mir-92 seed is mutated in the predicted *mir-92* seed sequence at position 28-

29 in *DKK3*-3'UTR. Similarly, mut let-7 seed1, mut let-7 seed2 and mut let-7 seed1+2 were mutated in the predicted *let-7* seed sequences at position 550-556, 180-185 and 550-556+180-185 in *DKK3*-3'UTR, respectively. A plasmid constitutively expressing Renilla luciferase was used for normalization of the data. Data shown are mean ± SD of the ratio of normalized luciferase activity in miRNA mimic and control transfections. *$P < 0.05$ vs. respective control.

Figure 4: DKK3 ELISA analyses of culture media from neuroblastoma cell lines treated with antagomirs and miRNA mimics - (A) MNA neuroblastoma cell lines SK-N-BE(2) and Kelly were transfected with antagomir-92a (antimir-92a), antagomir-92b (antimir-92b) or a negative control antagomir (antimir-NC). **(B)** Non-MNA neuroblastoma cell lines SH-SY-5Y and SK-N-AS were transfected with *mir-92a*, *mir-92b*, *let-7e* or negative control microRNA (mir-NC) mimics. Secretion of endogenous DKK3 proteins to the culture media was measured using an ELISA kit. Data shown are mean ± SD of the ratio of DKK3 proteins secreted to the culture media normalized to total protein in cell extracts of miRNA mimic/antagomir and control transfections. *$P < 0.05$ compared to antimir-NC or mir-NC.

Figure 5: Immunohistochemical staining of Dickkopf-3 (DKK3) in neuroblastoma tumors - (A) A human neuroblastoma tissue section stained with a red fluorescently labeled (Alexa 594) anti-DKK3 monoclonal antibody, together with a green fluorescently labeled (Alexa 488) anti-CD31 monoclonal antibody. The nuclei were stained with DAPI, which are represented in blue. The merge represents an

overlay view of the DKK3, CD31 and DAPI image; 600x magnification. **(B)** Immunohistochemical staining of DKK3 in neuroblastoma primary tumors and ganglioneuromas showing specific staining of DKK3 in tumor vasculature (left image, sample no. 8, Supplementary Table 2) and in differentiated ganglion cells of a benign ganglioneuroma (right image, sample no. 28, Supplementary Table 2); 600x magnification.

Supplementary Figure 1: MiRNA *in silico* analysis of *DKK3*-3'UTR - (A) Venn diagram displaying miRNAs computationally predicted to target the 3'UTR sequence of *DKK3* by Diana-microT3.0, TargetScan v5.1 and MicroCosm. **(B)** Schematic overview from TargetScan v5.1 of the *DKK3* 3'UTR with conserved miRNA binding sites. **(C)** TargetScan v5.1 predictions of miRNAs that bind to the seed sequences at positions 25-31 (Site-1) and 550-556 (Site-2) of the *DKK3* 3'UTR. The RNA22 miRNA software predicted *let-7e* to target *DKK3*-3'UTR at position 180-186 (Site-3).

Supplementary Figure 2: Luciferase reporter assay for mir-17-92 members - MicroRNA mimics of the individual members of the mir-17-92 cluster were cotransfected with the pMIR-DKK3 vector into HEK293 cells. A plasmid constitutively expressing Renilla luciferase was used for normalization of the data. Data shown are mean ± SD of the ratio of normalized luciferase activity in miRNA mimic and control transfections. $*P < 0.05$.

Supplementary Figure 3: ***Mir-92b*** **expression in SK-N-BE(2) and SH-SY-5Y cells treated with antagomir-92b and** ***mir-92b*** **mimic, respectively - (A)** Relative expression of *mir-92b* in SK-N-BE(2) cells transfected with *mir-92b* antagomirs (antimir-92b). Antimir-NC = negative control antagomir. **(B)** Relative expression of *mir-92b* in SH-SY-5Y cells transfected with miRNA mimics of *mir-92b*. *Mir-92b* was measured using a stem-loop qRT-PCR assay for *mir-92b*. **P* < 0.05.

Supplementary Figure 4: ***DKK3*** **mRNA levels in SH-SY-5Y cells treated with miRNA mimics of** ***mir-92a, mir-92b*** **and** ***let-7e*** **-** Relative expression of *DKK3* mRNA in SH-SY-5Y cells transfected with miRNA mimics of *mir-92b*. *Mir-92b* was measured using a stem-loop qRT-PCR assay for *mir-92b*. **P* < 0.05.

Supplementary Table 1: Overview of the oligonucleotide primers used in this study

Supplementary Table 2: Neuroblastoma tumor and ganglioneuroma samples used in this study - [a]Diagnosis, [b]INSS=International Neuroblastoma Staging System, [c]Patient fulfilling clinico-biological criteria to obtain high-risk therapy, [d]Neuroblastoma, [e]Ganglioneuroma, [f]Non-malignant adrenal gland, [g]Multifocal primary, [h]No evidence of disease, [i]Died from disease, [j]Alive with disease, [k]Died of surgical complications, *EC*: Endothelial cells, *GC*: Ganglion cells

Figure 1

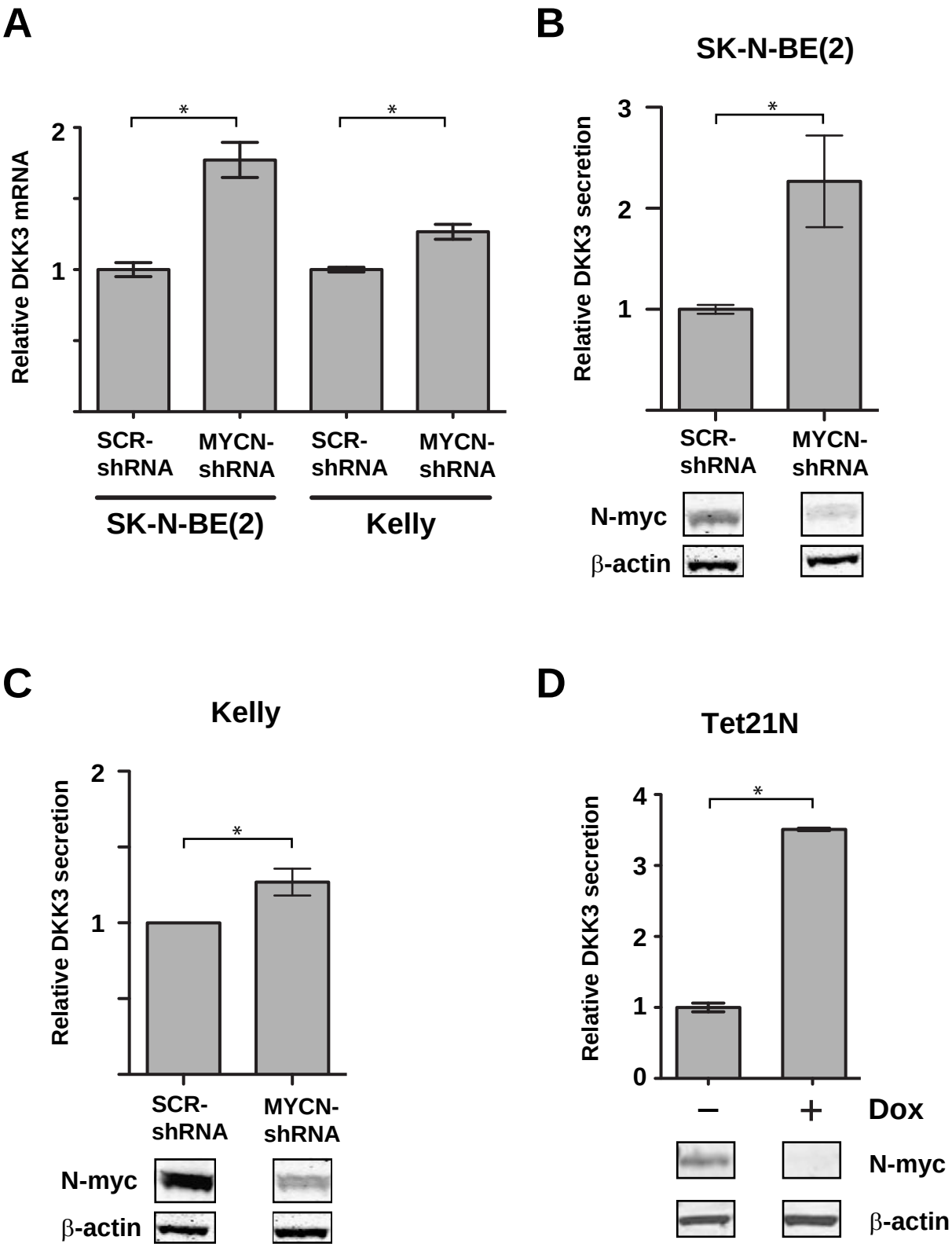

A
Relative DKK3 mRNA
2
1
*
*
SCR-shRNA
MYCN-shRNA
SCR-shRNA
MYCN-shRNA
SK-N-BE(2)
Kelly
B
SK-N-BE(2)
Relative DKK3 secretion
3
2
1
*
SCR-shRNA
MYCN-shRNA
N-myc
β-actin
C
Kelly
Relative DKK3 secretion
2
1
*
SCR-shRNA
MYCN-shRNA
N-myc
β-actin
D
Tet21N
Relative DKK3 secretion
4
3
2
1
0
*
–
+
Dox
N-myc
β-actin

Figure 2

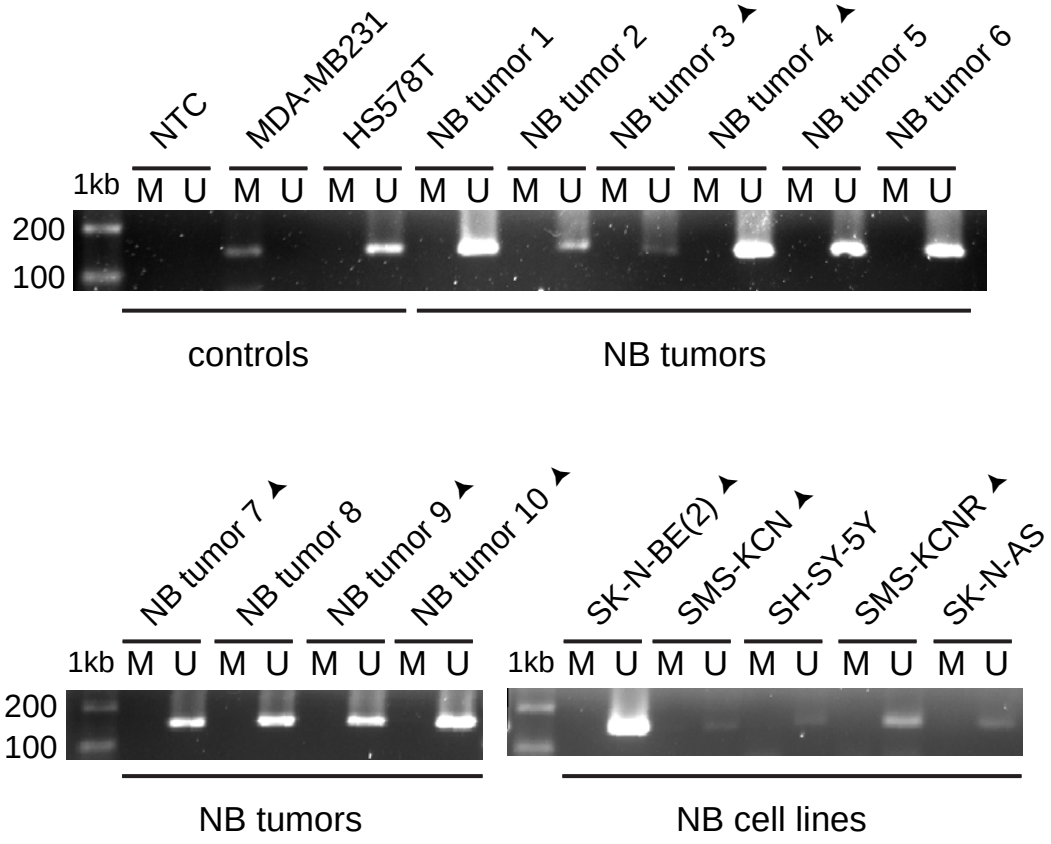

NTC
MDA-MB231
HS578T
NB tumor 1
NB tumor 2
NB tumor 3
NB tumor 4
NB tumor 5
NB tumor 6
1kb
M U M U M U M U M U M U M U M U M U M U
200
100
controls
NB tumors
NB tumor 7
NB tumor 8
NB tumor 9
NB tumor 10
SK-N-BE(2)
SMS-KCN
SH-SY-5Y
SMS-KCNR
SK-N-AS
1kb
M U M U M U M U
1kb
M U M U M U M U M U
200
100
NB tumors
NB cell lines

Figure 3

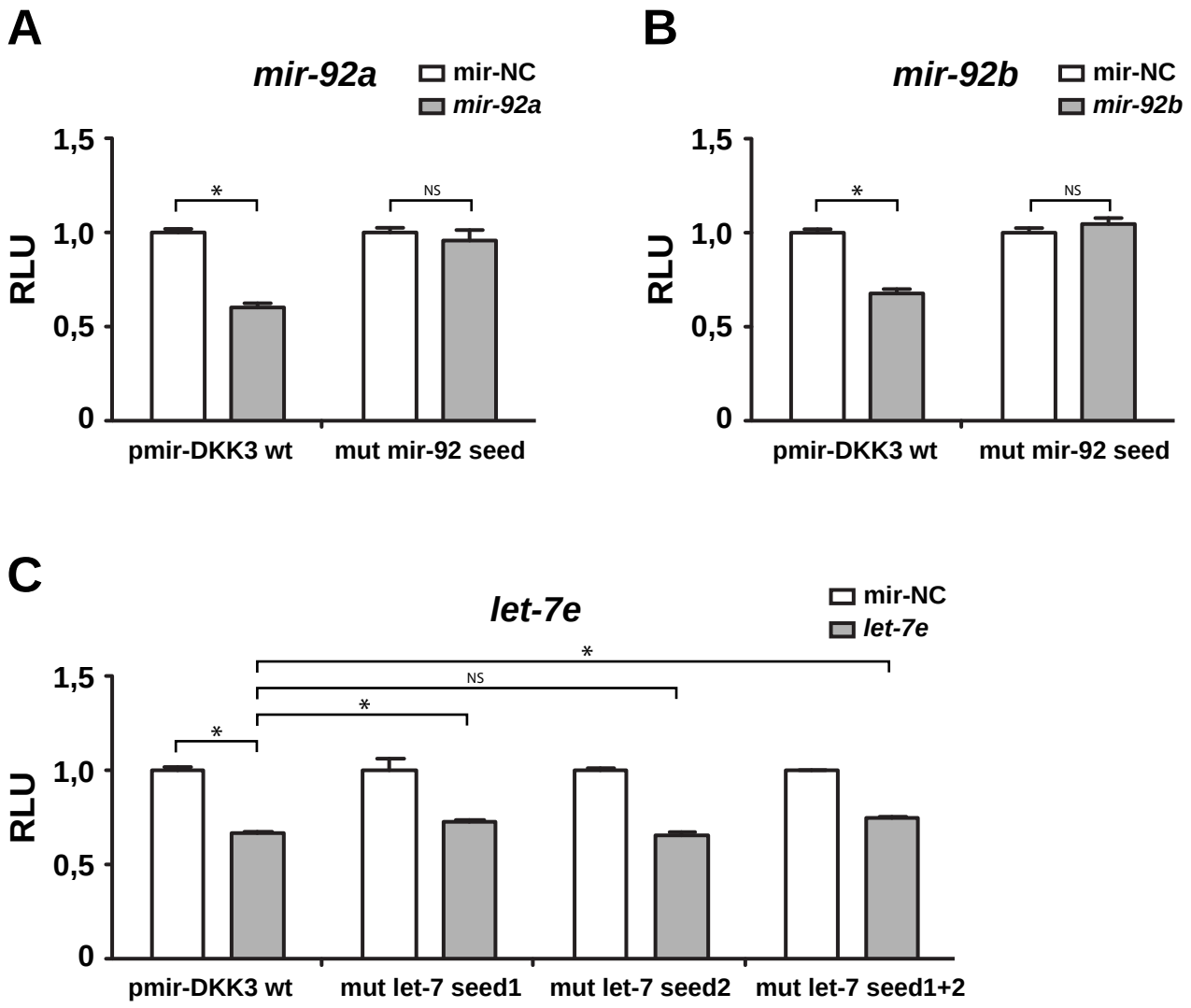

A
mir-92a
mir-NC
mir-92a
RLU
1,5
1,0
0,5
0
*
NS
pmir-DKK3 wt
mut mir-92 seed
B
mir-92b
mir-NC
mir-92b
RLU
1,5
1,0
0,5
0
*
NS
pmir-DKK3 wt
mut mir-92 seed
C
let-7e
mir-NC
let-7e
RLU
1,5
1,0
0,5
0
*
NS
*
*
pmir-DKK3 wt
mut let-7 seed1
mut let-7 seed2
mut let-7 seed1+2

Figure 4

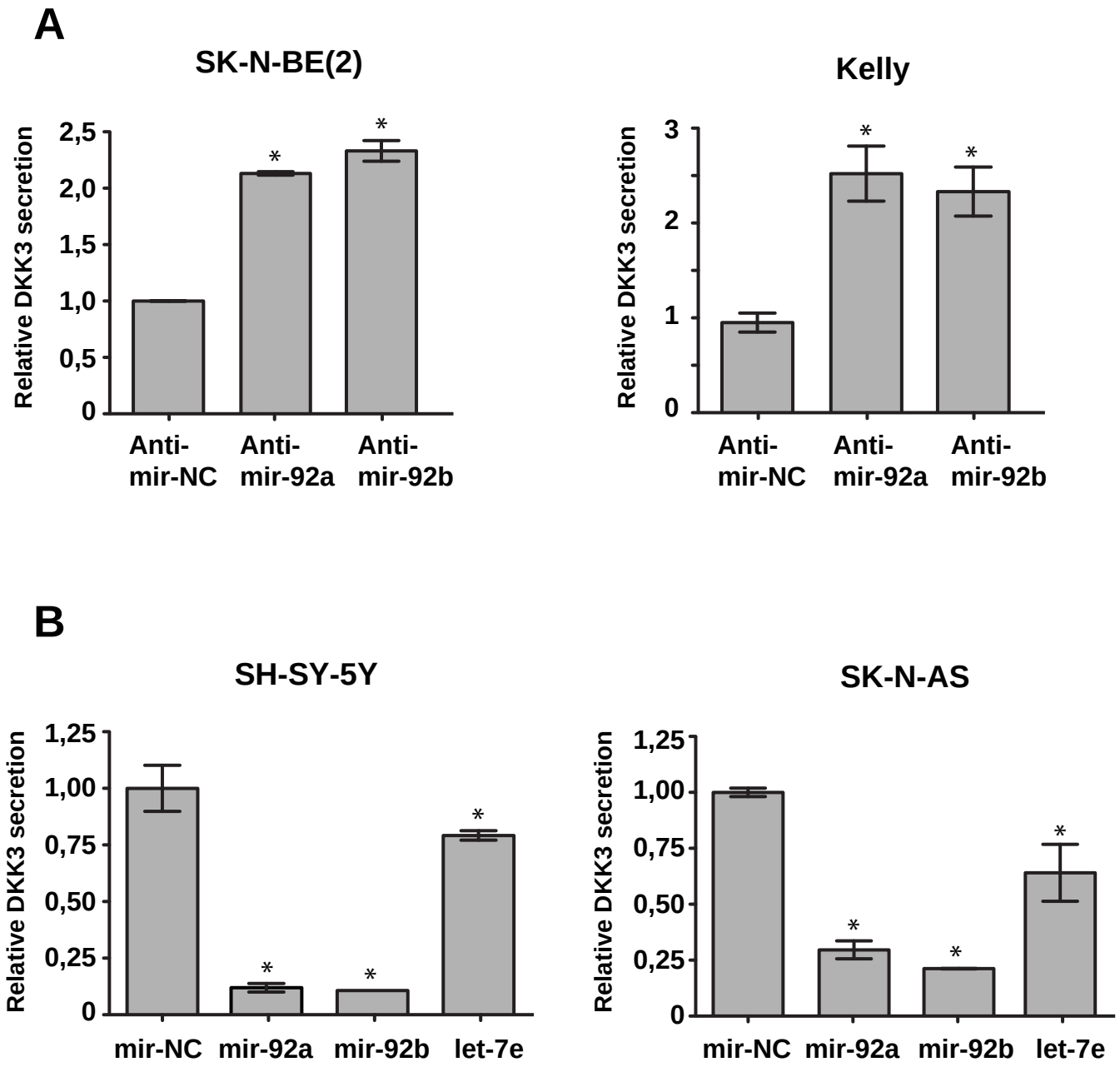
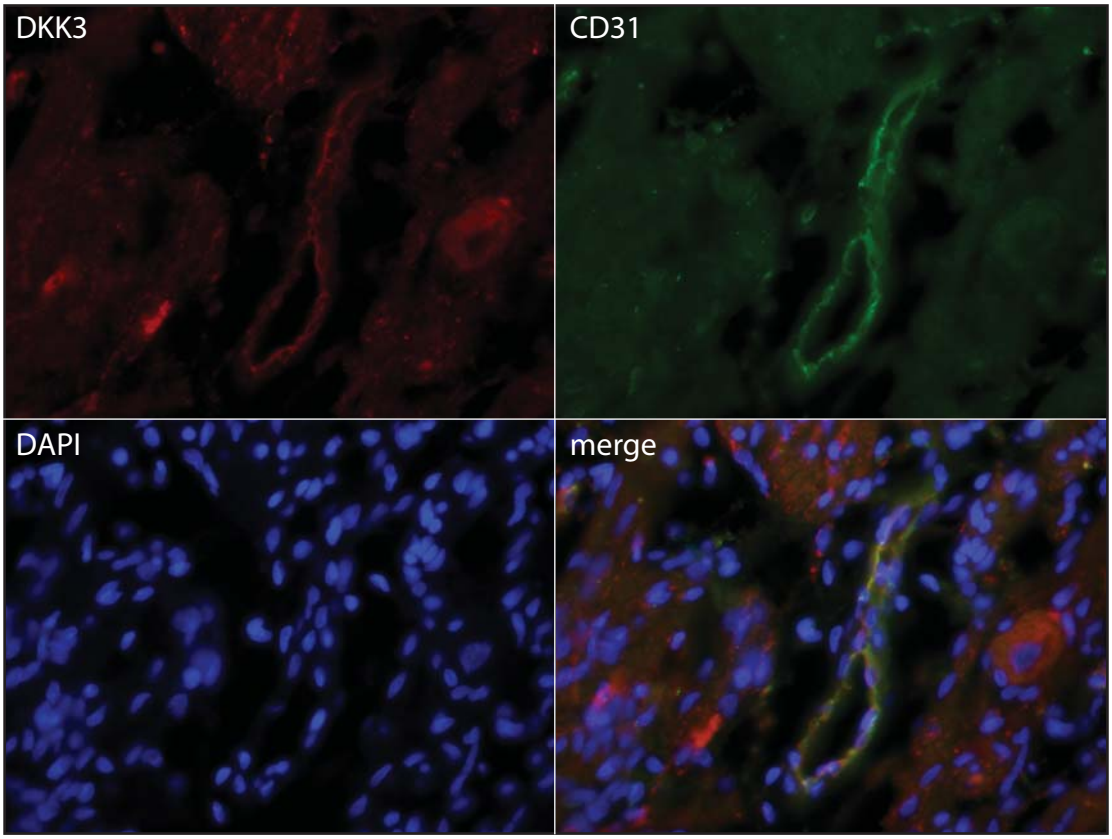
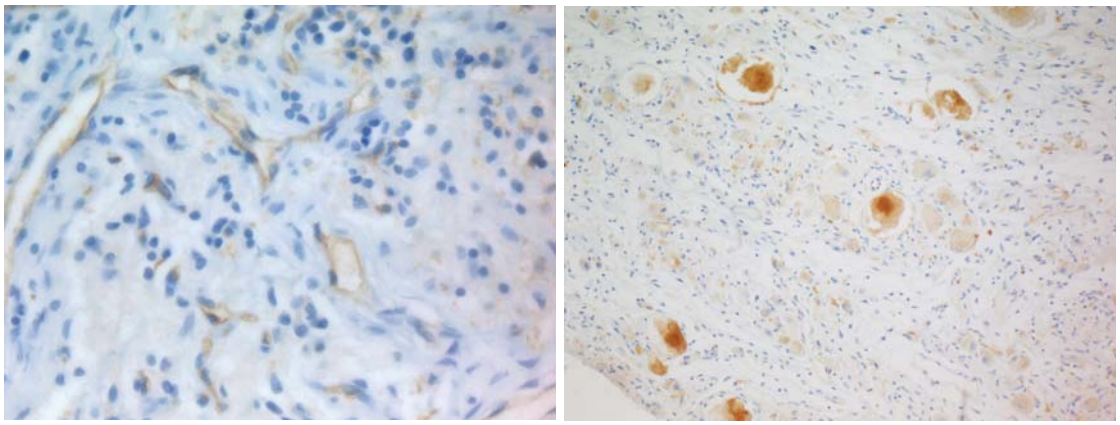

A
SK-N-BE(2)
Relative DKK3 secretion
2,5
2,0
1,5
1,0
0,5
0
*
*
Anti-mir-NC
Anti-mir-92a
Anti-mir-92b
Kelly
Relative DKK3 secretion
3
2
1
0
*
*
Anti-mir-NC
Anti-mir-92a
Anti-mir-92b
B
SH-SY-5Y
Relative DKK3 secretion
1,25
1,00
0,75
0,50
0,25
0
*
*
*
mir-NC
mir-92a
mir-92b
let-7e
SK-N-AS
Relative DKK3 secretion
1,25
1,00
0,75
0,50
0,25
0
*
*
*
mir-NC
mir-92a
mir-92b
let-7e

Figure 5

A

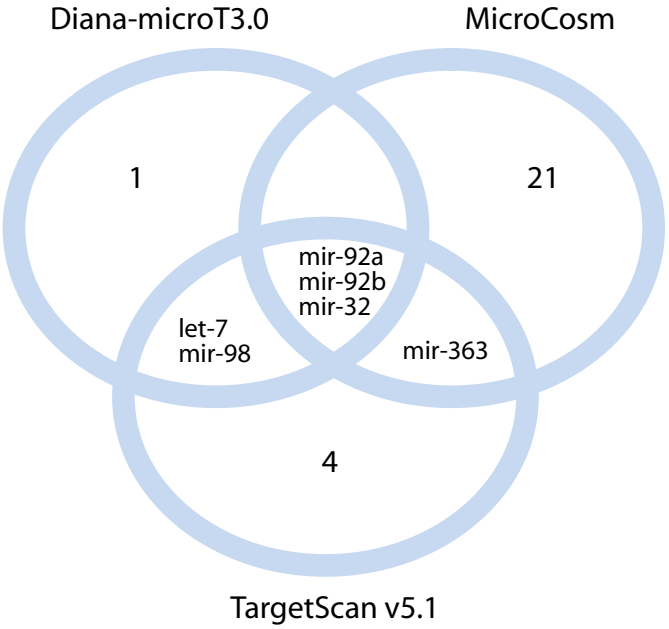


B



Supplementary figure 1

A



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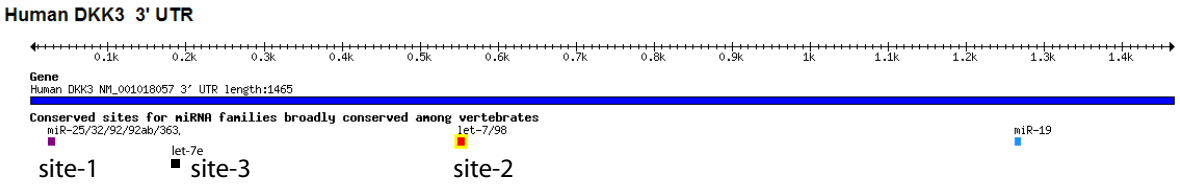


C

Site-1:

Position 25-31 of DKK3 3' UTR
hsa-miR-92a

```
5' ...AGGCUGUGGGUAGAUGUGCAAUA...
                |||      |||||||
3' ...UGUCCGGCCCUGUU-CACGUUAU...
```

Position 25-31 of DKK3 3' UTR
hsa-miR-92b

```
5' ...AGGCUGUGGGUAGAUGUGCAAUA...
                |||      |||||||
3' ...CCUCCGGCCCUGCU-CACGUUAU...
```

Position 25-31 of DKK3 3' UTR
hsa-miR-363

```
5' ...AGGCUGUGGGUAGAUGUGCAAUA...
                |||      |||||||
3' ...AUGUCUACCUAUGG-CACGUUAA...
```

Position 25-31 of DKK3 3' UTR
hsa-miR-32

```
5' ...AGGCUGUGGGUAGAU--GUGCAAUA...
                  |||    |||||||
3'    ...ACGUUGAAUCAUUACACGUUAU...
```

Site-2:

Position 550-556 of DKK3 3' UTR
hsa-let-7e

```
5'  ...AGUGUUGCUCAGCUCCUACCUCU...
                          |||||||
3'    ...UUGAUAUGUUGGAGGAUGGAGU...
```

Position 550-556 of DKK3 3' UTR
hsa-miR-98

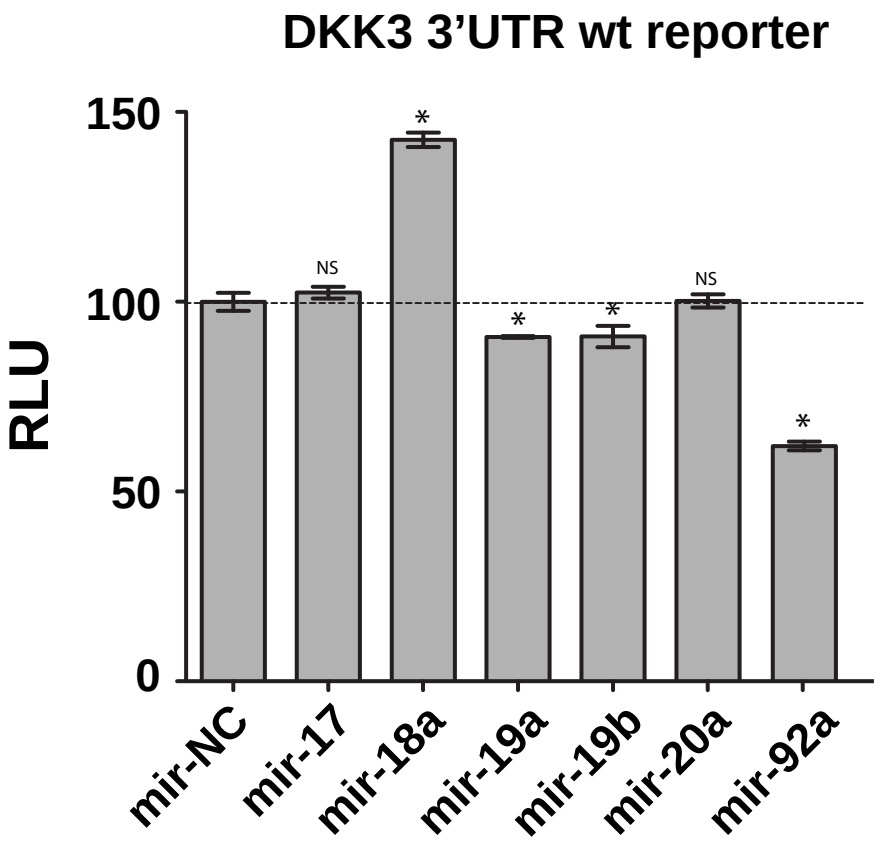
```
5'  ...AGUGUUGCUCAGCUCCUACCUCU...
                          |||||||
3'    ...UUGUUAUGUUGAAUGAUGGAGU...
```

Site-3 (RNA22 predicted):

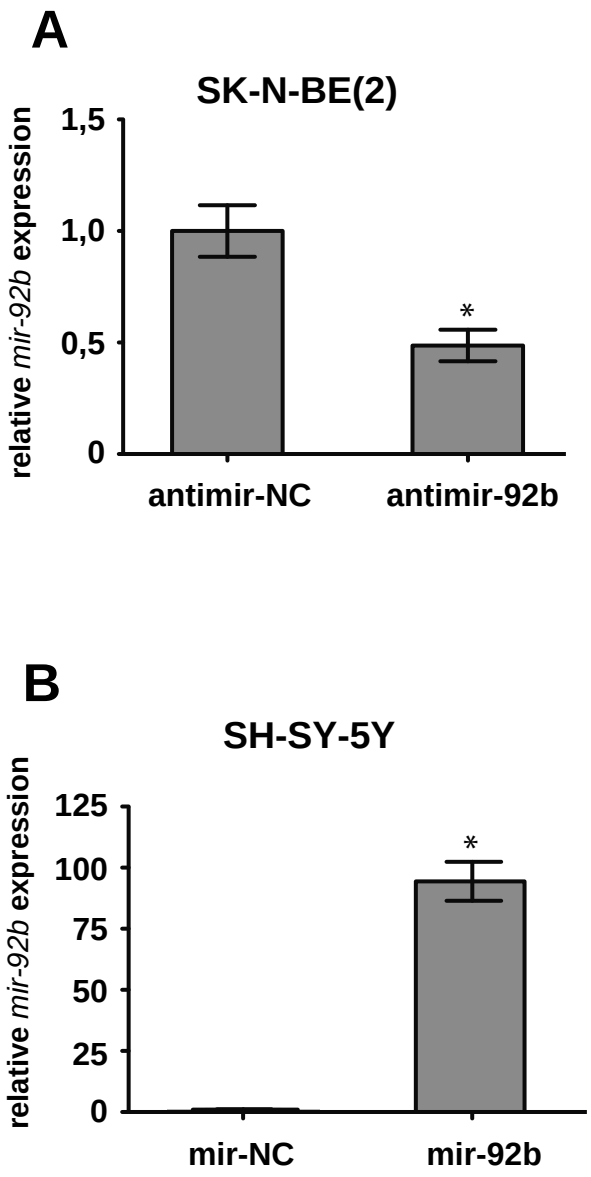
Position 180-185 of DKK3 3' UTR
hsa-let-7e

```
5' ...AGGCUGU----UCUCCAGGCUUCA...
      |||||||    |||||  |||||||
3' ...UUGAUAUGUUGGAGGA-UGGAGU...
```

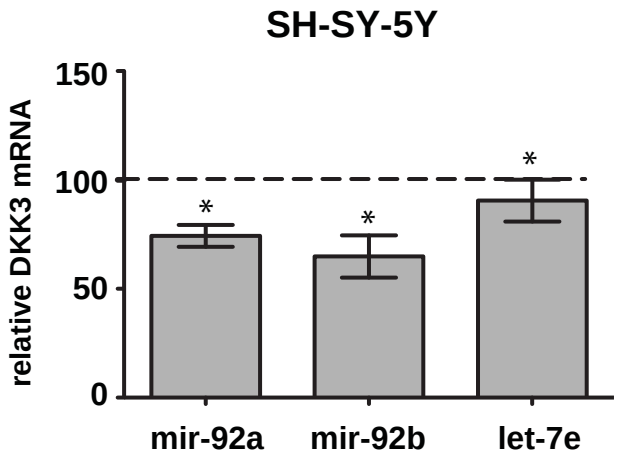
Supplementary figure 2



Supplementary figure 3



Supplementary figure 4



Supplementary Table 1.

Primer:	**Sequence (5'-3'):**	**Description:**
ON-005	ATGGGCGGTAGGCGTGTA	CMV sequencing primer
ON-056	ATTTGGGTCGCGGTTCTTG	qPCR UBC Forward
ON-057	TGCCTTGACATTCTCGATGGT	qPCR UBC Reverse
ON-176	TGACACTGGCAAAACAATGCA	qPCR HPRT1 Forward
ON-177	GGTCCTTTTCACCAGCAAGCT	qPCR HPRT1 Reverse
ON-327	GATCAAGCTTCTATGGAAGATTTTTAATACAGG	DKK3-3`UTR reverse
ON-342	GATGTTCCGCGAGGTTGAGG	qPCR DKK3 Forward
ON-343	CCAACCTTCGTGTCTGTGTTGG	qPCR DKK3 Reverse
ON-361	AAAGCTGCGCACTAGTATCTGGACCAGGCTGTGGGTAGA	Dkk3-3`UTR In-fusion cloning primer
ON-363	ATCCTCATAAAGGCCAAGAA	pMIR-report forward sequencing primer
ON-447	TGTGGGTAGATGTGGTATAGAAATAGCTAA	pMIR-report DKK3-mutagenesis 92a/b
ON-448	GCAGTGTTGCTCAGCTCCTACCAGTGTGCCAGGGCAGC	pMIR-report DKK3- mutagenesis let-7
ON-479	CGAGTAGATTTAGTTCGGTTCGTAGC	Methylated specific forward primer (DKK3)
ON-480	CTTAACGTCGAATCCTACTCGAACG	Methylated specific reverse primer
ON-481	GAGTGAGTAGATTTAGTTTGGTTTGTAGT	Unmethylated specific forward primer
ON-482	CCCCTTAACATCAAATCCTACTCAAACA	Unmethylated specific reverse primer

Supplementary Table 2.

Sample	*DIA*[a]	*AGE Mo*	*Gender*	*Stage INSS*[b]	*MYCN ampl.*	*1p del*	*DNA Ploidy*	*High-risk*[c]	*Outcome*	*DKK3 in EC*
1	NB[d]	21	M	1	no	no	4n	No	NED[h]	+++
2	NB	123	F	1	no	no	3n	No	NED	++
3	NB	7	F	1	yes	yes	2n	No	DOD[i]	-
4	NB	13	M	1	no	no		No	NED	+++
5	NB	18	F	1	no	no		No	NED	++
6	NB	31	M	2B	no	no	3n	No	NED	++
7	NB	33	F	2A	no	no	3n	No	NED	+
8	NB	8	F	2	no	no	3n	No	NED	+++
9	NB	110	M	2	no	no	2n	No	NED	+++
10	NB	5	F	2	no	no	3n	No	AWD[j]	(+)
11	NB	103	F	2B	no	no	2n	No	NED	++
12	NB	6	M	3	no	nd	3n	No	NED	++
13	NB	12	F	3	no	no	5n	No	NED	++
14	NB	0	M	3	no	no	3n	No	DOC[k]	+
15	NB	79	M	3	yes	yes	3n	Yes	NED	(+)
16	NB	136	M	4	yes	yes	2n	Yes	DOD	-
17	NB	39	F	4	yes	yes	2n	Yes	DOD	-
18	NB	35	F	4	no	yes		Yes	NED	-
19	NB	28	M	4	yes	yes	3n	Yes	NED	-
20a	NB	8	M	4_M[g]	no	no	3n	No	NED	+
20b	NB	8	M	4_M	no	no	4n/5n	No	NED	+
21	NB	22	M	4	yes	yes		Yes	DOD	(+)
22	NB	50	F	4	yes	yes		Yes	DOD	(+)
23	NB	0	M	4S	no	no	3n	No	NED	++
24	NB	10	M	4S	no	no	3n	No	NED	+++
25	NB	0	M	4S	no	nd	4n	No	NED	(+)
										DKK3 in GC
26	GN[e]	145	M						NED	+++
27	GN	30	F						AWD	++
28	GN	59	F						NED	+++
29	GN	137	M						NED	++

[a]Diagnosis, [b]INSS=International Neuroblastoma Staging System, [c]Patient fulfilling clinico-biological criteria to obtain high-risk therapy, [d]Neuroblastoma, [e]Ganglioneuroma, [f]Non-malignant adrenal gland, [g]Multifocal primary, [h]No evidence of disease, [i]Dead of disease, [j]Alive with disease, [k]Dead of surgical complications, *EC*: Endothelial cells, *GC*: Ganglion cells