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Epigenetic silencing of Kruppel like factor-3 increases expression of pro-metastatic miR-182

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Abstract

Accumulating evidence indicates that microRNAs (miRs) regulate cancer metastasis. We have shown that miR-182 drives sarcoma metastasis *in vivo* by coordinated regulation of multiple genes. Recently, we also demonstrated that in a subset of primary sarcomas that metastasize to the lung, miR-182 expression is elevated through binding of MyoD1 to the miR-182 promoter. However, it is not known if there are also transcription factors that inhibit miR-182 expression. Defining negative regulators of miR-182 expression may help explain why some sarcomas do not metastasize and may also identify pathways that can modulate miR-182 for therapeutic benefit. Here, we use an *in silico* screen, chromatin-immunoprecipitation, and luciferase reporter assays to discover that Kruppel like factor -3 (Klf-3) is a novel transcriptional repressor of miR-182. Knockdown of Klf-3 increases miR-182 expression, and stable overexpression of Klf-3, but not a DNA-binding mutant Klf-3, decreases miR-182 levels. Klf-3 expression is downregulated in both primary mouse and human metastatic sarcomas, and Klf-3 levels negatively correlate with miR-182 expression. Interestingly, Klf-3 also negatively regulates MyoD1, suggesting an alternative mechanism for Klf-3 to repress miR-182 expression in addition to direct binding of the

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6. Authors' contributions

M.S., R.D., Z.H and D.G.K. designed the experiments and analyzed the data. M.S., C.G and R.D. performed the experiments. Y.M. performed the histopathology. D.M.C. performed the pathological analysis for H&E staining. D.C.L. provided human sarcoma samples. M.S. and R.D. performed statistical analysis of the data. M.S., R.D., and D.G.K. wrote the manuscript. All authors proofread the manuscript.

8. Conflict of Interest

The authors declare that there are no conflicts of interest.

miR-182 promoter. Using Methylation Specific PCR (MSP) and pyrosequencing assays, we found that Klf-3 is epigenetically silenced by DNA hypermethylation both in mouse and human sarcoma cells. Finally, we show the DNA methylation inhibitor 5′Azacytidine (Aza) restores Klf-3 expression, while reducing miR-182 levels. Thus, our findings suggest that demethylating agents could potentially be used to modulate miR-182 levels as a therapeutic strategy.

Keywords

Gene regulation; Epigenetics; microRNA; Metastasis

1. Introduction

Soft tissue sarcomas (STS) are a diverse group of malignant tumors that arise from connective tissues such as bone, muscle, cartilage and nerve sheath. Because patients with STS generally present with localized disease, surgery and radiation therapy are common treatment modalities. However, in one third of STS patients, cancer spreads or metastasizes to distant sites and for many patients cure is not possible [1]. To metastasize, tumor cells locally invade into the stroma, intravasate into the circulation, extravasate into the secondary site, and finally colonize in a distant organ to form a secondary tumor. Currently there are no clinically-available molecular markers that predict which patient sarcomas will develop metastases.

To better understand the molecular mechanisms that mediate metastasis, we performed a screen in a genetically engineered mouse model (GEMM) of soft-tissue sarcoma (STS) using mice with conditional mutations in *Kras* and *p53* (KP mice) [2]. Intramuscular injection of these mice with an adenovirus that expresses Cre recombinase causes soft tissue sarcomas to develop at the site of injection within 2 to 3 months that resemble undifferentiated pleomorphic sarcomas [3]. Using this model system, we amputated primary sarcomas and followed the mice for the development of metastasis. In primary sarcomas that metastasized, we found that many miRNAs were downregulated. miRNAs are small non-coding RNA molecules that are transcribed by RNA polymerase (Pol II) to primary transcripts (pri-miRNAs), which are cleaved to produce stem-loop structured precursors (pre-miRNAs) that are subsequently processed by Dicer into mature miRNAs. Moreover, deleting one allele of Dicer in this model decreased global miRNA expression that promoted metastasis *in vivo* [4]. In addition, we observed that in a subset of mouse sarcomas that metastasized to the lungs, miR-182 is overexpressed [5]. Using novel genetically engineered conditional mutant mice to either delete or overexpress miR-182 in primary sarcomas, we showed that deletion of miR-182 in primary sarcomas significantly decreased, while overexpression of miR-182 significantly increased the rate of lung metastasis [5]. Also, we found that miR-182 was enriched in a subset of human metastatic sarcomas and miR-182 expression correlates with myogenic markers, such as Pax7 and MyoD1, in a subset of metastatic sarcomas [6].

Others have also correlated a high level of miR-182 to cancer progression in a number of other human cancers such as metastatic melanomas, gliomas and follicular carcinomas [7; 8;

9]. Elevated miR-182 correlated with aggressiveness and overall survival in invasive bladder cancer and high-grade prostate cancer [10; 11]. Interestingly, a genetic region spanning human miR-182 (7q32) is amplified in a variety of tumors including sarcomas, prostate cancer and neuroblastoma, which correlated with poor prognosis of these tumors [12]. In contrast, some studies have also found a tumor suppressor role of miR-182 in a tissue dependent manner. For example, one study reported an association of low miR-182 expression with osteosarcoma progression [13]. Another study found that miR-182 suppresses proliferation and invasion of human lung adenocarcinoma cells by targeting human cortical actin-associated protein [14]. Therefore, it is important to understand the regulation of miR-182 in a cancer specific manner, given its emerging dual role as a tumor suppressor and oncogene. Thus, miR-182 is an attractive therapeutic target in a variety of cancers. However the molecular pathways that regulate miR-182 expression are not well defined, which limits our ability to modulate miR-182 expression in a clinical setting.

In this study, we sought to gain insights into the molecular mechanisms that regulate miR-182 expression. Kruppel like factors (Klfs) are DNA-binding transcriptional regulators that control essential cellular processes such as proliferation, migration and differentiation [15]. There are 17 Klfs in mammals, including Klf-3, which was originally discovered as a *CACCC* box binding transcription factor in erythroid cells and is expressed in multiple cell types including brain and muscle [16; 17; 18]. Like other Klf members, Klf-3 exhibits positive transcriptional activity; studies have also demonstrated a role for Klf-3 in repressing transcription via its interaction with co-repressors such as mCtBP2 and FHL3 [19; 20]. However, a role for Klf-3 in regulating miR-182 has not been described. In this study, we used *in silico* analysis and reporter assays to show that Klf-3 functions as a repressor of miR-182 both in mouse and human sarcoma cells. In addition, Klf-3 also suppresses expression of MyoD1, an activator of miR-182. Moreover, we discovered that Klf-3 is epigenetically silenced by DNA methylation in primary sarcomas, and treatment with the DNA methylation inhibitor 5′-Azacytidine (Aza) can restore Klf-3 expression and reduce miR-182 levels. Thus, our findings suggest that demethylating agents are an attractive therapeutic approach to modulate miR-182 levels in cancer.

2. Materials and Methods

2a. Animals and *in vivo* metastatic study

All animal work was performed in accordance with Duke University Animal Care and Use Committee approved protocols. Primary soft tissue sarcomas were generated in LSL-$Kras^{G12D}$ and $p53^{flox/flox}$ mice (KP mice) [2] or in NF1 $^{flox/flox}$ and Ink-arf4 $^{flox/flox}$ mice [21] by injecting an adenovirus expressing Cre recombinase into the hind limb of mice. To delete miR-182 in primary sarcomas, KP mice were first crossed to mice expressing a Cre-activated Yellow Fluoroscent Protein (LSL-YFP) reporter to generate KPY mice. KPY mice were then crossed to miR-182 floxed mice, in which miR-182 is flanked by loxP sites and is expressed at wild-type levels [5]. Injection of adenovirus-expressing Cre recombinase into the hind limb of KPY; miR-182 flox/flox mice generated primary sarcomas with miR-182 deletion (miR-182 KO). Primary sarcomas between 250-300 cm3 were harvested and cell lines generated as we previously described [5]. For the orthotopic metastasis assay, athymic

nude (nu/nu) mice (5-6 weeks old) were purchased from Taconic labs (NCRNU-M) and were maintained in Duke University's accredited animal facility. Fifty thousand exponentially growing KP #A-vector, KP #B-vector, KP #A-Klf-3 or KP #B -Klf-3 cells were injected into the hind limb muscle of the nude mice. When the tumor size reached about 200 mm^3, the tumor-bearing limb was amputated, and mice were followed for the development of lung metastases. Mice sacrificed when they developed signs of systemic illness such as hunched posture, lethargy, and ruffled fur. At this point the mice were euthanized and the lungs analyzed for metastasis.

2b. Evaluation for Metastases

For all mice, lungs were fixed in formalin and 70% ethanol. After embedding the lungs in paraffin, sections were stained with hematoxylin and eosin for evaluation by a sarcoma pathologist (DMC) blinded to the experimental variable, such as the genotype of the mice. To score the number of lung metastases and the area of lung with metastases per mouse, 3 independent areas of lung sections were imaged using a Leica DM5500B microscope with a 10X objective. The area of lung containing metastatic growths was quantified on Image Pro.

2c. Migration & Invasion assay

The 24-Multiwell FluoroBlok Insert System, with a 24-well plate and lid and pore size of 8.0 μm (BD Biosciences, San Jose, CA) was used to determine the effect of Klf-3 on migration and invasiveness per the manufacturer's protocol. Infected cells were serum starved overnight, trypsinized the next day, resuspended in serum free medium and then transferred to the hydrated matrigel chambers (~25,000 cells/well). The chambers were then incubated for 24 hrs in DMEM supplemented with 10% FBS in the bottom chambers before examination. The cells on the upper surface were scraped and washed away while the invaded cells on the lower surface were stained with Calcein AM dye for 1h at 37°C. Finally, invaded cells were counted under a microscope, and the relative number was calculated. All the fluoroscence images were taken with 10X objective using Zeiss Axiovert 25 microscope.

2d. Human Sarcoma Samples

Human soft tissue sarcoma samples were acquired from MD Anderson under a protocol approved by the Duke University and MD Anderson Institutional Review Boards. The human sarcomas included in the study are Undifferentiated Pleomorphic Sarcomas (UPS). Total RNA was extracted from frozen tumor samples using TRIZOL, and cDNA synthesis was performed for mature miR-182 using the Taqman MicroRNAs Reverse Transcription kit, followed by quantitative PCR with its respective probe, per the manufacturer's recommendation (Applied Biosystems, Foster City, CA, USA). miR-182 expression was calculated using the delta-delta-CT method after normalization to SnoRNA234 expression. Klf-3 expression was measured by Taqman RT-PCR assay and change in expression was calculated using the delta-delta-CT method after normalization to Gapdh expression. All human sarcoma cell lines were originally diagnosed as UPS, except for STS145 was a pleomorphic rhabdomyosarcoma.

2e. Cell culture

All KP and NI cell lines were derived from primary sarcomas in KP and NI mice, as described previously [5; 21]. Cells were cultured in DMEM medium supplemented with 10% FBS and incubated at 37 °C with 5% CO_2 in a humidified cell culture incubator. Human sarcoma cell lines were cultured in DMEM/F12 medium supplemented with 10% FBS and incubated at 37 °C with 5% CO_2 in a humidified cell culture incubator.

2f. Plasmids

The cDNAs expressing Klf-3, Klf-11 and Klf-15 were cloned into the lentiviral vector pCDH-CMV-MCS-EF1-copGFP (System Biosciences). mRNA sequences were first PCR amplified using mouse cDNA as a template and Platinum Taq polymerase enzyme (Invitrogen) with corresponding specific primers. Primer sequences are shown in Supplementary Table 1. The amplified fragment was then cloned into a lentiviral vector (pCDH-CMV-MCS-EF1-copGFP from System Biosciences, Mountain View, CA) at EcoR1 and Not1 sites using the Choo-Choo cloning kit per the manufacturer's protocol (MCLAB South San Francisco, CA). The Luciferase reporter constructs were generated by introducing miR-182 or MyoD1 promoters into pGL3 basic vector (Promega). We first amplified the promoter sequence by PCR using gene specific primers (Supplementary Table 1) and directly cloned them into the Kpn1 and Xba1 site of the pGL3 using the Choo-Choo cloning kit per the manufacturer's protocol (MCLAB South San Francisco). Site-directed mutagenesis of the Klf-3 binding site in the miR-182 promoter was carried out by the two-step PCR approach as described previously [22] with the mutant primer sequences (included in Supplementary Table 1). All PCR products were verified by DNA sequencing. For siRNA studies, sarcoma cell lines were transfected with siRNAs against Klf-3, Klf-11, Klf-15, Dnmt-1, Dnmt3a, Dnmt3b or scramble, purchased from Dharmacon, GE life sciences, with Lipofectamine (Invitrogen) following the manufacturer's protocol. The cells were harvested after 36 hrs and 48 hrs post transfection for RNA and protein analysis, respectively.

2g. Transduction

The lentiviral vectors (System Biosciences) expressing vector, Klf-3, Klf-11 or Klf-15 were packaged using pPACHK1 lentiviral packaging system and used to infect KP cell lines, as described previously [23]. For stable knockdown experiments, two independent KP sarcoma cell lines were infected with a lentivirus expressing either scramble or shRNA against Klf-3 (Origene). Because cells infected with viruses either express green or red fluorescent markers, stably infected clones were identified using flow cytometry.

2h. Real-time RT-PCR

RNA from cell lines or the tumors was isolated with Trizol reagent (Invitogen), according to the manufacturer's protocol. Reverse transcription for specific mature miRNAs was performed using the Taqman MicroRNAs Reverse Transcription kit followed by quantitative PCR with their respective probes, per manufacturer's suggestion (Applied Biosystems, Foster City, CA,). Mature miRNA expression in primary mouse and human tumors was calculated using the delta-delta-CT method [24] after normalization to SnoRNA202 and Sno234 expression, respectively. Klf-3, Klf-11 and Klf-15 mRNA

expression were measured using gene and species specific Taqman probes purchased from Applied Biosystems. Relative change was measured using the delta-delta-CT method after normalizing gene expression with Gapdh expression.

2i. Northern blotting

15-20 ug RNA from cell lines was separated in a 10% denaturing acrylamide gel purchased from (Bio-Rad, Hercules, CA). Separated RNA was then wet transferred onto the Nylon membrane (N+ Bond, GE Health sciences, Pittsburgh, PA). miR-182 and U6 probes (Exiqon, MA) were labeled at the 5′ end with radioactive ^{32}P using T4 PNK (New England Biolabs, MA) using the manufacturer's protocol and subsequently cleaned with a Microspin G-25 column (GE Health sciences, Pittsburg, PA). After UV cross-linking, the blot was pre-hybridized for 30 mins and then incubated with the radioactive probe overnight at 55 °C in a hybridization oven. Washing was performed the next day and the membrane was exposed to X-ray film in the dark at −80 °C for 2 hrs.

2j. Chromatin Immunoprecipitation (ChIP) Assay

ChiP was performed as described previously [6]. KP cells stably expressing vector and Klf-3 were seeded at 50% confluence overnight. Nuclear proteins bound to the genomic DNA were cross-linked directly in the cell culture medium in 1% formaldehyde at room temperature. Chromatin was digested with Micrococcal Nuclease for 20 mins at 37° C and incubated with Klf-3, Klf-11 and Klf-15 antibodies overnight. Protein G agarose beads captured the antibody-chromatin complex. DNA was separated from beads and eluted using ChIP elution buffer (Cell signaling technology), purified with spin columns, and PCr amplified (35 cycles) using specific PCR primers (Supplementary Table 1).

2k. Luciferase Assay

Cells were transfected with different miR-182 or MyoD1 promoter constructs with or without the Klf-3 plasmid. Cells were harvested 36 h after transfection. Luciferase assays were performed as previously described [22]. Plasmids with different miR-182 or MyoD1 promoters were cloned into a luciferase vector (Promega). miR-182 promoter sequences were PCR amplified with high fidelity Taq polymerase enzyme (Invitrogen) using mouse genomic DNA as a template. Primer sequences are provided in Supplementary Table 1. The amplified fragment was cloned into a Pgl3-basic vector at *Kpn1* and *Xho1* sites (Cho-Cho Cloning Kit). To mutate the putative Klf-3 binding sites in the miR-182 promoter, we adopted a two-step PCR ligation method with two sets of overlapping primers (Supplementary Table 1) that span the new binding site with mutations. The two amplified PCR fragments were used as a template for the second PCR using primers miR-182-pro-5.4 -kpn1 and miR-182-pro-3.1-rev-xho1 and cloned using a similar strategy as described above.

2l. Western blotting

Cultured cells or primary tumors were harvested using RIPA lysis buffer and proteins were extracted. The protein concentration was determined using a BCA protein assay kit (Pierce, Thermo Scientific) and samples were separated in SDS polyacrylamide gels (4-20% gradient

concentrations) from Bio-Rad (Hercules, CA). After blocking the membrane with 10% milk for one hour, the membrane was probed with a primary antibody (Supplementary Table 2) overnight and subsequently incubated with an HRP-conjugated secondary antibody for one hour. Working solutions of the Pierce ECL Substrate was then added to the membrane for 4 minutes. The membrane was exposed to Thermo Scientific CL-XPosure Film and developed using an X-ray film developer. After converting X-ray films to computer files, the files were imported and analyzed using ImageJ.

2m. Methylation specific PCR (MSP) for mouse and *human KLF-3*

DNA methylation at the mouse or human KLF-3 promoter region was analyzed using MSP. Genomic DNA (800 ng) was bisulfite modified using the Zymo Easy DNA Methylation Kit (Zymo Research; Irvine, Ca). Following bisulfite modification, 40 ng were used for amplification by PCR, assuming complete recovery of the DNA. The primers for both the mouse and human MSP assay are presented in Supplementary Table 1. PCR for both regions was performed using the Qiagen HotStar PCR kit (Qiagen; Valencia, Ca) and thermocycler conditions as follows: 95°C for 10 minutes, followed by 35 cycles at 95°C for 15 seconds, 57°C for 30 seconds, and 72°C for 30 seconds, with a final extension at 72°C for 10 minutes. The PCR products were visualized on 2% agarose gels using ethidium bromide staining and the pictures were taken using an Alphaimager HP gel scanning system (San Jose, California).

2n. Pyrosequencing analysis for mouse Klf-3

Genomic DNA (800 ng) was bisulfite modified using the Zymo Easy DNA Methylation Kit. Following bisulfite modification, 40 ng were used for amplification by PCR, assuming complete recovery of the DNA. Two regions for the mouse Klf-3 promoter region were analyzed using pyrosequencing. The PCRs for region 1 and region 2 were performed using specific primers (Supplementary Table 1) and then, the sequences YGGYGTTAGG GGTYGTAGAG YGTGTGAYGT TYGGAGYGGG TTAAYGTYGT TTTTTTTTTT TTT and GGGYGGGGTY GYGGGTTTAG GGYGTYGGGY GGAGYGTTTA GGYGGGGTGG AGGTTTAGGG TGGAGAGT were analyzed using specific sequencing primers, respectively (Supplementary Table 1). The PCR reaction for both regions was performed at 95°C for 15 minutes followed by 55 cycles of 95°C 30 seconds, 65°C 30 seconds, and 72°C 30 seconds, then extension at 72°C for 10 minutes using Qiagen PyroMark PCR kit. Pyrosequencing was performed using 7 microliter PCR products followed by the protocol provided by the manufacturer (Qiagen, Valencia, CA).

2o. Immunohistochemistry (IHC) and In-situ hybridization

Primary sarcomas were fixed in 10% formalin and paraffin embedded. 5-micron sections were cut and subjected to haematoxylin and eosin staining. Immunohistochemistry was used to detect Klf-3 (SantaCruz (sc-30380), CA, USA) in paraffin-embedded sarcomas using anti-goat secondary antibody with the Vectastain Elite ABC Reagent (Vector Labs). In-situ hybridization of MIR-182 was performed with LNA-miR-182-5′-fluorescein probe (Exiqon, USA) with 1:500 anti-FITCHRP secondary antibody and then, developed with DAB substrate. The hybridization of probe was performed at 56 degree Celsius for one hour followed by 37 degree Celsius for two hours.

2p. Statistical analyses

Statistical significance of the studies was analyzed by Student's t- test, Mann-Whitney test, one-way ANOVA or two-way ANOVA. Differences with p values less than 0.05 are considered significant.

3. Results

3a. Klf-3 is a bona fide transcriptional suppressor of miR-182

We have previously shown that miR-182 drives sarcoma metastasis by targeting several genes that contribute to invasion, thereby resulting in increased intravasation of tumor cells and ultimately lung metastases *in vivo* [5]. Additionally, we recently identified that Pax-7 can positively regulate miR-182 expression through MyoD1 in a subset of metastatic sarcomas [6]. However, little is known about how miR-182 is regulated in the absence of MyoD1. To search for transcriptional repressor(s) of miR-182 transcription, we utilized *in silico* prediction programs such as *Genomatix* and *TFSEARCH.* These programs predicted several binding motifs for Kruppel like factors (Klfs) scattered within the 3.5 kb miR-182 promoter. The prediction programs revealed four Klf binding sites in the miR-182 promoter (Supplementary Figure 1A). As Klfs have been shown to both activate and repress transcription of genes in a context dependent manner, we further investigated the role of Klfs in regulating miR-182. Although there are 15 Klf homologs in mice, and many Klf members share a common DNA binding motif, we focused on studying the roles of Klf-3, Klf-11 and Klf-15 because these Klfs are expressed at high levels in skeletal muscle [18; 25]. Additionally, these Klfs are capable of repressing transcription [20; 26], and they can regulate proliferation in Kras mutant cells [27]. A ChiP assay was initially employed to determine whether Klfs could bind to the predicted binding sites on the miR-182 promoter. The results showed that Klf-3 binds to the site2 of miR-182 promoter, but failed to bind to a region lacking a Klf-3 binding site (Figure 1A). Also, siRNA-mediated transient knockdown of Klf-3or shRNA-mediated stable knockdown of Klf-3 in mouse sarcoma cells resulted in increased miR-182 transcript levels. No effect on miR-182 levels was seen with knockdown of Klf-11 nor Klf-15. (Figure 1B,C and Supplementary figure 1B & C). Notably, no change was observed in miR-183, which is another members of the miR-183/96/182 cluster at chr 7q32.2. Although expression of miR-183 and miR-96 were highly correlated in 15 primary sarcoma cell lines, miR-182 levels were not correlated with miR-183 and miR-96 (Figure 1D and Supplementary figure 1D). These results suggest that miR-182 is regulated independently from miR-183/96.

To complement these loss of function experiments, we overexpressed Klf-3 in mouse sarcoma cells. Ectopic expression of Klf-3 decreases miR-182 levels in multiple mouse sarcoma cell lines, as indicated by RT-PCR (Figure 1E,F & G) while overexpression of either Klf-11 or Klf-15 does not change miR-182 expression (Supplementary figure 1E). In addition, RT-PCR and northern blotting further confirmed that both pri-miR-182 and pre-miR-182 transcripts were suppressed in Klf-3 expressing KP cells (Figure 1H & I). Interestingly, ectopic expression of a DNA-binding mutant Klf-3 [28] failed to decrease miR-182 expression, indicating that the DNA binding function of Klf-3 is required to regulate miR-182 expression (Figure 1J & K). To investigate whether KLF-3 also represses

MIR-182 in human sarcoma cells, we ectopically expressed KLF-3 in two primary human sarcoma cell lines (Figure 1L). RT-PCR showed that KLF-3 decreases MIR-182 levels in human sarcoma cells (Figure 1M). To determine if expression of Klf-3 and miR-182 correlated in primary sarcoma cell lines and metastatic mouse tumors *in vivo*, we performed RT-PCR for Klf-3 and miR-182 in a cohort of primary mouse sarcoma cells (n=15) and mouse sarcomas from KP mice (n=30). Klf-3 expression was significantly reduced in metastatic primary sarcomas, and expression of Klf-3 negatively correlated with miR-182 levels in both primary cell lines and primary sarcomas (Figure 1N, O & P). miR-182 levels did not correlate with Klf-11 in mouse sarcoma cell lines (Supplementary figure 1F) Also, levels of miR-96 or miR-183 were not correlated with Klf-3 in mouse sarcoma cell lines or primary mouse sarcomas (Supplementary figure 1G & H). To gain insight into the potential clinical relevance of KLF-3 in sarcoma metastasis, we performed real time RT-PCR for KLF-3 on patient sarcoma samples. We found that primary human UPS sarcomas from patients with lung metastases had reduced levels of KLF-3 (Figure 1Q). Although reduced levels of KLF-3 correlated with increased MIR-182, in this sample size the correlation does not reach statistical significance (Figure 1R). In a complementary experiment in human soft tissue sarcomas, we performed immunohistochemistry for KLF-3 and *in-situ* hybridization for MIR-182 on 10 clinical samples. We found a strong negative correlation between KLF-3 protein expression and MIR-182 transcript levels in primary human sarcomas (Supplementary figure 1I&J). To examine the induction of miR-182 target genes in samples that express Klf-3, we examined the levels of miR-182 targets Rsu-1 and Pai-1, in addition to downstream protease targets MMP-2, MMP-9, and uPA (Supplementary figure 2). A western blot showed that Klf-3 overexpression induces levels of Rsu-1 in two independent primary mouse sarcoma cells (Supplementary figure 2A). To further show that Klf-3 levels correlate with the induction of miR-182 target genes, we analyzed the expression of Klf-3 and miR-182 targets in both primary mouse and human sarcomas. As seen in Supplementary figure 2 B-E, a significant positive correlation was seen in both mouse and human sarcomas between Klf-3 and the miR-182 targets Rsu-1 and Pai-1. In addition, a western blot performed on protein lysates from primary mouse sarcomas, which are annotated for metastasis, further shows a negative correlation between Klf-3 and metastatic proteases including MMP2, MMP9, and uPA *in vivo* (Supplementary figure 2F).

To further investigate the regulation of the miR-182 promoter by Klf-3, we cloned different regions of the promoter region of the miR-182 promoter into a luciferase reporter construct (Pgl3-basic). Co-transfection of these reporter constructs and Klf-3 into mouse primary sarcoma cells and 293T cells demonstrated that Klf-3 inhibited the activity of the miR-182 promoter, but had no effect on the construct that lacks Klf-3 binding sites (Supplementary figure 3A-C). To test the activity of each putative Klf binding site, we cloned a series of constructs containing promoters with mutations in one or more Klf binding sites. Cotransfection of these mutated constructs with Klf-3 indicated that sites 2 and 4 are required for Klf-3 mediated repression of miR-182 promoter activity (Supplementary figure 3D).

3b. Klf-3 negatively regulates MyoD1

We recently showed that MyoD1 positively regulates miR-182 expression [6]. Thus, while performing a western blot to validate Klf-3 overexpression in the experiments described above, we also blotted for MyoD1 and noticed that Klf-3 overexpressing cells have reduced MyoD1 levels (Figure 2A). Therefore, we hypothesized that Klf-3 represses miR-182 not only by directly binding to its promoter, but also indirectly by suppressing the expression of its positive regulator, MyoD1. So, we searched for Klf binding motifs in the MyoD1 promoter that might be responsible for the regulation of MyoD1. Based on bioinformatics analysis using the Genomatix MatInspector (http://www.genomatix.de), we found several putative Klf binding sites in the MyoD1 promoter (Figure 2B, *Top panel*). To test whether Klf-3 can regulate MyoD1 promoter activity, we generated two MyoD1 luciferase reporter constructs either spanning 1Kb or 2Kb of the upstream MyoD1 promoter region. Experiments with ectopic expression of Klf-3 showed that Klf-3 decreases MyoD1 promoter activity in both C2C12 and 293T cells, but the results with the 1Kb reporter construct did not reach statistical significance in 293T cells (Figure 2B, *Bottom panel*). Furthermore, ChiP assays confirmed that Klf-3, but not Klf-11, binds to the MyoD1 promoter, but exhibited no binding to a region lacking a Klf binding site, as shown by negative primers (Figure 2C). Consistent with these results, western blot detected a significant reduction of MyoD1 protein and RNA in the mouse sarcoma cells stably expressing Klf-3, but not Klf-11 and Klf-15 (Figure 2D-F), suggesting that Klf-3 is a negative regulator of MyoD1. Western blot further showed that KLF-3 decreases MYOD1 levels in human sarcoma cells (Supplementary figure 3E). Noticeably, ectopic expression of a DNA-binding mutant Klf-3 failed to suppress MyoD1 protein indicating that the DNA binding activity of Klf-3 is required to regulate MyoD1 expression (Figure 2G). In loss of function experiments, siRNA mediated knockdown of Klf-3 induced MyoD1 expression (Figure 2H). Taken together, these data suggest that Klf-3 controls miR-182 expression by directly binding to the miR182 promoter and by indirect mechanisms via the regulation of MyoD1 (Figure 2I).

3c. Klf-3 suppresses sarcoma metastasis *in vitro* and *in vivo*

With our finding that Klf-3 suppresses miR-182 expression and our previous work that established a role for miR-182 in promoting sarcoma metastasis [5], we next tested if Klf-3 overexpression can impact *in vitro* metastatic phenotypes in mouse KP cells using transwell migration and invasion assays. Overexpression of Klf-3 significantly decreased both migration and invasion of KP cells through matrigel chambers, with no effect on cell proliferation (Supplementary figure 4A & B and data not shown). To determine if Klf-3 requires miR-182 to suppress *in vitro* metastatic phenotypes, we overexpressed Klf-3 in sarcoma cells with miR-182 deleteion (derived from primary sarcomas in miR-182$^{flox/flox}$; LSL-Kras^{G12D}; p53$^{flox/flox}$ mice). While Klf-3 expression significantly decreased both migration and invasion in control cells, Klf-3 overexpression had no significant effect on metastatic phenotypes of miR-182 knockout (KO) cells (Supplementary figure 4C & D). Taken together, these data suggest that Klf-3 modulates *in vitro* metastatic phenotypes at least partially through regulation of miR-182.

To study the impact of Klf-3 expression on sarcoma metastasis *in vivo*, two independent sarcoma cell lines with stable overexpression of Klf-3 were orthotopically injected into the

flank of nude mice. Stable overexpression of Klf-3 had no impact on primary tumor growth (Supplementary figure 4E & F). After tumors reached 200 mm^3, the tumor-bearing limb was amputated and the mice were followed for the development of lung metastases. Overexpression of Klf-3 in the orthotopic sarcomas was confirmed by immunohistochemistry (Supplementary Figure 4G) and these tumors showed a lower level of miR-182 by RT-PCR compared to cells expressing vector alone (Supplementary Figure 4H). Mice with sarcomas overexpressing Klf-3 had improved metastasis-free survival after amputation (Supplementary Figure 4I). Klf-3 overexpression reduced the number of metastatic lung colonies and the metastatic burden in the lung (Supplementary Figure J & K). Taken together, these data suggest that the Klf-3 regulates sarcoma metastasis *in vivo*.

3d. DNA-methylation alters Klf-3 and miR-182 levels in primary sarcoma cells

Because miR-182 regulates intravasation of sarcoma cells [5], strategies to inhibit miR-182 at early stages of sarcoma development could prevent metastasis. Therefore, in an attempt to discover therapeutics that can alter Klf-3, and thereby miR-182 expression, we treated four independent primary sarcoma cell lines derived from KP mice with different therapeutic agents including doxorubicin, a chemotherapy used to treat sarcoma patients. In this unbiased drug screen, we found that only the DNA methyltransferase inhibitor, 5′-azacytidine (Aza) and Doxorubicin induced Klf-3 expression by three fold ($p<0.005$) (Figure 3A). In contrast, a dual PI3K/mTOR inhibitor, BEZ235, and a Mek inhibitor, PD0325901, increased Klf-3 expression by approximately two fold ($p<0.01$) (Figure 3A). No induction in Klf-11 expression was observed with Aza treatment, suggesting a Klf-3 specific phenotype (Supplementary Figure 5A). In contrast to Klf-3 induction, Aza caused more than a 50% reduction in miR-182 expression ($p<0.05$). Although two different PI3K inhibitors BEZ235 and BKM120 reduced miR-182 expression by 40% and 30%, respectively and doxorubicin caused a 20% reduction in miR-182 expression, these differences did not reach statistical significance (Figure 3B). Interestingly, Aza does not decrease miR-183 or miR-96 expression suggesting that Aza specifically targets miR-182, which is likely because miR-182 is transcribed by an independent promoter (Figure 3B & Supplementary figure 5B). Because Aza was able to modulate both Klf-3 and miR-182 levels in a statistically significant manner, we sought to understand whether Aza-mediated reduction in miR-182 is mediated by Klf-3 induction. Thus, we treated sarcoma cells with Aza in the presence of either scramble siRNA or siRNA against Klf-3. Notably, RT-PCR data shows that Aza can suppress miR-182 in the cells treated with scramble siRNA, but the effect in the cells treated with siRNA Klf-3 was blunted, suggesting that Aza suppresses miR-182 expression at least partially through Klf-3 (Figure 3C). Additionally, mouse MyoD1 levels were also reduced by Aza treatment (Supplementary figure 5C). To investigate whether Aza can reduce KLF-3 and increase MIR-182 expression in primary human sarcoma cells, we treated three different primary human sarcoma cell lines with Aza and measured KLF-3 protein levels by western blot and Klf-3 mRNA and MIR-182 expression by RT-PCR. Similar to mouse sarcoma cells, KLF-3 expression was induced while the level of MIR-182 was significantly reduced with Aza treatment, implying that demethylating agents like Aza could potentially be used to modulate MIR-182 levels in human tumor cells (Supplementary Figure 5D-F).

3e. Methylation of Klf-3 promoter occurs in a context dependent manner

To explore Aza-mediated induction of Klf-3, we characterized the cis element(s) and putative trans-acting factor(s) that regulate transcription from the Klf-3 promoter. We analyzed *in silico* CpG island prediction programs such as the UCSC genome browser, MethPrimer and Emboss to search for the presence of CpG islands in the Klf-3 promoter. CpG islands are located within 1kb of the mouse and human KLF-3 promoters (Figure 3D and Supplementary Figure 5G). In contrast, the same *in silico* CpG prediction programs did not find any CpG islands in either mouse or human miR-182 or MyoD1 promoter regions (Supplementary Figure 5I-J). To test whether Klf-3 is epigenetically silenced via DNA hypermethylation, we designed a methylation-specific PCR (MSP) assay for two independent regions within the 1Kb promoter of mouse and human KLF-3 promoters. MSP detected PCR bands with both methylated and unmethylated primer pairs suggesting that methylation occurs at the Klf-3 promoter in multiple primary cells derived from KP mouse sarcomas and in primary human STS cells (Figure 3E & Supplementary Figure 5H). To our surprise, we did not see any products using the methylation-specific primers in primary cell lines derived from sarcomas generated with deletion of NF1 and Ink4a/Arf (NI) (Figure 3E). Pyrosequencing was then performed on the DNA isolated from primary sarcoma cells either from KP or NI mice. Pyrosequencing determined average methylation of 40% and 17% for two different Klf-3 promoter regions in primary sarcoma cells derived from KP mice (n=5) compared to methylation of ~1.5% in sarcoma cells isolated from NI mice (n=5) (Figure 3F). To further investigate the level of promoter hypermethylation, we compared Klf-3 and miR-182 levels between KP and NI cells treated with Aza. As expected, Aza treatment caused a significant induction in Klf-3 mRNA expression in all the KP cell lines tested while a significant induction was observed only in 2 of 5 NI cell lines (Figure 3G-H & Supplementary Figure 5K-L). Interestingly, we observed that the endogenous level of Klf-3 is greater in NI cells, both at the transcript and protein levels. To test whether the elevated level of Klf-3 in NI cells is sufficient to suppress miR-182, we treated NI cells with Klf-3 siRNA and measured miR-182 levels. RT-PCR showed a significant induction of miR-182 in the Klf-3 siRNA cells compared to scramble treated cells (Supplementary Figure 5M).

3f. Dnmt3b methylates the Klf-3 promoter

Because three distinct DNA methyltransferases (Dnmt1, Dnmt3a And Dnmt3b) are involved in methylation of cytosine-guanine dinculeotides and the level of DNA methyltransferases is altered in cancers [29], we measured both the protein and RNA levels of various Dnmts in cell lines derived from KP and NI sarcomas. RT-PCR detected significantly higher expression of Klf-3 in NI cells, while Dnmt3b expression was found to be significantly lower in the same cells (Figure 4A-C). No difference in Dnmt1 or Dnmt3a was observed between KP and NI cells (Figure 4A-C). Western blotting further showed higher levels of endogenous Klf-3 expression in NI cells compared to KP cells (Figure 4A). In contrast, Dnmt3b protein levels were lower in NI cells (Figure 4A) suggesting that higher level of Dnmt3b in KP cells may be responsible for hypermethylation of Klf-3. Also, a strong negative correlation between Klf-3 and Dnmt3b was observed for their mRNAs (Figure 4D). To further explore the relationship between Klf-3 and Dnmts, we treated KP cells with siRNA against Dnmt3a or Dnmt3b and measured Klf-3 expression. Klf-3 induction was

highest in the cells treated with siRNA against Dnmt3b compared to the siRNA against Dnmt3a (Figure 4E-F). Moreover, miR-182 was most suppressed in cells treated with siRNA to Dnmt3b. Interestingly, in one of the two cell lines; a siRNA against Dnmt3a increases Klf-3 expression and decreases miR-182 expression. Taken together, our findings suggest that Dnmt3b methylates the Klf-3 promoter in KP sarcomas, which can lead to high expression of miR-182.

4. Discussion

In this study, we sought to identify modulators of miR-182 that could be therapeutic targets for cancer therapy. Using a combination of *in silico* and *in vitro* approaches, we discovered Kruppel like factor-3 (Klf-3) as a novel repressor of pro-metastatic miR-182 in sarcomas. Klf-3 not only binds to the miR-182 promoter, but also binds to the promoter of MyoD1 to suppress MyoD1 expression. Because MyoD1 is a positive regulator of miR-182 [6], Klf-3 can suppress miR-182 expression via a dual mechanism. Importantly, we also present evidence that the CpG island in the Klf-3 promoter is highly methylated in KP sarcoma cells, which is reversed by Azacytidine and thus, demethylating agents such as Azacytidine could be used to suppress miR-182 in sarcomas.

Klf-3 was originally discovered as a CACCC box binding transcription factor in erythroid cells [17]. Although Klf-3 can promote positive transcriptional activity, studies have also demonstrated a role for Klf-3 in repressing transcription via its interaction with multiple co-repressors [19; 20]. In fact, we found that overexpression of Klf-3 significantly decreases miR-182 expression in both mouse and human sarcoma cells. Using Chip assays and complementary luciferase reporter assays, we show that Klf-3 binds to the miR-182 promoter and decreases the transcription of miR-182, as determined by decreased levels of pri, pre and mature miR-182 transcripts. Moreover, a strong negative correlation was observed between Klf-3 and miR-182 in both primary mouse and human sarcomas. Additionally, Klf-3 also suppresses an activator of miR-182, MyoD1, suggesting a secondary mechanism that can be employed by cells to suppress miR-182 levels. Other studies have identified other mechanisms of transcriptional activation of miR-182 in different cancers. For example, TGF-ß has been shown to induce miR-182 in gliomas [30], while Sp1 induces miR-182 in lung cancer cells [31], and the RNA Helicase DDX5 has been shown to activate miR-182 in basal breast cancer cells [32]. Interestingly, SREBP2 was shown to induce the miR-183/96/182 cluster after binding to an upstream responsive element, which connects the regulation of miR-182 to intracellular lipid metabolism [33]. Understanding the mechanisms governing miR-182 regulation in different tissues and cancer type is important for developing approaches to modulate miR-182 levels therapeutically.

The role of Klf-3 has been extensively studied in development of the hematopoietic system where it is known to regulate the expression of multiple globulin genes. In fact, Klf3-/-mice display a myoproliferative disorder and Klf-3 is highly expressed in erythroid cells [34]. A recent study indicates that Klf-3 is critical during the later stages of erythroid maturation *in vivo*. Also, targeted disruption of Klf-3 has revealed a role in adipogenesis [35]. Although not much is known about Klf-3 in cancer, a study reported significantly lower levels of Klf-3 in acute myeloid leukemia patients compared with Klf3 expression in neutrophils from

healthy donors [36]. The downregulation of Klf-3 in a majority of sarcomas prompted us to investigate the mechanisms underlying its reduced expression. We detected conserved CpG islands in both mouse and human KLF-3 promoters. Because DNA hypermethylation in CpG rich promoters blocks the initiation of transcription of a gene, and is now established as a common mechanism for silencing of tumor suppressor genes such as INK4a-ARF and MLH1 in human cancers [37; 38; 39], we studied the CpG islands in the Klf-3 promoter. Using MSP combined with pyrosequencing, we discovered that the Klf-3 promoter is hypermethylated. Consistent with our findings, a recent report also detected methylation of Klf-3 after flow-dependent stress in endothelial cells [40]. Interestingly, we observed a difference in Klf-3 methylation in primary mouse cells derived from sarcomas with different tumor-initiating mutations. For example, primary sarcomas cells expressing oncogenic K-ras and deletion of p53 have high methylation of the Klf-3 promoter compared to sarcoma cells with deletion of NF1 and Ink4a-Arf. As a consequence, we found a lower Klf-3 mRNA and protein in KP cells compared with NI cells. Similar to our finding, a study investigating the expression of various Klfs in mutated Kras cell lines reported lower expression of Klf-3 in Kras mutated cells [27], however the mechanism for Klf-3 expression was not identified. Of note, a recent study using an unbiased RNA interference screen found that KRAS promotes epigenetic silencing through upregulation of ZNF304, which after binding to DNA recruits a complex of co-repressors that included DNA methyltransferase DNMT1 [41]. In another study, investigators found that Braf increases the transcriptional repressor MAFG, which drives DNA binding and recruits a co-repressor complex that includes its heterodimeric partner BACH1, the chromatin remodeling factor CHD8 and the DNA methyltransferase DNMT3b, resulting in hypermethylation [42]. Therefore, we compared the expression of all three enzymatically active Dnmts between KP and NI sarcoma cells. We found that Dnmt3b, but not Dnmt1 or Dnmt3a, was highly expressed in KP cells and negatively correlated with Klf-3 at the mRNA levels. In addition, knockdown of Dnmt3b in KP cells rescued Klf-3 expression. Thus, our results show a unique pathway where sarcomas with Ras and p53 mutations are more prone to methylate Klf-3 than sarcomas with loss of NF1 and Ink4a-Arf. Because downregulation of Klf-3 in sarcoma occurs due to DNA hypermethylation, it would be interesting to investigate whether low Klf-3 expression due to hypermethylation exists in other cancers, particularly if they harbor mutations in Ras and p53.

Pharmacological agents and dietary supplements can modulate DNA methylation steady state, which makes DNA methylation an attractive therapeutic target. Several DNMT inhibitors have been developed. 5-azacytidine and its deoxy derivative 5-aza-2′ deoxycytidine are two FDA approved drugs used in the treatment of AML [43]. Along with the role of these drugs blocking cell proliferation *in vitro* and *in vivo*, several studies have suggested a potential role for these drugs in suppressing the metastatic phenotype [44; 45; 46; 47]. For example, Aza has been reported to inhibit invasiveness of human breast, ovarian and non-small lung cancer cell lines [48; 49]. With our data that Aza can induce Klf-3 to suppress pro-metastatic miR-182, we propose that demethylating agents like Aza could be use as a therapy to decrease miR-182 and potentially ameliorate sarcoma metastasis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Highlights

Klf-3 is a novel transcriptional repressor of pro-metastatic miR-182 in sarcomas.

Klf-3 suppresses a myogenic transcription factor MyoD1.

Klf-3 is epigenetically silenced by DNA hypermethylation.

Demethylating agents could potentially be used to modulate miR-182 levels as a therapeutic strategy.

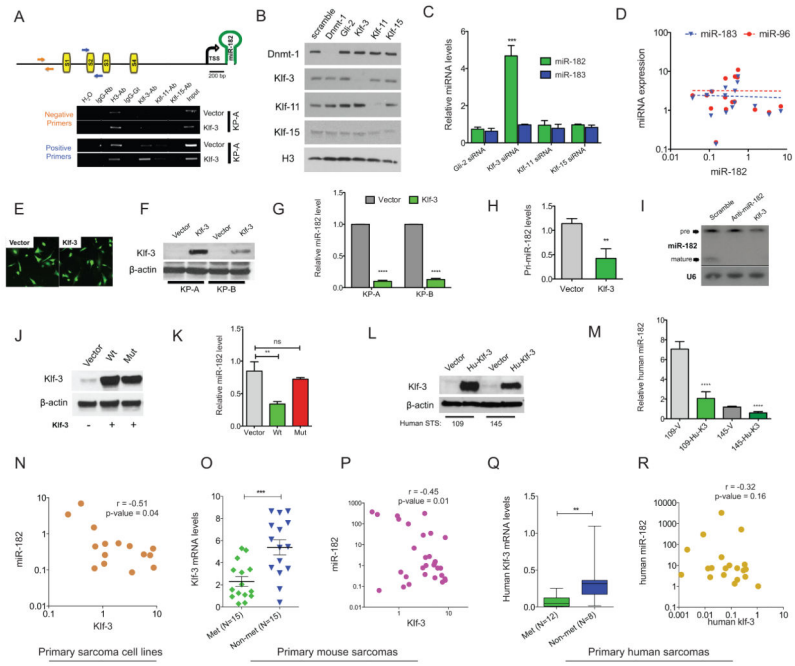

Figure 1. Klf-3 suppresses miR-182 expression

A) *Top panel*: Schematic of miR-182 promoter highlighting putative Klf-3 binding sites (yellow); *Bottom panel*: Chromatin immunoprecipitation of miR182 promoter in primary mouse sarcoma cells overexpressing Klf-3 (Klf-3) or control (Vector), which demonstrates direct binding of Klf-3, but not Klf-15. B) Western blot confirming siRNA-mediated knockdown of Klf-3 in a primary mouse sarcoma cell line. C) Knockdown of Klf-3, but not Klf-11, increases miR-182 expression (Averaged data from two different cell lines performed in triplicate). D) miR-182 expression does not correlate with miR-96 or miR-183 levels in primary mouse sarcoma cells (N=15). E) Image showing stably transduced sarcoma cell line expressing copGFP ± Klf-3. F) Western blot confirms ectopic expression of stably transduced Klf-3. G) Klf-3 overexpression decreases miR-182 expression in multiple sarcoma cell lines. H) RT-PCR shows reduction in primary miR-182 transcript in Klf-3 transduced cells (Averaged data from three different cell lines performed in triplicate). I) Northern blot demonstrates reduction in miR-182 levels in Klf-3 overexpressing cells. J) Western blot confirms ectopic expression of stably transduced DNA-binding mutant Klf-3. K) RT-PCR shows no change in miR-182 expression with DNA-binding mutant Klf-3 (Average of two cell lines performed in triplicate). L) Western blot confirms ectopic expression of human KLF-3. M) Overexpression of human KLF-3 decreases MIR-182 expression in primary human sarcoma cell lines. N) miR-182 shows a significant negative correlation with Klf-3 in primary mouse sarcoma cell lines. O) Klf-3 expression is significantly lower in primary sarcomas that metastasized in KP mice. P) Negative correlation between miR-182 and Klf-3 in mouse sarcomas. Q) KLF-3 expression is significantly lower in human metastatic sarcomas and (R) negatively correlates with MIR-182. One-way ANOVA is used for statistical analysis. All experiments are performed in triplicate and repeated three times. Data are mean ± SEM. *P<0.05, **P<0.01, ***P<0.005, ****P<0.0001.

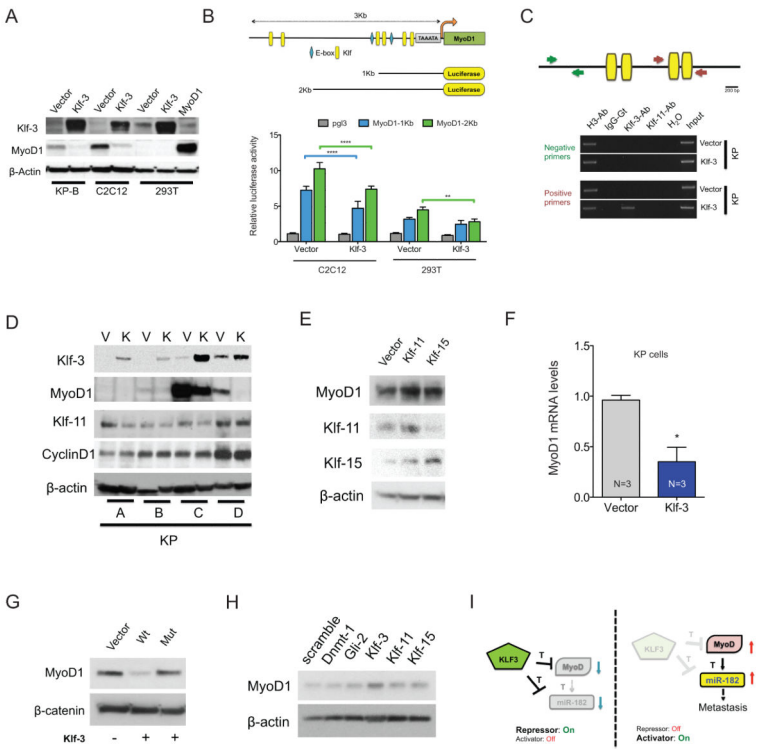

Figure 2. Klf-3 suppresses MyoD1

A) Western blot shows that ectopic expression of Klf-3 suppresses endogenous MyoD1 expression in KP sarcoma and C2C12 cell lines. B) *Top panel*: Schematic of constructs of mouse MyoD1 promoter showing putative Klf-3 binding sites; *Bottom panel*: Luciferase assay showing that Klf-3 decreases MyoD1 promoter activity in both C2C12 and 293T cells. C) Chromatin immunoprecipitation of MyoD1 promoter demonstrating direct binding of Klf-3. D) Western blot shows that ectopic expression of Klf-3 suppresses endogenous MyoD1 in mouse sarcoma cells, while overexpression of Klf-11 or Klf-15 has no effect on MyoD1 expression (E). F) Klf-3 overexpression decreases MyoD1 mRNA transcripts. G) Western blot showing no change in MyoD1 expression with overexpression of DNA-binding mutant Klf-3. H) siRNA-mediated knockdown of Klf-3, but not Klf-11 or Klf-15, increases MyoD1 expression. I) Schematic of the direct and indirect mechanisms by which Klf-3 regulates miR-182 (T: transcription, “over inhibitory arrows”, Blue arrows: repression and Red arrows: activation). All experiments were performed in triplicate and repeated at least twice and data are mean ± SEM. *P<0.05, **P<0.01, ***P<0.005, ****P<0.0001.

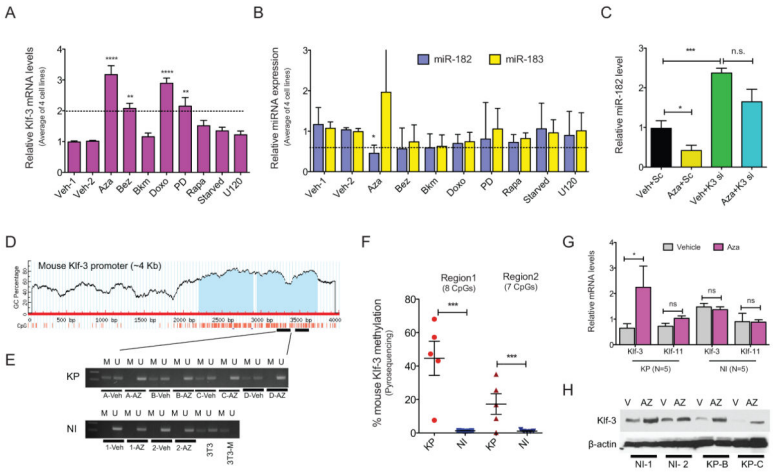

Figure 3. DNA-methylation of Klf-3 in a context dependent manner

A) RT-PCR results show a significant induction of Klf-3 and decrease in miR-182 (B), after 5′ Azacytidine (Aza) treatment, but not with any other treatment (Average of four different cell lines are represented in the graph). Mouse sarcoma cells were treated with different treatments for 48 hrs except Aza, which was treated for 72 hrs at 500 nM. Veh-1: DMSO, Veh-2: Ethanol, Aza: 5′-Azacytidine, Bez: PI3K inhibitor-BEZ235, Bkm: PI3K inhibitor-BKM450, Doxo: Doxorubicin, PD: Mek inhibitor PD 0325901:, Rapa: Rapamycin, Starved: 1% serum-media, U120: Mek inhibitor U120. C) Aza decreases miR-182 levels in cells with wild-type Klf-3, but the effect of Aza on miR-182 expression is blunted in cells with Klf-3 knockdown. D) Schematic showing CpG islands in mouse Klf-3 promoter. E) Methylation specific PCR and (F) Pyrosequencing demonstrates methylation of Klf-3 in KP mutant, but not NI mutant, sarcoma cells. G) Aza induces Klf-3 both at the mRNA and protein level (H) in different cell types. All experiments were performed in triplicate and repeated three times and data are mean ± SEM. *P<0.05, **P<0.01, ***P<0.005, ****P<0.0001.

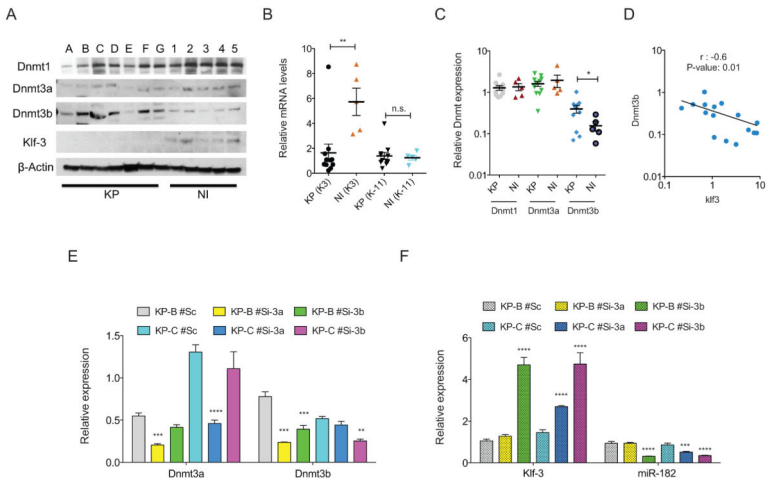

Figure 4. Dnmt3 regulates expression of Klf-3 and miR-182

A) Western blot showing expression of Klf-3 and various Dnmts in primary sarcoma cells from KP (N=7) and NI (N=5) mice. B) RNA levels of Klf-3 and Klf-11 in primary sarcoma cells. (C) RNA levels of Dnmts in primary sarcoma cells. D) A significant negative correlation between Klf-3 and Dnmt3b in mouse sarcomas. E) siRNA mediated knockdown of Dnmt3a and Dnmt3b in KP cells. F) Knockdown of Dnmt3b induces Klf-3 and reduces miR-182 levels in sarcoma cells. All experiments were performed in triplicate and repeated twice. Data are mean ± SEM. *P<0.05, **P<0.01, ***P<0.005, ****P<0.0001