Manuscript Details

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Abstract

Myocardial Infraction Associated Transcript (MIAT) is a subnuclear lncRNA that interferes with alternative splicing and is associated with increased risk of various heart conditions and nervous system tumours. The current study aims to elucidate the role of MIAT in cell survival, apoptosis and migration in neuroblastoma and glioblastoma multiforme. To this end, MIAT was silenced by MIAT-specific siRNAs in neuroblastoma and glioblastoma cell lines, and RNA sequencing together with a series of functional assays were performed. The RNA sequencing has revealed that the expression of an outstanding number of genes is altered, including genes involved in cancer-related processes, such as cell growth and survival, apoptosis, reactive oxygen species (ROS) production and migration. Furthermore, the functional studies have confirmed the RNA sequencing leads, with our key findings suggesting that MIAT knockdown eliminates long-term survival and migration and increases basal apoptosis in neuroblastoma and glioblastoma cell lines. Taken together with the recent demonstration of the involvement of MIAT in glioblastoma, our observations suggest that MIAT could possess tumour-promoting properties, thereby acting as an oncogene, and has the potential to be used as a reliable biomarker for neuroblastoma and glioblastoma and be employed for prognostic, predictive and, potentially, therapeutic purposes for these cancers.

Keywords	MIAT; neuroblastoma; glioblastoma
Manuscript category	Proteins and Nucleic acids

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Research Data Related to this Submission

There are no linked research data sets for this submission. The following reason is given:
Data will be made available on request

Keele University

Editorial Office
INTERNATIONAL JOURNAL OF BIOLOGICAL MACROMOLECULES
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20 January 2019

Dear Editor,

I would like to submit the article: 'RNA sequencing reveals a key role for the long non-coding RNA MIAT in regulating the survival of neuroblastoma and glioblastoma cells', by Aikaterini Bountali, Daniel P. Tonge and Mirna Mourtada-Maarabouni for consideration for publication as full-length research article in the International Journal of Biological Macromolecules.

There is currently much interest in the roles that long non-coding RNAs (lncRNAs) play in physiology and pathology. Several lncRNAs play key, rate-limiting roles in oncogenesis, and the mechanisms involved are now being elucidated. We and others have identified MIAT lncRNA as a key regulator of cell survival and apoptosis in different cell types. The expression of MIAT has been reported to be up-regulated in a number of cancers including breast cancer and its down-regulation has been shown to negatively affect cancer cell survival.

In this study, we investigated the role of MIAT in the regulation of cell survival, apoptosis and migration in neuroblastoma and glioblastoma multiforme. We used RNA sequencing to determine the specific transcriptional signatures regulated by MIAT and studied the effects of MIAT silencing on the survival of neuroblastoma and glioblastoma cells. Our work demonstrates, for the first time, that silencing of endogenous MIAT silencing leads to significant changes in the expression of an

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outstanding number of genes, including genes involved in cancer-related processes, such as cell growth and survival, apoptosis, reactive oxygen species (ROS) production and migration. Functional studies have confirmed the RNA sequencing leads, with our key findings suggesting that MIAT knockdown eliminates long-term survival and migration and increases basal apoptosis in neuroblastoma and glioblastoma cell lines. Thus the present work demonstrates that MIAT lncRNA may constitute a novel therapeutic target for neuroblastoma and glioblastoma. Such findings provide essential information leading to the exploitation of this new area to improve the therapy of neuroblastoma and glioblastoma and other cancers. We therefore feel that this paper will be of wide interest to your readership. I can confirm that all authors have approved the manuscript for submission and that this work is novel and will not be submitted elsewhere pending the outcome of the review process.

Yours faithfully

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Response to Reviewer

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We thank the Reviewers for their constructive comments and have addressed all the points made as detailed below:

Reviewer 1

Point 2

In defining MIAT in the Abstract the authors use "infraction" and it should be "infarction".

This typo has been identified and corrected in the manuscript.

Point 4

Since multiple cell lines are used the various Figure panels should indicate which cell line is being used.

The name of the cell line used is clearly mentioned in each figure legend. However, extra labels have been added on top of every figure, so that it is easier to distinguish among the different cell lines.

Point 6

The various gap closure experiments are impossible for me to decipher in my copy of the manuscript. The authors should prepare some quantitative data to compare effects of MIAT knockdown to controls

The requested quantitative data have been added to the corresponding figures (Fig. 5, 8, 10).

Point 8

The authors demonstrate the functional pro-oncogenic properties of MIAT and these are straightforward and have previously been demonstrated for MIAT and many other lncRNAs. However the Discussion initially focuses on ROS based on results of their genomic studies. Therefore they should at least determine if MIAT silencing induces ROS and determine if antioxidants (eg NAC, GSH) can reverse some of the MIAT knockdown responses.

Although the functional effects of MIAT silencing have been previously demonstrated in breast cancer, retina cells and leukemia, our work shows for the first time that MIAT regulate neuroblastoma and glioma cell survival. Our RNA sequencing results implicate MIAT in the regulation of many biological pathway including apoptosis and migration. While the present work confirms the role of MIAT in regulating survival, apoptosis and migration, validating the role of MIAT in the biological pathways identified by RNA sequencing is beyond the scope of this paper and is currently being investigated.

Point 10

The authors should note that ROS is known to decrease Myc via epigenetic pathways (Cancer Cell 20, 606, 20?4) and Myc triggers a downstream cascade of events leading to downregulation of Sp transcription factors (rev. in Cancer Prev Res 11, 371, 2018). ROS-inducing agents and their mechanism of action have been extensively investigated (see review) and some of the affected genes are probably modulated in this study.

We would like to thank the reviewer for drawing our attention to the aforementioned articles. They provided useful insights, and after reading them, some pieces of relevant information have been added to the discussion (highlighted below in bold), accompanied by two supplementary tables listing changes in relevant gene/miRNA expression (Supplementary tables 2, 3).

"Given that cell migration comprises one of the first steps towards tumour metastasis, *MIAT* downregulation could be a potent therapeutic approach towards the prevention of metastasis.

To link the aforementioned observations, it could be speculated that *MIAT* exerts its effects through a ROS-induced Sp (Specificity protein) TF (transcription factor) mechanism. Sps belong to the Sp/Krüppel-like factor (KLF) family of TFs and play important roles in healthy and pathological settings, including cancer [52]. Among the various family members, Sp1, Sp3 and Sp4 have gained attention, with Sp1 being the subject of thorough investigation [53], and importantly all three members displayed at least a three-fold decrease upon *MIAT* knockdown in our RNA sequencing, and in addition, numerous regulators of Sp1, including various miRNAs (Supplementary table 2) and eighteen members of the ZBTB (zing finger and

BTB) family were significantly deregulated. Since the elevated activity of Sp1 has been associated with malignancy and tumour progression in various cancers including glioma [53]–[55], it could be assumed that its downregulation could prevent this effect. In fact, a reasonable mechanism would suggest that *MIAT* knockdown induces an increase in ROS production, which in turn induces a ROS-mediated epigenetic downregulation of c-MYC [56] leading to the downregulation of Sp1 via the regulation of miRNAs and ZBTB proteins. Interestingly, the downstream effectors of Sp1 include a variety of crucial cancer-related genes involved in survival, apoptosis and migration, such as cMET (tyrosine-protein kinase Met), survivin, Fas, bcl-2, VEGFs and MMPs (matrix metallopeptidases) , and notably, a variety are deregulated in our study (Supplementary table 3).

In conclusion, the current study suggests that the downregulation of *MIAT* reduces the long-term survival of neuroblastoma and GBM cells, while it promotes basal apoptosis, as well as deteriorates the cells' ability to migrate."

Abstract

Myocardial Infraction Associated Transcript (MIAT) is a subnuclear lncRNA that interferes with alternative splicing and is associated with increased risk of various heart conditions and nervous system tumours. The current study aims to elucidate the role of MIAT in cell survival, apoptosis and migration in neuroblastoma and glioblastoma multiforme. To this end, MIAT was silenced by MIAT-specific siRNAs in neuroblastoma and glioblastoma cell lines, and RNA sequencing together with a series of functional assays were performed. The RNA sequencing has revealed that the expression of an outstanding number of genes is altered, including genes involved in cancer-related processes, such as cell growth and survival, apoptosis, reactive oxygen species (ROS) production and migration. Furthermore, the functional studies have confirmed the RNA sequencing leads, with our key findings suggesting that MIAT knockdown eliminates long-term survival and migration and increases basal apoptosis in neuroblastoma and glioblastoma cell lines. Taken together with the recent demonstration of the involvement of MIAT in glioblastoma, our observations suggest that MIAT could possess tumour-promoting properties, thereby acting as an oncogene, and has the potential to be used as a reliable biomarker for neuroblastoma and glioblastoma and be employed for prognostic, predictive and, potentially, therapeutic purposes for these cancers.

RNA sequencing reveals a key role for the long non-coding RNA MIAT in regulating neuroblastoma and glioblastoma cell fate

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Abstract

Myocardial Infarction Associated Transcript (MIAT) is a subnuclear lncRNA that interferes with alternative splicing and is associated with increased risk of various heart conditions and nervous system tumours. The current study aims to elucidate the role of MIAT in cell survival, apoptosis and migration in neuroblastoma and glioblastoma multiforme. To this end, MIAT was silenced by MIAT-specific siRNAs in neuroblastoma and glioblastoma cell lines, and RNA sequencing together with a series of functional assays were performed. The RNA sequencing has revealed that the expression of an outstanding number of genes is altered, including genes involved in cancer-related processes, such as cell growth and survival, apoptosis, reactive oxygen species (ROS) production and migration. Furthermore, the functional studies have confirmed the RNA sequencing leads, with our key findings suggesting that MIAT knockdown eliminates long-term survival and migration and increases basal apoptosis in neuroblastoma and glioblastoma cell lines. Taken together with the recent demonstration of the involvement of MIAT in glioblastoma, our observations suggest that MIAT could possess tumour-promoting properties, thereby acting as an oncogene, and has the potential to be used as a reliable biomarker for neuroblastoma and glioblastoma and be employed for prognostic, predictive and, potentially, therapeutic purposes for these cancers.

Key words**:** *MIAT*, RNA sequencing, oxidative stress, apoptosis, neuroblastoma, glioblastoma

Funding: Aikaterini Bountali is supported by a studentship awarded by the Faculty of Natural Sciences, Keele University.

1. Introduction

Non-coding RNAs (NcRNAs) are RNAs that lack an apparent open reading frame (ORF) [1], [2] and can be further divided into two subcategories: small and long ncRNAs, based primarily on their sequence length. Long ncRNAs (lncRNAs) are RNA molecules of >200nt length with limited or no protein-coding capacity [1], [3]. Their expression is elegantly regulated, following a spatiotemporal- and environmental stimulus-specific fashion [4], [5]. They are transcribed by RNA polymerase II, they are often 5' capped and 3' polyadenylated and multi-exonic, as well as subject to alternative splicing, resembling mRNAs [3], [6], [7]. The majority of lncRNAs display low levels of evolutionary sequence conservation [3], [8] and being very heterogeneous molecules, they are classified based on their genomic position relative to neighbouring protein-coding genes [e.g. long/large intergenic/intervening RNAs (lincRNAs), enhancer RNAs (eRNAs), intronic lncRNAs etc.)] [6], [9].

Myocardial Infarction Associated Transcript (*MIAT*), also known as *Gomafu* and *RNCR2* (retinal non-coding RNA 2) is a nuclear lncRNA that is localised across the nucleoplasm in a spotted pattern, but these 'spots' do not co-localise with any of the known sub-nuclear bodies. *MIAT* is a ~10kb long transcript transcribed from chromosome 22q12.1 [10]–[14]. *MIAT* is mainly expressed in some cells of the fetal brain and the adult brain/CNS (including Müller glia, neurons and endothelial cells [15]), especially in the retinal tissue. In addition, *MIAT* is polyadenylated at the 3' end and has at least 4 and 10 alternatively spliced variants for human and mouse, respectively [12], [16]. *MIAT* is thought to participate in pre-mRNA splicing through its binding to splicing factor 1 (SF1), although this interaction is not essential for the localisation of *MIAT* [11]. Apart from its physiological roles in a healthy setting, *MIAT* is associated with various heart conditions [10], [16], [17], eye disorders [15], [18], brain disorders such as Alzheimer's Disease and Schizophrenia [17], [19], as well as various cancers, including CLL (chronic lymphocytic leukaemia) and DLBL (diffuse large B-cell lymphoma) [20].

Neuroblastoma (NB) is the most common extracranial paediatric cancer that primarily affects very young children and accounts for 7-10% of all paediatric tumours [21], [22]. On the other hand, gliomas are the most common malignant tumours of the CNS in adults and are neuroectodermal in origin [23]. The most malignant, yet most common, glioma is glioblastoma, also known as glioblastoma multiforme (GBM). A long list of lncRNAs, including *ncRAN* (non-coding RNA expressed in aggressive neuroblastoma), *T-UC 300A, NBAT* (neuroblastoma associated transcript 1), *lncUSMycN, CAI2* (CDKN2A/ARF Intron 2 lncRNA)*, Paupar, and MALAT1* (metastasis-associated lung adenocarcinoma transcript 1) have been considered as perfect candidates to be involved in neuroblastoma [24]–[28]. Similarly, several studies have implicated lncRNAs, such as *linc-POU3F3, HOTAIR (Hox transcript antisense intergenic RNA*) *, H19, TUG1* (taurine upregulated gene 1), *GAS5* (growth arrest-specific 5), *NEAT1* (nuclear enrichment abundant transcript 1) *and MALAT1* in glioma pathogenesis and progression [7], [29]–[31].

Several lines of evidence have already implicated *MIAT* in glioma pathogenesis and progression. High *MIAT* expression has been associated with prolonged survival in GBM patients [32]. Further, the expression of *MIAT* is upregulated after de-methylation treatment, a common therapeutic approach, in GBM patients [33]. Together with that, *MIAT*'s prognostic value has been validated for the stratification of GBM patients, as part of a six- lncRNA signature [31]–[33]. Nevertheless, our knowledge of the mechanisms mediating these effects, as well as of the eligibility of this molecule as a biomarker for neuroblastoma and/or glioma, is still lacking, since the precise role of *MIAT* in these systems is far from being thoroughly investigated and fully elucidated.

To this end, the aim of the current study is to unveil the underpinning role of the subnuclear body-associated lncRNA MIAT, in cellular proliferation and survival of neuroblastoma and glioma cells. Through sequencing of the whole transcriptome, we investigated gene expression changes and key molecular pathways modulated in response to altered MIAT levels. Furthermore, the role of MIAT in the regulation of both short- and long-term cell survival, cell death and cell migration of neuroblastoma and glioma cells was investigated.

2. Materials and Methods

2.1 Cell culture and passaging

Three cell lines were used to perform the studies reported herein: the human neuroblastoma SH-SY5Y cell line, purchased from the ATCC (ATCC® CRL-2266™), the human astrocytoma/GBM 1321N1 cell line and the human GBM T98G cell line, kindly donated by Dr. N. Leslie, Heriot-Watt University. SH-SY5Y and 1321N1 were cultured using the HyClone™ DMEM/F12 1:1 growth media (GE Healthcare Life Sciences), supplemented with 10% heat-inactivated fetal bovine serum (Biosera), 2μM L-Glutamine, 1μM Sodium Pyruvate and 10mg/ml gentamicin solution (Sigma-Aldrich). For T98G the same recipe was used, supplemented with an extra 10% FBS, 15% cell-conditioned growth media and 1% MEM non-essential amino acid solution (Sigma-Aldrich). All cells were incubated in a humidified incubator at 37°C and 5% CO_2 and upon reaching ~80% confluence, were washed twice with phosphate buffered saline (PBS), trypsinised by adding 3ml of 0.25% Trypsin/EDTA solution (Sigma-Aldrich), centrifuged (1500 rpm, 7 minutes) and finally the cell pellet was resuspended in the appropriate volume of growth media to acquire a $8x10^4$ cells/ml cell density for SH-SY5Y, a $15x10^4$ cells/ml cell density for 1321N1 and T98G. . Cell lines were replaced with fresh stocks after a maximum culture period of 2 months.

2.2 *MIAT* downregulation

RNA interference

The experiments were performed using Nucleofection as a method of transfection. The siRNAs used included the *Silencer*® Negative control siRNA, and three different *MIAT* specific siRNAs: MIAT_1 siRNA, MIAT_2 siRNA and MIAT_3 siRNA (QIAGEN) targeting different sites of the fifth exon of the full length *MIAT* transcript [NR_003491 (10193 bp)] (Supplementary table 1). The Amaxa™ Cell Line Nucleofector™ Kit V (LONZA) and the program A-023 were used for the SH-SY5Y cell line, and the Ingenio® kit (Mirus) and the programmes T-016 and X-001 were used for the 1321N1and T98G cell lines, respectively. $1.5x10^6$ (SH-SY5Y) and $1.2\ x10^6$ (1321N1 and T98G) cells were transfected according to the manufacturer's protocol, and were incubated and re-plated at $1x10^5$ and $0.5\ x10^5$ cells/ml (SH-SY5Y, and 1321N1/T98G, respectively).

LNA GapmeRs

In addition to siRNAs, LNA GapmeRs were used in a series of experiments to knockdown *MIAT.* Antisense LNA GapmeRs are highly potent, single-stranded antisense oligonucleotides (ASO) for silencing of lncRNAs in cell cultures. The GapmeRs that were used included the Negative control A Antisense LNA GapmeR (QIAGEN) and three custom designed GapmeRs (namely 1_1, 2_1, 2_2) (QIAGEN) targeting different sites of the full length MIAT transcript [NR_003491 (10193 bp) (Supplementary table 1). The conditions of these experiments were identical to those used for the siRNA-mediated knockdown.

2.3 RNA extraction

Total RNA was extracted from cells using the Direct-zol™ RNA MiniPrep kit (ZYMO RESEARCH, Cat # R2050), according to the manufacturer's protocol. The assessment of RNA quality and the quantity was performed by spectrophotometric analysis (NanoDrop™ 1000, ThermoFisher Scientific). Samples with NanoDrop 260nm/280nm absorbance ratio between1.8-2 were considered of high purity.

2.4 Real-Time PCR

The effects of the RNA interference on MIAT expression levels was quantified by Real-Time PCR. To this end, RNA extracted (as described in section 2.3) from transfected cells was reverse transcribed into cDNA using the Omniscript® RT kit (QIAGEN), 10 μM random primers (Invitrogen) and 10 units/μl RNaseOUT recombinant ribonuclease inhibitor (Invitrogen), following the manufacturer's instructions.

Real-Time PCR was subsequently performed for the synthesized cDNA using the SensiFast Probe Hi-ROX kit and TaqMan Gene Expression Assays (Assay code Hs00402814_m1 for MIAT Hs99999901_s1 for eukaryotic 18S rRNA, Applied Biosystems), according to the manufacturer's instructions. The ABI Prism 7000 (Applied Biosystems) was used for the measurement of real-time fluorescence and the ABI Prism 7000 SDS software was used to perform the data analysis. Expression

comparisons were made relative to the negative (-ve) siRNA transfected cells, using the $2^{-\Delta\Delta Ct}$ method.

2.5 RNA Sequencing

Global gene expression changes in response to *MIAT* silencing were determined by sequencing the whole transcriptome. This approach has key advantages over equivalent microarray analyses including identification and quantification of unknown transcripts and novel splice variants. Total RNA was extracted as detailed above. Next-generation sequencing was conducted by the Earlham Institute; sequencing libraries were prepared using the NEXTflex directional RNA-Seq Library Kit, and following stringent quality control measures, sequenced to a depth of approximately 30 million reads per sample, 150bp PE read metric, on the HiSeq 4000 platform.

Raw sequencing data was trimmed of sequencing adapters and low quality reads discarded using the Trimgalore package, a wrapper that incorporates CutAdapt and FastQC. Quality controlled reads were aligned to Human Genome build (hg19) using Tophat, a splice-junction aware mapping utility necessary for the successful mapping of any intron-spanning (multi-exon) transcripts, transcripts were assembled using Cufflinks (with GTF support) and the number of reads mapping to each feature counted and expressed as FPKM using the CuffNorm package. Differentially expressed mRNAs were condensed into gene networks representing biological and disease processes using iPathwayGuide, with the aim of elucidating key mechanisms responsible for mediating the phenotypic effects of gene knockdown.

2.6 Assessment of apoptosis-mediated cell death

At specific time intervals after transfection (48 and 72h) apoptosis was determined by assessment of the nuclear morphology using fluorescence microscopy after staining with acridine orange (5µg/ml).

2.7 Assessment of long-term cell survival

The long term survival of the cells depends on their ability to form colonies. Therefore, siRNA transfected cells were re-plated 48/72h post transfection in 6-well plates at optimized densities (500/75/100 cells/ml for SH-SY5Y/1321N1/T98G, respectively) and were incubated for two to three weeks. The colonies were stained with 1% w/v Crystal Violet (Sigma-Aldrich), air-dried and counted.

2.8 Assessment of cell migration

The ability of cell migration was assessed by the wound healing assay. Cells were re-plated in 12-well plates in triplicates at $2x10^5$ and $1x10^5$ cells/ml (SH-SY5Y, and 1321N1/T98G, respectively), were incubated for 24 and 48h for 1321N1/T98G and SH-SY5Y, respectively, and a small linear scratch was introduced. The cells were then washed with PBS and fresh media was added to the wells. The cells were observed under transmitted light using the EVOS FL Cell Imaging System (Life

Technologies) at 0/18/24h and 0/24/48h for 1321N1/T98G and SH-SY5Y, respectively, and the gap closure was calculated using the formula [(Pre-migration)$_{area}$-(Migration)$_{area}$/ (Pre-migration)$_{area}$] x100 for 15 measurements per sample. Image analysis was performed using the ImageJ software.

2.9 Statistical Analysis

Statistical analyses were performed using GraphPad Prism 6 (GraphPad Software). Data are presented as the mean±SEM; the number of observations (n) refers to different transfected samples, each transfection being conducted on a separate culture of cells. Comparisons were made using an unpaired Student's t-test or One-Way ANOVA with Bonferroni's multiple comparison test (MCT). Statistical significance was set at the 0.05 level. Differences were considered statistically significant when *P-value* was <0.05 (95% confidence intervals).

3. Results

3.1 RNA Sequencing reveals cancer-related differentially expressed pathways and perturbed biological processes following *MIAT* downregulation in SH-SY5Y cells

In order to elucidate the molecular mechanism through which MIAT exerts its biological effects, we identified those genes that exhibited the most pronounced expression changes in response to *MIAT* knockdown. Figure 1 presents the 10000 most variable genes as a heatmap and revealed a large number of genes that are deregulated in response to reduced *MIAT* levels. Moreover, global gene expression analysis also revealed that the two *MIAT* knockdown experiments (MIAT_2 and MIAT_4) did not perform equally, with very pronounced effects of the MIAT_2 siRNA noted in comparison with almost non-existent effects of the MIAT_4 siRNA, due to the fact that MIAT_4 siRNA knockdown did not lead to the desired downregulation levels of *MIAT* in these specific experiments (Supplementary figure 1). Therefore MIAT_4 was excluded from further functional assays.

		Upregulated Genes	Downregulated Genes	Cancer Hallmark	Oncogene/ TSG	Significance (*p-value*)
Pathway name	MAPK signalling pathway	*DDIT3*		Sustaining Proliferative Signalling/ Resisting Cell Death/ Activating Invasion and Metastasis	Oncogene/TSG	0.032
			MYC	Evading Growth Suppressors	Oncogene/TSG	
			FAS	Resisting Cell Death	Oncogene	
			GADD45A	Evading Growth Suppressors/ Resisting Cell Death	Oncogene/TSG	
			DAXX	Resisting Cell Death	TSG	
	EGFR tyrosine kinase inhibitor resistance	*NRG1*		Sustaining Proliferative Signalling	Oncogene	0.035
		HRAS		Evading Growth Suppressors	Oncogene	
		AKT3		Resisting Cell Death	Oncogene	
		STAT3		Sustaining Proliferative Signalling/ Resisting Cell Death/ Activating Invasion and Metastasis/ Inducing Angiogenesis	Oncogene	
	TGF-beta signalling pathway	*DCN*		Inducing Angiogenesis/ Avoiding Immune Destruction	TSG	0.046
			SMAD5	Evading Growth Suppressors	TSG	
			TGFBR1/2	Evading Growth Suppressors	TSG	
	Phospholipase D signalling pathway	*HRAS*		Evading Growth Suppressors	Oncogene	0.046
		MAPK3		Sustaining Proliferative Signalling	Oncogene	
			SYK	Evading Growth Suppressors/ Resisting Cell Death	Oncogene/TSG	
	NOD-like receptor signalling pathway	*CASP1/8*		Resisting Cell Death	TSG	0.048
		IKBKB/E		Sustaining Proliferative Signalling/ Resisting Cell Death/ Activating Invasion and Metastasis	Oncogene	
		RELA		Sustaining Proliferative Signalling/ Resisting Cell Death/ Activating Invasion and Metastasis	Oncogene	
		VDAC2		Resisting Cell Death	TSG	
			XIAP	Resisting Cell Death	Oncogene	
	cell death in response to oxidative	*MAPK7*		Sustaining Proliferative Signalling/	Oncogene	1.800e-4

Biological Process	stress		*HIF1A* *MCL1*	Resisting Cell Death/ Activating Invasion and Metastasis Sustaining Proliferative Signalling/ Inducing Angiogenesis/ Deregulating cellular energetics Resisting Cell Death	Oncogene Oncogene	
	regulation of MAPK cascade	*ADAM8* *FGF1* *HRAS*	*FAS* *GADD45A*	Inducing Angiogenesis/ Activating Invasion and Metastasis Evading Growth Suppressors/ Inducing Angiogenesis Evading Growth Suppressors Resisting Cell Death Evading Growth Suppressors/ Resisting Cell Death	Oncogene Oncogene Oncogene Oncogene Oncogene/TSG	8.400e-4
	cellular response to radiation	*TP73*	*GADD45A* *MME*	Resisting Cell Death/ Deregulating cellular energetics Evading Growth Suppressors/ Resisting Cell Death Deregulating cellular energetics/ Activating Invasion and Metastasis	TSG Oncogene/TSG Oncogene	0.001
	tissue migration	*FGF1* *CDH13*	*TGFBR1/2*	Evading Growth Suppressors/ Inducing Angiogenesis Sustaining Proliferative Signalling /Evading Growth Suppressors/ Resisting Cell Death/ Activating Invasion and Metastasis Evading Growth Suppressors	Oncogene TSG TSG	0.004
	cellular response to oxidative stress	*ETS1* *RELA*		Resisting Cell Death/ Activating Invasion and Metastasis/ Inducing Angiogenesis Sustaining Proliferative Signalling/ Resisting Cell Death/ Activating Invasion and Metastasis	Oncogene Oncogene	0.008

			MCL1 *FUT8*	Resisting Cell Death Activating Invasion and Metastasis	Oncogene Oncogene	
	angiogenesis	*ADAM2/8* *CDH13* *FGF1* *HYAL1* *TERT* *VEGFB*		Inducing Angiogenesis/ Activating Invasion and Metastasis Sustaining Proliferative Signalling /Evading Growth Suppressors/ Resisting Cell Death/ Activating Invasion and Metastasis Evading Growth Suppressors/ Inducing Angiogenesis Inducing Angiogenesis/ Activating Invasion and Metastasis Enabling Replicative Immortality Inducing Angiogenesis	Oncogene TSG Oncogene Oncogene Oncogene Oncogene	0.012
	vascular endothelial growth factor production	*BRCA1*	*NDRG2* *HIF1A*	Evading Growth Suppressors Evading Growth Suppressors/ Resisting Cell Death/ Inducing Angiogenesis/ Deregulating cellular energetics Sustaining Proliferative Signalling/ Inducing Angiogenesis/ Deregulating cellular energetics	TSG TSG Oncogene	0.013
	regulation of cell adhesion mediated by integrin	*MUC1*	*SYK* *ITGAV*	Activating Invasion and Metastasis Evading Growth Suppressors/ Resisting Cell Death Sustaining Proliferative Signalling/ Activating Invasion and Metastasis	Oncogene Oncogene/TSG Oncogene	0.016
	regulation of cellular response to oxidative stress	*MAPK7*	*FUT8* *MCL1*	Sustaining Proliferative Signalling/ Resisting Cell Death/ Activating Invasion and Metastasis Activating Invasion and Metastasis Resisting Cell Death	Oncogene Oncogene Oncogene	0.021

	regulation of cell migration	*ADAM8* *FGF1* *NRG1* *TERT* *LAMA4*		Inducing Angiogenesis/ Activating Invasion and Metastasis Evading Growth Suppressors/ Inducing Angiogenesis Sustaining Proliferative Signalling Enabling Replicative Immortality Inducing Angiogenesis/ Activating Invasion and Metastasis	Oncogene Oncogene Oncogene Oncogene Oncogene	0.045
	regulation of oxidative stress-induced intrinsic apoptotic signalling pathway	*MAPK7*	*MCL1* *BAG5*	Sustaining Proliferative Signalling/ Resisting Cell Death/ Activating Invasion and Metastasis Resisting Cell Death Resisting Cell Death	Oncogene Oncogene Oncogene	0.046
	autophagosome assembly		*MTMR3* *RAB23*	Sustaining Proliferative Signalling/ Resisting Cell Death/ Activating Invasion and Metastasis Evading Growth Suppressors	Oncogene Oncogene	0.049

Table 1. Molecular and biological perturbations in response to *MIAT* downregulation (via MIAT_2 siRNA)

TSG: tumour suppressor gene; FAS: Fas cell surface death receptor; GADD45A: growth arrest and DNA damage inducible alpha; DDIT3: DNA damage inducible transcript 3; MYC: MYC proto-oncogene, bHLH transcription factor; DAXX: death domain associated protein; NRG1: Neuregulin 1; HRAS: HRas Proto-Oncogene, GTPase; AKT3: AKT Serine/Threonine Kinase 3; STAT3: Signal Transducer And Activator Of Transcription 3; SMAD5: SMAD Family Member 5; TGFBR1/2: Transforming Growth Factor Beta Receptor 1/2; DCN: Decorin; SYK: Spleen Associated Tyrosine Kinase; MAPK3: Mitogen-Activated Protein Kinase 3; XIAP: X-Linked Inhibitor Of Apoptosis; CASP1/8: Caspase 1/8; IKBKB/E: Inhibitor Of Nuclear Factor Kappa B Kinase Subunit Beta/Epsilon; RELA: RELA Proto-Oncogene, NF-KB Subunit; VDAC2: Voltage Dependent Anion Channel 2; HIF1A: Hypoxia Inducible Factor 1 Alpha Subunit; MCL1: BCL2 Family Apoptosis Regulator; MAPK7: Mitogen-Activated Protein Kinase 7; ADAM2/8: ADAM Metallopeptidase Domain 2/8; FGF1: Fibroblast Growth Factor 1; TP73: Tumor Protein P73; MME: Membrane Metalloendopeptidase; CDH13: Cadherin 13; ETS1: ETS Proto-Oncogene 1, Transcription Factor; FUT8: Fucosyltransferase 8; HYAL1: Hyaluronoglucosaminidase 1; TERT: Telomerase Reverse Transcriptase; NDRG2: N-Myc Downstream Regulated 2; BRCA1: BRCA1, DNA Repair Associated; ITGAV: Integrin Subunit Alpha V; MUC1: Mucin 1, Cell Surface Associated; LAMA4: Laminin Subunit Alpha 4; BAG5; BCL2 Associated Athanogene 5; MTMR3: myotubularin related protein 3; RAB23: RAB23, Member RAS Oncogene Family

Given the superior effects of our MIAT_2 knockdown siRNA, we next investigated those genes that were deregulated by a factor of at least 1.5 fold (log2 = 0.6) between the control and MIAT_2 knockdown samples. In excess of 10,000 genes were identified as being deregulated by at least 1.5 fold and thus we elected to condense the individual gene changes into common biological processes and pathways prior to interpretation.

Pathway analysis revealed that a number of cellular processes were affected by the downregulation of *MIAT*. Among them, numerous cancer-related processes were significantly affected, including apoptosis-mediated cell death, oxidative stress, cell migration, angiogenesis and autophagy. These perturbations were also reflected in the corresponding molecular pathways. Eight pathways were found to be significantly impacted and of these, five were cancer-related (Table 1). Figure 2 shows the expression of genes implicated in each of these five pathways.

3.2. siRNA-mediated *MIAT* knockdown increases basal apoptosis and decreases long-term survival and migration in SH-SY5Y neuroblastoma cells

Given the fact that among the perturbed pathways as revealed by the RNA sequencing cell survival- and cell growth-associated pathways, such as the MAPK and EGFR pathways, were significantly deregulated, we were prompted to examine the effects of *MIAT* silencing on long-term survival of our cell lines. For this, SH-SY5Y neuroblastoma cells were transfected with one of three different siRNAs which target different *MIAT* sequences. Of the three *MIAT*-specific siRNAs used only MIAT_2 and MIAT_3 led to the downregulation of *MIAT* as assessed by qRT-PCR in these cells, and therefore MIAT_1 was excluded from this series of functional assays.

The long-term survival of SH-SY5Y cells, as measured by clonogenic assays, was decreased due to the downregulation of *MIAT.* Specifically, the findings show an overall significant decrease in the number of colonies for the two of the three *MIAT*-specific siRNAs (Figures 3b, 3c) (34.3% for MIAT_2 and 45.8% for MIAT_3). In addition, the levels of apoptosis were measured 48h, 72h and 96h after transfection. Impressively, the levels of apoptotic cells were immensely increased at all time intervals after downregulation by both *MIAT*-specific siRNAs, especially 72h post-Nucleofection, with apoptotic cells comprising about 20% of the cell population (Figures 3d-3f). To further validate the effect of *MIAT* downregulation on apoptosis, a LNA GapmeR-mediated downregulation approach was adopted. The obtained results confirmed the siRNA results, suggesting that the downregulation of *MIAT* indeed induces a 2-fold increase of apoptotic cells for all of the three different GapmeRs (namely 1_1, 2_1, 2_2) used in this case, both after 48h and 72h (supplementary figure 2).

The observation of an altered morphology of the SH-SY5Y cells under the microscope following transfection (Figure 4) led to the assumption that the migrating ability of the cells could be as well affected. To this end, this hypothesis was tested via the wound healing assay. The results provided confirmation that the migration capability of the cells is deteriorated due to the silencing of *MIAT* at both time points tested (24 and 48h) for both *MIAT*-specific siRNAs. Specifically, there was

significantly less gap closure, with the greatest results being observed after 48h for MIAT_2 (33% less gap closure) (Figures 5a-c).

3.3 siRNA-mediated *MIAT* knockdown increases basal apoptosis and decreases long-term survival and migration in glioblastoma cells

The effects of *MIAT* knockdown on the levels of apoptosis, long-term survival and migration observed in the neuroblastoma cells, together with previous literature that strongly associated *MIAT* with cell survival [31]–[33], generated the question whether the same effects would be observed in GBM cell lines. To this end, and similar to the response of the SH-SY5Y neuroblastoma cells to *MIAT* downregulation, the 1321N1 cells displayed a similar response pattern. In terms of long-term survival, the number of colonies was significantly decreased for all three *MIAT*-specific siRNAs (Figures 6b, 6c) (18.5% for MIAT_1, 23.1% for MIAT_2 and 26.2% for MIAT_3, n=5 experiments). Following the same procedure as for the SH-SY5Y cells, apoptosis-mediated cell death was assessed by acridine orange. The acquired results have revealed an outstanding increase of apoptosis for all the three siRNAs at all time points (48h/72h/96h), especially for MIAT_2. Similarly to SH-SY5Y, the greatest effects were observed 72h after the Nucleofection (Figures 6d-6f). In line with the confirmation strategy followed for the neuroblastoma cells, GapmeRs were employed to confirm the effect on apoptosis. In this case, as well, all the three GapmeRs induced the downregulation of *MIAT* and consequently induced elevated apoptosis levels after 48h and 72h (Supplementary figure 3).

Similar to the SH-SY5Y cells, an unexpected change in morphology was observed in the 1321N1 cells as well (Figure 7), giving a lead to assess the cells' migration ability using the wound healing assay. The results provided again confirmation that the migration capability of the cells is reduced due to the silencing of MIAT at both time points tested (18 and 24h) for all the three *MIAT*-specific siRNAs. Specifically, there was significantly less gap closure, with the greatest results being observed after 24h for MIAT_2 (~38% less gap closure) and MIAT_3 (~37% less gap closure) (Figures 8a-c).

Consistent with these observations, the T98G glioblastoma cells also displayed a decrease in the colony number for all three *MIAT*-specific siRNAs (Figures 9b, 9c), however this decrease was statistically significant only for MIAT_1 and MIAT_3 (24.2% for MIAT_1, and 34.9% for MIAT_3, n=4 experiments), while the decrease in MIAT_3 was not significant. As far as the apoptosis levels -as assessed by acridine orange- are concerned, the results acquired for the T98G cell line are in agreement with the other two studied cell lines. More specifically, in this instance the apoptosis levels were assessed 48 and 72h post the re-plating of the cells. The results have revealed a significant increase of apoptosis for all the three siRNAs at both time points. The magnitude of the effect was similar at both time points, ranging from ~11% to ~13% of apoptotic cells for siRNA-treated cells versus ~4% for the negative control (Figures 9d-9f). In addition, validation of the effect of *MIAT* downregulation on apoptosis was performed using *MIAT*-specific GapmeRs. The results revealed that the knockdown of *MIAT* causes an elevation in the number of apoptotic cells comparing to the negative control (2-3-fold) for all the GapmeRs used at both time points assessed (48h and 72h) (Supplementary figure 4).

Following the same rationale we followed for the other two cell lines, we also decided to assess the impact of the downregulation of *MIAT* on the migratory activity of the T98G cells. The wound healing assay results have shown that the ability of the cells to migrate is affected by the downregulation of *MIAT*. In detail, their invasive ability is decreased following the downregulation of *MIAT* by all three *MIAT*-specific siRNAs. This effect is again observed at both time points tested (18 and 24h). Specifically, the greatest results were observed after 18h for MIAT_2 (~38% less gap closure) (Figures 10a-c).

4. Discussion

Due to their versatility, as well as their diverse and elegantly controlled roles as key regulators in every level of gene expression, lncRNAs have been recognised as essential players both in normal cell physiology and numerous diseases and disorders, including a variety of cancers, and are currently being thoroughly investigated [34], [35]. lncRNAs are involved, among others, in the regulation of cell survival, apoptosis [34], [36] and migration [37]. To this end, the current study aimed at unravelling the role of *MIAT* in the regulation of these processes in neuroblastoma and glioblastoma by *MIAT* downregulation using a neuroblastoma (SH-SY5Y) and two GBM (1321N1 and T98G) cell lines, producing a number of interesting observations. Interestingly, these include the deregulation of several vital cancer-related processes as revealed by the elimination of the long-term survival and the migratory ability of the cells, as well as their increased basal apoptosis.

The RNA sequencing results have provided very useful and diverse insights of the role of *MIAT* in a variety of cancer-related procedures. Of special interest was the fact that numerous processes associated with oxidative stress-induced cell death, such as the regulation of cellular response to oxidative stress and the regulation of oxidative stress-induced intrinsic apoptotic signalling pathway, were remarkably perturbed in response to *MIAT* knockdown. It has long been established that cancer cells are metabolically active and undergo severe oxidative stress, leading to the production of ROS (reactive oxygen species)[38]. ROS have also long been speculated to be associated with diverse cellular responses of the cancer cell depending on the cell background, ranging from a transient growth arrest and adaptation, increase in cellular proliferation, permanent growth arrest or senescence, apoptosis, and necrosis [39]. For example, the notorious *c-myc,* whose expression is slightly downregulated in our dataset, has been implicated in the production of ROS through oncogenic processes [40]. From a lncRNA perspective, more recent findings suggest that an extensive list of lncRNAs, including *NEAT1* (nuclear enrichment abundant transcript 1)*, lincRNA-p21, UCA1* (Urothelial Cancer Associated 1)*, H19,* and *MALAT1* (metastasis-associated lung adenocarcinoma transcript 1)*,* are implicated in oxidative stress and the consequent hypoxia [41]. In turn, a lot of other cellular processes are affected by oxidative stress, including angiogenesis, migration and metastasis, through different mechanisms [42]. Therefore, it comes as no surprise that *MIAT* seems to be involved in oxidative stress regulation and its downstream effects.

Following the lead of our RNA sequencing results, we performed a series of functional assays to validate the suggested effects. In fact, as the sequencing reveals in one of the most pronounced deregulated pathways, the NOD-like receptor signalling pathway, there is a tremendous upregulation of the initiator Caspase 8,

together with a significant downregulation of the anti-apoptotic *XIAP* (X-linked inhibitor of apoptosis protein). In line with that, our functional studies' results show an overall tendency of *MIAT*-specific siRNA knockdown to cause reduced long-term cell viability and an impressive multi-fold increase of the apoptosis levels in all the tested cell lines. These effects are in agreement with a number of studies looking into the role of *MIAT* in various healthy and unhealthy cells. For example, *MIAT* knockdown leads to reduced viability and increased apoptosis in endothelial cells, Müller glia and neurons, causing neurovascular dysfunctions and neurodegenerative disorders [15]. In addition, Shen *et al*.[18] found that the downregulation of *MIAT* leads to reduced survival of lens epithelial cells in cataract patients. In a cancerous context, Sattari *et al*. [20] found that siRNA-mediated suppression led to increased apoptosis in malignant B cells in patients with aggressive chronic lymphocytic leukaemia, while a recent study has revealed augmented basal apoptosis in various breast cancer cell lines in response to *MIAT* downregulation [43].

Notably, the effect on the long-term survival was not very pronounced in all cases, whereas apoptosis was vastly affected. Cancer is a complex disease in which there exist numerous pathway cross-talks. As the RNA sequencing suggests, a number of cell growth and pro-survival pathways are affected following *MIAT* downregulation, for example the overlapping MAPK, TGF-β, EGFR and Phospholipase D pathways, with a lot of crucial genes being perturbed in both directions (*HRAS* and *SMAD 5*, for example), making it extremely hard to decipher the direction in which the system is balancing. Another such example is c-MYC, a transcription factor that transcribes several target genes, mainly associated with cell survival and proliferation. However, it has also been found that when deregulated, it participates in both the intrinsic apoptotic pathway, therefore promoting apoptosis via anti-apoptotic molecule suppression (e.g. Bcl-2 family) and pro-apoptotic molecule induction, and the extrinsic apoptotic pathway [44], [45]. Additionally, *MYC*, which was found to be slightly downregulated (as part of the perturbed MAPK signalling cascade) following *MIAT* knockdown in SH-SY5Y cells in our RNA sequencing, had been previously found to cause *MIAT*'s significant upregulation when inhibited in GBMs [46]. This suggests a high versatility of these genes' functions, as well as a great network complexity, especially among different tumour types. Whether *MIAT* or its downregulation is somehow involved in these pathways leading to these phenotypes, or whether there is a regulatory loop between the two molecules in neuroblastoma and GBM cells remains to be investigated.

The next step incorporated the assessment of the effect of *MIAT* downregulation on cell migration, as implicated by the sequencing results. Similarly to our sequencing, prior functional annotation had implied that *MIAT* is associated with EMT-related canonical pathways in hepatocellular carcinoma cells [47], including the TGF-β pathway, justifying the perturbations in this pathway following the perturbation in *MIAT* expression in our study. Furthermore, consistent with other studies reporting that *MIAT* is involved in cell migration and invasion and its downregulation inhibits this effect in other cancer types, for example in NSCLC (non-small-cell lung cancer) [48], colorectal cancer [49], clear cell renal cell carcinoma [50] and breast cancer [51], our results reveal that the ability of neuroblastoma and GBM cells to migrate is as well inhibited to an important extent when *MIAT* is knocked down. Given that cell migration comprises one of the first steps towards tumour metastasis, *MIAT* downregulation could be a potent therapeutic approach towards the prevention of metastasis.

To link the aforementioned observations, it could be speculated that *MIAT* exerts its effects through a ROS-induced SP (Specificity protein) TF (transcription factor) mechanism. Sps belong to the Sp/Krüppel-like factor (KLF) family of TFs and play important roles in healthy and pathological settings, including cancer [52]. Among the various family members, Sp1, Sp3 and Sp4 have gained attention, with Sp1 being the subject of thorough investigation [53], and importantly all three members displayed at least a three-fold decrease upon *MIAT* knockdown in our RNA sequencing, and in addition, numerous regulators of Sp1, including various miRNAs (Supplementary table 2) and eighteen members of the ZBTB (zing finger and BTB) family were significantly deregulated. Since the elevated activity of Sp1 has been associated with malignancy and tumour progression in various cancers including glioma [53]–[55], it could be assumed that its downregulation could prevent this effect. In fact, a reasonable mechanism would suggest that *MIAT* knockdown induces an increase in ROS production, which in turn induces a ROS-mediated epigenetic downregulation of c-MYC [56] leading to the downregulation of Sp1 via the regulation of miRNAs and ZBTB proteins. Interestingly, the downstream effectors of Sp1 include a variety of crucial cancer-related genes involved in survival, apoptosis and migration, such as cMET (tyrosine-protein kinase Met), survivin, Fas, bcl-2, VEGFs and MMPs (matrix metallopeptidases) , and notably, a variety are deregulated in our study (Supplementary table 3).

In conclusion, the current study suggests that the downregulation of *MIAT* reduces the long-term survival of neuroblastoma and GBM cells, while it promotes basal apoptosis, as well as deteriorates the cells' ability to migrate. These findings highlight the crucial role of *MIAT* in a variety of cancer-promoting processes in neuroblastoma and glioblastoma pathogenesis. However, further research is essential to establish that *MIAT* could be used as a biomarker in neuroblastoma and GBM patient samples in the future, like numerous other lncRNAs for a variety of tumours [34], [35], [57] and open new prognostic, predictive and even therapeutic avenues for patients suffering from these tumours.

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Conflicts of Interest

The authors declare no competing interests.

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Figure 1: Heatmap of Log2 normalised expression values for the 10000 most variable genes. Data are Log normalised read counts expressed as Fragments Per Kilobase of transcript per Million mapped reads (FPKM). Dark blue colouration represents higher expression, whilst light green colouration denotes lower expression for each given gene. Y-axis clustering identifies groups of genes with similar expression patterns. The key (top left) represents the density of data (y) against expression level (x). ; -ve: negative siRNA.

Figure 2. The expression of individual genes implicated in cancer-related pathways: (a) MAPK signalling, (b) EGFR tyrosine kinase inhibitor resistance, (c) TGF-beta signalling, (d) Phospholipase D signalling and (e) NOD-like receptor signalling as determined by sequencing and analysis of the whole transcriptome. Data are the difference in expression between MIAT_2 knockdown and control cells expressed as a log fold change. Blue bars represent downregulated genes, whilst red bars represent upregulated genes.

Figure 3. *MIAT*-specific downregulation reduces the long-term survival and increases the levels of apoptosis of SH-SY5Y. SH-SY5Y cells were transfected with the negative siRNA or one of the two *MIAT*-specific siRNAs using Nucleofection, and were assessed 48h/72h/96h post-Nucleofection. The relative expression of *MIAT* was measured by Real-Time PCR 48h post-transfection, and was lower for the two siRNAs (a); Cells were also seeded and incubated (37°C, 5% CO_2) for two weeks, and the colonies formed were stained with Crystal Violet (1%w/v) and counted. Overall (n=3), MIAT-specific downregulation induced by *MIAT*-specific siRNAs leads to a decrease in the number of colonies formed (b); representative illustration of a clonogenic assay (c); The levels of apoptosis are overall (n=3) importantly elevated, especially 72h after Nucleofection, reaching even a 3-fold increase (d,e); representative illustration of apoptotic cells 72h post-Nucleofection, stained with acridine orange and observed using fluorescent microscopy (f). Grey arrows indicate cells undergoing apoptosis; -ve: negative siRNA, M2: MIAT_2, M3: MIAT_3. * indicates a *p-value*<0.05; ** indicate a *p-value*<0.01; **** indicate a *p-value*<0.001, as measured by One-way ANOVA tests. Data are represented as mean +/- SEM.

Figure 4. *MIAT*-specific downregulation alters the cellular morphology of SH-SY5Y. SH-SY5Y cells were transfected with the negative siRNA or one of the three *MIAT*-specific siRNAs using Nucleofection, and were subsequently stained with acridine orange and observed using light and fluorescent microscopy. The morphology of the cells is changed after transfection. The cells display less elongated, neuronal-like structures, as well as a more sparse spatial distribution pattern. Representative illustration of cells treated with the negative siRNA (a) and MIAT_2 siRNA (b); -ve: negative siRNA, M2: MIAT_2.

Figure 5. *MIAT*-specific downregulation reduces the migrating ability of SH-SY5Y. SH-SY5Y cells were transfected with the negative siRNA or one of the three *MIAT*-specific siRNAs using Nucleofection, re-plated, and a linear scratch was introduced 48h post re-plating. The % gap closure of the scratch was measured after 24 and 48h. The migrating ability of the cells is overall (n=3) reduced for all three *MIAT*-specific siRNAs at both time points assessed, especially for MIAT_2 at 48h (a); relative gap closure of *MIAT*-specific siRNAs versus the –ve siRNA (b); representative illustration of a wound healing (“scratch”) assay (c); -ve: negative siRNA, M2: MIAT_2, M3: MIAT_3. * indicates a *p-value*<0.05; ** indicate a *p-value*<0.01; *** indicate a *p-value*<0.001, as measured by One-way ANOVA tests. Data are represented as mean +/- SEM.

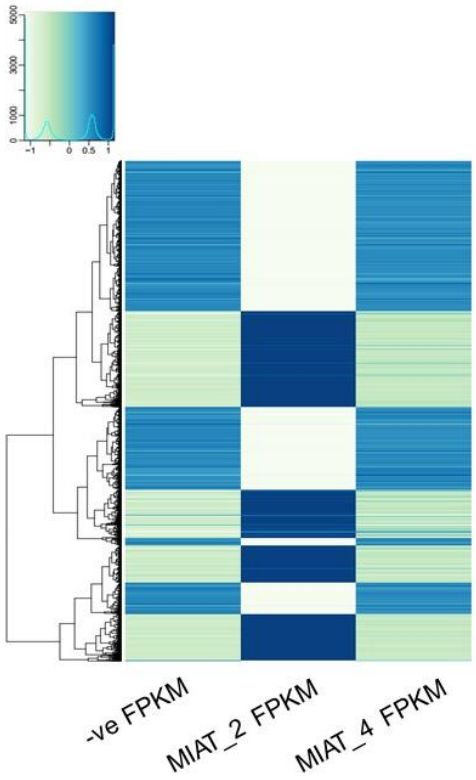
Figure 6. *MIAT*-specific downregulation reduces the long-term survival and increases the levels of apoptosis of 1321N1. 1321N1 cells were transfected with the negative siRNA or one of the three *MIAT*-specific siRNAs using Nucleofection, and were assessed 48h/72h/96h post-Nucleofection The relative expression of *MIAT* was measured by Real-Time PCR 48h post-transfection, and was lower for all three siRNAs (a); Cells were also seeded and incubated (37°C, 5% CO_2) for two weeks, and the colonies formed were stained with Crystal Violet (1%w/v) and counted. Overall (n=5), MIAT-specific downregulation induced by *MIAT*-specific siRNAs leads to a decrease in the number of colonies formed (b); representative illustration of a clonogenic assay (c); The levels of apoptosis are overall (n=3) importantly elevated, especially 72h after Nucleofection, reaching a 3-fold increase for MIAT_2-induced downregulation (d,e); representative illustration of apoptotic cells 72h post-Nucleofection, stained with acridine orange and observed using fluorescent microscopy (f). Grey arrows indicate cells undergoing apoptosis; -ve: negative siRNA, M1: MIAT_1, M2: MIAT_2, M3: MIAT_3. * indicates a *p-value*<0.05; ** indicate a *p-value*<0.01; ***/**** indicate a *p-value*<0.001, as measured by One-way ANOVA tests. Data are represented as mean +/- SEM.

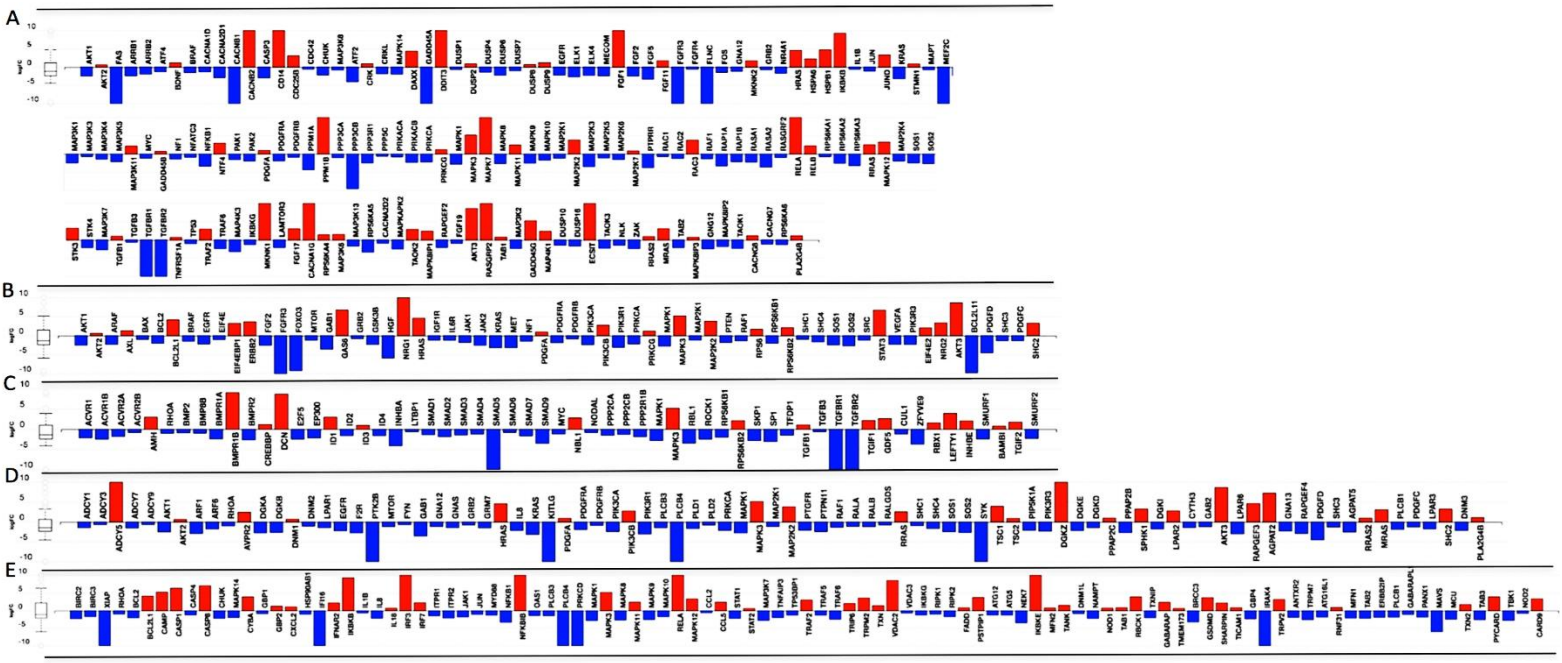
Figure 7. *MIAT*-specific downregulation alters the cellular morphology of 1321N1. 1321N1 cells were transfected with the negative siRNA or one of the three *MIAT*-specific siRNAs using Nucleofection, and were subsequently stained with acridine orange and observed using light and fluorescent microscopy. The morphology of the cells is changed after transfection. The cells display less elongated, neuronal-like structures, as well as a more sparse spatial distribution pattern. Representative illustration of cells treated with the negative siRNA (a) and MIAT_2 siRNA (b); -ve: negative siRNA, M2: MIAT_2.

Figure 8. *MIAT*-specific downregulation reduces the migrating ability of 1321N1. 1321N1 cells were transfected with the negative siRNA or one of the three *MIAT*-specific siRNAs using Nucleofection, re-plated, and a linear scratch was introduced 24h post re-plating. The % gap closure of the scratch was measured after 18 and 24h. The migrating ability of the cells is overall (n=3) reduced for all three *MIAT*-specific siRNAs at both time points assessed, especially for MIAT_2 and MIAT_3 at 24h (a); relative gap closure of *MIAT*-specific siRNAs versus the –ve siRNA (b); representative illustration of a wound healing (“scratch”) assay (c); -ve: negative siRNA, M1: MIAT_1, M2: MIAT_2, M3: MIAT_3. * indicates a *p-value*<0.05; ** indicate a *p-value*<0.01; ***/**** indicate a *p-value*<0.001, as measured by One-way ANOVA tests.

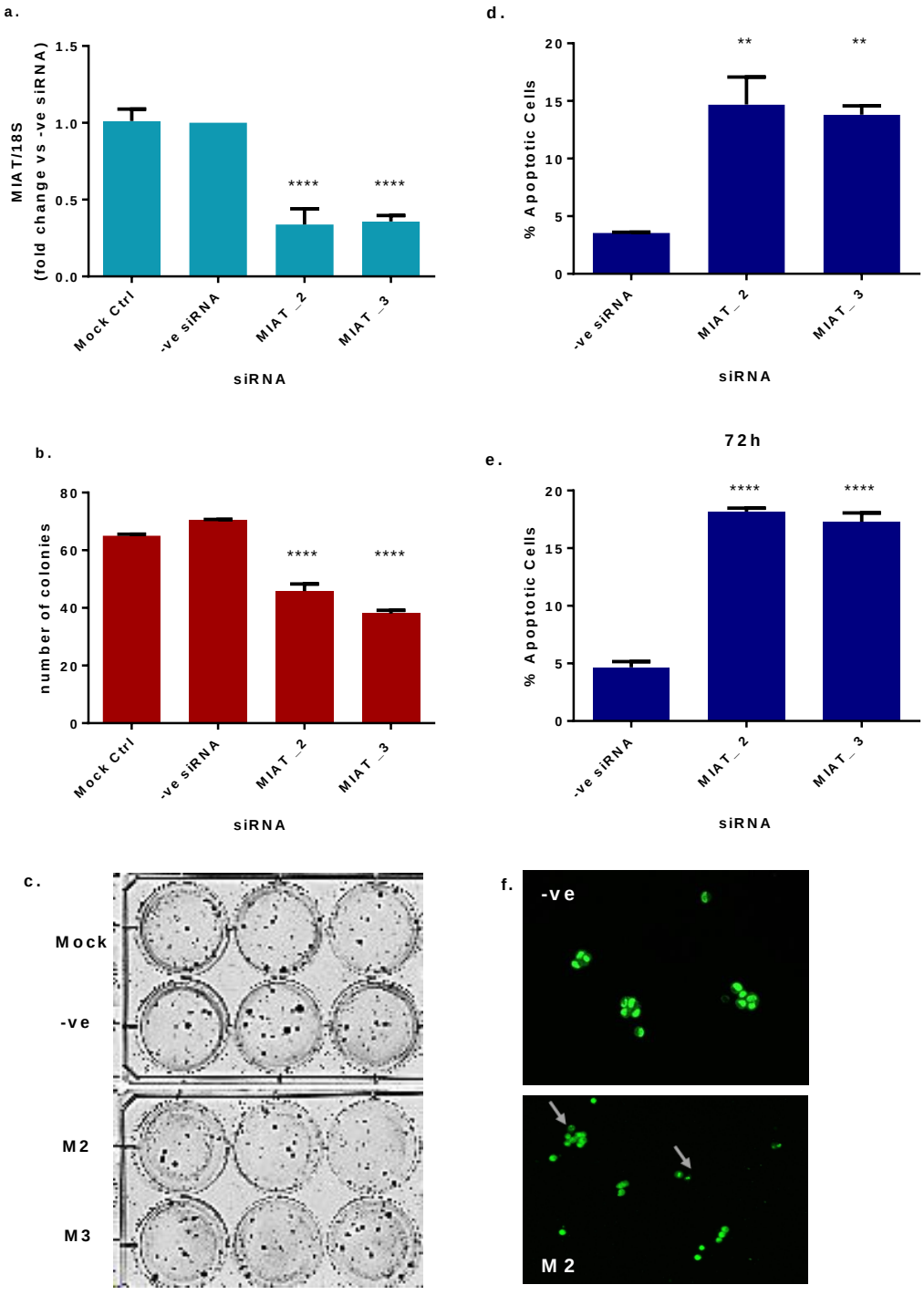
Figure 9. *MIAT*-specific downregulation reduces the long-term survival and increases the levels of apoptosis of T98G. T98G cells were transfected with the negative siRNA or one of the three *MIAT*-specific siRNAs using Nucleofection, re-plated, and assessed 48h/72h post re-plating. The relative expression of *MIAT* was measured by Real-Time PCR 48h post-transfection, and was lower for all three siRNAs (a); Cells were also seeded and incubated (37°C, 5% CO_2) for two weeks, and the colonies formed were stained with Crystal Violet (1%w/v) and counted. Overall (n=4), *MIAT*-specific downregulation induced by *MIAT*-specific siRNAs leads to a decrease in the number of colonies formed (b); representative illustration of a clonogenic assay (c); The levels of apoptosis are overall (n=4) importantly elevated, reaching a 2-fold increase for all three *MIAT*-specific siRNAs (d, e); representative illustration of apoptotic cells 48h post-Nucleofection, stained with acridine orange and observed using fluorescent microscopy (f). Grey arrows indicate cells undergoing apoptosis; -ve: negative siRNA, M1: MIAT_1, M2: MIAT_2, M3: MIAT_3. * indicates a *p-value*<0.05; ** indicate a *p-value*<0.01; ***/**** indicate a *p-value*<0.001, as measured by One-way ANOVA tests. Data are represented as mean +/- SEM.

Figure 10. *MIAT*-specific downregulation reduces migrating ability of T98G. T98G cells were transfected with the negative siRNA or one of the three *MIAT*-specific siRNAs using Nucleofection, re-plated, and a linear scratch was introduced 24h post re-plating. The % gap closure of the scratch was measured after 18 and 24h. The migrating ability of the cells is overall (n=3) tremendously reduced for all three *MIAT*-specific siRNAs at both time points assessed, especially for MIAT_2 (a); relative gap closure of *MIAT*-specific siRNAs versus the –ve siRNA (b); representative illustration of a wound healing ("scratch") assay (c); -ve: negative siRNA, M1: MIAT_1, M2: MIAT_2, M3: MIAT_3. * indicates a *p-value*<0.05; ** indicate a *p-value*<0.01; ***/**** indicate a *p-value*<0.001, as measured by One-way ANOVA tests. Data are represented as mean +/- SEM.


5000
3000
1000
0
−1
0
0.5
1
-ve FPKM
MIAT_2 FPKM
MIAT_4 FPKM


A
B
C
D
E

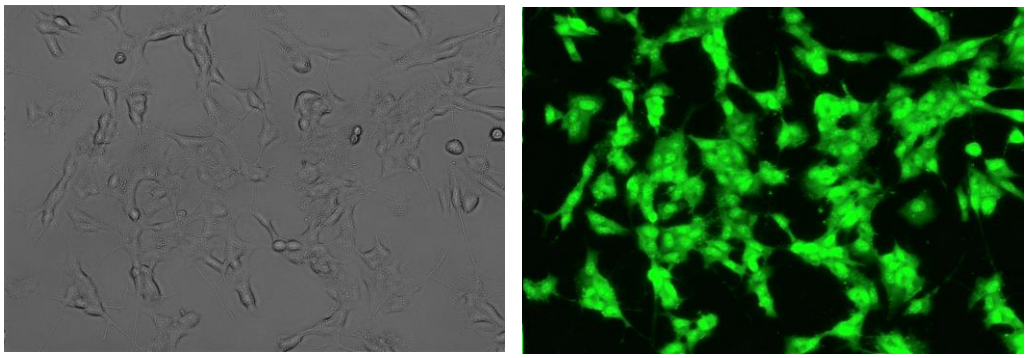
SH-SY5Y


a.
MIAT/18S
(fold change vs -ve siRNA)
1.5
1.0
0.5
0.0
****
****
Mock Ctrl
-ve siRNA
MIAT_2
MIAT_3
siRNA
d.
48h
% Apoptotic Cells
20
15
10
5
0
**
**
-ve siRNA
MIAT_2
MIAT_3
siRNA
b.
number of colonies
80
60
40
20
0
****
****
Mock Ctrl
-ve siRNA
MIAT_2
MIAT_3
siRNA
e.
72h
% Apoptotic Cells
20
15
10
5
0
****
****
-ve siRNA
MIAT_2
MIAT_3
siRNA
c.
Mock
-ve
M2
M3
f.
-ve
M2

SH-SY5Y

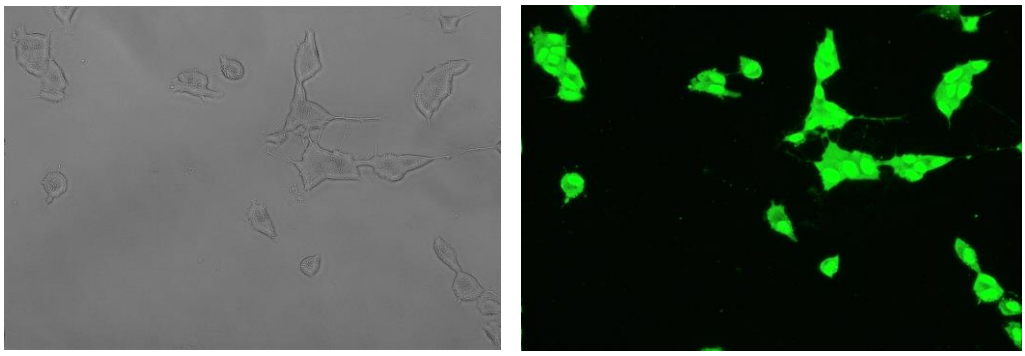
a.

-ve



b.

M2



SH-SY5Y

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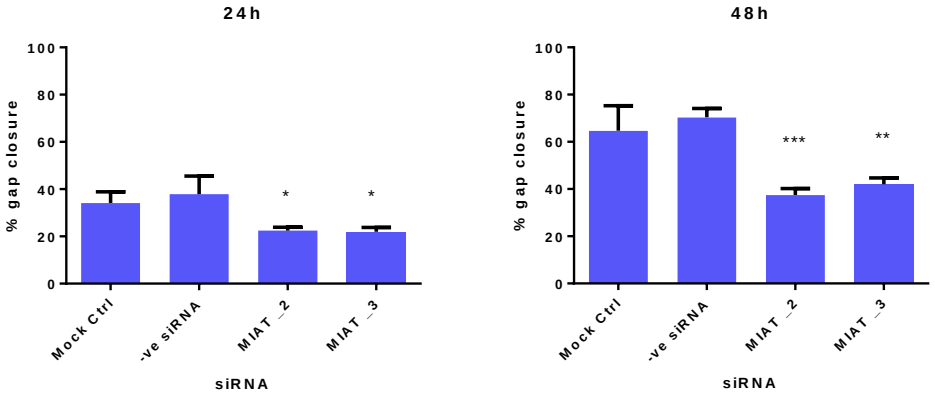
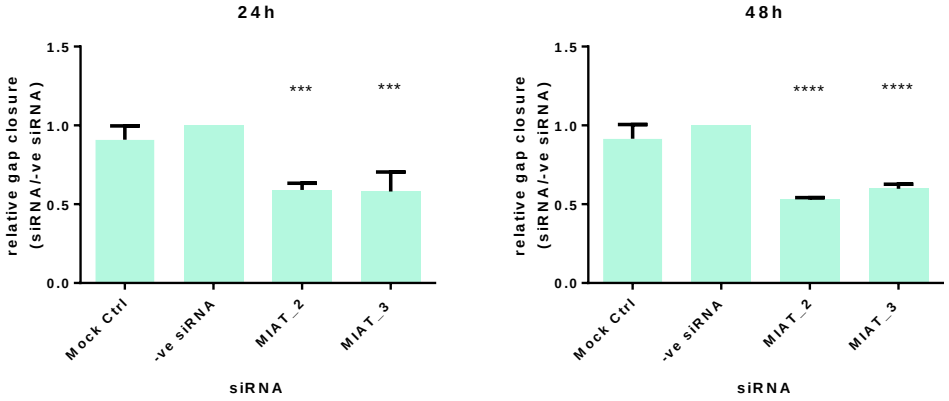
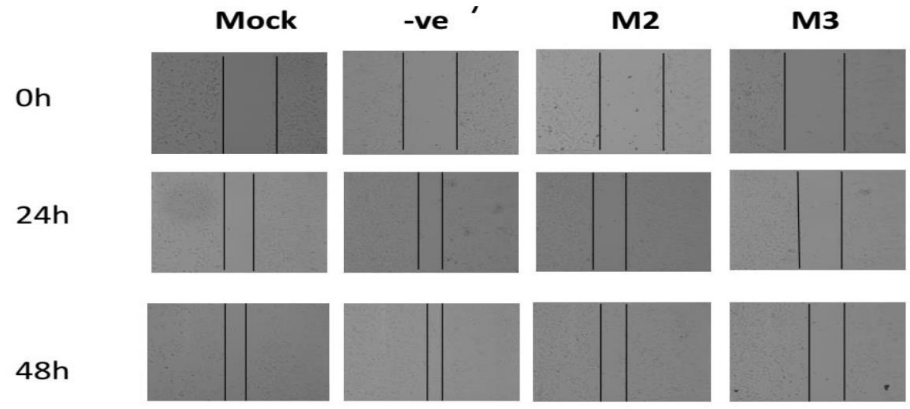

24h
48h
% gap closure
100
80
60
40
20
0
*
*
***
**
Mock Ctrl
-ve siRNA
MIAT_2
MIAT_3
siRNA

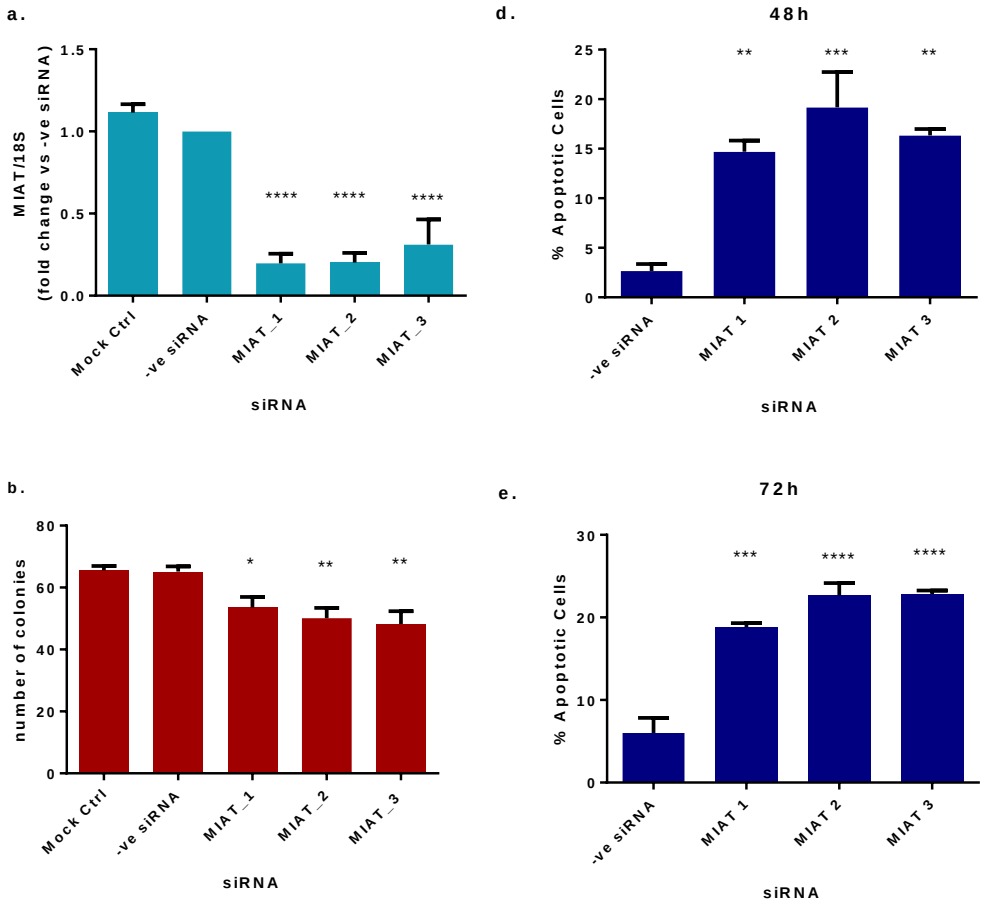
b.

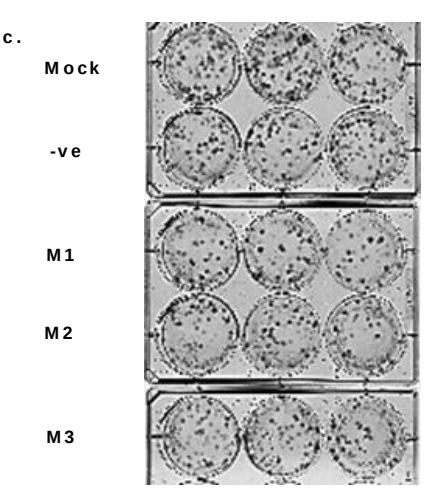

24h
48h
relative gap closure (siRNA/-ve siRNA)
1.5
1.0
0.5
0.0
***
***
****
****
Mock Ctrl
-ve siRNA
MIAT_2
MIAT_3
siRNA

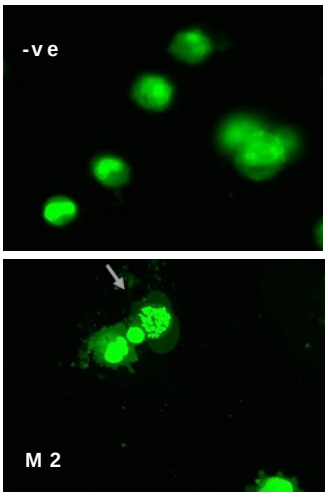
c.


Mock
-ve
M2
M3
0h
24h
48h

1321N1


a.
MIAT/18S
(fold change vs -ve siRNA)
1.5
1.0
0.5
0.0
****
****
****
Mock Ctrl
-ve siRNA
MIAT_1
MIAT_2
MIAT_3
siRNA
d.
48h
% Apoptotic Cells
25
20
15
10
5
0
**
***
**
-ve siRNA
MIAT 1
MIAT 2
MIAT 3
siRNA
b.
number of colonies
80
60
40
20
0
*
**
**
Mock Ctrl
-ve siRNA
MIAT_1
MIAT_2
MIAT_3
siRNA
e.
72h
% Apoptotic Cells
30
20
10
0
***
****
****
-ve siRNA
MIAT 1
MIAT 2
MIAT 3
siRNA

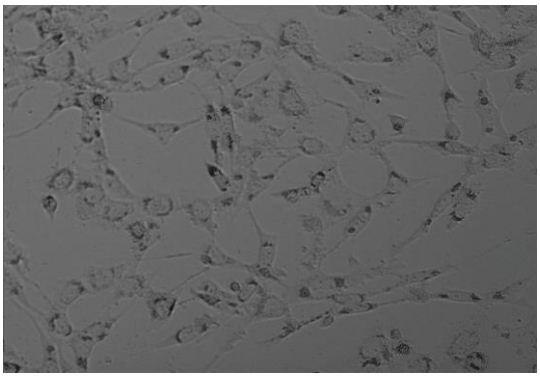

c.
Mock
-ve
M1
M2
M3

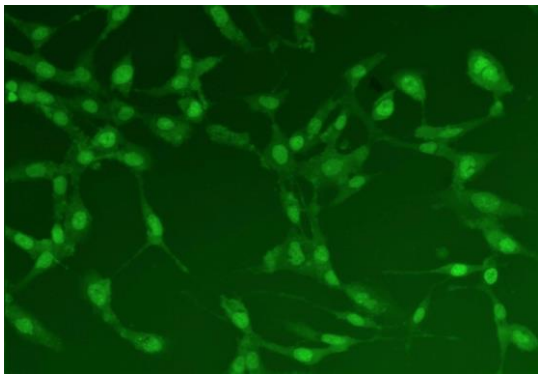

f.
-ve
M 2

1321N1

a.

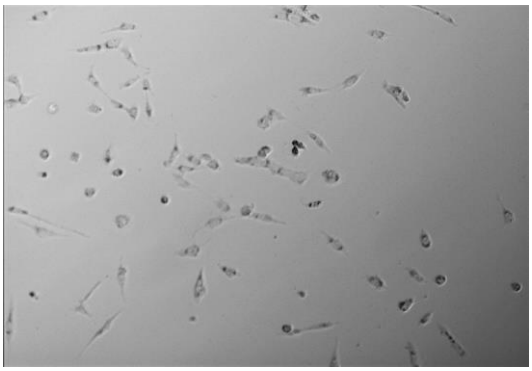
-ve

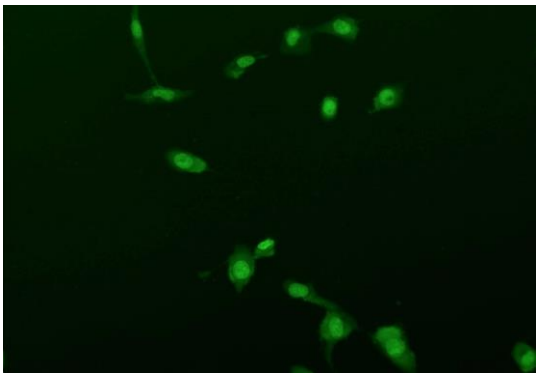




b.

M2





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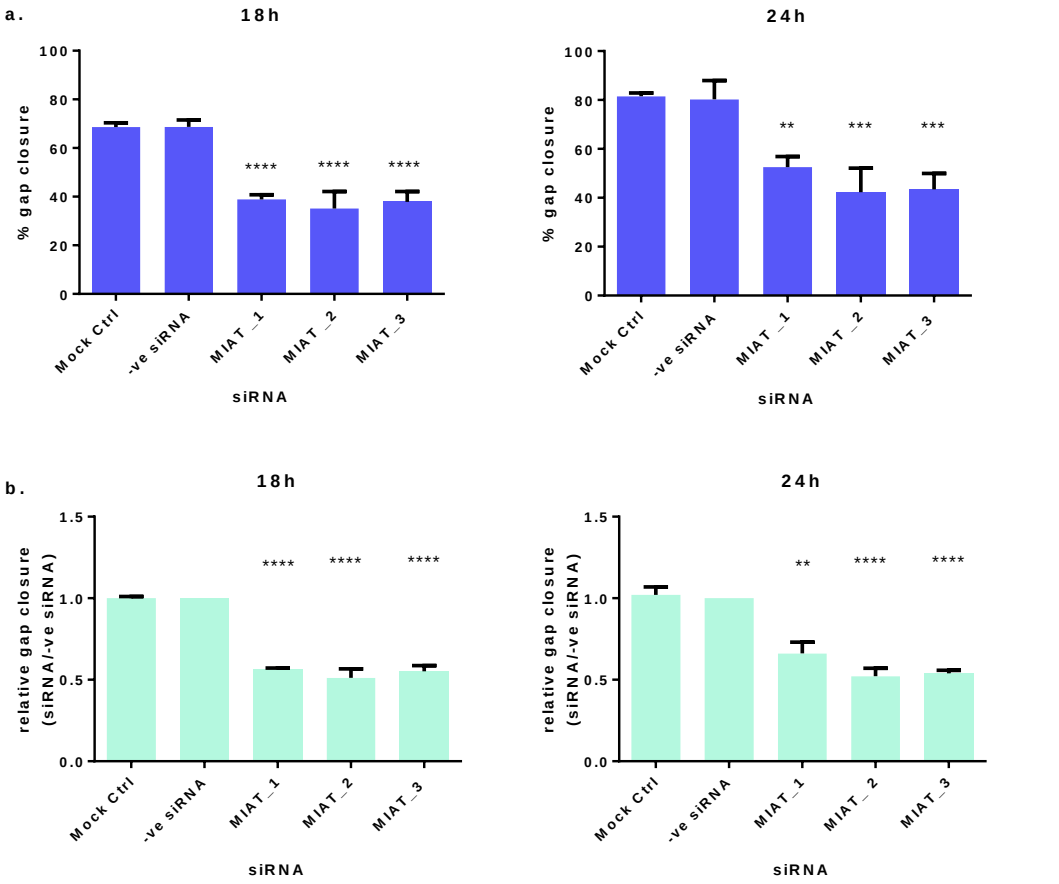
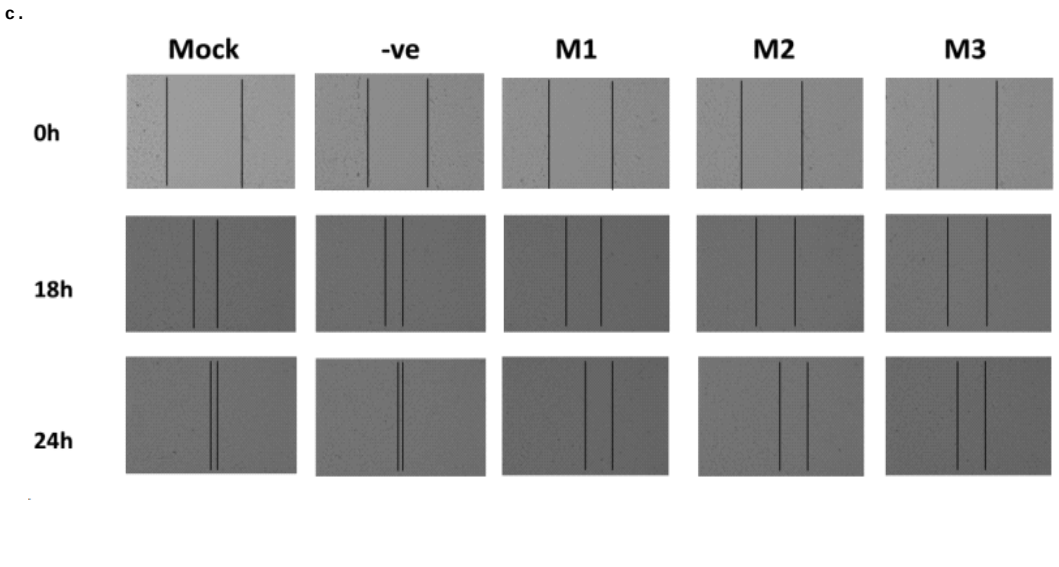
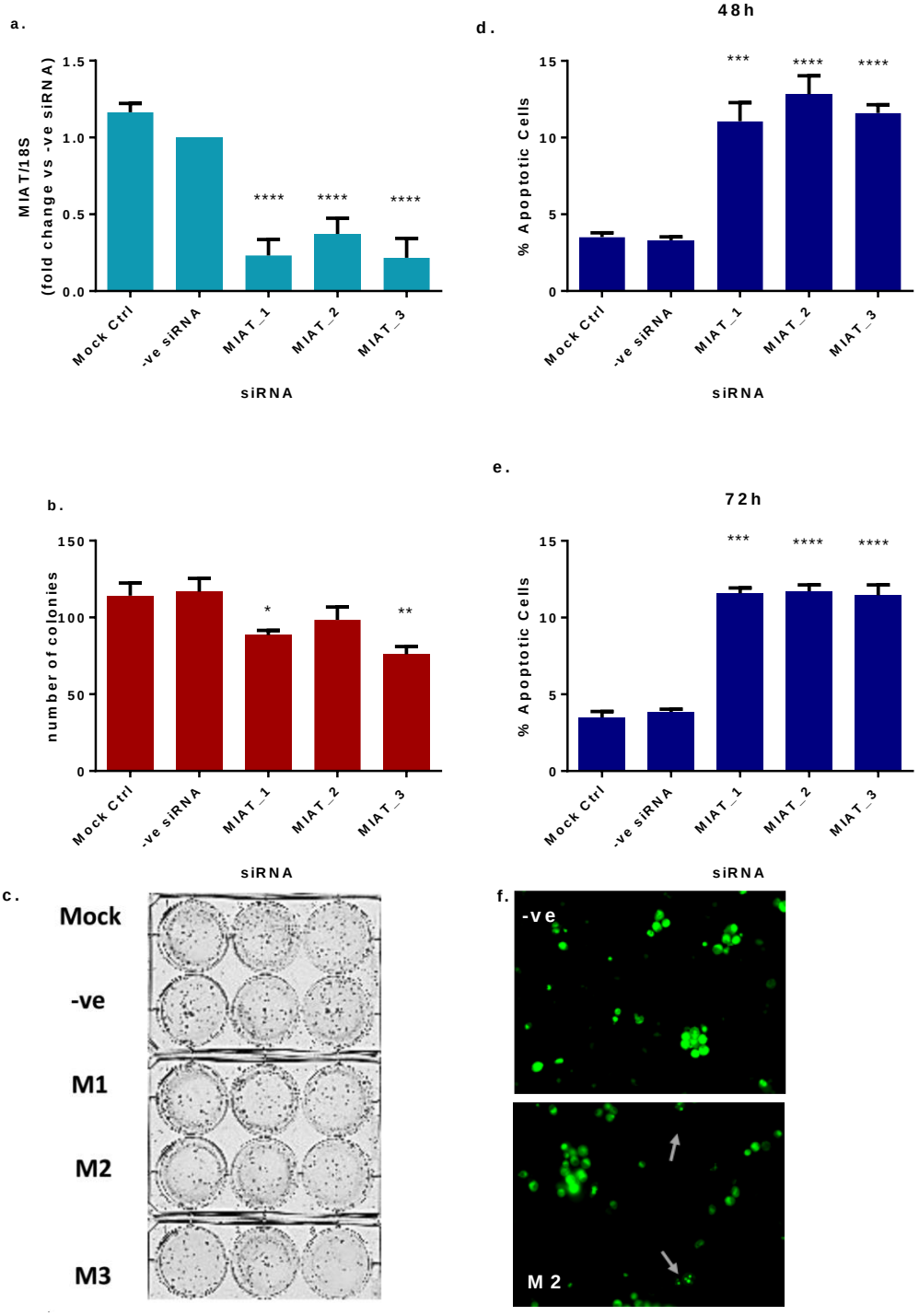

a.
18h
24h
% gap closure
100
80
60
40
20
0
****
**
***
Mock Ctrl
-ve siRNA
MIAT_1
MIAT_2
MIAT_3
siRNA
b.
relative gap closure (siRNA/-ve siRNA)
1.5
1.0
0.5
0.0


c.
Mock
-ve
M1
M2
M3
0h
18h
24h

T98G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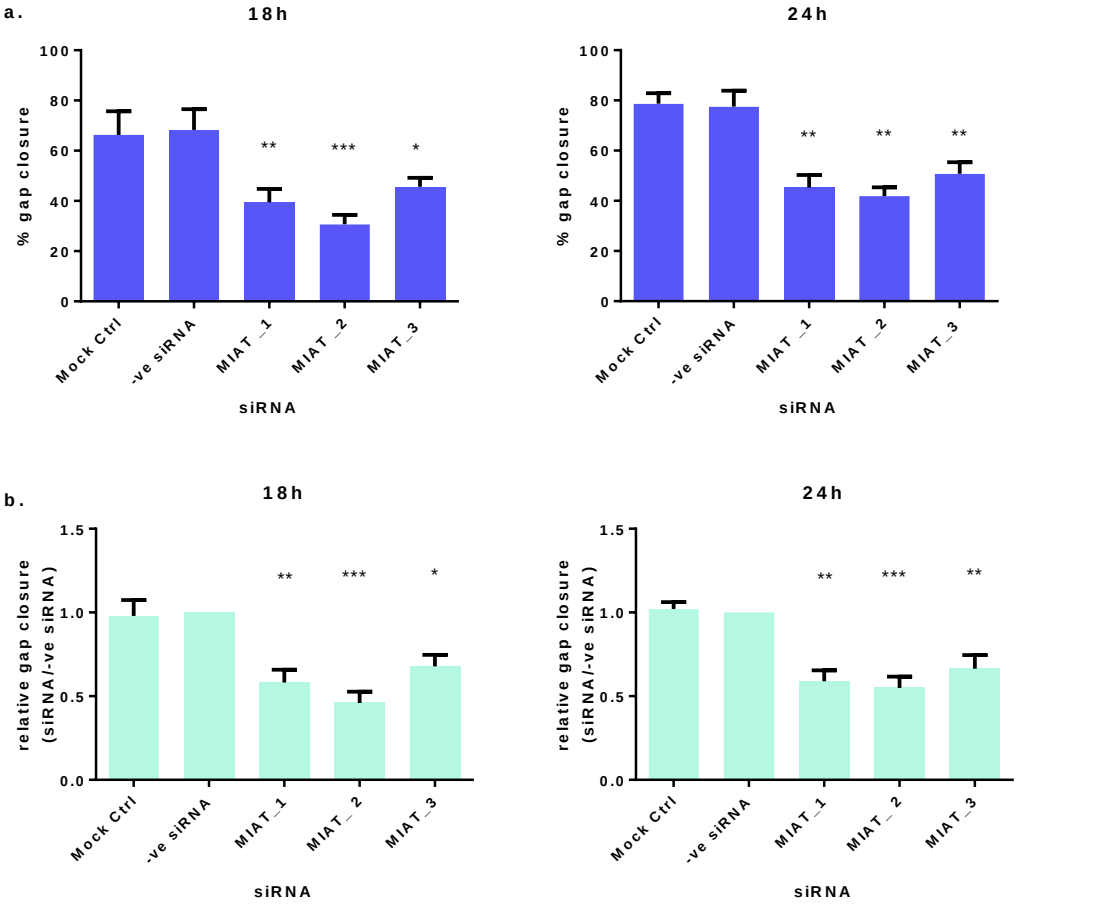
MIAT/18S
(fold change vs -ve siRNA)
1.5
1.0
0.5
0.0
****
****
****
Mock Ctrl
-ve siRNA
MIAT_1
MIAT_2
MIAT_3
siRNA
d.
48h
% Apoptotic Cells
15
10
5
0
***
****
****
Mock Ctrl
-ve siRNA
MIAT_1
MIAT_2
MIAT_3
siRNA
b.
number of colonies
150
100
50
0
*
**
Mock Ctrl
-ve siRNA
MIAT_1
MIAT_2
MIAT_3
siRNA
e.
72h
% Apoptotic Cells
15
10
5
0
***
****
****
Mock Ctrl
-ve siRNA
MIAT_1
MIAT_2
MIAT_3
siRNA
c.
Mock
-ve
M1
M2
M3
f.
-ve
M 2

T98G


a.
18h
24h
% gap closure
100
80
60
40
20
0
**
***
*
Mock Ctrl
-ve siRNA
MIAT_1
MIAT_2
MIAT_3
siRNA
b.
18h
24h
relative gap closure (siRNA/-ve siRNA)
1.5
1.0
0.5
0.0
**
***
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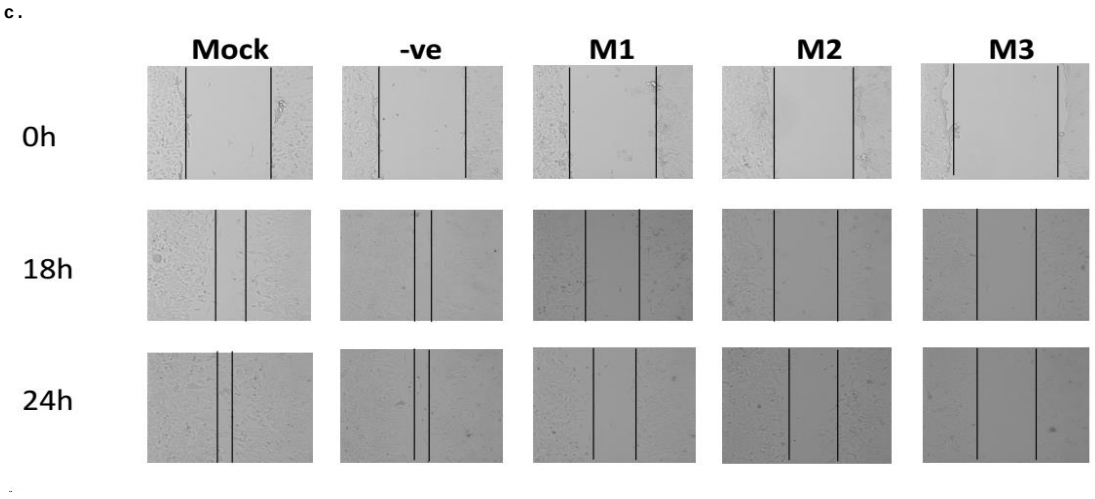
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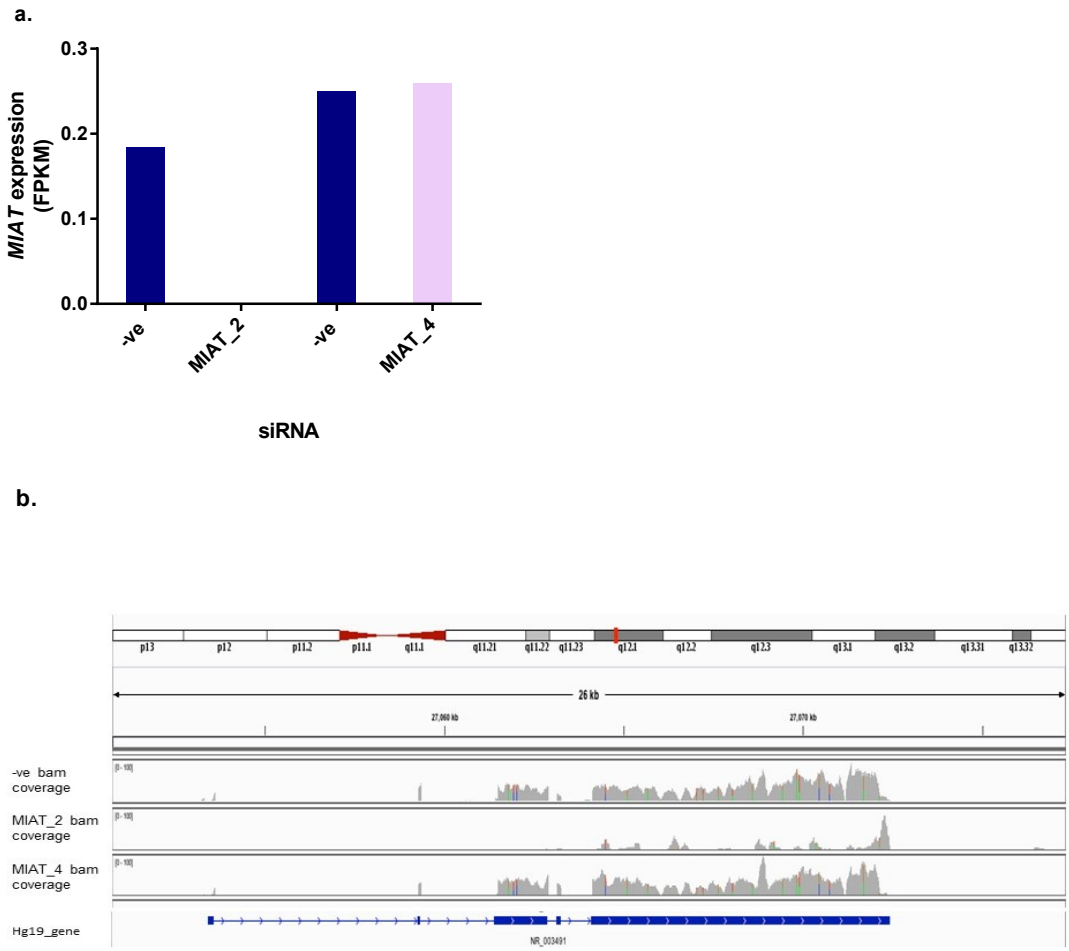
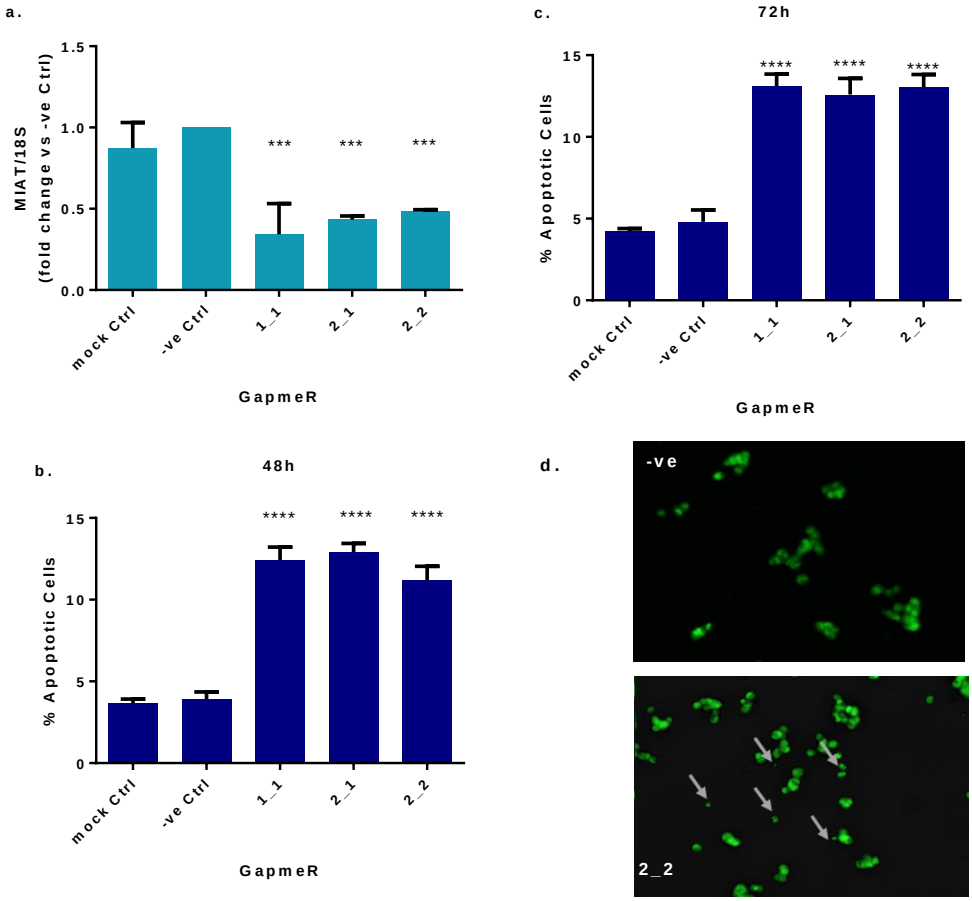
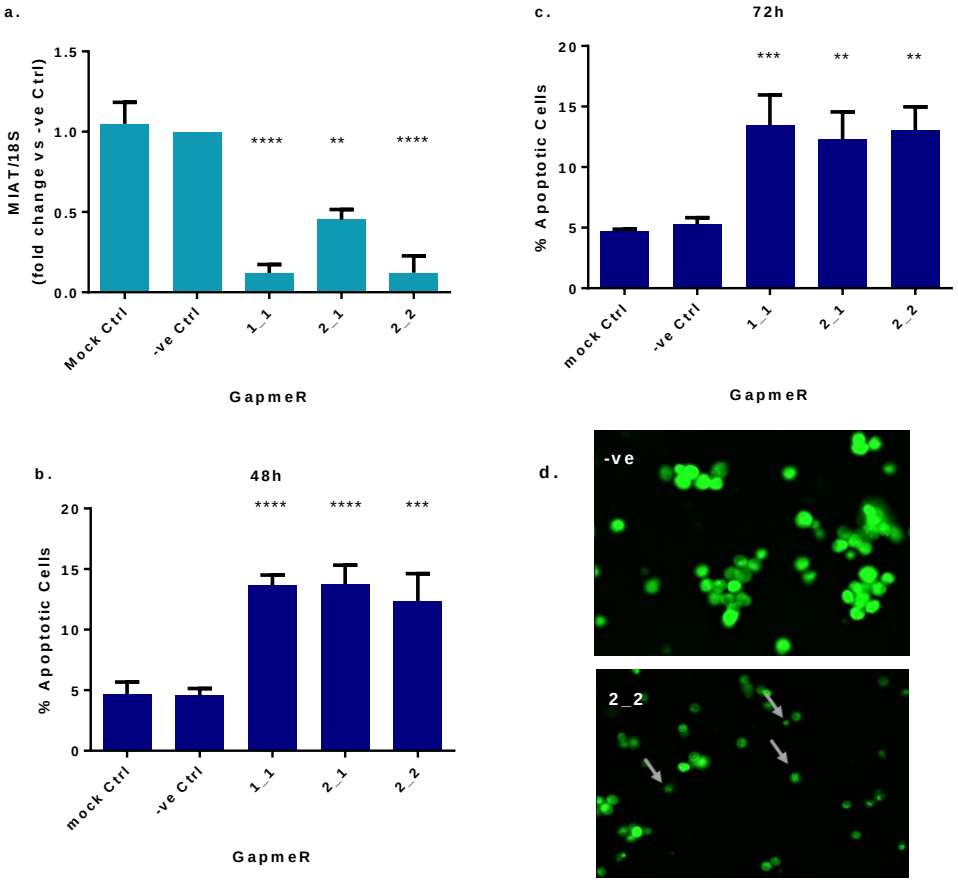
Mock
-ve
M1
M2
M3
0h
18h
24h



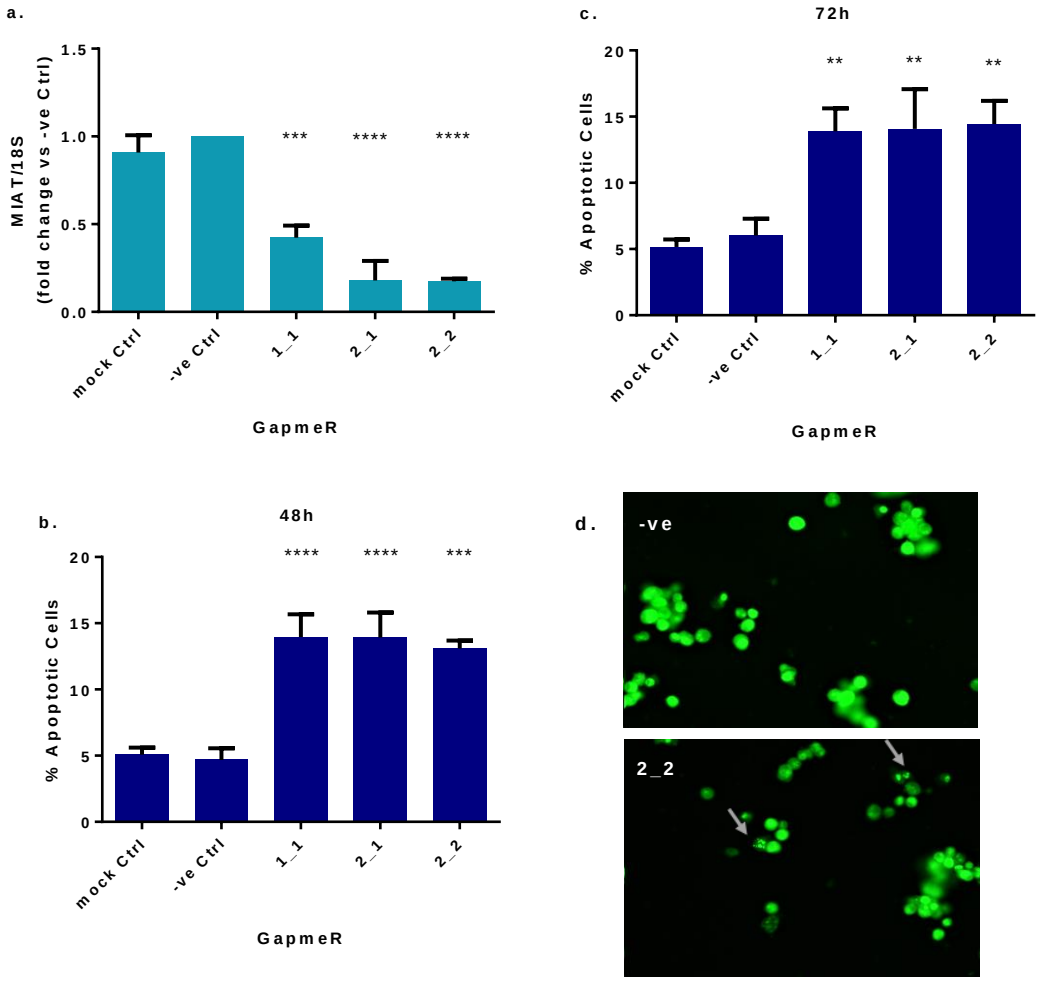
Supplementary figure 1. Expression values of *MIAT*. Data are expressed as Fragments Per Kilobase of transcript per Million mapped reads (FPKM) for the full length *MIAT* transcript [NR_003491 (10193 bp)] (a); *MIAT* sequence coverage in the different samples (b); -ve: negative siRNA.



Supplementary figure 2. LNA GapmeR- mediated *MIAT*-specific downregulation increases the levels of apoptosis of SH-SY5Y. SH-SY5Y cells were transfected with the negative Ctrl GapmeR or one of the three *MIAT*-specific LNA GapmeRs using Nucleofection, and were assessed 48h/72h/96h post-Nucleofection. The relative expression of *MIAT* was measured by Real-Time PCR 48h post-transfection, and was lower for all the three GapmeRs (a); Cells were also seeded and incubated (37°C, 5% CO_2) for two weeks, and the colonies formed were stained with Crystal Violet (1%w/v) and counted. Overall (n=3), *MIAT*-specific downregulation induced by *MIAT*-specific LNA GapmeRs leads to a significant increase of apoptosis levels, especially 72h after Nucleofection, reaching even a 2-fold increase (b,c); representative illustration of apoptotic cells 72h post-Nucleofection, stained with acridine orange and observed using fluorescent microscopy (f). Grey arrows indicate cells undergoing apoptosis; -ve: negative Control LNA GapmeR, 1_1, 2_1, 2_2: *MIAT*-specific LNA GapmeRs. * indicates a *p-value*<0.05; ** indicate a *p-value*<0.01; **** indicate a *p-value*<0.001, as measured by One-way ANOVA tests. Data are represented as mean +/- SEM.



Supplementary figure 3. LNA GapmeR- mediated *MIAT*-specific downregulation increases the levels of apoptosis of 1321N1. 1321N1 cells were transfected with the negative Ctrl GapmeR or one of the three *MIAT*-specific LNA GapmeRs using Nucleofection, and were assessed 48h/72h/96h post-Nucleofection. The relative expression of *MIAT* was measured by Real-Time PCR 48h post-transfection, and was lower for all the three GapmeRs (a); Cells were also seeded and incubated (37°C, 5% CO_2) for two weeks, and the colonies formed were stained with Crystal Violet (1%w/v) and counted. Overall (n=3), *MIAT*-specific downregulation induced by *MIAT*-specific LNA GapmeRs leads to a significant increase of apoptosis levels, especially 72h after Nucleofection, reaching even a 2-fold increase (b,c); representative illustration of apoptotic cells 72h post-Nucleofection, stained with acridine orange and observed using fluorescent microscopy (f). Grey arrows indicate cells undergoing apoptosis; -ve: negative Control LNA GapmeR, 1_1, 2_1, 2_2: *MIAT*-specific LNA GapmeRs. * indicates a *p-value*<0.05; ** indicate a *p-value*<0.01; **** indicate a *p-value*<0.001, as measured by One-way ANOVA tests. Data are represented as mean +/- SEM.



Supplementary figure 4. LNA GapmeR- mediated *MIAT*-specific downregulation increases the levels of apoptosis of T98G. T98G cells were transfected with the negative Ctrl GapmeR or one of the three *MIAT*-specific LNA GapmeRs using Nucleofection, and were assessed 48h/72h/96h post-Nucleofection. The relative expression of *MIAT* was measured by Real-Time PCR 48h post-transfection, and was lower for all the three GapmeRs (a); Cells were also seeded and incubated (37°C, 5% CO_2) for two weeks, and the colonies formed were stained with Crystal Violet (1%w/v) and counted. Overall (n=3), *MIAT*-specific downregulation induced by *MIAT*-specific LNA GapmeRs leads to a significant increase of apoptosis levels, especially 72h after Nucleofection, reaching even a 2-fold increase (b,c); representative illustration of apoptotic cells 72h post-Nucleofection, stained with acridine orange and observed using fluorescent microscopy (f). Grey arrows indicate cells undergoing apoptosis; -ve: negative Control LNA GapmeR, 1_1, 2_1, 2_2: *MIAT*-specific LNA GapmeRs. * indicates a *p-value*<0.05; ** indicate a *p-value*<0.01; **** indicate a *p-value*<0.001, as measured by One-way ANOVA tests. Data are represented as mean +/- SEM.

Supplementary table 3. Sp (Specificity protein) TF- regulated genes with significant (*p-value* <0.05) differential expression upon *MIAT* knockdown.

Sp1 target	Upregulated/ Downregulated	Pathway/ Process
FAS	Downregulated	Apoptosis
Bcl-2	Downregulated	Apoptosis
BIRC5	Upregulated	Survival/ Proliferation
EGFR	Downregulated	Membrane signalling
FGFR3	Downregulated	Membrane signalling
cMET	Downregulated	Membrane signalling
VEGFA	Downregulated	Migration/ invasion/metastasis
VEGFB	Upregulated	Migration/ invasion/metastasis
MMP9	Upregulated	Migration/ invasion/metastasis

Supplementary table 1. MIAT siRNAs and LNA GapmeRs details

Method	Cat #/ ID	Name/ Symbol	Sequence
siRNA	AM4611	-ve	N/A
	SI04287423	MIAT_1	N/A
	SI04314919	MIAT_2	N/A
	SI04344158	MIAT_3	N/A
LNA GapmeRs	LG00000002	-ve	N/A
	LG00188240	1_1	ACGGGTTAGTAATCGA
	LG00188250	2_1	CAGCGTGAATTGATTT
	LG00188251	2_2	TACAATTGGTTAGCTC

Supplementary table 2. Significantly deregulated miRNAs inhibiting Sp1 upon *MIAT* knockdown.

miRNA	*p-value*
miR-200b	1.960e-11
miR-200c	1.960e-11
miR-335	0.003
miR-23b	6.761e-9
miR-29b	4.896e-7
miR-29c	4.896e-7
miR-145	2.982e-8
miR-133a	5.354e-5
miR-133b	1.878e-5
miR-137	1.505e-11
miR-223	1.069e-4
miR-330	1.407e-5
miR-375	0.036
miR--429	