Aus dem Institut für Tumorimmunologie
des Fachbereichs Medizin der Philipps-Universität Marburg

Direktor: Prof. Dr. Elke Pogge von Strandmann

Deciphering the transcription factors that regulate the NKG2D ligand MICA in cancer cells

zur Erlangung des Doktorgrades der Naturwissenschaften

(Dr. rer. nat.)

vorgelegt von

Reem Al khayer

aus Latakia (Syrien)

Marburg, 2022

Angenommen vom Fachbereich Medizin der Philipps-Universität Marburg
am: 06. 04. 2023

Gedruckt mit Genehmigung des Fachbereichs Medizin

Dekanin: Prof. Dr. Denise Hilfiker-Kleiner

Referentin: Prof. Dr. Elke Pogge von Strandmann

Korreferent: Prof. Dr. Andreas Burchert

Content

Abstract

1. Zusammenfassung

Natürliche Killer (NK-) Zellen stellen einen wichtigen Teil des angeborenen Immunsystems dar und zeigen eine spontane zytolytische Aktivität gegen Tumorzellen. Ihre Funktion wird durch eine Vielzahl von hemmenden und aktivierenden Rezeptoren genauestens reguliert. NKG2D ist einer der bekanntesten aktivierenden Rezeptoren. Liganden für NKG2D (NKG2D-Ls), wie z.B. die MHC Class I Chain-Related Protein A (MICA), werden im Allgemeinen auf der Oberfläche bösartiger Zellen induziert. Tumorzellen entwickeln jedoch Mechanismen, um sich der Überwachung durch das angeborene Immunsystem zu entziehen. Zu diesen Mechanismen gehören die Herunterregulierung der Ligandenexpression, das proteolytische Shedding und die Freisetzung von löslichem NKG2D-L, um die Zielzellen für die NKG2D-abhängige NK-Zellaktivität unsichtbar zu machen. Wir haben bereits gezeigt, dass die Behandlung mit dem Histon-Deacetylase-Inhibitor (HDACi) LBH589 die Expression von NKG2D-L in Maus- und Humanzellen hochreguliert. Die Mechanismen, die die Expression von NKG2D-L nach der Behandlung mit LBH589 regulieren, sind jedoch nach wie vor nicht bekannt. Um einen Einblick in die komplexe Regulierung des NKG2D-L MICA-Promotors auf Chromatinebene zu erhalten, wurde in dieser Arbeit auf dem CRISPR/dCas9-System basierend eine ChromatinImmunopräzipitation (enChIP) in Kombination mit Massenspektrometrie als leistungsstarke neue Methode zur Identifizierung von DNA-bindenden Molekülen eingeführt. Dadurch konnte unter anderem der Zinkfinger-Transkriptionsfaktor KLF4 identifizieren werden, der je nach Tumorart oder -stadium entweder als Onkogen oder als Tumorsuppressor in verschiedenen Krebsarten wirken kann.

Genetische/pharmakologische Experimente zum Funktionsgewinn und -verlust in Zellen der akuten myeloischen Leukämie (AML) zeigten, dass die induzierbare MICAExpression mit der Expression von KLF4 assoziiert war. Insbesondere das in der klinischen Phase 1 befindliche Molekül APTO253, von dem bekannt ist, dass es den Zellzyklusarrest und die Apoptose fördert, induziert die Expression von KLF4 in AMLZellen. Diese Induktion ging mit einer erhöhten Expression von NKG2D-L einher, wodurch resistente AML-Zelllinien für die NK-Zell-vermittelte Abtötung empfänglich wurden. Diese Ergebnisse zeigen, dass es sinnvoll ist, AML-Patienten durch KLF4Induktion zu behandeln und APTO253 in Kombination mit einem adoptiven NKZelltransfer zur Eliminierung von AML-Blasten einzusetzen.

2. Abstract

Natural Killer (NK) cells constitute a significant part of the innate immune system that show spontaneous cytolytic activity against tumor cells. Their function is tightly regulated by a variety of inhibitory and activating receptors. NKG2D is one of the most prominent activating receptors. Ligands for NKG2D (NKG2D-Ls), such as MHC class I polypeptide-related sequence A (MICA), are generally induced on the surface of malignant cells. However, tumor cells develop mechanisms to evade innate immune surveillance. The mechanisms include down-regulation of ligand expression, proteolytic shedding, and release of soluble NKG2D-L to render the target cells invisible for the NKG2D-dependent NK cell activity. Previously we showed that the treatment with the histone deacetylase inhibitor (HDACi) LBH589 upregulates the expression of NKG2D-L in mouse and human cells.

Nevertheless, the mechanisms regulating NKG2D-L expression upon LBH589 treatment remain elusive. To gain insight into the complex regulation of NKG2D-L MICA promoter on a chromatin level, a CRISPR/dCas9 system-based chromatin immunoprecipitation (enChIP) in combination with mass spectrometry was established as a powerful novel method for identifying DNA-binding molecules. As a result, I could identify, among others, the zinc finger transcription factor KLF4, which can act either as an oncogene or as a tumor suppressor in different cancers depending on the tumor types or stages. Genetic/pharmacological gain and loss of function experiments in acute myeloid leukemia (AML) cells revealed that the inducible MICA expression was associated with the expression of KLF4. Notably, the phase 1 clinical-stage molecule APTO253, known to promote cell cycle arrest and apoptosis, induces the expression of KLF4 in AML cells. This induction was associated with an increased expression of NKG2D-L, thus rendering resistant AML cell lines susceptible to NK cell-mediated killing. These findings shed light on a strong rationale for targeting AML patients by KLF4 induction, using APTO253 in combination with adoptive NK cell transfer to eliminate AML blasts.

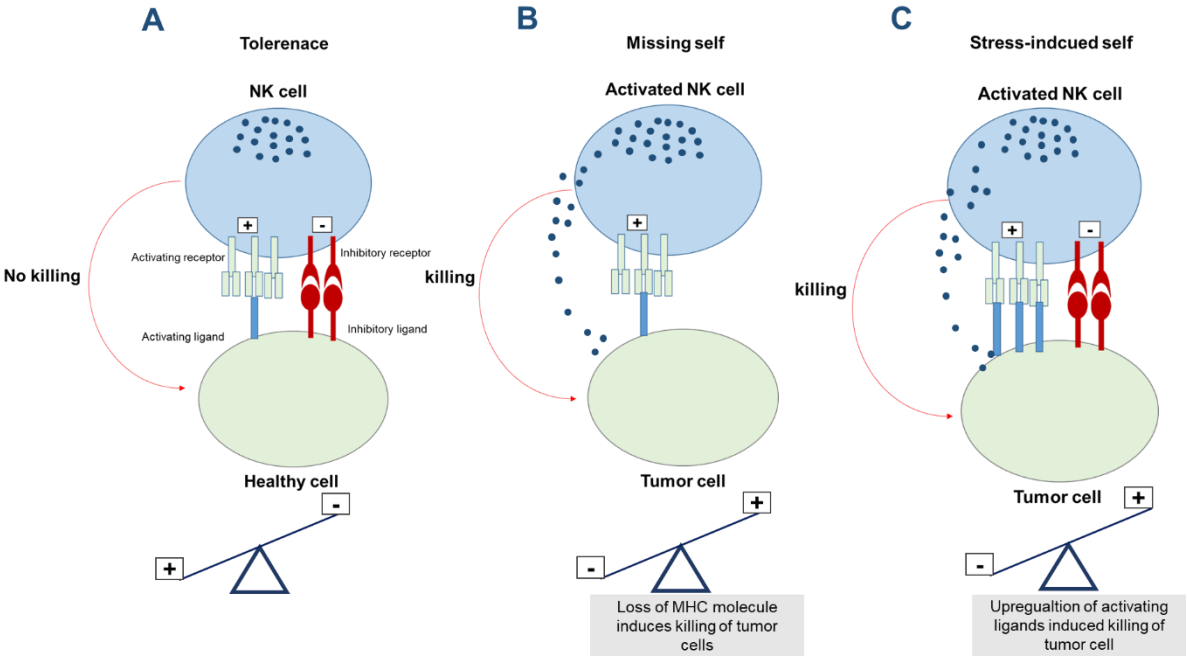
Introduction

The human immune system consists of the innate immune system (e.g., NK cells) and the adaptive immune system (e.g., T cells), which play an essential role in fighting and controlling tumors (Gonzalez, Hagerling and Werb, 2018). Several therapies, including histone deacetylase inhibitors, have shown promising effects in suppressing tumor growth mediated by modulating immune responses such as NK cells (Shanmugam, Rakshit and Sarkar, 2022). It has been widely reported that tumor suppression partially depends on the expression of ligands for the activating natural killer group 2 member D receptor (NKG2D-Ls), and the histone deacetylase inhibitors (HDACis) are known to induce these ligands in cancer cells (Diermayr *et al.*, 2008)(Sauer *et al.*, 2017)(Skov *et al.*, 2005)(Armeanu *et al.*, 2005). However, the mechanism behind this process has yet to be fully elucidated. Therefore, this project aims to decipher the regulatory mechanisms of NKG2D ligands in response to histone deacetylase inhibitors therapy. Because of the high medical need to identify novel targets aiming to enhance NKG2D ligands expression in tumor cells.

1. NK cells: function and regulation

Natural killer cells (NK) are a vital component of the innate immune system. They exhibit spontaneous cytolytic activity against virus-infected and malignant cells by secreting cytotoxic granules containing enzymes/proteins such as perforins and granzymes (Mazzurana *et al.*, 2018)(Voskoboinik, Smyth and Trapani, 2006)(Cooper, Fehniger and Caligiuri, 2001)(Vivier *et al.*, 2008). While perforins form holes in the membrane of the malignant cells, granzymes enter the cell and induce programmed cell death (apoptosis)(Voskoboinik et al., 2010)(Trinchieri, 1989). Furthermore, NK cells can also release chemokines and inflammatory cytokines such as interferon gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α), which in turn can promote and modulate adaptive immune responses (De Sanctis, Blanca and Bianco, 1997)(Vivier et al., 2011). Both mouse models and clinical studies have shown that decreased activity of NK cells is associated with tumor growth and metastasis, highlighting their central role in cancer immunosurveillance (Warren, Stembridge and Gardner, 1985)(Imai et al., 2000)(Talmadge et al., 1980)(Angka et al., 2017). NK cells express a set of activating and inhibitory surface receptors, and signals emanating from these receptors tightly regulate NK cell function (Kumar, 2018). The balance between inhibitory and activating signals allows NK cells to distinguish between ‘self’

(healthy cell), 'missing self' (foreign cell), and 'induced self' (malignant cell) (Moretta *et al.*, 2014)(Paul and Lal, 2017) (Introductory figure 1). Most activating receptors contain an immunoreceptor tyrosine-based activation motif (ITAM), whereas the inhibitory receptors have an immunoreceptor tyrosine-based inhibition motif (ITIM) (Vivier et al., 2008)(Brooks et al., 1997)(López-Botet et al., 1997). Healthy cells express the major histocompatibility complex class I (MHC-I) molecules that bind to the inhibitory receptors on NK cells and suppress their activity. In virus-infected or tumor cells, downregulation or loss of the expression of MHC-I molecules on their surface facilitates escape from the cytotoxic T-cell immune response. As a result, the loss of inhibitory signals allows activating signals to initiate NK cell-mediated lysis of the target cell. This mechanism is known as the 'missing self' detection hypothesis. Furthermore, in another situation, the 'stress-induced self' mechanism, tumor cells and virally infected cells may keep expressing MHC-I molecules on their surface. At the same time, they upregulate the expression of activating ligands recognized by the activating NK cell receptors so that the activating signals override any inhibitory signals and trigger NK cell-mediated lysis of the target cell (Paul and Lal, 2017)(Abel *et al.*, 2018). In summary, the balance between activating and inhibitory receptors signals tightly regulates the NK cell function.



Introductory figure 1: The mechanisms of NK cell-mediated recognition and killing of aberrant cells. **A.** "Tolerance towards a healthy cell. The engagement between MHC-I on healthy cells with inhibitory receptors on NK cells dampens the activating signals. **B.**

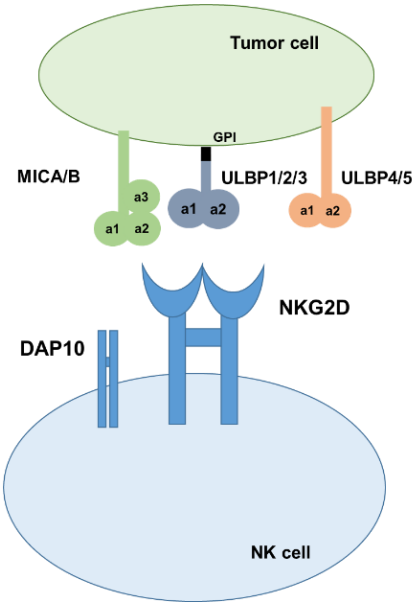
"Missingself" in this mechanism, the tumor cells lose the expression of MHC-I, leading to activation of NK cells and lysis of tumor cells. **C.** "Induced-self" tumor cells keep expressing MHC-I but simultaneously upregulate expression of activating ligands that activate NK cells to kill the tumor cell.

2. The activating NKG2D receptor and its ligands: An NK cell master regulator in tumor immune surveillance

NKG2D is one of the most critical activating receptors expressed on NK cells in terms of recognition and elimination of tumor cells. It is also expressed on CD8 T and γδ T cells and serves as a co-stimulatory receptor (Wensveen, Jelenčić and Polić, 2018). NKG2D is a lectin-like type 2 transmembrane receptor that needs adaptors in order to transduce a signal via a signal cascade from the surface to the target molecules in the cell (Raulet et al., 2013)(Wu et al., 1999, p. 10)(Gilfillan et al., 2002). The NKG2D receptor recognizes eight different ligands on target cells. In humans, ligands consist of two families, the major histocompatibility complex proteins MHC class I chainrelated A and B (MICA and MICB) and six UL-16 binding proteins (ULBP1-6). NKG2D ligands (NKG2D-Ls) identified in mice, in comparison to humans, include one murine UL16-binding protein-like transcript 1 (MULTI 1), three different isoforms of histocompatibility H60, and five isoforms of retinoic acid early transcript 1 (RAET1) (from α to ε)(Raulet et al., 2013)(Lazarova and Steinle, 2019). These ligands are not or only rarely expressed on healthy cells, but their expression is induced in response to cellular stress such as DNA damage, infection, heat shock, or malignancy. Therefore, they are also called stress-induced ligands (Groh et al., 2001)(Watson et al., 2006). Combined data from experimental animal models and clinical trials demonstrated the importance of the NKG2D/NKG2D-L pathway in controlling tumor growth and immune response, indicating their potential role in cancer immunotherapy (Guerra et al., 2008)(Smyth et al., 2005). Besides, many agents used for cancer treatment, such as chemotherapy, radiotherapy, and additional immune-modulating stimuli such as interleukin 2 (IL2), have been reported to potentiate the cytotoxic effect of NK cells by regulating NKG2D and/or NKG2D-Ls expression (Okita et al.,2016)(Konjević et al., 2010)(Lee et al., 2018)(Zhang et al., 2020)(Okita et al., 2015). It has been reported that NKG2D-deficient mice develop more aggressive tumor growth than the control mice (Raulet et al., 2013)(Guerra et al., 2008). In this context, Smyth et al. showed that deactivation of NKG2D function using antibodies leads to an increased susceptibility to methylcholanthrene- (MCA-) induced fibrosarcoma *in vivo* (Smyth *et al.*, 2005). The

expression of the NKG2D receptor is regulated in response to cytokines. For example, IL2, IL18, and IL15 can induce the expression of the NKG2D receptor (Huergo-Zapico *et al.*, 2014)(Marçais *et al.*, 2013). In contrast, the transforming growth factor beta (TGF-β) and IL10 have the opposite effect (Burgess *et al.*, 2006). Furthermore, the treatment of NK cells isolated from cancer patients with IL2 or IL15 reinforced NK cell anti-tumor activity depending on the upregulation of NKG2D receptor expression (Song *et al.*, 2006)(Konjević *et al.*, 2010). Altogether, the data from animal models and clinical studies confirm the critical role of the NKG2D receptor in tumor-immune surveillance. In addition to the NKG2D receptor, the ligands for this receptor play also a crucial role in tumor-immune surveillance. Their expression makes cancer cells more sensitive to cytotoxicity-mediated by immune cells (Sauer *et al.*, 2017)(Ponath *et al.*, 2020). However, cancer cells have developed a strategy to evade the immune system by cleaving the membrane-bound NKG2D ligands with proteases, such as ADAM10/17 or tumor-derived exosomes. In different types of cancer, elevated levels of soluble NKG2D-Ls, cleaved from the cell surface, are correlated with a poor prognosis and decreased NK cell-mediated cytotoxicity (Zhao *et al.*, 2015)(Yamaguchi *et al.*, 2012)(Mincheva-Nilsson and Baranov, 2014). Furthermore, in mouse models, only membrane-bound NKG2D-Ls stimulate immunity and inhibit tumor progression, contrary to their soluble forms, which impair host immunity (Raffaghello *et al.*, 2004)(Coudert *et al.*, 2005)(Holdenrieder *et al.*, 2006). High levels of the soluble form of these ligands, such as MICA, are detected in sera from patients with many cancers, including acute myeloid leukemia, and positively correlated with tumor development (Salih *et al.*, 2003)(Koguchi *et al.*, 2015)(Jinushi *et al.*, 2008)(Huergo-Zapico *et al.*, 2012). MICA/B ligands consist of three domains: the distal α1 and α2 domains that bind to the NKG2D receptor and the proximal α3 domain, which is considered the site of proteolytic shedding (as shown in introductory figure 2)(Ferrari de Andrade *et al.*, 2018). Some studies showed that using monoclonal antibodies to specifically block the proximal MICA/B α3 domain not only inhibits the loss of MICA/B membrane ligands in cancer cells but also reactivates the anti-tumor immunity mainly by enhancing the NK cell function. Moreover, these antibodies suppress tumor growth in fully immunocompetent mouse models and reduce melanoma as well as lung metastases in a humanized mouse model (Ferrari de Andrade *et al.*, 2018)(Ferrari de Andrade *et al.*, 2020). Thus, NKG2D ligands in both forms -surface and soluble- play a role in tumor immunity, but in opposite ways. Therefore, the regulation of NKG2D-Ls is a

significant target for cancer immunotherapy mediated by NKG2D/NKG2D-L axis. Different approaches targeting the NKG2D/NKG2D-L pathway are under investigation, especially in the case of AML, which is used as a model in this thesis (reviewed in (Wu *et al.*, 2021)).



Introductory figure 2: The NKG2D activating receptor on NK cells and its various ligands on human tumor cells. The activating receptor NKG2D is a type II transmembrane glycoprotein that binds to DAP10 in the transmembrane domain. There are eight ligands: MICA and MICB, which have three domains (α1-3), a transmembrane structure, and a cytoplasmic tail, whereas ULBP1-6 have only α1-2 domains. ULBP1, 2, 3, and 6 have a GPI anchoring structure. Both ULBP4 and ULBP5 have a transmembrane structure and cytoplasmic tail. The engagement between the NKG2D activating receptor on the NK cell and its ligands on the tumor cell plays an essential role in immune surveillance.

3. Targeting the NKG2D/NKG2D-Ls pathway in AML pathogenesis is a promising therapeutic strategy

AML is one of the most common malignant diseases of the hematopoietic system in adults, associated with a poor prognosis and/or a short survival rate. It is characterized by abnormal proliferation of undifferentiated myeloid cells as well as genetic heterogeneity (Wu *et al.*, 2021)(Shallis *et al.*, 2019). Various cell signaling pathways that are involved in cell growth, proliferation, and somatic mutations or chromosome abnormalities play a critical role in the development of AML, such as p53, Wnt/β catenin, FLT3-ITD, and adenosine monophosphate-activated protein kinase (AMPK) signaling (Wu *et al.*, 2021), MYC deregulation and amplification (Salvatori *et al.*, 2011, p. 2)(Ohanian *et al.*, 2019)(Slovak *et al.*, 1994). Many AML patients achieved remission after a short time of standard chemotherapy based on cytarabine (Ara-C) and anthracyclines (such as daunorubicin or idarubicin). However, they suffer from relapse caused by resistance to chemotherapeutic drugs and poor immune function (Krauss *et al.*, 2019)(Tallman, Gilliland and Rowe, 2005). Data from different studies have revealed an association between drug resistance, tumor immune evasion, and the NKG2D/NKG2D-L axis in AML (Wu *et al.*, 2021)(Paczulla *et al.*, 2019)(Baragaño Raneros *et al.*, 2015). In many AML patients, a significant downregulation or even

absence of some activating receptors on NK cells, including NKG2D, has been detected (Hilpert *et al.*, 2012)(Sandoval-Borrego *et al.*, 2016)(Baragaño Raneros, López-Larrea and Suárez-Álvarez, 2019). Furthermore, shedding the surface expression of NKG2D-Ls is one of the strategies for immune evasion. In two separate studies, the soluble form of these ligands was detectable in the sera of AML patients, which also promotes the downregulation of the NKG2D receptor (Salih *et al.*, 2003)(Hilpert *et al.*, 2012). Besides, the level of the soluble ligands was negatively associated with the survival rate (Hilpert *et al.*, 2012). In AML, a population of leukemia stem cells (LSCs) is generally characterized by the unlimited ability to divide and self renew. They are involved in the development of chemotherapy resistance, among other factors, and the accumulation of these cells has been correlated with clinical relapse in AML (Valent *et al.*, 2020)(Siveen, Uddin and Mohammad, 2017). Several studies have indicated that even a small population of LSCs that survive after chemotherapy can promote AML either by decreasing the survival rate or leading to relapse by the end of the therapy (Stiehl *et al.*, 2015). LSCs defined by their ability to initiate leukemia in immunodeficient mice after transplantation (Valent *et al.*, 2020)(Thomas and Majeti, 2017). Moreover, the LSCs are recently characterized by the absence of the NKG2D-Ls in AML. AML cells expressing NKG2D-Ls are recognized and killed by NK cells, in contrast to cells lacking NKG2D-Ls from the same individual (Paczulla *et al.*, 2019). The treatment with drugs that affect restoring the expression of NKG2D-Ls on AML cells enhanced the sensitivity to NK cell-mediated clearance (Rohner *et al.*, 2007)(Paczulla *et al.*, 2019)(Diermayr *et al.*, 2008). In addition, using the NKG2D-chimeric antigen receptor (CAR) NK cells significantly enhances survival rate. It reduces tumor growth in both AML human cell lines and a xenograft model compared to mock NK cells, emphasizing their anti-leukemic role (Du *et al.*, 2021). In this regard, targeting the expression of NKG2D-Ls could be a promising therapeutic direction for improving NK cell-based immunotherapy.

4. The regulation of NKG2D-Ls expression: a target for cancer therapy

Given the vital role of NKG2D-Ls in immunoreactivity and surveillance, various attempts have been made in recent years to induce their expression for cancer immune therapy (Schmiedel and Mandelboim, 2018)(Jones *et al.*, 2022). Thus, elucidating the mechanism of NKG2D-Ls regulation may help to exploit the NKG2D/NKG2D-Ls axis for cancer based-immunotherapy. So far, the regulatory mechanisms of NKG2D-Ls expression on the cell surface are poorly understood.

Some studies have reported that the expression of NKG2D-Ls is influenced by various cancer-associated transcription factors such as p53, SP family transcription factors, heat shock transcription factor 1 (HSF1), and NF-κB. Nevertheless, their impact on NKG2D-Ls is ligand-specific, and also depends on the cell lines and the model system used (Lanier, 2015)(Raulet *et al.*, 2013)(Sauer *et al.*, 2017). For example, p53 binds to ULBP1 and ULBP2 genes in response to DNA damage, inducing their expression in a human non-small lung cancer cell line (Textor *et al.*, 2011, p. 2). In contrast, the SP transcription family binds and induces the expression of MICA, MICB, and ULBP1 in proliferating cells (Rodríguez-Rodero *et al.*, 2007, p. 1)(Schmiedel and Mandelboim, 2018). Moreover, among the current anti-cancer drugs used, some can significantly induce the surface expression of NKG2D-Ls on tumor cells, including the histone deacetylase inhibitors (HDACi) (e.g., valproic acid and LBH589), DNA damaging agents (e.g., Ara-C), and poly (ADP-ribose) polymerase (PARP) inhibitors (e.g., Veliparib)(Jensen *et al.*, 2013)(Poggi *et al.*, 2009)(Klein *et al.*, 2013)(Ullrich *et al.*, 2013)(Yamanegi, 2010)(Koguchi *et al.*, 2015)(Gasser *et al.*, 2005)(Skov *et al.*, 2005)(Sauer *et al.*, 2017). Some of the HDAC inhibitors as a single agent or in combination with other agents have been approved by the United States Food and Drug Administration (FDA) for therapeutic use, particularly in patients with hematological malignancies (multiple myeloma and T cell lymphomas). However, they are associated with severe side effects such as myelosuppression, diarrhea, and cardiotoxicity, which limit their use and require further improvements (Shah, 2019)(Grant *et al.*, 2010)(Sawas, Radeski and O'Connor, 2015)(Moore, 2016). Thus, a better understanding of the particular pathway by which HDACi regulate the expression of NKG2D-Ls bears a high potential to improve and develop immune based therapies.

5. HDACi are potentially used as anticancer agents in various cancer types

HDACi have been successfully used as anti-cancer agents. They have shown potent efficacy in inhibiting tumor cell proliferation and inducing apoptosis with relatively little effect on normal cells (Bolden *et al.*, 2013)(Ungerstedt *et al.*, 2005). Accumulating evidence indicates that the acetylation and deacetylation of histones are involved in gene regulation (Grunstein, 1997)(Gujral *et al.*, 2020). Generally, but not always, histone acetylation is associated with a transcriptionally active gene, whereas deacetylation is associated with repression (Khan and Tomasi, 2008). The balance

between these processes is usually controlled by two groups of enzymes: histone acetyltransferases (HATs) and histone deacetylases (HDACs) (Gujral *et al.*, 2020). While HATs transfer an acetyl group to the lysine residues of the histone tail, HDACs remove it (Sun *et al.*, 2015). HDACs regulate several cellular processes, such as proliferation, development, and differentiation (Zupkovitz *et al.*, 2010). Furthermore, HDACs with abnormal expression and/or activity have been observed in various cancers (Schneider *et al.*, 2011)(Müller *et al.*, 2013, p. 3)(Wilson *et al.*, 2006)(Ropero *et al.*, 2006) (Sauer *et al.*, 2017). Many studies have described that dysregulation of HDACs drives uncontrolled cell proliferation by inhibiting some tumor suppressor genes, such as cyclin-dependent kinase (CDK) inhibitors, p21, and p53 in numerous cancers (Lagger, 2002)(Gui *et al.*, 2004)(Juan *et al.*, 2000). There are 18 HDAC enzymes in humans, and they use two mechanisms to deacetylate acetyl-lysine substrates depending on either zinc or NAD^+. The zinc-dependent group includes three classes: Class I (HDAC1, HDAC2, HDAC3, and HDAC8); Class II (HDAC4, HDAC5, HDAC6, HDAC7, HDAC9, and HDAC10), and Class IV (HDAC11), and the NAD^+-dependent class III sirtuin enzymes (Seto and Yoshida, 2014)(Marks and Xu, 2009)(Barneda-Zahonero and Parra, 2012). The inhibitors used for the different HDAC classes are divided into four groups according to their unique chemical structure: Hydroxamic acids, benzamides, cyclic peptides, and short-chain fatty acids. Hydroxamic acids are one of the most common anti-cancer agents used in preclinical and clinical studies (Mottamal *et al.*, 2015). HDACi can act either selectively against specific HDAC (e.g., ricolinostat (ACY-1215) for HDAC 6) or as a pan-inhibitor against several classes of HDACs (e.g., LBH589)(Cosenza *et al.*, 2017)(Singh *et al.*, 2016).

5.1. The HDACi LBH589 used for cancer treatments

The pan-HDACi Panobinostat (LBH589) is a hydroxamic acid-based compound. It shows the ability to inhibit the activity of classes I, II, and IV HDAC enzymes at low nanomolar concentrations. Its dominant effect is on HDAC 1, 2, 3, and 6, indicating its pan-deacetylase (pan-DAC) potency (Prince, Bishton and Johnstone, 2009)(Atadja, 2009). LBH589 has been reported to upregulate CDK1A (p21) levels and induce hyperacetylation of histone H3 as well as H4 (Wagner *et al.*, 2010)(Vilas-Zornoza *et al.*, 2012). In addition, LBH589 enhances the accumulation of the acetylated histone and non-histone proteins by inhibiting the activity of HDAC enzymes, which in turn, causes cell cycle arrest and apoptosis (Vilas-Zornoza *et al.*, 2012)(San José-Enériz *et al.*, 2019)(Van Veggel, Westerman and Hamberg, 2018). The FDA has approved

LBH589 as an effective anti-cancer agent for treating multiple myeloma. Its clinical application for a variety of hematological and solid tumors is currently evaluated (Li and Seto, 2016)(Fortunati *et al.*, 2014)(Ceccacci and Minucci, 2016). In addition, numerous preclinical studies have demonstrated the effective anti-leukemic activity of LBH589 against primary AML samples and cell lines (Ocio *et al.*, 2015)(Mottamal *et al.*, 2015). Early-stage clinical trials have indicated the modest efficacy of LBH589 as a single drug (Schlenk *et al.*, 2018). Therefore, a combination of LBH589 with other anti-cancer drugs seems crucial for effective treatment (San José-Enériz *et al.*, 2019). In detail, LBH589 is approved for treating patients with multiple myeloma in combination with bortezomib and dexamethasone (Laubach *et al.*, 2015). For the treatment of AML, the combination of LBH589 with several anti-cancer drugs has been investigated, including the DNA damaging agent doxorubicin, the hypomethylating agent azacitidine (AZA) as well as decitabine, and the chemotherapy drugs cytarabine, and idarubicin, and bortezomib (Ocio *et al.*, 2015)(Jiang *et al.*, 2012)(Blagitko-Dorfs *et al.*, 2019)(Maiso *et al.*, 2009). Nevertheless, only some of them have been clinically tested up to now. Compared to a single drug treatment, the combination treatment enhances the anti-leukemic effect by inhibiting cell growth and inducing apoptosis in AML cell lines and primary cells from patients. Additionally, the combination has improved the survival of AML xenograft mouse models in some cases (San JoséEnériz *et al.*, 2019). Treatment with LBH589 plus the demethylating agent decitabine significantly reduced the proliferation of primary AML cells compared to one of the agents alone (Fiskus *et al.*, 2009). LBH589 treatment of AML patients older than 65 years with a combination of cytarabine and anthracyclines was safe and effective, as reported in the phase Ib/II panobidara study and phase I clinical trials (Wieduwilt *et al.*, 2019)(Ocio *et al.*, 2015). Though, the combination of LBH589 with cytarabine and mitoxantrone failed to enhance treatment efficacy. Overall, the combination of LBH589 with chemotherapy has stopped because of the limited response in AML patients.

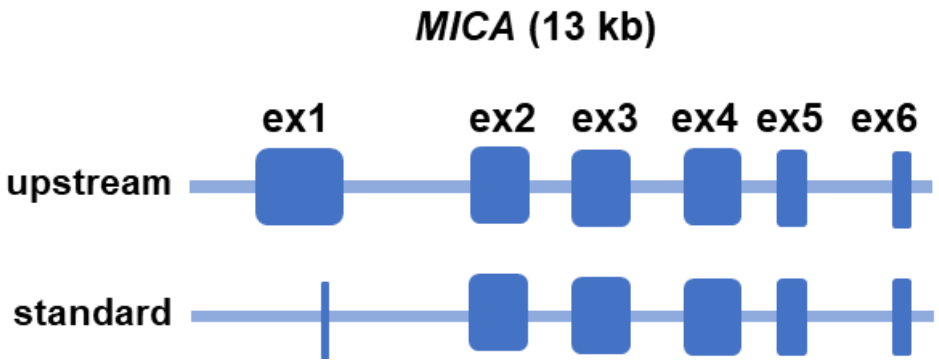
Further studies using LBH589 in combination with immune therapy could be more promising for cancer treatment. A recent study showed that the pre-treatment of various cancer cell lines with LBH589, followed by NK cells, significantly increased the sensitivity to NK cell cytolysis compared to treatment with NK cells or LBH589 alone. The anti-tumor activity of LBH589 was NK cell-dependent *in vitro* as well *in vivo* (Afolabi *et al.*, 2021). Furthermore, using LBH589 in combination with antiProgrammed Cell Death 1-Lligand (PD-L1) also enhanced the anti-tumor immune response (Afolabi

et al., 2021). Ferrari de Andrade *et al*. have shown that adding LBH589 to antibodies which inhibit the shedding of MICA/B surface expression, increased the expression of NKG2D-Ls and also the killing of tumor cells by NK cells in some *in vitro* and *in vivo* models (Ferrari de Andrade *et al.*, 2020). These data highlight the potential role of LBH589 in cancer treatment and shed light on its role in immune based-therapies, especially NK cell-based immunotherapy. However, the exact mechanism responsible for the NK cell response mediated by LBH589 in tumor surveillance remains largely unclear**.**

5.2 LBH589 promotes the regulation of NKG2D-Ls on the surface of tumor cells

Our group reported that the LBH589 could induce the expression of NKG2D-Ls, mainly MICA and MICB, in several human cell lines and increase the sensitivity to NK cellmediated lysis (Sauer *et al.*, 2017). Moreover, this induction occurs at the transcription level, as preincubation of cells with transcription inhibitor actinomycin D or translation inhibitor cycloheximide significantly inhibited the induction of MICA/B on the cell surface in response to treatment with LBH589 (Sauer *et al.*, 2017). The same study has provided evidence for the crucial role of the acetyltransferases CBP/p300 in the LBH589-mediated NKG2D-L induction because this induction was abolished after chemical inhibition or genetic ablation of CBP/p300 both *in vitro* and *in vivo* (Sauer *et al.*, 2017). CREB-binding protein (CBP) and its paralogue p300 are acetyltransferases. They can act as a bridge and promote transcription activity by bringing transcriptional machinery to the promoter (Wang, Marshall and Ikura, 2013). In addition, they have a HAT domain in their structure and can acetylate histones and more than 70 nonhistone protein substrates. Some essential proteins gain transcriptional function after acetylation by CBP/p300, including p53, NF-κB, STAT3, and EKLF (Castillo *et al.*, 2019)(Hull, Montgomery and Leyva, 2016). The main goal of this project is to uncover the transcription factors and other regulators that are implicated in the regulation of NKG2D-Ls after treatment with LBH589. Therefore, the NKG2D-L MICA was used as a model to map the MICA promoter's regulators in response to LBH589 treatment. The MICA gene is highly polymorphic with more than 80 coding alleles and has two different promoters, an upstream promoter and a standard promoter (Introductory figure 3)(Robinson *et al.*, 2015)(Lin, Hiron and O'Callaghan, 2018). The transcription factors involved in the MICA regulatory mechanism have not been well studied. In this

study, the enChIP approach was applied to identify transcription factors specifically involved in the regulation of MICA promoter.



Introductory figure 3: The structure of the *MICA* gene, the model used for enChIP assay.

MICA consists of two promoters (upstream and standard) and six exons.

6. Isolation and purification of genomic loci

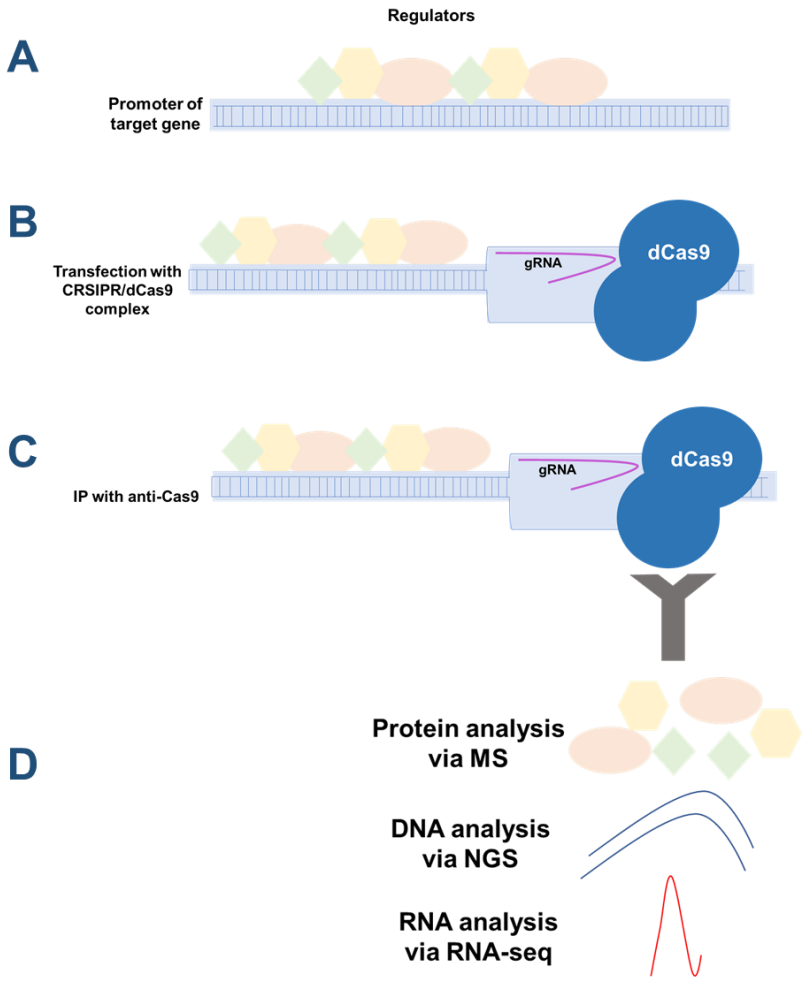
Chromatin in the eukaryotic nucleus consists of DNA tightly wrapped around proteins. This mixture of DNA and protein plays a crucial role in the regulation of gene expression, such as transcription and epigenetic regulation (Li, Carey and Workman, 2007)(Mariño-Ramírez *et al.*, 2005). To understand chromatin regulation, it is necessary to identify proteins, which bind to the regulatory regions in the genome, such as promoters and enhancers. Such proteins are called transcription factors and mediate either the activation or repression of the transcription of a particular gene (Narlikar and Ovcharenko, 2009). Purification of chromatin at genomic-specific loci has always been necessary for chromatin research but remains a significant technical challenge (Wierer and Mann, 2016)(Korthout *et al.*, 2018). Chromatin immunoprecipitation (ChIP) is a powerful technique commonly used to discover DNAprotein interactions *in vivo*. It provides a snapshot of transcription factors, histones, and other proteins involved in the regulation of a single specific gene or multiple genes together. Furthermore, a significant advantage of ChIP is that it can be combined with several molecular biology techniques such as real-time polymerase chain reaction (PCR), Western blot, Next-generation sequencing, and other analyses (Gade and Kalvakolanu, 2012)(Gade and Kalvakolanu, 2012)(Das *et al.*, 2004). However, like any other molecular biology technique, the ChIP technique has its limitations: not only does it depends on the prior identification of molecular targets, but

it also is limited to previously studied single transcription factors (Déjardin and Kingston, 2009).

Moreover, it depends on the antibody's specificity and quality (Déjardin and Kingston, 2009). Consequently, the development of a site-specific chromatin purification method has become essential.

6.1. enChIP and capture for the purification of a single genomic locus

Over the last few years, technologies to purify specific genomic regions for analyzing locus-specific genome functions have been developed. For example, the insertional chromatin immunoprecipitation (iChIP) method is a powerful technique to purify proteins and RNAs associated with specific genomic regions. However, it needs the insertion of the recognition sequences of an exogenous DNA-binding molecule such as LexA into the target locus (Hoshino and Fujii, 2009)(Fujita and Fujii, 2011)(Fujita and Fujii, 2013b). Engineered DNA-binding proteins such as transcription-activator-like effector (TAL) proteins, zinc-finger proteins, and the clustered regularly interspaced short palindromic repeats (CRISPR/dCas9) system have been successfully used for various purposes such as genome editing, transcriptional regulation, and the isolation of a genomic locus of interest. The CRISPR system consists of a catalytically inactive form of the Cas9 endonuclease (dCas9) and a small guide RNA (gRNA) that targets a specific sequence (Pabo, Peisach and Grant, 2001)(Bogdanove and Voytas, 2011)(Qi *et al.*, 2013)(Fujita, Yuno and Fujii, 2018). Among all tools, the CRISPR system is the most flexible, inexpensive, and time-efficient way to create engineered DNA-binding molecules (Barrangou *et al.*, 2007). Furthermore, enChIP using the CRISPR system can be coupled to mass spectrometry (enChIP-MS) to identify proteins associated with the isolated locus (Introductory figure: 4)(Fujita and Fujii, 2013a). For example, CRISPRmediated enChIP technology has been used to detect proteins associated with the promoter region of interferon- (IFN-) inducible IFN regulatory factor 1 *(IRF1)* gene (Fujita and Fujii, 2013a). Moreover, enChIP-Real-Time-PCR and next-generation sequencing (enChIP-seq) are also helpful for the identification of genomic DNA or interactions between genomic regions, respectively (Introductory figure: 4). Thus, enChIP presents a powerful and flexible method for isolating and identifying molecules associated with specific genomic regions. In this study, using the enChIP/MS approach, we identified several regulatory candidates for the *MICA* promoter region and the most prominent of them is Krüppel-like factor 4 (KLF4).



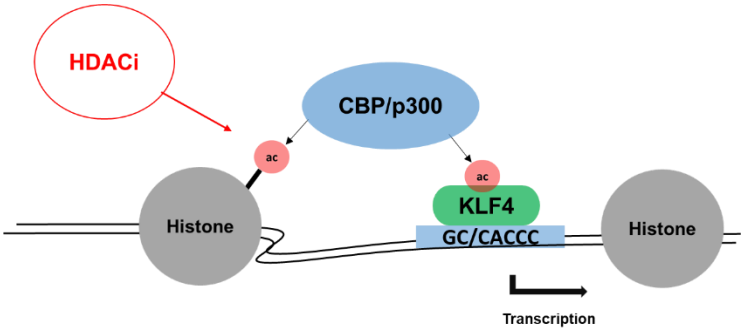
Introductory figure 4: Scheme of enChIP approach for isolating a single genomic locus. **A.** The proper gRNAs are designed for the specific genomic locus. **B.** Cells are transfected with the CRISPR/dCas9 complex, which consists of the targeting gRNA and the catalytically inactive Cas9. Chromatin is cross-linked with formaldehyde and is fragmented by sonication. **C.** The dCas9-containing complex is precipitated with Cas9 antibody. **D.** Finally, DNA, RNA, and proteins associated with the target genomic locus are identified by Next generation sequencing (NGS), RNA-seq, and mass spectrometry (MS), respectively. IP = Immunoprecipitation

7. KLF4 expression, function, and regulation

Krüppel-like factor 4 is a zinc finger transcription factor expressed in a wide range of human tissues and is involved in the regulation of numerous cellular processes, including proliferation, differentiation, and apoptosis (McConnell *et al.*, 2007)(Evans *et al.*, 2007)(Wei *et al.*, 2005)(Bieker, 2001). It plays different roles in various kinds of cancer, and functions as a tumor suppressor or an oncogene depending on the tumor type or stage (Tetreault, Yang and Katz, 2013)(Riverso, Montagnani and Stecca,

2017). KLF4 can recognize and bind to the GC- and CACCC- gene regulatory in promoters and enhancers (Ray, 2016)(Swamynathan, 2010)(Ghaleb and Yang, 2017). Moreover, KLF4, a well-known Yamanaka factor, is one of the four transcription factors involved in reprogramming somatic cells to become induced pluripotent stem cells (iPS) (Marpaung and Sinaga, 2020). It has two adjacent domains within its amino terminus, a transactivation domain, and a repression domain, which regulate its activity (Geiman, 2000)(Yet *et al.*, 1998)(Ghaleb and Yang, 2017). Consequently, according to the cellular context, KLF4 exhibits different functional roles: it can act as a transcriptional activator or transcriptional repressor (Ray, 2016)(Shie *et al.*, 2000). Many splice variants of the KLF4 gene have been detected in normal and cancer human cells (Ghaleb and Yang, 2017). The human KLF4 gene is 91 % identical to the mouse KLF4 gene (Ghaleb and Yang, 2017). Additionally, the knockout of the KLF4 gene in mice leads to death after birth due to defects in the skin barrier function (Segre, Bauer and Fuchs, 1999). The transcriptional coactivators CBP/p300 can bind directly to KLF4 both *in vitro* as well as *in vivo*, acetylating it at lysine residues 225 and 229 and subsequently activating transcription (Introductory figure: 5)(Evans *et al.*, 2007). Furthermore, mutation at these lysine residues reduced the ability of KLF4 to activate the target gene. This suggests that acetylation is important for KLF4-mediated transactivation (Evans *et al.*, 2007). KLF4 also plays a vital role in the regulation of hematopoietic cells such as monocyte development, $CD8^+$ T cell differentiation, and NK cell survival (Feinberg *et al.*, 2007)(Shik Park *et al.*, 2012)(Mamonkin *et al.*, 2013). However, the function of KLF4 is cancer-type dependent. The reduction of KLF4 has been reported in different cancer types. As in human AML patients, KLF4 expression was significantly lower than in normal controls (Huang *et al.*, 2014). Additionally, KLF4 expression in AML correlates with the inhibition of cell proliferation through induction of the cell cycle arrest *in vitro* and *in vivo* (Huang *et al.*, 2014). The same study also found that the expression of HDACs was higher in AML patients than in the controls. Furthermore, a negative correlation was observed between KLF4 and HDAC1 expression. AML patients with a lower level of HDAC1 and consequently overexpression of KLF4 had a better prognosis (Huang *et al.*, 2014). Several studies suggest that KLF4 is a downstream target of HDACi, which induces cell cycle arrest and apoptosis (Huang *et al.*, 2014)(Chen, Rex and Tseng, 2004). As in ovarian SKOV3, colon HT-29, and lung A549 cancer cells, the treatment with the HDACi trichostatin A (TSA) induced transcription of KLF4 and induced apoptosis *in vitro*

(Zohre *et al.*, 2014). Moreover, this induction was associated with hyperacetylation of the KLF4 promoter (Chen, Rex and Tseng, 2004). APTO253, also known as LOR253, is a small molecule that exhibits potent anti-cancer activity in treating solid and hematologic cancers, including AML. It is currently used in a phase 1a/b clinical trial to treat AML patients with relapse or refectory (Rice *et al.*, 2015). In addition, in the KG1, THP1, and Kasumi-1 xenograft models of human AML, the tumor growth was significantly inhibited by APTO253 treatment. Interestingly, the cytotoxic activity against AML is regulated by induction of the KLF4 (Rice *et al.*, 2015). Furthermore, treatment of cultured AML cells with APTO253 induced the expression of p21 and consequently cell death by apoptosis. Treatment of KG1 cells with APTO253 for 24 h markedly upregulated the expression of KLF4 and p21 at both mRNA and protein levels (Rice *et al.*, 2015).



Introductory figure 5: The acetylation of Krüppel-like factor (KLF4) via CBP/p300 mediates the transactivation of gene transcription. Treatment with HDACi induces acetylation of histones and non-histone proteins such as KLF4, which in turn leads to transcriptional activation of downstream target genes.

Aim of this thesis

The NK cells activating receptor NKG2D and its ligands (NKG2D-Ls) that are induced on tumor cells upon malignant transformation play a crucial role in tumor immune surveillance. However, tumor cells evolve different mechanisms to evade immune clearance and NK cell cytotoxicity by reducing or even losing the surface expression of NKG2D-Ls, which is an important immune escaping strategy in AML. To date, several anti-cancer drugs, including the HDACi, have significantly increased the expression of NKG2D-Ls on tumor cells, which in turn enhanced their susceptibility to killing by NK cells. Among the HDACi, LBH589 has been shown to induce the expression of NKG2D-Ls, including the MICA gene. This induction occurs at the transcription level and is mediated by the acetyltransferases CBP/p300. Nevertheless, the mechanism for regulating NKG2D-Ls, such as MICA in response to LBH589 has not been fully elucidated. My dissertation aims to identify druggable transcription factors involved in MICA gene expression in response to LBH589 treatment. To this end, the following questions will be investigated:

I. Which of the two promoters of the MICA gene is responsible for MICA upon LBH589 treatment?
II. Is the novel enChIP approach followed by mass spectrometry able to target the MICA promoter and identify the transcriptional regulators under treatment with LBH589?
III. Do the proteins isolated by the enChIP/MS approach play an important role in regulating the MICA gene?
    - Does the HDACi LBH589 regulate the expression of the proteins isolated and identified by enChIP/MS?
    - Does the expression of MICA and our enChIP/ MS candidates differ in chemotherapy-sensitive and–resistant AML cell lines?

Answering these questions will be helpful in two ways: first, to demonstrate the efficacy of enChIP/MS to understand gene regulation at the genomic level. Second, deciphering the basic mechanism of MICA gene regulation in response to LBH589 treatment, thereby opening the way for cancer therapy targeting the NKG2D/NKG2DLs axis.

Materials

1. Table of buffers

Name	Ingredients and supplier
PBS	Thermo Fisher
DMEM media	Thermo Fisher
RPMI media	Thermo Fisher
Opti-MEM I Reduced Serum Media	Gibco
Formaldehyde 37 %	Carl Roth 7398.4
Glycine (2.5 M stock solution)	Carl Roth 3908.2
Lysis buffer I (hypotonic lysis buffer)	5 mM PIPES pH 8.0, 85 mM KCl, 0.5 % (v/v) NP40
Lysis buffer II (RIPA buffer)	10 mM Tris–HCl pH 7.5, 150 mM NaCl, 1 % (v/v) NP40, 1 % (w/v) sodium deoxycholate, 0.1 % (w/v) SDS, 1 mM EDTA
Wash buffer I (mixed micelle buffer)	20 mM Tris pH 8.1, 150 mM NaCl, 2 mM EDTA, 0.1 % (w/v) SDS, 1 % (v/v) Triton X-100
Wash buffer II (buffer 500)	20 mM Tris pH 8.1, 500 mM NaCl, 2 mM EDTA, 0.1 % SDS, 1 % (v/v) Triton X-100
Wash buffer III (lithium chloride detergent buffer)	10 mM Tris pH 8.1, 250 mM LiCl, 1 % (v/v) NP40, 1 % (w/v) sodium deoxycholate, 1 mM EDTA
Elution buffer (prepare fresh)	1 % (w/v) SDS, 100 mM $NaHCO_3$
Elution buffer (EB)	10 mM Tris-Cl, pH 8.5 Qiagen 19086
Reversion mix (Per sample)	16 µL (5 M NaCl), 16 µL (1 M Tris pH 6.8), 8 µL (0.5 M EDTA), 10 mg RNase A (10 g/L stock), 20 µg Proteinase K (20 g/L stock)

Binding buffer (PB)	contains a high concentration of guanidine hydrochloride and isopropanol

PE buffer	Qiagen 19065
Albumin Fraktion V, NZOrigin, 50 g	Fraction V, 20 g/L, dissolved in TE pH 8.0 Carl Roth 8076.2
Sonicated salmon sperm DNA (stock at 10 g/L)	Thermo Fisher 15632011
Sodium deoxycholate 1 % (w/v)	Sigma-Aldrich D6750-25G
RNase I (10 U/µL)	Thermo Fisher EN0602
Proteinase K	Thermo Fisher EO0491
SDS 0.1 % (w/v)	Carl Roth CN30.3
Separation buffer	67 % (v/v) Rotiphorese Gel 30 AA/BA 1.5 M Tris/HCl pH 8.9 10 % (g/v) SDS
Stacking gel (4 %)	13 % (v/v) Rotiphorese Gel 30 AA/BA 1.5 M Tris/HCl pH 6.8 10 % (g/v) SDS
Ammonium persulfate (APS) 10 %	AppliChem 131138
Tris/HCl buffer for SDS gels, pH 8.9	1.5 M Tris/HCl 10 % (g/v) SDS
RIPA buffer	50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.1 % SDS (w/v), 0.5 % (v/v) TritonX-100, 0.5 % DOC (Deoxycholic acid)
SDS-running buffer	25 mM Tris-HCl, 192 mM glycine, 0.1 % (w/v) SDS, pH 8.3
Blotting buffer	250 mM Na_2HPO_4
TBS-T buffer	150 mM NaCl, 50 mM Tris-HCl, 0.1 % (v/v) Tween-20, pH 7.5

Tween20	Applichem
Trypsin-EDTA Solution 10X	Sigma-Aldrich
5x Laemmli sample buffer, pH 6.8	0.5 M Tris/HCl (pH 7) 50 % (v/v) glycerin 5 % (w/v) SDS
	0.25 M DTT 0.05 % (w/v) bromphenol blue
RIPA-Buffer	50 mM Tris-HCl 1 % Triton X100 0.1 % SDS (sodium dodecyl sulfate) 0.5 % SOD (sodium deoxycholate*) 150 mM NaCl 1 mM EDTA
TEMED (N N N' N' Tetramethylethylenediamine)	Carl Roth
Isopropanol	Carl Roth
LB Medium	1 % (w/v) Peptone, 1 % (w/v) NaCl, 0.5 % (w/v) Yeast extract
Absolute qPCR SYBR Green Mix	Thermo Fisher Scientific/Life Technologies
Ethanol	Carl Roth
Penicillin/Streptomycin (Pen/Strep), 100x	Capricorn
Ampicillin, Ready-Made Solution, 100 mg/mL, 0.2 µm filtered	Sigma-Aldrich
Kanamycin	Sigma-Aldrich (K1377)
Methanol	Carl Roth
Fast Digest Buffer, 10x	Thermo Fisher Scientific/Life Technologies
Nuclease free water	Carl Roth

2: Table of inhibitors and enzymes

Name	Function	Used concentration	supplier
Panobinostat (LBH589)	HDACi (HDAC1-11)	100 nM	SCBT
Protease inhibitorCocktail	It contains inhibitors with a broad specificity for serine, cysteine, and acid proteases, and aminopeptidases	1:1000	Sigma P8340
Kenpaullone	GSK-3β inhibitor; also inhibits CDKs and KLF4	1, 5, or 10 µM	Selleck Chemicals S7917
APTO253	Inducer of KLF4	100, 200, 300, 400, and 500 nM	Selleck Chemicals S6963
U0126	MEK1/2 inhibitor	10 µM	Cell signaling 9903
Cytarabine Ara-C	an anti-cancer ("antineoplastic" or "cytotoxic") and known as "antimetabolite" chemotherapy drug used to treat different form of leukemia including AML	100 and 500 nM	AG Prof. Dr. med. Andreas Neubauer
Hind III	*HindIII* restriction enzyme recognizes A^AGCTT sites and cuts	1 µL	Fisher Scientific ER0501
Nde I	restriction enzyme recognizes CA^TATG sites and cuts	1 µL	Fisher Scientific FD0583
Kpn I	restriction enzyme recognizes GGTAC^C sites and cuts	1 µL	Fisher Scientific ER0521

T4 Ligase	Catalyzes the formation of a phosphodiester bond between juxtaposed 5' phosphate and 3' hydroxyl termini in duplex DNA or RNA	1 µL	Fisher Scientific 10723941
BbsI	restriction enzyme *BpiI* (*BbsI*) recognizes GAAGAC (2/6) ^ sites and cuts	1 µl	Fisher Scientific ER1011

3: Table of antibodies and beads

Name	**Used concentration**	**supplier**
H3K27 ac	2 µg	Diagenode C15410174
IgG	4 µg	Sigma i5006
KLF4	0.5 µg/mL	R&D AF3640-SP
KLF4	1:1000	Cell Signaling Technology 12173S
Cas9	4 µg	Sigma-Aldrich SAB4200701
Beta-Actin(C4)	1:10,000	Santa Cruz Biotechnology sc-47778
GAPDH	1:5,000	Sigma-Aldrich G9545-100
PARP	4 µg	Cell Signaling Technology 9532S
PARP	4 µg	Thermo Fisher Scientific PA5-34803
Streptavidin Agarose	50 µL	Thermo Fisher Scientific SA10004

Dynabeads™ MyOne™ Streptavidin T1	10 µL	Thermo Fisher Scientific 65601
Agarose A	Further details in the method section	AG Prof. Dr. Uta-Maria Bauer
Protein G Sepharose	Further details in the method section	GE Healthcare 17-0618-05
MICA/B, AF 647	1 µL	Biolegend 320914
IgG2a,k, AF 647	1 µL	Biolegend 400234
Mouse Gamma Globulin	(1:100) 50 µL per sample	Jackson ImmunoResearch 015-000-002

4: Table of kits

Name	**supplier**
PCR-purification kit	Qiagen 28106
RevertAid First Strand cDNA Synthesis Kit	Thermo Fisher Scientific 10387979
GeneJET PCR Purification Kit	Thermo Fisher Scientific 10400450
NucleoSpin RNA, Mini kit for RNA purification	Macherey-Nagel REF 740955.50
Dual-Glo Luciferase Assay System, 10 x 100 mL, Promega, Dual-Glo Luciferase Assay System	Promega E2920
PierceTM BCA protein assay Kit	Thermo Fisher Scientific
DNeasy Blood & Tissue Kits	Qiagen 157023568

5: Table of cell lines and competent cells

Cell lines/competent cells	origin	Cultural conditions
HEK293	Human embryonic kidney cells (DSMZ)	DMEM + 10 % (v/v) FBS + 1 % (v/v) P/S
Ara-C sensitive HL60	Human acute promyelocytic leukemia cell line (DSMZ) (from AG Prof. Andreas Neubauer´s lab, Marburg, Germany)	RPMI, GlutaMAX™ Supplement 10 % (v/v) FBS + 1 % (v/v) P/S
Ara-C resistant HL60	Ara-C resistant human acute promyelocytic leukemia cell line (DSMZ). (Here the cells seeded in	RPMI, GlutaMAX™ Supplement 10 %
	medium with 400 nM Ara-C. (from AG Prof. Andreas Neubauer´s lab, Marburg, Germany)	(v/v) FBS + 1 % (v/v) P/S
MonoMac 6	Human acute monocytic leukemia from the peripheral blood of a 64year-old man with recurrent acute monocytic leukemia (DSMZ) (from AG Prof. Daniel Lacorazza, Texas, Houston, USA)	RPMI 1640, GlutaMAX™ Supplement 20 % (v/v) FBS + 1 % (v/v) P/S. 1 % Non-Essential Amino Acids (NEAA) + 1 µg/mL Insulin + 1 % Sodium Pyruvate
NB4	Human acute promyelocytic leukemia from the bone marrow of a 23-yearold woman (DSMZ) (from AG Prof. Daniel Lacorazza, Texas, Houston, USA)	RPMI 1640, GlutaMAX™ Supplement 10 % (v/v) FBS + 1 % (v/v) P/S

6: Table of gRNA – oligos

Name	Sequence (5’→ 3’)

MICA 1	sense	CACCGTTAATGGGGCGGCCGGCGAA
	anti-sense	AAACTTCGCCGGCCGCCCCATTAAC
MICA 2	sense	CACCGTAATGGGGCGGCCGGCGAAA
	anti-sense	AAACTTTCGCCGGCCGCCCCATTAC
MICA 3	sense	CACCGCTTTAGGCTGCGCTCCCGC
	anti-sense	AAACGCGGGAGCGCAGCCTAAAGC
MICA 4	sense	CACCGTTTCATTGGATGAGCGGTCG
	anti-sense	AAACCGACCGCTCATCCAATGAAAC
MICA 5	sense	CACCGCCGCCCTCTCCGCTCGTGAT
	anti-sense	AAACATCACGAGCGGAGAGGGCGGC
MICA 6	sense	CACCGTTCATTGGATGAGCGGTCGG
	anti-sense	AAACCCGACCGCTCATCCAATGAAC

MICA 7	sense	CACCGCTGAGAGGGTGGCGACGTCG
	anti-sense	AAACCGACGTCGCCACCCTCTCAGC
MICA 8	sense	CACCGCCAGAAAATGGGGAGCACGC
	anti-sense	AAACGCGTGCTCCCCATTTTCTGGC
HBG	sense	CACCGCTAAACTCCACCCATGGGT
	anti-sense	AAACACCCATGGGTGGAGTTTAGC
GFP	sense	CACCTTCAGCTCGATGCGGTTCAC
	anti-sense	AAACGTGAACCGCATCGAGCTGAA

7: Table of RT-qPCR primers

Name		**Sequence (5' → 3')**
MICA	Forward	CTGCAGGAACTACGGCGATATCT
	Reverse	CCCTCTGAGGCCTCGCTG
MICB	Forward	AGAAGAAAACATCAGCGGCAG
	Reverse	CATCCCTGTGGTCTCCTGTC

ULBP2	Forward	GCCGCTACCAAGATCCTTCT
	Reverse	GCAAAGAGAGTGAGGGTCGG
HBG	Forward	TGGATGATCTCAAGGGCAC
	Reverse	TCAGTGGTATCTGGAGGACA
RPL27	Forward	AAAGCTGTCATCGTGAAGAAC
	Reverse	GCTGTCACTTTGCGGGGGTAG
UP MICA	Forward	GGCGCCTAAAGTCTGAGAGA
	Reverse	CAGCAAGAAACCCTGACTGC
SP MICA	Forward	GGCTGGCATCTTCCCTTTTG
	Reverse	CAGCAAGAAACCCTGACTGC
KLF4	Forward	GAAATTCGCCCGCTCCGATGA
	Reverse	CTGTGTGTTTGCGGTAGTGCC
c-MYC	Forward	CCTCCACTCGGAAGGACTATC
	Reverse	TGTTCGCCTCTTGACATTCTC

8: Table of ChIP-qPCR primers

Name		**Sequence (5’ → 3’)**
MICA	Forward	CGTGCTTATGAAGTTGGA
	Reverse	AGACCTGGGGAGATTTAG
HBG	Forward	CTTGTCAAGGCTATTGGT
	Reverse	TATCTCAATGCAAATATCTG
PDK4RE1	Forward	GTATGTGTACTGGGGGGAC
	Reverse	CAGATGGCTCTTTTCGTTCC
	Forward	GCTGGAGTTGAAGGGAAGTG

TSC22D3	Reverse	AGGAGCCAAAATATCTCCGAGT
RPL30	Forward	ATTTGCCCATTTTGGTTCCCA
	Reverse	TAGTATTTTCCGCATGCTGTGC

9: Table of vectors

Name	Function	Addgene catalog #
gRNA-GFP-T1	Express gRNA to target GFP	41819
SP-dCas9-VPR	SP-dCas9 with VP64-p65-Rta (VPR) fused to its Cterminus to activate the target gene	63798
pEF1a-FB-dCas9puro	Expresses N-terminal FLAG and biotin-acceptor-site (FB)-tagged dCas9 protein	100547
pEF1a-BirA-V5-neo	Expresses C-terminal V5-tagged BirA biotin ligase	100548
pX330-U6Chimeric_BB-CBhhSpCas9	A human codon-optimized SpCas9 and chimeric guide RNA expression plasmid.	42230
pGL3- Basic Vector	The pGL3-Basic Vector does not contain any promoter and enhancer sequences. Activation of luciferase expression in the cell depends on transfection with this plasmid after inserting a functional promoter upstream from luc^+.	U47295 Promega
pRL-TK- Vector	The pRL Vector is a wild-type renilla luciferase reporter vector. It induces renilla luciferase expression.	E2241 Promega

Methods

1. *In vitro* cell culture treatment

Human embryonic kidney cells (HEK293) were maintained in a DMEM medium with 10 % FBS and 1 % penicillin/streptomycin. Cells were passaged twice to three times per week and always used for experiments when the passage number was between two to five. Cells were incubated under standard culture conditions at 37°C with 5 % CO_2. To detach HEK293 cells, trypsin was added for 5 min. Frequently, the mycoplasma contamination test was performed, and only mycoplasma-free cells were used. 2.5×10^5 were cultured in 6 wells plate one day before treatment. The day after, cells were treated with different agents.

2. Transient transfection

$5\text{-}6\times10^6$ cells were seeded in a 15 cm dish containing 20 mL DMEM medium with 10 % FBS and 1 % penicillin/streptomycin 20 h before transfection. After one day, the medium was removed, and FBS and penicillin/streptomycin-free DMEM medium was added. For transfection, 40 µL of Lipofectamine 2000 DNA transfection reagent (Life Technologies) was diluted in 750 µL serum-free Opti-MEM (Thermo Fisher), and 16 µg vector DNA was also diluted in 750 µL serum-free Opti-MEM. After 5 min incubation at room temperature, the diluted Lipofectamine was combined with the diluted DNA. Lipofectamine-DNA complexes were incubated for 20 min at room temperature before the solution was dropped onto the cells and incubated at 37°C with 5 % CO_2. The medium was replaced again after 18 h with a DMEM medium containing 10 % FBS and 1 % penicillin/streptomycin. Transfection efficiency was monitored by transfection with pEGFP-C1 as control and visualized by a fluorescence microscope (Olympus).

3. gRNA Cloning

The gRNA-GFP-T1 plasmid containing a kanamycin resistance cassette was ordered from Addgene (see material section). The gRNA sequence was modified by adding an A-U pair flip and a 5 bp hairpin extension. The modified hairpin sequence was ordered from IDT (Integarted and technologies). Next, the gRNA-GFP-T1 plasmid and the modified hairpin were digested by using *HindIII* and *NdeI* restriction enzymes for 2 h at 37°C. Later, the plasmid with the new hairpin was ligated in a ratio of 1:3 using T4 ligase for 1 h at 22°C. 8 µL of ligation mixture was used for transformation into 100 µL aliquot of *E.coli* XL1- blue competent bacteria and incubated on ice for 30 min. Next, they were incubated at 42°C for 90 s, followed by incubation on ice for 2 min for

recovery. 500 µL of LB medium was added for cells. Then, cells were incubated on a shaker for 20 min at 37°C. Later, 100 µL of cells were spread on pre-warm LB/ Kanamycin agar plates. Finally, colonies were picked, and DNA was extracted and sequenced by LGC sequencing services (LGC Genomics GmbH, Berlin).

gRNA-original:

5'-
GTTTTAGAGCTAGAAATAGCAAGTTAAAATaaggctagtccgttatcaacttgaaaaagtggca

c cgagtcggtgc-3' gRNA-modified A:U flip and a 5bp stem extension:

5'-
GTTTAAGAGCTATGCTGGAAACAGCATAGCAAGTTTAAATaaggctagtccgttatcaactt
gaaaaagtggcaccgagtcggtgc-3'

The generated plasmid was used as a backbone to clone the individual gRNAs. Single guide RNAs were designed to target the promoter region of a specific gene using the Zhang Lab design tool (Guide Design Resources). The two annealed oligo duplex of gRNA were cloned into the backbone plasmid by digestion with a *BpiI* (*BbsI*) restriction enzyme at 37°C for 2 h and ligation using T4 ligase at 22°C for 1 h. As previously described, 8 µL of ligation mixture is directly used for transformation with *E.coli* XL1-blue competent cells. Finally, colonies were picked, and DNA was extracted and sequenced by LGC sequencing services.

4. The transcription activation based on the CRISPR/dCas9 activation system

2.5×10^5 HEK293 cells were seeded in 6 well plates. After 24 h, cells were transiently co-transfected with 2.5 µg of SP-dCas9-VPR activation vector and 2.5 µg of mixed gRNA vectors targeting MICA standard promoter region or gRNA targeting HBG promoter region (as positive) and gRNA targeting GFP (negative control). Transfection was performed using 5 µL of Lipofectamine 2000 as previously described (see transient transfection paragraph). 48 h post-transfection cells were harvested for RTqPCR analysis.

5. RNA isolation and cDNA preparation

Total RNA was extracted from the cells using the NucleoSpin RNA Kit from MachereyNagel. The concentration of extracted RNA was determined by measuring

light absorbance at 260 nm (A260) and the ratio of (A260/A280) via NanoDrop2000 (Thermo Scientific Fisher). Total RNA (500 ng) was used for first-strand cDNA synthesis in a 20 µL reaction volume using the RevertAid RT Reverse Transcription Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. 1 µL oligo(dT) primers and 1 µL random hexamer primers were used. Last, cDNA was diluted at 1:20 in nuclease-free water.

6. RT-qPCR

Primers used were designed via the NCBI Primer Blast (table 7) and tested for specificity before use. A technical triplicate PCR reaction was carried out using the ABsolute qPCR SYBR Green Mix (Thermo Fisher Scientific) and a Thermo Cycler Mx3005P from Stratagene. The program setup used was as the following: initially for 15 min at 95°C followed by 40 cycles of 95°C for 15 s, 60°C for 20 s, 72°C for 15 s, and 95°C for 60 s were, followed by a melting curve analysis. The level of target gene expression was measured using the delta-Ct method using RPL27 as a housekeeping gene. Briefly, △Ct was calculated as △Ct = Ct (gene of interest) – Ct (housekeeping gene, which is RPL27). △△Ct was calculated as △△Ct = △Ct (experimental group) – △Ct (control group). The fold change of the gene of interest vs. the control gene was calculated as fold change C = $2^{(-\triangle\triangle Ct)}$. Each gene was tested in three technical replicates in every independent experiment, and all experiments were independently repeated for a minimum of three.

7. Preparing the blocked protein A and G sepharose beads slurry

50:50 of protein A and protein G sepharose beads (GE Healthcare) were mixed and incubated for blocking with 0.4 g/L sonicated salmon sperm DNA and 1 g/L BSA in lysis Buffer II with 1x protease inhibitor cocktail at 4°C on a roller overnight. The following day, dynabeads were washed twice with Lysis buffer II with a fresh protease inhibitor cocktail, and a portion of the supernatant was discarded to make a 50 % slurry. The blocked dynabeads were incubated at 4°C for ChIP method.

8. enChIP- qPCR

5×10^6 HEK293 cells were seeded in a 15 cm culture dish for 20 h. The following day, the medium was replaced with FCS/PS-free medium. After 2 h, cells were transfected with 8 µg of SP- pEF1a-FB-dCas9-puro expression vector and 8 µg of non-targeting gRNA (GFP) vector or a mix of gRNAs to target the MICA promoter region or gRNA targeting HBG promoter region with 40 µL Lipofectamine 2000 and cells were

incubated at 37°C overnight. Fresh FCS/PS-containing medium was added to the transfected cells the following day, and cells were incubated for 24 h. Cells were treated with 100 nM LBH589 for 4 h. Later, cells were fixed with 1 % formaldehyde for 10 min at RT and neutralized with 125 mM final concentration of glycine at RT for 5 min. The supernatant was discarded, and the fixed cells were washed twice with cold PBS. Cells were detached with a cell scraper, harvested in a 15 mL falcon, and centrifuged at 2000 g, 4°C for 5 min. Gently but thoroughly, the pellet was diluted in 2 mL Lysis buffer I (hypotonic lysis buffer) with 1x protease inhibitor cocktail, incubated on ice for 20 min to disrupt the cellular membrane and centrifuged as before. Then, the pellet was resuspended in 2 mL lysis buffer II (RIPA buffer) with 1x protease inhibitor cocktail and incubated on ice for 10 min. The extracted chromatin was fragmented by sonication using 52 pulses of 1 s with 4 s of pause at 20 % amplitude in a 15 mL tube cooler (Active Motif, no. 53077) by active motif sonifier (The average length of fragments was between 500-750 bp). Subsequently, the sonicated chromatin was transferred to a new tube and centrifuged at 13000 g, 4°C for 15 min. Carefully the sheared chromatin was transferred to a new tube again without disrupting the pellet. 100 µL of protein A+G dynabeads (prepared as described in the previous paragraph) per 1 mL of shared chromatin was freshly blocked with 10 µg rabbit IgG for 30 min at 4 °C on a roller. Next, blocked beads were washed twice with lysis buffer II, and the supernatant was discarded to make a 50 % slurry. Blocked beads were added to the shared chromatin for pre-clearing and incubated for 45 min at 4°C on a roller. Later, samples were centrifuged at 1200 g for 5 min, and the cleared chromatin was carefully transferred to a new tube. 1 µL of the shared chromatin was saved at 4°C as the 1 % input DNA sample. 300 µL of shared chromatin was incubated either with anti-FLAG M2 Ab (Sigma–Aldrich) or anti-Cas9 Ab (Sigma-Aldrich) and IgG (Sigma–Aldrich) at 4°C on a roller overnight. The next day, 50 µL of conjugated Protein G+A per dynabeads sample was added and incubated for 1 h at 4°C on a roller. The dynabeads were washed with 1 mL of wash buffer I (mixed micelle buffer), centrifuged at 100 g for 1 min, wash buffer II twice with wash buffer III lithium chloride detergent buffer and twice with EB elution buffer (also centrifuged at 100 g for 1 min). Next, the dynabeads were suspended in 200 µL of elution buffer freshly prepared per sample, and incubated at RT for 15 min with vigorous agitation. Samples were centrifuged at a 13000 g for 2 min and carefully transferred the supernatant to a new vessel. This step was repeated and 400 µL of the elution buffer was also added to the input sample

that was saved at 4°C. Next, for each sample 42 µL of reversion mix was added. For DNA – proteins reverse cross-links, samples were incubated at 65°C overnight. Finally, DNA was purified using a PCR purification kit (Qiagen) according to the manufacturer's instructions. 1 µL of the sample was used as a DNA template for qPCR. The specific DNA enrichment after precipitation with the selected antibody was calculated as percentage of the input. The input sample represented 1 % of the soluble chromatin used in the method. The percentage of input was calculated as the following: **Input % = 2$^{-(CT\ sample - CT\ input)}$.**

9. CAPTURE-qPCR

The captured DNA was isolated using the protocol described for enChIP-RT-qPCR, except that the shared chromatin was pulled by incubation with 10 µl of MyOne Streptavidin T1 dynabeads or 50 µL Streptavidin agarose (Thermo Fisher Scientific) at 4°C overnight on a roller. Following the same washing steps (see enChIP-qPCR), DNA was purified using a PCR purification Kit (Qiagen).

10. enChIP–MS

For the enChIP–MS analysis, the enChIP procedure was performed as previously described for enChIP-qPCR. 10 dishes of 6 × 10^6 cells per condition were seeded. 2000 µL of blocked A+G dynabeads-Protein for pre-clearing the sheared chromatin was used. 120 µg of Cas9 antibody (Thermo Fisher) and 1500 µL of A+G dynabeads Protein were used. Then, samples were treated as per the protocol previously described in detail. Later, samples were stored in EB buffer (Qiagen) with protease inhibitor cocktail at -80°C until they were proceeded by off-bead digest and high pH reversed-phase separation, followed by LC-MS2 analysis utilizing label-free quantitation (in cooperation with Prof. Johannes Graumann, Marburg).

11. Western Blot

Whole cell lysates were prepared in RIPA lysis buffer containing a fresh protease inhibitor cocktail (1:1000) and phosphatase inhibitors (1:10). 50-200 µL of lysis buffer was used depending on cell pellet volume, and after 20 min of rotation at 4°C, the cell lysate was cleared by centrifugation at 15000 xg for 15 min at 4 °C. Protein concentration was determined by the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). 10 - 20 µg of protein were subjected to 8, 10, or 12 % SDS polyacrylamide gels, depending on the protein size. Before loading, the sample was boiled at 95°C for

5 min, then immediately cooled. Then, the sample was transferred onto a 0.2 µm nitrocellulose membrane (GE Healthcare) using 1x blotting transfer buffer. Blocking non-specific binding is achieved by incubating the membrane in a dilute 3–5 % bovine serum albumin (BSA) or non-fat dry milk in Tris-buffered saline with 0.1 % Tween20 detergent (TBS-T) for 1 h at RT. After that, the membrane was washed with TBS-T buffer and incubated with HRP-labelled secondary antibody in BSA or milk in TBS-T buffer for at least 2 h at RT or overnight at 4 °C on a roller. After several washing steps with TBS-T, the membrane was subjected to Immobilon Forte western HRP substrate to the chemiluminescence imager ChemiDoc (Bio-Rad) solution (Millipore).

12. Transfection with siRNA

HEK293 cells were transfected with 50 nM siRNA KLF4 (Santa Cruz Biotechnology sc-35480), and siRNA control using lipofectamine 2000. Transfected cells were incubated for 24 h at 37°C and 5 % CO_2 for 24 h. Next, cells were treated with 100 nM LBH589 for 4 h, and subsequently, RT-qPCR analysis was performed to determine the expression of KLF4 in cells treated with different conditions.

13. Cloning of the standard promoter of MICA

The human promoter region of MICA (583 bp) was amplified from HEK293 genomic DNA by PCR using the following primers: sense primer '5AAAGGTACCAGGTCTGCAAATCTGGTCACT-3', antisense primer: '5AAAAAGCTTGAGGTGCAAAAGGGAAGATGC-3'. Next, the promoter sequence of KLF4 was cloned into a pGL3-Basic vector with double-digestion using *Kpn*I and *Hind*III and transformed into *E.coli* XL1 blue. Positive clones were verified by Sanger sequencing.

MICA promoter sequence:

5'-

AGGTCTGCAAATCTGGTCACTGGTCTCATTGCCAGTAACGCTGTGCGCGGTTG
AGGGAGTGTGTTGGGAGAATAGCCACGCGTTGTCTGTCCTGGAAGGAACAAGC
CAGTGAGAGCCGGTTTAATGGGGCGGCCGGCGAAAGGGGCTTGGTGAGGCCC
GCGCTCCTCGGGGTGGGGGCGCGGGGATGGGTGGTCGCGATGCCGGGAGGG
CAGGCAGGGCCCTGGCCGTGCTTATGAAGTTGGAGCTGTACTCTCAGCTACTC
GAAGCTGGTCCCTGCTTTAGGCTGCGCTCCCGCGTGCTCCCCATTTTCTGGGC
CCCAGGTCCCGCCTTCTAAATCTCCCCAGGTCTCCAGCCCACTGGAATTTTCTC
TTCCAAGCGTGGCCCCGCCCTCTCCGCTCGTGATTGGCCCTAAGTTCCGGGCC

CCAGTTTCATTGGATGAGCGGTCGGGGGACCGGGCCAGGTGACTAAGTTTCCG
CGGCGCCTTCTCCCCGGCCACTGCTTGAGCCGCTGAGAGGGTGGCGACGTCG
GGGCCATGGGGCTGGGCCCGGTCTTTCTGCTTCTGGCTGGCATCTTCCCTTTT
GCA-3’ (583 bp)

14. Dual-Glo Luciferase Assay System

3.10^5 HEK293 cells per well were seeded in 6 wells plate overnight. The next day, cells were co-transfected with a 2.5 µg pGL3-MICA-luciferase vector, 100 ng pRL-TK-renilla vector (Promega), and 200 ng of KLF4 expressing plasmid, YY1, or pcDNA3.1 that was used as a control using Lipofectamine 2000. The following day, the transfection medium was changed. 48 h post-transfection cells were harvested and analyzed using the Dual-Glo Luciferase reporter system (Promega, Madison, WI, USA) according to the manufacturer's recommendations: cells were harvested and centrifuged at 300 g for 5 min. The medium was discarded, and cells were resuspended in 750 µL DMEM medium. 96 wells plate was used to lyse the cells, 75 µL Dual-Glo Reagent was added to 75 µL culture medium and mixed in each well. Cells were incubated for 10 min at RT. Then, the firefly luminescence was measured with a luminometer. Afterward, 75 µL of Dual-Glo Stop & Glo reagent was added and incubated for 10 min at RT. Renilla luminescence was measured. Relative luciferase units (RLU) were calculated by measuring the ratio of firefly and renilla luciferase and compared to appropriate controls.

15.Flowcytometry

7x105/2 ml HL60 cells were seeded in 6 well plates and treated with LBH589, APTO253, or DMSO for 18 h. Then, cells were washed with 1 mL buffer (1x PBS containing 1% FSC) and centrifuged at 300 g for 5 min. Cell pellets were resuspended in 600 µL same buffer. 100 µL of each sample was used and blocked with 50 µL (diluted 1:100 in staining buffer) of mouse gamma-globulin per sample for 15 min at 4°C. Cells were incubated with 1 µL MICA/B or IgG2a1 for 30 min at 4°C in the dark. Cells were washed with staining buffer and centrifuged at 300 g for 5 min. For each sample 1 µl of 50 µg/ml propidium iodide (PI) was added. Cell death and MICA/B expression was measured by flow cytometry using a FACS Canto II cytometer (BD Bioscience) and analyzed by FACS Diva software.

16. Statistics

Experiments were performed in three independent biological replicates or otherwise indicated in the figure. Data are presented as mean ± SEM values. GraphPad Prism 7 software was used to generate the figures and calculate the significance between replicates. An unpaired student´s *t*-test was used to calculate the significance. ns= not significant, *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$.

Results

1. MICA transcription initiated from the standard promoter region in response to LBH589

To date, the precise regulation of the ligands for the activating receptor NKG2D is still largely unknown. A previous study demonstrated that the histone deacetylase inhibitor LBH589 significantly induced the expression of these ligands on tumor cells and consequently enhanced the susceptibility to NK cell-mediated cytotoxicity (Sauer *et al.*, 2017). Thus, this study aims to identify the regulators involved in this mechanism.

The NKG2D ligand MICA encodes two main transcripts, one of which initiates transcription from the standard promoter and the other from the alternative promoter located around 1 kb upstream of the standard promoter. Previously, it was observed that the MICA cell surface expression correlates with the expression of the standard transcript but not the upstream transcript (Lin, Hiron and O'Callaghan, 2018).

To investigate which of the promoters is responsible for transcription, HEK293 cells were treated with LBH589 for 4 h, and total RNA was isolated for RT-qPCR analysis. Interestingly, data showed that transcription was predominantly initiated by the standard MICA promoter and not by the upstream one (Figure 1). Hence, LBH589 induced the transcription of the MICA gene through the standard promoter locus.

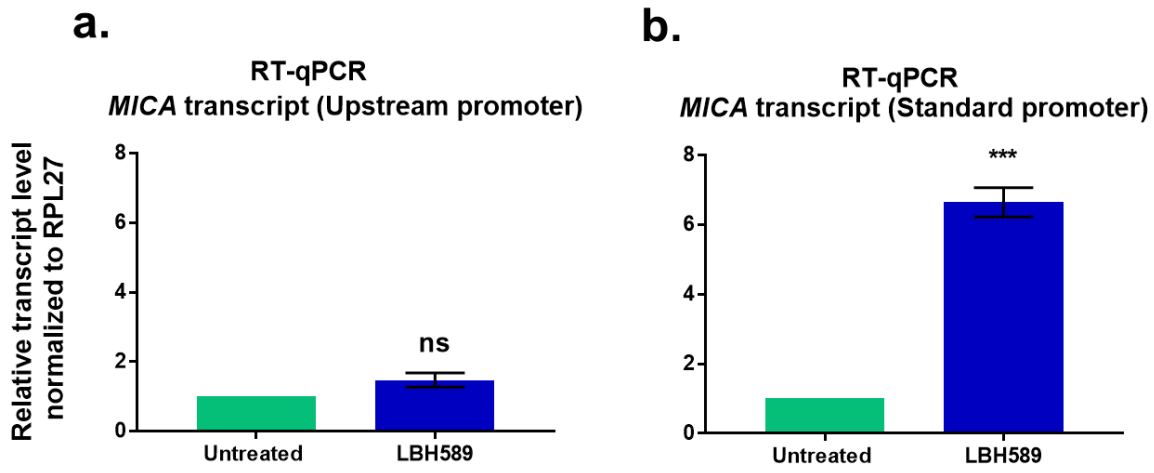


Figure 1: LBH589 stimulated the transcription of MICA from the standard promoter but not from the upstream promoter. HEK293 cells were treated with 100 nM LBH589 for 4 h. RT-qPCR analysis was performed to detect the expression of the **a.** upstream transcript and

b. standard transcript. Data represented the mean ± SEM of three biological experiments. Statistical significance was calculated using an unpaired student's *t*-test. ***$p < 0.001$, ns = not-significant.

2. LBH589 induced H3K27 acetylation at the MICA standard promoter region

After revealing that LBH589 promotes MICA transcription from the standard promoter region, a ChIP assay was conducted to explore the histone acetylation level in this region as a result of LBH589. Previously our group has shown that LBH589- mediated upregulation of MICA depends on the acetyltransferases CBP/p300 in both mice and humans. Using CBP/p300-deficient cells resulted in a significant reduction in MICA upregulation (Sauer *et al.*, 2017). Both CBP and p300 are specifically responsible for the acetylation of histone H3 at lysine 27 (H3K27)(Martire *et al.*, 2020). Additionally, the acetylation of H3K27 is found at both active enhancers as well as promoters and is associated with active gene transcription (Gao *et al.*, 2020). Therefore, HEK293 cells were treated with LBH589 for 4 h and then subjected to a ChIP-qPCR assay using an H3K27ac antibody and IgG as a negative control for non-specific binding. Interestingly, LBH589 enhanced the acetylation of histone H3 at lysine 27 in the MICA standard promoter region (Figure 2a). RPL30 was used as a positive control and high enrichment was observed (Figure 2b). The PDK4 locus was used as a negative control and did not show any enrichment of H3K27 acetylation (Figure 2c). Altogether, the MICA standard promoter region was used as a target in this study to identify putative regulators in response to LBH589 stimulation.

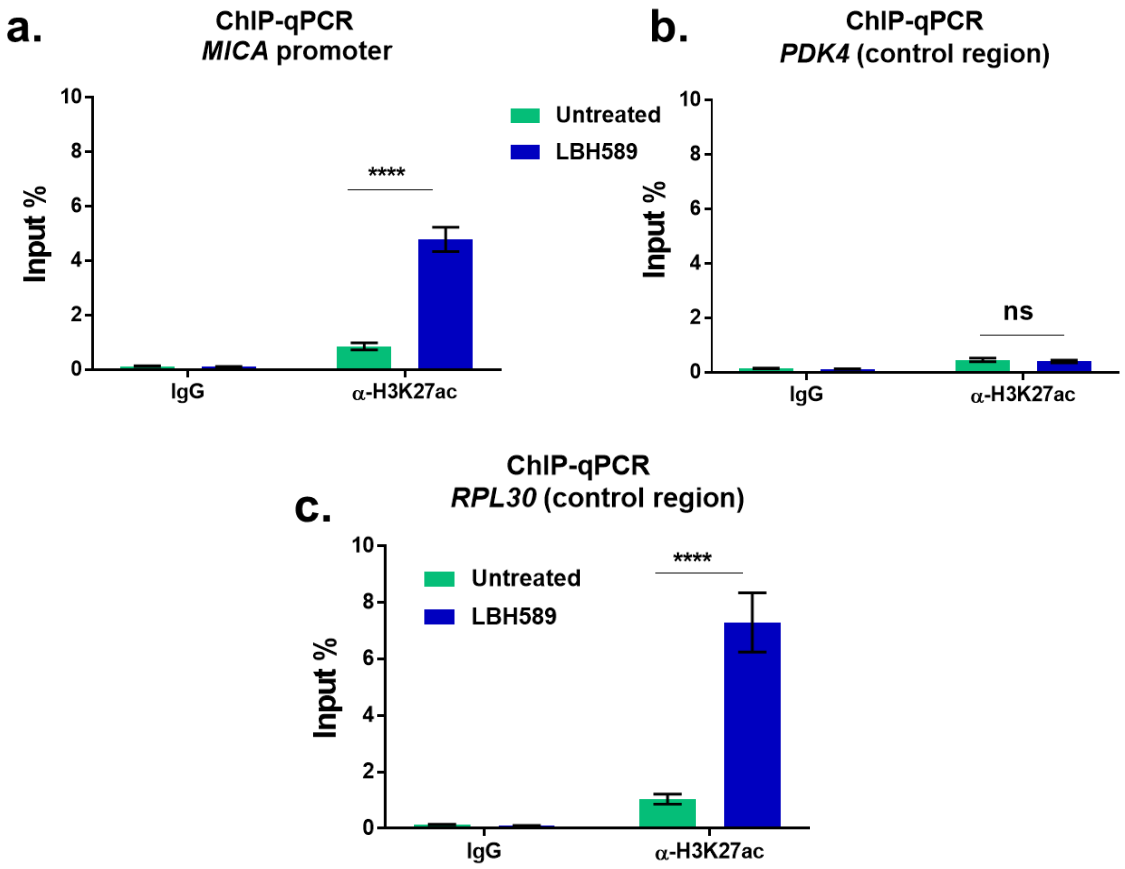


Figure 2: Enhanced acetylation of histone H3 at the MICA promoter region upon LBH589 treatment. ChIP-qPCR of HEK293 cells treated with 100 nM LBH589 for 4 h was performed. H3K27ac antibody against histone H3 was used for immunoprecipitation of **a.** the MICA promoter region, **b.** the RPL30 positive control locus, and **c.** the PDK4 locus as a negative control. Analysis was done using the input % method (details in the method section). Data are shown as mean ± SEM of three independent experiments, and Statistical significance was calculated using an unpaired student's *t*-test. ns = not-significant, ****$p < 0.0001$.

3. Engineered DNA-binding molecule-mediated chromatin immunoprecipitation (enChIP) to target MICA single locus using the CRISPR/dCas9 system: method development

Although the identification of proteins associated with a specific genomic locus is necessary to understand particular gene regulation, developing such approaches is still challenging and needs to be improved. Recently, a novel method called engineered DNA-binding molecule-mediated chromatin immunoprecipitation (enChIP) has been developed to identify chromatin-associated proteins at a specific locus (Fujita and Fujii, 2013a). enChIP utilizes the CRISPR/dCas9 system. This system consists of the inactive Cas9 (dCas9) expressed together with a single guide RNA (gRNA) targeting a specific site, which in this project is the region of the *MICA* standard promoter.

Chromatin complexes are cross-linked with formaldehyde, and then samples are sonicated. Afterward, complexes containing dCas9 are pulled down via Cas9 antibody. Finally, molecules (DNA and proteins) associated with the target genomic region can be detected by qPCR and mass spectrometry, respectively (Fujita and Fujii, 2013a). Further, the approach named CRISPR affinity purification *in situ* of regulatory elements (CAPTURE) was improved to achieve better enrichment of a specific locus while reducing the background of proteins bound to Cas9 antibodies. To this end, a FLAG and biotin-tagged dCas9 protein was used, and protein isolation was performed with anti-FLAG antibodies or streptavidin against biotin (Figure 3)(Liu *et al.*, 2017, p. 9). In this project, this method was established and applied, as will be explained in detail later.

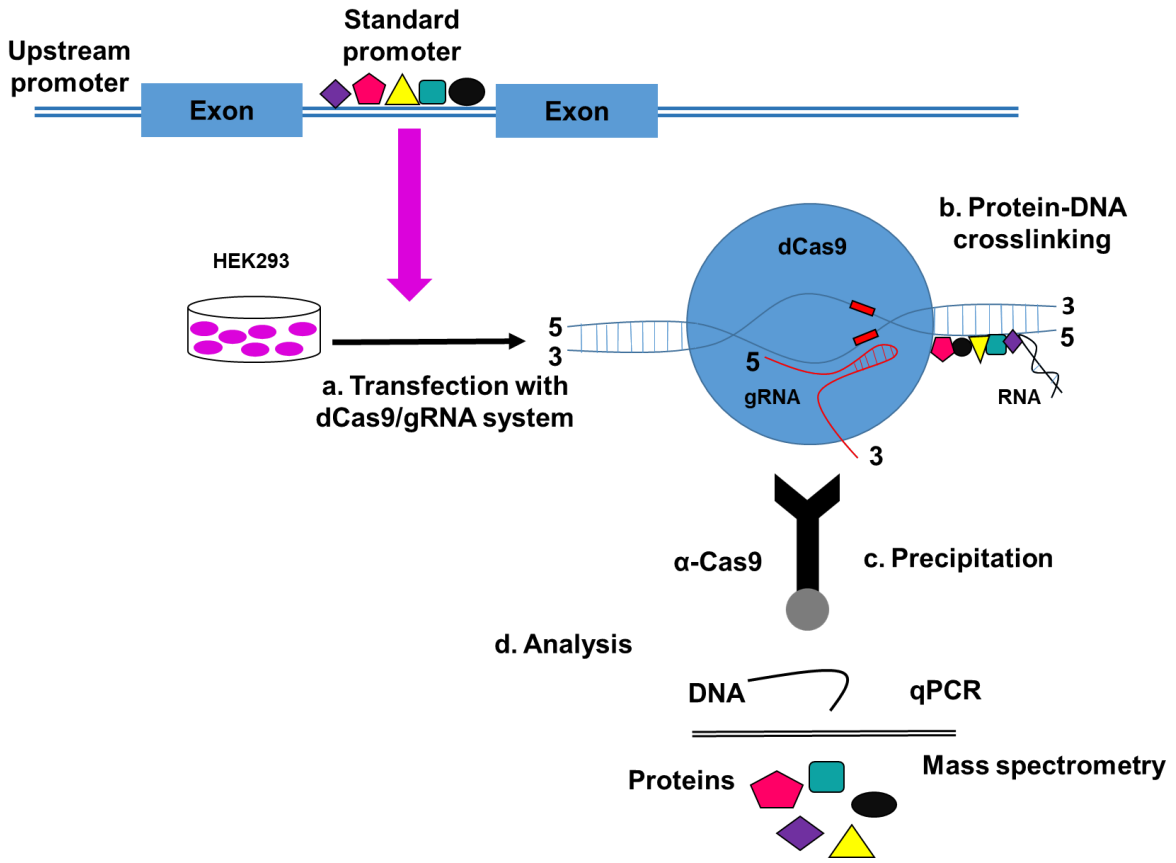


Figure 3: Scheme of enChIP to target a specific genomic locus using the CRISPR/dCas9 system. a. HEK293 cells were transfected with dCas9/gRNA (CRISPR) plasmids to target a specific genomic region, and then **b.** Protein-DNA cross-linking was generated using formaldehyde. Next, **c.** immunoprecipitation of specific genomic regions was performed using either an antibody against Cas9, FLAG, or streptavidin beads recognizing biotinylated

proteins. Finally, **d.** The associated DNA, RNA, and proteins were identified through qPCR and mass spectrometry analysis, respectively.

4. The CRISPR/Cas9-based transcriptional activation system confirmed that the generated gRNAs successfully targeted the MICA promoter region

This experiment aimed to develop gRNA sequences that precisely target the locus of interest, the MICA standard promoter region, to avoid genomic off-target interactions. The CRISPR activation (CRISPRa) system was used for transcriptional activation of the target gene, and then the specificity of the designed gRNA to target the MICA locus was assessed. The CRISPRa system is composed of a catalytically inactivated Cas9 (dCas9) fused with the transcriptional activators VP64, p65, and Rta (VPR) (Figure 4a). Upon binding, the CRISPRa system will induce transcription of the target gene (Casas-Mollano *et al.*, 2020). Initially, 8 individual gRNAs were generated targeting the MICA standard promoter region at different sites upstream of the transcription start site. In addition, a gRNA targeting *GFP* (non-targeting gRNA) and a gRNA targeting the human β-Globin (HBG) gene were used as a negative and positive control, respectively. The gRNA targeting the human β-Globin (HBG) locus has already been validated in a previous study (Liu *et al.*, 2017, p. 9). In HEK293 cells, transient co-transfection of the dCas9-VPR expression plasmid (Figure 4b) was performed with a single gRNA targeting the MICA standard promoter region. Consistent with our expectations, RT-qPCR analysis confirmed significant activation of MICA transcription compared to the non-targeting gRNA (GFP). The gRNA targeting GFP (control) did not achieve transcription as expected. 6 out of the 8 gRNAs, which were designed to target the MICA standard promoter region, lead to a significant transcriptional activation of the MICA gene. Additionally, none of the eight gRNAs targeting the MICA region induced the transcription of HGB nor GFP, which demonstrated the specificity of the gRNAs to target the MICA locus. Then, the five gRNAs with the highest transcriptional activation for the MICA region were selected and combined/mixed for the following experiments (Figure 4c). As expected, the gRNA targeting HBG significantly activated the transcription of its target gene *HBG*, but not MICA or GFP (Figure 4d). In summary, the data indicate that the designed gRNAs specifically target the MICA promoter region.

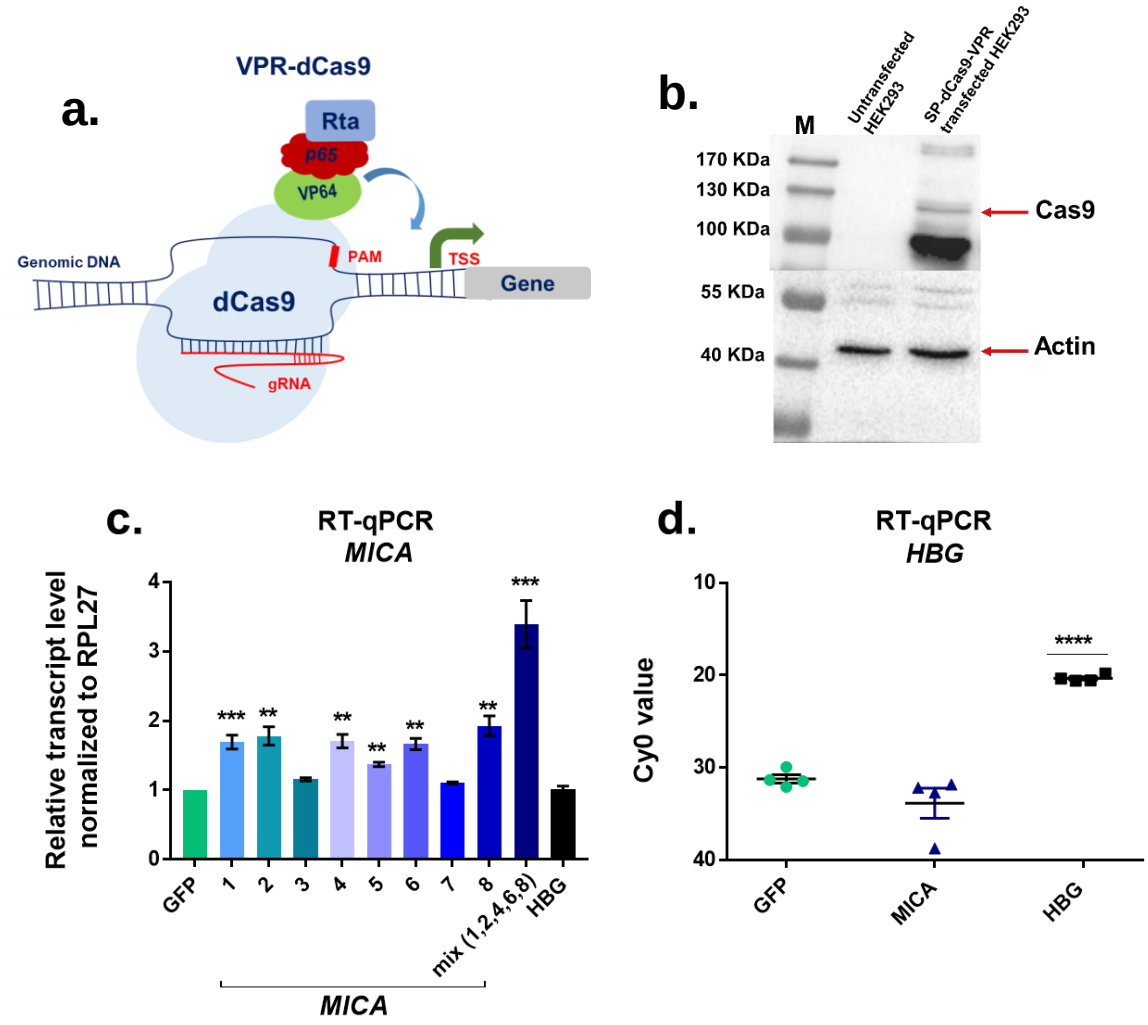


Figure 4: Activation of MICA transcription using the SP-dCas9-VPR/gRNA activation system. **a.** A scheme of the CRISPR activation system. **b.** Western blot showed dCas9 expression in SP-dCas9-VPR transfected HEK293 cells. HEK293 cells were transiently cotransfected with SP-dCas9-VPR and gRNA targeting a specific locus. 48 h post-transfection RT-qPCR analysis was performed to detect the mRNA expression of **c.** the MICA promoter locus and **d.** the HBG promoter locus (a positive control locus). Non-targeting gRNA (*GFP*) was used as a negative control. Data represent the mean (±SEM) of three or four biological experiments. Significance was calculated using an unpaired student's *t*-test. ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001.

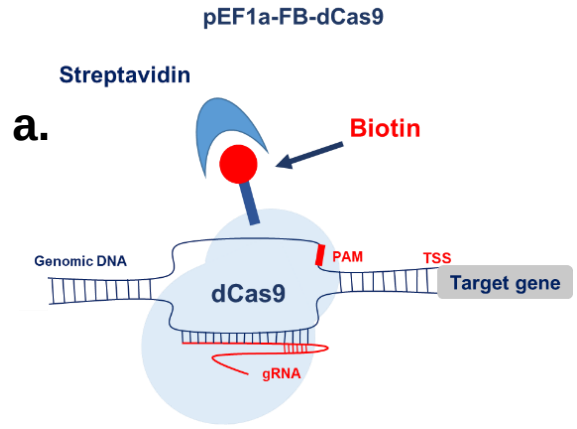
5. Selection of the antibody to isolate the MICA promoter loci: Cas9 antibody showed the highest yield

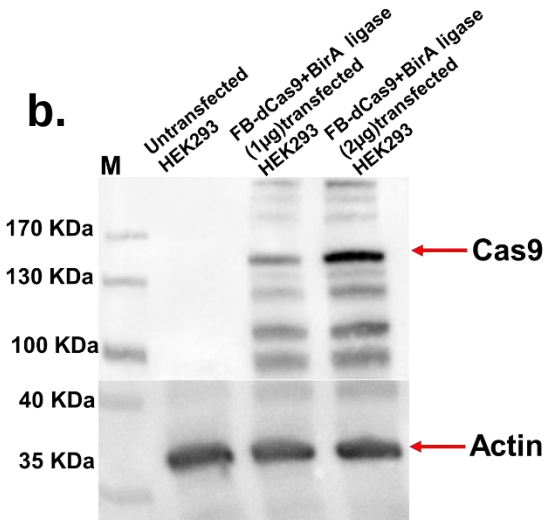
The aim of the experiment was not only to target and purify a single genomic locus, the MICA standard promoter, but also to enrich the target locus most efficiently. Achieving a substantial enrichment of the locus of interest is essential to obtain sufficient co-

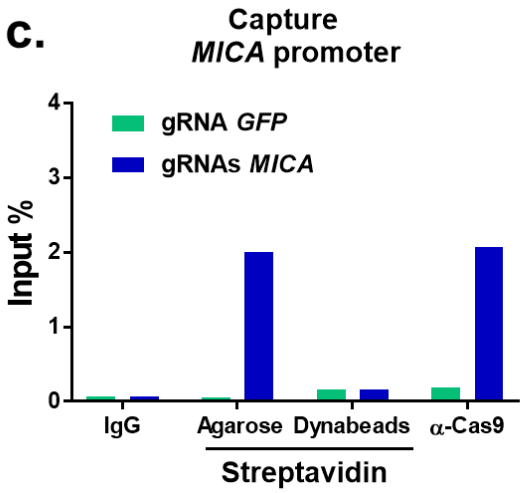
purified proteins for subsequent proteome analysis. Therefore, two systems were applied in this study to set the appropriate protocol for purifying the target locus. The enChIP approach (using Cas9 antibody for precipitating the locus as already described in the paragraph above) and the CRISPR affinity purification *in situ* of regulatory elements (CAPTURE) method. The CAPTURE system has three core components: an N-terminal FLAG fused to a dCas9, a biotin acceptor tandem peptide (FB-dCas9), and an *E.coli*. Biotin holoenzyme synthetase (BirA) and a gRNA targeting a specific locus (Figure 5a)(Liu *et al.*, 2018, p. 9). The biotin acceptor peptide on the expressed FB-dCas9 proteins acts as a substrate for the BirA biotin ligase *in vivo*. Then, the biotinylated dCas9 proteins are directed to the desired locus by a specific gRNA. DNA complexes interacting with dCas9 were precipitated in different ways to select the most efficient approach: either with Cas9 antibodies against dCas9, or by high-affinity streptavidin purification with streptavidin agarose, or streptavidin dynabeads against biotinylated dCas9. Subsequently, the target genomic region of interest was detected by qPCR analysis.

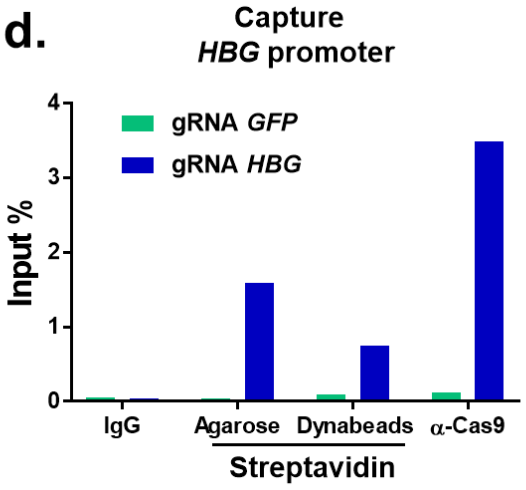
HEK289 cells were transiently co-transfected with a pEF1a-FB-dCas9 expression vector, biotin ligase BirA vector, and gRNAs targeting the gene of interest or a non-targeting gRNA (GFP) as a negative control. Two days later, the expression of biotindCas9 was confirmed by Western blot using HRP-conjugated streptavidin (Figure 5b). Then, CAPTURE followed by qPCR analysis was performed and showed a higher enrichment of the MICA promoter region using Cas9 antibody and streptavidin agarose compared to the locus isolated by streptavidin dynabeads (Figure 5c). No enrichment of the *MICA* locus was detected in the negative control sample. Similar results were obtained with gRNA targeting the *HBG* promoter locus (Figure 5d).

Next, this method was tested in the LBH589-treated HEK293 cells. Cells were cotransfected with the CRISPR/dCas9 system and gRNAs targeting the MICA locus. 48 h post-transfection cells were treated with LBH589 for 4 h and then were subjected to CAPTURE/qPCR analysis. Data showed higher enrichment of the MICA promoter region that was pulled down with Cas9 antibody compared to streptavidin agarose in both LBH589 treated and untreated cells (Figure 5e). Thus, the Cas9 antibody was used to isolate the MICA promoter region for further assays, which will be mentioned as an enChIP approach in the following section.


a.
pEF1a-FB-dCas9
Streptavidin
Biotin
Genomic DNA
PAM
TSS
Target gene
dCas9
gRNA


b.
M
Untransfected HEK293
FB-dCas9+BirA ligase (1μg)transfected HEK293
FB-dCas9+BirA ligase (2μg)transfected HEK293
170 KDa
130 KDa
100 KDa
40 KDa
35 KDa
Cas9
Actin


c.
Capture
MICA promoter
gRNA GFP
gRNAs MICA
Input %
4
3
2
1
0
IgG
Agarose
Dynabeads
α-Cas9
Streptavidin


d.
Capture
HBG promoter
gRNA GFP
gRNA HBG
Input %
4
3
2
1
0
IgG
Agarose
Dynabeads
α-Cas9
Streptavidin

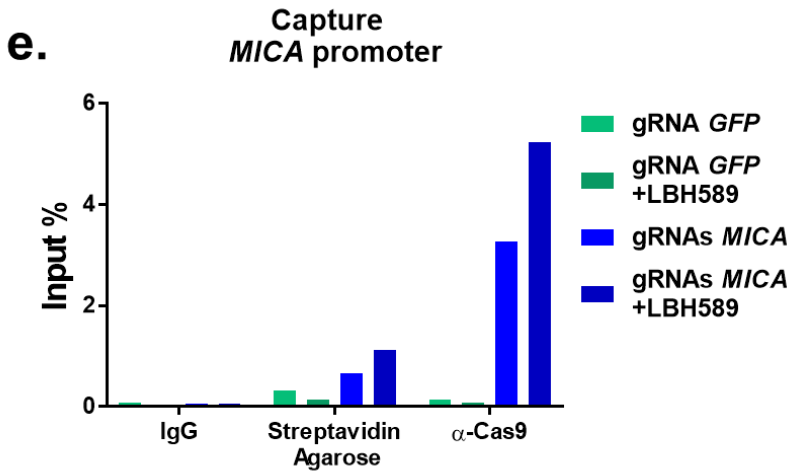


Figure 5: The isolation of the MICA promoter locus was most efficient using Cas9 antibody. a. A scheme illustrating the components of the CAPTURE system. HEK293 cells were co-transfected with pEF1a-FB-dCas9 expression vector, a biotin ligase BirA expression vector, and either a mix of gRNAs to target MICA promoter locus or HBG targeting sgRNA as a control or sgRNA targeting GFP as a negative control. 48 h post-transfection, part of the cells was subjected to **b.** Western blotting to validate the expression of the biotinylated dCas9. Then, the CAPTURE method was performed using the rest of the cells to isolate the locus of interest by utilizing several ways for pulling down the locus of interest: Streptavidin Agarose, Streptavidin dynabeads, and dCas9 antibody and IgG (as a negative control) were used. The precipitated genomic DNA was subjected to a qPCR analysis to check the enrichment of **c.** the MICA promoter region and **d.** the HBG promoter region. **e.** HEK293 cells were cotransfected with the previous CRISPR/dCas9 and gRNAs system, 48 h post-transfection cells were treated with 100 nM LBH589 for 4 h, and then cells were subjected to CAPTURE followed by qPCR to isolate MICA promoter locus.

6. Detection of the proteins associated with the MICA standard promoter region: Mass spectrometry analysis identified many of the proteins isolated by enChIP method

After several steps for method establishment and validation, the enChIP approach followed by mass spectrometry was applied to identify the regulators associated with the MICA locus. Achieving a sufficient amount of single gene copies for mass spectrometry analysis is challenging. Therefore, the experiment was performed with a large number of HEK293 cells. In total, 10 15 cm dishes per condition were used for transfection. The validated gRNAs targeting the MICA standard promoter region or the non-targeting gRNA (GFP) were co-transfected with dCas9 expression vector for 48 h. Then, cells were treated with LBH589 for 4 h. Afterward, HEK293 chromatin was cross-

linked, sonicated, and purified either with Cas9 antibody or with IgG (as a control). An aliquot of each sample was used for DNA purification. As expected, qPCR analysis of the purified chromatin showed a high and specific enrichment of the MICA target region, whereas no signal was detected in the control samples (Figure 6a). In addition, the control region PDK4 did not show any enrichment in both targeting and non-targeting control samples, as well as in LBH589-treated and untreated cells (Figure 6b). Hence, a significant and specific enrichment of the MICA single locus was achieved.

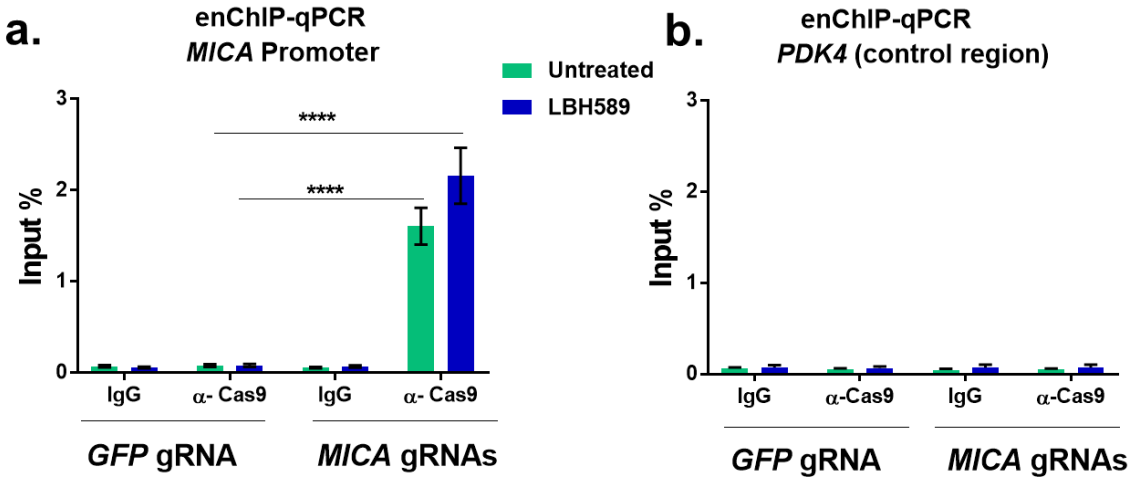


Figure 6: enChIP using pEF1a-FB-dCas9/gRNA system to isolate the MICA promoter genomic region. HEK293 cells were transiently co-transfected with pEF1a-FB-dCas9 expression vector and a mix of (1, 2, 4, 6, 8) gRNA vectors to target the MICA promoter locus or the non-targeting gRNA vector (GFP). 48 h post-transfection cells were treated with 100 nM LBH589 for 4 h and then cells were subjected to the enChIP method. The chromatin fragments were precipitated using Cas9 antibody. Next, qPCR analysis was performed to check the enrichment of the **a.** MICA locus and **b.** PDK4 locus (control region). Data represent four or more biological replicates ± SEM. Statistical significance was calculated using an unpaired student's *t*-test. ****$p < 0.0001$.

In parallel to qPCR analysis, the purified DNA/protein complexes (samples) were sent for mass spectrometry analysis to detect MICA-associated proteins. Mass spectrometry analysis was performed in three independent replicates, using off-bead in-gel digestion and high pH reversed-phase separation, followed by LC-MS2 analysis utilizing label-free quantitation (cooperation with Prof. Johannes Graumann MPI Marburg). About 1500 proteins were identified, including histones, SWI/SNF components, RNA polymerases, metabolic enzymes, heat shock proteins, and some

transcription factors. As expected, the enChIP/MS method revealed a strong variation between samples (data not shown), and the challenge was to filter out the proteins that were functionally related to MICA regulation. Therefore, we focused on proteins enriched in MICA samples or generally involved in gene regulation and known to be targets for CBP/p300 and also interact with each other as depicted (Figure 7a and b).

There are published ChIP-seq data (ENCODE database) for some of the factors identified via enChIP/mass spectrometry, demonstrating their binding to the MICA standard promoter region near the CPG island (Figure 7c). Additionally, using JASPAR, the open-access transcription factors database, we could predict binding sites for some of the transcription factors. Of note, among these transcription factors, KLF4 and YinYang (YY1) have binding motifs on the MICA standard promoter within the region for which the gRNAs were generated (Figure 7d). Myeloid leukemia factor 2 (MLF2) is also detected, but its binding motif is yet unknown.

Based on enChIP/MS results and our previous data, we aimed to analyze the role of KLF4 in more detail. It has been reported that both KLF4 and YY1 recruit HAT or HDAC cofactors to activate or repressor transcription (Evans *et al.*, 2007)(Yao, Yang and Seto, 2001). Furthermore, they can be acetylated by CBP and p300, which regulate their transcriptional activity (Evans *et al.*, 2007)(Yao, Yang and Seto, 2001). They also can bind directly to CREB-binding protein, which is known to be involved in MICA regulation (Zhou, Gedrich and Engel, 1995)(Stavri *et al.*, 2015). The association of these factors with the SWI/SNF complex has been described predominantly in the context of their role in embryonic stem cells (Singhal *et al.*, 2010)(Wang *et al.*, 2018). Overall, this suggested a possible network for the regulatory mechanisms of MICA that needs to be confirmed by further investigation.

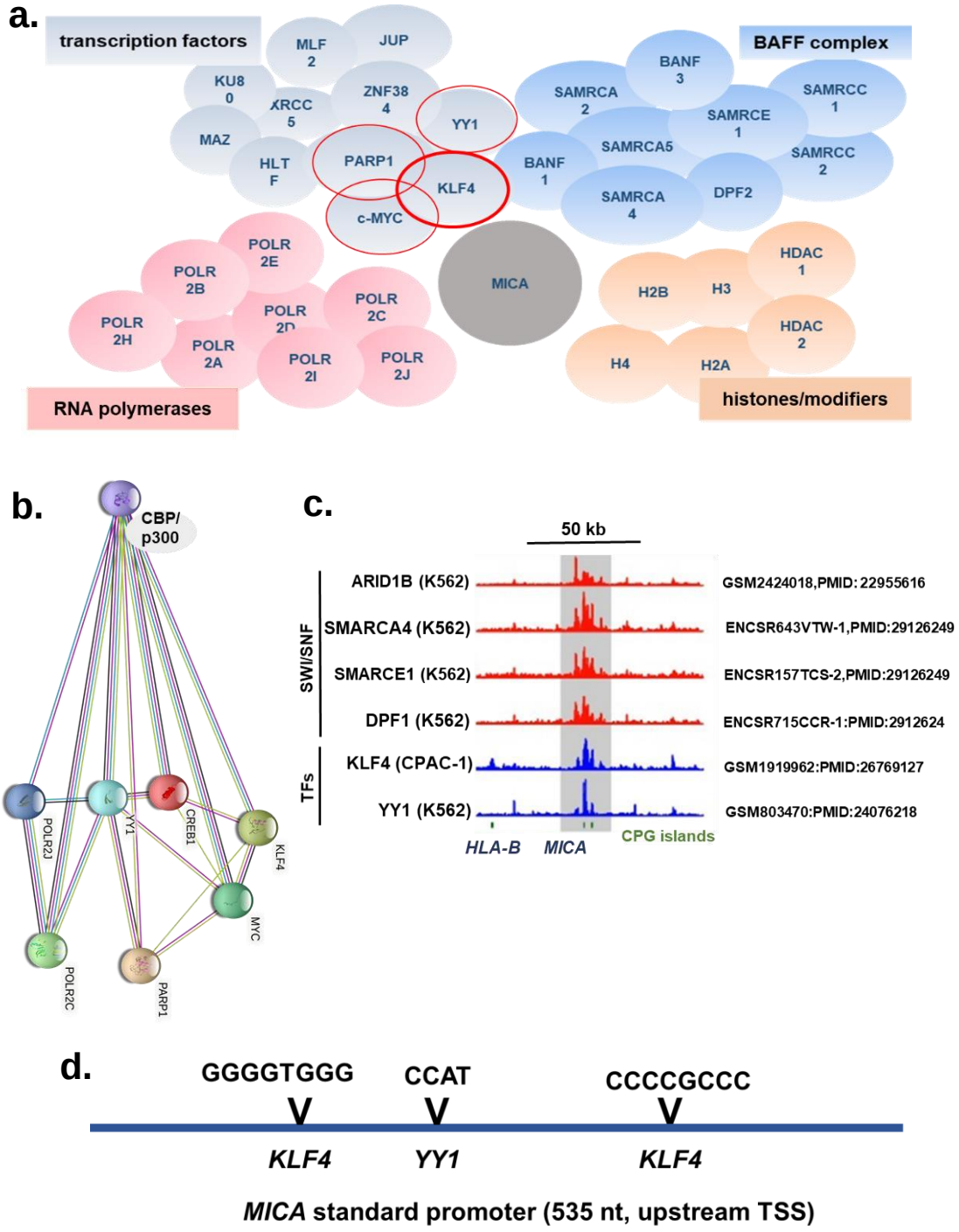


Figure 7: Identification of putative regulators associated with the MICA standard promoter region by enChIP/MS. Transient co-transfection of HEK293 cells with pEF1a-FBdCas9 expression vector and mix (1, 2, 4, 6, 8) gRNAs to target MICA promoter locus or nontargeting gRNA (GFP) as a control. Transfected cells were subjected to enChIP followed by mass spectrometry (MS). **a.** A figure representing the proteins detected by MS analysis. **b.** Using the STRING database (https://string-db.org), the protein-protein interaction network for candidate transcription factors was generated. Interactions are shown as experimentally

determined (pink), from curated databases (blue), predicted from gene neighborhood (green), gene co-occurrence (dark blue), or protein homology (purple). **c.** ChIP-seq analysis of enChIP/MS candidates binding sites in MICA standard promoter region. **d.** A schematic drawing of the MICA standard promoter region illustrates the predicted binding sites of transcription factors. Three biological replicates were used for enChIP/MS analysis. TSS = Transcription start site.

7. The transcription factors KLF4 and YY1 may be involved in regulating MICA expression

By bioinformatics analysis using JASPAR as well as published data, we identified two possible binding sites for KLF4 (Figure 8a) and two for YY1 upstream of the transcription start site (TSS) in the human MICA standard promoter (Xu *et al.*, 2021)(Yao, Yang and Seto, 2001). The binding motif of MLF2 is still elusive. To test whether these candidates are able to functionally regulate the expression of MICA, a luciferase reporter gene activity assay was performed. 583 nucleotides upstream of the TSS in the MICA standard promoter region were cloned into the multiple cloning sites of the pGL3 basic Vector (reporter plasmid). Then, HEK293 cells were cotransfected with the reporter plasmid either with KLF4, YY1, or MLF2 expression vector, and a GFP expression vector was used as a negative control. 48 h post transfection cells were subjected to luciferase reporter gene activity assay and luciferase activity was measured. Intriguingly, cells transfected with KLF4 and YY1 vectors independently or in a combination markedly induced the luciferase activity compared with MLF2 and GFP expression vectors (Figure 8b). An unrelated promoter sequence was used as a control (pGL3-YAP1, 443 nucleotides upstream of the transcription start site). The KLF4-induced activation was also detectable but significantly lower compared with the pGL3-MICA, suggesting that MICA-dependent luciferase activity was specifically activated by KLF4. In conclusion, KLF4 could functionally activate the MICA promoter.

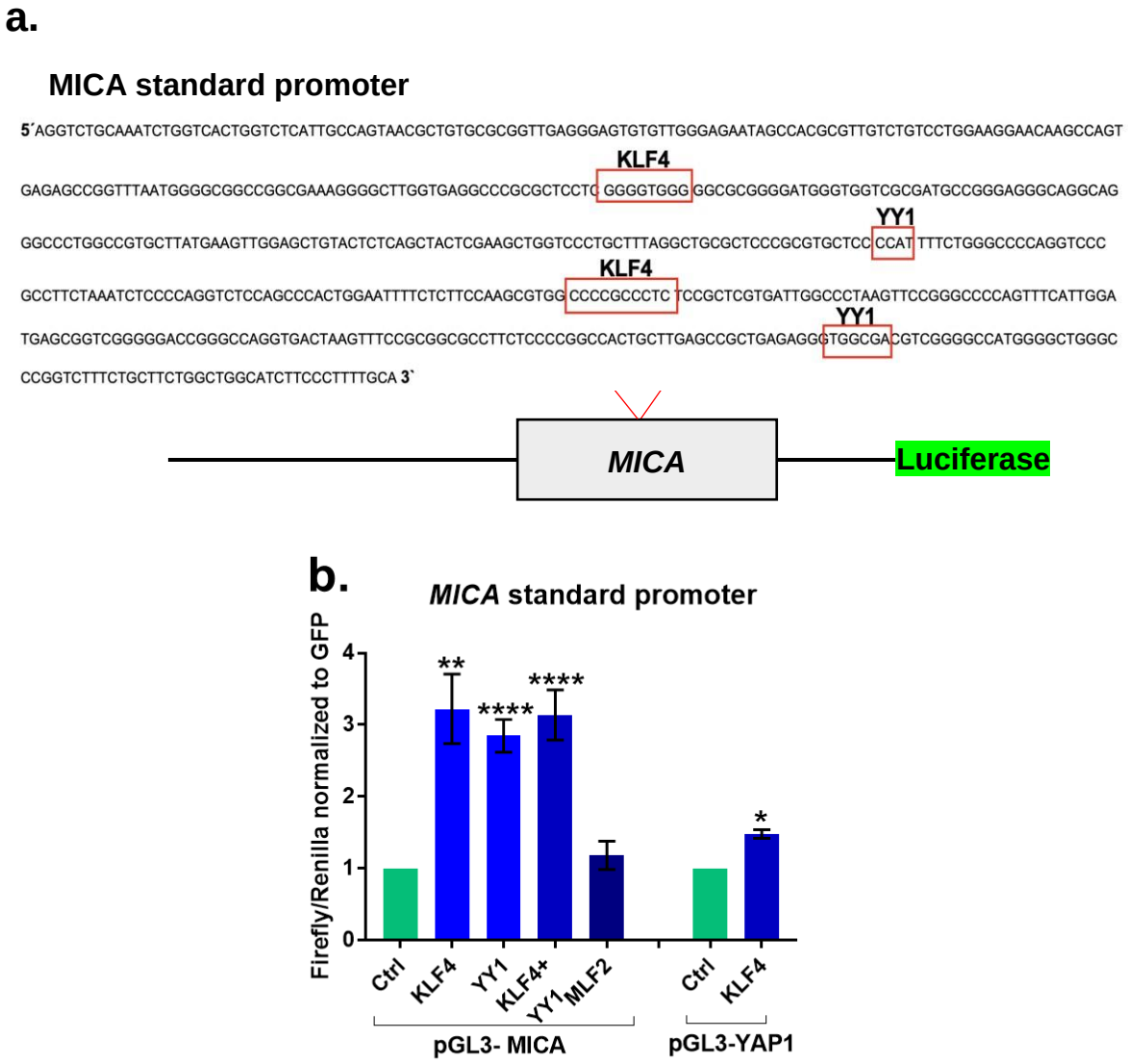


Figure 8: KLF4 and YY1 regulated and induced the expression of the MICA standard promoter. a. The target region of the MICA standard promoter contains binding motifs for transcription factors KLF4 and YY1. The target region of MICA was cloned into pGL3-Basic vector (reporter vector) **b.** HEK293 cells were transiently co-transfected with the pGL3-MICA vector along with either a KLF4 or YY1 or MLF2 expression vector and a GFP expression vector as a negative control. Additionally, another related sequence (pGL3-YAP1 vector) was also used as a control and co-transfected with the KLF4 expression vector into the HEK293 cells. 48 h post-transfection cells were harvested and the luciferase reporter assay was performed. To correct the differences in transfection efficiencies, the renilla luciferase vector was included in each transfection, and firefly-dependent luciferase activity was normalized to renilla signals. Data are shown as the mean (±SEM) of three independent experiments. Significance was calculated using an unpaired *t*-test. $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

In collaboration with Prof. Dr. Lars, French, the University of Munich, we have obtained a FLAG-tagged wild-type KLF4 vector (WT-KLF4) and a mutant vector lacking the

DNA-binding domain (DN-KLF4). It is known that the DN-KLF4 acts as a dominantnegative KLF4 mutant. Doxycycline is used to induce KLF4 expression in both vectors (Satoh *et al.*, 2020). HEK293 cells were transiently co-transfected with the reporter plasmid containing the target region of the MICA promoter (pGL3-*MICA* basic) and either the DN-KLF4 or WT-KLF4 vector. As a positive control, the FLAG- KLF4 expression vector, and as a negative control, the cDNA 3.1 expression vector (Ctrl) were used. 24 h post-transfection, only cells transfected with WT-KLF4 and DN-KLF4 were treated with 1 µg/mL doxycycline for an additional 24 h. Then, KLF4 expression was detected by Western blot. As expected, KLF4 expression was induced only in the cells transfected with WT-KLF4 vector and the positive control (Figure 9a). Consistent with the transfected cells, a reporter gene assay was performed to check if KLF4 was functionally able to regulate the MICA expression. The obtained data demonstrated that the luciferase activity was induced only in the samples expressing KLF4 compared to the controls (Figure 9b), indicating that KLF4 is a putative inducer of MICA expression.

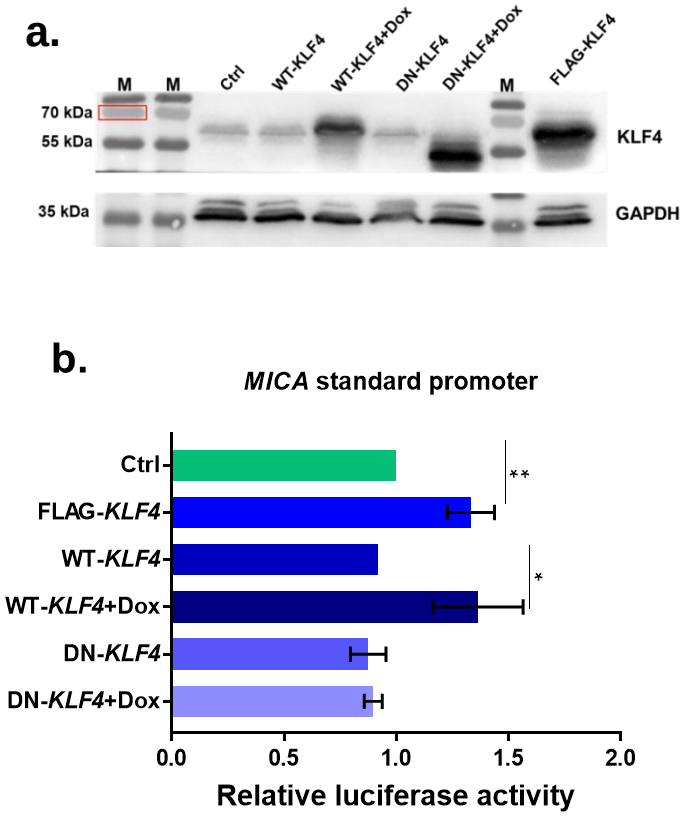


Figure 9: KLF4 induced and regulated the expression of the MICA standard promoter region. HEK293 cells were transiently co-transfected with the indicated reporter construct along with either a FLAG-KLF4 (KLF4) expression vector or cDNA3.1 expression vector as a

negative control or using a Tet-on system: WT-KLF4 or its negative control DN-KLF4 that overexpress the KLF4 after treatment with doxycycline (Dox) for 24 h. Cells were harvested 48 h post-transfection. Part of the cells was used for **a.** Western blot to detect KLF4 expression. **b.** The rest of the cells were introduced to luciferase reporter assay. To correct the differences in transfection efficiencies, the renilla luciferase vector was included in each transfection, and firefly-dependent luciferase activity was normalized to renilla signals. Data are shown as the mean (±SEM) of three independent experiments. Significance was calculated using an unpaired *t*-test. **p* < 0.05, ***p* < 0.01.

8. Reduced KLF4 expression diminished LBH589-mediated MICA upregulation

The next series of experiments were performed to unravel whether KLF4 has a role in regulating MICA expression. KLF4 has distinct functional and structural domains for binding to promoters and enhancers (Park *et al.*, 2016). Furthermore, it can be acetylated by the coactivators CBP/p300, resulting in transcriptional activation (Evans *et al.*, 2007). KLF4 is essential for the cellular response to DNA damage, which also induces the expression of NKG2D-Ls (Yoon, Chen and Yang, 2003)(Gasser *et al.*, 2005). To investigate whether reducing the expression of KLF4 could affect the MICA expression, a small molecule (kenpaullone) was applied. Kenpaullone is an inhibitor with different targets, including KLF4 (Yu *et al.*, 2011). Therefore, HEK293 cells were treated with kenpaullone (5 µM and 10 µM) for 6 h. The expression of KLF4 with both concentrations was significantly inhibited (Figure 10a). Interestingly, kenpaullone also significantly decreased the induction of MICA expression under treatment with LBH589 (Figure 10b). Similar results were observed when KLF4 was knocked down using small interfering RNA (siRNA). The downregulation of KLF4 expression was associated with a significant reduction in MICA expression after treatment with LBH589 (Figures 10c and d). These results suggested that KLF4 is involved in regulating MICA expression upon treatment with LBH589. Therefore, HEK293 cells were treated with kenpaullone (5 µM and 10 µM) for 6 h. The expression of KLF4 with both concentrations was significantly inhibited (Figure 10a). Interestingly, kenpaullone also significantly decreased the induction of MICA expression under treatment with LBH589 (Figure 10b). Similar results were observed when KLF4 was knocked down using small interfering RNA (siRNA). The downregulation of KLF4 expression was associated with a significant reduction in MICA expression after treatment with LBH589 (Figures 10c and d). These results suggested that KLF4 is involved in regulating MICA expression upon treatment with LBH589.

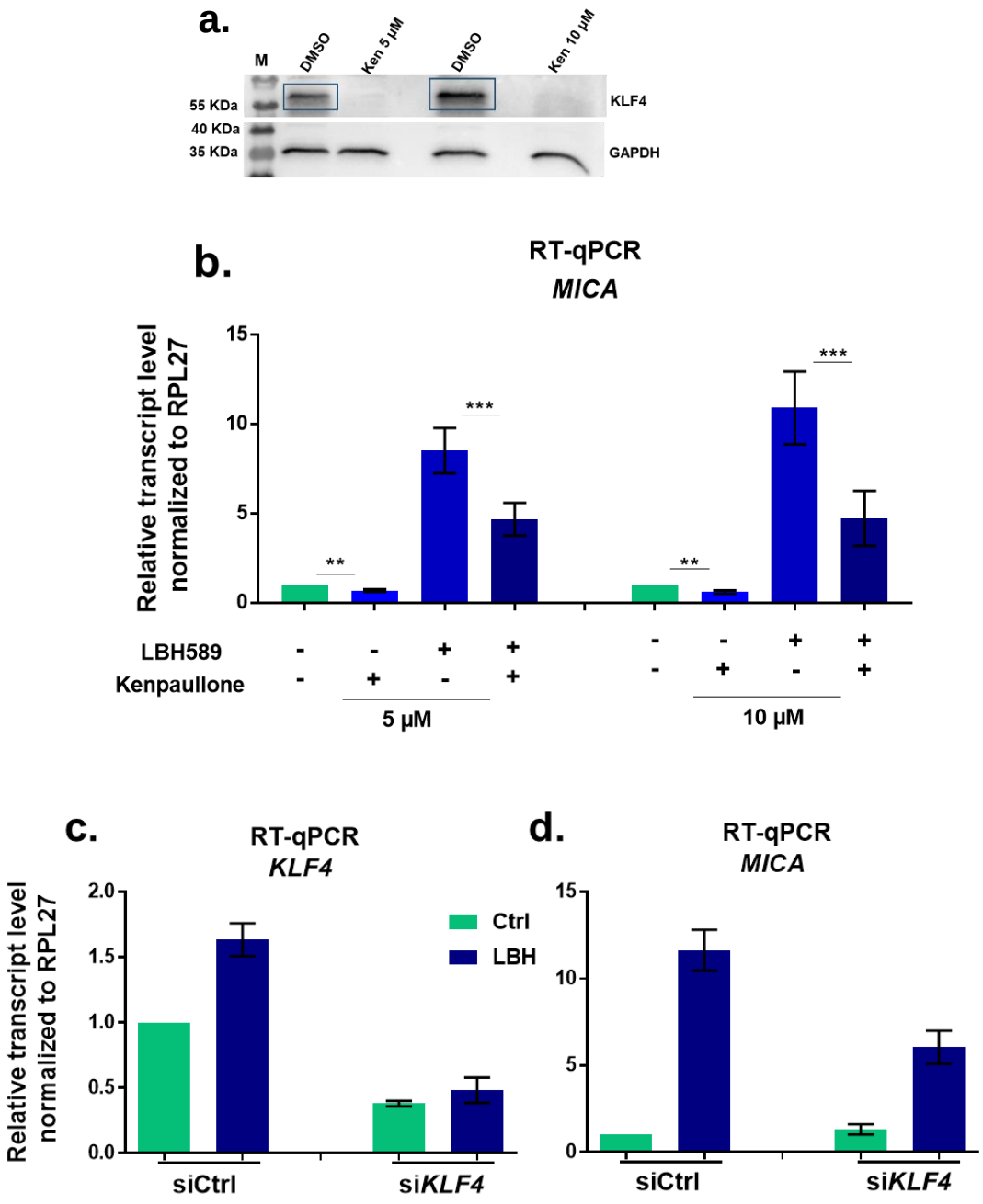


Figure 10: Modulation of KLF4 expression, significantly affected the induction of the MICA gene in response to LBH589. HEK293 cells were seeded in 6-well plates and treated with kenpaullone (5 µM and 10 µM) for 6 h followed by 100 nM LBH589 for 4 h. Then, total RNA and proteins were extracted. Protein lysates were subjected to **a.** Western blot analysis to check the inhibition of KLF4 protein expression. And the total RNAs were subjected to **b.** RT-qPCR analysis to check the expression of the MICA gene. **c.** and **d.** HEK293 cells were treated with siRNA (control or KLF4) for 24 h followed by treatment with 100 nM LBH589 for 4 h. RT-qPCR analysis was performed for the mRNA expression of KLF4 and MICA. (The experiments represented in figures **c.** and **d.** were performed and analyzed by Dr. Viviane

Ponath). Data represent the mean (±SEM) of two or three biological experiments. Significance was calculated using an unpaired *t*-test. ** $p < 0.01$, *** $p < 0.001$.

9. The expression of KLF4 and MICA varied between Ara-C sensitive and resistant AML cell lines

To date, the treatment of leukemia patients still depends mainly on chemotherapeutic drugs such as Ara-C. However, this treatment cannot eradicate leukemia stem cells (LSCs), which promote drug resistance in AML (Wu *et al.*, 2021). Several studies have demonstrated that AML cells especially LSCs lack the expression of NKG2D-Ls, including MICA, which impair the recognition and killing by NK cells (Paczulla *et al.*, 2019)(Salih *et al.*, 2003). The question arose whether the expression of MICA associated with KLF4 differs in response to chemotherapy. Therefore, two HL60 cell lines, one sensitive and another resistant to Ara-C, were used. The Ara-C-resistant cell line was generated by exposing the parental cells to a constant concentration of Ara-C. (The cells were established and checked by the group of Prof. Dr. Andreas Neubauer). First, the expression of MICA and KLF4 in response to 24 h Ara-C treatment with two non-toxic concentrations (100 and 500 nM) was examined in both cell lines. Interestingly, the expression of MICA was significantly increased in the AraC-sensitive HL60 cells. At the same time, it was not detected in the resistant cells at both concentrations compared to the untreated cells used as a control (Figure11a). Similarly, KLF4 expression was induced only in the Ara-C sensitive HL60 cells (Figure 11b). These data showed a possible correlation between MICA and KLF4 expression in response to Ara-C treatment.

As previously reported, the induction of MICA in response to the LBH589 was independent of the DNA damage response (DDR) pathway. ATR/ATM inhibitors hinder the expression of MICA upon Ara-C treatment, whereas they only partially block the induction of LBH598-mediated MICA expression (Sauer *et al.*, 2017). Therefore, I decided to answer whether LBH589 can enhance the expression of both MICA and KLF4 in the Ara-C resistant cell line. Interestingly, the treatment with the LBH589 as a single agent or in combination with Ara-C significantly increased the expression of MICA associated with KLF4 in the Ara-C sensitive cell line (Figures 11c and d). Whereas in the Ara-C resistant cell line, LBH589 slightly induced the expression of both genes and the combination did not enhance the induction (Figure 11d). In conclusion,

LBH589 played an influential role in inducing the expression of MICA and KLF4 in Ara-C-sensitive and resistant cell lines.

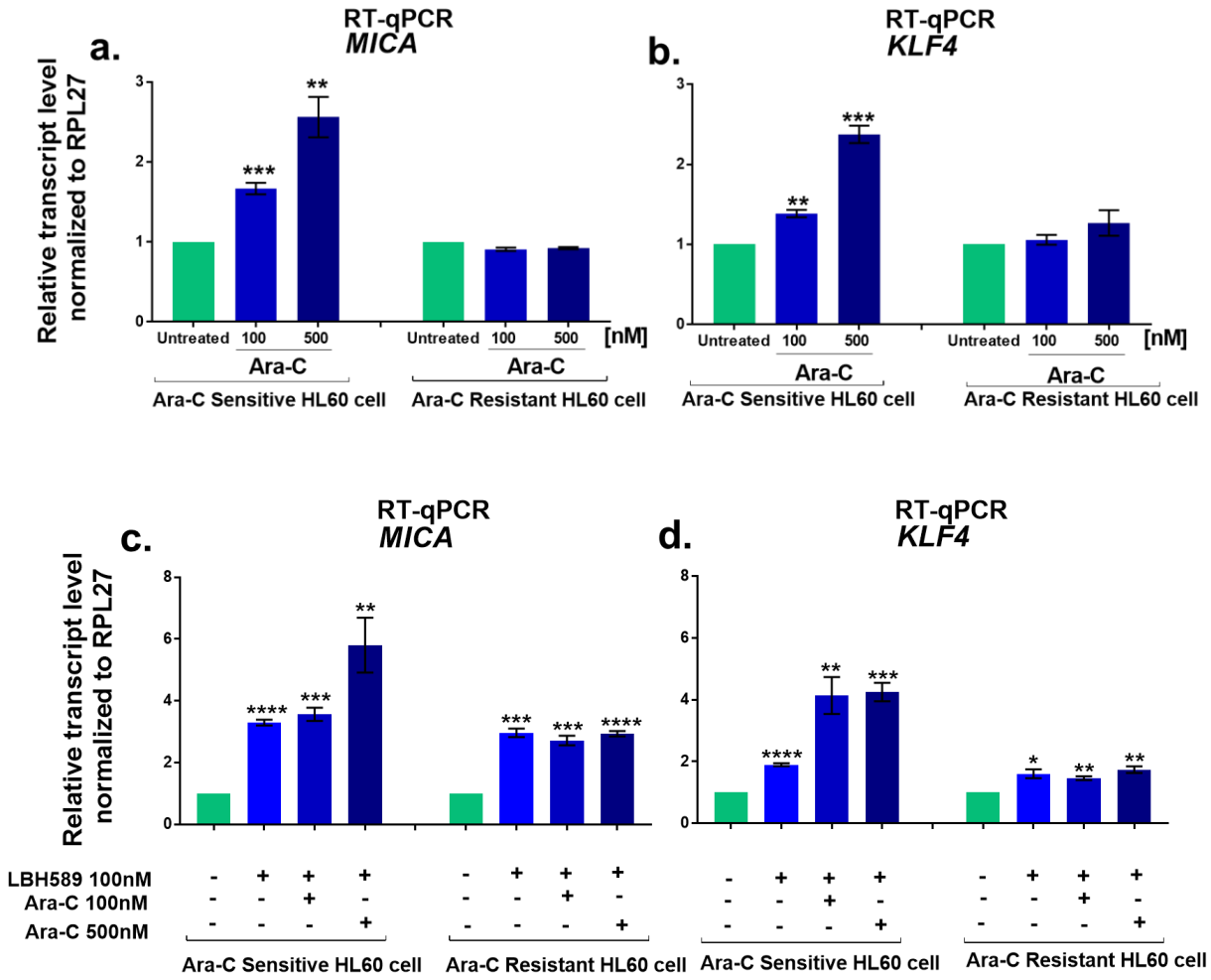


Figure 11: The expression of KLF4 along with MICA differed between Ara-C- resistant and sensitive AML cell lines. Ara-C sensitive and resistant HL60 were treated with 100 and 500 nM Ara-C for 24 h. Next, cells were subjected to RT-qPCR analysis to check the expression of **a.** KLF4 and **b.** MICA on mRNA levels. The same cell lines were treated with 100 and 500 nM Ara-C for 24 h, followed by 100 nM LBH589 for 4 h. RT-qPCR analysis was performed, and the expression level of **c.** KLF4 and **d.** MICA was detected. Data represent the mean (±SEM) of three biological replicates. Significance was calculated with an unpaired *t*test. **p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001.

10. LBH589 effectively induced the expression of MICA and KLF4 in a panel of AML cell lines

Human leukemia is heterogeneous and has genetic and epigenetic causes (Gambacorta *et al.*, 2019)(Li, Mason and Melnick, 2016). Moreover, the Cancer Genome Atlas has provided that the KLF4 expression is not uniform among AML patients (Morris *et al.*, 2016)(Lewis *et al.*, 2021). Therefore, to define whether the observed correlation between MICA and KLF4-induced expression is not restricted to the HL60 AML cell line (Figure12a upper panel MICA left and KLF4 right). Two other AML cell lines, NB4 and MonoMac6, were tested. The AML cell lines were treated with LBH589 at a final concentration of 100 nM for 4 h and then subjected to RT-qPCR analysis. Interestingly, LBH589 induces the expression of both MICA and KLF4 in the HL60 cell line (Figure12a upper panel MICA left and KLF4 right) and the NB4 cell line (Figure12b middle panel MICA left and KLF4 right). The same results were also observed in the MonoMac6 cell line (Figure12a lower left panel MICA and right one KLF4). In conclusion, the obtained results highlight the role of LBH589 in the induction of MICA and KLF4 expression in different AML cell lines.

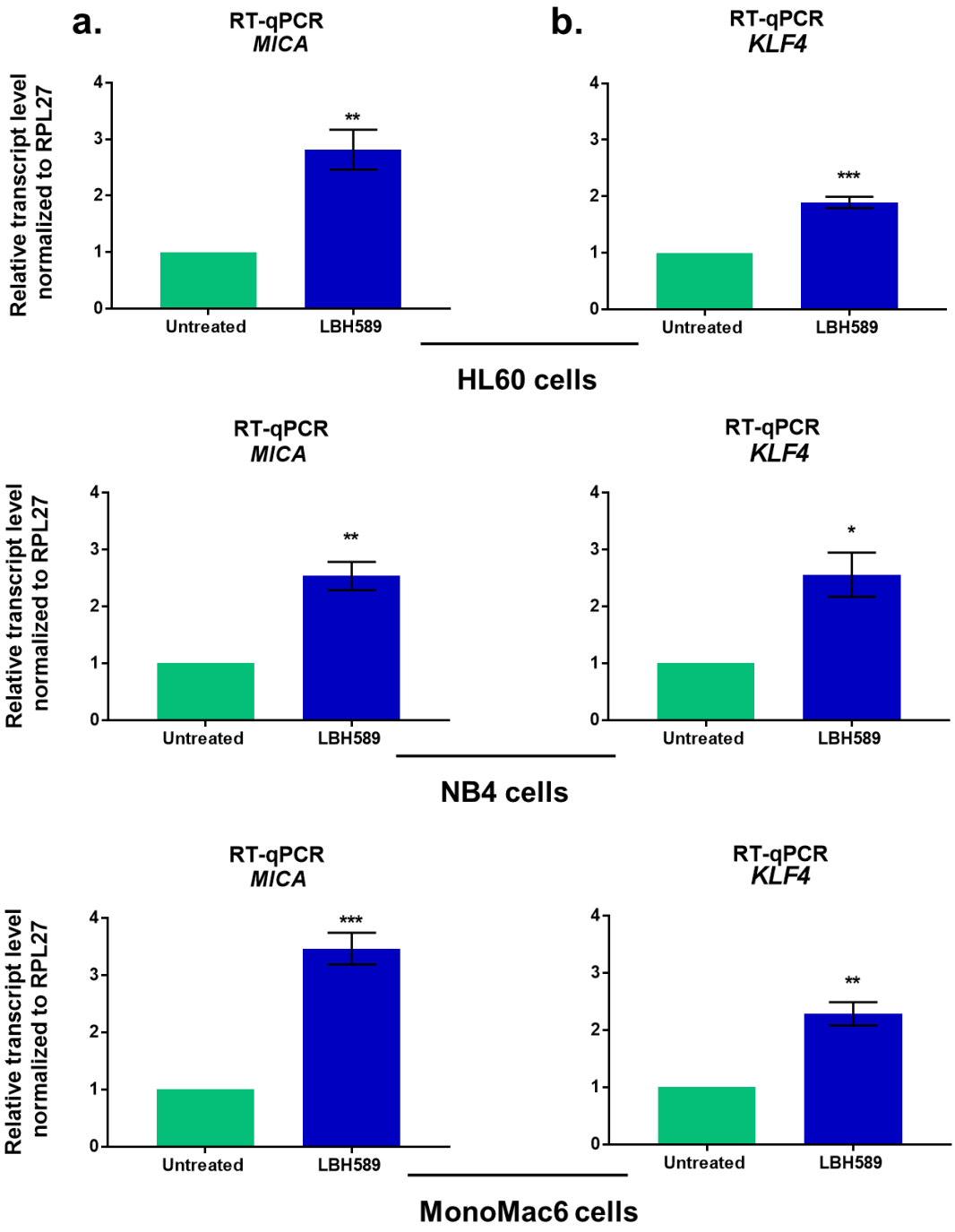


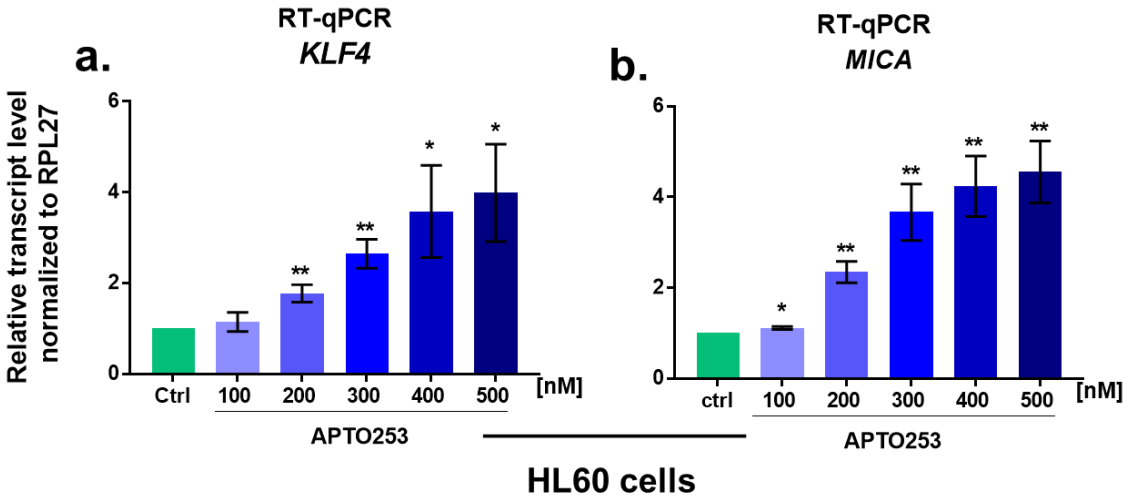
Figure 12: LBH589 induced MICA in line with KLF4 in different AML cell lines

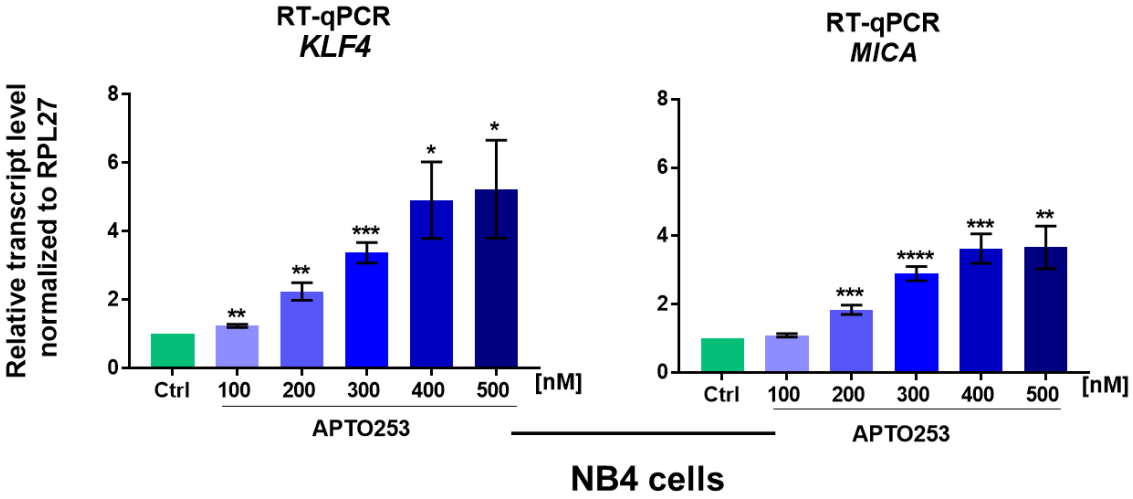
HL60, NB4, and MonoMac6 AML cell lines were treated with 100 nM LBH for 4 h. Next, total RNA was extracted, and RT-qPCR was performed to check the mRNA expression levels of **a.** MICA and **b.** KLF4, respectively. Data represent the mean (±SEM) of three or four independent experiments, and significance was calculated using an unpaired *t*-test. **p* < 0.05, ***p* < 0.01, ****p* < 0.001.

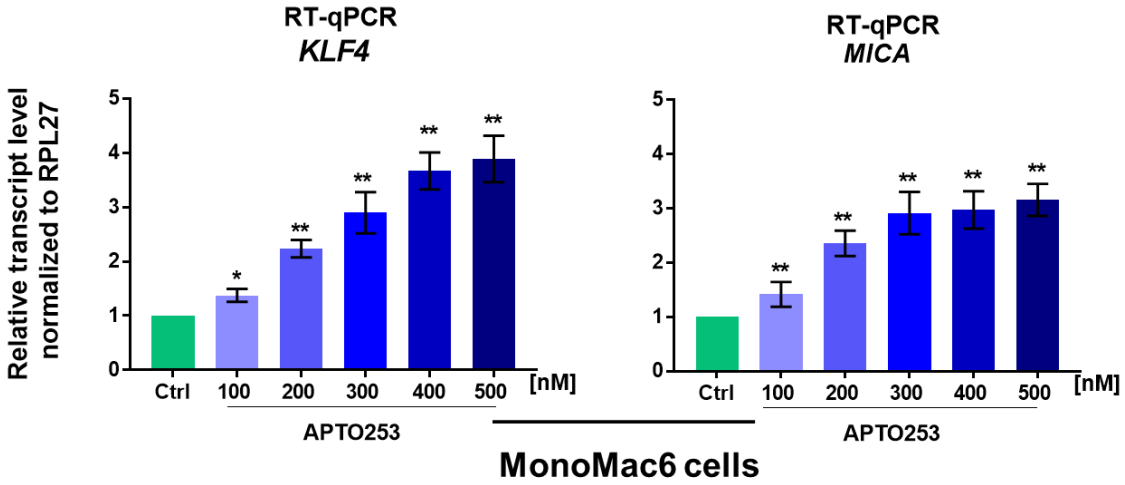
11. The KLF4 inducer APTO253 stimulated the expression of MICA in different AML cell lines

Finally, in order to investigate the functional effect of KLF4 on MICA expression APTO253 was used to induce KLF4 expression. APTO253, a small molecule, has been clinically developed for treating hematologic malignancies, including leukemia. It shows a potent anti-tumor activity through the induction of the tumor suppressor KLF4, as KLF4 expression is often silenced in several hematologic cancers. Furthermore, APTO253 inhibited tumor growth in AML xenograft models (Rice *et al.*, 2014)(Rice *et al.*, 2015). Therefore, HL60, NB4, and MonoMac6 AML cell lines were treated with APTO253 for 18 h at final concentrations of 100, 200, 300, 400, and 500 nM. Later, RT-qPCR analysis was performed to quantify the induction of KLF4 and MICA. Surprisingly, inducing the expression of KLF4 was associated with a marked upregulation of MICA expression in all AML cell lines. Additionally, the induction of both genes was dose-dependent and started mainly from 200 nM, as shown (Figures 13a left panel KLF4 and 13b right panel MICA).

Furthermore, to detect the expression of MICA on protein level under APTO253 treatment, HL60 cells were treated with APTO253 for 18 h at concentrations of 150 nM and 200 nM. Then, cells were subjected to flow cytometry analysis. For technical issues, we used the MICA/B antibody. Interestingly and for the first time, data showed that APTO253, the KLF4 inducer, significantly upregulates MICA/MICB expression on mRNA levels and protein levels (Figure 13c and supplementary figure 3a MICB on mRNA level), linking APTO253 to the innate immune response.


a.
RT-qPCR
KLF4
Relative transcript level normalized to RPL27
Ctrl
100
200
300
400
500
[nM]
APTO253
b.
RT-qPCR
MICA
ctrl
100
200
300
400
500
[nM]
APTO253
HL60 cells


RT-qPCR
KLF4
Relative transcript level normalized to RPL27
Ctrl
100
200
300
400
500
[nM]
APTO253
RT-qPCR
MICA
Ctrl
100
200
300
400
500
[nM]
APTO253
NB4 cells


RT-qPCR
KLF4
Relative transcript level normalized to RPL27
Ctrl
100
200
300
400
500
[nM]
APTO253
RT-qPCR
MICA
Ctrl
100
200
300
400
500
[nM]
APTO253
MonoMac6 cells

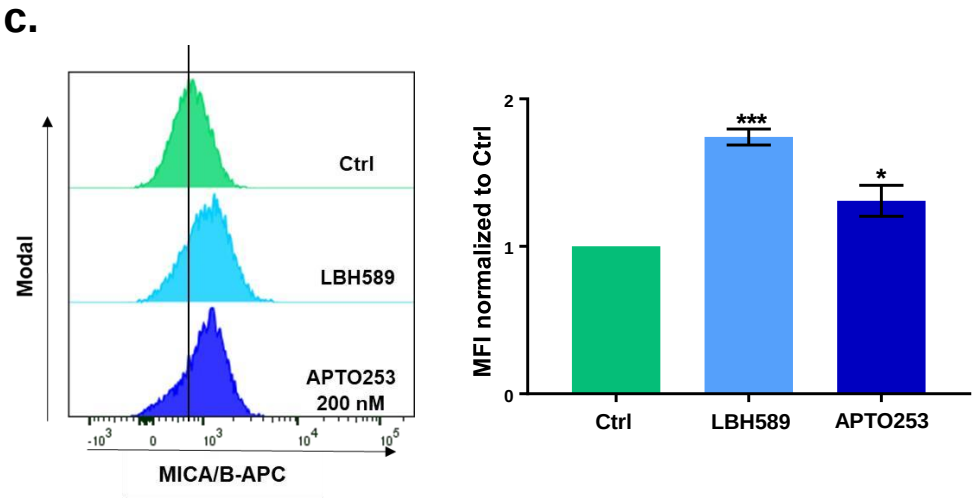


Figure 13: Overexpression of KLF4 via APTO253 induced the expression of MICA in AML cell lines. HL60, NB4, and MonoMac6 cell lines were incubated with APTO253 at different concentrations of 100, 200, 300, 400, and 500 nM for 18 h. Then cells were subjected to RT-qPCR analysis to detect the mRNA level of a. KLF4 and b. MICA. c. HL60 cells were treated with LBH589 100 nM or APTO253 200 nM for 18 h. Flow cytometry analyses were performed for MICA/B surface expression. Data are shown as the mean (±SEM) of three independent experiments. Significance was calculated using an unpaired *t*-test. **p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001.

Discussion

1. enChIP is a powerful method for the identification of gene regulatory factors but has technical limitations

The HDAC inhibitors have been used in several clinical and preclinical studies as a treatment for a variety of hematological malignancies, including leukemia (San JoséEnériz *et al.*, 2019)(Wieduwilt *et al.*, 2019)(Vilas-Zornoza *et al.*, 2012)(Ocio *et al.*, 2015) (Piekarz *et al.*, 2009). However, their use is limited due to severe side effects and/or poor efficacy (Shah, 2019)(Wieduwilt *et al.*, 2019)(Piekarz *et al.*, 2009). In addition, HDACis are known to have the ability to modulate immune responses through different mechanisms (Schotterl, Brennenstuhl and Naumann, 2015)(Lisiero *et al.*, 2014)(Oki *et al.*, 2014). They significantly induce the expression of NKG2D-Ls in tumor cells and enhance their sensitivity to NK cells (Sauer *et al.*, 2017)(Cho *et al.*, 2021). Therefore, the identification of the mechanisms by which HDACi induce NKG2D ligand expression is of high importance but difficult, so we developed a modified enChIP method (see below). Previously our group has demonstrated the role of LBH589 in the regulation of NKG2D-Ls, including MICA, and shown that this depends on CBP/p300 acetyltransferases (Sauer *et al.*, 2017). In this study, we detected hyperacetylation of the H3 on lysine K27 in the MICA promoter region (Figure 2). H3K27ac is a marker for CBP/p300 and is found at both promoters and enhancers for transcriptionally active genes (Gao *et al.*, 2020)(Wang *et al.*, 2008). This is consistent with another study showing that the use of HDACi results in increased acetylation of H3 on the *MICA* promoter region not only in cell lines but also in cells from leukemia patients and enhances the surface expression of this ligand (Kato *et al.*, 2007). CBP/p300 can regulate gene transcription through binding and acetylating several targets including chromatin components and transcription factors (Weinert *et al.*, 2018)(Gu and Roeder, 1997). One of them is the transcription factor CREB, which was detected to target CBP/p300 in the MICA promoter region upon LBH589 treatment (Sauer *et al.*, 2017). MICA, a gene with tandem promoters, consists of an upstream promoter and a standard promoter, encoding two major transcripts. It has been reported that the surface expression of MICA is only correlated with the expression of the standard transcript (Lin, Hiron and O'Callaghan, 2018). The upstream promoter, however, can be regulated by different stimuli, such as the IL-4 and IFN-γ, and also represses MICA surface expression through the standard promoter (Lin, Hiron and O'Callaghan, 2018). Based on our data, LBH589 upregulates the expression of *MICA* predominantly by inducing the transcription from the standard promoter without affecting the upstream

promoter, thus, clearly defining the MICA standard promoter as the target region for LBH589-mediated MICA upregulation (Figure 1). As the chromatin composition is very complex, the identification of transcription factors associated with a target locus is still challenging. Consequently, we have established the novel enChIP method to identify regulators involved in the mechanism described above. Fujita and his colleagues were first able to isolate the interferon regulatory factor 1 (*IRF1*) promoter locus by enChIP/MS and identify both known and novel proteins that interact with this locus (Fujita and Fujii, 2013a). Our data show that the enChIP-based CRISPR/dCas9 approach did not interfere with the effect of LBH589 in MICA induction (Supplementary figure 1), thereby confirming that dCas9 did not locate at the MICA promoter in the region required for transcription factors and RNA polymerase II. Chromatin is rich in different structural factors such as histones, transcription factors, histone modifying enzymes, ATP-dependent chromatin remodellers, DNA and RNA polymerases, and DNA repair enzymes (Vermeulen and Déjardin, 2020). In contrast to histones, the transcription factors mainly regulate the gene function (expression) and are present in several orders of magnitude less than histones (Vermeulen and Déjardin, 2020)(Gauchier *et al.*, 2020). Therefore, the amount of starting material (number of cells) is a crucial point in the enChIP/MS method to identify transcription factors associated with a single genomic locus (Gauchier *et al.*, 2020). In this study, the cell number used for enChIP/MS was approximately 2-3x10^8 cells. In comparison, Fujita and colleagues had shown that 6x10^7 HEK293T human cells for transient transfection are sufficient for subsequent mass spectrometry analysis (Fujita and Fujii, 2013a). In contrast, Gauchier and his colleagues have claimed that at least half a billion cells are needed as starting material to allow the detection of proteins using enChIP/MS, and they further discussed that the number of cells required also depends on the respective target (Gauchier *et al.*, 2020). These results represent the high background and the challenge is to filter out the potential regulators. Our data demonstrated that the MICA locus was effectively isolated by enChIP-qPCR, with an enrichment of approximately 2-3 % compared to the input genomic DNA (Figure 6a). Furthermore, precipitation of the *MICA* locus using Cas9 antibody showed the highest locus enrichment compared to controls such as streptavidin agarose, streptavidin dynabeads, or anti-FLAG antibody (Figure 5 and supplementary figure 2), although streptavidin agarose was reported to have a higher sensitivity and affinity for biotin tagged dCas9 than Cas9 antibody-based precipitation (Liu *et al.*, 2017, p. 9). In addition, our enChIP/qPCR

results showed that the gRNAs used, were highly specific in targeting the MICA locus, as no signal was detected in the various controls used (Figures 6a and b). Using a mixture of five different gRNAs, each targeting specifically different regions with the MICA locus, improved the specificity. In addition, the off targets could be reduced as each gRNA had a different off-target, which was also suggested by Fujita et al (Fujii and Fujita, 2015).

Based on our enChIP/MS approach, we were able to detect several proteins involved in gene regulation, including RNA polymerases (POLR2C, POLR2A, and POLR2I), histones (H2, H3, and H4), histone deacetylases (HDAC1 and HDAC2), chromatin remodeling complexes such as SWI/SNF complex, and some other transcription factors (Figure 7a). We selected a set of putative candidates that could be involved in *MICA* regulation based on our data obtained by enChIP/MS analysis. In addition to cMYC, PARP1, H2A.Z, MLF2, YY1, we mainly focused in this study on KLF4 (Figure 7a). All these factors have in common, that they are reported to be acetylated by CBP/p300 and play an important role in chromatin remodeling and gene transcription (Evans *et al.*, 2007)(Yao, Yang and Seto, 2001)(Colino-Sanguino *et al.*, 2019)(Vervoorts *et al.*, 2003)(Hassa *et al.*, 2005).

enChIP/MS approach is a useful method, but still improvements are possible, such as generating stable cell lines with the CRISPR/dCas9 system. Moreover, isolating the locus of interest with two precipitating steps, the first of which involves pulling the locus with a Cas9 antibody, followed by a FLAG antibody or streptavidin targeting the biotin tagged dCas9 to reduce the background as much as possible and enrich the transcription factors comparing to other chromatin components.

2. Identifying KLF4 as a target for LBH89-mediated MICA upregulation

The transcription factor KLF4 has a dual effect as a tumor suppressor or oncogene in a context-dependent manner (Tetreault, Yang and Katz, 2013)(Riverso, Montagnani and Stecca, 2017). The expression of KLF4, like the NKG2D-Ls, can be induced in response to DNA damage and also by all-trans-retinoic acid (Meng *et al.*, 2009)(Rohner *et al.*, 2007)(Poggi *et al.*, 2009)(Sauer *et al.*, 2017) (Yoon, Chen and Yang, 2003). Furthermore, it has been reported to be a target for HDACi (Kee and Kook, 2009) (Kee and Kook, 2009). For example, in a KLF4-dependent manner, treatment of A549 lung cancer cells with HDACi partially suppresses tumor growth (Yu *et al.*, 2016). Of note, KLF4 can be acetylated by CBP/p300, which in turn promotes the transactivation of

some other target genes (Zhang *et al.*, 2010)(Evans *et al.*, 2007). Recent, ChIP-seq data (ENCODE data) highlighted the presence of KLF4 on the MICA promoter region (Figure 7c). Consistent with this, our prediction using the JASPAR database also revealed that the MICA promoter region harbors binding sites for KLF4 and YY1 (Figure 7d). This was further validated using a dual reporter luciferase assay where regulation of the MICA promoter region by KLF4 could be demonstrated (Figure 8b and Figure 9b). Although the data were reproducible and significant, the dual reporter system has some limitations such as high background stimulation of the empty vector compared to the vector-related sequence. Therefore, the use of an alternative system with deletion of most regulatory sequences such as the vector pGL4 or mutation of KLF4 motifs within the MICA promoter region would be beneficial.

However, independent approaches confirmed the role of KLF4 as a regulator for MICA. Of note, pharmacological inhibition or genetic deletion of KLF4 significantly reduced LBH589-mediated MICA upregulation. Kenpaullone, a small molecule, has shown the ability to inhibit the expression of KLF4 in several studies (Yu *et al.*, 2011)(Montecillo-Aguado *et al.*, 2021). It has been reported that kenpaullone inhibits the expression of KLF4 on transcription levels in breast cancer cell lines (Yu *et al.*, 2011). We could demonstrate that kenpaullone not only inhibited the expression of KLF4 (Figure 10a) but also significantly reduced the upregulation of MICA by LBH589 (Figure 10b). However, it should be noted that kenpaullone exhibits other functions, such as suppression of cyclin-dependent kinases (CDKs) and glycogen synthase kinase-3 (GSK-3), which could be responsible for this reduction (Schultz *et al.*, 1999)(Yeo *et al.*, 2021). Reducing the expression of KLF4 using siRNA also significantly decreased the upregulation of *MICA* upon LBH589 stimulation (Figure 10c and d). In sum, we have elucidated for the first time the regulation of MICA by KLF4, which is presumed to be modulated by LBH589.

3. The expression of MICA and KLF4 was similarly regulated in AML cell lines by a stress factor

In AML blasts, the surface expression of NKG2D-Ls is heterogenous between cells and is positively correlated with enhanced survival rate and controlling the relapse in AML patients (Mastaglio *et al.*, 2018). LSCs are one of the reasons for chemoresistance-related relapse (Valent *et al.*, 2020)(Siveen, Uddin and Mohammad, 2017). Our data showed induced expression of MICA associated with KLF4 in chemotherapy-sensitive cells compared to the resistant ones (Figures 11a and b). This is in line with a previous

study performed in AML cells from patients, defining LSCs as lacking the surface expression of NKG2D-Ls ligands (Paczulla *et al.*, 2019). Besides, the NKG2D-Ls^{neg} status, LSCs exhibit a significant reduction in the expression of KLF4 compared to the NKG2D-Ls^{pos} LSCs (*p* value = $4.4x10^{-44}$ see supplementary figure of RNA-seq data (Paczulla *et al.*, 2019). Interestingly, the expression of NKG2D-Ls on
LSCs renders them sensitive to NK cell cytotoxicity and increases survival rate in the AML-transplanted mice after treatment with Ara-C (Paczulla *et al.*, 2019). The role of KLF4 in leukemia is still controversially discussed and depends on the cellular context (Wei *et al.*, 2016)(Lewis *et al.*, 2021)(Ghaleb and Yang, 2017). Several studies have reported downregulation of the KLF4 expression in AML patients as well as in cell lines (Huang *et al.*, 2014)(Morris *et al.*, 2016). In addition, KLF4 has been suggested to function as a tumor suppressor in T cell acute lymphoblastic leukemia cells and some B cells malignancies (Yasunaga *et al.*, 2004)(Guan *et al.*, 2010)(Kharas *et al.*, 2007)(Li *et al.*, 2015). KLF4, a known Yamanaka factor, can induce pluripotent stem cells and recently was reported to promote the division and stemness of LSCs (Lewis *et al.*, 2022). Which can be explained regarding the expression of the MICA ligand. As, it is a stress-inducible ligand that alters the innate immune system to dangerous cells characterized by the high expression of KLF4 (Paczulla *et al.*, 2019).Taken together, these results highlight the importance of the *MICA* and *KLF4* in the chemotherapy mediated response. AML is frequently associated with epigenetic dysregulation(Fennell, Bell and Dawson, 2019), and multiple attempts have been made to use HDACi as a monotherapy or in combination with chemotherapy to treat patients with AML (Maiso *et al.*, 2009)(Wieduwilt *et al.*, 2019). Although the treatment showed promising results in preclinical studies, it was discontinued in clinical trials due to severe side effects and limited response (Ocio *et al.*, 2015)(Wieduwilt *et al.*, 2019)(Richardson *et al.*, 2013)(San-Miguel *et al.*, 2014). In contrast, LBH589 is an FDA-approved drug in 2015 for the treatment of multiple myeloma and in addition inhibits HDAC classes I, II, and IV (Prince, Bishton and Johnstone, 2009)(Atadja, 2009)(Laubach *et al.*, 2015). A high level of HDAC1 is observed in some AML patients and is correlated with a poor prognosis (Huang *et al.*, 2014). Moreover, LBH589 significantly induces NKG2D ligand expression, including MICA (Sauer *et al.*, 2017). Here we could demonstrate that the enhanced expression of MICA is also associated with upregulated expression of KLF4 in numerous AML cell lines after treatment with LBH589 (Figure 12) .In two separate studies, HDAC1 is described as a negative

regulator of both MICA and KLF4 expression in lung and AML cancer cells, respectively (Cho *et al.*, 2021)(Huang *et al.*, 2014). Inhibition or knockdown of HDAC1 by HDACi or small interfering RNA (siRNA) results in higher expression of KLF4 and suppress AML cell proliferation (Huang *et al.*, 2014). Similarly, in lung cancer cell lines suppressing the expression of HDAC1 leads to induced expression of NKG2D-Ls, including MICA, and increase the susceptibility of cells to NK cell cytotoxicity (Cho *et al.*, 2021). This also suggests that MICA and KLF4 both play a crucial role in the HDACi-mediated cancer treatment. For subsequent studies, it would be interesting to evaluate if the inhibition of HDAC1 together with KLF4 in AML cells impairs the sensitivity to recognition and killing by NK cells.

In this study, similar results were obtained regarding the expression of KLF4 and MICA in response to the combination of LBH589 with Ara-C. The combination treatment significantly increases the expression of MICA associated with KLF4 compared to LBH589 single treatment in the Ara-C-sensitive but not in the resistant HL60 cell line (Figures 11c and d). Previously, it was shown that the use of LBH589 in combination with Ara-C or doxorubicin significantly inhibited AML cell growth compared to either drug alone and enhanced the antileukemic effect in blasts from AML patients (Maiso *et al.*, 2009). Overall, the data show that the modulation of MICA is correlated with KLF4 in response to stress factors.

4. APTO253, a potent KLF4 inducer, boosted the expression of NKG2D-Ls in AML cells: a novel therapeutic strategy to treat AML

The KLF4 activator, APTO253, is a small molecule that has been clinically in development to treat solid tumors and hematological malignancies (Rice *et al.*, 2015)(Rice *et al.*, 2014). Interestingly, our data demonstrated for the first time that the forced induction of KLF4, using APTO253, upregulated not only KLF4 but also MICA expression in several AML cell lines in a dose-dependent manner (Figures 13a and b). Previously, APTO253 has been reported to selectively eradicate AML cells with no cytotoxic effect on both normal peripheral blood mononuclear cells (PBMCs) as well as bone marrow cells in animal models and humans (Local, no date). Moreover, APTO253 effectively inhibits tumor growth in xenograft models of human AML (Rice *et al.*, 2015)(Rice *et al.*, 2014). Its potent anti-tumor activity was attributed to the induction of the KLF4 (Rice *et al.*, 2014). Furthermore, the induction upon treatment with APTO253 appeared not to be limited to MICA but also to upregulate other NKG2D-Ls including, MICB and ULBP1 (Supplementary figure 3).

However, several studies have been conducted to elucidate the mechanism underlying the role of APTO253 in cancer treatment. To our knowledge investigating the role of APTO253 in promoting immune cell cytotoxicity is still elusive. For example, Cercek and colleagues have illustrated that the sensitivity of lung cancer cells to APTO253 is mediated by the induction of KLF4 (Cercek *et al.*, 2015). Blocking the expression of KLF4 attenuates the response to APTO253 cytotoxicity (Cercek *et al.*, 2015). These results are in contrast to those of Lewis et al, who observed that KLF4 expression plays an oncogenic role in the two AML cell lines, NB4 and MonoMac6 (Lewis *et al.*, 2021). Nevertheless, this study lacks detection of the NKG2D ligand expression and the response of tumor cells to the immune cell cytotoxicity, including NK cells (Lewis *et al.*, 2021). Moreover, Lewis et al. claimed that KLF4 is not involved in the response to APTO253 in AML cells, but through other mechanisms, such as the MYC regulation (Lewis *et al.*, 2021). Some other mechanisms of APTO253 function include induction of DNA damage, apoptosis, and down-regulation of c-MYC expression (Local, no date)(Tsai *et al.*, 2018). Our observations indicate that the treatment of AML cell lines resulted in a dose-dependent decrease in c-MYC levels (Supplementary figure 4). c-MYC is a transcription factor also identified in our enChIP/MS data. It plays a crucial role in cell growth, proliferation, and apoptosis (Dang *et al.*, 2006). Mutation, amplification, and activation of the oncogene MYC family are common and correlated with cancer. In AML, c-MYC is often activated and involved in cancer development (Salvatori *et al.*, 2011). It has been reported that KLF4 could act as a negative regulator of MYC in the AML cell line (Morris *et al.*, 2016). Thus, it could also be that KLF4 has an indirect way of regulating MICA by decreasing MYC expression. Nevertheless, the role of MYC in regulating the expression of NKG2D-Ls has not been well studied. As shown in two different studies, c-MYC modulates the expression of NKG2D-Ls in a ligand-dependent manner (Nanbakhsh *et al.*, 2014)(Lee *et al.*, 2019). However, these two studies provided completely contrary data. A study performed in AML cell lines suggests c-MYC as an inducer of NKG2DL expression, but the study has shown that MICA was not affected by the activating effect (Nanbakhsh *et al.*, 2014). On the other hand, another study claims that c-MYC has an inhibitory effect on the expression of NKG2D-Ls, including MICA (Lee *et al.*, 2019), which is more consistent with the data presented in this thesis indicating c-MYC as an inhibitor of MICA in different AML cell lines. Furthermore, HDACi have been reported to acetylate c-MYC and decrease its expression in AML cells (Nebbioso *et al.*, 2017). Interestingly, our data showed a

substantial reduction of c-MYC in several AML cells after treatment with LBH589 (Supplementary figure 5). So LBH589 treatment increases the expression of KLF4, downregulates c-MYC, and upregulates MICA expression. Therefore, it will be fundamental to further dissect the role of c-MYC in LBH589-mediated MICA induction and also the regulation of NK cell cytotoxicity.

Taken together, the use of HDACi in cancer is still limited because of low response and side effects but recently promising results have been achieved using LBH589 in combination with adoptive NK cells (Afolabi *et al.*, 2021). APTO253 is also used in clinical trials to treat AML patients but shows low response without severe side effects. Here we established that APTO253 has the ability to modulate the expression of the NKG2D ligand, especially MICA. Thus, the combination treatment of APTO253 in AML patients followed by adoptive NK cell transfer is a potential therapeutic strategy, and this thesis is the first to uncover the link between APTO253 and the NKG2D/NKG2DL axis.

Conclusions

The HDACi LBH589 induced MICA ligand expression mainly through the standard promoter region. Using the novel enChIP method followed by mass spectrometry analysis to isolate the regulators associated with a single genomic locus had some limitations. However, the transcription factor KLF4 was identified as a target for LBH589-mediated MICA upregulation. Moreover, the expression of MICA correlated with KLF4 after the treatment with various stress factors, including LBH589, the chemotherapeutic agent Ara-C and the KLF4 inducer APTO253. This study highlights the role of KLF4 as a putative regulator for MICA. In addition, it reveals for the first time a novel link between the APTO253 and the NKG2D/NKG2D-L axis in AML that may help improve the AML patients' survival in the context of NK cell-based immunotherapy.

Supplementary data

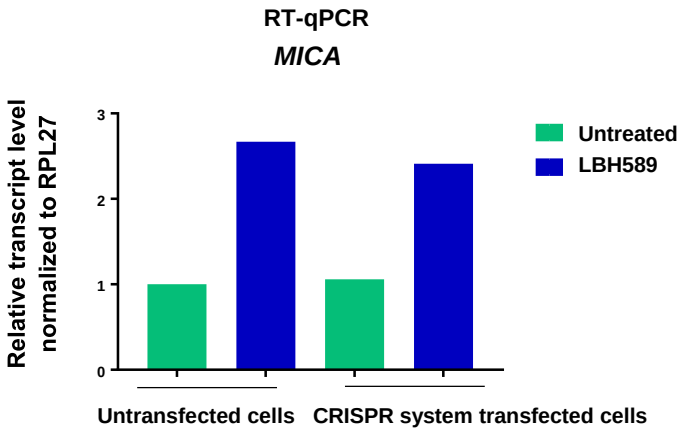


Figure 1: Transfection of HEK293 cells with the pEF1a-FB-dCas9/gRNA system to isolate the *MICA* promoter genomic region did not interfere with the induction of MICA transcription through LBH589 treatment. HEK293 cells were transfected with pEF1a-FBdCas9 plasmid and gRNAs targeting the MICA promoter region. 48 h post-transfection cells were treated with 100 nM LBH589 for 4 h. Then cells were subjected to RT-qPCR to measure the expression of MICA.

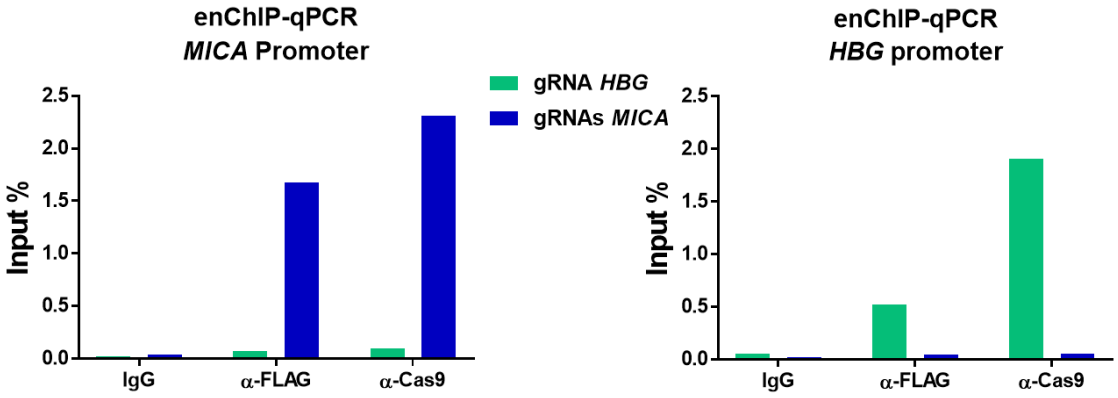


Figure 2: Pull-down of CRISPR/dCas9-containing DNA by α-Cas9 or α-FLAG. Transient co-transfection of HEK293 cells with pEF1a-FB-dCas9, and mix (1, 2, 4, 6, 8) gRNAs to target MICA promoter locus or HBG targeting sgRNA as a control. 48 h post-transfection, cells were subjected to ChIP assay, the target gene was isolated using α-Cas9 or α-FLAG against the FLAG-tagged dCas9, and IgG was used as a negative control. qPCR analysis to check the enrichment of the **a.** MICA promoter and **b.** HBG promoter region.

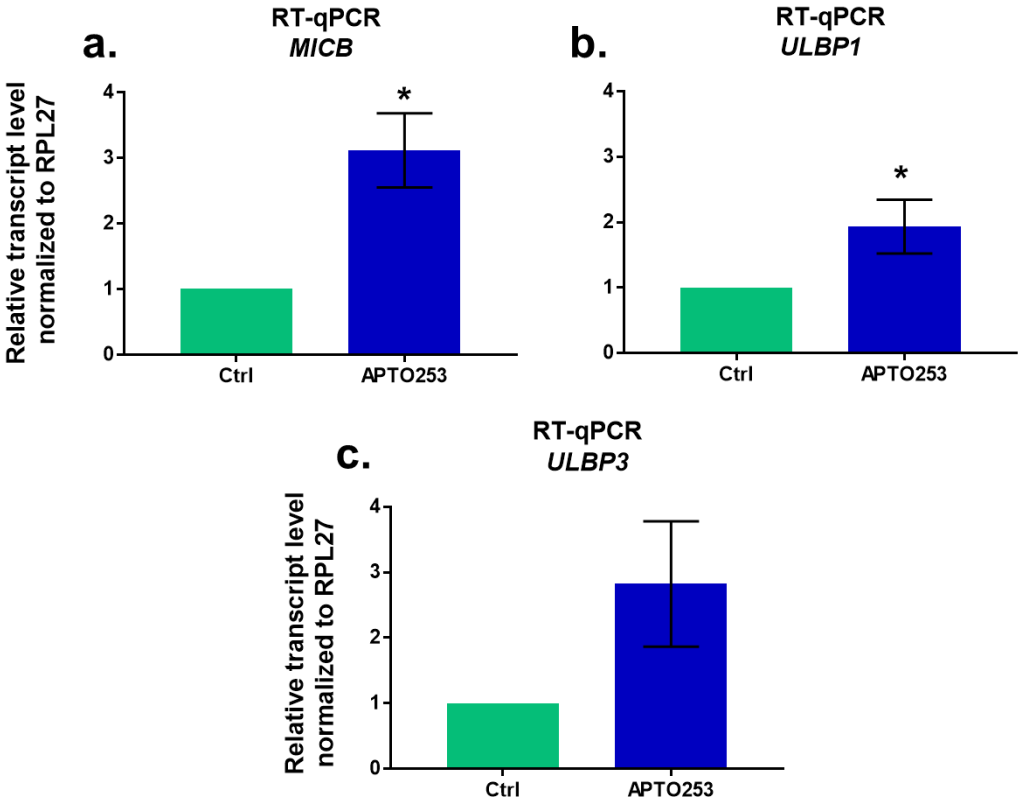


Figure 3: APTO253 induced the expression of NKG2D-Ls in the HL60 AML cell line. The HL60 cell line was incubated with APTO253 at a concentration of 300 nM for 18 h. RT-qPCR analysis was performed to detect the mRNA level of **a.** MICB, **b.** ULBP1, and **c.** ULBP3**.** Data show the mean (±SEM) of three biological replicates. Significance was calculated using an unpaired *t*-test. *$p < 0.05$.

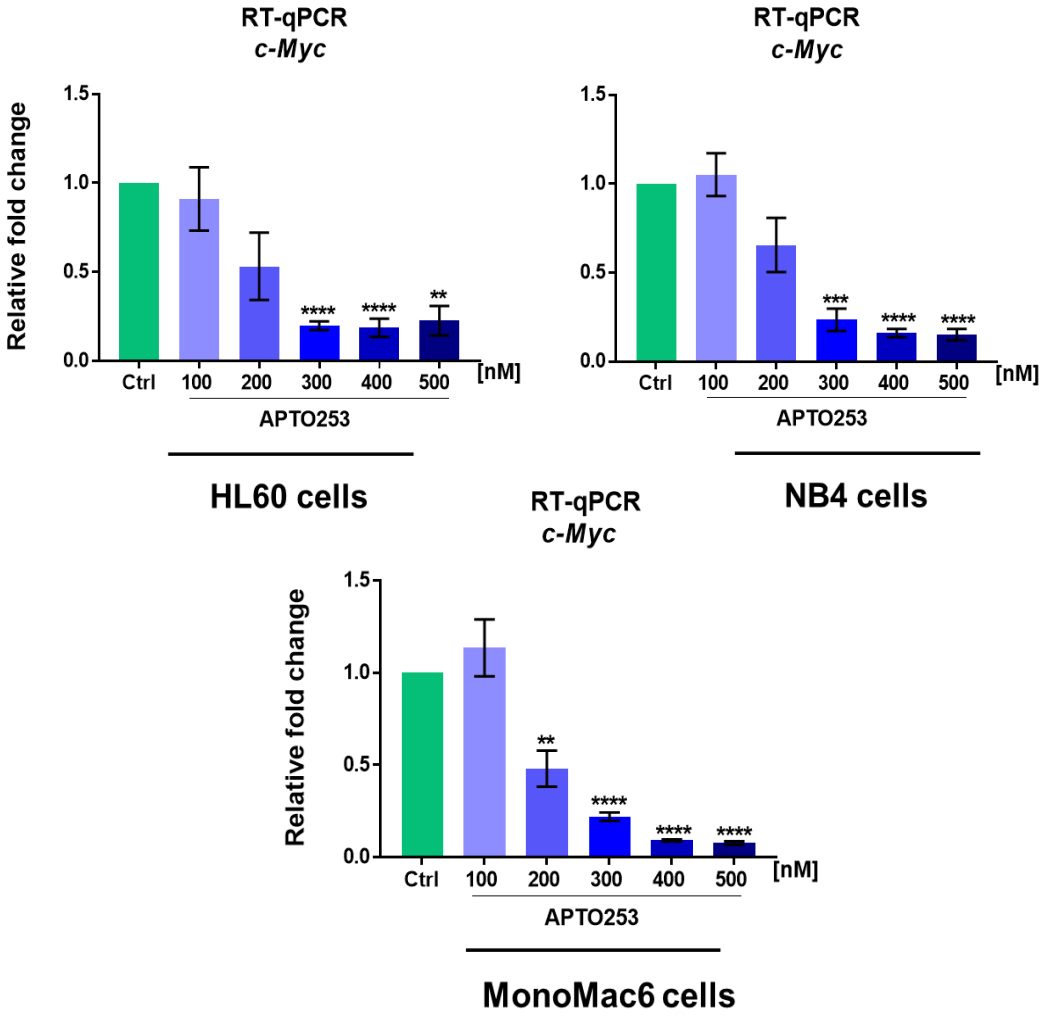


Figure 4: APTO253 significantly reduced the expression of c-MYC in several AML cell lines. AML cell lines were treated with APTO253 at concentrations of 100, 200, 300, 400, and 500 nM for 18 h. RT-qPCR analysis was performed to detect the mRNA level of c-MYC in **a.** HL60 cells, **b.** NB4 cells, and **c.** MonoMac6**.** Data show the mean (±SEM) of three biological replicates. Significance was calculated using an unpaired *t*-test. ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001 (Prof. Neuberger's group performed only the qPCR analysis using their *c-MYC* primers).

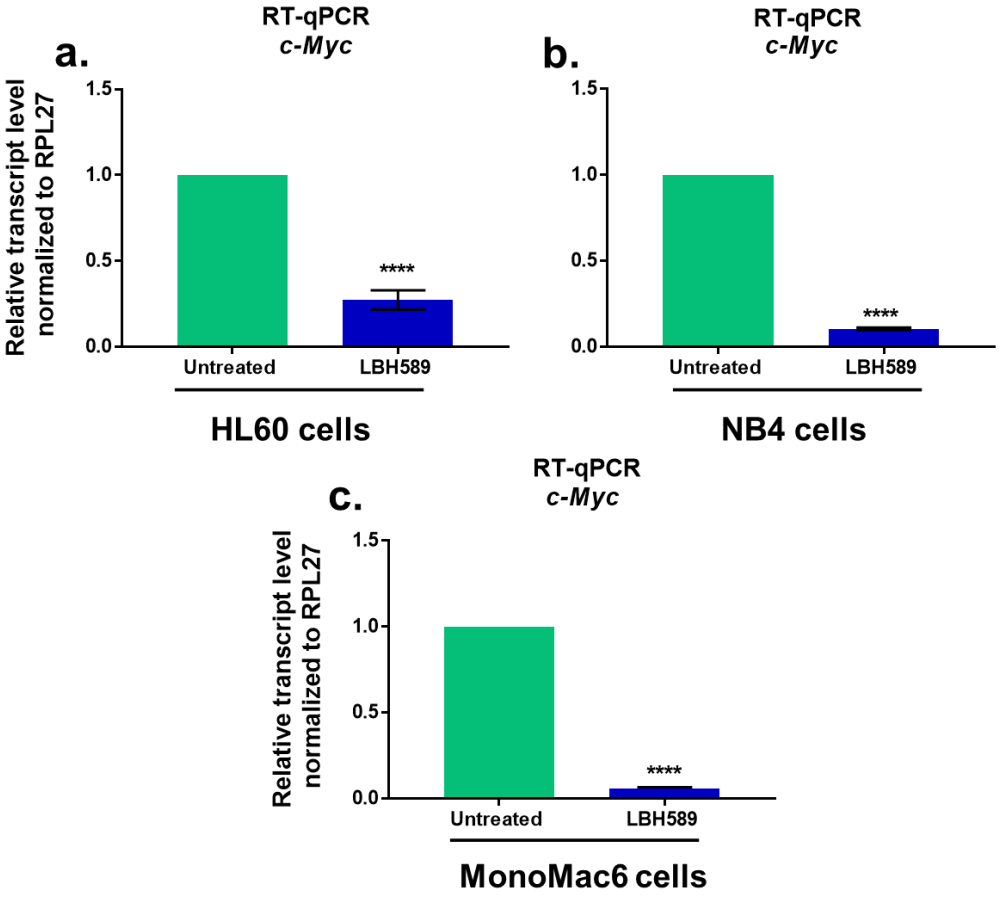


Figure 5: LBH589 was a potent inhibitor of the c-MYC in several AML cell lines. AML cell lines were treated with 100 nM LBH589 for 4 h. RT-qPCR analysis was performed to detect the mRNA level of c-MYC in **a.** HL60 cells, **b.** NB4 cells, and **c.** MonoMac6**.** Data show the mean (±SEM) of three biological replicates. Significance was calculated using an unpaired *t*test. ****$p < 0.0001$.

References

Abel, A.M. *et al.* (2018) 'Natural Killer Cells: Development, Maturation, and Clinical Utilization', *Frontiers in Immunology*, 9, p. 1869. Available at: https://doi.org/10.3389/fimmu.2018.01869.

Afolabi, L.O. *et al.* (2021) 'Synergistic Tumor Cytolysis by NK Cells in Combination With a Pan-HDAC Inhibitor, Panobinostat', *Frontiers in Immunology*, 12, p. 701671. Available at: https://doi.org/10.3389/fimmu.2021.701671.

Angka, L. *et al.* (2017) 'Dysfunctional Natural Killer Cells in the Aftermath of Cancer Surgery', *International Journal of Molecular Sciences*, 18(8), p. 1787. Available at: https://doi.org/10.3390/ijms18081787.

Armeanu, S. *et al.* (2005) 'Natural Killer Cell–Mediated Lysis of Hepatoma Cells via Specific Induction of NKG2D Ligands by the Histone Deacetylase Inhibitor Sodium Valproate', *Cancer Research*, 65(14), pp. 6321–6329. Available at: https://doi.org/10.1158/0008-5472.CAN-04-4252.

Atadja, P. (2009) 'Development of the pan-DAC inhibitor panobinostat (LBH589): Successes and challenges', *Cancer Letters*, 280(2), pp. 233–241. Available at: https://doi.org/10.1016/j.canlet.2009.02.019.

Baragaño Raneros, A. *et al.* (2015) 'Methylation of NKG2D ligands contributes to immune system evasion in acute myeloid leukemia', *Genes & Immunity*, 16(1), pp. 71–82. Available at: https://doi.org/10.1038/gene.2014.58.

Baragaño Raneros, A., López-Larrea, C. and Suárez-Álvarez, B. (2019) 'Acute myeloid leukemia and NK cells: two warriors confront each other', *OncoImmunology*, 8(2), p. e1539617. Available at: https://doi.org/10.1080/2162402X.2018.1539617.

Barneda-Zahonero, B. and Parra, M. (2012) 'Histone deacetylases and cancer', *Molecular Oncology*, 6(6), pp. 579–589. Available at: https://doi.org/10.1016/j.molonc.2012.07.003.

Barrangou, R. *et al.* (2007) 'CRISPR Provides Acquired Resistance Against Viruses in Prokaryotes', *Science*, 315(5819), pp. 1709–1712. Available at: https://doi.org/10.1126/science.1138140.

Bieker, J.J. (2001) 'Krüppel-like Factors: Three Fingers in Many Pies', *Journal of Biological Chemistry*, 276(37), pp. 34355–34358. Available at: https://doi.org/10.1074/jbc.R100043200.

Blagitko-Dorfs, N. *et al.* (2019) 'Combination treatment of acute myeloid leukemia cells with DNMT and HDAC inhibitors: predominant synergistic gene downregulation associated with gene body demethylation', *Leukemia*, 33(4), pp. 945–956. Available at: https://doi.org/10.1038/s41375-018-0293-8.

Bogdanove, A.J. and Voytas, D.F. (2011) 'TAL Effectors: Customizable Proteins for DNA Targeting', *Science*, 333(6051), pp. 1843–1846. Available at: https://doi.org/10.1126/science.1204094.

Bolden, J.E. *et al.* (2013) 'HDAC inhibitors induce tumor-cell-selective pro-apoptotic transcriptional responses', *Cell Death & Disease*, 4(2), pp. e519–e519. Available at: https://doi.org/10.1038/cddis.2013.9.

Brooks, A.G. *et al.* (1997) 'NKG2A Complexed with CD94 Defines a Novel Inhibitory Natural Killer Cell Receptor', *Journal of Experimental Medicine*, 185(4), pp. 795–800. Available at: https://doi.org/10.1084/jem.185.4.795.

Burgess, S.J. *et al.* (2006) 'IL-21 Down-Regulates NKG2D/DAP10 Expression on Human NK and CD8 $^{+}$ T Cells', *The Journal of Immunology*, 176(3), pp. 1490–1497. Available at: https://doi.org/10.4049/jimmunol.176.3.1490.

Casas-Mollano, J.A. *et al.* (2020) 'CRISPR-Cas Activators for Engineering Gene Expression in Higher Eukaryotes', *The CRISPR Journal*, 3(5), pp. 350–364. Available at: https://doi.org/10.1089/crispr.2020.0064.

Castillo, J. *et al.* (2019) 'CBP/p300 Drives the Differentiation of Regulatory T Cells through Transcriptional and Non-Transcriptional Mechanisms', *Cancer Research*, 79(15), pp. 3916– 3927. Available at: https://doi.org/10.1158/0008-5472.CAN-18-3622.

Ceccacci, E. and Minucci, S. (2016) 'Inhibition of histone deacetylases in cancer therapy: lessons from leukaemia', *British Journal of Cancer*, 114(6), pp. 605–611. Available at: https://doi.org/10.1038/bjc.2016.36.

Cercek, A. *et al.* (2015) 'Phase 1 study of APTO-253 HCl, an inducer of KLF4, in patients with advanced or metastatic solid tumors', *Investigational New Drugs*, 33(5), pp. 1086–1092. Available at: https://doi.org/10.1007/s10637-015-0273-z.

Chen, Z.Y., Rex, S. and Tseng, C.-C. (2004) 'Krüppel-Like Factor 4 Is Transactivated by Butyrate in Colon Cancer Cells', *The Journal of Nutrition*, 134(4), pp. 792–798. Available at: https://doi.org/10.1093/jn/134.4.792.

Cho, H. *et al.* (2021) 'Differential Effects of Histone Deacetylases on the Expression of NKG2D Ligands and NK Cell-Mediated Anticancer Immunity in Lung Cancer Cells', *Molecules*, 26(13), p. 3952. Available at: https://doi.org/10.3390/molecules26133952.

Colino-Sanguino, Y. *et al.* (2019) 'A Read/Write Mechanism Connects p300 Bromodomain Function to H2A.Z Acetylation', *iScience*, 21, pp. 773–788. Available at: https://doi.org/10.1016/j.isci.2019.10.053.

Cooper, M.A., Fehniger, T.A. and Caligiuri, M.A. (2001) 'The biology of human natural killer-cell subsets', *Trends in Immunology*, 22(11), pp. 633–640. Available at: https://doi.org/10.1016/S1471-4906(01)02060-9.

Cosenza, M. *et al.* (2017) 'Ricolinostat, a selective HDAC6 inhibitor, shows anti-lymphoma cell activity alone and in combination with bendamustine', *Apoptosis*, 22(6), pp. 827–840. Available at: https://doi.org/10.1007/s10495-017-1364-4.

Coudert, J.D. *et al.* (2005) 'Altered NKG2D function in NK cells induced by chronic exposure to NKG2D ligand–expressing tumor cells', *Blood*, 106(5), pp. 1711–1717. Available at: https://doi.org/10.1182/blood-2005-03-0918.

Dang, C.V. *et al.* (2006) 'The c-Myc target gene network', *Seminars in Cancer Biology*, 16(4), pp. 253–264. Available at: https://doi.org/10.1016/j.semcancer.2006.07.014.

Das, P.M. *et al.* (2004) 'Chromatin immunoprecipitation assay', *BioTechniques*, 37(6), pp. 961–969. Available at: https://doi.org/10.2144/04376RV01.

De Sanctis, J.B., Blanca, I. and Bianco, N.E. (1997) 'Secretion of cytokines by natural killer cells primed with interleukin-2 and stimulated with different lipoproteins', *Immunology*, 90(4), pp. 526–533. Available at: https://doi.org/10.1046/j.1365-2567.1997.00174.x.

Déjardin, J. and Kingston, R.E. (2009) 'Purification of Proteins Associated with Specific Genomic Loci', *Cell*, 136(1), pp. 175–186. Available at: https://doi.org/10.1016/j.cell.2008.11.045.

Diermayr, S. *et al.* (2008) 'NKG2D ligand expression in AML increases in response to HDAC inhibitor valproic acid and contributes to allorecognition by NK-cell lines with single KIR-HLA class I specificities', *Blood*, 111(3), pp. 1428–1436. Available at: https://doi.org/10.1182/blood-2007-07-101311.

Du, Z. *et al.* (2021) 'piggyBac system to co-express NKG2D CAR and IL-15 to augment the in vivo persistence and anti-AML activity of human peripheral blood NK cells', *Molecular Therapy - Methods & Clinical Development*, 23, pp. 582–596. Available at: https://doi.org/10.1016/j.omtm.2021.10.014.

Evans, P.M. *et al.* (2007) 'Krüppel-like Factor 4 Is Acetylated by p300 and Regulates Gene Transcription via Modulation of Histone Acetylation', *Journal of Biological Chemistry*, 282(47), pp. 33994–34002. Available at: https://doi.org/10.1074/jbc.M701847200.

Feinberg, M.W. *et al.* (2007) 'The Kruppel-like factor KLF4 is a critical regulator of monocyte differentiation', *The EMBO Journal*, 26(18), pp. 4138–4148. Available at: https://doi.org/10.1038/sj.emboj.7601824.

Fennell, K.A., Bell, C.C. and Dawson, M.A. (2019) 'Epigenetic therapies in acute myeloid leukemia: where to from here?', *Blood*, 134(22), pp. 1891–1901. Available at: https://doi.org/10.1182/blood.2019003262.

Ferrari de Andrade, L. *et al.* (2018) 'Antibody-mediated inhibition of MICA and MICB shedding promotes NK cell–driven tumor immunity', *Science*, 359(6383), pp. 1537–1542. Available at: https://doi.org/10.1126/science.aao0505.

Ferrari de Andrade, L. *et al.* (2020) 'Inhibition of MICA and MICB Shedding Elicits NK-Cell–Mediated Immunity against Tumors Resistant to Cytotoxic T Cells', *Cancer Immunology Research*, 8(6), pp. 769–780. Available at: https://doi.org/10.1158/23266066.CIR-19-0483.

Fiskus, W. *et al.* (2009) 'Panobinostat treatment depletes EZH2 and DNMT1 levels and enhances decitabine mediated de-repression of JunB and loss of survival of human acute leukemia cells', *Cancer Biology & Therapy*, 8(10), pp. 939–950. Available at: https://doi.org/10.4161/cbt.8.10.8213.

Fortunati, N. *et al.* (2014) 'The pan-histone deacetylase inhibitor LBH589 (panobinostat) alters the invasive breast cancer cell phenotype', *International Journal of Oncology*, 44(3), pp. 700–708. Available at: https://doi.org/10.3892/ijo.2013.2218.

Fujii, H. and Fujita, T. (2015) 'Isolation of Specific Genomic Regions and Identification of Their Associated Molecules by Engineered DNA-Binding Molecule-Mediated Chromatin Immunoprecipitation (enChIP) Using the CRISPR System and TAL Proteins', *International Journal of Molecular Sciences*, 16(9), pp. 21802–21812. Available at: https://doi.org/10.3390/ijms160921802.

Fujita, T. and Fujii, H. (2011) 'Direct Identification of Insulator Components by Insertional Chromatin Immunoprecipitation', *PLoS ONE*. Edited by R. Jothi, 6(10), p. e26109. Available at: https://doi.org/10.1371/journal.pone.0026109.

Fujita, T. and Fujii, H. (2013a) 'Efficient isolation of specific genomic regions and identification of associated proteins by engineered DNA-binding molecule-mediated chromatin immunoprecipitation (enChIP) using CRISPR', *Biochemical and Biophysical Research Communications*, 439(1), pp. 132–136. Available at: https://doi.org/10.1016/j.bbrc.2013.08.013.

Fujita, T. and Fujii, H. (2013b) 'Locus-Specific Biochemical Epigenetics/Chromatin Biochemistry by Insertional Chromatin Immunoprecipitation', *ISRN Biochemistry*, 2013, pp.
1–8. Available at: https://doi.org/10.1155/2013/913273.

Fujita, T., Yuno, M. and Fujii, H. (2018) 'enChIP systems using different CRISPR orthologues and epitope tags', *BMC Research Notes*, 11(1), p. 154. Available at: https://doi.org/10.1186/s13104-018-3262-4.

Gade, P. and Kalvakolanu, D.V. (2012) 'Chromatin Immunoprecipitation Assay as a Tool for
Analyzing Transcription Factor Activity', in A. Vancura (ed.) *Transcriptional Regulation*. New York, NY: Springer New York (Methods in Molecular Biology), pp. 85–104. Available at: https://doi.org/10.1007/978-1-61779-376-9_6.

Gambacorta, V. *et al.* (2019) 'Epigenetic Therapies for Acute Myeloid Leukemia and Their Immune-Related Effects', *Frontiers in Cell and Developmental Biology*, 7, p. 207. Available at: https://doi.org/10.3389/fcell.2019.00207.

Gao, Y. *et al.* (2020) ‘Acetylation of histone H3K27 signals the transcriptional elongation for estrogen receptor alpha’, *Communications Biology*, 3(1), p. 165. Available at: https://doi.org/10.1038/s42003-020-0898-0.

Gasser, S. *et al.* (2005) ‘The DNA damage pathway regulates innate immune system ligands of the NKG2D receptor’, *Nature*, 436(7054), pp. 1186–1190. Available at: https://doi.org/10.1038/nature03884.

Gauchier, M. *et al.* (2020) ‘Purification and enrichment of specific chromatin loci’, *Nature Methods*, 17(4), pp. 380–389. Available at: https://doi.org/10.1038/s41592-020-0765-4.

Geiman, D.E. (2000) ‘Transactivation and growth suppression by the gut-enriched Kruppellike factor (Kruppel-like factor 4) are dependent on acidic amino acid residues and proteinprotein interaction’, *Nucleic Acids Research*, 28(5), pp. 1106–1113. Available at: https://doi.org/10.1093/nar/28.5.1106.

Ghaleb, A.M. and Yang, V.W. (2017) ‘Krüppel-like factor 4 (KLF4): What we currently know’, *Gene*, 611, pp. 27–37. Available at: https://doi.org/10.1016/j.gene.2017.02.025.

Gilfillan, S. *et al.* (2002) ‘NKG2D recruits two distinct adapters to trigger NK cell activation and costimulation’, *Nature Immunology*, 3(12), pp. 1150–1155. Available at: https://doi.org/10.1038/ni857.

Gonzalez, H., Hagerling, C. and Werb, Z. (2018) ‘Roles of the immune system in cancer: from tumor initiation to metastatic progression’, *Genes & Development*, 32(19–20), pp. 1267–1284. Available at: https://doi.org/10.1101/gad.314617.118.

Grant, C. *et al.* (2010) ‘Romidepsin: a new therapy for cutaneous T-cell lymphoma and a potential therapy for solid tumors’, *Expert Review of Anticancer Therapy*, 10(7), pp. 997– 1008. Available at: https://doi.org/10.1586/era.10.88.

Groh, V. *et al.* (2001) ‘Costimulation of CD8αβ T cells by NKG2D via engagement by MIC induced on virus-infected cells’, *Nature Immunology*, 2(3), pp. 255–260. Available at: https://doi.org/10.1038/85321.

Grunstein, M. (1997) ‘Histone acetylation in chromatin structure and transcription’, *Nature*, 389(6649), pp. 349–352. Available at: https://doi.org/10.1038/38664.

Gu, W. and Roeder, R.G. (1997) ‘Activation of p53 Sequence-Specific DNA Binding by Acetylation of the p53 C-Terminal Domain’, *Cell*, 90(4), pp. 595–606. Available at: https://doi.org/10.1016/S0092-8674(00)80521-8.

Guan, H. *et al.* (2010) ‘KLF4 is a tumor suppressor in B-cell non-Hodgkin lymphoma and in classic Hodgkin lymphoma’, *Blood*, 116(9), pp. 1469–1478. Available at: https://doi.org/10.1182/blood-2009-12-256446.

Guerra, N. *et al.* (2008) 'NKG2D-Deficient Mice Are Defective in Tumor Surveillance in Models of Spontaneous Malignancy', *Immunity*, 28(4), pp. 571–580. Available at: https://doi.org/10.1016/j.immuni.2008.02.016.

Gui, C.-Y. *et al.* (2004) 'Histone deacetylase (HDAC) inhibitor activation of p21 ^{WAF1} involves changes in promoter-associated proteins, including HDAC1', *Proceedings of the National Academy of Sciences*, 101(5), pp. 1241–1246. Available at: https://doi.org/10.1073/pnas.0307708100.

Gujral, P. *et al.* (2020) 'Histone acetylation and the role of histone deacetylases in normal cyclic endometrium', *Reproductive Biology and Endocrinology*, 18(1), p. 84. Available at: https://doi.org/10.1186/s12958-020-00637-5.

Hassa, P.O. *et al.* (2005) 'Acetylation of Poly(ADP-ribose) Polymerase-1 by p300/CREBbinding Protein Regulates Coactivation of NF-κB-dependent Transcription', *Journal of Biological Chemistry*, 280(49), pp. 40450–40464. Available at: https://doi.org/10.1074/jbc.M507553200.

Hilpert, J. *et al.* (2012) 'Comprehensive Analysis of NKG2D Ligand Expression and Release in Leukemia: Implications for NKG2D-Mediated NK Cell Responses', *The Journal of Immunology*, 189(3), pp. 1360–1371. Available at: https://doi.org/10.4049/jimmunol.1200796.

Holdenrieder, S. *et al.* (2006) 'Soluble MICA in malignant diseases', *International Journal of Cancer*, 118(3), pp. 684–687. Available at: https://doi.org/10.1002/ijc.21382.

Hoshino, A. and Fujii, H. (2009) 'Insertional chromatin immunoprecipitation: A method for isolating specific genomic regions', *Journal of Bioscience and Bioengineering*, 108(5), pp. 446–449. Available at: https://doi.org/10.1016/j.jbiosc.2009.05.005.

Huang, Y. *et al.* (2014) 'HDAC1 and Klf4 interplay critically regulates human myeloid leukemia cell proliferation', *Cell Death & Disease*, 5(10), pp. e1491–e1491. Available at: https://doi.org/10.1038/cddis.2014.433.

Huergo-Zapico, L. *et al.* (2012) 'Expression of ERp5 and GRP78 on the membrane of chronic lymphocytic leukemia cells: association with soluble MICA shedding', *Cancer Immunology, Immunotherapy*, 61(8), pp. 1201–1210. Available at: https://doi.org/10.1007/s00262-011-1195-z.

Huergo-Zapico, L. *et al.* (2014) 'Expansion of NK Cells and Reduction of NKG2D Expression in Chronic Lymphocytic Leukemia. Correlation with Progressive Disease', *PLoS ONE*. Edited by S.B. Gibson, 9(10), p. e108326. Available at: https://doi.org/10.1371/journal.pone.0108326.

Hull, E.E., Montgomery, M.R. and Leyva, K.J. (2016) 'HDAC Inhibitors as Epigenetic Regulators of the Immune System: Impacts on Cancer Therapy and Inflammatory Diseases', *BioMed Research International*, 2016, pp. 1–15. Available at: https://doi.org/10.1155/2016/8797206.

Imai, K. *et al.* (2000) 'Natural cytotoxic activity of peripheral-blood lymphocytes and cancer incidence: an 11-year follow-up study of a general population', *The Lancet*, 356(9244), pp. 1795–1799. Available at: https://doi.org/10.1016/S0140-6736(00)03231-1.

Jensen, H. *et al.* (2013) 'Regulation of NKG2D-ligand cell surface expression by intracellular calcium after HDAC-inhibitor treatment', *Molecular Immunology*, 53(3), pp. 255–264. Available at: https://doi.org/10.1016/j.molimm.2012.08.011.

Jiang, X.-J. *et al.* (2012) 'Synergistic effect of panobinostat and bortezomib on chemoresistant acute myelogenous leukemia cells via AKT and NF-κB pathways', *Cancer Letters*, 326(2), pp. 135–142. Available at: https://doi.org/10.1016/j.canlet.2012.07.030.

Jinushi, M. *et al.* (2008) 'MHC class I chain-related protein A antibodies and shedding are associated with the progression of multiple myeloma', *Proceedings of the National Academy of Sciences*, 105(4), pp. 1285–1290. Available at: https://doi.org/10.1073/pnas.0711293105.

Jones, A.B. *et al.* (2022) 'Regulation of NKG2D Stress Ligands and Its Relevance in Cancer Progression', *Cancers*, 14(9), p. 2339. Available at: https://doi.org/10.3390/cancers14092339.

Juan, L.-J. *et al.* (2000) 'Histone Deacetylases Specifically Down-regulate p53-dependent Gene Activation', *Journal of Biological Chemistry*, 275(27), pp. 20436–20443. Available at: https://doi.org/10.1074/jbc.M000202200.

Kato, N. *et al.* (2007) 'Regulation of the expression of MHC class I-related chain A, B (MICA, MICB) via chromatin remodeling and its impact on the susceptibility of leukemic cells to the cytotoxicity of NKG2D-expressing cells', *Leukemia*, 21(10), pp. 2103–2108. Available at: https://doi.org/10.1038/sj.leu.2404862.

Kee, H.J. and Kook, H. (2009) 'Krüppel-like factor 4 mediates histone deacetylase inhibitorinduced prevention of cardiac hypertrophy', *Journal of Molecular and Cellular Cardiology*, 47(6), pp. 770–780. Available at: https://doi.org/10.1016/j.yjmcc.2009.08.022.

Khan, A.N.H. and Tomasi, T.B. (2008) 'Histone deacetylase regulation of immune gene expression in tumor cells', *Immunologic Research*, 40(2), pp. 164–178. Available at: https://doi.org/10.1007/s12026-007-0085-0.

Kharas, M.G. *et al.* (2007) 'KLF4 suppresses transformation of pre-B cells by ABL oncogenes', *Blood*, 109(2), pp. 747–755. Available at: https://doi.org/10.1182/blood-200603-011106.

Klein, J.M. *et al.* (2013) 'The Histone Deacetylase Inhibitor LBH589 (Panobinostat) Modulates the Crosstalk of Lymphocytes with Hodgkin Lymphoma Cell Lines', *PLoS ONE*. Edited by F. Bertolini, 8(11), p. e79502. Available at: https://doi.org/10.1371/journal.pone.0079502.

Koguchi, Y. *et al.* (2015) 'Serum Immunoregulatory Proteins as Predictors of Overall Survival of Metastatic Melanoma Patients Treated with Ipilimumab', *Cancer Research*, 75(23), pp. 5084–5092. Available at: https://doi.org/10.1158/0008-5472.CAN-15-2303.

Konjević, G. *et al.* (2010) 'In-vitro IL-2 or IFN-α-induced NKG2D and CD161 NK cell receptor expression indicates novel aspects of NK cell activation in metastatic melanoma patients', *Melanoma Research*, 20(6), pp. 459–467. Available at: https://doi.org/10.1097/CMR.0b013e32833e3286.

Korthout, T. *et al.* (2018) 'Decoding the chromatin proteome of a single genomic locus by DNA sequencing', *PLOS Biology*. Edited by T. Misteli, 16(7), p. e2005542. Available at: https://doi.org/10.1371/journal.pbio.2005542.

Krauss, A.C. *et al.* (2019) 'FDA Approval Summary: (Daunorubicin and Cytarabine) Liposome for Injection for the Treatment of Adults with High-Risk Acute Myeloid Leukemia', *Clinical Cancer Research*, 25(9), pp. 2685–2690. Available at: https://doi.org/10.1158/1078-0432.CCR-18-2990.

Kumar, S. (2018) 'Natural killer cell cytotoxicity and its regulation by inhibitory receptors', *Immunology*, 154(3), pp. 383–393. Available at: https://doi.org/10.1111/imm.12921.

Lagger, G. (2002) 'Essential function of histone deacetylase 1 in proliferation control and CDK inhibitor repression', *The EMBO Journal*, 21(11), pp. 2672–2681. Available at: https://doi.org/10.1093/emboj/21.11.2672.

Lanier, L.L. (2015) 'NKG2D Receptor and Its Ligands in Host Defense', *Cancer Immunology Research*, 3(6), pp. 575–582. Available at: https://doi.org/10.1158/23266066.CIR-15-0098.

Laubach, J.P. *et al.* (2015) 'Panobinostat for the Treatment of Multiple Myeloma', *Clinical Cancer Research*, 21(21), pp. 4767–4773. Available at: https://doi.org/10.1158/10780432.CCR-15-0530.

Lazarova, M. and Steinle, A. (2019) 'The NKG2D axis: an emerging target in cancer immunotherapy', *Expert Opinion on Therapeutic Targets*, 23(4), pp. 281–294. Available at: https://doi.org/10.1080/14728222.2019.1580693.

Lee, Y. *et al.* (2019) 'Upregulation of Myc promotes the evasion of NK cell-mediated immunity through suppression of NKG2D ligands in K562 cells', *Molecular Medicine Reports* [Preprint]. Available at: https://doi.org/10.3892/mmr.2019.10583.

Lee, Y.S. *et al.* (2018) 'The combination of ionizing radiation and proteasomal inhibition by bortezomib enhances the expression of NKG2D ligands in multiple myeloma cells', *Journal of Radiation Research*, 59(3), pp. 245–252. Available at: https://doi.org/10.1093/jrr/rry005.

Lewis, A.H. *et al.* (2021) 'Krüppel-like factor 4 promotes survival and expansion in acute myeloid leukemia cells', *Oncotarget*, 12(4), pp. 255–267. Available at: https://doi.org/10.18632/oncotarget.27878.

Lewis, A.H. *et al.* (2022) 'Krüppel-like Factor 4 Supports the Expansion of Leukemia Stem
Cells in MLL-AF9-driven Acute Myeloid Leukemia', *Stem Cells*, 40(8), pp. 736–750. Available at: https://doi.org/10.1093/stmcls/sxac033.

Li, B., Carey, M. and Workman, J.L. (2007) 'The Role of Chromatin during Transcription', *Cell*, 128(4), pp. 707–719. Available at: https://doi.org/10.1016/j.cell.2007.01.015.

Li, S., Mason, C.E. and Melnick, A. (2016) 'Genetic and epigenetic heterogeneity in acute myeloid leukemia', *Current Opinion in Genetics & Development*, 36, pp. 100–106. Available at: https://doi.org/10.1016/j.gde.2016.03.011.

Li, W. *et al.* (2015) 'Genome-wide analyses identify KLF4 as an important negative regulator in T-cell acute lymphoblastic leukemia through directly inhibiting T-cell associated genes', *Molecular Cancer*, 14(1), p. 26. Available at: https://doi.org/10.1186/s12943-014-0285-x.

Li, Y. and Seto, E. (2016) 'HDACs and HDAC Inhibitors in Cancer Development and Therapy', *Cold Spring Harbor Perspectives in Medicine*, 6(10), p. a026831. Available at: https://doi.org/10.1101/cshperspect.a026831.

Lin, D., Hiron, T.K. and O'Callaghan, C.A. (2018) 'Intragenic transcriptional interference regulates the human immune ligand MICA', *The EMBO Journal*, 37(10). Available at: https://doi.org/10.15252/embj.201797138.

Lisiero, D.N. *et al.* (2014) 'The histone deacetylase inhibitor, LBH589, promotes the systemic cytokine and effector responses of adoptively transferred CD8+ T cells', *Journal for ImmunoTherapy of Cancer*, 2(1), p. 8. Available at: https://doi.org/10.1186/2051-1426-2-8.

Liu, X. *et al.* (2017) 'In Situ Capture of Chromatin Interactions by Biotinylated dCas9', *Cell*, 170(5), pp. 1028-1043.e19. Available at: https://doi.org/10.1016/j.cell.2017.08.003.

Liu, X. *et al.* (2018) 'CAPTURE: *In Situ* Analysis of Chromatin Composition of Endogenous Genomic Loci by Biotinylated dCas9', *Current Protocols in Molecular Biology*, 123(1), p. e64. Available at: https://doi.org/10.1002/cpmb.64.

Local, A. (no date) 'APTO-253 Interaction with G-Quadruplex DNA Is Linked to Inhibition of c-Myc Expression, Induction of DNA Damage, and Generation of Synthetic Lethality in Cells with BRCA1/2 Impairment', p. 2.

López-Botet, M. *et al.* (1997) 'The CD94/NKG2 C-type lectin receptor complex: Involvement in NK Cell-Mediated Recognition of HLA Class I Molecules', *Immunologic Research*, 16(2), pp. 175–185. Available at: https://doi.org/10.1007/BF02786361.

Maiso, P. *et al.* (2009) 'The synergy of panobinostat plus doxorubicin in acute myeloid leukemia suggests a role for HDAC inhibitors in the control of DNA repair', *Leukemia*, 23(12), pp. 2265–2274. Available at: https://doi.org/10.1038/leu.2009.182.

Mamonkin, M. *et al.* (2013) 'Differential roles of KLF4 in the development and differentiation of CD8+ T cells', *Immunology Letters*, 156(1–2), pp. 94–101. Available at: https://doi.org/10.1016/j.imlet.2013.09.008.

Marçais, A. *et al.* (2013) 'Regulation of Mouse NK Cell Development and Function by Cytokines', *Frontiers in Immunology*, 4. Available at: https://doi.org/10.3389/fimmu.2013.00450.

Mariño-Ramírez, L. *et al.* (2005) 'Histone structure and nucleosome stability', *Expert Review of Proteomics*, 2(5), pp. 719–729. Available at: https://doi.org/10.1586/14789450.2.5.719.

Marks, P.A. and Xu, W.-S. (2009) 'Histone deacetylase inhibitors: Potential in cancer therapy', *Journal of Cellular Biochemistry*, 107(4), pp. 600–608. Available at: https://doi.org/10.1002/jcb.22185.

Marpaung, D.S.S. and Sinaga, A.O.Y. (2020) 'A mini review on production of pluripotency factors (Oct4, Sox2, Klf4 and c-Myc) through recombinant protein technology', *Communications in Science and Technology*, 5(1), pp. 1–4. Available at: https://doi.org/10.21924/cst.5.1.2020.171.

Martire, S. *et al.* (2020) 'Differential contribution of p300 and CBP to regulatory element acetylation in mESCs', *BMC Molecular and Cell Biology*, 21(1), p. 55. Available at: https://doi.org/10.1186/s12860-020-00296-9.

Mastaglio, S. *et al.* (2018) 'Natural killer receptor ligand expression on acute myeloid leukemia impacts survival and relapse after chemotherapy', *Blood Advances*, 2(4), pp. 335– 346. Available at: https://doi.org/10.1182/bloodadvances.2017015230.

Mazzurana, L. *et al.* (2018) 'The roles for innate lymphoid cells in the human immune system', *Seminars in Immunopathology*, 40(4), pp. 407–419. Available at: https://doi.org/10.1007/s00281-018-0688-7.

McConnell, B.B. *et al.* (2007) 'The diverse functions of Krüppel-like factors 4 and 5 in epithelial biology and pathobiology', *BioEssays*, 29(6), pp. 549–557. Available at: https://doi.org/10.1002/bies.20581.

Meng, F. *et al.* (2009) 'All-trans retinoic acid increases KLF4 acetylation by inducing HDAC2 phosphorylation and its dissociation from KLF4 in vascular smooth muscle cells', *Biochemical and Biophysical Research Communications*, 387(1), pp. 13–18. Available at: https://doi.org/10.1016/j.bbrc.2009.05.112.

Mincheva-Nilsson, L. and Baranov, V. (2014) 'Cancer exosomes and NKG2D receptor– ligand interactions: Impairing NKG2D-mediated cytotoxicity and anti-tumour immune surveillance', *Seminars in Cancer Biology*, 28, pp. 24–30. Available at: https://doi.org/10.1016/j.semcancer.2014.02.010.

Montecillo-Aguado, M. *et al.* (2021) 'KLF4 inhibition by Kenpaullone induces cytotoxicity and chemo sensitization in B-NHL cell lines via YY1 independent', *Leukemia & Lymphoma*, 62(6), pp. 1422–1431. Available at: https://doi.org/10.1080/10428194.2020.1869960.

Moore, D. (2016) 'Panobinostat (Farydak): A Novel Option for the Treatment of Relapsed Or Relapsed and Refractory Multiple Myeloma', *P & T: A Peer-Reviewed Journal for Formulary Management*, 41(5), pp. 296–300.

Moretta, L. *et al.* (2014) 'Human NK Cells: From Surface Receptors to the Therapy of Leukemias and Solid Tumors', *Frontiers in Immunology*, 5. Available at: https://doi.org/10.3389/fimmu.2014.00087.

Morris, V.A. *et al.* (2016) 'Deregulated KLF4 Expression in Myeloid Leukemias Alters Cell
Proliferation and Differentiation through MicroRNA and Gene Targets', *Molecular and Cellular Biology*, 36(4), pp. 559–573. Available at: https://doi.org/10.1128/MCB.00712-15.

Mottamal, M. *et al.* (2015) 'Histone Deacetylase Inhibitors in Clinical Studies as Templates for New Anticancer Agents', *Molecules*, 20(3), pp. 3898–3941. Available at: https://doi.org/10.3390/molecules20033898.

Müller, B.M. *et al.* (2013) 'Differential expression of histone deacetylases HDAC1, 2 and 3 in human breast cancer - overexpression of HDAC2 and HDAC3 is associated with clinicopathological indicators of disease progression', *BMC Cancer*, 13(1), p. 215. Available at: https://doi.org/10.1186/1471-2407-13-215.

Nanbakhsh, A. *et al.* (2014) 'c-Myc regulates expression of NKG2D ligands ULBP1/2/3 in AML and modulates their susceptibility to NK-mediated lysis', *Blood*, 123(23), pp. 3585– 3595. Available at: https://doi.org/10.1182/blood-2013-11-536219.

Narlikar, L. and Ovcharenko, I. (2009) 'Identifying regulatory elements in eukaryotic genomes', *Briefings in Functional Genomics and Proteomics*, 8(4), pp. 215–230. Available at: https://doi.org/10.1093/bfgp/elp014.

Nebbioso, A. *et al.* (2017) 'c-Myc Modulation and Acetylation Is a Key HDAC Inhibitor Target in Cancer', *Clinical Cancer Research*, 23(10), pp. 2542–2555. Available at: https://doi.org/10.1158/1078-0432.CCR-15-2388.

Ocio, E.M. *et al.* (2015) 'Panobinostat as part of induction and maintenance for elderly patients with newly diagnosed acute myeloid leukemia: phase Ib/II panobidara study', *Haematologica*, 100(10), pp. 1294–1300. Available at: https://doi.org/10.3324/haematol.2015.129577.

Ohanian, M. *et al.* (2019) 'MYC protein expression is an important prognostic factor in acute myeloid leukemia', *Leukemia & Lymphoma*, 60(1), pp. 37–48. Available at: https://doi.org/10.1080/10428194.2018.1464158.

Oki, Y. *et al.* (2014) 'Immune regulatory effects of panobinostat in patients with Hodgkin lymphoma through modulation of serum cytokine levels and T-cell PD1 expression', *Blood Cancer Journal*, 4(8), pp. e236–e236. Available at: https://doi.org/10.1038/bcj.2014.58.

Okita, R. *et al.* (2015) 'Contrasting Effects of the Cytotoxic Anticancer Drug Gemcitabine and the EGFR Tyrosine Kinase Inhibitor Gefitinib on NK Cell-Mediated Cytotoxicity via Regulation of NKG2D Ligand in Non-Small-Cell Lung Cancer Cells', *PLOS ONE*. Edited by A.R.M.R. Amin, 10(10), p. e0139809. Available at: https://doi.org/10.1371/journal.pone.0139809.

Okita, R. *et al.* (2016) 'MHC class I chain-related molecule A and B expression is upregulated by cisplatin and associated with good prognosis in patients with non-small cell lung cancer', *Cancer Immunology, Immunotherapy*, 65(5), pp. 499–509. Available at: https://doi.org/10.1007/s00262-016-1814-9.

Pabo, C.O., Peisach, E. and Grant, R.A. (2001) 'Design and Selection of Novel Cys $_2$ His $_2$ Zinc Finger Proteins', *Annual Review of Biochemistry*, 70(1), pp. 313–340. Available at: https://doi.org/10.1146/annurev.biochem.70.1.313.

Paczulla, A.M. *et al.* (2019) 'Absence of NKG2D ligands defines leukaemia stem cells and mediates their immune evasion', *Nature*, 572(7768), pp. 254–259. Available at: https://doi.org/10.1038/s41586-019-1410-1.

Park, C.S. *et al.* (2016) 'Role of the reprogramming factor KLF4 in blood formation', *Journal of Leukocyte Biology*, 99(5), pp. 673–685. Available at: https://doi.org/10.1189/jlb.1RU1215-539R.

Paul, S. and Lal, G. (2017) 'The Molecular Mechanism of Natural Killer Cells Function and Its Importance in Cancer Immunotherapy', *Frontiers in Immunology*, 8, p. 1124. Available at: https://doi.org/10.3389/fimmu.2017.01124.

Piekarz, R.L. *et al.* (2009) 'Phase II Multi-Institutional Trial of the Histone Deacetylase Inhibitor Romidepsin As Monotherapy for Patients With Cutaneous T-Cell Lymphoma', *Journal of Clinical Oncology*, 27(32), pp. 5410–5417. Available at: https://doi.org/10.1200/JCO.2008.21.6150.

Poggi, A. *et al.* (2009) 'Effective in vivo induction of NKG2D ligands in acute myeloid leukaemias by all-trans-retinoic acid or sodium valproate', *Leukemia*, 23(4), pp. 641–648. Available at: https://doi.org/10.1038/leu.2008.354.

Ponath, V. *et al.* (2020) 'The Oncoprotein SKI Acts as A Suppressor of NK Cell-Mediated Immunosurveillance in PDAC', *Cancers*, 12(10), p. 2857. Available at: https://doi.org/10.3390/cancers12102857.

Prince, H.M., Bishton, M.J. and Johnstone, R.W. (2009) 'Panobinostat (LBH589): a potent pan-deacetylase inhibitor with promising activity against hematologic and solid tumors', *Future Oncology*, 5(5), pp. 601–612. Available at: https://doi.org/10.2217/fon.09.36.

Qi, L.S. *et al.* (2013) 'Repurposing CRISPR as an RNA-Guided Platform for SequenceSpecific Control of Gene Expression', *Cell*, 152(5), pp. 1173–1183. Available at: https://doi.org/10.1016/j.cell.2013.02.022.

Raffaghello, L. *et al.* (2004) 'Downregulation and/or Release of NKG2D Ligands as Immune Evasion Strategy of Human Neuroblastoma', *Neoplasia*, 6(5), pp. 558–568. Available at: https://doi.org/10.1593/neo.04316.

Raulet, D.H. *et al.* (2013) 'Regulation of Ligands for the NKG2D Activating Receptor', *Annual Review of Immunology*, 31(1), pp. 413–441. Available at: https://doi.org/10.1146/annurev-immunol-032712-095951.

Ray, S.K. (2016) 'The Transcription Regulator Kruppel-Like Factor 4 and Its Dual Roles of
Oncogene in Glioblastoma and Tumor Suppressor in Neuroblastoma', *Forum on Immunopathological Diseases and Therapeutics*, 7(1–2), pp. 127–139. Available at: https://doi.org/10.1615/ForumImmunDisTher.2016017227.

Rice, W.G. *et al.* (2014) 'APTO-253 Induces KLF4 to Promote Potent in Vitro Pro-Apoptotic Activity in Hematologic Cancer Cell Lines and Antitumor Efficacy As a Single Agent and in Combination with Azacitidine in Animal Models of Acute Myelogenous Leukemia (AML)', *Blood*, 124(21), pp. 4813–4813. Available at: https://doi.org/10.1182/blood.V124.21.4813.4813.

Rice, W.G. *et al.* (2015) 'Clinical Pharmacokinetics of Apto-253 Support Its Use As a Novel Agent for the Treatment of Relapsed or Refractory Hematologic Malignancies', *Blood*, 126(23), pp. 4934–4934. Available at: https://doi.org/10.1182/blood.V126.23.4934.4934.

Richardson, P.G. *et al.* (2013) 'PANORAMA 2: panobinostat in combination with bortezomib and dexamethasone in patients with relapsed and bortezomib-refractory myeloma', *Blood*, 122(14), pp. 2331–2337. Available at: https://doi.org/10.1182/blood-201301-481325.

Riverso, M., Montagnani, V. and Stecca, B. (2017) 'KLF4 is regulated by
RAS/RAF/MEK/ERK signaling through E2F1 and promotes melanoma cell growth', *Oncogene*, 36(23), pp. 3322–3333. Available at: https://doi.org/10.1038/onc.2016.481.

Robinson, J. *et al.* (2015) 'The IPD and IMGT/HLA database: allele variant databases', *Nucleic Acids Research*, 43(D1), pp. D423–D431. Available at: https://doi.org/10.1093/nar/gku1161.

Rodríguez-Rodero, S. *et al.* (2007) 'Transcriptional regulation of MICA and MICB: A novel polymorphism in MICB promoter alters transcriptional regulation by Sp1', *European Journal of Immunology*, 37(7), pp. 1938–1953. Available at: https://doi.org/10.1002/eji.200737031.

Rohner, A. *et al.* (2007) 'Differentiation-promoting drugs up-regulate NKG2D ligand expression and enhance the susceptibility of acute myeloid leukemia cells to natural

killer cell-mediated lysis', *Leukemia Research*, 31(10), pp. 1393–1402. Available at: https://doi.org/10.1016/j.leukres.2007.02.020.

Ropero, S. *et al.* (2006) 'A truncating mutation of HDAC2 in human cancers confers resistance to histone deacetylase inhibition', *Nature Genetics*, 38(5), pp. 566–569. Available at: https://doi.org/10.1038/ng1773.

Salih, H.R. *et al.* (2003) 'Functional expression and release of ligands for the activating immunoreceptor NKG2D in leukemia', *Blood*, 102(4), pp. 1389–1396. Available at: https://doi.org/10.1182/blood-2003-01-0019.

Salvatori, B. *et al.* (2011) 'Critical Role of c-Myc in Acute Myeloid Leukemia Involving Direct Regulation of miR-26a and Histone Methyltransferase EZH2', *Genes & Cancer*, 2(5), pp. 585–592. Available at: https://doi.org/10.1177/1947601911416357.

San José-Enériz *et al.* (2019) 'HDAC Inhibitors in Acute Myeloid Leukemia', *Cancers*, 11(11), p. 1794. Available at: https://doi.org/10.3390/cancers11111794.

Sandoval-Borrego, D. *et al.* (2016) 'Overexpression of CD158 and NKG2A Inhibitory Receptors and Underexpression of NKG2D and NKp46 Activating Receptors on NK Cells in Acute Myeloid Leukemia', *Archives of Medical Research*, 47(1), pp. 55–64. Available at: https://doi.org/10.1016/j.arcmed.2016.02.001.

San-Miguel, J.F. *et al.* (2014) 'Panobinostat plus bortezomib and dexamethasone versus placebo plus bortezomib and dexamethasone in patients with relapsed or relapsed and refractory multiple myeloma: a multicentre, randomised, double-blind phase 3 trial', *The Lancet Oncology*, 15(11), pp. 1195–1206. Available at: https://doi.org/10.1016/S14702045(14)70440-1.

Satoh, T.K. *et al.* (2020) 'IL-36γ drives skin toxicity induced by EGFR/MEK inhibition and commensal Cutibacterium acnes', *Journal of Clinical Investigation*, 130(3), pp. 1417–1430. Available at: https://doi.org/10.1172/JCI128678.

Sauer, M. *et al.* (2017) 'CBP/p300 acetyltransferases regulate the expression of NKG2D ligands on tumor cells', *Oncogene*, 36(7), pp. 933–941. Available at: https://doi.org/10.1038/onc.2016.259.

Sawas, A., Radeski, D. and O'Connor, O.A. (2015) 'Belinostat in patients with refractory or relapsed peripheral T-cell lymphoma: a perspective review', *Therapeutic Advances in Hematology*, 6(4), pp. 202–208. Available at: https://doi.org/10.1177/2040620715592567.

Schlenk, R.F. *et al.* (2018) 'Panobinostat monotherapy and combination therapy in patients with acute myeloid leukemia: results from two clinical trials', *Haematologica*, 103(1), pp. e25–e28. Available at: https://doi.org/10.3324/haematol.2017.172411.

Schmiedel, D. and Mandelboim, O. (2018) 'NKG2D Ligands–Critical Targets for Cancer Immune Escape and Therapy', *Frontiers in Immunology*, 9, p. 2040. Available at: https://doi.org/10.3389/fimmu.2018.02040.

Schneider, G. *et al.* (2011) 'Acetylation as a Transcriptional Control Mechanism—HDACs and HATs in Pancreatic Ductal Adenocarcinoma', *Journal of Gastrointestinal Cancer*, 42(2), pp. 85–92. Available at: https://doi.org/10.1007/s12029-011-9257-1.

Schotterl, S., Brennenstuhl, H. and Naumann, U. (2015) 'Modulation of Immune Responses by Histone Deacetylase Inhibitors', *Critical Reviews in Oncogenesis*, 20(1–2), pp. 139–154. Available at: https://doi.org/10.1615/CritRevOncog.2014012393. Schultz, C. *et al.* (1999) 'Paullones, a Series of Cyclin-Dependent Kinase Inhibitors: Synthesis, Evaluation of CDK1/Cyclin B Inhibition, and in Vitro Antitumor Activity', *Journal of Medicinal Chemistry*, 42(15), pp. 2909–2919. Available at: https://doi.org/10.1021/jm9900570.

Segre, J.A., Bauer, C. and Fuchs, E. (1999) 'Klf4 is a transcription factor required for establishing the barrier function of the skin', *Nature Genetics*, 22(4), pp. 356–360. Available at: https://doi.org/10.1038/11926.

Seto, E. and Yoshida, M. (2014) 'Erasers of Histone Acetylation: The Histone Deacetylase Enzymes', *Cold Spring Harbor Perspectives in Biology*, 6(4), pp. a018713–a018713. Available at: https://doi.org/10.1101/cshperspect.a018713.

Shah, R.R. (2019) 'Safety and Tolerability of Histone Deacetylase (HDAC) Inhibitors in Oncology', *Drug Safety*, 42(2), pp. 235–245. Available at: https://doi.org/10.1007/s40264018-0773-9.

Shallis, R.M. *et al.* (2019) 'Epidemiology of acute myeloid leukemia: Recent progress and enduring challenges', *Blood Reviews*, 36, pp. 70–87. Available at: https://doi.org/10.1016/j.blre.2019.04.005.

Shanmugam, G., Rakshit, S. and Sarkar, K. (2022) 'HDAC inhibitors: Targets for tumor therapy, immune modulation and lung diseases', *Translational Oncology*, 16, p. 101312. Available at: https://doi.org/10.1016/j.tranon.2021.101312.

Shen, Y. *et al.* (2017) 'Inactivation of KLF4 promotes T-cell acute lymphoblastic leukemia and activates the MAP2K7 pathway', *Leukemia*, 31(6), pp. 1314–1324. Available at: https://doi.org/10.1038/leu.2016.339.

Shie, J.-L. *et al.* (2000) 'Role of gut-enriched Krüppel-like factor in colonic cell growth and differentiation', *American Journal of Physiology-Gastrointestinal and Liver Physiology*, 279(4), pp. G806–G814. Available at: https://doi.org/10.1152/ajpgi.2000.279.4.G806.

Shik Park, C. *et al.* (2012) 'Krüppel-like factor 4 (KLF4) promotes the survival of natural killer cells and maintains the number of conventional dendritic cells in the spleen', *Journal of Leukocyte Biology*, 91(5), pp. 739–750. Available at: https://doi.org/10.1189/jlb.0811413.

Singh, A. *et al.* (2016) 'Panobinostat as Pan-deacetylase Inhibitor for the Treatment of Pancreatic Cancer: Recent Progress and Future Prospects', *Oncology and Therapy*, 4(1), pp. 73–89. Available at: https://doi.org/10.1007/s40487-016-0023-1.

Singhal, N. *et al.* (2010) 'Chromatin-Remodeling Components of the BAF Complex Facilitate Reprogramming', *Cell*, 141(6), pp. 943–955. Available at: https://doi.org/10.1016/j.cell.2010.04.037.

Siveen, K.S., Uddin, S. and Mohammad, R.M. (2017) 'Targeting acute myeloid leukemia stem cell signaling by natural products', *Molecular Cancer*, 16(1), p. 13. Available at: https://doi.org/10.1186/s12943-016-0571-x.

Skov, S. *et al.* (2005) 'Cancer Cells Become Susceptible to Natural Killer Cell Killing after
Exposure to Histone Deacetylase Inhibitors Due to Glycogen Synthase Kinase-3–Dependent Expression of MHC Class I–Related Chain A and B', *Cancer Research*, 65(23), pp. 11136– 11145. Available at: https://doi.org/10.1158/0008-5472.CAN-05-0599.

Slovak, M.L. *et al.* (1994) 'Localization of amplified MYC gene sequences to double minute chromosomes in acute myelogenous leukemia', *Genes, Chromosomes and Cancer*, 9(1), pp.
62–67. Available at: https://doi.org/10.1002/gcc.2870090111.

Smyth, M.J. *et al.* (2005) 'NKG2D function protects the host from tumor initiation', *Journal of Experimental Medicine*, 202(5), pp. 583–588. Available at: https://doi.org/10.1084/jem.20050994.

Song, H. *et al.* (2006) 'IL-2/IL-18 prevent the down-modulation of NKG2D by TGF-β in NK cells via the c-Jun N-terminal kinase (JNK) pathway', *Cellular Immunology*, 242(1), pp. 39– 45. Available at: https://doi.org/10.1016/j.cellimm.2006.09.002.

Stavri, S. *et al.* (2015) 'Krüppel-like factor 4 synergizes with CREB to increase the activity of apolipoprotein E gene promoter in macrophages', *Biochemical and Biophysical Research Communications*, 468(1–2), pp. 66–72. Available at: https://doi.org/10.1016/j.bbrc.2015.10.163.

Stiehl, T. *et al.* (2015) 'Cell Division Patterns in Acute Myeloid Leukemia Stem-like Cells Determine Clinical Course: A Model to Predict Patient Survival', *Cancer Research*, 75(6), pp. 940–949. Available at: https://doi.org/10.1158/0008-5472.CAN-14-2508.

Sun, X.-J. *et al.* (2015) 'The Role of Histone Acetyltransferases in Normal and Malignant Hematopoiesis', *Frontiers in Oncology*, 5. Available at: https://doi.org/10.3389/fonc.2015.00108.

Swamynathan, S.K. (2010) 'Krüppel-like factors: Three fingers in control', *Human Genomics*, 4(4), p. 263. Available at: https://doi.org/10.1186/1479-7364-4-4-263.

Tallman, M.S., Gilliland, D.G. and Rowe, J.M. (2005) 'Drug therapy for acute myeloid leukemia', *Blood*, 106(4), pp. 1154–1163. Available at: https://doi.org/10.1182/blood-200501-0178.

Talmadge, J.E. *et al.* (1980) 'Role of NK cells in tumour growth and metastasis in beige mice', *Nature*, 284(5757), pp. 622–624. Available at: https://doi.org/10.1038/284622a0.

Tetreault, M.-P., Yang, Y. and Katz, J.P. (2013) 'Krüppel-like factors in cancer', *Nature Reviews Cancer*, 13(10), pp. 701–713. Available at: https://doi.org/10.1038/nrc3582.

Textor, S. *et al.* (2011) 'Human NK Cells Are Alerted to Induction of p53 in Cancer Cells by Upregulation of the NKG2D Ligands ULBP1 and ULBP2', *Cancer Research*, 71(18), pp. 5998–6009. Available at: https://doi.org/10.1158/0008-5472.CAN-10-3211.

Thomas, D. and Majeti, R. (2017) 'Biology and relevance of human acute myeloid leukemia stem cells', *Blood*, 129(12), pp. 1577–1585. Available at: https://doi.org/10.1182/blood2016-10-696054.

Trinchieri, G. (1989) 'Biology of Natural Killer Cells', in *Advances in Immunology*. Elsevier, pp. 187–376. Available at: https://doi.org/10.1016/S0065-2776(08)60664-1.

Tsai, C.-Y. *et al.* (2018) 'APTO-253 Is a New Addition to the Repertoire of Drugs that Can Exploit DNA BRCA1/2 Deficiency', *Molecular Cancer Therapeutics*, 17(6), pp. 1167–1176. Available at: https://doi.org/10.1158/1535-7163.MCT-17-0834.

Ullrich, E. *et al.* (2013) 'New prospects on the NKG2D/NKG2DL system for oncology', *OncoImmunology*, 2(10), p. e26097. Available at: https://doi.org/10.4161/onci.26097.

Ungerstedt, J.S. *et al.* (2005) 'Role of thioredoxin in the response of normal and transformed cells to histone deacetylase inhibitors', *Proceedings of the National Academy of Sciences*, 102(3), pp. 673–678. Available at: https://doi.org/10.1073/pnas.0408732102.

Valent, P. *et al.* (2020) 'Cell-based and antibody-mediated immunotherapies directed against leukemic stem cells in acute myeloid leukemia: Perspectives and open issues', *Stem Cells Translational Medicine*, 9(11), pp. 1331–1343. Available at: https://doi.org/10.1002/sctm.200147.

Van Veggel, M., Westerman, E. and Hamberg, P. (2018) 'Clinical Pharmacokinetics and Pharmacodynamics of Panobinostat', *Clinical Pharmacokinetics*, 57(1), pp. 21–29. Available at: https://doi.org/10.1007/s40262-017-0565-x.

Vermeulen, M. and Déjardin, J. (2020) 'Locus-specific chromatin isolation', *Nature Reviews Molecular Cell Biology*, 21(5), pp. 249–250. Available at: https://doi.org/10.1038/s41580020-0217-0.

Vervoorts, J. *et al.* (2003) 'Stimulation of c-MYC transcriptional activity and acetylation by recruitment of the cofactor CBP', *EMBO reports*, 4(5), pp. 484–490. Available at: https://doi.org/10.1038/sj.embor.embor821.

Vilas-Zornoza, A. *et al.* (2012) 'Preclinical activity of LBH589 alone or in combination with chemotherapy in a xenogeneic mouse model of human acute lymphoblastic

leukemia', *Leukemia*, 26(7), pp. 1517–1526. Available at: https://doi.org/10.1038/leu.2012.31.

Vivier, E. *et al.* (2008) 'Functions of natural killer cells', *Nature Immunology*, 9(5), pp. 503– 510. Available at: https://doi.org/10.1038/ni1582.

Vivier, E. *et al.* (2011) 'Innate or Adaptive Immunity? The Example of Natural Killer Cells', *Science*, 331(6013), pp. 44–49. Available at: https://doi.org/10.1126/science.1198687.

Voskoboinik, I. *et al.* (2010) 'Perforin: structure, function, and role in human immunopathology: Perforin structure and function', *Immunological Reviews*, 235(1), pp. 35– 54. Available at: https://doi.org/10.1111/j.0105-2896.2010.00896.x.

Voskoboinik, I., Smyth, M.J. and Trapani, J.A. (2006) 'Perforin-mediated target-cell death and immune homeostasis', *Nature Reviews Immunology*, 6(12), pp. 940–952. Available at: https://doi.org/10.1038/nri1983.

Wagner, J.M. *et al.* (2010) 'Histone deacetylase (HDAC) inhibitors in recent clinical trials for cancer therapy', *Clinical Epigenetics*, 1(3–4), pp. 117–136. Available at: https://doi.org/10.1007/s13148-010-0012-4.

Wang, F., Marshall, C.B. and Ikura, M. (2013) 'Transcriptional/epigenetic regulator CBP/p300 in tumorigenesis: structural and functional versatility in target recognition', *Cellular and Molecular Life Sciences*, 70(21), pp. 3989–4008. Available at: https://doi.org/10.1007/s00018-012-1254-4.

Wang, Jia *et al.* (2018) 'YY1 Positively Regulates Transcription by Targeting Promoters and Super-Enhancers through the BAF Complex in Embryonic Stem Cells', *Stem Cell Reports*, 10(4), pp. 1324–1339. Available at: https://doi.org/10.1016/j.stemcr.2018.02.004.

Wang, Z. *et al.* (2008) 'Combinatorial patterns of histone acetylations and methylations in the human genome', *Nature Genetics*, 40(7), pp. 897–903. Available at: https://doi.org/10.1038/ng.154.

Warren, R.P., Stembridge, A.M. and Gardner, E.J. (1985) 'Deficient immune function of peripheral blood mononuclear cells from patients with Gardner syndrome', *Clinical and Experimental Immunology*, 60(3), pp. 525–531.

Watson, N.F.S. *et al.* (2006) 'Expression of the stress-related MHC class I chain-related protein MICA is an indicator of good prognosis in colorectal cancer patients', *International Journal of Cancer*, 118(6), pp. 1445–1452. Available at: https://doi.org/10.1002/ijc.21510.

Wei, D. *et al.* (2005) 'Emerging role of KLF4 in human gastrointestinal cancer', *Carcinogenesis*, 27(1), pp. 23–31. Available at: https://doi.org/10.1093/carcin/bgi243.

Wei, D. *et al.* (2016) 'KLF4 Is Essential for Induction of Cellular Identity Change and Acinar-to-Ductal Reprogramming during Early Pancreatic Carcinogenesis', *Cancer Cell*, 29(3), pp. 324–338. Available at: https://doi.org/10.1016/j.ccell.2016.02.005.

Weinert, B.T. *et al.* (2018) 'Time-Resolved Analysis Reveals Rapid Dynamics and Broad Scope of the CBP/p300 Acetylome', *Cell*, 174(1), pp. 231-244.e12. Available at: https://doi.org/10.1016/j.cell.2018.04.033.

Wensveen, F.M., Jelenčić, V. and Polić, B. (2018) 'NKG2D: A Master Regulator of Immune Cell Responsiveness', *Frontiers in Immunology*, 9, p. 441. Available at: https://doi.org/10.3389/fimmu.2018.00441.

Wieduwilt, M.J. *et al.* (2019) 'Histone Deacetylase Inhibition with Panobinostat Combined with Intensive Induction Chemotherapy in Older Patients with Acute Myeloid Leukemia: Phase I Study Results', *Clinical Cancer Research*, 25(16), pp. 4917–4923. Available at: https://doi.org/10.1158/1078-0432.CCR-19-0171.

Wierer, M. and Mann, M. (2016) 'Proteomics to study DNA-bound and chromatin-associated gene regulatory complexes', *Human Molecular Genetics*, 25(R2), pp. R106–R114. Available at: https://doi.org/10.1093/hmg/ddw208.

Wilson, A.J. *et al.* (2006) 'Histone Deacetylase 3 (HDAC3) and Other Class I HDACs Regulate Colon Cell Maturation and p21 Expression and Are Deregulated in Human Colon Cancer', *Journal of Biological Chemistry*, 281(19), pp. 13548–13558. Available at: https://doi.org/10.1074/jbc.M510023200.

Wu, J. *et al.* (1999) 'An Activating Immunoreceptor Complex Formed by NKG2D and DAP10', *Science*, 285(5428), pp. 730–732. Available at: https://doi.org/10.1126/science.285.5428.730.

Wu, Z. *et al.* (2021) 'Targeting the NKG2D/NKG2D-L axis in acute myeloid leukemia', *Biomedicine & Pharmacotherapy*, 137, p. 111299. Available at: https://doi.org/10.1016/j.biopha.2021.111299.

Xu, Q. *et al.* (2021) 'KLF4 Inhibits the Differentiation of Goat Intramuscular Preadipocytes Through Targeting C/EBPβ Directly', *Frontiers in Genetics*, 12, p. 663759. Available at: https://doi.org/10.3389/fgene.2021.663759.

Yamaguchi, K. *et al.* (2012) 'Diagnostic and prognostic impact of serum-soluble UL16binding protein 2 in lung cancer patients', *Cancer Science*, 103(8), pp. 1405–1413. Available at: https://doi.org/10.1111/j.1349-7006.2012.02330.x.

Yamanegi (2010) 'Sodium valproate, a histone deacetylase inhibitor, augments the expression of cell-surface NKG2D ligands, MICA/B, without increasing their soluble forms to enhance susceptibility of human osteosarcoma cells to NK cell-mediated cytotoxicity', *Oncology Reports*, 24(6). Available at: https://doi.org/10.3892/or_00001026.

Yao, Y.-L., Yang, W.-M. and Seto, E. (2001) 'Regulation of Transcription Factor YY1 by Acetylation and Deacetylation', *Molecular and Cellular Biology*, 21(17), pp. 5979–5991. Available at: https://doi.org/10.1128/MCB.21.17.5979-5991.2001.

Yasunaga, J. *et al.* (2004) 'Identification of Aberrantly Methylated Genes in Association with Adult T-Cell Leukemia', *Cancer Research*, 64(17), pp. 6002–6009. Available at: https://doi.org/10.1158/0008-5472.CAN-04-1422.

Yeo, M. *et al.* (2021) 'Repurposing cancer drugs identifies kenpaullone which ameliorates pathologic pain in preclinical models via normalization of inhibitory neurotransmission', *Nature Communications*, 12(1), p. 6208. Available at: https://doi.org/10.1038/s41467-02126270-3.

Yet, S.-F. *et al.* (1998) 'Human EZF, a Krüppel-like Zinc Finger Protein, Is Expressed in Vascular Endothelial Cells and Contains Transcriptional Activation and Repression Domains', *Journal of Biological Chemistry*, 273(2), pp. 1026–1031. Available at: https://doi.org/10.1074/jbc.273.2.1026.

Yoon, H.S., Chen, X. and Yang, V.W. (2003) 'Krüppel-like Factor 4 Mediates p53dependent G1/S Cell Cycle Arrest in Response to DNA Damage', *Journal of Biological Chemistry*, 278(4), pp. 2101–2105. Available at: https://doi.org/10.1074/jbc.M211027200.

Yu, F. *et al.* (2011) 'Kruppel-like factor 4 (KLF4) is required for maintenance of breast cancer stem cells and for cell migration and invasion', *Oncogene*, 30(18), pp. 2161–2172. Available at: https://doi.org/10.1038/onc.2010.591.

Yu, T. *et al.* (2016) 'KLF4 regulates adult lung tumor-initiating cells and represses K-Rasmediated lung cancer', *Cell Death & Differentiation*, 23(2), pp. 207–215. Available at: https://doi.org/10.1038/cdd.2015.85.

Zhang, R. *et al.* (2010) 'Krüppel-like factor 4 interacts with p300 to activate mitofusin 2 gene expression induced by all-trans retinoic acid in VSMCs', *Acta Pharmacologica Sinica*, 31(10), pp. 1293–1302. Available at: https://doi.org/10.1038/aps.2010.96.

Zhang, X. *et al.* (2020) 'Low-Dose Gemcitabine Treatment Enhances Immunogenicity and Natural Killer Cell-Driven Tumor Immunity in Lung Cancer', *Frontiers in Immunology*, 11, p. 331. Available at: https://doi.org/10.3389/fimmu.2020.00331.

Zhao, Y.-K. *et al.* (2015) 'Expression and clinical value of the soluble major histocompatibility complex class I-related chain A molecule in the serum of patients with renal tumors', *Genetics and Molecular Research*, 14(2), pp. 7233–7240. Available at: https://doi.org/10.4238/2015.June.29.16.

Zhou, Q., Gedrich, R.W. and Engel, D.A. (1995) 'Transcriptional repression of the c-fos gene by YY1 is mediated by a direct interaction with ATF/CREB', *Journal of Virology*, 69(7), pp. 4323–4330. Available at: https://doi.org/10.1128/jvi.69.7.4323-4330.1995.

Zohre, S. *et al.* (2014) ‘Trichostatin A-induced Apoptosis is Mediated by Krüppel-like Factor
4 in Ovarian and Lung Cancer’, *Asian Pacific Journal of Cancer Prevention*, 15(16), pp. 6581–6586. Available at: https://doi.org/10.7314/APJCP.2014.15.16.6581.

Zupkovitz, G. *et al.* (2010) ‘The Cyclin-Dependent Kinase Inhibitor p21 Is a Crucial Target for Histone Deacetylase 1 as a Regulator of Cellular Proliferation’, *Molecular and Cellular Biology*, 30(5), pp. 1171–1181. Available at: https://doi.org/10.1128/MCB.01500-09.

Abbreviations

‘i’	Inhibitor
Ara-C	Cytarabin
‘ac-’	Acetyl-
ATM	Ataxia telangiectasia mutated
ATR	Ataxia telangiectasia and Rad3-related
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
dCas9	Nuclease-dead Cas9
CBP	CREB-binding protein
CGK	CGK733 (ATM/ATRi)
ChIP	Chromatin immunoprecipitation
MS	Mass spectrometry
NGS	Next-generation sequencing
enChIP	Engineered DNA-binding molecule-mediated ChIP
CAPTURE	CRISPR affinity purification in situ of regulatory elements
c-MYC	Avian myelocytomatosis virus oncogene cellular homolog
CREB	cAMP response element-binding protein
p300	E1A-associated protein p300
CRISPR	Clustered regularly interspaced short palindromic repeats
CRISPRa	CRISPR activation
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
CDK	Cyclin-dependent kinase
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
H3	Histone H3
H4	Histone H4
H2A.Z	Histone variant H2A
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HDACi	HDAC inhibitor
HEK293	Human embryonic kidney
IgG	Immunoglobulin G

MHC	Major histocompatibility complex
MICA/B	MHC class I polypeptide-related sequence A/B
NF-κB	Nuclear factor κ-light-chain-enhancer of activated B cells
NK cell	Natural killer cell
NKG2D	Natural killer group 2, member D
PARP1	Poly [ADP-ribose] polymerase 1
RPL30	60S ribosomal protein L30
TP53	Tumor protein p53
LBH589	LBH589/Panobinostat (HDACi)
TSA	Trichostatin A (HDACi)
VPA	Valproic acid
ULBP	UL16-binding protein
WB	Western Blot
TF	Transcription factor
TGFβ	Transforming growth factor β
TNFα	Tumor necrosis factor α
RT-qPCR	Real time quantitative polymerase chain reaction
PAM	Protospacer adjacent motif
TSS	Transcriptional start site
APTO253	LOR 253
NKG2D-L	NKG2D Ligand
KLF4	Krüppel- like factor
YY1	Yin Yang 1
DDR	DNA damage response
AML	Acute myeloid leukemia
MLF2	Myeloid leukemia factor 2
LSCs	Leukemia stem cells
iPS	induced pluripotent stem cell

IFNy	Interferon-gamma
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
mRNA	Messenger-RNA
sgRNA	Single guide RNA

siRNA	Small interfering RNA
HSF 1	Heat shock transcription factor
CGK	CGK733 (ATM/ATRi)
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibition motif
PBS	Phosphate buffered saline
mAb	Monoclonal antibody
GFP	Green Fluorescent Protein
HBG	Human-beta-Globin
Ken	Kenpaullone
nM	Nano Molar
µL	Micro liter
WT	Wild type
WT-KLF4	Doxycycline-responsive transactivator KLF4
DN-KLF4	Dominant-negative KLF4
Dox	Doxycycline
FDA	Food and Drug Administration
ns	Not-significant
TNF-α	Tumor nicrosis factor-α
TGF	Transforming growth factor
KD	Knock down
KO	Knock out
KDa	Kilo Dalton
HRP	Horseradish peroxidase
pb	Base pairs
Ctrl	Control
IgG	Immunoglobulin G
IL	Interleukin
DNA	Deoxyribonucleic acid
DMEM	Dulbecco`s modified eagle medium
FCS	Fetal calf serum
Pen/Strep	Penicillin/Streptomycin

Curriculum vitae

Name: Reem Al khayer
Date of birth: 25.06.1988
Place of birth: Latakia, Syria
Nationality: Syrian
E. mail: Reem.alkhayer88@gmail.com

Education

20.06.2017-Today: Ph.D. Student at the Philipps University, Marburg.
2015-2016: Master of Science in Pharmacy at Saarland University, Saarbrücken, Germany.
2008-2013: Bachelor of Pharmacy and Pharmaceutical Chemistry at Tishreen University, Latakia, Syria.

Publications

1. Reem Alkhayer 1,2, Viviane Ponath 1,2, Miriam Frech 2, Till Adhikary 3, Johannes Graumann 4, Andreas Neubauer 2 and Elke Pogge von Strandmann 1,2*,"KLF4-mediated upregulation of the NKG2D ligand MICA in acute myeloid leukemia: a novel therapeutic target identified by enChIP". **In revision: Cell** Communication and Signaling.

2. Viviane Ponath 1, Miriam Frech 2, Mathis Bittermann 1, **Reem Al Khayer** 1, Andreas Neubauer 2, Cornelia Brendel 2, Elke Pogge von Strandmann 1, "The Oncoprotein SKI Acts as A Suppressor of NK Cell-Mediated Immunosurveillance in PDAC," Cancers, vol. 12, no. 10, p. 2857, Oct. 2020, doi: 10.3390/cancers12102857.

3. Sharoon Griffin 1,2, **Reem Alkhayer 1**, Seda Mirzoyan 3, Astghik Turabyan 3, Paolo Zucca 4 ID, Muhammad Sarfraz 1, Muhammad Jawad Nasim 1, Armen Trchounian 3, Antonio Rescigno 4 Cornelia M. Keck 2 and Claus Jacob 1*, "Nanosizing Cynomorium: Thumbs up for Potential Antifungal Applications," Inventions, vol. 2, no. 3, p. 24, Sep. 2017, doi: 10.3390/inventions2030024.

4. Sharoon Griffin 1 2, Nassifatou Koko Tittikpina 3 4 5 6, Adel Al-Marby 7, **Reem Alkhayer 8**, Polina Denezhkin 9, Karolina Witek 10, Koffi Apeti Gbogbo 11, Komlan Batawila 12, Raphaël Emmanuel Duval 13 14 15, Muhammad Jawad Nasim 16, Nasser A Awadh-Ali 17, Gilbert Kirsch 18 19, Patrick Chaimbault 20 21, Karl-Herbert Schäfer 22, Cornelia M Keck 23, Jadwiga Handzlik 24, Claus Jacob 25, "Turning Waste into Value: Nanosized Natural Plant Materials of Solanum incanum L. and Pterocarpus erinaceus Poir with Promising Antimicrobial Activities," Pharmaceutics, vol. 8, no. 2, p. 11, Apr. 2016, doi: 10.3390/pharmaceutics8020011.

Verzeichnis der akademischen Lehrer

Meine akademischen Lehrenden in Latakia, Syrien waren: Makhus, Alkhayer, Awama, Salama, Nasser, Haroun, Al-Haushey, Abbood, Ames, Morie, Zrieki, Assad, Mansour, Youssef, Hammad, Yazji, Araaj, kamoua, Hajouze, Sliman, Aboud, Ammar, Diab, Abbas, Yaseen, Mohammad, Ibrahem, AL-Hanuon, Alshamaa, Madani, Hussein, Zyzafoun, Suleiman, Bassma, Deeb.

Mein akademischer Lehrer im Saarland, Deutschland war: Claus Jacob.

Acknowledgment

First and foremost, I would like to express my sincere gratitude to my advisor Prof. Dr. Elke Pogge von Strandmann for the unlimited support, patience, motivation, and immense knowledge. Her guidance has helped me throughout the research and the writing of this thesis. Her office door had always been open whenever I encountered any difficulties or had questions. I could not have asked for a better supervisor as I have learned a lot from her during my Ph.D. time. If it had not been for her invaluable guidance, the endeavor would not have been accomplished. She has positively inspired me in all aspects of life and has become my idol over time.

Undoubtedly, such a great mentor must have a brilliant group. Therefore, I also thank the Pogges´ group one by one for the warm familial atmosphere that makes this group what it is. Kathrin, much thanks for always being so supportive and bringing joy into my life over the past five years with your astonishing surprises. I am also grateful for having you beside me, Steffi. Your sweet words and warm hugs have always helped to push me forward. Viviane, I am so grateful for your scientific contribution and for proofreading this thesis. Christian thanks a lot for always being supportive and friendly. I wholeheartedly thank each of Manuel, Maria (my office mate), Bilal, Karen, Petra, Klaus and Leoni for being such encouraging and lovely friends.

A special thank goes to Dr. Till Adhikary for his great pieces of advice and his unlimited support, and I would extend thanks to his wife, Dr. Sovana.

I would like to thank all the great people on the third floor of ZTI who were always ready to help and support us as much as possible. Thank you, Bernhard, for the support and the positive energy you spread everywhere at ZTI.

Thank you, Leah, for being there when I needed you the most.

Also, thanks to Anna, Anna Brischkina, Karie, Margitta, Sonja, Roman and Suresh.

This acknowledgment cannot go without an appreciation from the bottom of my heart for my best friend, Bastian, for his continued support and motivation.

I would like to thank Michelle for being my best friend in Germany. You will be forever remembered as long as I live. Thank you, Maulik; it was such a great time during the first year of my Ph.D. You have been a one-of-a-kind team member. Thank you, Helga; you have proved that friendship is not limited to a time or place.

I am incredibly thankful to Dr. Samr Mkhlof and Somia, who were like a family to me here in Marburg. I sincerely thank my cousin Dr. Rabeea Hasan and his family for helping me to be in Germany and for their support at the beginning of my journey here. At the end, nothing would have been possible without my dear parents, Ali and Hind. Dad; I know you are watching over me from up above. You were always my soul source of strength and pride whenever I felt down. My heroic mom, I could not have undertaken this journey without your constant belief and the huge amounts of unconditional love that you have passed to me. Many thanks to my whole family and friends in Syria. Special thanks to Iyad for being such a great brother, friend, and partner who has my back. Lastly, I am beyond grateful to my homeland Syria for everything.

Ehrenwörtliche Erklärung

Ich erkläre ehrenwörtlich, dass ich die dem Fachbereich Medizin Marburg zur

Promotionsprüfung eingereichte Arbeit mit dem Titel „Deciphering the transcription factors that regulate the NKG2D ligand MICA in cancer cells“ im Institut für Tumorimmunologie unter Leitung von Prof. Dr. Elke Pogge von Strandmann ohne sonstige Hilfe selbst durchgeführt und bei der Abfassung der Arbeit keine anderen als die in der Dissertation aufgeführten Hilfsmittel benutzt habe. Ich habe bisher an keinem in- oder ausländischen Medizinischen Fachbereich ein Gesuch um Zulassung zur Promotion eingereicht, noch die vorliegende oder eine andere Arbeit als Dissertation vorgelegt. Ich versichere, dass ich sämtliche wörtlichen oder sinngemäßen Übernahmen und Zitate kenntlich gemacht habe.

Mit dem Einsatz von Software zur Erkennung von Plagiaten bin ich einverstanden.

Vorliegende Arbeit wurde in folgenden Publikationsorganen veröffentlicht oder eingereicht:

Ort, Datum, Unterschrift Doktorandin/Doktorand

Die Hinweise zur Erkennung von Plagiaten habe ich zur Kenntnis genommen.

Ort, Datum, Unterschrift Referentin/Referent
