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Histone deacetylase 2-mediated deacetylation of the Ribonuclease 1 promoter in inflamed human endothelial cells

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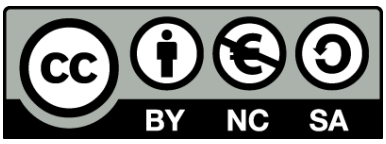

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Summary

Endothelial cells (ECs) function as protective barrier to separate the blood from the surrounding tissue by conducting crucial roles in regulation and maintenance of vascular homeostasis, such as control of vessel permeability or coagulation. Therefore, dysfunction of the EC barrier due to inflammation, infection or injury can cause a variety of vascular pathologies, such as thrombosis or atherosclerosis. In this context, the circulating extracellular endonuclease Ribonuclease 1 (RNase1) was identified as a vessel- and tissue-protective enzyme and a potent regulator of vascular homeostasis. Upon acute inflammation, RNase1 functions as a natural counterpart to extracellular RNA (eRNA), a damage-associated molecular pattern, via degradation to protect the EC cell layer from excessive inflammation. However, long-term inflammation disrupts the RNase1-eRNA system. Thereby, eRNA accumulates in the extracellular space to induce massive proinflammatory cytokine release from circulating inflammatory cells, such as tumor necrosis factor alpha (TNF-α) or interleukin 1 beta (IL-1β). These cytokines negatively affect the EC layer by downregulation of RNase1 presumably through activation of histone deacetylases (HDACs). In this regard, this study investigated whether inflammation-mediated deacetylase function of HDACs suppresses RNase1 expression in human ECs through modulation of chromatin modifications. Proinflammatory stimulation with TNF-α or IL-1β of human umbilical vein endothelial cells significantly reduced RNase1 expression. Thus, identification of the *RNASE1* promoter region and analysis of its chromatin state revealed the association of *RNASE1* repression with deacetylation of histone 3 at lysine 27 and histone 4. The important role of HDACs in this process was further confirmed by administration of the specific class I HDAC1-3 inhibitor MS275 that successfully restored *RNASE1* promoter acetylation and mRNA abundance upon TNF-α or IL-1β treatment. These results indicate an essential impact of HDAC1-3 in RNase1 regulation. Additionally, identification of specific HDACs involved in RNase1 regulation was obtained by chromatin immunoprecipitation kinetics confirming significant accumulation of HDAC2 at the *RNASE1* promoter upon TNF-α stimulation. These findings were further validated by siRNA double knockdown of HDAC2 and its redundant enzyme HDAC1, which also recovered RNase1 mRNA abundance upon proinflammatory stimulation. In conclusion, our data identified HDAC2 as a crucial factor in RNase1 regulation in human ECs. HDAC2 is recruited to the *RNASE1* promoter site to attenuate histone acetylation and suppress subsequent gene repression. This effect can be blocked by the specific HDAC inhibitor MS275 implicating the potential of HDAC inhibitors as novel therapeutic strategy to promote vascular integrity by preventing RNase1 downregulation in EC inflammation.

Zusammenfassung

Endothelzellen fungieren als schützende Barriere zwischen Blut und Gewebe und sind maßgeblich an der Regulation und Aufrechterhaltung der vaskulären Homöostase beteiligt. Demzufolge kann eine Fehlfunktion des Endothels auf Grund von Entzündungen, Infektionen oder Verletzungen zu einer Vielzahl von Krankheitszuständen, wie Thrombosen oder Atherosklerose, führen. In diesem Kontext wurde Ribonuklease 1 (RNase1), eine zirkulierende, extrazelluläre Endonuklease, als gefäß- und gewebeschützendes Enzym und wichtiger Regulator der vaskulären Homöostase identifiziert. Bei akuten Entzündungsprozessen fungiert RNase1 als natürlicher Gegenspieler der extrazellulären RNA (eRNA), einem „*damage-associated molecular pattern*“. Hierbei degradiert RNase1 akkumulierende eRNA, um die Zellen vor einer übermäßigen Entzündungsreaktion zu schützen. Bei lang anhaltenden Entzündungen kommt es jedoch zu einer Störung des RNase1-eRNA Gleichgewichtes. Dabei induziert die Akkumulation von eRNA die Freisetzung großer Mengen proinflammatorischer Zytokine durch zirkulierende Entzündungszellen, wie zum Beispiel Tumornekrosefaktor alpha (TNF-α) oder Interleukin 1 beta (IL-1β). Diese Zytokine können das Endothel entzündungsbedingt beeinflussen und führen unter Anderem zur Repression der RNase1-Expression, welche möglicherweise durch die Aktivierung von Histon-Deacetylasen (HDACs) vermittelt wird. Die hier vorliegende Studie untersuchte, ob die entzündungsvermittelte Deacetylasefunktion der HDACs durch Veränderung von Chromatinmodifikationen mit der RNase1-Repression in humanen Endothelzellen einhergeht. Dabei führte die Stimulation humaner Nabelschnurvenenendothelzellen mit TNF-α oder IL-1β zu einer signifikanten Reduktion der RNase1-Expression. Durch Identifizierung der *RNASE1*-Promotorregion und Analysen des Chromatinstatus wurde weiterhin eine Verbindung zwischen der RNase1-Repression und der Deacetylierung von Histon 3 Lysin 27 und Histon 4 aufgezeigt. Zusätzlich konnte durch Inhibierung der Klasse I-HDACs 1-3, mittels des spezifischen Inhibitors MS275, die RNase1 mRNA sowie die damit verbundene Promotoracetylierung selbst nach Zytokinstimulation der Endothelzellen wiederhergestellt werden. Zur Identifizierung der funktionellen HDAC wurden Chromatin-Immunopräzipitations-Kinetiken durchgeführt, welche eine signifikante Akkumulation von HDAC2 am *RNASE1*-Promotor nach TNF-α-Stimulation aufzeigten. Diese Ergebnisse wurden durch siRNA-vermittelte Hemmung der HDAC2-Expression und dessen redundanten Enzyms HDAC1 bestätigt, da auch diese Behandlung die RNase1 mRNA-Expression nach proinflammatorischer Stimulation wiederherstellen konnte. Diese Ergebnisse identifizieren HDAC2 als einen Hauptfaktor in der RNase1-Regulation in humanen Endothelzellen. Dabei wird HDAC2 nach proinflammatorischer Stimulation zum *RNASE1*-Promotor rekrutiert, um die Histonacetylierung zu verhindern und so die Genexpression zu reprimieren. Demzufolge eröffnet der hier aufgezeigte protektive Effekt des spezifischen HDAC-Inhibitors MS275 neuartige Therapieansätze zur Förderung der Gefäßintegrität durch Verhinderung der RNase1-Repression in entzündeten Endothelzellen.

List of Abbreviations

ac	acetylation
bp	base pairs
ChIP	Chromatin immunoprecipitation
CK2	casein kinase 2
CoREST	REST co-repressor complex
DNA	deoxyribonucleic acid
EC	endothelial cell
eRNA	extracellular RNA
E-selectin	endothelial selectin
e.g.	*exempli gratio,* for example
et al.	*et alii*
Fig.	Figure
H	histone
H3K27	histone 3 lysine 27
H4	histone 4
HAT	histone acetyltransferase
HDAC	histone deacetylase
HUVEC	Human Umbilical Vein Endothelial Cells
ICAM	intracellular adhesion molecule
IFN	interferon
IL	interleukin
JNK	c-Jun N-terminal kinase
KD	knockdown
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinase
mRNA	messenger RNA
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NuRD	Nucleosome Remodeling and Deacetylase complex
p38	p38 mitogen activated protein kinase
Pol II	RNA Polymerase II
POLR2A	Pol II subunit 2A
Poly I:C	polyinosinic polycytidylic acid
Pr	promoter
PTM	post translational modification
qRT-PCR	quantitative reverse transcription polymerase chain reaction
RNase1	Ribonuclease 1
RNaseA	Ribonuclease A
Sin3	Sin3 co-repressor complex
siRNA	small interfering RNA
TGF	Transforming growth factor
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha
TSS	Transcriptional start site
UCSC	University of California, Santa Cruz
WHO	World Health Organization
WPB	Weibel-Palade Body

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1 Introduction

1.1 Inflammation of endothelial cells

The endothelial cell (EC) layer forms the inner lining of all blood vessels and functions as physical barrier to separate the blood from the surrounding tissue. Thereby, ECs conduct multiple functions in regulating maintenance and integrity of vascular homeostasis, such as control of vessel permeability, blood fluidity, or coagulation and platelet function (Pober and Sessa, 2007; Rajendran *et al.*, 2013). Under physiological conditions, resting ECs avoid interaction with circulating leukocytes. Thereby, they sequester leukocyte interactive proteins, like adhesion molecules or chemokines, within their secretory vesicles called Weibel-Palade Bodies (WPBs) (Pober and Sessa, 2007; Rondaij *et al.*, 2006), and suppress transcription of membrane bound adhesion molecules, (*e.g.* endothelial selectin (E-selectin), intracellular adhesion molecule (ICAM)-1) or proinflammatory cytokines to prevent leukocyte interaction (De Caterina *et al.*, 1995; Pober and Sessa, 2007). Upon inflammation, ECs get rapidly activated to support the recruitment, attachment and interaction with leukocytes, for instance via initiation of WPB exocytosis and activation of proinflammatory gene expression to release leukocyte attractant chemokines and transport adhesion molecules to the EC surface. Consequently, leukocytes interact with the EC layer to leave the vessel and infiltrate the tissue, thereby secreting further proinflammatory cytokines, such as tumor necrosis factor alpha (TNF-α) or interleukin (IL)-1β (Pober and Sessa, 2007). Due to this inflammation, the homeostatic function of the EC layer may be affected resulting in dysfunction of the endothelium, which favors progression of vascular diseases, such as atherosclerosis, thrombosis or myocardial infarction (Poredos, 2002; Sitia *et al.*, 2010; Zernecke and Preissner, 2016). In this regard, cardiovascular pathologies depict one of the main causes of death worldwide with several million fatalities per year according to the World Health Organization (WHO) (WHO, 2017).

1.2 RNase1 function in endothelial cells

Previous research concerning cardiovascular pathologies, such as atherosclerosis or thrombosis, described the important function of a novel protective factor during EC inflammation called Ribonuclease (RNase1) (Cabrera-Fuentes *et al.*, 2014; Fischer *et al.*, 2007; Kannemeier *et al.*, 2007; Simsekyilmaz *et al.*, 2014; Zernecke and Preissner, 2016). RNase1 is one of eight secretable members of the mammalian ribonuclease A (RNaseA) superfamily that possesses ribonuclease activity (Koczera *et al.*, 2016; Sorrentino, 2010). The biological relevance of the RNaseA superfamily varies from host defense to angiogenesis and digestion. However, the precise physiological functions of RNases need to be further investigated (Koczera *et al.*, 2016). RNase1, also known as RNaseA or pancreatic-type RNase, is produced in various tissues, *e.g.* pancreas, testis, ovary or brain (Futami *et al.*, 1997; Koczera *et al.*, 2016; Landre *et al.*, 2002; Moenner *et al.*, 1997), and was shown to be predominantly expressed by vascular ECs where it is stored and released from WBPs (Fischer *et al.*, 2011; Futami *et al.*, 1997; Landre *et al.*, 2002; Lu *et al.*,

2018). Additionally, previous studies indicated that especially human umbilical vein endothelial cells (HUVEC) express and secrete high amounts of active RNase1 (Fischer *et al.*, 2011; Landre *et al.*, 2002), offering an ideal model system to study RNase1 function and regulation. Due to the high secretory levels of RNase1, a non-digestive function of this enzyme was suggested in humans and its endothelial origin pointed towards a regulatory role in maintenance of vascular homeostasis (Fischer *et al.*, 2007; Kannemeier *et al.*, 2007; Landre *et al.*, 2002; Sorrentino *et al.*, 2003).

RNase1 function in acute and long-term EC inflammation

The predicted role of RNase1 in regulation and maintenance of vascular homeostasis was further confirmed by Gansler *et al.* who firstly described a regulatory mechanism for RNase1 in inflamed endothelial cells, thereby revealing its importance as regulatory factor for maintenance and integrity of vascular homeostasis (Gansler *et al.*, 2014). In acute inflammation (Figure IA)[1], ECs release leukocyte interactive proteins and chemokines as well as a subset of damage-associated molecular patterns, such as extracellular RNA (eRNA). Thereby, the danger-associated signal eRNA is released to the extracellular space to induce the immune response[2] (Fischer *et al.*, 2013; Fischer *et al.*, 2012; Gansler *et al.*, 2014; Zernecke and Preissner, 2016), and further mediate degranulation of WPBs[3] from ECs resulting in release of proinflammatory agents and RNase1[4]. Once released, RNase1 acts as a natural counterpart to eRNA via degradation[5] to protect the EC layer from eRNA-mediated overwhelming inflammatory responses (Cabrera-Fuentes *et al.*, 2015b; Cabrera-Fuentes *et al.*, 2014; Gansler *et al.*, 2014). However, upon long-term inflammation, the balance of the RNase1-eRNA system gets disturbed (Figure IB). Prolonged inflammation of ECs results in accumulation of eRNA in the extracellular space, which promotes recruitment of inflammatory cells, such as monocytes, to the site of inflammation[1] (Cabrera-Fuentes *et al.*, 2015a; Fischer *et al.*, 2013; Gansler *et al.*, 2014). These inflammatory cells release high amounts of proinflammatory cytokines, like TNF-α and IL-1β[2], which further contribute to a disturbed EC barrier function by increasing vascular permeability through redistribution of adhesion molecules[3] as well as massive downregulation of RNase1[4] (Gansler *et al.*, 2014). Consequently, RNase1 protein translation, its storage and release from WPBs[5] as well as its function in eRNA degradation is impaired resulting in subsequent eRNA accumulation and EC dysfunction[6] (Gansler *et al.*, 2014).

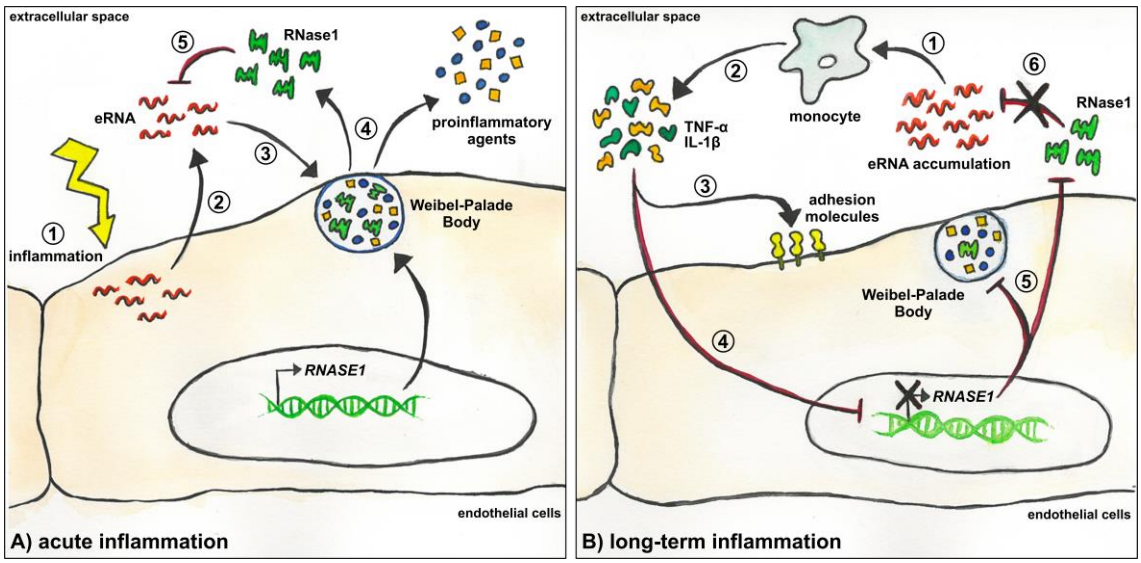

Figure I: RNase1 function in acute and long-term endothelial cell inflammation. Schematic illustration of RNase1 function in inflamed endothelial cells (ECs). A) eRNA release upon acute inflammation of ECs mediates degranulation of Weibel-Palade Bodies to release RNase1 and proinflammatory agents to protect vascular homeostasis. B) Upon long-term inflammation, eRNA accumulation promotes proinflammatory cytokine secretion of recruited monocytes that further affect EC function by redistribution of adhesion molecules and downregulation of RNase1 to block eRNA degradation. Abbreviations: eRNA, extracellular RNA; IL-1β, interleukin 1 beta; RNase1, Ribonuclease1; TNF-α, tumor necrosis factor alpha.

1.3 Chromatin structure and dynamics

Compaction of the human genome is a necessity in order to pack the large amount of DNA into a single nucleus and to ensure the precise function of all regulatory processes in the cell, such as DNA replication, repair or transcription (Luger *et al.*, 2012). Thereby, the DNA passes through different stages of compaction, starting with the DNA double helix, through chromatin and chromatin fibers of packed nucleosomes, to sections of extended and condensed chromosomes, ending up with the entire mitotic chromosome structure (Figure IIA) (Fyodorov *et al.*, 2018). A particularly important packaging level is the so called “beads on a string” structure of chromatin, where the DNA is coiled on beads to form the nucleosomes, the basic repeat units of the chromatin. Each nucleosome core particle consists of approximately 146 base pairs (bp) of DNA, which are wrapped around an octameric core of histone (H) proteins (Luger *et al.*, 2012; Luger *et al.*, 1997). There exist five histone proteins, H1, H2A, H2B, H3 and H4, that built up the octameric nucleosome core by two H2A-H2B dimers and one H3-H4 tetramer, while the linker histone H1 together with the linker DNA connects the individual nucleosomes to form higher organizational units of DNA (Jenuwein and Allis, 2001; Luger *et al.*, 2012; Luger *et al.*, 1997). These small, highly conserved, basic proteins contain positively charged N-terminal tails that are highly subjected to post-translational modifications (PTMs), such as phosphorylation of serine residues, methylation of arginine residues or acetylation of lysine residues (Kouzarides, 2007). Thereby, PTMs of the core histones are decisive for regulation of the chromatin structure and function and

adjust several DNA-related processes, such as transcription or replication (Bannister and Kouzarides, 2011; Fyodorov *et al.*, 2018; Grewal and Elgin, 2007; Portela and Esteller, 2010).

HATs and HDACs as modulators of histone acetylation

One of the first identified histone modifications, discovered by Allfrey *et al.* in 1964, was the acetylation (ac) of lysine residues (Allfrey *et al.*, 1964; Gershey *et al.*, 1968), which acts as critical regulator of gene expression (Durrin *et al.*, 1991; Megee *et al.*, 1990; Roh *et al.*, 2005). Acetylations thus function as specific markers for active and transcribed chromatin, such as histone 4 acetylation (H4ac) and histone 3 lysine 27 acetylation (H3K27ac) (Kouzarides, 2007; Wang *et al.*, 2008). The enzymes that modulate the pattern of histone acetylation are histone acetyl transferases (HATs) and histone deacetylases (HDACs) (Figure IIB). Due to their enzymatic function, HATs and HDACs facilitate the addition or removal of acetyl groups on lysine residues of N-terminal histone tails to modulate the intensity of histone-DNA interactions (Bannister and Kouzarides, 2011; Fyodorov *et al.*, 2018; Grewal and Elgin, 2007). HATs transfer acetyl groups to lysine residues to decrease the positive histone charge and weaken the interaction of histones with the negatively charged DNA. Thereby, providing access for the transcriptional machinery to the DNA by opening the densely packed chromatin structure (Zentner and Henikoff, 2013). *Vice versa*, deacetylation of lysine residues is catalyzed through HDACs to amplify the histone-DNA interaction by enhancing the positive histone charge. This provokes strong histone-DNA interactions and a tightly packed, inactive chromatin structure, which ultimately leads to transcriptional repression (Grewal and Elgin, 2007; Seto and Yoshida, 2014).

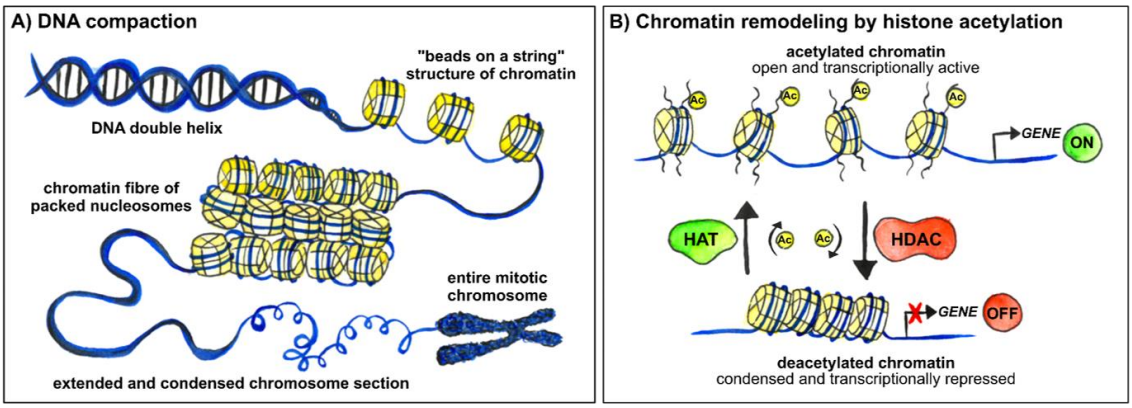

Figure II: Chromatin structure and dynamics. Schematic illustration of A) DNA packaging and B) chromatin remodeling by histone acetylation. A) Different stages of DNA compaction to mediate packaging of a large amount of DNA into the nucleus of a cell. B) Histone acetylation on lysine residues of N-terminal histone tails is mediated by HATs to promote an open chromatin structure and gene expression. *Vice versa*, histone deacetylation is mediated by HDACs to promote a condensed chromatin structure and transcriptional repression. Abbreviations: Ac, acetylation; DNA, deoxyribonucleic acid; HAT, histone acetyl transferase; HDAC, histone deacetylase.

Histone deacetylases as erasers of histone acetylation

HDAC enzymes were firstly described in 1969 (Inoue and Fujimoto, 1969), however, it took several decades until the first enzyme, HDAC1, was isolated by Taunton and colleagues and identified as orthologue of yeast Rpd3 protein (Taunton *et al.*, 1996). The family of histone deacetylases consists of so far 18 members, divided into 4 distinct classes of enzymes that are distinguished based on their sequence similarity (de Ruijter *et al.*, 2003; Micelli and Rastelli, 2015): Class I enzymes, HDAC1, HDAC2, HDAC3 and HDAC8, share sequence homology with the yeast Rpd3 protein, while class II enzymes, including class IIa (HDAC4, HDAC5, HDAC7 and HDAC9) and class IIb (HDAC6 and HDAC10), share sequence similarity with yeast Hda1 protein. Compared to that, the class III HDAC proteins SIRT1-7 belong to the conserved family of sirtuins (Sir2 deacetylases), sharing homology with yeast Sir2, while class IV consists of only one enzyme, HDAC11, with limited overall sequence identity to other HDAC classes (Micelli and Rastelli, 2015; Seto and Yoshida, 2014; Yang and Seto, 2008). Besides their important function in histone post-translational modification, HDACs can also post-translationally modulate a variety of non-histone proteins, such as transcription factors to regulate a multitude of cellular processes (Seto and Yoshida, 2014). Especially class I HDACs were described to conduct a critical role in regulation of diverse inflammatory processes by influencing for example regulation of Toll-like receptor (TLR) signaling, interferon (IFN)-mediated signaling to modulate immune cell proliferation (Kong *et al.*, 2009; Yamaguchi *et al.*, 2010), expression of inflammatory mediators (Elsharkawy *et al.*, 2010; Nusinzon and Horvath, 2003; Pakala *et al.*, 2010) or immune responses against pathogens (Nusinzon and Horvath, 2006; Shakespear *et al.*, 2011). Additionally, several studies also indicated important class I HDAC functions in vascular pathologies, like myocardial infarction or atherosclerosis (Yoon and Eom, 2016; Zampetaki *et al.*, 2010) and are therefore of special interest in inflammation-mediated regulation of gene expression in endothelial cells. Class I HDACs HDAC1, HDAC2, HDAC3 and HDAC8 are ubiquitously expressed enzymes that are mainly located in the nucleus and consist of approximately 400 amino acids (de Ruijter *et al.*, 2003; Micelli and Rastelli, 2015). All these enzymes share high sequence homology to each other, while HDAC1 and HDAC2 are described to be almost identical. The N-terminal catalytic domain of class I HDACs is conserved and requires a zinc ion for catalytic activity (de Ruijter *et al.*, 2003; Yang and Seto, 2008), although, the detailed catalytic mechanism is still unclear and needs to be investigated (Micelli and Rastelli, 2015).

1.4 Aim of this study

Gansler *et al.* found massive downregulation of the protective vascular factor RNase1 upon proinflammatory stimulation of human ECs and provided first insights on RNase1 regulation in this context. They observed the recovery of RNase1 mRNA expression by pretreatment of HUVEC with the universal HDAC inhibitor, Trichostatin A, even upon proinflammatory stimulation. Based on these findings, the authors postulated an HDAC-dependent mechanism for RNase1 regulation in inflamed ECs (Gansler *et al.*, 2014). HDACs are known as important regulators of gene expression by modifying the chromatin structure due to histone deacetylation. In this regard, this study aimed to clarify the underlying mechanism of RNase1 suppression during vascular inflammation on chromatin level and the impact of HDACs in this process. Specific objectives were to identify and localize the *RNASE1* promoter region, analyze its acetylation status and further investigate the regulatory impact of responsible HDACs for histone deacetylation in this region.

2 Results

2.1 Regulation of RNase1 mRNA abundance in inflamed endothelial cells

To investigate the precise regulation of RNase1 mRNA abundance in inflamed ECs, HUVEC were stimulated with proinflammatory cytokines TNF-α and IL-1β [10 ng/ml] over a time course of 0.5 to 24 h. mRNA levels of RNase1 as well as the known EC marker E-selectin were investigated by quantitative real-time polymerase chain reaction (qRT-PCR). Successful stimulation was confirmed by significant increase in E-selectin mRNA expression over time for both stimuli (Supplemental Fig. S1A; see page 40) (Edelstein *et al.*, 2005). In comparison, RNase1 mRNA abundance was decreased after 6 h of cytokine treatment, and further extended to significant downregulation after 9 h and 24 h TNF-α and IL-1β stimulation (Fig. 1A; see page 30). To investigate whether RNase1 downregulation is generally induced upon proinflammatory stimulation or whether this effect seems to be a more precise inflammatory response, HUVEC were treated with a subset of proinflammatory stimulants for 24 h, namely IL-13 [50 ng/ml], IFN-γ [250 ng/ml], transforming growth factor (TGF)-β [1 µg/ml], polyinosinic polycytidylic acid (Poly I:C) [10 µg/ml], lipopolysaccharide (LPS) [100 ng/ml] as well as the trifluoroacetate salt Pam3CSK4 [200 ng/ml]. Although with less extend than TNF-α, a significant decrease in RNase1 mRNA abundance was only observed upon treatment with the TLR3 ligand Poly I:C (Fig. 1B; see page 30). These results suggest, that only certain proinflammatory stimuli severely repress RNase1 mRNA production in inflamed human ECs.

2.2 Inflammation-mediated histone deacetylation of the *RNASE1* promoter

To investigate underlying regulatory mechanisms of *RNASE1* gene expression in inflamed ECs, Gansler *et al.* suggested an HDAC-dependent mechanism (Gansler *et al.*, 2014), implying the participation of altered histone acetylation at the *RNASE1* promoter region. Therefore, *in silico* analysis using Chromatin immunoprecipitation (ChIP)-sequencing data from HUVEC, obtained by the University of California, Santa Cruz, (UCSC) genome browser, were performed to localize the promoter region of *RNASE1*. Here, we focused on specific marks that represent a functional promoter site. Besides a binding site for the RNA polymerase II (Pol II) subunit 2A (POLR2A), the major subunit of the transcription machinery (Cramer *et al.*, 2008; Cramer *et al.*, 2001), the active chromatin mark H3K27ac was identified in close proximity to the different *RNASE1* transcript variants, suggesting a potential transcriptional start site (TSS) in this region (Fig. 2A; see page 31). To further examine whether the aforementioned regions also possess promoter activity, our cooperation partners from the Department of Biochemistry at the Faculty of Medicine at Justus-Liebig-University Giessen generated different *RNASE1* promoter constructs (C1-C4) and analyzed them for their potential to actively induce transcription by luciferase reporter assay (Fig. 2B; see page 31). Thereby, the obtained results indicated transcriptional activity of the *RNASE1* gene regions amplified by construct C2 and C3. Interestingly, both regions harbor not only a binding site for the Pol II transcription machinery and the active chromatin mark H3K27ac

but also the potential TSS of *RNASE1* (Fig. 2A). In addition to the transcriptionally active part of promoters (TSS), whole promoter regions are described to be very large and usually divided into three parts (Wang *et al.*, 2009): the core promoter, which is known to be located approximately 100 bp around the TSS, designated as *region A* for *RNASE1*, and the proximal and distal promoter that are located approximately 500 bp and 1000 bp upstream of the TSS, respectively, designated as *region B* and *region C* of the *RNASE1* promoter region (Fig. 2A). Therefore, *region A-C* of the predicted *RNASE1* promoter were included in the following investigations.

Since chromatin modifications in promoter regions highly influence accessibility of the transcription machinery to the DNA, the composition of histone PTMs at promoter sites is significantly involved in the regulation of gene expression (Bannister and Kouzarides, 2011; Grewal and Elgin, 2007). Here, we investigated the influence of the proinflammatory cytokines TNF-α and IL-1β on chromatin modifications of the predicted *RNASE1* promoter regions. To determine the accessibility of the transcription machinery to chromatin, Pol II recruitment as well as the active histone modifications, H4ac and H3K27ac, were examined in *region A-C* of the *RNASE1* promoter by ChIP. HUVEC were treated for 30 min with TNF-α or 60 min with IL-1β and successful experimental settings were validated by recruitment of Pol II as well as H4ac and H3K27ac at the promoter region of *E-SELECTIN* ($Pr_{E\text{-}SELECTIN}$). *E-SELECTIN* expression is known to be upregulated upon proinflammatory stimulation in HUVEC, accompanied by increased Pol II recruitment and histone acetylation (Edelstein *et al.*, 2005). Consisting with these data, our results revealed an increase in Pol II recruitment as well as H4ac and H3K27ac at $Pr_{E\text{-}SELECTIN}$ upon TNF-α and IL-1β treatment (Supplemental Fig. S1B, C; see page 40). In case of *RNASE1*, increased Pol II recruitment and significantly elevated H4ac and H3K27ac were determined in control cells (Fig. 3; see page 32), predominantly at the TSS (*region A*), while impaired accessibility of Pol II recruitment to *region A* goes along with significant reduction in H4Ac and H3K27ac upon proinflammatory stimulation (Fig. 3A). Similar effects were obtained by analysis of *region B* (Fig. 3B) and *region C* (Fig. 3C), although to a lesser extent. Here, Pol II recruitment was almost absent in control as well as stimulated cells, while only slight reductions of H4ac and H3K27ac were obtained upon proinflammatory stimulation compared to control cells. In conclusion, proinflammatory stimulation of HUVEC mediated reduction of Pol II recruitment and the active chromatin marks, H4ac and H3K27ac, at the core promoter of *RNASE1* (*region A*), implicating their potential role in inflammation-mediated downregulation of RNase1.

2.3 Class I HDACs are responsible for TNF-α-mediated RNase1 repression

Our data nicely demonstrated the importance of H4ac and H3K27ac on *RNASE1* expression and is consistent with previous results, suggesting participation of HDACs in RNase1 regulation (Gansler *et al.*, 2014). To further identify, which distinct HDACs are responsible for RNase1 regulation in ECs, the endothelial cell line EA.hy926 and HUVEC were treated with different HDAC inhibitors prior to 24 h cytokine stimulation. Therefore, the HDAC6 specific inhibitor

Tubacin (Haggarty *et al.*, 2003a; Haggarty *et al.*, 2003b) and the class I HDAC inhibitor MS275, which specifically targets HDAC1-3 (Kalin *et al.*, 2018; Leus *et al.*, 2017) were tested, while DMSO served as solvent control. In Tubacin treated EA.hy926, almost no regulation of RNase1 mRNA was observed in control as well as TNF-α treated cells, compared to the solvent control (Fig. 4A, left panel; see page 33). By contrast, MS275 treatment slightly increases RNase1 mRNA abundance in EA.hy926 and HUVEC under physiological conditions. Interestingly, cytokine stimulation significantly decreased RNase1 mRNA in DMSO treated cells, while MS275 treatment completely rescued RNase1 mRNA expression (Fig. 4A, right panels). These results indicated a prominent role of the class I HDACs HDAC1-3 in regulation of RNase1.

To further validate the impact of HDAC1-3 on *RNASE1* promoter acetylation, HUVEC treated with MS275 prior to TNF-α stimulation were used for ChIP analyses (Fig. 4B-C; see page 33). Successful stimulation was validated by investigation of $Pr_{E\text{-}SELECTIN}$ (Supplemental Fig. S1D; see page 40). Here, cytokine stimulation increases Pol II recruitment to $Pr_{E\text{-}SELECTIN}$ and histone acetylation of H4 and H3K27 was significantly elevated compared to controls, both in DMSO as well as MS275 treated cells. In comparison, analyses of the *RNASE1* core promoter *region A* in DMSO treated cells revealed a significant decrease in Pol II recruitment, H4ac, and H3K27ac upon TNF-α stimulation. These effects were abolished by MS275 treatment resulting in recovery of Pol II recruitment, H4 and H3K27 acetylation of *region A* even upon TNF-α treatment (Fig. 4B). Although no significant changes were detected for Pol II binding in the more upstream regions of the TSS, *region B* and *region C*, comparable results were obtained for H4ac and H3K27ac. Hence, TNF-α stimulation induced significant deacetylation of H4 and H3K27 in DMSO treated cells, but the acetylation was restored by MS275 treatment (Fig. 4C, D; see page 33). Altogether, inhibition of HDAC1-3 function by MS275 restored histone acetylation (H4, H3K27) at the *RNASE1* promoter *region A-C* predicting an essential regulatory impact of HDAC1-3 on *RNASE1* in inflamed ECs.

2.4 TNF-α treatment promotes HDAC2 recruitment to the *RNASE1* promoter

To examine, which class I HDACs (HDAC1-3) occupy a predominant role in *RNASE1* promoter deacetylation, recruitment of HDAC1-3 to the *RNASE1* promoter was investigated by ChIP kinetics. Therefore, HUVEC were treated for 5-30 min with TNF-α and accumulation of HDAC1-3 to *RNASE1 region A-C* was investigated in comparison to control cells. Initially, significantly increased Pol II recruitment to $Pr_{E\text{-}SELECTIN}$ over time validated successful stimulation (Supplemental Fig. S2A; see page 42). In respect to *RNASE1*, a slight increase in Pol II recruitment was detected after 10 min of stimulation at *region A* that was subsequently decreased over time, while almost no Pol II was recruited to upstream *regions B* and *C* (Supplemental Fig. S2B; see page 42). Interestingly, HDAC2 (green line) significantly accumulated after 10 min of cytokine stimulation at the *RNASE1* core promoter *region A*, while almost no HDAC1 (blue line) and HDAC3 (red line) recruitment was detected in control and TNF-α treated HUVEC.

Although HDAC2 accumulation decreased over time, it still exceeded HDAC1 and HDAC3 levels (Fig. 5A: left panel, qualitative analysis; right panel, quantitative analysis; see page 34). Comparable HDAC accumulation was obtained for *region B* upon TNF-α treatment (Fig. 5B; see page 34), while no distinct HDAC recruitment could be observed at *region C* (Supplemental Fig. S2D; see page 42). To exclude that the detected HDAC accumulation at the *RNASE1* promoter was reliant on differential abundance in the cells, mRNA expression of HDAC1-3 was analyzed by qRT-PCR in TNF-α as well as IL-1β stimulated HUVEC over time (Supplemental Fig. S3; see page 42). Since no significant changes in mRNA expression of all three HDACs were detected upon stimulation, HDAC recruitment to the *RNASE1* promoter was suggested to be independent of HDAC mRNA abundance in the cells. In conclusion, these results demonstrated a significant impact of HDAC2 in inflammation-mediated *RNASE1* regulation in human ECs through its accumulation to the *RNASE1* promoter.

2.5 HDAC2 and HDAC1 redundantly act on RNase1 expression in inflamed endothelial cells

To further verify the specific impact of HDAC2 in RNase1 regulation, small interfering RNA (siRNA) knockdown (KD) was performed in the hybrid endothelial cell line EA.hy926. Besides HDAC2 single KD, HDAC1 single KD as well as double KD of HDAC1 and HDAC2 was conducted, allocable to the known functional redundancy of HDAC1 and HDAC2 and their operation in the same co-repressor complexes (Sengupta and Seto, 2004; Yang and Seto, 2008). KD efficiency was validated by quantitative mRNA analysis of HDAC1 and HDAC2 in control and TNF-α treated cells, resulting in significant downregulation of the respective mRNAs upon siRNA treatment (Fig. 6A; see page 35). To clearly point out the impact of HDAC1 and HDAC2 siRNA KD on RNase1 mRNA abundance, results were depicted as fold-change values normalized to siRNA control in unstimulated cells (Fig. 6B, left panel; see page 35) as well as relative percentages of mRNA abundance in TNF-α treated cells, normalized to the respective KD in untreated cells (Fig. 6B, right panel). Thus, cytokine stimulation of EA.hy926 in siRNA control as well as single siRNA KD cells resulted in significant reduction of RNase1 mRNA to 65-70% of the respective control cells. This effect was only prevented by double KD of HDAC1 and HDAC2 (Fig. 6B, left panel). In addition, relative RNase1 mRNA expression upon TNF-α stimulation was significantly decreased in control and single transfected cells. However, this effect could be significantly restored in HDAC1/2 double KD cells compared to siRNA control, HDAC2 siRNA and almost significantly to HDAC1 siRNA single KD cells (Fig. 6B, right panel).

Altogether, HDAC2 was suggested to be the most prominent class I HDAC involved in RNase1 regulation by its accumulation (Fig. 5) and subsequent deacetylation of the *RNASE1* promoter (Fig. 4). However, only double KD of HDAC1 and HDAC2 successfully restored RNase1 abundance in inflamed ECs (Fig. 6), indicating that loss of HDAC2 presumably enables HDAC1 to regulate RNase1 expression redundantly.

2.6 Indication of work shares in the publication considered for dissertation

The publication considered for dissertation was published as follows:

Bedenbender, K., Scheller, N., Fischer, S., Leiting, S., Preissner, K. T., Schmeck, B. T., and Vollmeister, E. (2019) Inflammation-mediated deacetylation of the ribonuclease 1 promoter via histone deacetylase 2 in endothelial cells. *FASEB journal: official publication of the Federation of American Societies for Experimental Biology* **33**, 9017-9029

My personal contribution to the aforementioned publication (see 5. Supplements, section 5.1, page 27-42) considered for dissertation encompasses the collection of primary material (human umbilical cords) and isolation of primary cells (HUVEC) used for indicated experiments. Besides that, I conducted research design, experiments, and data analysis for Figure 1, Figure 2A, Figure 3, Figure 4 (except of Figure 4A, left panel, Tubacin treatment), Figure 5, Figure 6, Supplemental Figure S1, Supplemental Figure S2 and Supplemental Figure S3. Additionally, I wrote the manuscript and created all illustrations presented here.

3 Discussion

Cardiovascular pathologies cause nearly 18 million deaths per year worldwide representing approximately 31% of all global deaths (WHO, 2017). The development and progression of such diseases, *e.g.* thrombosis, atherosclerosis or myocardial infarction, caused by vascular inflammation, infection or injury, are tightly associated to the loss of EC barrier function which usually occupies a crucial role in maintenance and integrity of vascular homeostasis (Poredos, 2002; Sitia *et al.*, 2010; Zernecke and Preissner, 2016). Therefore, investigation of underlying mechanisms of EC inflammation and dysfunction is of global interest. Here, we investigated the inflammation-mediated regulation of RNase1 in human ECs, a critical regulator of vascular integrity and protective factor in cardiovascular pathologies (Gansler *et al.*, 2014; Zernecke and Preissner, 2016). We identified the transcriptionally active *RNASE1* promoter region via luciferase reporter assays and further investigated the chromatin state of the *RNASE1* promoter with focus on deacetylation and the responsible mediating enzymes, class I HDACs, by ChIP, inhibitor treatment and siRNA KD. Finally, we confirmed an HDAC2-dependent mechanism of *RNASE1* deacetylation upon inflammation of human ECs.

RNase1 has been discovered as a potent regulator of EC function and protective factor in maintenance of vascular homeostasis. In this regard, RNase1 counteracts the function of eRNA for balancing the RNase1-eRNA system protecting thereby the vascular homeostasis to prevent eRNA-associated progression of vascular pathologies (Figure I). By doing so, RNase1 administration was shown to improve the outcome of different vascular pathologies, such as reduction of thrombus or edema formation by eliminating proinflammatory cytokine expression as well as plaque development and supporting beneficial disease outcome of ischemic heart disease (Cabrera-Fuentes *et al.*, 2015b; Cabrera-Fuentes *et al.*, 2014; Fischer *et al.*, 2007; Kannemeier *et al.*, 2007; Walberer *et al.*, 2009). However, Gansler *et al.* demonstrated that prolonged inflammation of vascular ECs results in impaired RNase1 expression and function. Thereby, long-term inflammation induces significant downregulation of RNase1 mRNA and increasing eRNA levels in the extracellular space. Followed by a loss of vascular integrity, for instance by redistribution of the vascular adhesion molecule vascular endothelial cadherin and subsequent increase in vascular permeability. Moreover, it was demonstrated that inflammation-mediated downregulation of RNase1 can be blocked by addition of the universal HDAC inhibitor trichostatin A, suggesting an HDAC dependent regulatory mechanisms of RNase1 on chromatin level (Gansler *et al.*, 2014). Consequently, the development of new therapeutic strategies to fight cardiovascular diseases presupposes the identification of underlying molecular mechanisms of RNase1 regulation in inflamed ECs.

Here, we demonstrated that the significant downregulation of RNase1 mRNA abundance upon TNF-α and IL-1β stimulation increases over a time course of 24 h in primary human vascular ECs (Fig. 1A), confirming previous results by Gansler *et al*. (Gansler *et al.*, 2014). Interestingly, other

general proinflammatory agents failed to block RNase1 expression with exception of the TLR3 ligand Poly I:C, a synthetic double-stranded RNA analog that induced comparable downregulation of RNase1 after 24 h stimulation (Fig. 1B). Hence, RNase1 downregulation in ECs might not be a general proinflammatory reaction but rather a specific inflammatory response to only a small subset of stimuli. Based on these findings, a common signaling pathway, activated upon TNF-α, IL-1β and Poly I:C treatment, is suggested to be required for RNase1 regulation. All three stimuli can mediate the regulation of gene expression by two distinct signaling cascades via nuclear factor kappa B (NF-κB) or mitogen activated protein kinases (MAPK) (Brenner *et al.*, 2015; Kawai and Akira, 2006; Weber *et al.*, 2010). Here, Gansler *et al.* already confirmed an NF-κB-independent mechanism for RNase1 regulation, since the NF-κB inhibitor BAY 11-7082 did not prevent TNF-α- or IL-1β-mediated RNase1 downregulation (Gansler *et al.*, 2014). Besides that, TNF-α, IL-1β and Poly I:C can induce MAPK signaling through activation of p38 MAPK or c-Jun N-terminal kinase (JNK) (Brenner *et al.*, 2015; Kawai and Akira, 2006; Weber *et al.*, 2010). Consequently, it is proposed that signaling via MAPK is responsible for regulation of RNase1 in ECs. Therefore, identification of specific signaling pathways will be of future interest to offer a broad spectrum of novel potential targets for treatment of RNase1-associated vascular dysfunction. Interestingly, although not significant, our data suggested a tendency of IFN-γ-mediated RNase1 upregulation (Fig. 1B). The IFN-γ immune response is tightly associated to host defense and clearance of viral as well as bacterial infections (Shtrichman and Samuel, 2001), which is consistent with already described functions of RNase1. Besides its function as vessel protective factor, RNase1 is also associated to host defense, for example by its antiviral activity against human immunodeficiency virus 1 or its antibacterial activity against pneumococcal infections (Lee-Huang *et al.*, 1999; Zakrzewicz *et al.*, 2016). Therefore, it would be interesting to further elucidate the role of IFN-γ as an RNase1 promoting factor in IFN-γ-mediated antimicrobial immune responses as well as vascular inflammation.

To investigate the influence of proinflammatory stimulation on the chromatin state of *RNASE1* in ECs, identification of the *RNASE1* promoter region was necessary. In this regard, core promoters usually comprises several specific marks for open and therewith actively transcribed chromatin, like H3K27ac marks (Creyghton *et al.*, 2010; Karlic *et al.*, 2010; Wang *et al.*, 2009; Wang *et al.*, 2008), while the proximal and distal promoter regions also contain additional regulatory elements (Wang *et al.*, 2009). One major factor to identify actively transcribed promoter sites is the chromatin accessibility of the Pol II transcription complex, a crucial factor for successful transcription. Thereby, the largest subunit of this complex, POLR2A, is essential for complex binding and its catalytic activity (Cramer *et al.*, 2008; Cramer *et al.*, 2001; Wang *et al.*, 2009). Based on high-throughput screenings, provided by the UCSC Genome Browser, a binding site for POLR2A was predicted directly upstream of the different RNase1 transcript variants (Fig. 2A). In addition, further marks for active promoter regions, namely a DNase hypersensitivity site, a

CTCF (CCCTC-binding factor) and c-fos (fos protooncogene) transcription factor binding site as well as the H3K27ac histone mark (Fig. 2A), were identified in close proximity to this area. Additionally, the precise location of the core promoter of *RNASE1* was further confirmed by significant transcriptional activity of construct C2 and C3 of the *RNASE1* gene in luciferase reporter assays (Fig. 2B). Altogether, these results support the localization of the potential core promoter and TSS in *region A* of the *RNASE1* gene. The suggested transcriptional activity of *region A* was further confirmed by ChIP that revealed increased Pol II recruitment to this region under physiological conditions that was subsequently vanished upon proinflammatory stimulation (Fig. 3). This loss of Pol II recruitment to the TSS of *RNASE1* further corresponds to the decrease in RNase1 mRNA expression in inflamed ECs (Fig. 1). Besides Pol II binding, chromatin modifications display a crucial role in regulation of gene expression. Thereby, especially the acetylation status of promoter regions is of great importance to distinguish between transcriptional activity and repression (Figure IIB) (Karlic *et al.*, 2010; Wang *et al.*, 2009; Wang *et al.*, 2008). Here, not only the core promoter *region A* but also the more upstream regions of the proximal and distal promoter, depicted as *region B* and *region C*, respectively, were investigated in terms of active histone marks. Similar to Pol II, H4 and H3K27 acetylation was increased at the *RNASE1* promoter under physiological conditions and significantly deacetylated upon proinflammatory stimulation of HUVEC (Fig. 3). These findings are consistent with the literature, which described tight relations of chromatin acetylation and active transcription (Shahbazian and Grunstein, 2007). Thereby, H3K27 as well as H4 acetylation were associated with gene expression and especially acetylation of H3K27 located in close proximity to TSS of transcribed genes was proposed to prevent repressive effects of trimethylation at the same lysine residue of histone 3 (Karlic *et al.*, 2010; Wang *et al.*, 2008). Moreover, Roh et *al.* demonstrated in activated T-cells, a tight correlation of chromatin accessibility and gene expression with increased acetylation at promoter regions by using high-resolution genome wide mapping strategies (Roh *et al.*, 2005). Specifically in ECs, high-throughput analysis of TNF-α treated HUVEC identified increased H3K27ac levels as active marker for upregulated gene expression, to correlate gene transcription and histone modifications (Li *et al.*, 2012). *Vice versa*, transcriptional repression was tightly associated to histone deacetylation: in HUVEC, the transcription factor BACH1 (BTB domain and CNC homolog 1) mediated HDAC1 recruitment to the *IL-8* promoter, resulting in repressed gene expression due to H3 and H4 deacetylation (Jiang *et al.*, 2015), while HDAC1 also facilitated H3 and H4 deacetylation at target gene promoters in NF-κB-mediated angiogenesis of vascular ECs (Aurora *et al.*, 2010). Accordingly, *RNASE1* promoter deacetylation of H4 and H3K27 augmented histone-DNA interactions to promote a tightly packed chromatin structure, resulting in diminished Pol II binding to the TSS of *RNASE1* and subsequent gene repression (Fig. 3).

Due to significant deacetylation of the *RNASE1* promoter in inflamed human ECs, HDAC enzymes that mediate histone deacetylation were of great importance for the underlying

investigations. Our results demonstrated the particular impact of class I HDACs, HDAC1, HDAC2, and HDAC3 in *RNASE1* regulation. In this respect, treatment with the HDAC1-3-specific inhibitor MS275 successfully restored RNase1 mRNA abundance in TNF-α treated HUVEC (Fig. 4A) by recovering not only H4 and H3K27ac in the *RNASE1* promoter *region A-C*, but also restoring Pol II recruitment to the *RNASE1* TSS in *region A* (Fig. 4B-D). These results are in line with already described experiments were MS275 administration successfully enhanced H4 and H3 acetylation in pediatric tumor cell lines or LPS/IFN-γ stimulated macrophages (Jaboin *et al.*, 2002; Leus *et al.*, 2017), as well as counteracted hypo-acetylation in the immature hippocampus in a rat model system (Joksimovic *et al.*, 2018). Therefore, HDAC1-3 are suggested to be crucial regulators in inflammation-mediated RNase1 repression in human ECs and validation of specific HDAC recruitment to the *RNASE1* promoter in inflamed ECs was of major interest. Here, we demonstrated that HDAC1 and HDAC3 were almost not recruited to the *RNASE1* promoter, while HDAC2 significantly accumulated to *region A* and *B* of *RNASE1* upon proinflammatory stimulation (Fig. 5). Accordingly, proinflammatory stimulation of human ECs mediates HDAC2 recruitment to the *RNASE1* promoter to enable local histone deacetylation and transcriptional repression. Thereby, indicating a significant impact of HDAC2 in RNase1 regulation in inflamed ECs.

Interestingly, the literature describes the possibility of functional redundancy between HDAC2 and HDAC1, so we included HDAC1 in the functional characterization of HDAC-mediated RNase1 regulation by siRNA KD. Our results revealed that only double KD of HDAC1 and HDAC2 significantly prevented RNase1 downregulation upon TNF-α stimulation (Fig. 6). These findings indicate functional redundancy of HDAC1 and HDAC2 in RNase1 regulation, where HDAC1 is able to partially takeover the RNase1 regulatory function in absence of HDAC2. The functional redundancy of HDAC1 and HDAC2 implicates that both enzymes conduct by similar molecular mechanisms and is promoted by the high sequence similarity of HDAC1 and HDAC2 of ~75% and even more identity on protein level. Thus, both enzymes are known to associate and function either as homo- or heterodimers in the same multiprotein co-repressor complexes to mediate transcriptional repression, namely Sin3 complex, REST co-repressor complex (CoREST) and the Nucleosome Remodeling and Deacetylase (NuRD) complex (Sengupta and Seto, 2004; Tong *et al.*, 1998; Yang and Seto, 2008; You *et al.*, 2001; Zhang *et al.*, 1997). Moreover, the functional redundancy of HDAC1 and HDAC2 has been also described in literature, for instance in the context of cardiac morphogenesis or oocyte development in mice as well as B-cell development (Ma *et al.*, 2012; Montgomery *et al.*, 2007; Yamaguchi *et al.*, 2010). Nevertheless, even double KD of HDAC1 and HDAC2 was not able to obtain a complete recovery of RNase1 mRNA expression (Fig. 6). Since, HDAC proteins are essential regulators of gene transcription, it is hardly surprising that they conduct high protein stability. Thus, these enzymes might still exist even after 48 h of siRNA treatment or gene inactivation (Jamaladdin *et al.*, 2014; Kallsen *et*

al., 2012; Lee *et al.*, 2014; Mercado *et al.*, 2011), which might explain the still partially regulated RNase1 expression.

Although there is indication for functional redundancy between HDAC1 and HDAC2 in inflammation-mediated RNase1 regulation, HDAC2 yet persist as the major regulator, due to its significant accumulation at the *RNASE1* promoter. The crucial impact of class I HDACs, particularly HDAC2, in inflammation supports our current findings. Zhang and colleagues described that LPS induced inflammation of myeloid cells resulted in HDAC2-mediated deacetylation of H4 and H3 of the *IL-6* promoter and subsequent gene repression (Zhang *et al.*, 2015). Comparable results for HDAC2 function were observed in context of chronic inflammatory diseases, such as rheumatoid arthritis or multiple sclerosis. Here, HDAC2 is recruited to the *IL-17* promoter to mediate its downregulation due to dysregulation of the RORγ (RAR-related orphan receptor γ) transcription factor (Singh *et al.*, 2018). Moreover, HDAC2 also acts specifically in the context of vascular inflammation, where it influences progression of atherogenesis by deacetylation-mediated repression of the transcription factor CIITA (class II transactivator) in macrophages and smooth muscle cells (Kong *et al.*, 2009). All these studies emphasize the important function of HDAC2 in inflammatory processes, demonstrating the requirement to attend inflammatory and vascular disorders by targeting HDAC2 with novel therapeutic strategies.

In this regard, HDAC inhibitors specifically target HDAC function and have already been defined as upcoming medical treatments for inflammatory disorders. Thereby, cell survival and proliferation in several cancer types has been described to be influenced by highly elevated class I HDAC expression, *e.g.* HDAC2 in cervical or gastric cancer (Huang *et al.*, 2005; Song *et al.*, 2005). In this context, clinical treatment with universal HDAC inhibitors is already applied for diverse cancers such as ovarian cancer or cutaneous T-cell lymphoma. Thereby, robust induction of cancer cell differentiation due to histone acetylation was mediated by HDAC inhibition to increase susceptibility of these cells to chemotherapeutics (Caslini *et al.*, 2006; Mann *et al.*, 2007; Yoon and Eom, 2016). Universal HDAC inhibitors were also validated to improve progression of cardiovascular diseases, such as inhibition of fibrosis and cardiac hypertrophy, preserving cardiac function after myocardial infarction and ischemia-reperfusion injury, as well as preventing progression of atherosclerosis (Eom *et al.*, 2011; Findeisen *et al.*, 2011; Granger *et al.*, 2008; Kee *et al.*, 2011; Kee *et al.*, 2006; Kong *et al.*, 2006; Okamoto *et al.*, 2006). Additionally, new promising therapies to treat HDAC-related disorders using more specific HDAC inhibitors are already under approval in clinical trials, for example MS275 that functions as antiepileptic or breast/lung cancer treatment. However, a persistent problem occurred: Even specific HDAC inhibitors not only target one distinct HDAC, for instance HDAC2, but rather targeting a distinct group of closely related HDACs, such as HDAC1-3 in terms of MS275. This issue is further attenuated due to the close relation of HDAC enzymes and their diverse functions and interaction

partners in different cellular contexts, making development and generation of HDAC specific inhibitors even more challenging (Bantscheff *et al.*, 2011; Falkenberg and Johnstone, 2014; Roche and Bertrand, 2016). In addition, functional redundancies between different enzymes, as demonstrated for HDAC1 and HDAC2, amplify these problematics (Haberland *et al.*, 2009; Jurkin *et al.*, 2011), but there is already ongoing progress in the development of enzyme-specific HDAC inhibitors or specifically interfering with HDAC functions at different cellular levels, for example by inhibition of interaction partners (Wagner *et al.*, 2015; Yamakawa *et al.*, 2017).

Irrespective of the described issues, this study revealed a protective function of MS275 in recovery of the essential vascular factor RNase1 during inflammation of human ECs. In combination with the vessel-protective role of RNase1 in vascular pathologies, like reduction of myocardial infarction size in a rat model system or protecting the heart against ischemia-reperfusion injury (Cabrera-Fuentes *et al.*, 2015b; Lu *et al.*, 2018; Simsekyilmaz *et al.*, 2014), the observed function of MS275 offers new therapeutic strategies to fight cardiovascular disorders.

Concerning the transfer of these results to a clinical setting, certain limitations have to be considered: The presented study is based on *in vitro* experiments. Cultured EC monolayers are widely used to investigate diverse scientific objectives of EC function and dysfunction. However, selection of an accurate EC model system is essential, since ECs from different origins might behave differently (Kvietys and Granger, 1997). In this regard, we used HUVEC as primary human EC model. HUVEC express and secrete high amounts of RNase1 (Fischer *et al.*, 2011; Landre *et al.*, 2002) and are the most often used EC model for investigation of inflammatory issues in humans (Bouis *et al.*, 2001; Kvietys and Granger, 1997). Additionally, compared to other primary tissue, human umbilical cords are more easily accessible and HUVEC can be isolated with a relatively high yield of cell number and purity (Jaffe *et al.*, 1973; Kvietys and Granger, 1997). However, HUVEC can be used only in low passages to preserve their primary EC character, comprise a high donor variancy (Bouis *et al.*, 2001) and HDAC siRNA transfection turned out to induce unintended side effects in this study. In this regard, alternative methods to obtain HDAC-specific depletion in HUVEC could be suggested, *e.g.* lentiviral transduction, which was already successfully performed in these cells (Margariti *et al.*, 2010). Due to these limitations, additionally the human hybrid EC cell line EA.hy926 was included in our studies, as they enable siRNA transfections and express and secrete decisive amounts of RNase1 (Landre *et al.*, 2002). Despite their hybrid origin (a fusion of HUVEC and the cancerogenic epithelial cell line A549), EA.hy926 are generally used as human vascular EC line model due to its well-characterized endothelial phenotype (Bouis *et al.*, 2001). Nevertheless, *in vitro* EC monolayers differ from an intact endothelium and validation of the presented data in *in vivo* studies will be of future interest. Here, the demonstrated protective function of MS275 on RNase1 has to be investigated in context of cardiovascular pathologies. Additionally, siRNA KD of

HDACs appeared to be difficult due to their global function and high protein stability. Thus, the impact of HDAC2 on RNase1 in a cardiovascular disease model in EC-specific HDAC2 knockout mice can be suggested, since HDAC2-null mice appeared to be lethal (Montgomery *et al.*, 2007).

Summary and Outlook

Altogether, the data presented here proposes the following mechanistic model of RNase1 regulation in human ECs **(**Figure III**)**: Under physiological conditions, the *RNASE1* promoter region (*region A-C*) is acetylated at H4 and H3K27, resulting in a relaxed and open chromatin structure. This structure provides access of the Pol II transcription machinery to the DNA and promotes *RNASE1* expression (Figure IIIA). In comparison, proinflammatory stimulation, for instance by TNF-α, IL-1β or Poly I:C, induces HDAC2 recruitment to the *RNASE1* promoter by a so far unidentified co-repressor complex. Hence, HDAC2 mediates deacetylation of H4 and H3K27 at the *RNASE1* promoter to support a tightly packed, inactive chromatin structure. Consequently, binding of the Pol II transcription machinery to the *RNASE1* TSS and subsequent *RNASE1* expression is suppressed (Figure IIIB).

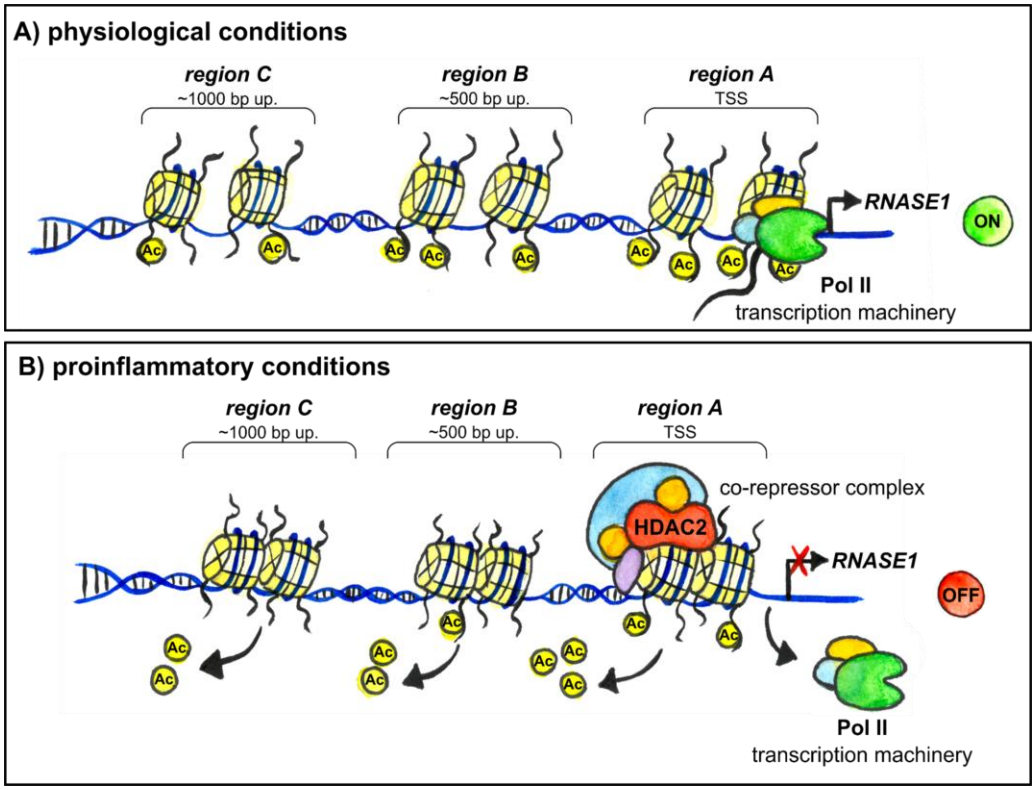

Figure III: HDAC2-mediated deacetylation of the *RNASE1* promoter. Model of *RNASE1* promoter acetylation state in human endothelial cells under A) physiological and B) proinflammatory conditions. A) Under physiological conditions, *RNASE1* promoter acetylation at histone 4 and histone 3 lysine 27 in *region A-C* supports an open chromatin structure, RNA Polymerase II (Pol II) transcription machinery binding and *RNASE1* expression. B) Proinflammatory stimulation induces deacetylation of *RNASE1* promoter *region A-C* through co-repressor complex-mediated HDAC2 recruitment, resulting in a condensed chromatin structure, loss of Pol II binding and *RNASE1* repression. Abbreviations: Ac, acetylation; bp, base pairs; HDAC2, histone deacetylase 2; Pol II, RNA polymerase II; *RNASE1*, Ribonuclease 1 gene; TSS, transcriptional start site; up., upstream.

This study partially describes the underlying molecular mechanism of HDAC2-mediated RNase1 regulation in inflamed human ECs. Nevertheless, many aspects of this mechanism remain unknown and need to be investigated in future studies: First, the involved signaling pathways from EC stimulation to *RNASE1* promoter deacetylation are still obscure and should be of future interest. Our data suggested a common signaling mechanism for RNase1 regulation via MAPK p38 and JNK (Brenner *et al.*, 2015; Kawai and Akira, 2006; Weber *et al.*, 2010). Therefore, it would be interesting to elucidate the participation of these signaling pathways in RNase1 regulation. Besides that, the analysis of IFN-γ-mediated signaling and its function as a potential novel RNase1 protective factor should be further investigated. Second, apart from signaling pathways, it is still unclear how HDAC2 is activated and recruited to the *RNASE1* promoter in our model. In this respect, no changes in mRNA abundance upon proinflammatory stimulation were detected for HDAC2 over HDAC1 and HDAC3. This indicates that specific accumulation of HDAC2 to the *RNASE1* promoter might depend on changes in its protein abundance or function. According to literature, activity and complex formation of HDAC2 is regulated through PTMs, especially phosphorylation of serine residues that are mediated by the protein kinase casein kinase 2 (CK2) (Brandl *et al.*, 2009; Tsai and Seto, 2002). Therefore, it would be interesting to investigate, whether CK2 is responsible for RNase1 regulation through modulation of HDAC2 phosphorylation in EC inflammation. Thirdly, the recruitment of HDAC2 to the *RNASE1* promoter region needs to be investigated. HDACs themselves do not comprise any DNA-binding ability and require the function of co-repressor complexes to successfully mediate histone deacetylation (Sengupta and Seto, 2004). Thus, it would be necessary to investigate, which co-repressor complex, Sin3, CoREST or NuRD (Tong *et al.*, 1998; You *et al.*, 2001; Zhang *et al.*, 1997), associates with HDAC2 upon EC inflammation to promote its recruitment to the *RNASE1* promoter for subsequent gene repression. Additionally, identification of a specific co-repressor complex can further provide information about HDAC2-associated proteins, such as DNA-binding proteins, transcription factors, or other complex components essential for RNase1 regulation.

Altogether, further investigations are required to unravel the underlying molecular mechanisms of proinflammatory regulation of the vessel-protective factor RNase1 in ECs. Thereby, leading to the identification of new potential targets, such as signaling pathway components or HDAC2 interaction partners, to develop novel therapeutic strategies to fight cardiovascular pathologies by protecting RNase1 to preserve the vascular integrity.

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5 Supplements

5.1 Inflammation-mediated deacetylation of the ribonuclease 1 promoter via histone deacetylase 2 in endothelial cells.

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Inflammation-mediated deacetylation of the ribonuclease 1 promoter *via* histone deacetylase 2 in endothelial cells

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ABSTRACT: Ribonuclease 1 (RNase1) is a circulating extracellular endonuclease that regulates the vascular homeostasis of extracellular RNA and acts as a vessel- and tissue-protective enzyme. Upon long-term inflammation, high amounts of proinflammatory cytokines affect endothelial cell (EC) function by down-regulation of RNase1. Here, we investigated the transcriptional regulation of RNase1 upon inflammation in HUVECs. TNF-α or IL-1β stimulation reduced the expression of RNase1 relative to the acetylation state of histone 3 at lysine 27 and histone 4 of the *RNASE1* promoter. Inhibition of histone deacetylase (HDAC) 1, 2, and 3 by the specific class I HDAC inhibitor MS275 abolished the TNF-α– or IL-1β–mediated effect on the mRNA and chromatin levels of RNase1. Moreover, chromatin immunoprecipitation kinetics revealed that HDAC2 accumulates at the *RNASE1* promoter upon TNF-α stimulation, indicating an essential role for HDAC2 in regulating RNase1 expression. Thus, proinflammatory stimulation induced recruitment of HDAC2 to attenuate histone acetylation at the *RNASE1* promoter site. Consequently, treatment with HDAC inhibitors may provide a new therapeutic strategy to stabilize vascular homeostasis in the context of inflammation by preventing RNase1 down-regulation in ECs.—Bedenbender, K., Scheller, N., Fischer, S., Leiting, S., Preissner, K. T., Schmeck, B. T., Vollmeister, E. Inflammation-mediated deacetylation of the ribonuclease 1 promoter *via* histone deacetylase 2 in endothelial cells. FASEB J. 33, 9017–9029 (2019). www.fasebj.org

KEY WORDS: chromatin immunoprecipitation · vascular homeostasis · MS275 · histone acetylation · TNF-α

Multiple endothelial cell (EC) functions contribute to maintain vascular homeostasis under physiologic as well as inflammatory conditions. Upon inflammation, ECs get activated and rapidly attract leukocytes, which leave the vessel, infiltrate the tissue, and secrete further proinflammatory agents (1). In this process, the integrity and homeostatic functions of the EC layer may be impaired, promoting vascular diseases, such as atherosclerosis, thrombosis, and myocardial infarction (2, 3). Upon acute inflammation due to infection or injury, ECs release extracellular RNA (eRNA) as danger signals to induce the immune response (4–6). However, increasing amounts of eRNA can result in EC damage and dysfunction (7, 8). An important counteracting enzyme that helps to maintain vascular homeostasis is ribonuclease 1 (RNase1) (6, 9). RNase1 is a secreted protein that belongs to the extracellular RNase A superfamily (9–11). It is mainly expressed and released by vascular ECs (10, 12) to act as a natural counterpart of eRNA *via* degradation to protect the EC layer (6, 13, 14). However, upon long-term inflammation of ECs, eRNA accumulates in the extracellular space, recruiting inflammatory cells, such as monocytes, to the site of inflammation (5, 6). These monocytes release proinflammatory cytokines, such as TNF-α or IL-1β, which induce a massive down-regulation of RNase1 mRNA expression in ECs that can be blocked by inhibitors of histone deacetylases (HDACs) (6). RNase1 suppression results in impaired degradation of eRNA that contributes to disturbed EC-barrier function (6). However, the detailed

ABBREVIATIONS: ChIP, chromatin immunoprecipitation; CTRL, either unstimulated or untreated control; EC, endothelial cell; eRNA, extracellular RNA; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; H3, histone 3; H3K27ac, histone 3 lysine 27 acetylation; H4, histone 4; H4ac, histone 4 acetylation; HDAC, histone deacetylase; HUVEC, human umbilical vein endothelial cell; ICAM-2, intercellular adhesion molecule 2; KD, knockdown; Pol II, RNA polymerase II; POLR2A, Pol II subunit 2A binding site; Poly I:C, polyinosinic polycytidylic acid; qPCR, quantitative PCR; qRT-PCR, quantitative reverse transcription PCR; RNase1, ribonuclease 1; RPS18, ribosomal protein S18; siC, scramble transfected; siRNA, small interfering RNA; TSS, transcriptional start site; UCSC, University of California–Santa Cruz

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mechanism of TNF-α–induced down-regulation of RNase1 has not been elucidated.

Histone modifications, such as acetylation, are post-translational modifications at the N-terminal tails of histone proteins. These modifications are critical for the regulation of chromatin structure and function (15, 16) and highly affect DNA-related processes such as transcription, replication, or chromosomal organization (17). One of the first reported histone modifications was acetylation of lysine residues (18, 19) that play a crucial role in regulating gene activation and repression (20–22). Besides others, histone 4 acetylation (H4ac) and histone 3 lysine 27 acetylation (H3K27ac) function as specific markers for active, transcribed chromatin, whereas the deacetylation of these residues results in transcriptional repression (23, 24). HDACs conduct important functions in modifying chromatin structure and accessibility by removing acetyl groups from lysine residues on histone tails (15, 25, 26). As a consequence, the positive charge of the histones increases, augmenting their interaction with DNA and resulting in a condensed chromatin structure and transcriptional repression (15). HDACs consist of 4 distinct classes of enzymes, which can be distinguished because of their specific sequence similarity (26). In particular, the class I HDACs HDAC1–3 play an important role in regulation of inflammatory processes and vascular diseases by influencing, for instance, TLR-dependent signaling or atherosclerosis, respectively (27–31).

RNase1 regulation upon proinflammatory stimulation in ECs is proposed to be regulated by HDACs (6). In this study, we aimed to investigate the underlying mechanism of reduced RNase1 expression during vascular inflammation, addressing the acetylation status and the regulatory potential of HDACs for histone deacetylation in the promotor region of the *RNASE1* gene, using luciferase reporter assay, chromatin immunoprecipitation (ChIP), HDAC-inhibitor treatment, and HDAC small interfering RNA (siRNA) knockdown (KD) in human ECs. Our findings provide new insights into the mechanism of proinflammatory down-regulation of RNase1 by HDACs and may help to counteract RNase1 silencing under long-term inflammatory conditions and potentially improve vascular homeostasis.

MATERIALS AND METHODS

Ethical statement

All umbilical cords were donated from healthy individuals who were fully informed and consented to donation. Donated tissue was handled in accordance with local ethics regulations (Philipps-University Marburg; AZ 20/16).

Cell isolation, culture, and treatment

All cells used in this study were cultivated at 37°C with 5% CO_2. HUVECs were isolated and cultured as previously described by Jaffe *et al.* (32) with the following modifications: umbilical veins were flushed with 1× HBSS with magnesium and calcium (HyClone, GE Healthcare, Waukesha, WI, USA) and 1× PBS with magnesium chloride and calcium chloride (Merck, Darmstadt, Germany). HUVECs were isolated by administration of 0.1% collagenase D solution (from *Clostridium histolyticum*; Merck) into the vein for 20 min at 37°C, cultured in EC growth medium purchased from PromoCell (Heidelberg, Germany) supplemented with 1% penicillin and streptomycin (Thermo Fisher Scientific, Waltham, MA, USA), and used up to passage 4 for all indicated experiments.

For stimulation experiments, 3.8×10^4 cells/cm^2 were seeded overnight. Cells were stimulated with 10 ng/ml human recombinant TNF-α, IL-1β (R&D Systems, Minneapolis, MN, USA), IL-13 (50 ng/ml), IFN-γ (250 ng/ml) (PromoCell), or TGF-β (1 μg/ml) (PeproTech, Rocky Hill, NJ, USA) as well as LPS from *Salmonella minnesita* R595 (100 ng/ml) (Enzo Life Sciences, Farmingdale, NY, USA), polyinosinic polycytidylic acid (Poly I:C) (10 μg/ml), or trifluoroacetate salt Pam3CSK4 (200 ng/ml) (InvivoGen, San Diego, CA, USA) as indicated. For HDAC inhibitor tests, HUVECs were treated with indicated concentrations of MS275 (2, 4 μM) for 4 h prior to cytokine stimulation. For ChIP assays, HUVECs were stimulated with TNF-α or IL-1β (10 ng/ml) as indicated. For MS275-ChIP assays, cells were prestimulated with the HDAC inhibitor MS275 (4 μM; Merck) or DMSO (Carl Roth, Karlsruhe, Germany) as solvent control for 2 h prior to TNF-α treatment.

The hybrid EC line EA.hy926 [American Type Culture Collection (ATCC), Manassas, VA, USA] was cultured in DMEM with high glucose (Merck) supplemented with 10% fetal calf serum (Merck). Cells were used up to passage 20 for all indicated experiments. For stimulation experiments, 3.8×10^4 cells/cm^2 were seeded overnight and stimulated with 10 ng/ml human recombinant TNF-α (R&D Systems) as indicated. For HDAC inhibitor tests, EA.hy926 cells were treated with indicated concentrations of tubacin (0.5, 1 μM; Merck) or MS275 (0.3, 1, 2 μM; Merck) 4 h prior to cytokine stimulation.

HDAC siRNA KD

EA.hy926 cells (3.8×10^4 cells/cm^2) were seeded overnight and transfected for 24 h with 60 pmol Silencer Select siRNAs (Ambion; Thermo Fisher Scientific) against HDAC1 (AssayID s73), HDAC2 (AssayID s6493), a combination of both (each 30 pmol), or scrambled transfected (siC) control siRNA (AssayID 4390843) using Lipofectamine RNAiMax (Thermo Fisher Scientific) in Opti Minimum Essential Media I reduced serum medium (Thermo Fisher Scientific) and DMEM with 10% fetal calf serum. After 24 h, fresh medium was added, and cells were stimulated for an additional 24 h with 10 ng/ml TNF-α or left untreated as control.

RNA isolation and quantitative reverse transcription PCR

Total RNA was isolated from HUVECs or EA.hy926 cells using a phenol-chloroform based method with Tri Reagent (Merck) as described by the manufacturer's protocol. Afterwards, cDNA was generated from RNA using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) according to the manufacturer's protocol. Transcript expression of RNase1, E-selectin, HDAC1, HDAC2, HDAC3, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or ribosomal protein S18 (RPS18) as internal control was quantified by quantitative reverse transcription PCR (qRT-PCR) with respective primer pairs (**Table 1**, Metabion International, Planegg/Steinkirchen, Germany) using the Fast Sybr Green Master Mix (Thermo Fisher Scientific) and the QuantStudio System and QuantStudio Design & Analysis Software v.1.3.1 (Thermo Fisher Scientific) according to the manufacturer's instructions. The fold induction was

TABLE 1. *Primer sequences*

Primer	Primer sequence, 5′–3′		Application, (RE)
	Forward	Reverse	
RNase 1	GCTGCAGATCCAGGCTTTTCTGGG	AATTTCTTGGCCCGGGATTC	mRNA analysis
E-selectin	TGAAGCTCCCACTGAGTCCAA	GGTGCTAATGTCAGGAGGGAGA	mRNA analysis
HDAC1	CTACGACGGGGATGTTGGAA	CAGCATTGGCTTTGTGAGGG	mRNA analysis
HDAC2	TGCTACTACTACGACGGTGA	TCGGCAGTGGCTTTATGGG	mRNA analysis
HDAC3	GCTGCTCAAGTACCACGCCTC	TCTCTGCCCCGAGTTCATACA	mRNA analysis
GAPDH	CCACATCGCTCAGACACCAT	CGCAACAATATCCACTTTACCAGAG	mRNA analysis
RPS18	GCGGCGGAAAATAGCGTTTG	GATCACAGGTTCGACCTGATC	mRNA analysis
Region A	TGAGGAAGGAGTGGTGAATC	TTTCTCTGCTGCTCCTTGTG	ChIP
Region B	CATTAGATCGGCCTGTTG	TTTACACGACACGGGAGCCTTC	ChIP
Region C	CTGGCCCTAGGAATCCTGAAAC	CTGCAGTAAGGGCTTCTGATGG	ChIP
$Pr_{E\text{-}SELECTIN}$	ACCGACCTGAGAGATCCTGTGT	GGCTGCCCTTATAAAGCGTTCT	ChIP
C1	TATGAGCTGCTGAATACACAGGACGAAGGG	TATGATATCTTCTGTAAGCCTGCTTCACTTAGTT	Cloning, (*Sac*I, *Nhe*I)
C2	TATGAATTCTAGAAAGACGGCGGCCTGGG	TATAAGCTTCCTGGATCTGCAGCTTGGTGTCT	Cloning, (*Eco*RI, *Hind*III)
C3	TATGAATTCTAGAAAGACGGCGGGCGTGGG	TATAAGCTTCTGGAATGACTCAGCTCCCGG	Cloning, (*Eco*RI, *Hind*III)
C4	TATGAGCTCAGACCTCGCTTTGGAGAGTG	AAAGCTAGCGGAACTGTCTGAGTCCATAT	Cloning, (*Sac*I, *Nhe*I)
$Pr_{ICAM\text{-}2}$	TATGAGCTCCCAGGGCATGACTCCAACAATGC	AAAGCTAGCGACATCTCTGGCAGTGTCCACG	Cloning, (*Sac*I, *Nhe*I)

RE, restriction enzymes used for cloning.

calculated using the $2^{-\Delta\Delta Ct}$ method, and qRT-PCR results were normalized to the corresponding control cells (33).

ChIP

Chemicals used for ChIP were purchased from Carl Roth unless otherwise stated. Confluent cells were stimulated as indicated and fixed with 1% formaldehyde (methanol-free; Polysciences, Warrington, PA, USA) for 5 min at room temperature and stopped by the addition of 0.125 M glycine for 5 min at room temperature. Cells were washed and scraped with ice-cold PBS (1×, without magnesium and calcium, HyClone; GE Healthcare) and centrifuged twice at 300 *g* for 10 min at 4°C. Cells were lysed chemically with lysis buffer I and II [I: 5 mM PIPES pH 8, 85 mM KCl, 0.5% Nonidet P40 (AppliChem, Darmstadt, Germany); II: 10 mM Tris hydrochloride pH 7.5, 150 mM NaCl, 1% sodium deoxycholate, 0.1% SDS, 1% Nonidet P40], and chromatin was sheared *via* sonication (Biorupter Plus; Diagenode, Seraing, Belgium). As a control, a portion of the sonicated chromatin was used for Proteinase K (AppliChem) digestion followed by DNA purification using the QiaQuick PCR Purification Kit (Qiagen, Hilden, Germany), and the fragment size was analyzed on a 1.5% agarose gel. Sepharose A (GE Healthcare) beads were blocked with 1 mg/ml bovine serum albumin (Carl Roth) and 400 μg sonicated salmon sperm DNA (AppliChem) overnight at 4°C. Sonicated chromatin was precleared by incubation with blocked beads, and 10–20 μg of precleared chromatin was used for immunoprecipitation with protein-specific antibodies: anti–RNA polymerase II (Pol II) (ab26721), anti-H3K27ac (ab4729), anti-HDAC1 (ab7028), anti-HDAC2 (ab7029), anti-HDAC3 (ab7030), anti-rabbit IgG (ab46540; Abcam, Cambridge, MA, USA), or anti-H4ac (06-598; Merck) overnight at 4°C. Beads were added for 2 h, washed with 1× PBS without magnesium and calcium (HyClone; GE Healthcare), and chromatin-antibody complexes were chemically eluted (1% SDS, 0.1 M sodium hydrogen carbonate). Reversal of crosslinking was conducted by Proteinase K digestion overnight with immunoprecipitation samples prior to DNA purification using the QiaQuick PCR Purification Kit according to the manufacturer's instructions. Purified chromatin was eluted in H_2O and analyzed by quantitative PCR (qPCR) as previously described using specific primers (Table 1, Metabion International) for human *RNASE1* promoter regions *region A*, *region B*, *region C* and human *E-SELECTIN* promoter [$Pr_{E\text{-}SELECTIN}$ (34)]. Results of ChIP experiments were normalized to the input control, whereas results of ChIP kinetics were normalized to the input control and the IgG control. All values are depicted as a percentage of input. For qualitative analyses of ChIP experiments, qPCR samples of one representative replicate were run on a 1.5% agarose gel for 2 h at 100 V and visualized by UV light with the gel documentation system Gel iX imager (Intas Science Imaging Instruments, Göttingen, Germany).

Reporter plasmids and luciferase reporter assay

For generation of luciferase reporter plasmids, parts of the *RNASE1* gene region (C1–C4) as well as the positive control intercellular adhesion molecule (*ICAM*)-2 promoter ($Pr_{ICAM\text{-}2}$) were amplified by PCR from genomic DNA of the EC line EA.hy926 using respective primer pairs (Table 1, Microsynth Seqlab, Göttingen, Germany). Amplified sequences were cloned into the luciferase vector backbone pGL4.10[luc2] (Promega, Madison, WI, USA) to generate C1–C4-specific *RNASE1* and *ICAM-2* positive control reporter vectors.

For luciferase reporter assay, 2.1×10^4 cells/cm^2 EA.hy926 cells were seeded overnight. Cells were transfected in Opti Minimum Essential Medium I with 0.5 μg of the aforementioned luciferase reporter plasmids and the vehicle control pGL4.10 [luc2] together with the *Renilla* luciferase control plasmid pRL-TK (Promega) using Lipofectamine 2000 (Thermo Fisher Scientific) according to the manufacturer's instructions. After 24 h, medium was changed to DMEM (Merck) with 1% penicillin streptomycin and incubated for another 24 h. Firefly and *Renilla* luciferase activity of cell lysates was measured with Tecan Infinite 200 (Tecan Trading, Männedorf, Switzerland) or TriStar2S (Berthold Technologies, Bad Wildbad, Germany) as previously described by Dyer *et al.* (35). Results were normalized to *Renilla* control plasmid as transfection control and depicted as relative luminescence units.

Statistical analysis

Results are expressed as means ± SD of linear data (ChIP, luciferase reporter assay) or log2-transformed data (qRT-PCR). One- or 2-way ANOVA and subsequent multiple comparison Holm-Sidak posttest were conducted on linear data (ChIP, luciferase reporter assay) or log2-transformed data (qRT-PCR). Results were considered significant at a value of $P < 0.05$. Statistical analyses were performed using GraphPad Prism v.6.05 (GraphPad Software, La Jolla, CA, USA).

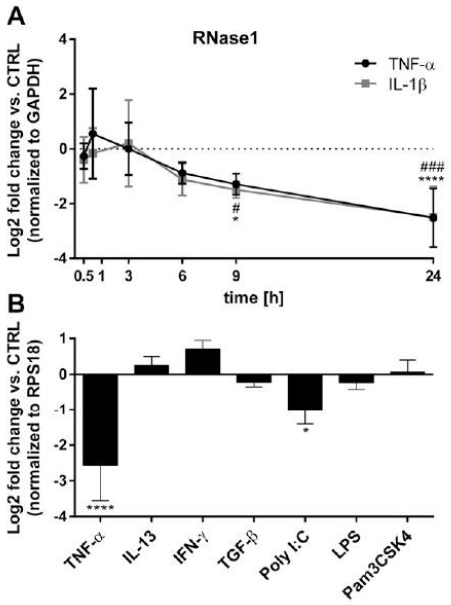

Figure 1. Regulation of RNase1 mRNA expression in HUVECs. *A*) RNase1 mRNA expression kinetics upon 10 ng/ml TNF-α or IL-1β stimulation for 0.5, 1, 3, 6, 9, or 24 h in HUVECs. Expression of RNase1 mRNA was analyzed by qRT-PCR. Results were normalized to GAPDH and the respective CTRL samples; n = 4–5. Means ± SD were calculated using log2-transformed data. Two-way ANOVA using Holm-Sidak posttest. Significance for TNF-α: $^{*}P < 0.05$, $^{****}P < 0.0001$; significance for IL-1β: $^{\#}P < 0.05$, $^{\#\#\#}P < 0.0001$. *B*) RNase1 mRNA expression upon 24 h of stimulation with TNF-α (10 ng/ml), IL-13 (50 ng/ml), IFN-γ (250 ng/ml), TGF-β (1 μg/ml), Poly I:C (10 μg/ml), LPS (100 ng/ml), or Pam3CSK4 (200 ng/ml) in HUVECs. Expression of RNase1 mRNA was analyzed by qRT-PCR. Results were normalized to RPS18 and the respective CTRL samples; n = 3–5. Means ± SD were calculated using log2-transformed data; 1-way ANOVA using Holm-Sidak posttest. $^{*}P < 0.05$, $^{****}P < 0.0001$.

RESULTS

RNase1 mRNA expression is severely reduced in inflamed ECs

We first examined RNase1 mRNA expression over time in HUVECs stimulated with TNF-α or IL-1β. E-selectin mRNA, which is known to be up-regulated upon TNF-α or IL-1β treatment, was tested by qRT-PCR as a hallmark for successful stimulation (34). Expression of E-selectin mRNA was significantly up-regulated already 30 min after stimulation (Supplemental Fig. S1*A*). A decrease in RNase1 mRNA abundance was detected 6 h post-stimulation (**Fig. 1*A***). This effect reached significance after 9 h and increased until 24 h post stimulation. To identify whether regulation of RNase1 is also mediated by other proinflammatory agents, HUVECs were stimulated with IL-13 (50 ng/ml), IFN-γ (250 ng/ml), TGF-β (1 μg/ml), Poly I:C (10 μg/ml), LPS (100 ng/ml), or Pam3CSK4 (200 ng/ml) for 24 h, and RNase1 mRNA expression was analyzed. Only stimulation with the TLR-3 ligand Poly I:C down-regulated RNase1 mRNA abundance significantly (Fig. 1*B*) compared with TNF-α.

Identification of the RNASE1 promoter region

To define the active *RNASE1* promoter region, *in silico* analyses were performed based on the University of California–Santa Cruz (UCSC) Genome Browser (**Fig. 2*A***). We focused on respective marks that indicate a functional promoter site obtained by ChIP-sequencing analysis in HUVECs (UCSC Genome Browser): a Pol II subunit 2A binding site (POLR2A) and active chromatin marks such as H3K27ac. These 2 prominent marks suggested a transcriptional start site (TSS) in close proximity (36), upstream of the different transcript variants of *RNASE1*.

For identification of promoter activity in this specific region, different promoter constructs (C1–C4) were generated and tested for their potential to activate transcription in luciferase reporter assays. Therefore, translation of firefly luciferase in the EC line EA.hy926 under physiologic conditions was analyzed (Fig. 2*B*). As a control, we used the *ICAM-2* promoter that is active for transcription (37) or an empty vehicle control, respectively. Almost no luciferase luminescence was detected in cells transfected with vehicle control, whereas cells transfected with the *ICAM-2* promoter construct showed a significant increase in luciferase luminescence. Compared with that, *RNASE1* promoter constructs C1 and C4 exhibited no induction of luciferase activity compared with vehicle control. Only constructs C2 and C3 significantly induced luciferase luminescence, which was even higher than *ICAM-2* promoter–mediated activity.

Proinflammatory stimulation reduces histone acetylation at the predicted RNASE1 promoter

To further analyze the influence of proinflammatory stimulation on chromatin level at the predicted

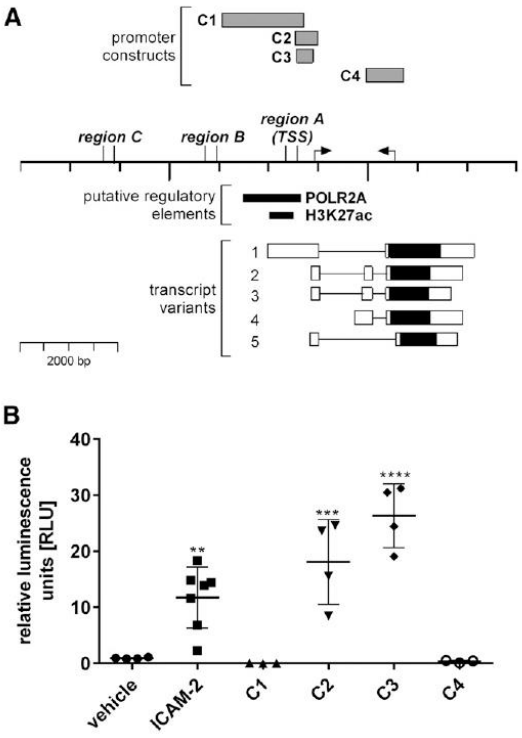

Figure 2. Identification of the *RNASE1* promoter region. *A*) Schematic diagram of *RNASE1* genomic region based on UCSC Genome Browser [Human February 2009 (Genome Reference Consortium human genome build 37/human genome 19) Assembly]. Three primer pairs for promoter analysis, named *region A*, *region B*, and *region C*, were selected. Arrows indicate qRT-PCR primer binding sites. Upper part depicts promoter constructs (C1–4, gray bars) that were used for luciferase assay. Lower part depicts 5 different transcript variants of *RNASE1* (1–5; black bars: protein coding regions; white bars: UTRs). Known marks for promoter regions are shown: an H3K27ac site and a POLR2A (narrow black bars). *B*) Promoter constructs (C1–C4) of *RNASE1* were used for luciferase reporter analysis. EA.hy926 cells were transfected for 48 h with empty firefly luciferase vehicle control, the *ICAM-2* reporter plasmid as positive control, or reporter constructs C1–C4 as well as with *Renilla* luciferase control vector as transfection control. Luciferase activity depicted in relative luminescence units (RLU) was calculated as ratio of firefly/*Renilla* luminescence; n = 3–7. Means ± SD; 1-way ANOVA using Holm-Sidak posttest, compared with vehicle control. $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

RNASE1 promoter regions, ChIP was performed in HUVECs. Promoter regions are divided into 3 different parts: the core promoter, the proximal promoter, and the distal promoter (36). Therefore, specific sets of promoter primers were generated to screen the different parts of the *RNASE1* promoter region upstream of the predicted TSS: *region A*, located in close proximity to the predicted TSS; *region B*, located ~500 bp; or *region C*, located ~1000 bp upstream of the TSS of *RNASE1* (Fig.

2*A*). For analyses of Pol II recruitment and active acetylated histone marks (H4ac and H3K27ac) at *regions A*, *B*, and *C* of the *RNASE1* promoter, ChIP was performed in HUVECs treated with TNF-α or IL-1β for 30 or 60 min, respectively. As a control for successful stimulation, Pol II recruitment as well as H4ac and H3K27ac were investigated at the promoter region of *E-SELECTIN* ($Pr_{E\text{-}SELECTIN}$; Supplemental Fig. S1*B*, *C*). Comparable to literature (34), Pol II recruitment, H4ac, and H3K27ac were significantly increased upon TNF-α and IL-β treatment in HUVECs at $Pr_{E\text{-}SELECTIN}$. In *region A* of *RNASE1*, TNF-α as well as IL-1β stimulation decreased Pol II recruitment nearly to the IgG control level compared with untreated cells (**Fig. 3*A***). Moreover, significant results were obtained by analysis of the histone marks H4ac and H3K27ac, which were significantly reduced upon TNF-α (Fig. 3*A*; left panels) and IL-1β (Fig. 3*A*; right panels) treatment in *region A*. Analysis of the *RNASE1* promoter *regions B* (Fig. 3*B*) and *C* (Fig. 3*C*) obtained similar results. Almost no Pol II recruitment was detected in these regions in control and TNF-α– or IL-1β–treated cells. Similar to *region A*, a reduction of H4ac and H3K27ac after TNF-α or IL-1β treatment was detected in *regions B* and *C* (Fig. 3*B*, *C*), although these results were not significant. Accordingly, in unstimulated (CTRL) cells, acetylation was present predominantly at the TSS. Hence, reduction of the active chromatin marks H4ac and H3K27ac in *region A* upon proinflammatory stimulation may be involved in the down-regulation of RNase1.

Inhibition of class I HDACs prevents the negative effect of TNF-α on RNase1 mRNA and chromatin level

Previous results demonstrated that treatment of HUVECs with HDAC inhibitor trichostatin A prior to proinflammatory stimulation rescued RNase1 mRNA expression (6). To identify which specific HDACs are involved in RNase1 regulation, EA.hy926 cells and HUVECs were pretreated with different concentrations of specific HDAC inhibitors prior to stimulation: HDAC6 inhibitor tubacin and class I HDAC inhibitor MS275, which specifically inhibits HDAC1–3. DMSO-treated cells served as solvent control. In the EC line EA.hy926, almost no regulation of RNase1 mRNA expression was detected in control cells treated with tubacin compared with solvent control. Upon TNF-α stimulation, RNase1 mRNA expression was significantly down-regulated in the solvent control as well as in tubacin-treated cells (**Fig. 4*A***). In comparison, MS275 treatment of control cells slightly increased the basal RNase1 mRNA expression compared with the solvent control in both EA.hy926 cells and HUVECs. Upon TNF-α stimulation, RNase1 mRNA expression was significantly down-regulated in cells treated with the solvent control, and MS275 treatment restored the negative effect of TNF-α on RNase1 mRNA expression (Fig. 4*A*).

To assess whether HDAC1–3 is important for RNase1 regulation, the influence of MS275 on histone acetylation at the *RNASE1* promoter was investigated. Prior to TNF-α stimulation, HUVECs were treated with

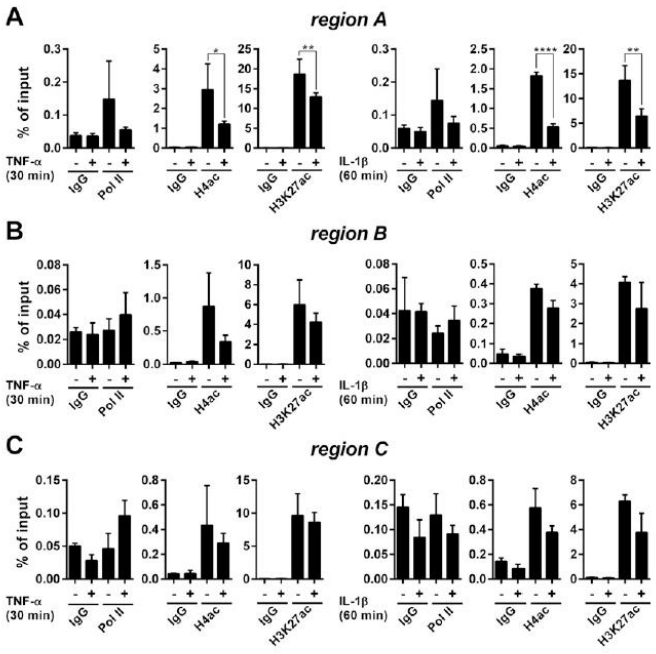

Figure 3. ChIP analysis of the predicted *RNASE1* promoter *regions A–C*. HUVECs were stimulated with 10 ng/ml TNF-α (left panels) or IL-1β (right panels) for 30 min or 60 min (+), respectively, or left untreated as control (−). Immunoprecipitation using specific antibodies was performed for Pol II, H4ac, H3K27ac, or an unspecific IgG control, respectively. *Region A* (*A*), *B* (*B*), or *C* (*C*) of predicted *RNASE1* promoter was pulled down by respective antibodies and analyzed by qPCR using respective primers. Results were normalized to input control (1%); $n = 3$–4. Means ± SD; 1-way ANOVA using Holm-Sidak posttest. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$.

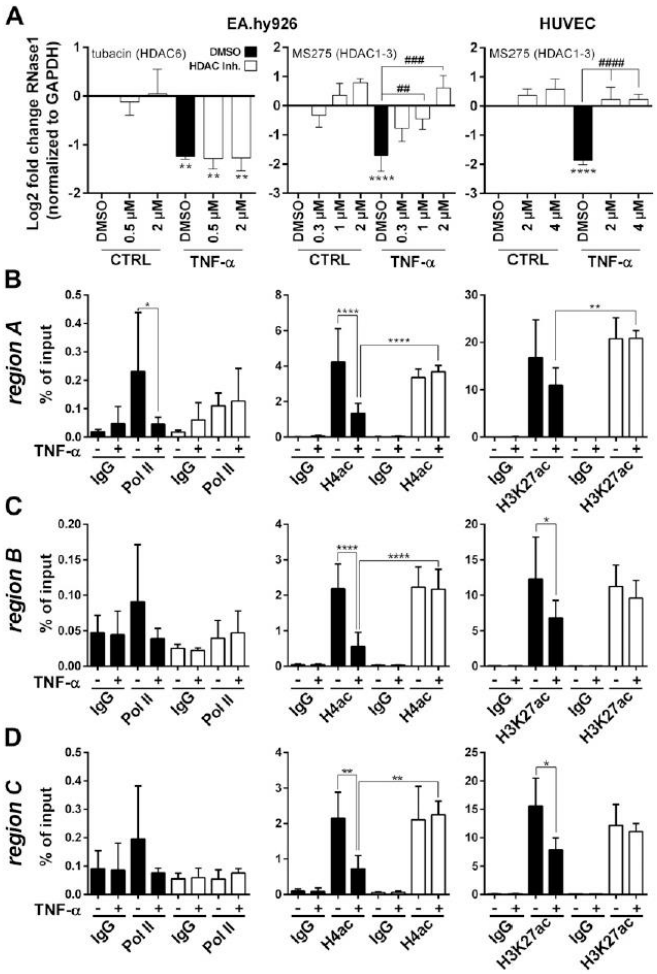

Figure 4. MS275 treatment reverses the negative effect of TNF-α on RNase1. *A*) EA.hy926 cells or HUVECs were pretreated for 4 h with indicated concentrations of tubacin (HDAC6 inhibitor), MS275 (HDAC1–3 inhibitor; white bars), or DMSO (black bars) as solvent control prior to control (CTRL) or TNF-α (10 ng/ml) stimulation for 24 h. RNA was isolated, and mRNA expression of RNase1 was analyzed by qRT-PCR. Results were normalized to endogenous GAPDH and CTRL DMSO samples. HDAC Inh., HDAC inhibitor. Statistics were performed on log2-transformed data. Mean ± SD; 1-way ANOVA using Holm-Sidak posttest; n=3–4. Influence of TNF-α: $^{**}P < 0.01$, $^{****}P < 0.0001$; influence of inhibitor: $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, $^{\#\#\#\#}P < 0.0001$. *B–D*) For MS275 ChIP assays, HUVECs were pretreated with 4 μM MS275 (white bars) or DMSO (black bars) as solvent control for 2 h and subsequently stimulated with 10 ng/ml TNF-α for 30 min (+) or left untreated as control (−). Immunoprecipitation using specific antibodies against Pol II, H4ac, and H3K27ac or an unspecific IgG control was performed. *Region A* (*B*), *region B* (*C*), and *region C* (*D*) of the predicted *RNASE1* promoter were pulled down by respective antibodies and analyzed by qPCR. Results were normalized to input control (1%); n = 3–4. Means ± SD; 1-way ANOVA using Holm-Sidak posttest. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$.

MS275 (4 μM, 2 h) or DMSO as solvent control, respectively. Successful stimulation was validated by Pol II recruitment to $Pr_{E\text{-}SELECTIN}$, which was increased after stimulation upon both treatments (Supplemental Fig. S1*D*). At *region A* of *RNASE1*, treatment with the solvent control resulted in significantly downregulated Pol II recruitment after TNF-α stimulation. Interestingly, upon MS275 pretreatment, Pol II was almost equally recruited to *region A* in control as well as TNF-α–stimulated samples (Fig. 4*B*, left panel). Moreover, treatment with the solvent control prior to TNF-α stimulation did not prevent significant downregulation of both histone marks H4ac (Fig. 4*B*, middle panel) and H3K27ac (Fig. 4*B*, right panel) compared with control cells, whereas MS275 treatment recovered histone acetylation significantly to equal or even higher levels than in control cells. Likewise, in the *RNASE1* promoter regions upstream of the putative TSS, *regions B* and *C* (Fig. 4*C*, *D*), significant deacetylation of histone 4 (H4) and H3K27 was detected in solvent control–treated cells upon TNF-α stimulation, and this effect was blocked by MS275 (Fig. 4*C*, *D*, middle and right panel). Compared with *region A*, no significant changes could be observed for Pol II recruitment in *regions B* and *C* (Fig. 4*C*, *D*, left panel). In summary, MS275 recovered histone acetylation at the predicted *RNASE1* promoter (*regions A–C*) upon proinflammatory stimulation, suggesting a regulatory function of HDAC1–3 on *RNASE1*.

TNF-α mediates HDAC2 accumulation at the predicted RNASE1 promoter region

To analyze which specific class I HDACs are essential for deacetylation of the *RNASE1* promoter region, ChIP kinetic experiments were performed. Binding of HDAC1–3 to *regions A* and *B* of the predicted *RNASE1*

promoter was analyzed in HUVECs stimulated for 5, 10, 20, or 30 min with TNF-α or left untreated as control. Successful stimulation was validated by Pol II recruitment to $Pr_{E\text{-}SELECTIN}$, which was significantly increased over time (Supplemental Fig. S2*A*). Moreover, Pol II recruitment at *region A* was slightly increased after 10 min of stimulation and subsequently decreased afterwards, whereas no Pol II recruitment was detected at the upstream regions of TSS, *regions B* and *C* (Supplemental Fig. S2*B*). Kinetic ChIP measurements revealed almost no HDAC1 (blue line) and HDAC3 (red line) accumulation in *region A* of *RNASE1* after control or TNF-α stimulation of HUVECs. Compared with that, HDAC2 significantly accumulated at *region A* after 10 min of TNF-α treatment. This binding declined over time; however, it still remained elevated compared with HDAC1 and HDAC3 (**Fig. 5*A***). Similar results were obtained by analysis of *region B* for all 3 HDACs (Fig. 5*B*). In *region C*, no specific accumulation of HDAC1–3 was observed upon TNF-α stimulation (Supplemental Fig. S2*D*). To ensure that HDAC accumulation to the *RNASE1* promoter region was independent of their abundance in the cells, mRNA regulation of HDAC1–3 was analyzed by qRT-PCR. No significant regulation of HDAC1–3 mRNA was detected in TNF-α stimulation kinetics (Supplemental Fig. S3), indicating that recruitment of HDAC1–3 to the *RNASE1* promoter region was independent of mRNA abundance.

Taken together, HDAC2 significantly accumulated in *regions A* and *B* of the *RNASE1* promoter upon proinflammatory stimulation of ECs.

HDAC1 and HDAC2 double KD restores RNase1 mRNA abundance upon TNF-α stimulation

To validate the specific impact of HDAC2 on RNase1 expression, siRNA KD of HDAC2 and the functionally redundant enzyme HDAC1, which often coexists in the same repressor complexes as HDAC2 (38, 39), were performed in EA.hy926 cells. Successful KD of both HDAC1 and HDAC2 mRNA as well as double KD of HDAC1 and HDAC2 were confirmed in CTRL cells as well as after TNF-α treatment (**Fig. 6*A***). To highlight the differences in RNase1 mRNA abundance upon siRNA KD, fold-change values normalized to siC CTRL cells (Fig. 6*B*, left panel) and the relative percentage of mRNA abundance upon stimulation to the respective KD in CTRL cells (Fig. 6*B*, right panel) were analyzed. TNF-α stimulation resulted in a significant down-regulation of RNase1 mRNA, corresponding to a reduction to ~65–70% of respective CTRL cells. This RNase1 down-regulation was prevented by double KD of HDAC1 and HDAC2, but not by siC as well as siRNAs targeting HDAC1 or HDAC2 alone (Fig. 6*B*). Additionally, upon TNF-α stimulation, relative RNase1 mRNA abundance was significantly increased in the double KD compared to cells transfected with siC, siRNA targeting HDAC2 and almost significantly to siRNA targeting HDAC1 (Fig. 6*B*, right panel).

These results suggest that HDAC2 is the most important HDAC involved in RNase1 regulation by binding to the *RNASE1* promoter (Fig. 5). However, in the absence of HDAC2 mRNA, HDAC1 might act redundantly on RNase1 because only double KD of

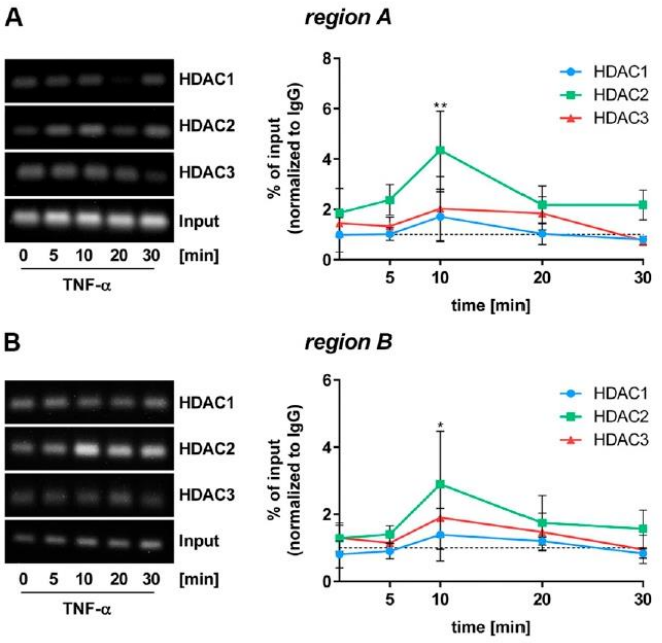

Figure 5. HDAC2 accumulates at the *RNASE1* promoter *regions A* and *B* upon TNF-α stimulation. HUVECs were stimulated with 10 ng/ml TNF-α for 5, 10, 20, or 30 min or left untreated as control, respectively. ChIP using specific antibodies against HDAC1 (blue), HDAC2 (green), HDAC3 (red), or an unspecific IgG control was performed. HDAC accumulation to *region A* (*A*) and *region B* (*B*) was analyzed by qPCR. For qualitative analysis, qPCR samples of one representative replicate were loaded on an agarose gel (left panels), and qPCR results of 3 representative replicates (right panel) were quantitatively analyzed and depicted as percentage of the input control (1%) normalized to IgG; $n = 3$. Means ± SD; 2-way ANOVA using Holm-Sidak posttest. $^{*}P < 0.05$, $^{**}P < 0.01$.

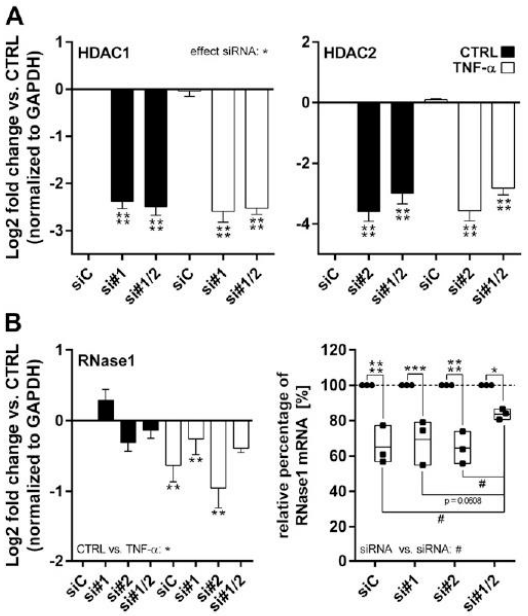

Figure 6. HDAC siRNA KD restores RNase1 mRNA from TNF-α–mediated down-regulation. EA.hy926 cells were transfected with 60 pmol siRNA against HDAC1 (si#1), HDAC2 (si#2), a combination of both (si#1/2; each 30 pmol), or siC siRNA control for 24 h followed by additional 24 h of stimulation with 10 ng/ml TNF-α or left untreated as control (CTRL). RNA was isolated, and mRNA expression of HDAC1 and HDAC2 (*A*) and RNase1 (*B*) was analyzed by qRT-PCR. Results were normalized to endogenous GAPDH and CTRL siC samples. Statistics were performed on log2-transformed data; relative percentage of RNase1 mRNA level was calculated for each transfection relative to the respective control in untreated cells, set to 100% [(*B*), right panel]; $n = 3$. Means ± SD; 2-way ANOVA (*A*, *B*, left panel) or 1-way ANOVA (*B*, right panel) using Holm-Sidak posttest. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$, $^{\#}P < 0.05$.

HDAC1 and HDAC2 restored RNase1 mRNA abundance upon proinflammatory stimulation.

DISCUSSION

EC functions play an important role in the regulation and maintenance of vascular homeostasis and integrity. The loss or disruption of this function is closely linked to cardiovascular pathologies such as atherosclerosis, thrombosis, and myocardial infarction (2, 3, 40), which according to the World Health Organization, cause several million deaths per year worldwide. In this study, we identified HDAC2-associated chromatin modifications as a critical event in the regulation of RNase1, an important regulator of endothelial homeostasis.

RNase1 has been identified as a relevant factor to protect vascular homeostasis by regulation of EC function. RNase1 acts as a counterpart to eRNA, a known damage-associated molecular pattern that contributes to cardiovascular diseases (2), to maintain vascular integrity by balancing the RNase1-eRNA system. For instance, administration of RNase1 led to a better disease outcome in ischemic heart disease and thrombus or edema formation by eliminating proinflammatory cytokine expression and plaque development (8, 13, 14, 41, 42). Upon prolonged inflammation, RNase1 function is disturbed, resulting in a loss of vascular integrity, which was demonstrated, for example, by redistribution of the cell adhesion molecule vascular endothelial cadherin due to RNase1 down-regulation (6). Consequently, understanding RNase1 regulation in ECs under proinflammatory conditions is important for the development of new therapeutic strategies to fight cardiovascular diseases.

It has been reported that down-regulation of RNase1 by TNF-α was successfully blocked by pretreatment with the HDAC inhibitor trichostatin A in HUVECs. Based on this observation, a regulatory mechanism for RNase1 *via* HDACs on chromatin level was proposed (6). Hence, the role of HDACs in the regulation of RNase1 expression during inflammation in ECs was investigated.

In this study, we found that down-regulation of RNase1 mRNA occurred after TNF-α or IL-1β stimulation, with increasing magnitude over time, supporting previous findings by Gansler *et al.* (6). However, this effect might not be a general proinflammatory reaction. Only treatment with the TLR-3 ligand Poly I:C, a synthetic double-stranded RNA analog, was able to mimic the effect of TNF-α on RNase1. In that sense, a common signaling pathway activated by TLR-3, TNF-α, and IL-1β stimulation may be important for RNase1 regulation. These 3 stimuli share 2 common signaling pathways *via* NF-κB or MAPK (43–45). Gansler *et al.* (6) demonstrated that RNase1 mRNA expression is not dependent on NF-κB signaling, because treatment with the NF-κB inhibitor BAY 11-7082 did not restore RNase1 mRNA expression upon TNF-α or IL-1β stimulation. Accordingly, RNase1 regulation seems to depend on MAPK signaling *via* p38 MAPK or JNK (43–45).

The identification of the *RNASE1* promoter was necessary to gain information on the chromatin state of *RNASE1* upon proinflammatory stimulation. As described in the literature, promoter regions are separated into 3 different parts: the core promoter, the proximal promoter, and the distal promoter. The core promoter comprises a region of ~100 bp around the TSS and contains specific marks for actively transcribed chromatin (*e.g.*, H3K27ac histone marks) (23, 36, 46, 47). The proximal and the distal promoter are located several hundred and up to 1000 bp, respectively, upstream of the core promoter and contain additional regulatory elements (36). High-throughput screenings provided by the UCSC Genome Browser suggested a POLR2A binding site directly upstream of the RNase1 transcript variants. POLR2A is the largest subunit of the Polymerase II transcription complex and is essential for its catalytic activity (48, 49). Additionally, a DNase hypersensitivity site, a CCCTC-binding factor and FOS

proto oncogene transcription factor binding site, and the active histone mark H3K27ac are located in close proximity to this region. Together, these prominent markers strongly point toward a potential core promoter and TSS located in *region A* of *RNASE1*. These predictions are confirmed by the significant transcriptional activity in the luciferase reporter assay with construct C2 and C3 of the *RNASE1* gene region.

Besides the impaired Pol II binding in *region A* upon proinflammatory stimulation, the core promoter region as well as the proximal and distal promoter, depicted as *regions B* and *C*, respectively, display significant H4 and H3K27 acetylation marks, which were abrogated upon proinflammatory stimulation. Chromatin modifications are of great importance during gene regulation, and the acetylation state of promoter regions, in particular, is widely used to identify transcriptional activity or repression (23, 36, 47). The literature clearly demonstrates that acetylation of chromatin is tightly associated with actively transcribed genes (50). High-resolution genome-wide mapping of activated T-cells has demonstrated that chromatin accessibility and gene expression is correlated with increased acetylation of promoter regions (21). Moreover, acetylation of H4 and H3K27 correlated with gene expression, and H3K27ac was mainly located around the TSS of regulated genes and suggested to prevent repressive trimethylation of H3K27 in these regions (23, 47). In ECs, Li *et al.* (51) analyzed high-throughput data to correlate gene expression and histone modifications in TNF-α–treated HUVECs and also defined enriched H3K27ac as an active chromatin mark of up-regulated genes. In addition, deacetylation has been closely linked to transcriptional repression. In HUVECs, the transcription factor BTB domain and CNC homolog 1 recruits HDAC1 to the *IL-8* promoter to deacetylate H4 and histone 3 (H3) and subsequently repress gene expression (52). Additionally, Aurora *et al.* (53) demonstrated that NF-κB regulates angiogenesis in vascular ECs *via* recruitment of HDAC1 to target gene promoters to deacetylate H3 and H4. Accordingly, deacetylation of H4 and H3K27 at the *RNASE1* promoter corresponds to the impaired Pol II binding to the *RNASE1* TSS because of augmented histone-DNA interactions in a condensed chromatin structure.

Concerning the significant deacetylation at the *RNASE1* promoter site upon proinflammatory stimulation, the HDAC enzymes that mediate deacetylation are of particular interest (26). Treatment with inhibitor MS275, specifically targeting class I HDACs HDAC1–3 (54, 55), successfully rescued RNase1 mRNA expression after TNF-α treatment through significant recovery of H4ac and H3K27ac, leading to increased Pol II recruitment at the core promoter of *RNASE1* (*region A*). Additionally, even in the more upstream promoter regions, *regions B* and *C*, MS275 restored histone acetylation. These results are confirmed by previous findings, in which MS275 treatment has been observed to successfully increase H3 and H4 acetylation in LPS/IFN-γ–treated macrophages and pediatric tumor cell lines or reverse hypo-acetylation in the immature rat hippocampus (55–57). Therefore, restored *RNASE1* acetylation upon MS275 treatment demonstrates an important impact of HDAC1–3 on RNase1, which is further supported by a significant recovery of RNase1 mRNA by MS275 treatment upon inflammation. In this regard, the identification of specific HDACs, recruited to the *RNASE1* promoter upon inflammation, is of key interest. Our results demonstrate a significant accumulation of HDAC2 in *region A* and *B* of *RNASE1*, whereas HDAC1 and HDAC3 were hardly regulated, as well as suggest a role of HDAC2 in RNase1 regulation. Thereby, proinflammatory stimulation induced HDAC2 recruitment to the *RNASE1* promoter, local histone deacetylation, and transcriptional repression in ECs.

Interestingly, there is indication for functional redundancy between HDAC2 and HDAC1. Here, we demonstrated that only double KD of HDAC1 and HDAC2 prevented significant down-regulation of RNase1 mRNA by TNF-α treatment. These results suggest that HDAC1 might partially take over the function in absence of HDAC2 to maintain RNase1 regulation. This functional redundancy of HDAC1 and HDAC2 is supported by their high sequence homology and their frequent coexistence and function in multiprotein repressor complexes (39, 58). In this context, functional redundancy of these enzymes is already described in the literature [*e.g.*, during cardiac morphogenesis or oocyte development in mice (59, 60) and B-cell development (61)]. However, double KD of HDAC1 and HDAC2 did not completely restore RNase1 mRNA abundance. Several studies indicated that HDAC1 or HDAC2 proteins might persist even after 48 h of siRNA transfection or gene inactivation, demonstrating high stability of HDAC proteins (62–65). Thus, it might be possible that the remaining protein can still regulate RNase1 expression in part.

Despite the predicted functional redundancy of HDAC2 and HDAC1 in RNase1 regulation, HDAC2 still seems to be the most important HDAC in RNase1 regulation because of its significant binding to the *RNASE1* promoter upon proinflammatory stimulation. These findings are supported by the essential role of class I HDACs, especially HDAC2, in inflammation: in the context of LPS induced inflammation, HDAC2 has been observed to down-regulate expression of IL-6 by recruitment to its promoter and subsequent deacetylation of H4 and H3 in myeloid cells (66). Similar regulatory functions were detected in chronic inflammatory disorders like rheumatoid arthritis or multiple sclerosis, in which dysregulation of the transcription factor RAR-related orphan receptor γ has been described to result in HDAC2 recruitment to *IL-17* promoter and its subsequent repression (67). Besides that, HDAC2 also exerts a particular role in the context of vascular inflammation. For instance, down-regulation of the transcription factor class II transactivator in a deacetylation-dependent manner in smooth muscle cells and macrophages has been observed, thereby influencing the progression of atherogenesis (68). These examples demonstrate the impact of HDAC2 in

inflammation, emphasizing the necessity to develop novel therapeutic strategies to treat inflammatory and vascular diseases by targeting HDAC2.

HDAC inhibitors have already been described as upcoming therapeutics for inflammatory diseases. For instance, class I HDACs have been demonstrated to trigger cell proliferation and survival and are highly up-regulated in several cancers, as shown for HDAC2 in cervical or gastric cancer (69, 70). In this regard, universal HDAC inhibitors are already approved for clinical treatment of cutaneous T-cell lymphoma or ovarian cancer, in which inhibitor treatment resulted in robust increase of histone acetylation accompanied with cancer cell differentiation, making those cells more susceptible to chemotherapy (30, 71, 72). Besides that, global HDAC inhibitors also have been shown to be beneficial in cardiovascular diseases by blocking cardiac hypertrophy and fibrosis, preventing progression of atherosclerosis, or preserving cardiac performance after myocardial infarction and ischemia-reperfusion injury (73–79). However, more specific inhibitors such as MS275 are already tested in clinical trials (*e.g.*, as antiepileptic or breast/lung cancer drug), designating MS275 as a promising new candidate for HDAC inhibitor therapy. Nevertheless, MS275 targets HDAC1–3 rather than inhibiting one specific HDAC (*e.g.*, HDAC2), and the development of enzyme specific inhibitors evolves to be challenging. This problem can be linked to diverse functions and interaction partners of HDACs varying between different cell types (80–82) and also to functional redundancy between distinct enzymes, such as HDAC1 and 2, aggravating these difficulties (83, 84). However, there is ongoing progress in developing specific HDAC inhibitors (85) or, specifically, target HDAC2 function at different cellular levels (*i.e.*, by inhibition of its interaction partners) (86).

Despite the challenges described, the present work demonstrates the function of MS275 to successfully restore the protective vascular factor RNase1 after proinflammatory stimulation. These results combined with the protective function of RNase1 in vascular pathologies, such as heart protection against ischemia-reperfusion injury or reduction of myocardial infarction size in a rat model system, offer new promising therapeutic strategies for cardiovascular diseases (2, 12, 13, 87). Hence, treatment with MS275, identification and targeting of HDAC2 interaction partners and signaling pathways, and administration of RNase1 can be suitable to prevent the damaging effect of HDAC2 recruitment to the *RNASE1* promoter, its deacetylation, and its subsequent down-regulation.

In summary, we describe a mechanism by which inflammatory cytokines impaired endothelial RNase1-eRNA homeostasis regulation: proinflammatory stimulation of ECs primed the class I HDAC HDAC2 to deacetylate H4 and H3K27 in the predicted *RNASE1* promoter region. Consequently, Pol II recruitment and RNase1 mRNA expression were suppressed. This may be an important feature of future chromatin-centered therapies addressing vascular diseases. Our future analyses will focus on elucidation of underlying signaling pathways and factors by which HDAC2 is recruited to the *RNASE1* promoter to regulate RNase1 upon inflammation.

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AUTHOR CONTRIBUTIONS

K. Bedenbender, N. Scheller, S. Fischer, B. T. Schmeck, and E. Vollmeister designed research; K. Bedenbender, N. Scheller, and S. Leiting analyzed data; K. Bedenbender, N. Scheller, and S. Leiting performed research; K. Bedenbender and E. Vollmeister wrote the manuscript; and S. Fischer, N. Scheller, S. Leiting, K. T. Preissner, and B. T. Schmeck revised the manuscript.

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Supplemental material

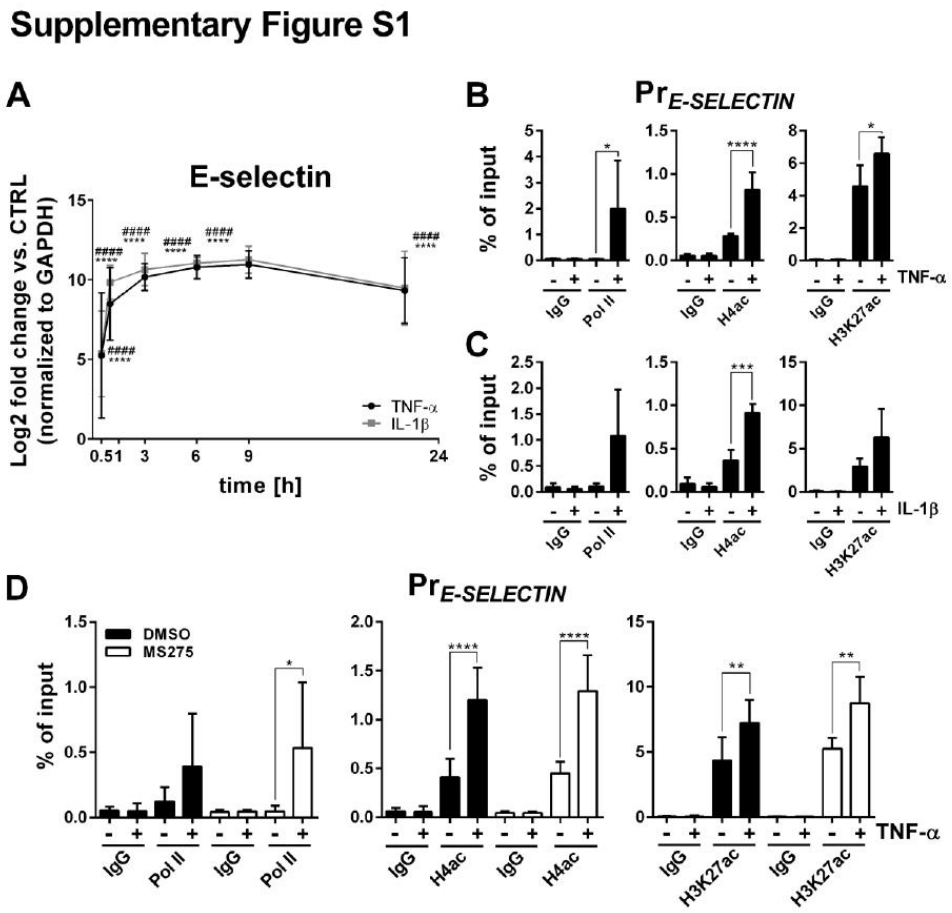

Figure S1. Effect of proinflammatory stimulation on E-selectin in HUVEC. A) HUVEC were stimulated with 10 ng/ml TNF-α or IL-1β for 0.5, 1, 3, 6, 9, or 24 h. Expression of E-selectin mRNA was analyzed by qPCR. Results were normalized to GAPDH and the respective CTRL sample. n = 3-4; mean ± standard deviation (SD) was calculated using log2-transformed data. Two-way ANOVA using Holm-Sidak posttest. Significance for TNF-α: ****$p < 0.0001$; Significance for IL-1β: $^{\#\#\#\#}p < 0.0001$. B-C) For chromatin immunoprecipitation (ChIP) analysis, HUVEC were stimulated with 10 ng/ml TNF-α (B) or IL-1β (C) for 30 or 60 min, respectively. For MS275 ChIP (D), HUVEC were pretreated with 4 µM MS275 (white bars) or DMSO (black bars) as solvent control for 2 h and subsequently stimulated with 10 ng/ml TNF-α for 30 min (+) or left untreated as control (-). Immunoprecipitation using specific antibodies against Pol II, H4ac, H3K27ac, or an unspecific IgG control was performed. The *E-SELECTIN* promoter region ($Pr_{\text{E-SELECTIN}}$), pulled down by antibodies, was analyzed by qPCR. Results were normalized to input control (1%). n = 3-4; mean ± SD, One-way ANOVA using Holm-Sidak posttest. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

Supplementary Figure S2

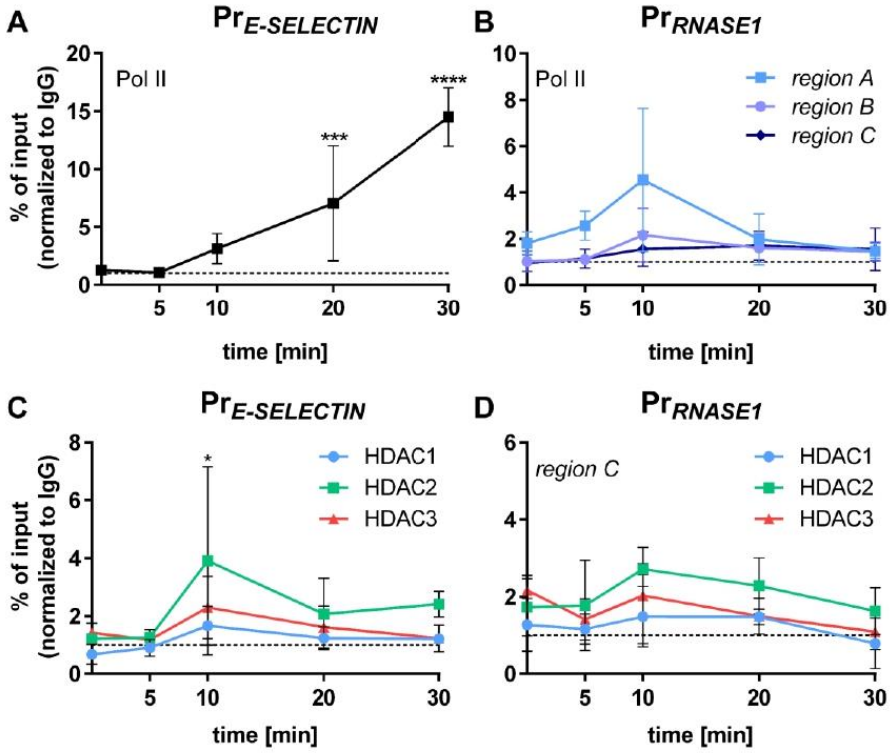

Figure S2. Chromatin immunoprecipitation kinetics in HUVEC. HUVEC were stimulated with 10 ng/ml TNF-α for 5, 10, 20, or 30 min or left untreated as control. ChIP using specific antibodies against Pol II, HDAC1 (blue), HDAC2 (green), HDAC3 (red), or an unspecific IgG control was performed. A, B) Pol II recruitment to the *RNASE1* promoter (Pr_{RNASE1}) *regions A*, *B*, *C* or the *E-SELECTIN* promoter ($Pr_{E\text{-}SELECTIN}$) as well as C, D) HDAC accumulation to Pr_{RNASE1} *region C* or $Pr_{E\text{-}SELECTIN}$ was analyzed by qPCR. Results were depicted as percent of input control (1%) and normalized to IgG. n = 3; mean ± SD, One-way ANOVA using Holm-Sidak posttest; *$p < 0.05$, ***$p < 0.001$, ****$p < 0.0001$.

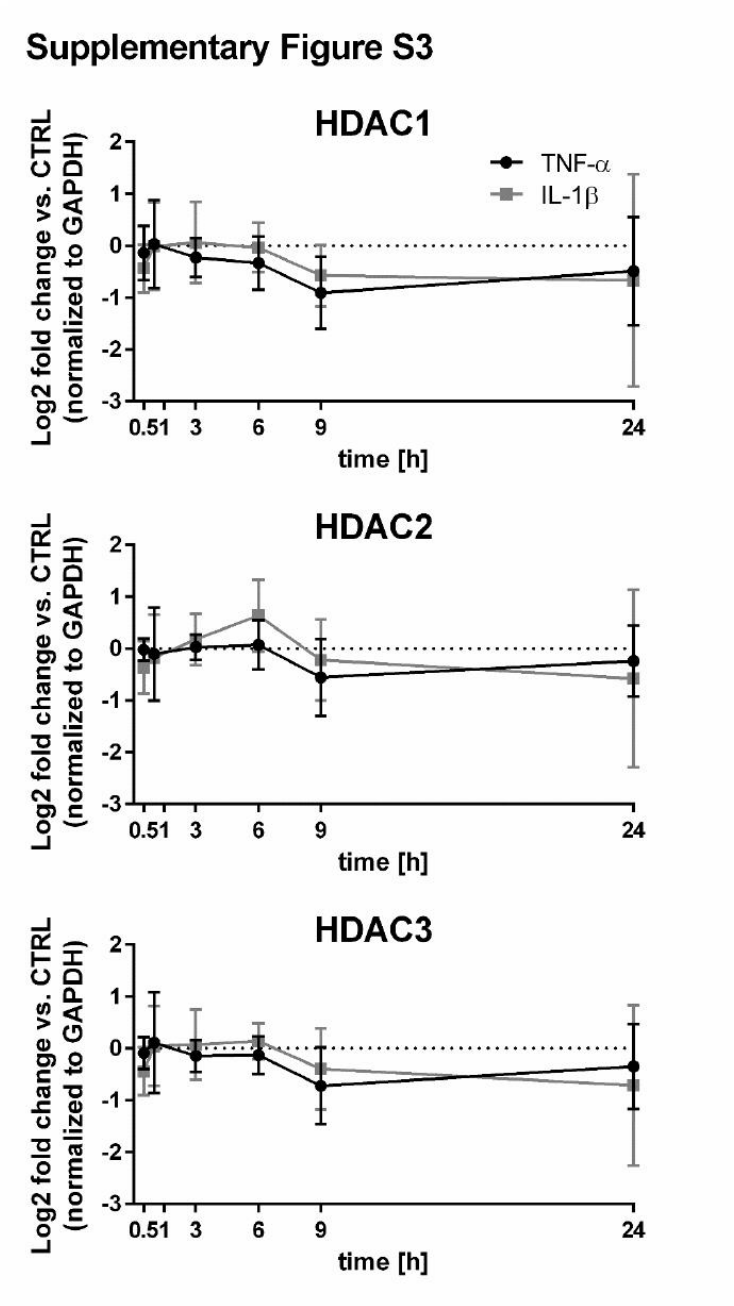

Figure S3. Regulation of HDAC1-3 mRNA upon proinflammatory stimulation. HUVEC were stimulated with 10 ng/ml TNF-α or IL-1β for 0.5, 1, 3, 6, 9, or 24 h. RNA was isolated and mRNA expression of HDAC1-3 was analyzed by quantitative PCR. Results were normalized to GAPDH and the respective CTRL samples. n = 3-4; mean ± SD was calculated on log2-transformed data, Two-way ANOVA using Holm-Sidak posttest, tested as not significant.

5.2 Conference contributions

2016

Bedenbender, K., Scheller, N., Fischer, S., Preissner, K. T., Schmeck, B. T.; Regulation of RNAse1 in endothelial cells via chromatin modifications and microRNAs (poster presentation) - 14th Annual Retreat of the International Graduate Programme Molecular Biology and Medicine of the Lung, 2016, Rauischholzhausen, Germany

Bedenbender, K., Scheller, N., Fischer, S., Preissner, K. T., Schmeck, B. T.; Regulation of RNAse1 in endothelial cells via chromatin modifications and microRNAs (poster presentation) - Symposium des SFB-TR84 2016, Berlin Germany

Bedenbender, K., Scheller, N., Schmeck, B. T.; Mechanisms of vascular remodeling in chronic obstructive pulmonary disease (COPD) (oral presentation) - 3. Symposium "Von der Inflammation zur Innovation", ein von der Behring Röntgen Stiftung geförderten Graduiertenkollegs ERAGON, 2016, Marburg, Germany

Bedenbender, K., Scheller, N., Fischer, S., Preissner, K. T., Schmeck, B. T.; Inflammatory regulation of RNAse1 in endothelial cells via chromatin modifications and microRNAs (poster and oral presentation) - Herbsttagung der Sektionen Infektiologie & Tuberkulose und Zellbiologie der DGP, 2016, Hannover, Germany

2017

Bedenbender, K., Scheller, N., Fischer, Vollmeister, E., S., Preissner, K. T., Schmeck, B. T., RNase1 regulation in endothelial cells via chromatin modifications (oral presentation) - 15th Annual Retreat of the International Graduate Programme Molecular Biology and Medicine of the Lung, 2017, Rauischholzhausen, Germany

Bedenbender, K., Vollmeister, E., Scheller, N., Fischer, S., Preissner, K. T., Schmeck, B. T., Mechanisms of vascular remodeling in chronic obstructive pulmonary disease (COPD) (poster presentation) - 4. Symposium "Von der Inflammation zur Innovation", ein von der Behring Röntgen Stiftung geförderten Graduiertenkollegs ERAGON, 2017, Marburg, Germany

Bedenbender, K., Vollmeister, E., Scheller, N., Fischer, S., Preissner, K. T., Schmeck, B. T., Inflammatory regulation of RNase1 in endothelial cells via chromatin modifications (poster presentation) - Herbsttagung der Sektionen Infektiologie & Tuberkulose und Zellbiologie der DGP, 2017, Giessen, Germany

2018

Bedenbender, K., Scheller, N., Fischer, Vollmeister, E., S., Preissner, K. T., Schmeck, B. T., Inflammation-mediated deacetylation of the RNASE1 promoter via HDAC2 (oral presentation) - 16th Annual Retreat of the International Graduate Programme Molecular Biology and Medicine of the Lung, 2018, Rauischholzhausen, Germany

Bedenbender, K., Vollmeister, E., Scheller, N., Fischer, S., Preissner, K. T., Schmeck, B. T., Mechanisms of vascular remodeling in chronic obstructive pulmonary disease (COPD) (oral presentation) - 5. Symposium "Von der Inflammation zur Innovation", ein von der Behring Röntgen Stiftung geförderten Graduiertenkollegs ERAGON, 2018, Marburg, Germany

2019

Bedenbender, K., Scheller, N., Fischer, S., Leiting, S., Preissner, K. T., Schmeck, B. T., and Vollmeister, E., Inflammation-mediated deacetylation of the ribonuclease 1 promoter via histone deacetylase 2 in endothelial cells (poster presentation) – Jahrestreffen des Deutschen Zentrums für Infektionsforschung (DZIF) 2019, Bad Nauheim, Germany

5.3 Directory of academic teaching

Academic teachers at the Philipps-University of Marburg

Course of study: Bachelor of Science in Biology:

Agarwal, Albers, Baranovski, Baumeister, Bölker, Braker, Brandis-Heep, Brandl, Brändle, Bremer, Brune, Buttgereit, Chatterjee, Conrad, Feuser, Galland, Greiner, Hassel, Heider, Higgs, Hoffmann, Homberg, Imhof, Kahmann, Kost, Kostron, Lingelbach, Lohöfer, Maier, Mathies, Mösch, Önel, Parak, Pryborski, Rathke, Reiß, Renkawitz-Pohl, Sandner, Sandrock, Schachtner, Soogard-Andersen, Thanbichler, Thauer, Weber, Zauner, Ziegenhagen

Academic teachers at the Johann Wolfgang Goethe-University of Frankfurt

Course of study: Master of Science in Cell Biology and Physiology

Acker-Palmer, Ebersberger, Gaese, Gampe, Grünewald, Kaufmann-Reiche, Kössel, Schleiff, Schliwa, Starzinski-Powitz, Stelzer, Strilic, Volknandt, Waibler

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