

**Cell-to-cell transcriptional remodelling: a nuclear role for myeloperoxidase (MPO) in
endothelial inflammation**

Inaugural Dissertation

zur

Erlangung des Doktorgrades
Dr. nat. med.

der Medizinischen Fakultät
und
der Mathematisch-Naturwissenschaftlichen Fakultät
der Universität zu Köln

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Abstract

Myeloperoxidase (MPO), formerly termed Verdoperoxidase, is a lysosomal protein produced mostly by activated neutrophils and monocytes. It functions in response to pathogens during bacterial and fungicidal infections and contributes to pathogen clearance both within the phagosome and upon secretion. Clinical studies identified both MPO deficiency and over presentation in a wide range of diseases, including cardiovascular diseases. However, patients lacking MPO were found healthy besides minor bacterial infection [111]. Hence, the demand to MPO functional studies apart from its role in the host defense has risen. Previous study had described the accumulation of MPO in the plasma upon secretion, as well as the afterwards uptake by endothelial cells (ECs) [123]. This finding opens up a promising aspect to dissect the vascular remodelling potential of MPO.

In this dissertation, EC remodelling upon MPO transcytosis was studied systematically by hiring RNA-seq and ATAC-seq techniques. Both transcriptional regulations and chromatin structural changes were identified in the MPO treated cells. For the first time, MPO association with EC chromatin was identified and verified via using multiple molecular biology methods, and its occupying regions realised via ChIP-seq. Moreover, by treating ECs with inactivated MPO, the EC remodelling was recapitalised, indicating that the effect is independent of MPO enzymatic activity.

With the help of mass-spectrometry analysis, potential MPO interacting proteins were identified, including ILF3, which previously was described in the context of angiogenesis. A significant loss of cytoplasmic and gain of nuclear ILF3 was detected. In the end, genes regulated by MPO via ILF3, and MPO-ILF3 synergistical effect on RNA stabilisation were studied via ILF3 knock down in ECs.

Zusammenfassung

Myeloperoxidase (MPO), früher als Verdoperoxidase bezeichnet, ist ein lysosomales Protein, das hauptsächlich von aktivierten Neutrophilen und Monozyten produziert wird. Es wird als Reaktion auf Krankheitserreger bei bakteriellen und pilztötenden Infektionen gebildet und trägt sowohl im Phagosom als auch bei der Sekretion zur Beseitigung von Krankheitserregern bei. In klinischen Studien wurden sowohl ein MPO-Mangel als auch ein übermäßiges Auftreten bei einer Vielzahl von Krankheiten, einschließlich Herz-Kreislauf-Erkrankungen, festgestellt. Allerdings wurden Patienten, denen MPO fehlte, abgesehen von leichten bakteriellen Infektionen als gesund befunden [111]. Daher ist die Nachfrage nach MPO-Funktionsstudien abgesehen von seiner Rolle in der Wirtsabwehr gestiegen. In einer früheren Studie wurde die Anreicherung von MPO im Plasma nach der Sekretion sowie die anschließende Aufnahme durch Endothelzellen (ECs) beschrieben [123]. Dieser Befund eröffnet einen vielversprechenden Aspekt, um das vaskuläre Umbaupotenzial von MPO zu untersuchen.

In dieser Dissertation wurde der EC-Umbau nach MPO-Transzytose systematisch mit Hilfe von RNA-seq- und ATAC-seq-Techniken untersucht. In den mit MPO behandelten Zellen wurden sowohl Transkriptionsregulationen als auch strukturelle Chromatinveränderungen identifiziert. Zum ersten Mal wurde die Assoziation von MPO mit EC-Chromatin mit Hilfe mehrerer molekularbiologischer Methoden identifiziert und verifiziert, und die von MPO besetzten Regionen wurden mit Hilfe von ChIP-seq ermittelt. Darüber hinaus konnte durch die Behandlung von ECs mit inaktiviertem MPO der EC-Umbau rekapitalisiert werden, was darauf hindeutet, dass der Effekt unabhängig von der enzymatischen Aktivität von MPO ist.

Mithilfe einer massenspektrometrischen Analyse wurden potenziell mit MPO interagierende Proteine identifiziert, darunter ILF3, das zuvor im Zusammenhang mit der Angiogenese beschrieben wurde. Es wurde ein signifikanter Verlust von zytoplasmatischem und ein Zuwachs von nukleärem ILF3 festgestellt. Schließlich wurden Gene, die durch MPO über ILF3 reguliert werden, und die synergistische Wirkung von MPO und ILF3 auf die RNA-Stabilisierung durch Ausschalten von ILF3 in ECs untersucht.

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List of Abbreviations

<i>Abbreviations</i>	<i>Definition</i>
<i>MPO</i>	Myeloperoxidase
<i>ChIP-seq</i>	Chromatin Immunoprecipitation sequencing
<i>RNA-seq</i>	RNA-sequencing
<i>GREAT</i>	Genomic Regions Enrichment of Annotations Tool
<i>NET</i>	Neutrophil Extracellular Traps
<i>MNase</i>	Micrococcal Nuclease
<i>IF</i>	Immunofluorescence
<i>IP</i>	Immunoprecipitation
<i>PAGE</i>	polyacrylamide gel electrophoresis
<i>HUVECs</i>	Human Umbilical Vein Endothelial Cells
<i>ATAC-seq</i>	Assay for Transposase-Accessible Chromatin using sequencing
<i>ctr</i>	control
<i>MPO2hr</i>	MPO 2 hours
<i>MPO8hr</i>	MPO 8 hours
<i>endMT</i>	Endothelial to mesenchymal transition
<i>SASP factors</i>	Senescence-associated secretory phenotype factors
<i>EMSA</i>	Electric mobility shift assay
<i>VEGFA</i>	Vascular endothelial growth factor A
<i>VEGFR2</i>	Vascular endothelial growth factor receptor 2
<i>ILF3</i>	Interleukin enhancer binding factor 3
<i>TSS</i>	Transcription start site
<i>kDa</i>	Kilo Dalton
<i>kbp</i>	Kilo base pair
<i>hr</i>	Hour
<i>min</i>	Minute
<i>sec</i>	Second
<i>SDS</i>	Sodium dodecyl-sulfate
<i>DRB</i>	5,6-Dichlorobenzimidazole 1-β-D-ribofuranoside
<i>KD</i>	Knock down
<i>LC</i>	Light chain
<i>HC</i>	Heavy chain
<i>AG</i>	Azurophilic granule
<i>SG</i>	Specific granule
<i>GG</i>	Gelatinase granule
<i>SV</i>	Secretory vesicle
<i>CAD</i>	Coronary artery disease
<i>HF</i>	Heart failure
<i>NADPH</i>	nicotinamide dinucleotide phosphate oxidase
<i>BCAEC</i>	Bovine artery endothelial cells
<i>NE</i>	Neutrophil elastase
<i>RNA</i>	Deoxyribonucleic acid
<i>DNA</i>	Ribonucleic acid
<i>FC</i>	Final concentration
<i>AA</i>	Amino Acid

<i>DBP</i>	DNA binding protein
<i>CAT</i>	Catalog
<i>No.</i>	Number
<i>P (Cell Culture)</i>	Passage
<i>FBS</i>	Fetal bovine serum
<i>DMSO</i>	Dimethyl Sulfoxide
<i>CMMC</i>	Center for Molecular Medicine Cologne
<i>L/ml</i>	Litre/ millilitre

Introduction

1.1 Chapter 1 Myeloperoxidase: gene expression, maturation, and chemical features

1.1.1 The gene expression control of MPO

Myeloperoxidase (MPO) is first isolated from leukocytes by Kjell Agner in 1941 [1], and was first given the name ‘Verdoperoxidase’ for its green color. It is majorly produced in Polymorphonuclear leukocytes (PMNs, or neutrophils) and inferiorly produced in monocytes [2], [3]. Orthologs of the human *MPO* gene can be found in a wide spectrum of animal species (Figure 1). Curiously, the very first ortholog is found in Australian saltwater crocodile (*Crocodylus porosus*) (Figure 1) [4]. Gene blast showed that human and the crocodile *MPO* genes share 39,19% consistency, and the consistent regions are protein-coding [4].

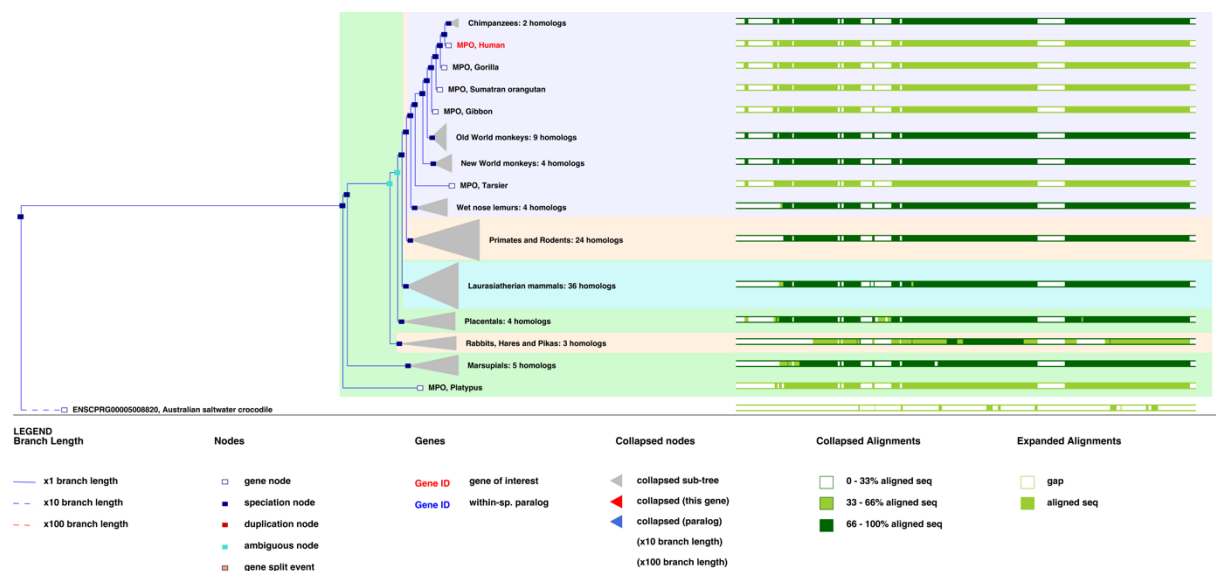


Figure 1. Phylogenetic tree showing MPO orthologs across species. The tree was generated by using ensemble online tool.

The *MPO* gene occupies human chromosome 17 and mouse chromosome 11. Its expression is strictly regulated during the maturation of neutrophils. Upon production, the mature MPO protein is stored in azurophilic granules (AG) of the neutrophils and neutrophil progenitors [2], [5], [6]. For a long time, the study to *MPO* gene expression has been tightly linked to that of the formation of azurophilic granules alongside neutrophil maturation. Illustration briefly showing the maturation process can be found in Figure 2. Other AG proteins are serine

proteases, neutrophil elastase (NE), protease 3, cathepsin G, and defensins [7], [8]. AGs were first considered to be specialized lysosomes due to their distinguishing biogenesis originating from the concave side of the Golgi body which faces the endoplasmic reticulum, like the lysosomes. Meanwhile, the other neutrophil granules are generated on the convex side of the Golgi body [9]. However, today this view is under discussion due to the fact that AG lacks lysosome specific markers such as LAMP I and II, while both were found in the multivesicular bodies (MVB) of neutrophils [10].

The formation of AG was observed starting from promyelocytes. Observations to myeloblasts revealed them to be undifferentiated unipotent cells having large oval nucleus and large nucleolus [2] where the AG is absent. In the next differentiation stage, specific granules (SGs) are formed that majorly contain lactoferrin and gelatinase associated lipocalin (NGAL) [11]. Gelatinase granules (GGs) that majorly contain gelatinase are formed laterally in further differentiated band cells [12]. Finally, secretory vesicles (SVs) that contain tetranectin are formed in mature neutrophils that contain all four types of granules [13]. Throughout the differentiation process, MPO had been detected abundantly in the AG, suggesting its specific expression in myeloblast and promyelocytic stages of myeloid lineage differentiation [2], [3], [14]. The tight regulated expression of the MPO gene makes it useful in the quality control of myeloid lineage differentiation [15].

Genetic studies to *MPO* expression confirmed the observations to MPO containing granules. Multiple *MPO* transcripts with different length and varied mRNA splicing was reported in an early study [16]. Chumakov et al. revealed a *MPO* promoter locating in between two *Alu* elements that locate at bp-1648 to bp-1345 and bp-531 to bp-200, individually. Later, Lin et al. identified other 2 promoters (Figure 4) [17]. Functional analysis to the 3 promoters revealed that only one locating at bp-128 and bp+11 is capable to produce the full-size *MPO* in myeloid differentiation *in vivo* [17]. Transcription factor SP1 binding to this promoter within *Alu* element was found to activate transcription of the *MPO* gene [18]. In the hunting to MPO promoters, Chumakov et al. noticed huge expressional variations driven by different constructs containing the same promoter, indicating the existence of both enhancers and silencers in some construct covered regions. In the same study, one silencer and one cell line specific enhancer containing 'GATA'-binding sequences were revealed [15]. Via performing DNase I footprinting assay, three cis-elements were defined (FTI, II, and III) within the enhancer region, which contains binding elements for transcription factors PU.1 and members of C/EBP family

[19], [20]. Ets family transcription factor PU.1 is known to control the expression of genes specifically in terminal differentiated hematopoietic cells, and its phosphorylation is required for its expression regulation function [21]. The association of unphosphorylated PU.1 to the *MPO* enhancer in multipotent hematopoietic cells was observed, suggesting potential regulation of *MPO* expression via PU.1 phosphorylation in myeloid differentiation [20]. On the contrary, both the association of hematopoietic differentiation regulatory transcription factor C/EBP- α with the *MPO* enhancer in multi- and unipotent hematopoietic cell lines, and the nucleus localization and phosphorylation of the C/EBP- α were verified, defining its role in positively regulating *MPO* expression [20]. Additionally, C/EBP- ϵ regulates the expression of specific granule enzymes and C/EBP- δ regulates gelatinase granule enzymes, respectively. The expression of C/EBP family transcription factors are corresponding to the differentiation stages [20], [22], [23]. RUNX1 has a known role in the granulopoiesis in hematopoietic cells, and its deletion or inhibition in mice resulted in decreased *c/ebp- α* transcription [24], [25]. It is proposed that C/EBP families and RUNX1 regulate the expression of hematopoietic granule components cooperatively [6]. Overall comparison of the upstream *MPO* gene enhancers in multipotent and unipotent hematopoietic progenitor cells revealed that although with similar accessibility, the former is functionally incompetent, confirming former studies to AG and suggesting the potential of the *MPO* enhancer in regulating myeloid differentiation [20]. A summary of the regulation build-up of *MPO* gene is shown in Figure 3 [4].

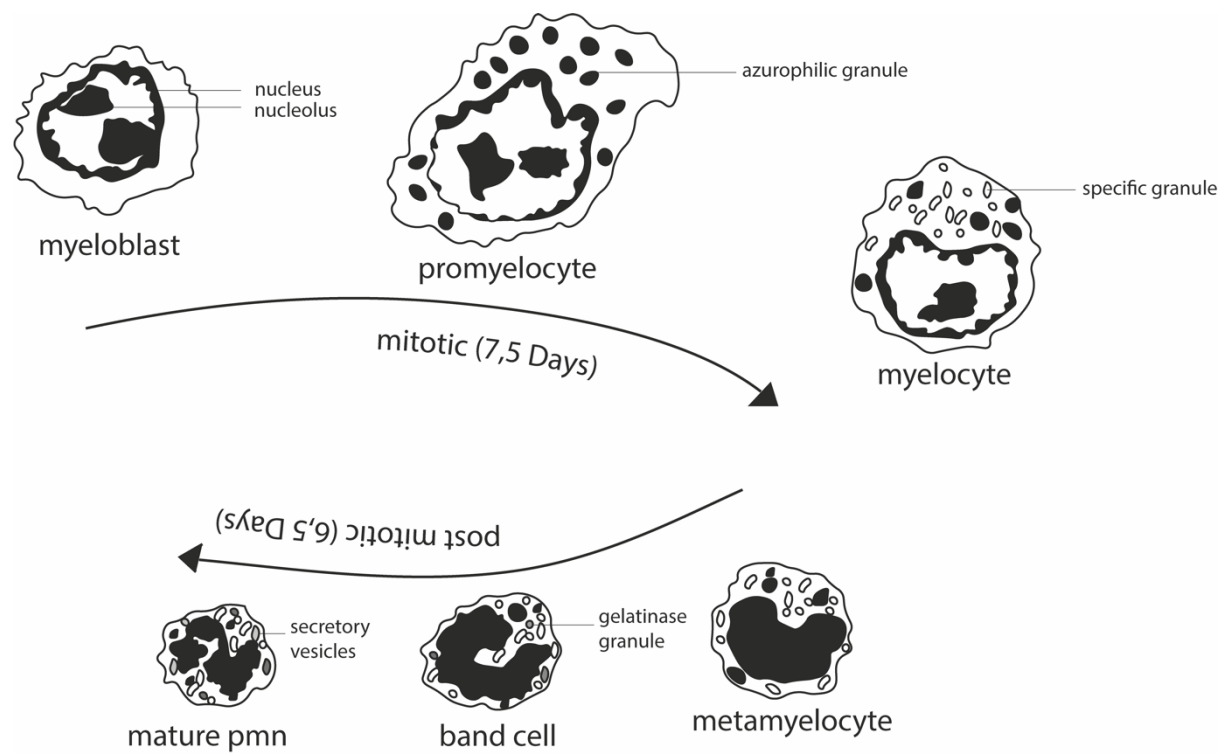


Figure 2. maturation of neutrophils with indications of granule formation time points. This is a redrawing of the illustration from the published article by Bainton et al. in 1971[2] with up-to-date modifications. PMN= polymorphonuclear neutrophil.

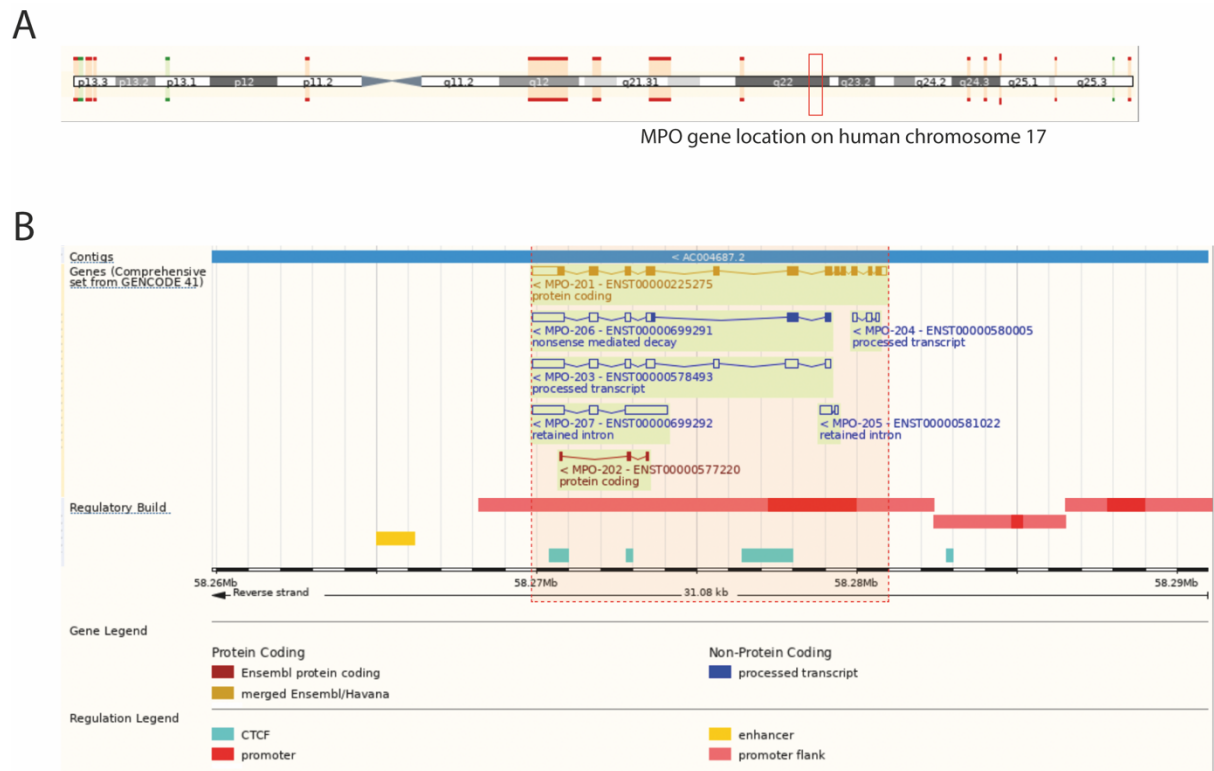


Figure 3. Landscape of human *MPO* gene. (A) *MPO* gene location on human chromosome 17 is showed in red box. (B) Relative locations of *MPO* transcripts and regulatory builds. full length *MPO* precursor transcript occupied region is showed in red box.

1.1.2 Dysregulated and aberrant *MPO* expression in human diseases

As described above, *MPO* expression is carefully controlled and nearly restricted to neutrophils and monocytes in homeostatic conditions. However, dysregulated and aberrant *MPO* expression were found in disease.

So far, studies to dysregulation of *MPO* gene expression in hematopoietic diseases revealed that epigenetic regulations play essential roles.

Acute myeloid leukemia (AML) is featured by abnormal and exceeding proliferation of immature hematopoietic cells filled in bone marrows of the patients. This event is clinically termed as hyperleukocytosis, and the immature hematopoietic cells leukemia blast. Cytochemistry determination of *MPO* expression in leukemia blasts has been carried out for AML prognosis, as it provides valuable information to the phenotype of leukemia cells. The fact that *MPO* is positively expressed in AML t(8;21) blast whilst the presence of dominant-

negative *MPO* gene positive regulators RUNX1 and C/EBP- α in those blast indicates of distinct factors regulating *MPO* gene expression in this case [26]–[28]. Studying to the methylation status of the *MPO* gene body, its promoter, and enhancer regions in 10 leukemia blast cell lines revealed demethylation of CpG sites in high *MPO* expression cell lines and hypermethylation in low *MPO* expression cell lines. DNA methylation microarray coupled with DNA methyltransferase inhibitors treatment helped to identify DNMT3B, that is responsible for the initiation of *de novo* DNA methylation, to regulate *MPO* gene expression in leukemia blasts [29].

Anti-neutrophil cytoplasmic autoantibody (ANCA)-associated small vessel vasculitis (AAV) is a systematic autoimmune disease features with vascular inflammation. The ANCA involved in AAV are found to target neutrophil granule components including MPO and proteinase-3 (PR3) [30]–[33]. Analysis to healthy donors and AAV patients mature neutrophils revealed an elevated MPO and PR3 transcription levels [34]. Functional annotations of microarray comparing leukocytes gene expression in healthy donors and AAV patients revealed their difference centred around histone, histone H3 and histone H4. In the same study, a reduction in transcription repression marker H3K9me2, and an elevation of transcription activation marker H4K16ac on the *MPO* gene were identified in AAV patient leukocytes [35].

Systemic lupus erythematosus (SLE) is an autoimmune disease involves antinuclear antibody production and immune complex deposition, where the development of lupus nephritis (LN) is one major cause of mortality [36]. Expression of a less transcribed *MPO* polymorphism allele was reported in SLE patients, and is suggested to associate with the risk of LN development in a dose-dependent manner [37]. Besides SLE, down regulation of *MPO* expression was reported in Kawasaki's disease, inflammatory bowel disease, and eosinophilic granulomatosis [38]–[40].

MPO functions positively in the innate immune system by producing oxidants to remove targeted microorganisms when produced in neutrophils and monocytes in a well-controlled fashion. However, when aberrantly expressed in certain tumor tissues, its peroxidase activity has versatile impacts. Its products, majorly hypochlorous acid, on one hand facilitates the clearance of cancer cells, whilst on the other hand, it imposes DNA damage by directly modifying nucleotides. Besides DNA damages, MPO enzymatic products were found to hinder DNA repair damage protein and lipids as well [41]–[43]. Additionally hypochlorous acid, the major MPO product, react with histones to generate chloramine and modify lipids.

Single nucleotide polymorphism (SNP) by its name is the polymorphism of a same gene defined by difference of one nucleotide [44]. It is one of the most common gene polymorphisms in human. As a result, SNPs may lead to drastic expressional changes, structural, or functional changes to the protein product [45].

One functional *MPO* polymorphism locates within the promoter region at position bp-463, and is defined by the substitution of a guanine by an adenine (-463 G/A). This substitution results in the decrease of the binding of the transcription factor SP1 to *MPO*, and finally leads to about 25 folds of transcriptional decrease. This low yielding allele (A/A and A/G) was found to negatively associated with the risk of lung, breast and ovarian cancer [46]–[50].

On the contrary, the homozygote G alleles (G/G) of the same *MPO* polymorphism that associated with active *MPO* expression was detected more frequently in the patients with early-stage ovarian cancer, suggesting a positive role of MPO in carcinoma development [48].

Aberrant *MPO* expression in central neuron system (CNS) leads to oxidant production, as a result cause tissue damage and disease conditions. In homeostatic condition, blood-brain barrier (BBB) functions as a shield to limit the infiltration of macrophages into CNS. However, studies revealed that this shield is weakened following the injury to CNS and in inflammation conditions via the activation of associated cells, such as endothelial cells and astrocytes [51]–[53]. Further studies found that this weakening effect is linked to neutrophil activation in and partially due to MPO-derived hypochlorous acid (HOCl) mediated modifications to lipids [54], [55]. In CNS, expression of *MPO* G allele by microglia, together with those MPO that brought by neutrophil infiltration into CNS, is associated with progressive multiple sclerosis and gender-specific risk increase for Alzheimer disease [56], [57]. Meanwhile, *MPO* expression in neurons was also observed in Alzheimer diseased brain, together with a three-fold increase of 3-chlorotyrosin, a derivative product of MPO [58]. Neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) induced Parkinson disease in mice is characterized by a loss of ventral midbrain dopaminergic neurons. Study to MPTP injected mice revealed upregulation of MPO and its derivative 3-chlorotyrosine in ventral midbrain. Moreover, mutant mice with defective *MPO* expression are more resistant to MPTP induced dopaminergic neuron loss. Likewise, *MPO* aberrantly expressed in astrocytes was detected in a mice Alzheimer disease model along with oxidized phospholipids [59], [60].

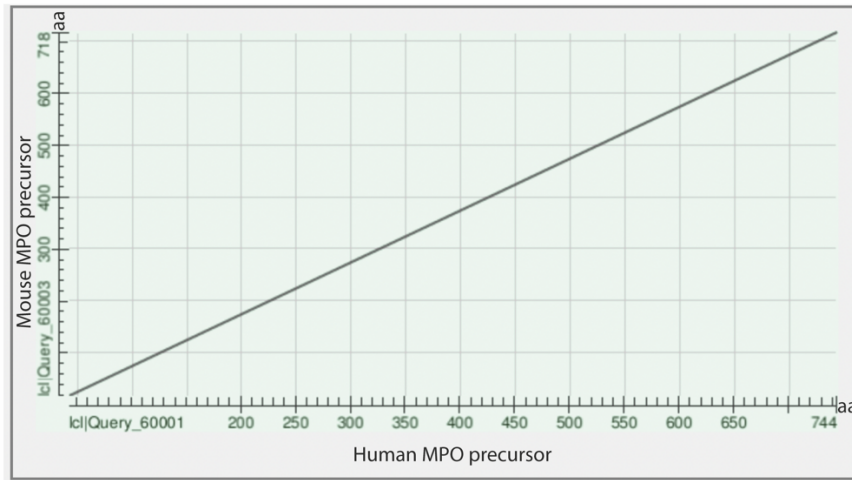
Additionally, an *in vitro* study revealed an inducible *MPO* expression in shear stressed endothelial cells [61], although the regulation to this induced expression remains unstudied.

1.1.3 Maturation of MPO in neutrophils

MPO belongs to a protein family with diverse peroxidases, including eosinophil peroxidase, lactoperoxidase, slivory peroxidase, thyroid peroxidase, ovoperoxidase, peroxidasin, peroxinexin, cyclooxygenase, and others. Originations of the members of this family spans from Monera to Animalia kingdoms [62], [63]. Studying to the open reading frames (ORF) of animalia kingdom originated members of this family revealed that the regions encoding for their heme group binding pockets share highly similarity [62].

The amino acid sequences of MPO precursors before post translational processing are highly conserved among species. Blast of the human and the mouse MPO precursor amino acid sequences using online tool showed that they share 85% identity (90% homology) [64] (Figure 4).

A



B

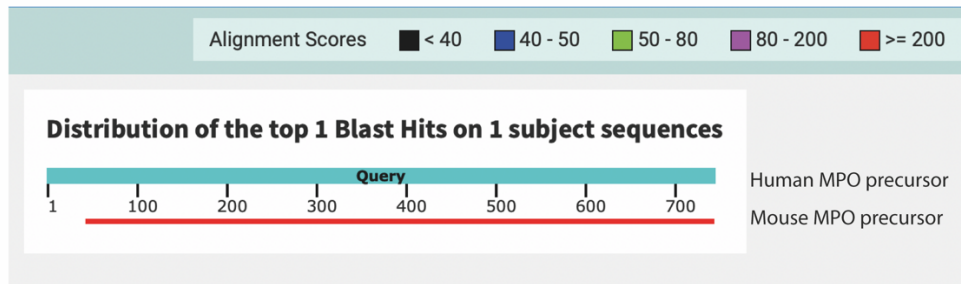


Figure 4. Human and mouse MPO precursor peptides share high identity. (A) Dox plot shows the regions of alignment between human and mouse MPO precursor amino acid sequences. (B) Overview of mouse MPO precursor aligned to human MPO precursor, color coded scores show the extend of alignment. Alignment done with NCBI online blast tool.

The 3D crystalizing structure of the mature MPO was characterized at 1,8 Å resolution, revealing it as a dimerized metalloprotein carrying two heme groups [65]. Years later, another crystal characterization of MPO at 1,25 Å resolution was casted [66]. The complete enzyme consists of two identical monomers, each carrying one heavy and one light chain (HC and LC). The monomers are linked via cysteine residues stretching from the heavy chains. The secondary structure of MPO is predominantly α -helical, with four in HC and one in LC. The molecular weight of the mature MPO dimer of around 140 kDa is much smaller compared to two times the previously mentioned precursor of around 168 kDa, indicating of post translational splicing.

The full length MPO precursor peptide, termed apo-pre-pro-MPO, undergoes a series of posttranslational modifications, intracellular trafficking, decorations and folding events before being casted into mature MPO (Figure 5). The first phase of posttranslational modification happens in the endoplasmic reticulum (ER) where both HC and LC are decorated with complex oligosaccharides and the signal peptide is removed. As a result, the 84 kDa precursor is casted into the 92 kDa apo-pro-MPO that lacks peroxidase activity [66]–[68].

In the second phase, a prosthetic heme group is attached to the Apo-pro-MPO in the ER, allowing the pro-MPO partial peroxidase activity [69]. In a study where heme synthesis is blocked by succinyl acetone, proMPO maturation was blocked. It was observed in the same study that the proMPO maturation in the ER was blocked via Golgi body disruption achieved by brefeldin A treatment. [68]. Two calcium binding ER molecular chaperones, calreticulin (CRT) and calnexin (CLN), were found to be important for the deglycosylation of the oligosaccharide side chains [70]. The pro-domain and the complex oligosaccharides chain on LC are removed from the apo-pro-MPO in the Golgi body, resulting in a 74 kDa peptide termed pro-MPO, which later folds into the MPO monomer [66] . Finally, two monomers dimerize into mature MPO before being transported to and stored in the AG of the neutrophils.

Interestingly and as a unique feature of MPO as a peroxidase family member, the proMPO that already acquired enzymatic activity but did not dimerize was not transported to AG or degraded, instead was secreted into the extracellular space. This observation suggests that the trafficking of mature MPO towards AG may be dimerization dependent [71].

Additionally, one recent research assessed the proteosome of mouse neutrophil after they leave bone marrow identified a circadian dynamic composition of the granules.

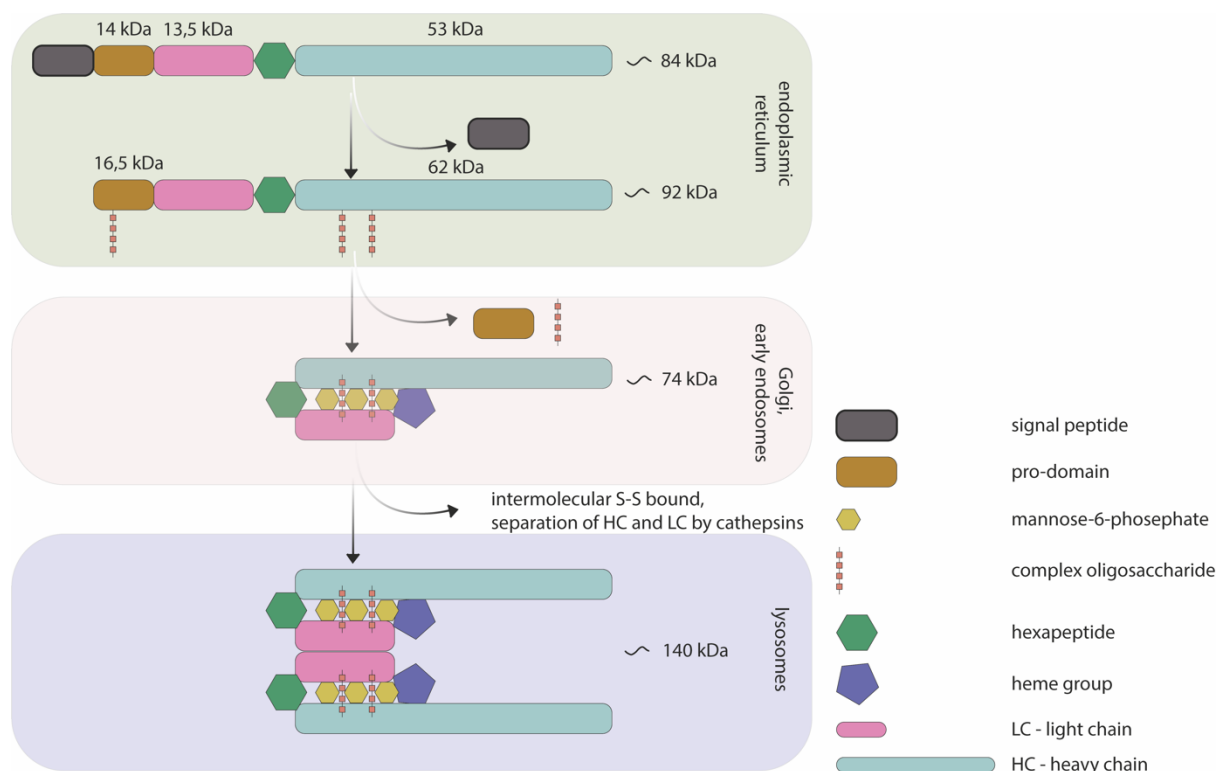


Figure 5. Maturation of MPO. Modified from Y Kargaplova et al., 2021 [72].

1.1.4 Enzymatic activity of MPO and its role in cardiovascular diseases

MPO catalysis the production of hypohalites from halides using hydrogen peroxide (H_2O_2) as major oxidizing agent, and the oxidizing potential is provided by the heme groups covalently bound to both HC and LC. During reaction, 3 different MPO compounds (compounds I, II, and III) and two forms (the native MPO and the ferrous MPO) transform from one to another, characterized with protein structural changes and prosthetic heme groups of different chemical valence, respectively (Figure 6).

MPO catalysis two redox reactions, halogenation cycle and peroxidase cycle. The first steps of both cycles involve the native MPO (Fe III) passes 2 electrons to the electron acceptor H_2O_2 , loses two electrons and becomes the more reductive MPO compound I. In the second step of the halogenation cycle, two electrons are donated halides, namely Cl^- , Br^- , I^- , and pseudohalide SCN^- . The later upon donating the electrons combine with one hydroxide (OH^-) and become hypohalites acids, namely HOCl , HOBr , HOI , and HOSCN . Meanwhile, the compound I returns to the native MPO. Since Cl^- is the most abundant halide in human circulation, the formation of HOCl is the most frequent. A sequential two-step electron transfer is involved in

the second step of the peroxidase cycle. At the beginning, one electron is given by an electron donor, whilst the compound I receives one electron and transits into compound II. Next, another electron is donated by the donor and the MPO compound II receives one electron thus return to native form. The known electro donors for peroxidase cycle includes hydrogen peroxide, nitrites, sulfhydryl, aromatic acids, indole derivatives, and potentially others [73]–[77].

Besides the halogenation cycle and peroxidase cycle, MPO also bears a superoxide dismutase activity. In physiological conditions, superoxide is formed by nicotinamide dinucleotide phosphate (NADPH) oxidase in the lysosomes of neutrophils. In the superoxide dismutase reaction, superoxide, instead of H_2O_2 , is taken as the electron acceptor by the native ferric MPO. As a result, an oxidised form of MPO, compound III, is formed. Superoxide competing with H_2O_2 for native MPO results in a decrease of both halogenation cycle and peroxidase cycle [78]–[81]. As mentioned previously, MPO uses H_2O_2 as reducing substrates when other sources are not available. Compounds II and III can transform back and forth using H_2O_2 as electron donator or electron acceptor, respectively [82].

Finally, exceeding H_2O_2 presentation to the compound III leads to irreversible damage to heme binding pocket and result in loss of the binding capacity and thus oxidative activity [82].

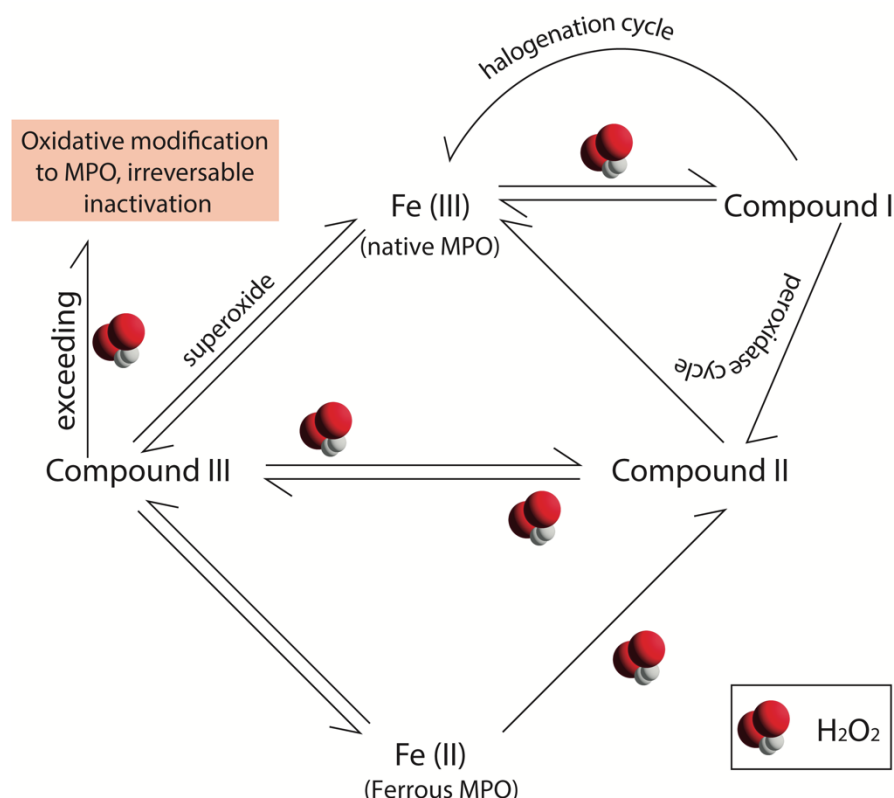


Figure 6. Myeloperoxidase catalysis halogenation cycle and peroxidase cycle reactions, and can be irreversibly inactivated by H₂O₂ saturation. Arrows indicate of capability to transform from one compound form to another.

The high oxidative capacity of MPO makes it a center of oxidative stresses in cardiovascular diseases. High levels of plasma MPO were reported in a spectrum of cardiovascular diseases including coronary artery disease (CAD), heart failure (HF), myocardial infarction and ischemia reperfusion injury (IRI) [83]–[86].

The first MPO deficient mouse model expressing mutant MPO (MPO^{-/-}) that fails to bind heme groups was constructed by Brennan et al. and published in 2001. After irradiation, low-density lipoprotein (LDL) receptor deficient mice (LDLR^{-/-}) that develop atherosclerosis either were cross-bred with MPO^{-/-} mice or received bone marrow of MPO^{-/-} mice. Measurements to proximal aortic lesions demonstrated that MPO deficiency led to more severe and larger lesions. Meanwhile, no MPO expression and low levels of 3-chlorotyrosine were detected in atherosclerotic tissue in both wild type and MPO^{-/-} mice [87]. The observations made in this

mouse model does not favor the previous hypothesis. However, at least 2 factors should be taken into account to interpret the result in MPO deficient mice. First, mice neutrophils produce much less MPO per se compared to human neutrophils, resulting in potential MPO effect covered. Secondly, the lesion development in CAD patients usually starts at young age and takes decades, while the LDLR deficient mice used in this study already bear lesions at five months when they were young. A much later study using ApoE^{-/-} mouse model combined either with MPO deficiency or inhibition recapitalized MPO deficiency dependent lesion size increase, and successfully verified MPO activity within the lesions with the help of MRI. The same study suggested that the remodelling of the lesions, rather than size, is important in predicting adverse events in atherosclerosis patients [88].

Matrix metalloproteinases (MMPs) are key regulators of extracellular matrix, they were found to participate in lesion architecture remodelling in cardiovascular diseases and can be activated by HOCl [89], [90]. MMP-2 and -9 enzymatic activity were both found decreased in angiotensin II (Ang II) treated MPO deficient mice compared to wild type mice with the same treatment, together with increased level of 3-chlorotyrosine. Studying to the same MPO deficient mouse model revealed that they are more resistant to Ang II challenges compared to wild type, which can be recapitalized by MPO supply via minipump [91]. This study supported the hypothesis that MPO worsens the situation in a peroxidase activity dependent way via its derivatives modifying proteins and lipids in cardiovascular diseases.

MPO modifications to both low- and high-density lipoproteins on lesion sites were reported in cardiac diseases, while modifications to LDL is more understood [92], [93]. MPO was found to modify both the lipid part and protein part of LDL and contribute to the oxidized LDL (oxLDL). Normally oxLDL trapped in the arterial subendothelial space is phagocytosed and contained by macrophages upon transcytosis into the dysfunctional endothelial cells. When the storing capacity of macrophages is met, the cells filled with lipid droplets are turned into 'foam cells', which is a hallmark of fatty acid stress [94], [95]. Additionally, MPO modified HDL was found in atherosclerosis plaque tissue isolated from patients [93].

Following the reperfusion of ischemic vasculature, a local endothelial cell dysfunction is characterised by the loss of Nitrate Oxide (NO) signal. NO functions as an important signalling molecule in stimulating the relaxation of vascular smooth muscle cells. Loss of vascular endothelium produced NO through acetylcholine (ACh) activation leads to increased level of cell adhesion molecules and adhering of neutrophils. [96], [97]. Using the aortic tissue obtained

from a rodent endotoxemia model, Baldus et al. revealed *ex vivo* that the presence of MPO in aortic microenvironment inhibited Ach induced vascular relaxation by depriving NO availability [98]. Further study revealed that MPO association with the vessel walls can be interrupted by heparin and thus leads to an increase of NO in the endothelium [99].

Tyrosine nitration product 3-nitrotyrosine (NO₂Tyr) has been considered as a hallmark of vascular inflammation, and its generation often correlates with increased oxidative stress [100]. Extracellular matrix (ECM) fibronectin tyrosine nitration upon MPO transcytosis into endothelial cells was detected in an *in vivo* study, suggesting endothelial cell ECM as a target of MPO in blood vessels [86].

To date and apart from the H₂O₂ mentioned above, multiple MPO inhibitors were proposed and used in molecular studies and on animal disease models. Examples are 4-aminobenzoic acid hydrazide (ABAH) whose use in mice successfully recapitulated MPO knock out amelioration of subacute stroke and AZM198 whose use in mice attenuated the rise of right ventricular pressure driven by hypoxia [101], [102]. The pharmaceutical use of these inhibitors needs to be tested due to limited specificity in targeting.

1.2 Chapter 2 MPO functions in innate immune system

1.2.1 Bactericidal function of MPO

MPO is majorly produced in neutrophils and inferior in monocytes, consisting around 2-4% total neutrophil cell weight and 0,9% monocyte cell weight [103], [104].

Neutrophils consist the main bactericidal force in the innate immune system, consisting 40-60% of all white blood cells in adult human circulation under homeostatic condition. The percentage varies with different sample collection and counting methods used, however, neutrophil presents as the most abundant white blood cells in human circulation across studies [105]–[108]. It was found that half of the neutrophils remain in bone marrow where they are produced and get mature, whilst the other half diffuse into blood vessels. Upon infection, the production of neutrophils may double [109]. As the terminal executing cell in immune defense, neutrophil is a source to bactericidal enzymes and employ diverse bacteria killing schemes. Invading microorganisms, after penetrating through the first line of physical defense comprised

with epidermis and getting in touch with tissues, would cause the release of signal molecules from activated macrophages which then activate and recruit neutrophils. Activated neutrophils penetrates into blood vessel, and destroy the invading microorganisms there via 3 well defined pathways: phagocytosis, secretion of bactericidal enzymes, and formation of the neutrophil extracellular traps (NETs).

MPO participates in all the 3 neutrophil derived microorganism killing strategies. When activated, neutrophil start degranulation and release bactericidal enzymes from peroxidase positive granules where they were stored into the cytosol. The degranulation process can be precisely analyzed with patch-clamp. Analysis to labelled granule membrane proteins found that the degranulation process is irreversible, since the labels cannot be retrieved once the granules membrane fuses with neutrophil membrane [110]. Upon released from AG, mature MPO fuses not only into the cytosol but also further into extracellular space. As mentioned in the previous chapter, heme group coupled peroxidase cycle and halogenation cycle reactions provide various oxidants that are capable of modifying surface proteins, lipids, and disrupt cell-cell junctions along their ways.

However, the pivotal role of MPO in human innate immune system is questioned by the fact that no innate immune impairment was reported in human with genetic MPO deficiency to date. The first case of MPO deficient individual was reported by Lehrer and colleagues in 1969. Checking to the neutrophils and monocytes of one patient with *Candida Albicans* infection suggested that the infection can be due to a lack of MPO in both cell types. Whilst loss of *Candida Albicans* bactericidal activity (generally referred to as candidicidal activity), the morphology and phagocytosis of the neutrophils were proved normal [111]. Chronic granulomatous disease (CGD) is caused by genetic NADPH oxidase deficiency [112], [113]. In a study comparing neutrophils isolated either from healthy donors or from two CGD children, the authors revealed a loss of candidicidal activity for diseased neutrophils [114]. This finding suggested that MPO is not the only source of candidicidal force in neutrophils. Another study on individuals with partial to total MPO loss suggested that they are with higher risks to have life-threatening infections [115]. Overall, these studies suggested that MPO plays a minor bactericidal role in neutrophil derived innate immune responses.

1.2.2 MPO triggers the formation of NETs

As mentioned in last chapter, MPO plays a role consisting NETs. First described by Brinkmann et al. in 2004, NETs appear to be fishnet-like structures consist mainly of negatively charged DNAs and associating histones of the neutrophil [116]. Triggered by various pathogens and inflammatory conditions, the formation of NETs involves the disintegration of neutrophil membrane and nucleus proteins, together with the homogenization of eu- and heterochromatins that usually result in netosis [117]. It is also reported that some Granulocyte/macrophage colony-stimulating factor (GM-CSF) prime treatment followed by complement factor 5a (C5a) stimulation induced NETs can be formed by living neutrophils with mitochondrial DNA alone, and in this way increase neutrophil survival rate [118]. Besides MPO, many other bactericidal agents from neutrophil granules also attached to NETs, including NE, cathepsin G, azurocidin, and others [117].

Apart from being a functional component of the NETs via killing microorganisms, recent studies revealed multiple NETs formation facilitating roles of MPO. Phorbol myristate acetate (PMA) is an agonist of protein kinase C (PKC), stimulation with PMA result in NADPH oxidase activation dependent NETs formation in neutrophils [119]. It was found that PMA induced NETs formation presents an MPO dependent manner. However, the same study also pointed out that NETs formation triggered by *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Escherichia coli* were not disturbed in the absence of MPO [120]. Studies to chromatin dynamics in NETs formation process revealed that the neutrophil chromatin decondensation is facilitated by NE and MPO together [121], [122]. Taken together, MPO may not be required for NETs formation in physiological conditions, rather, it plays an assistant role unfolding neutrophil DNA.

1.3 Chapter 3 Endothelial cell dysfunction and studying aim

In 2001, Stephan Baldus et.al described for the first time the attraction of MPO to the endothelial cell surface and transcytosis of MPO into endothelial cells in vitro, and revealed that the transcytosis process is regulated by heparin [123].

Based on this finding, further studies defined the endothelial cell remodelling role of MPO to hijack NO from endothelium and facilitate NO₂Tyr formation, both dependent on its enzymatic activity [86], [98].

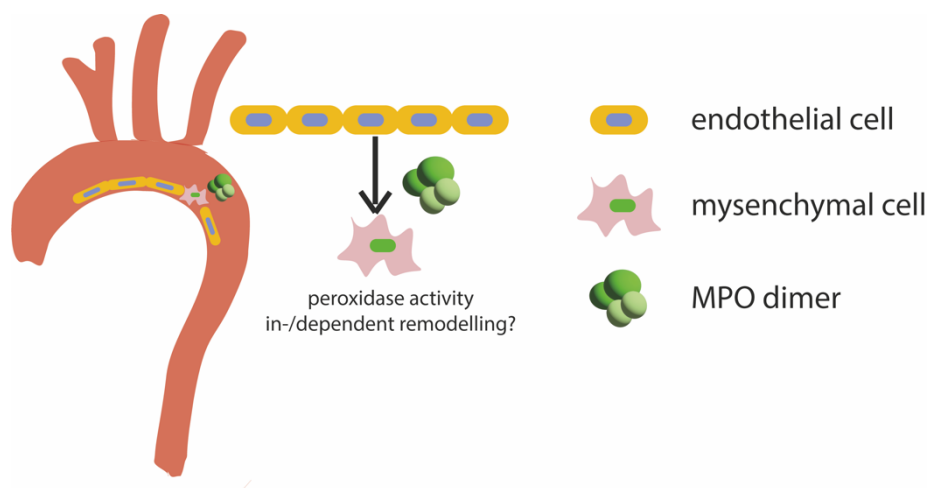


Figure 7. Illustration demonstrating the aim of this study. Illustration on the left shows MPO in human aorta attracted to endothelial surface via electronic force. Illustration on the right shows the hypothesis of MPO regulating endothelial cell remodelling, and the aim in studying the remodelling mechanism in both enzymatic dependent and independent manner.

A growing body of research on MPO revealed it as a molecule with multiple capacities and regulate endothelial cell remodelling in comprehensive ways beyond the its enzymatic activity dependent impacts. The accumulating evidences for MPO associating with DNA with specificity and facilitate transcriptional regulations suggest an enzymatic independent endothelial remodelling role bear by this molecule (Figure 7). This study roots from MPO transcytosis, combines chromatin research strategies and modern cell remodelling studying techniques, and aims at exploring and evaluating the impact of MPO on endothelial cells in a systematic manner.

Human umbilical vein endothelial cells (HUVECs) have been widely used in endothelial remodelling studies. Particularly, HUVECs is a favored model in studies to MPO-endothelial cell interactions. With cultured HUVECs as the studying model, it is assumed that this study can be robustly integrated into previously acquired knowledges.

List of studying goals and studying methods:

Goal 1: Determining MPO association with the endothelial cell chromatin

MPO transcytosis and subcellular localization was determined via Immunofluorescence (IF) staining. Its association with DNA was tested by performing IF, chromatin fractionation coupled with western blot, and electrophoretic mobility shift assay (EMSA). The binding specificity of MPO was realized via chromatin immunoprecipitation coupled with sequencing (ChIP-seq). The global condensation of endothelial chromatin upon MPO transcytosis was visualized by creating MNase-generated DNA ladder and local chromatin condensation was determined by conducting assay for transposase-accessible chromatin coupled with sequencing (ATAC-seq).

Goal 2: Assessing the endothelial cell remodelling role of MPO via transcription regulation

Endothelial transcriptome before and after MPO transcytosis was determined via performing RNA-seq, verification was performed via conducting qPCR and IF. Endothelial cell remodelling phenotype was determined by performing wound healing assay.

Goal 3: Identifying and verifying the potential MPO binding partners in endothelial cells

The binding partners of MPO in endothelial cytosol and nucleus were determined separately via performing immunoprecipitation (IP) and verified with western blot. The cooperating of MPO with binding partner was further explored by assessing endothelial transcriptome under control and binding partner knock down background.

Materials and Methods

2.1 Chapter 1. Cell Culture Techniques

2.1.1 General cell culture techniques

Pooled HUVECs produced by Longza (Cat No. C2519A) were passaged and used. Only P2-P8 cells were used for all the experiments described.

For general culture, cells were kept in incubators (Binder C170) at 37 ° and 5% CO₂ condition. Full Endopan 3 medium (PAN-Biotech) was used for culturing, and PBS (1x, Sigma Aldrich) was used for washing cell surfaces. Trypzean (Sigma Aldrich) was used for lifting the cells. Medium was changed every 2 days. Before applying to the cells, all liquid chemicals were thawed and afterwards warmed up in water bath (Mettler) at 37 ° for 15 min. For pelleting the cells, cells were centrifuged at 500g for 3 min at room temperature (Centrifuge: Eppendorf). To passage the cells, cells were cultured until an approximately 80% of confluency was reached and split in 1 to 3 or 1 to 4 manners.

2.1.2 Freezing and thawing

To freeze the cells, cells were cultured until an approximately 80% of confluency was reached. Confluent cells were first lifted and pelleted, afterwards resuspended in freezing medium (table 2-1) and kept in cryotubes (Thermo Fischer). The vials were immediately moved into freezing boxes (Nalgene Thermo Scientific) and kept in -80 °. Inactivated FBS (Sigma Aldrich) and DMSO (Sigma Aldrich) were used to prepare the freezing medium.

<i>Chemical</i>	<i>Volume (ml)</i>
<i>Endopan 3</i>	3
<i>FBS</i>	5
<i>DMSO</i>	2

Table 2- 1. Freezing medium recipe (10 ml).

To thaw the cells, 2 ml frozen vials were incubated in 37 ° water bath for 2 min before combined with 8 ml PBS and centrifuged. Pelleted cells were then resuspended with medium and put into an incubator.

2.1.3. Immunofluorescent Staining

Coverslips (15mm Ø, Marienfeld) were sterilized with 70% Ethanol before use. Cells were cultured in 24-well plates (Sarstedt) at 37 ° for less than 24 hrs for IF.

Cell surface was washed with PBS (Sigma) for 3 times before fixation with 4% PFA (diluted in PBS from 16% Methanol free PFA solution produced by Thermo Fisher) at room temperature for 10 min.

Fixed cells were washed with PBS for 3 times, before permeabilization. For each washing step, cells were rinsed in PBS for 5 min. Cells not stained on the same day were kept in PBS at 4° after washing. Cells were permeabilized with 1% Triton X-100 (diluted in PBS, Sigma) for 10 min at room temperature, and blocked for 1 hour at room temperature with 2% PBS-T-diluted BSA (PBS-T recipe see table 2-2, BSA was produced by Sigma). After blocking, cells were washed once with PBS and incubated with primary antibody at 4° overnight (dilutions of individual antibody see antibody list).

<i>PBS-T (1000 ml)</i>	<i>Volume</i>
<i>PBS</i>	995 ml
<i>Tween 20</i>	5 ml

Table 2- 2. PBS-T Recipe.

Cell surface was washed with PBS-T for 3 times before incubation with secondary antibody diluted in PBS-T for 2 hrs at room temperature. After incubation, cells were washed for another 3 times with PBS-T, and once with ddH₂O. DAPI (Sigma) was diluted 1:10000 in ddH₂O and applied to the cells for 10 minutes. Afterwards, cells were rinsed once with 70%, 80%, and pure Ethanol in order before mounted with ProLong gold (60ul/ coverslip, Thermo Fisher).

2.2 Chapter 2. DNA Techniques

2.2.1. MNase Assay

For the MNase experiment, permeabilization buffer and stop buffer were prepared freshly on the experimental day. Recipes of buffers are listed in table 2-3.

<i>Chemical</i>	<i>FC</i>
<i>Tris/HCl, pH=7.6</i>	15 mM
<i>KCl</i>	60 mM
<i>NaCl</i>	15 mM
<i>CaCl₂</i>	4 mM
<i>EGTA</i>	0.5 mM
<i>Sucrose</i>	300 mM (add freshly)
<i>NP-40</i>	0.2 % (add freshly)
<i>β-mercaptoethanol</i>	0.5 mM (add freshly)

Table 2- 3. MNase Assay Permeabilization buffer.

<i>Chemical</i>	<i>FC</i>
<i>Tris/HCl, pH=8.0</i>	50 mM
<i>EDTA</i>	20 mM
<i>SDS</i>	1 %

Table 2- 4. MNase Assay Stop buffer.

To prepare for the MNase treatment experiments, cells were cultured on 6-well plates (Figure 8). MPO treatment was applied before MNase treatment.

MNase (Merck) was added to previously prepared permeabilization buffer (100 U/ 1 ml). Cell surfaces were firstly washed with 10 ml PBS (Sigma) and in the following step covered with MNase dissolved in permeabilization buffer (MNase 0min group covered with permeabilize buffer without MNase). A figure showing MNase treatments can be found below. Cells covered with MNase were incubated for 2 min in the incubator before 1 ml/well stop buffer (table 2-4) were added to the MNase 2 min groups and mixed. SDS in the stop buffer helps to denature MNase and stop the enzymatic reaction. Next, the plates were put back to the

incubator for another 2 min before adding stop buffer to the MNase 4min groups. Likewise, after another 2 min incubation, stop buffer was added to the MNase 6min groups.

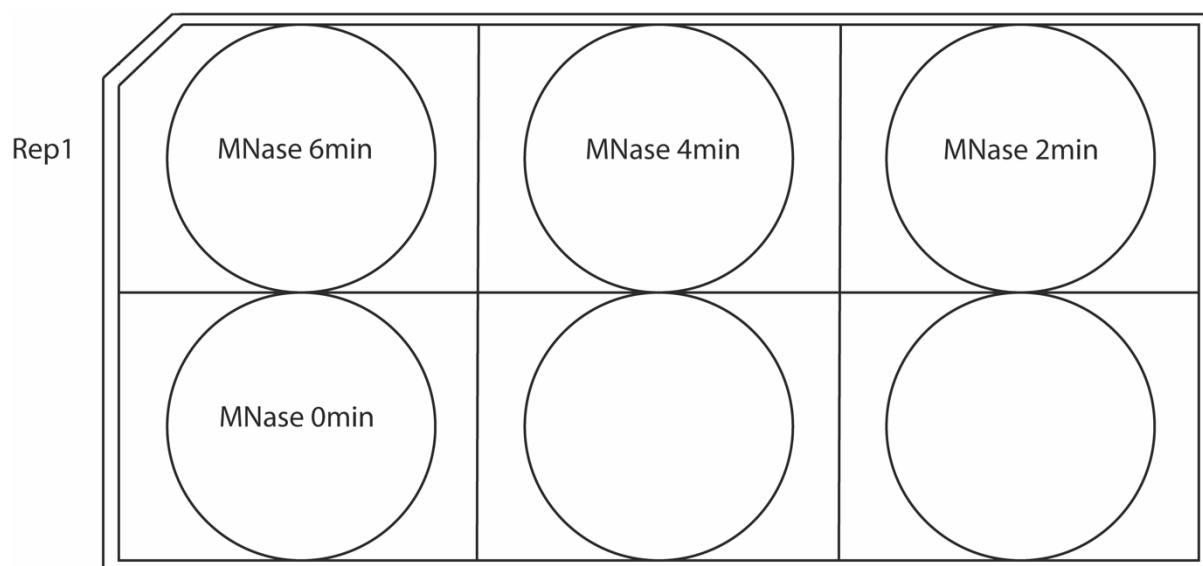


Figure 8. Illustration showing the distribution of cells with 0, 2, 4, and 6 min MNase treatment.

When all the enzymatic reactions were stopped, 250 µg/well RNase A was added and mixed, the lysis were incubated at 37 ° for 2 hrs. Following this step, 250 µg/well Proteinase K was added, mixed, and the lysis were incubated at 37 ° overnight. RNA and proteins were removed in these two steps, separately.

In the next day, DNA was collected using EtOH precipitation method. Firstly, the digested DNAs (2 ml) were transferred from 6-well plates into 15 ml Falcon tubes. Secondly, 200 µl/sample ammonium acetate and 4.4 ml 100% Ethanol were added in described order and mixed. The mixtures were frozen in -80 ° for 1 hour for better yielding. After thawing, the mixtures were centrifuged at 4000 g for 2 hours at 4 °. At this point, a white pellet should be able to be observed in each sample. Supernatants were discarded. On top of the white pellet, 2 ml 70% Ethanol was added. Samples were centrifuged at 4000 g for 30 min at 4 ° and supernatants discarded. The DNA pellets were air-dried for 30 min before dissolved with 50 µl ddH₂O.

For visualisation, the undigested genome and digested DNAs were resolved on 1.5 % agarose gel for 45 min at 100V followed by photo-taking under UV.

2.2.2. ATAC-Seq

Cells were cultured on 6-well plates (Corning) for ATAC-seq, and ATAC-seq kit produced by Active motif was used (Cat No. 53150). Cells were treated with MPO, counted and placed into 1,5 ml centrifuge tubes (Eppendorf) before sample preparation. For each reaction, 1×10^5 cells were used. After collection, cells were centrifuged 500 g for 5 min at 4°. Cell pellets were examined carefully under sunlight to visualize. When all the pellets are visible, they were further centrifuged for 5 min at 1000 g at 4°. After centrifuge, the supernatants were removed firstly with 1000 µl pipet tips and secondly with 200 µl pipet tips to ensure clear pellets. On top, 100 µl ice-cold PBS was added not disturbing the pellets. The pellets were centrifuged for another time at 500 g for 5 min at 4° and the supernatant was removed. Following this step, cell pellets were resuspended in 100 µl/pellet ice-cold ATAC lysis buffer. The lysates were transferred into PCR tubes on ice followed by an immediate centrifugation at 500 g for 10 min at 4°. During centrifugation, Tagmentation master mix was prepared as showed in the table 2-5.

<i>reagent</i>	<i>volume</i>
<i>2x Tagmentation Buffer</i>	25 µl
<i>10x PBS</i>	2 µl
<i>0,5% Digitonin</i>	0,5 µl
<i>10% tween 20</i>	0,5 µl
<i>H₂O</i>	12 µl
<i>Assembled Transposomes</i>	10 µl

Table 2- 5. Tagmentation master mix (per sample).

After centrifugation, the supernatant was removed from each sample with care and not disturbing the pellet. Firstly, 1000 µl pipet tips were used to clear the ATAC lysis buffer, followed by 200 µl tips and at last 10 µl tips. The pellets were observed against light again to make sure the collection was done successful. Without freezing to preserve the pellets, Tagmentation reaction and purification steps were conducted immediately following this step.

To each sample, 50 µl Tagmentation master mix was added. Nuclei were resuspended with 200 µl pipet tips whose tips were cut to avoid over-homogenizing. The Tagmentation reactions were incubated at 37 ° for 30 min at 800 rpm on a heat block and afterwards transferred into fresh 1,5 ml centrifuge tubes.

Directly after the incubation, 250 µl/sample DNA purification binding buffer (included in the kit) and 5 µl 3 M Sodium Acetate (included in the kit) were added to the samples. After mixing gently by inverting the tubes, the colour of the sample was observed carefully and compared to the spectrum provided online (for the spectrum, see the site: <https://www.activemotif.com/documents/2182.pdf>). If the colour of the sample is not bright yellow, additional 5 µl 3 M Sodium Acetate was added. For each sample, a labelled column was placed into a collection tube (included in the kit). Each sample was mixed thoroughly and transferred to a labelled column. Samples were then centrifuged in the columns at 17,000 g for 1 min at room temperature. The flow-through was discarded, and the columns were washed once with 750 µl/column Wash Buffer (included in the kit) by centrifugating at 17,000 g for 1 min at room temperature. With the cap of the column open, the columns were centrifuged at 17,000 g for 2 min to remove residual Wash Buffer. Columns were transferred from the collection tubes to fresh 1,5 ml centrifugation tubes. An amount of 25 µl DNA Purification Elution Buffer was added to the centre of the column matrix, and the columns were centrifuged at 17,000 g for 1 min at room temperature. The purified DNA were stored at -20 ° and the DNA concentrations were determined via conducting Qubit fluorometric quantification assays (Thermo Fisher).

2.2.3. ChIP-seq

Cells were cultured in 14 ml/plate medium on 15 cm plates (Corning) for ChIP-seq, 3 plates of cells were used to yield 1 sample (around $1 - 1.5 \times 10^7$ cells per plate). High sensitivity ChIP kit produced by Active motif was used in all the ChIP experiments (Cat No. 53040). Cells were treated as required and fixed with freshly prepared fix solution (for every 20 ml cell culture medium, 2.5 ml fixation solution was added containing 180 µl kit-provided fixation buffer, 1.57 ml ddH₂O and 750 µl 37% formaldehyde) for 15 min shaking at room temperature. The fixation was stopped by adding 1.2 ml provided stop solution and incubating 5 min at room temperature. After fixation, cells were scratched from the plates, transferred to 50 ml falcon and kept on ice. Next, cells were pelleted by centrifugation for 3 min at 1250 g at 4 °. The supernatant was removed and cell pellets were resuspended in 10 ml/sample ice-cold provided PBS Wash buffer. Cells were pelleted again and washed for a second time before resuspended in 5 ml provided Chromatin Prep Buffer containing freshly added 5 µl provided PIC (Proteinase Inhibitor Cocktail) and 5 µl provided 100 mM PMSF and rested for 10 min on ice. The resuspended cells were homogenised with 26G needle on ice for 30 strokes and pelleted in 15

ml falcons. Then, cell pellets were resuspended in 500 µl ChIP buffer containing freshly added 5 µl provided PIC and 5 µl provided 100 mM PMSF, transferred to 2 ml Eppendorf tubes and rested for 10 min on ice. Afterwards, the cells were sonicated in a water bath homogenizer at 30% power for 10 + 10 min. The samples were spun at 4 ° at maximum speed for 2 min to remove debris. To generate input and check shearing efficiency, 25 µl sample were separated.

To prepare for the input, 175 µl provided TE pH 8.0 and 1 µl RNase A were added to the 25 µl samples separated above in PCR tubes, and the samples were incubated at 37 ° for 30 min. 2 µl Proteinase K was added to each sample, and the samples were incubated at 55 ° for 30 min and 80 ° for 2 hrs. The samples were transferred to Eppendorf tubes, and 83 µl provided Precipitation buffer, 2 µl Carrier, and 750 µl absolute Ethanol were added. The samples were vortexed and stored at -80 ° overnight. On the next day, samples were pelleted by spinning at maximum speed at 4 ° for 15 min. The pellets were washed with 500 µl/sample 70% Ethanol and spinning at maximum speed for 5 min followed by air drying for 10 min. 25 µl/sample DNA purification Elution buffer was added, and the samples were incubated at room temperature for 10 min and vortexed.

The shearing efficiency was checked with 500 ng of DNA after input preparation. 500 ng DNA from each sample were combined with 1 µl 500 mM NaCl in a PCR tube, and the volumes of each reaction were adjusted to 10 µl. The samples were heated at 100 ° for 20 min and ramped down to 50 ° before rested in room temperature for 5 min. The DNAs were visualised on 1.5% agarose gels, only when a smear between 200-1200 bp was observed, was the sample used for ChIP-seq.

The rest of the samples were thawed for immunoprecipitation on ice. After thawing, the samples were centrifuged at 4 ° at maximum speed for 2 min. The ChIP reactions were set up as shown in the table 2-6 and incubated on an end-to-end rotator overnight at 4 °.

An amount of 4 µg MPO antibody (Calbiotech) was combined with 5 µl provided Blocker in a 1.5 ml Eppendorf tube, and incubated together for 1 min at room temperature.

Reagent

<i>Sheared Chromatin</i>	30 µg
<i>ChIP Buffer</i>	Adjust to 200 µl
<i>PIC</i>	5 µl
<i>Antibody/ Blocker mix</i>	10 µl
<i>Final Volume</i>	240 µl

Table 2- 6. ChIP set up.

Protein G agarose beads were washed with provided TE pH 8.0 twice and used (30 µl beads/sample). The samples were combined with the washed beads and incubated at 4 ° for 3 hrs. After incubation, the samples were shortly centrifuged at 1250 g for 1 min at room temperature. An amount of 600 µl ChIP buffer was added to each ChIP reaction, and the flow through by gravity was achieved with the use of provided filtration columns. Next, each sample was washed with 900 µl provided Wash Buffer AM1 for 3 min and for 5 times. The washed samples were dried by centrifugating at 1250 g for 3 min at room temperature in the columns assembled to collection tubes. The ChIP DNA was then collected by using pre-warmed (at 37 °, 50 µl/sample) provided Elution Buffer AM4. After centrifugating at 1250 g for 3 min at room temperature, collect the flow through and proceeded to DNA purification.

The eluted ChIP DNA was combined with 2 µl/sample Proteinase K, vortexed, incubated at 55 ° for 30 min, and eventually at 80 ° for 3 hours. The following DNA purification steps were carried out as described in ATAC-seq using the kit from the same company, and elute with 36 µl provided DNA Purification Elution Buffer prewarmed at 37 °.

2.3 Chapter 3. RNA Techniques

2.3.1. RNA isolation

RNA isolation for cDNA synthesis and RNA-seq were achieved using micro RNA isolation kit (Zymoresearch). HUVECs were cultured on 6 well plates for RNA collection. After washing with 2ml/well PBS, 1ml/well Trizol Reagent (Brand) was applied directly to the cell surface, incubate at room temperature for 5 minutes before homogenise and collect with 1ml pipette. Lysed cells were stored at -80°, and were stored for less than one week before RNA isolation. Surfaces of working benches were wiped with Bacillol AF (Bode Chemistry) and RNaseZAP (Sigma) before isolation to prevent potential RNA degradation by RNase. For isolation, an amount of 1ml Ethanol was added to the lysed cells in Trizol reagent and mixed well. The mixture was transferred to provided columns assembled to collection tubes and centrifuged at 14'000 g for 30 seconds to facilitate RNA binding to the silica membrane of the column. Samples were washed once with RNA Wash Buffer, and on column DNA removal was performed by incubation with provided DNase I dissolved in DNA digestion buffer at room temperature. Directly after DNA digestion, the samples were washed once with 400 µl/column RNA Pre-wash Buffer and once with 700 µl/column RNA Wash Buffer. At last, RNA was eluted from the columns with 15-25 µl ddH₂O.

2.3.2. cDNA Synthesis

For cDNA synthesis, 1- 2,5 µg RNA was used per 20 µl reaction. RNA templates, random primers, and dNTPs were mixed accordingly (see table 2-7) in a RNase/DNase free PCR tube (Thermo Fisher).

<i>Ingredient</i>	<i>Volume (μl)</i>
<i>Template (100-600 ng/μl)</i>	In total 1- 2,5 μ g
<i>ddH₂O</i>	To make the final volume 12 μ l
<i>dNTPs (10 mM)</i>	1
<i>Random Primer</i>	1

Table 2- 7. of cDNA synthesis mix1.

The mixture was incubated in a thermo cycler (Bio-Rad) for 5 min at 65°, then quickly chilled on ice and collected by short centrifugation. In the next step, chemicals were added (see table 2-8) and mixed gently with pipette.

<i>Ingredients</i>	<i>Volume (per 20 μl system)</i>
<i>5X First Strand Buffer (Thermo Fisher)</i>	4
<i>DTT (Invitrogen)</i>	2
<i>RiboLock RNase Inhibitor (Invitrogen)</i>	1
<i>SuperScript IV reverse transcriptase (Thermo Fisher)</i>	1

Table 2- 8. cDNA synthesis mix2.

The mixture was then incubated at 42 ° for 2 hours, and finally at 75 ° for 15 min to terminate the reaction. Synthesized cDNAs were kept at -20 ° for further use.

2.3.3. qPCR

For qPCR, cDNAs were diluted in a 1 to 200 manner, and for each qPCR reaction, 5 μ l cDNA was used. The qPCR mix was made as listed in table. For each condition or biological replica, a number of 3 technical replica were generated.

The qPCR protocol can be found in table 2-9.

<i>Stage</i>	<i>Temperature</i>
<i>Hold Stage</i>	Ramping up at 1.6 °/sec to 95 °, hold for 2 min
<i>PCR Stage (40 cycles)</i>	95 ° 3 sec
	Ramping down at 1.6 °/sec to 60 °, hold for 30 sec
<i>Melt Curve Stage</i>	Ramping up at 1.6 °/sec to 95 °
	Ramping down at 1.6 °/sec to 60 °, hold for 20 sec
	Ramping up at 0.15 °/sec to 95 °

Table 2- 9. qPCR protocol.

2.3.4. ILF3 knockdown via siRNA

Universal negative control siRNA (Sigma) and ILF3 siRNA (Sigma) were used in ILF3 knock down experiments, both diluted to 10uM in TE buffer (table 2-10). RNAimax (Thermo Fischer) and Opti-MEM (Thermo Fischer) were used for transfection. To ensure an ideal knock down rate, HUVECs were cultured in complete Endopan3 medium on 6-well plates (brand of 6 well plates) until they reached a 30% confluence, and shifted to no-antibiotic Endopan3 medium for siRNA transfection. Transfection was done according to the protocol provided by Thermo Fischer on thermofischer.com. Briefly, an amount of 6 µl ILF3 or control siRNA and 3 µl RNAimax were mixed with 150 µl Opti-MEM, separately. Next, siRNA/Opti-MEM mixture was combined with RNAimax/Opti-MEM mixture and incubated at room temperature for 5 minutes before added to the cells (300 µl of mixture per well). The mixture was homogenized by using 2 ml pipettes (Greiner) with pipette boy (Hirschmann), and cells were put back to the incubator. siRNA treated cells were collected after 24 hours for RNA isolation

and further mRNA level verification via qPCR, and after 72 hrs for protein level verification via western blot.

<i>Chemical</i>	<i>Final Concentration (f.c.)</i>
<i>Tris-HCl</i>	10 mM, adjust to pH = 7,6
<i>EDTA</i>	1 mM, adjust to pH = 8,1
<i>Autoclave before use</i>	

Table 2- 10. TE buffer.

2.3.5. RNA stability assay

For each biological replica, HUVECs were cultured on 2x 6-well plates for RNA stability assay. For transcription inhibition, 10 mg DRB (TCI Chemicals) was diluted in 1 ml DMSO to make 3.1 mM DRB solution, and a final concentration of 0.1 mM according to published review [124].

Cells were treated with ILF3 or control siRNA on day1 as described in ILF3 knock down session, and were cultured for 70 hours before MPO treatment and DRB treatment on day4. Finally, cells were collected on day 4 at 72 hours point for qPCR. Transcription levels were checked via qPCR.

2.4 Chapter 4. Protein Techniques

2.4.1. Protein Isolation

Protein isolation was performed using freshly prepared RIPA buffer (prepared as described in Cold Spring Harbour protocol, Table 2-11).

<i>Chemical</i>	<i>FC</i>
<i>NaCl</i>	150 mM
<i>Nonidet P-40</i>	1 %
<i>Sodium Deoxycholate (DOC)</i>	0,5 %
<i>SDS</i>	0,1 %
<i>Tris-HCl (pH = 7,4)</i>	25 mM
<i>Stored at -20</i>	

Table 2- 11. RIPA Buffer Recipe (Cold Spring Harbour Protocol).

Cells were collected and pelleted, an amount of 100-200 µl ice-cold RIPA buffer with PIC (Roche) was added to the cell pellet. The pellets were resuspended and homogenized gently with 200 µl pipet tips. The lysates were placed on ice for 5 min before agitation (Scientific Industries Vortex-Genie mixer). The incubation and agitation steps were repeated for three times for each isolation. Following this step, the lysates were centrifuged at 14,000 g for 30 min at 4 °. After centrifugation, the supernatants were transferred into fresh 1,5 ml centrifugation tubes and the debris were discarded.

For western blot, the protein samples were homogenized with water bath homogenizer at low power for 30 sec with 30 sec rest, and for 3 cycles.

2.4.2. Western Blot

Protein levels were determined via BCA assay (Sigma). BCA standards were diluted with the same diluent as the protein samples. Protein samples were diluted 1:3 or 1:5 with diluents when the diluent contains SDS, for determination.

Casted gels (4-15% gradient pre-casted gels, 15 or 10 well, Bio-Rad) were used for SDS PAGE. Electrophoresis was carried out for 30 min at 90 V, maximum 25 mA before 60-90 min at 120 V, maximum 25 mA. Western Blot running buffer was diluted from 10x SDS running buffer (table 2-12). Following the electrophoresis, dry transfer was performed using transfer buffer (table 2-13) with Turbo transfer system (Bio-Rad).

<i>Chemicals</i>	<i>Weight</i>
<i>Tris base</i>	30 g
<i>Glycine</i>	144 g
<i>SDS</i>	10 g
<i>ddH₂O</i>	Up to 1 L

Table 2- 12. 10x SDS running buffer recipe.

<i>Chemical</i>	<i>Volume (ml)</i>
<i>10x Tris/Glycine Transfer buffer (Bio-rad)</i>	100
<i>Methanol</i>	200
<i>ddH₂O</i>	700
<i>Transfer condition</i>	7V, 2.5 A, 2 hrs with Bio-Rad Trans-Blot Turbo system

Table 2- 13. 1 L Transfer buffer recipe (Bio-rad) and transfer condition.

2.4.3. Chromatin Fractionation

HUVECs were cultured in complete Endopan3 medium (Pan) on 15cm plates (Sarstedt), and washed once with 10ml/plate ice-cold PBS (Sigma) before collection with cell scraper (Sarstedt) in PBS (10 ml/plate). Next, cells were pelleted by centrifugation at 1000 rpm for 5 minutes and immediately lysed with 300ul/2plates extraction buffer (20mM Tris (pH = 7.5, Roth), 100mM NaCl (Chemsolute), 5mM MgCl₂ (Sigma), 10% glycerol (Sigma), 0.2% NP-40 (Sigma), 1mM NaF (Sigma), 0.5 mM DTT (Invitrogen), 1tablet/10ml protease inhibitor cocktail (Roche), diluted with ddH₂O (Anpuwa)). In the following step, lysates were collected in 2 ml Eppendorf tubes (Eppendorf) and homogenised in a water bath homogenizer at 30% power for 10 rounds (30 s homogenizing, 30 s rest, homogeniser: Bandelin Sonopuls), checking under a light microscope is required after homogenising to verify the exposing of nuclei). An amount of 50 µl/sample were separated and stored as whole fractions for further Western Blot verification. To separate chromatin, rest of the lysates were centrifuged twice at 1'000 rpm for 10 minutes in pre-cooled centrifuge (4°, Eppendorf). Supernatants were collected as soluble fractions in Western Blot. Pellets were washed with centrifugation in extraction buffer (500 µl/pellet, 13'000 rpm, 4°). Chromatin pellets were dissolved with PBS as chromatin fractions.

2.4.4. Nucleus Fractionation

Nucleus isolation was performed using the Chromatin Prep Buffer with provided PIC and 100uM PMSF in the ChIP-IT High Sensitivity kit (Activ Motif, Catalog No. 53040). HUVECs were cultured in complete Endopan3 medium on 15cm plates. For collection, cell surfaces were washed once with 10 ml/plate ice-cold PBS and scraped in fresh PBS (10 ml/plate). Pellets

were collected by centrifugation (3000g, 3min, 4°), and washed once with ice-cold PBS. Next, cells were lysed in Chromatin Prep Buffer (1 ml/pellet), rest for 10min on ice before homogenised 30sec for 3 cycles with water bath homogenizer at mild mode (brand of the homogeniser). An amount of 100 µl lysate for each sample were separated and used as whole fraction in Western Blot verification. Rest of the lysates were centrifuged (1250 g, 3 min, 4°), supernatants were kept as cytoplasmic fraction for Western Blot detection. Pellets were washed once with Chromatin Prep Buffer (1ml/pellet) and stored as nucleus fraction in 60 µl/pellet in RIPA buffer.

2.4.5. Immunoprecipitation

For immunoprecipitation (IP), cells were cultured on 15 cm plates, MPO treatments, and whole cell lysis or nuclei isolation were carried out according to experimental set-ups. Cells were collected by cell scrapers (Corning) on ice and pelleted by centrifugating at 500 g at 4 °.

Nuclei isolation was achieved by incubating the cells with NIP buffer (table 2-14) for 15 min on ice followed by pelleting by centrifugation at 800 g at 4 °. Pelleted nucleus were washed twice with NIP buffer before incubated for 10 min with IP buffer (table 2-15) and proceed with antibody incubation.

<i>Chemical</i>	<i>F.C.</i>
<i>Tris-HCl pH= 7.5</i>	15 mM
<i>KCl</i>	60 mM
<i>NaCl</i>	15 mM
<i>MgCl₂</i>	5 mM
<i>CaCl₂</i>	1 mM
<i>Sucrose</i>	250 MM
<i>NP-40</i>	0.3%

Table 2- 14. NIB buffer recipe.

Whole cell lysis was achieved by incubation with 1ml/sample IP buffer for 10 min on ice, and cell debris was removed by centrifugating at 5000 g for 5 min at 4 °. In between the lysates were homogenised 5 times using 26G syringes (Sigma Aldrich).

Finally, 1 mg of each sample (whole cell lysates or nucleus) was incubated overnight with 5 µl MPO antibody (Calbiochem, 475915) at 4 °. Magnetic beads (Active Motif) were prepared by washing with PBS-T on a magnetic stand (Thermo Fisher). Samples and antibodies were combined with prepared magnetic beads and incubated for 2 hrs at 4 °. After incubation, magnetic beads were washed for 5 times (each time for 5 min) with IP wash buffer (table 2-16) with NP-40, and another two times without NP-40.

<i>Chemical</i>	<i>F.C.</i>
<i>Protease Inhibitor (cOmplete mini, Roche)</i>	1 tablet
<i>Tris-HCl, pH= 7.5</i>	50 mM
<i>EDTA</i>	1mM
<i>Empigen BB (Sigma Aldrich)</i>	0.5%
<i>Benzonase (Sigma Aldrich)</i>	50 units/ml

Table 2- 15. IP buffer recipe.

<i>Chemical</i>	<i>F.C.</i>
<i>KCl</i>	150 mM
<i>MgCl₂</i>	5 mM
<i>Tris-HCl, pH= 7.5</i>	50 mM
<i>NP-40</i>	0.5%

Table 2- 16. IP wash buffer.

<i>Chemical</i>	<i>F.C. (or mass)</i>
<i>Urea (from CECAD Proteomics Facility)</i>	2 M
<i>Tris-HCl, pH= 7.5</i>	50 mM
<i>DTT (Sigma Aldrich)</i>	1 mM
<i>Trypsin</i>	150 ng

Table 2- 17. IP digestion solution.

<i>Chemical</i>	<i>F.C.</i>
<i>Urea</i>	2 M
<i>Tris-HCl, pH= 7.5</i>	50 mM
<i>Chloroacetamide (from CECAD Proteomics Facility)</i>	5 mM

Table 2- 18. IP elution solution.

After washing, pre-digestion and elution of the captured protein were carried out with incubation with IP digestion buffer (table 2-17) and elution buffer (table 2-18) supplemented

with LysC. The eluted proteins were sent out for mass-spectrometry on a Q-Exactive Plus Orbitrap platform coupled to an EASY nLC (Thermo Scientific) in CECAD Proteomics Facility.

2.4.6. MPO Inactivation

MPO (Planta Natural Products) were inactivated via H₂O₂ treatment as described in published protocol (Paumann-Page et al., 2013). 30% H₂O₂ was diluted to 1M with ddH₂O. Meanwhile, 100 µg MPO were diluted in 100 µl ddH₂O to reach a concentration of 1 µg/µl. Next, 100 µl 1M H₂O₂ were combined with MPO vial and the mixture was incubated for 30 min at 37 ° on a heat block (Eppendorf).

2.4.7. MPO activity determination

iMPO enzymatic activity was checked based on the fluorescence response of 3'-(*p*-aminophenyl) Fluorescein (referred to as APF, Thermo Fischer) reacting to the Reactive ROS. For activity determination, protein concentrations of MPO and iMPO were determined separately using nanodrop (brand of nanodrop). 5 mM APF solution was diluted with ddH₂O to 10 µM working solution. 2 µg MPO or iMPO were combined with 4 µl APF working solution and 8 µl of ddH₂O and incubated at 37 ° for 15 min. The fluorescence was tested in a plate reader (Beckmann Coulter).

<i>Primary</i>	<i>Host</i>	<i>Manufacturer</i>	<i>Product</i>	<i>Dilution</i>
<i>MPO</i>	Rabbit	Calbiotech	475915	WB 1:1000
<i>MPO</i>	Rabbit	Thermofisher	RB373A	WB 1:1000,
<i>HISTONE H3</i>	Goat	Santa Cruz	sc-8654	WB 1:1000
<i>GAPDH</i>	Rabbit	Cell Signaling	2118I	WB 1:1000
<i>H3K27AC</i>	Rabbit	Activ Motif	39133	WB 1:1000
<i>H3K4ME1</i>	Mouse	Activ Motif	ab32356	WB 1:1000
<i>ILF3</i>	Rabbit	Abclonal	A2496	WB 1:1000,
<i>TOPO1</i>	Mouse	Santa Cruz	sc-271285	WB 1:1000
<i>H3K79-ME1</i>	Rabbit	Abcam	ab3594	WB 1:1000
<i>CD31</i>	Mouse	Abcam	ab24590	WB 1:1000,
<i>CD34</i>	Rabbit	Abcam	ab81289	WB 1:1000,
<i>ICAM</i>	Rabbit	Abcam	ab53013	WB 1:1000,
<i>CD45</i>	Rabbit	Abcam	ab40763	WB 1:1000,
<i>NF-45</i>	Mouse	Santa Cruz	sc-365068	WB 1:1000

Table 2- 19. Antibody List.

<i>name</i>	<i>fwd</i>	<i>rev</i>	<i>length</i>	<i>source</i>
<i>CD31</i>	AACAGTGT TGACATGA AGAGCC	TGTAAAAC AGCACGTC ATCCTT	148	PrimerBank ID 313760623c1
<i>CD34</i>	CTACAACA CCTAGTAC CCTTGGA	GGTGAACA CTGTGCTG ATTACA	185	PrimerBank ID 68342037c1
<i>ICAM1</i>	ATGCCCAG ACATCTGT GTCC	GGGGTCTC TATGCCCA ACAA	112	PrimerBank ID 167466197c1
<i>CD45</i>	ACCACAAG TTTACTAA CGCAAGT	TTTGAGGG GGATTCCA GGTAAT	126	PrimerBank ID 18641362a1
<i>VWF</i>	CCGATGCA GCCTTTTC GGA	TCCCCAAG ATACACGG AGAGG	171	PrimerBank ID 89191867c1
<i>MMP-9</i>	GGGACGCA GACATCGT CATC	TCGTCATC GTCGAAA TGGGC	139	PrimerBank ID 74272286c3
<i>Colla2</i>	GGCCCTCA AGGTTTCC AAGG	CACCCTGT GGTCCAAC AACTC	166	PrimerBank ID 48762933c2
<i>CXCL8</i>	ACTGAGAG TGATTGAG AGTGGAC	AACCCTCT GCACCCAG TTTTTC	112	PrimerBank ID10834978a2
<i>PPDPF</i>	TTCCACTT CCAGCAA CAGCTC	AGAAGCTG GCCCAACA ATGAC	110	Designed using NCBI Primer-blast
<i>YWHAZ</i>	GCTTCACA AGCAGAGA GCAAAGT	TCTTG GTA TGCTTGTT GTGACTG	126	Designed using igv browser
<i>18srRNA</i>	CCCTATCA ACTTTTCA TGGTAGTG	CCAATGGA TCCTCGTA AAGGATTT	242	[125]

Table 2- 20. Primers List.

Results

3.1 Chapter 1 Visualizing MPO transcytosis, translocation into HUVECs nucleus, and associating to chromatin *in vitro*

3.1.1 Inactivation of MPO

In vivo study revealed that around 40% of MPO in the circulations of rats with pyogenic infection is enzymatically inactivated 24 hours post exposing to bacteria [126]. One central goal of this research is to dissect the peroxidase activity independent role of MPO. In order to achieve this goal, *in vitro* inactivation of the enzyme was carried out. By saturating with its major substrate, hydrogen peroxide (H_2O_2), MPO irreversibly loses its binding affinity to heme groups which enables oxidizing reactions [82], [127]. The inactivation scheme used in this script was modified from a published protocol [82], inactivated MPO will be referred to as iMPO in this script. In addition to the protocol, a dialysis step was performed to get rid of H_2O_2 remain.

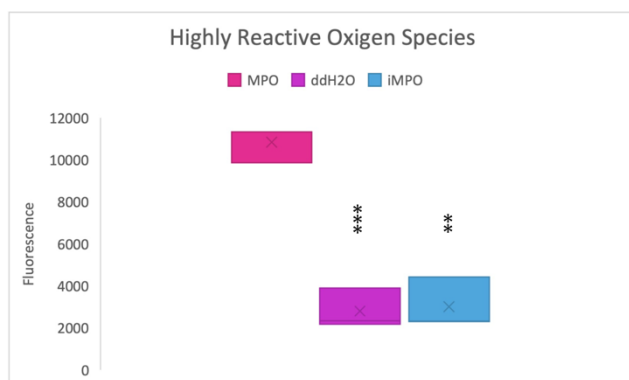


Figure 9. MPO inactivation via H_2O_2 saturation. Bar plot demonstrates level of fluorescence produced by APF reacting with highly oxidative oxygen species detected via fluorescence detecting parameter. “x” indicates of mean value, “—” indicates of median. $p < 0,005$ (**), $p < 0,0005$.

To measure enzymatic activity, synthesized 2-[6-(4-amino) phenoxy-3H-xanthen-3-on-9-yl] benzoic acid (APF) was incubated with objects being measured, which reacts to highly reactive oxygen species, including $\cdot OCl$, and form fluorescein. This method warrens the detection of major MPO products with a separation from H_2O_2 .

A mixture of equal volume of ddH₂O and H₂O₂ was measured in parallel as a negative reference while MPO and H₂O₂ mixture as positive reference. It was showed that after inactivation, the amount of fluorescence captured in iMPO sample was comparable to that of ddH₂O (Figure 9), indicating of a complete loss of enzymatic activity.

3.1.2 Establish MPO transcytosis studying model using HUVECs cell line

This *in vitro* research aims at revealing potential impacts of MPO transcytosis into endothelial cells and further exploring the molecular mechanisms lying behind.

HUVECs have been used to study cardiovascular disease and endothelial cell remodeling and migration. In this research, cultured HUVECs are used as the studying model.

First, an MPO translocating model using cultured HUVECs was established to determine MPO transcytosis process time-wisely and verify MPO subcellular location at different time points.

To achieve this, an immunofluorescence (IF) staining was performed on HUVECs treated with a final concentration of 1 µg/ml MPO, and visualized using confocal microscopy.

An overall scheme of IF experiment can be found in Figure 10 A.

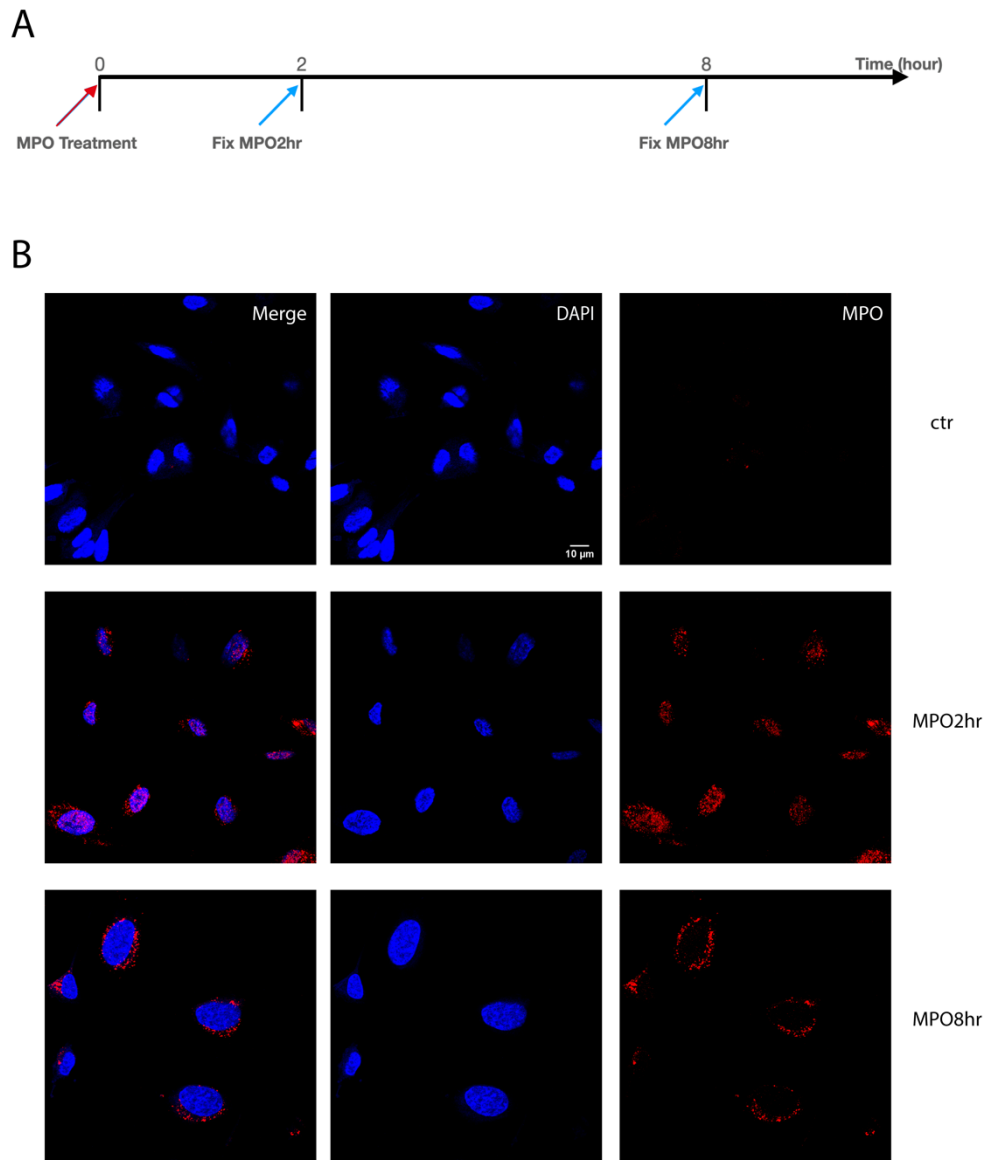


Figure 10. MPO translocation into HUVECs cytosol and nucleus *in vitro* detected with immunofluorescence staining. (A) Protocol shows MPO treatment and fixation time, pink arrow: time for MPO treatment, blue arrow: time for fixation. (B) Representative IF staining from one of three bio-replica for MPO (Red), DAPI (Blue) and merged images on HUVECs with non- (ctr) or indicated MPO treatments (MPO2hr, MPO8hr). Scale bar = 10 μ m.

After 2 hours of treatment, weaker MPO signal was observed in HUVECs cytosol, and stronger signal was observed in nucleus (Figure 10 B). Furthermore, staining at 8 hours after treatment showed less MPO signal in the nucleus compared to 2 hours, while major MPO signal

appeared gathering around HUVECs nucleus envelope. This observation by images is supported by analysis. The decrease of MPO signal at 8 hours is significant (Figure 11 B).

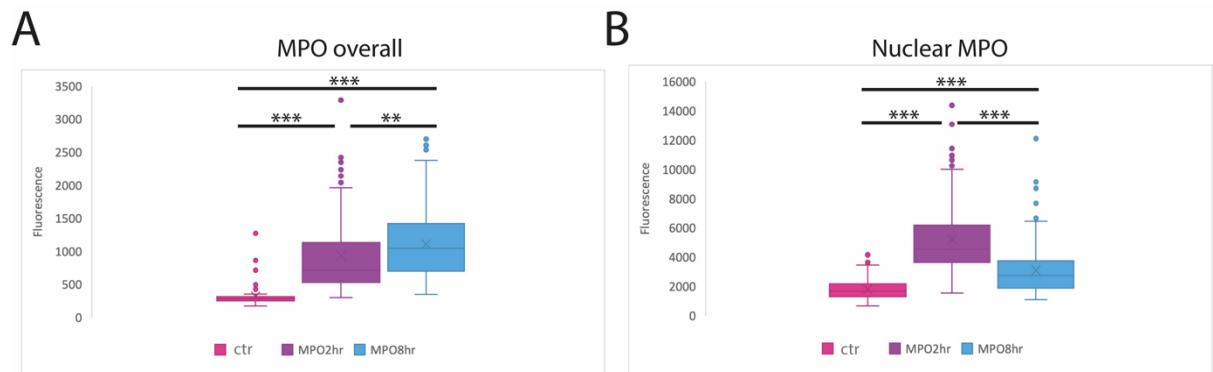


Figure 11. Analysis of immunofluorescence staining reflecting MPO transcytosis and nucleus translocation. (A) Quantification of overall MPO signal using MPO signal as mask in control and treated cells reflecting whole cell MPO. (B) Quantification of nucleus MPO signal using DAPI as mask in control and treated cells. **= $p < 0,005$; ***= $p < 0,0005$.

Analysis to overall MPO signal revealed a significant increase at 8 hours compared to 2 hours post treatment (Figure 11 A), indicating that MPO transcytosis went on during 8 hours of treatment.

Conclusively, MPO transcytosis into HUVECs 2 hours after treatment was verified. What's new, MPO translocation into nucleus at 2 hours and gradually fading away within 8 hours of treatment was defined, with an increased whole cell MPO amount.

3.1.3 Detection of MPO binding to HUVECs nucleus DNA

While confocal microscopic images (Figure 10) clearly demonstrated the translocation of MPO into HUVECs nucleus, it has limitations to provide further information on sub-nucleus MPO location. To find out if the peroxidase merely diffuses through the nucleoplasm or rather associates with potential binding partners on the chromatin, chromatin fractionation visualized with western blot was performed. A simplified protocol is shown in Figure 12 A.

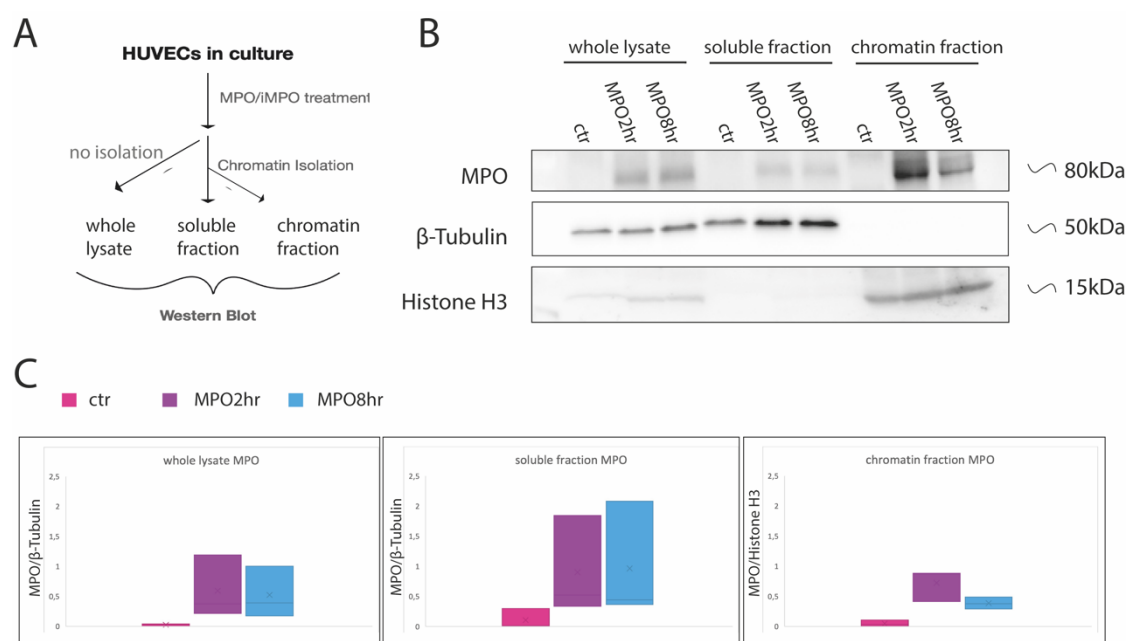


Figure 12. MPO associates with HUVECs chromatin showed by chromatin fractionation. (A) Protocol of chromatin fractionation experiment. (B) Representative western blot of whole lysate, soluble fraction and chromatin fraction of control and MPO treated HUVECs. (C) Analysis of chromatin fractionation western blot with ImageJ. MPO signal in whole and soluble fractions was shown relative to β-Tubulin while in chromatin fraction relative to Histone H3. “x” indicates of mean value, “—” indicates of median value.

HUVECs was treated with a final concentration of 1μg/ml MPO for 2 and 8 hours for chromatin isolation, and non-treated cells was used as control. β-Tubulin was detected in both whole cell lysate and soluble fraction, reflecting the subcellular distribution of the housekeeping protein on the plasma membrane and in the nucleus. Histone H3 was not detectable in soluble fraction while being enriched in chromatin fraction, proving that the chromatin isolation was efficient (Figure 12 B). MPO was detected on all fragments in 2 and 8 hours treated and not on the non-treated HUVECs, reflecting MPO association with HUVECs chromatin.

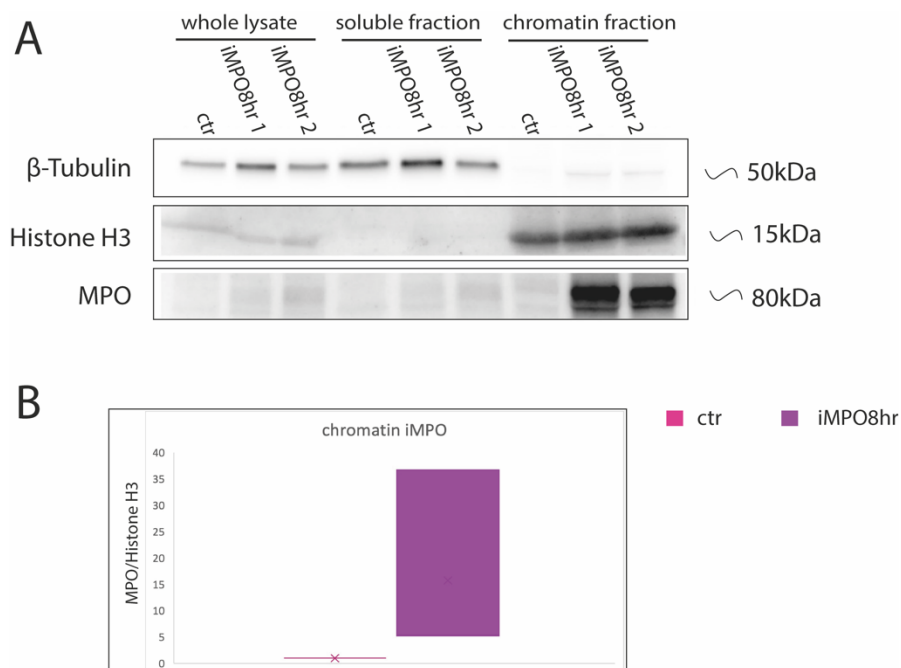


Figure 13. iMPO associates with HUVECs chromatin showed by chromatin fractionation. (A) Representative western blot image with β -Tubulin, Histone H3, and MPO staining. (B) Analysis of MPO chromatin fraction western blot via imageJ showed by box plot. “x” indicates of mean value, “—” indicates of median.

In order to study whether MPO enzymatic activity is required for its association with HUVECs chromatin, previous prepared iMPO was used. HUVECs were treated with iMPO for 8 hours for chromatin isolation and non-treated cells were used as control. β -Tubulin was detectable in all fractions, with a little amount in the chromatin fraction. The distribution of Histone H3 were observed as not different from MPO fractionation blot. Blot of iMPO using anti-MPO antibody revealed its binding to HUVECs chromatin (Figure 13). Taken together, it can be concluded that MPO binding to HUVECs chromatin is independent of its enzymatic activity.

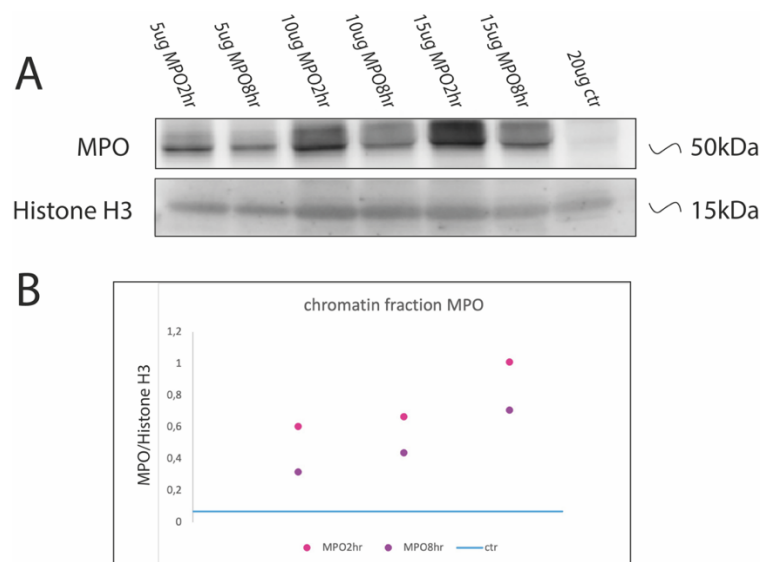


Figure 14. MPO associates with chromatin majorly 2 hours post treatment. (A) chromatin fractionation western blot loaded with 5, 10, 15, 20 µg chromatin fractions individually as indicated. (B) analysis of western blot via ImageJ showed with dot plot. MPO level in ctr chromatin fraction is indicated at the bottom with light blue line.

It was observed that the level of MPO in 2 hours treatment is higher than in the 8 hours treatment exclusively in chromatin fractions, which is in line with IF result. In order to verify if the difference is linear to chromatin fraction loaded on acrylamide gel, an additional western blot with different amount of chromatin fractions was performed, and a linear distribution was observed (Figure 14). Thus, it can be concluded that MPO accumulation in HUVECs nucleus 2 hours upon treatment, making it the best time point for capturing MPO association with chromatin.

Eukaryotic chromatin can be classified hierarchically into primary, secondary, tertiary and quaternary structures. The primary structure comprises how DNA is organized on nucleosomes, the basic units of chromatin. Previous result showing MPO association with chromatin indicates of its association to nucleosomes. In cooperation with Dr. Simon Pöpsel and his team, Electromobility Shift Assay (EMSA) was carried out to further realize nucleosome-binding capacity of MPO.

Nucleosomes were reconstituted as described in previous studies [128], [129] and incubated with a dilution of different concentrated MPO from 50 to 1000nM. For a

synchronization with MPO concentration used for cell culture, the dilutions used here can be equally described as 7,5 to 150 $\mu\text{g/ml}$.

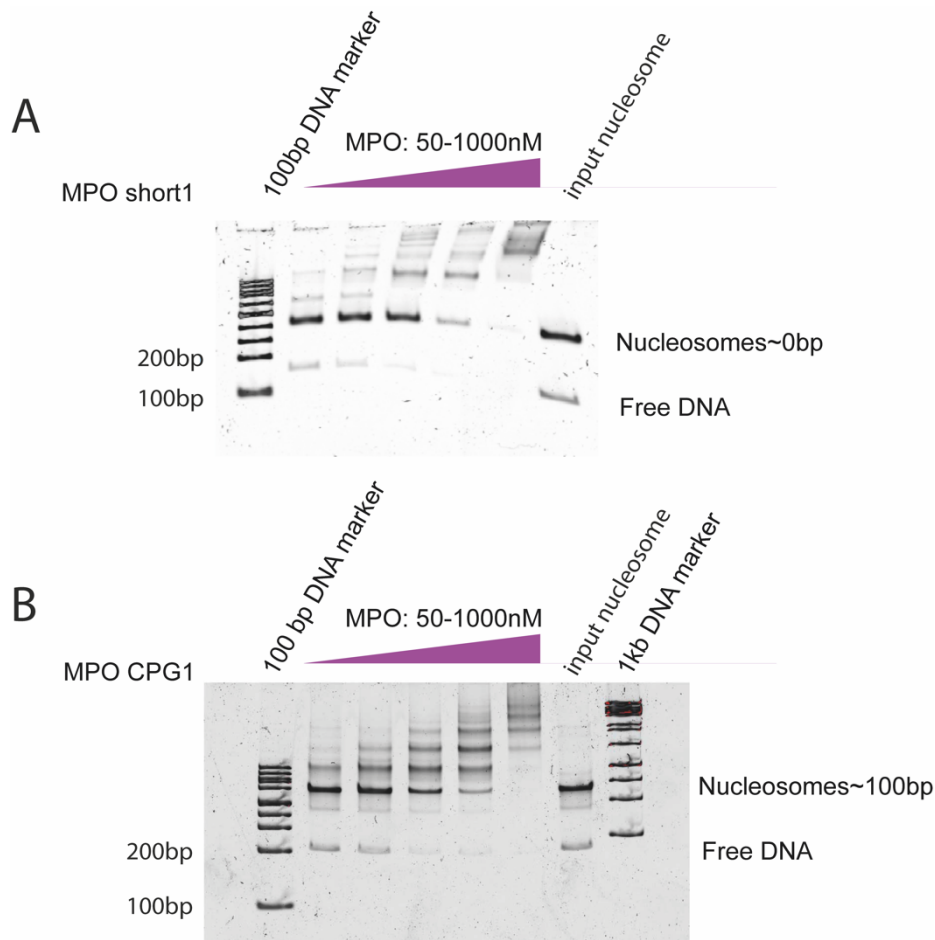
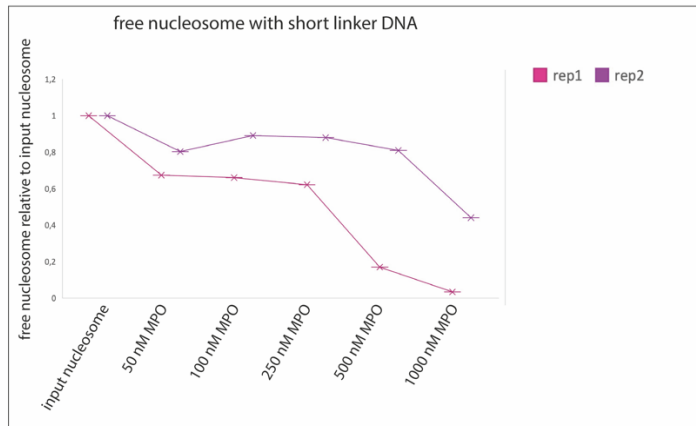


Figure 15. MPO binding to nucleosome with both short and long linker DNA detected via EMSA. (A) MPO concentrated from 50 to 1000nM was incubated for a same period of time with nucleosome with short linker DNA (ideally 0bp) and free DNA, the mixture was visualized on a non-denaturing polyacrylamide gel. (B) MPO incubated with nucleosome with short linker DNA (ideally 0bp) and free DNA visualized on a non-denaturing polyacrylamide gel.

In the EMSA using nucleosomes with short linker DNA and MPO incubation, a shift of nucleosome on acrylamide gel can be observed with all incubations from 50 to 1000 nM MPO (Figure 15 A, 16 A), revealing that MPO binds to nucleosome despite of the linker DNA.

The shift of nucleosome with long linker DNA upon 50, 250, 500, and 1000 nM MPO is significant, and the remaining free nucleosome decreased in a dose dependent manner (Figure 15 B, 16 B), revealing that MPO binds to nucleosome with long linker DNA with higher affinity.

A



B

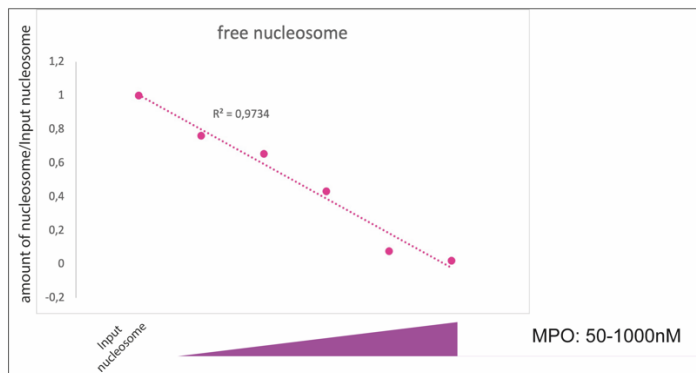
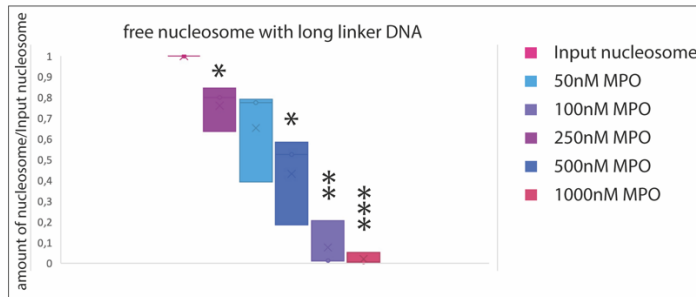


Figure 16. Analysis of EMSA using nucleosome with long and short linker DNA with different concentrations of MPO. (A) Relative amount of free nucleosome with short linker DNA normalized to input nucleosome showed in 2D-line plot. (B) Relative amount of free nucleosome with long linker DNA normalized to input nucleosome showed in box plot and dot plot with a reference trendline. Rep1, rep2: replica 1 and 2 from two independent and converging experiments. “x” indicates of mean value, “—” indicates of median. showed in dot plot with reference line ($r^2=0,9734$). $p<0,05$ (*), $p<0,005$ (**), $p<0,0005$ (***).

Same as in the chromatin fractionation experiment, iMPO was used to verify if the nucleosome binding activity of MPO is dependent on its enzymatic activity.

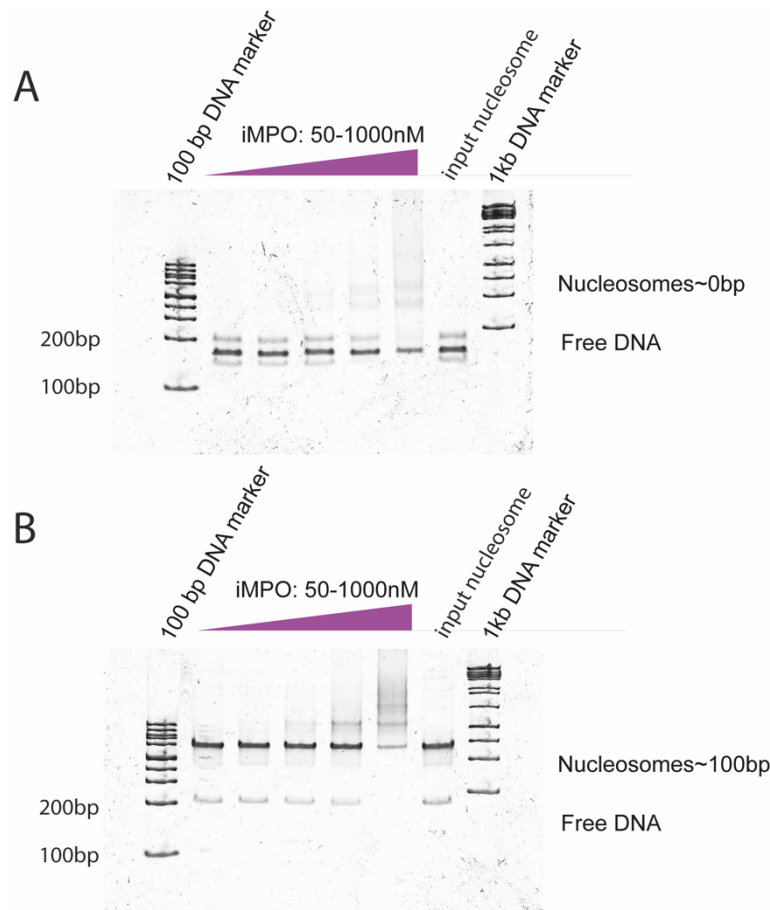


Figure 17. iMPO binding to nucleosome with both short and long linker DNA detected via EMSA. (A) iMPO concentrated from 50 to 1000 nM was incubated for a same period of time with nucleosome with short linker DNA (ideally 0bp) and free DNA, the mixture was visualized on a non-denaturing polyacrylamide gel. (B) iMPO incubated with nucleosome with short linker DNA (ideally 0bp) and free DNA visualized on a non-denaturing polyacrylamide gel.

A shift of the nucleosome with short linker DNA was observed exclusively with 1000 nM MPO incubation (Figure 17 A, 18), revealing that in comparison to MPO, iMPO binds with less affinity to nucleosome and independent of linker DNA.

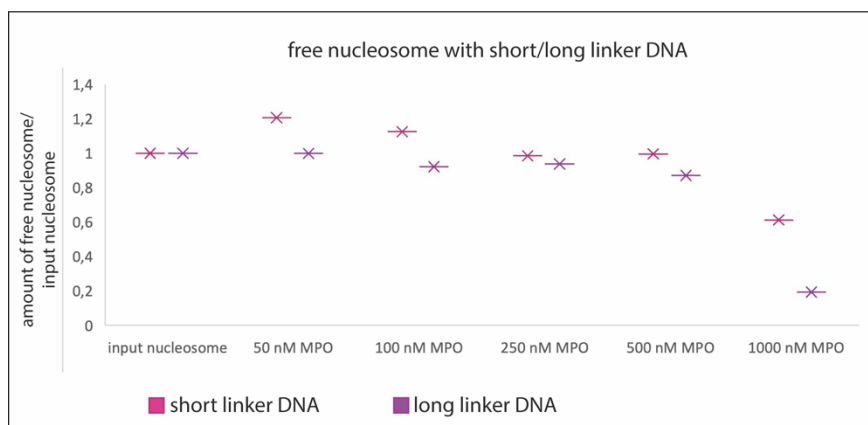


Figure 18. Analysis of EMSA using nucleosome with long and short linker DNA with different concentrations of iMPO. Relative amount of free nucleosome with short linker DNA normalized to input nucleosome showed in 2D-line plot.

EMSA analysis showed that 500 nM iMPO incubation resulted in a shift of the nucleosome with long linker DNA. Further, less free nucleosome was detected with 1000 nM iMPO incubation. This observation further revealed that iMPO binding to nucleosome does not depend on linker DNA, but prefers nucleosome with long linker DNA like that with MPO.

3.1.4 Determining MPO-specific binding to HUVECs chromatin genome-wide

In previous sessions, MPO translocation into HUVECs nucleus and association with chromatin and nucleosome was determined. The enzyme is quite cationic with positive charge. Therefore, it is possible that upon translocation, MPO is attracted by and further associate with acidic DNA. The aim of this session is to find out the binding capacity of MPO to bind HUVECs chromatin genome-wide.

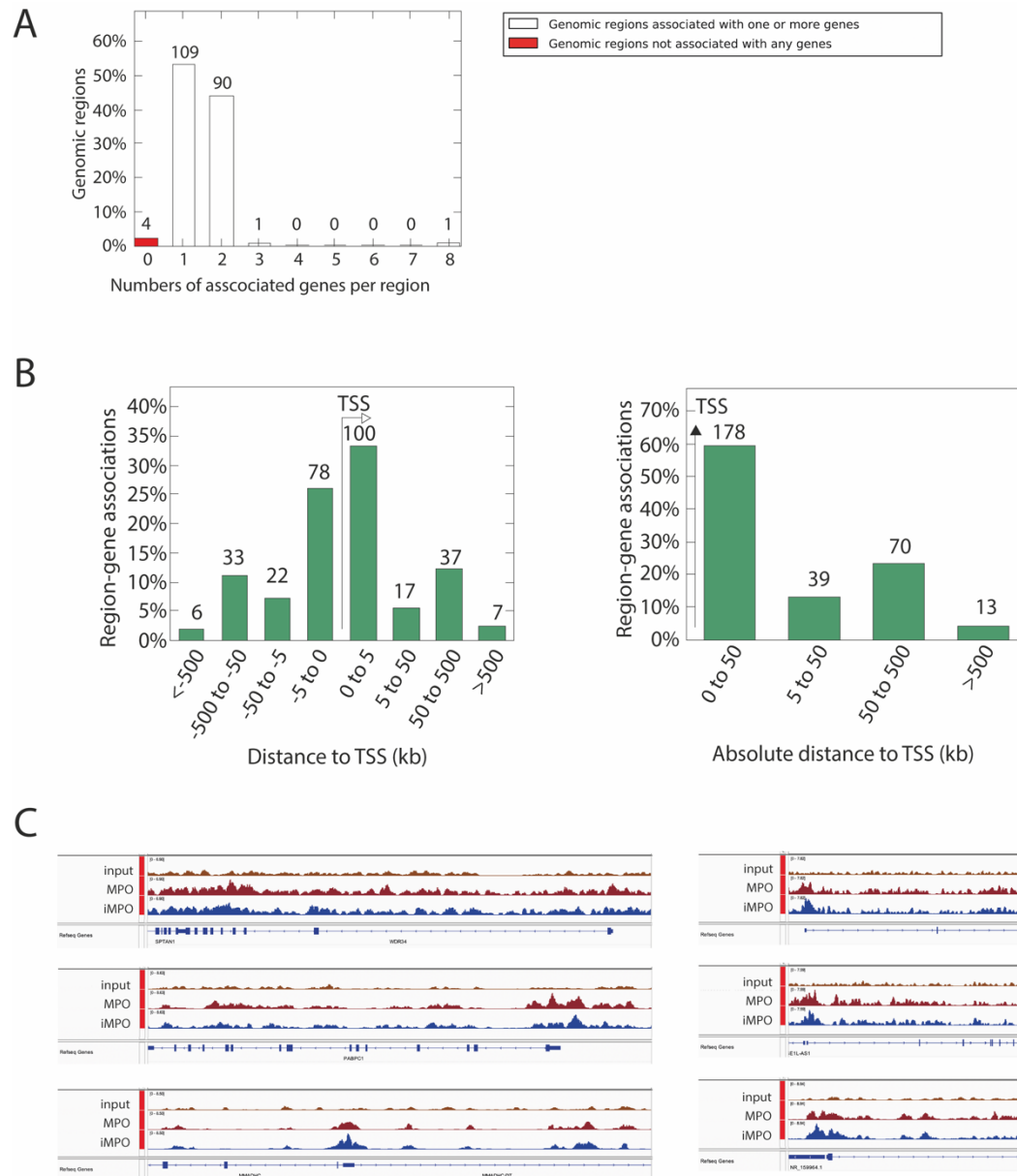


Figure 19. GREAT annotations MPO ChIP-seq showed MPO binding close to transcription starting sites. (A) number of associate genes per genomic regions displayed with box plot. (B) distance and absolute distance of MPO binding sites to TSS showed in bar plots. (C) In total 6 representative MPO binding peaks closest to TSS browsed in IGV (group scale). IGV= Integrative Genomics Viewer. GREAT= Genomic Regions Enrichment of Annotations tool.

To this end, ChIP-seq was performed on HUVECs treated with MPO for 2 hours, using MPO antibody working for IF and chromatin fractionation experiments. Non-treated HUVECs genome were cross-linked, sheared and immunoprecipitated with same MPO antibody was sequenced as input. Annotations of MPO binding sites was performed by using Genomics

Regions Enrichment of Annotations tool (GREAT) version 4.04[130]. By annotations, 97% genomic regions associate with one or more genes (Figure 19 A). 59% gene associated regions locates within 50bp from transcription start sites (TSS), 13% and 23% locates 5-50, and 50-500 bps away from TSS (Figure 19 B). To sum up, MPO binds mostly to TSS neighboring regions. Examples of MPO binding peaks that are closest to TSS around 6 gene regions are showed (Figure 19 C).

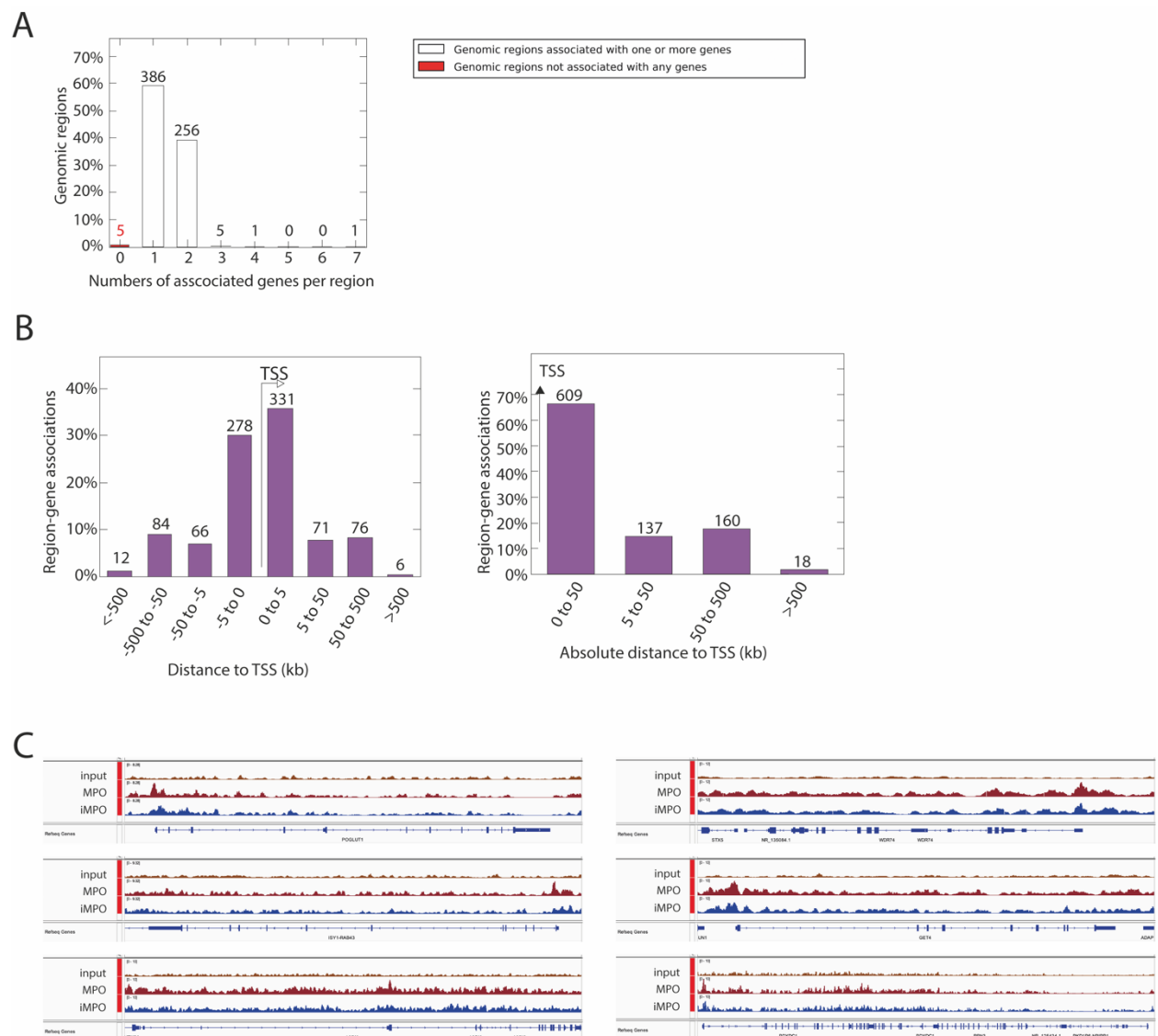


Figure 20. GREAT annotations iMPO ChIP-seq showed iMPO binding close to transcription starting sites. (A) number of associate genes per genomic regions displayed with box plot. (B) distance and absolute distance of MPO binding sites to TSS showed in bar plots. (C) In total 6 representative MPO binding peaks closest to TSS browsed in IGV (group scale).

In parallel, ChIP-seq with iMPO was performed and annotated. 98% genomic regions associate with one or more genes (Figure 20 A). 66% gene associated regions locates within 50bp from transcription start sites (TSS), 15% and 17% locates 5-50, and 50-500 bps away from TSS (Figure 20 B). Like MPO, iMPO also binds mostly to TSS neighboring regions. Examples of iMPO binding peaks that are closest to TSS around 6 gene regions are showed.

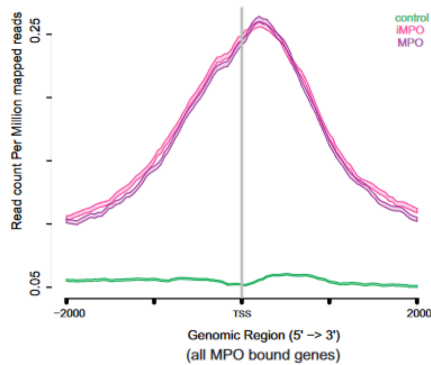


Figure 21. Alignment of MPO and iMPO occupied genomic regions relative to TSS (bp).

The alignment of MPO and iMPO occupied region further confirmed the preference of both associating to regions ± 500 bp from TSS (Figure 21). Moreover, a high consistency of chromatin association between MPO and iMPO was observed (Figure 21).

3.2 Chapter 2 MPO alters HUVECs chromatin structure

3.2.1 Global HUVECs chromatin condensation upon MPO treatment

It is known that protein-nucleosome and protein-protein interactions affect chromatin dynamics and have potentials to drive chromatin structure change [131]. MPO translocation into nucleus and binding to specific regions on genome were verified in chapter 1. In this chapter, investigation to MPO regulated HUVECs chromatin dynamics is shown.

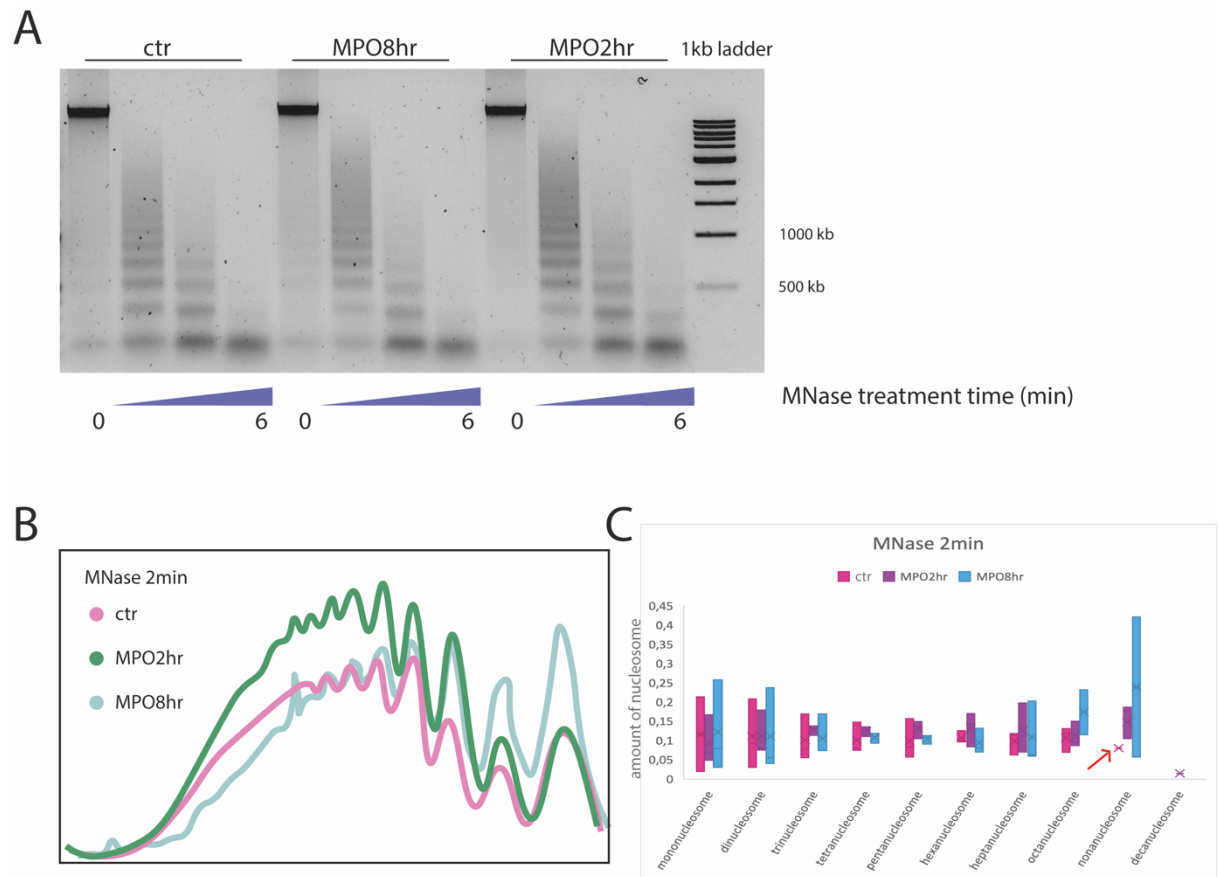


Figure 22. Global HUVECs chromatin condensation upon MPO treatment detected via MNase Assay. (A) Representative nucleosome ladder as a result of MNase treatment (0, 2, 4, 6min individually as indicated on the figure) visualized on agarose gel. From bottom to top bands: mono-, di-, tri-, etc. nucleosomes. (B) Analysis of the nucleosome bands of each nucleosome ladders generated by 2 min MNase treatment using ImageJ, plot of peaks merged in the same scale. First peak on the right reflects the amount of mononucleosomes, and the second dinucleosomes, etc. (C) The amount of each nucleosome bands normalized with that of the input genomes (0 min MNase treatment) showed with box plot. Red arrow: nonanucleosome of ctr nucleosome ladder.

To realize global HUVECs chromatin changes upon MPO uptake, cells were treated with MPO for 0, 2, and 8 hours before MNase treatment. MNase cut in between nucleosomes and produce a mixture of nucleosomes with various numbers of mononucleosome subunits. The mixtures were visualized on agarose gel as DNA ladders (Figure 22 A).

Analysis to DNA ladders of 2 min MNase treatment showed an overall chromatin condensation upon both 2 and 8 hours MPO treatment (Figure 22 B, C).

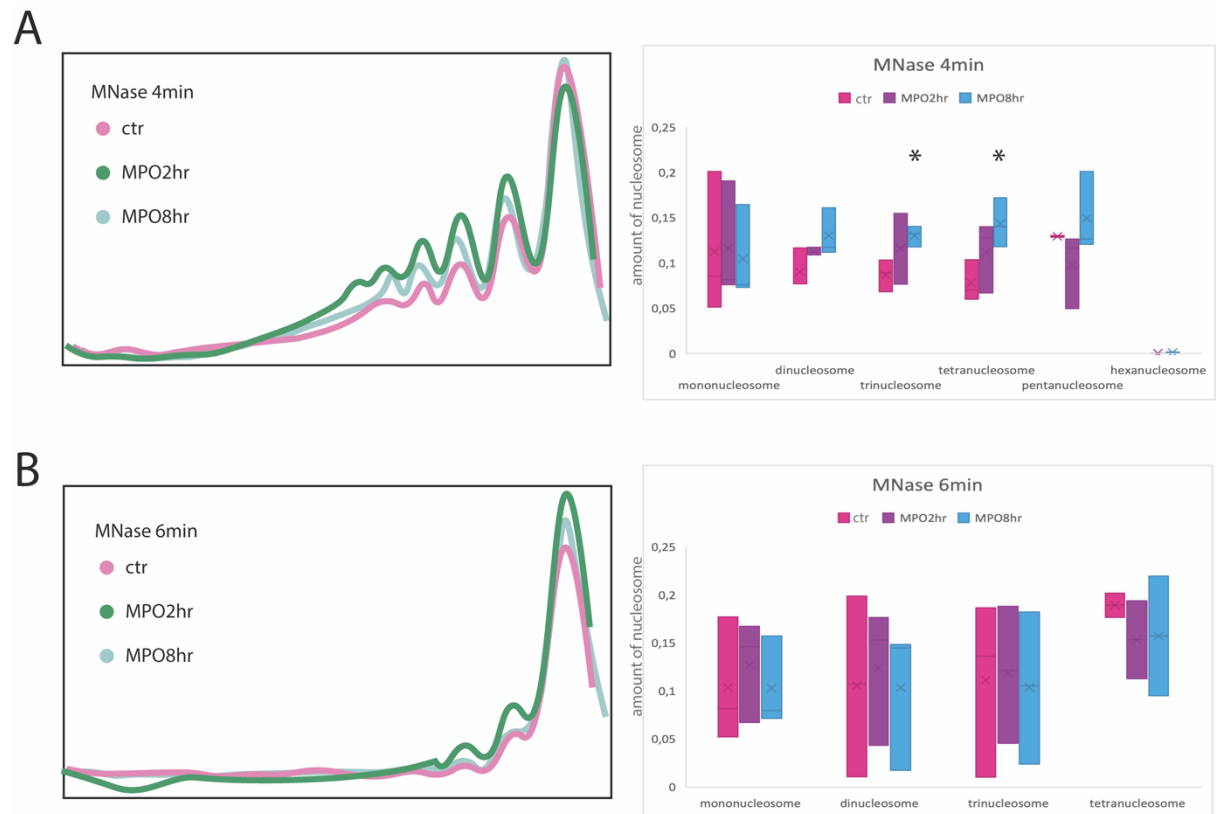


Figure 23. Analysis of the nucleosome ladders with 4 and 6 min MNase treatments. (A) left: Plot of nucleosome bands generated by 4 min MNase treatment. Right: The amount of each nucleosome normalized with that of the input genomes (0 min MNase treatment) showed with box plot. $p < 0,05$ (*).

Analysis to 4 and 6 min MNase treatment showed the same trend like 2 min, but in a lower scale (Figure 23 A, B), indicating that the longer the MNase treatment is, the more synchronized the genomes are after the treatments.

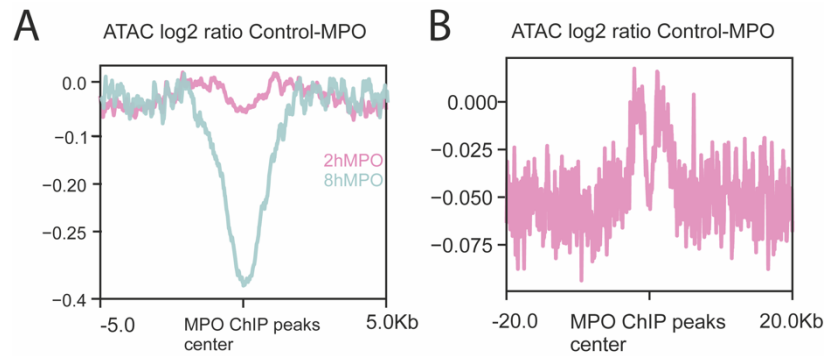


Figure 24. Local chromatin de-condensation on MPO binding region showed by ATAC-seq. (A) ATAC-seq peaks of HUVECs with MPO2hr and MPO8hr treatments normalized to control peaks and aligned with MPO binding sites learned from ChIP-seq. (B) Zoom in of MPO2hr peaks. Aligned by Dr. Yulia Kargapolova.

In order to have a closer view of how MPO impacts HUVECs chromatin, ATAC-seq was performed using HUVECs with 0, 2, and 8 hours of MPO treatments, separately. Both 2 and 8 hours MPO treatment led to local de-condensation of MPO occupied regions (Figure 24 A). A further zoomed in view of 2 hours treatment peaks further strengthened this observation (Figure 24 B).

3.3 Chapter 3 MPO regulates HUVECs Transcriptome

3.3.1 MPO transcytosis regulated endothelial transcriptional changes

Chromatin structural changes can associate with expressional changes. In this chapter, gene expression changes upon MPO transcytosis were studied.

To gain a general picture of HUVECs transcriptome before and after MPO treatment, RNA-seq was performed using HUVECs treated with MPO for 0, 2 and 8 hours, separately.

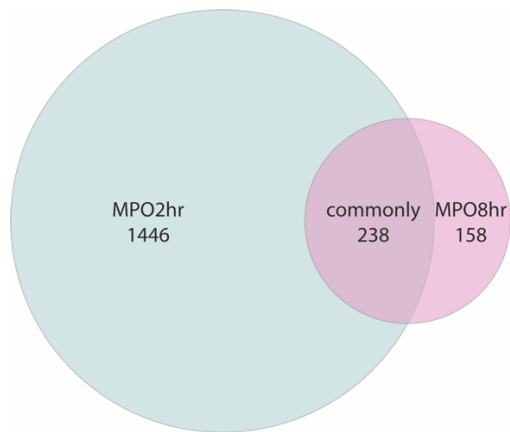


Figure 25. Venn diagram shows the numbers of significantly regulated genes in HUVECs with MPO2hr or MPO8hr treatments normalized to ctr, and commonly regulated.

In total, expression of 1684 gene were found to change with 2 hours MPO treatment compared to non-treated, among which 650 were up regulated, 1034 were down regulated. Expression of 396 genes were found to change with 8 hours MPO treatment compared to non-treated, among which 145 were up regulated, 251 were down regulated. A number of 238 genes were regulated in common among two MPO treatments (Figure 25).

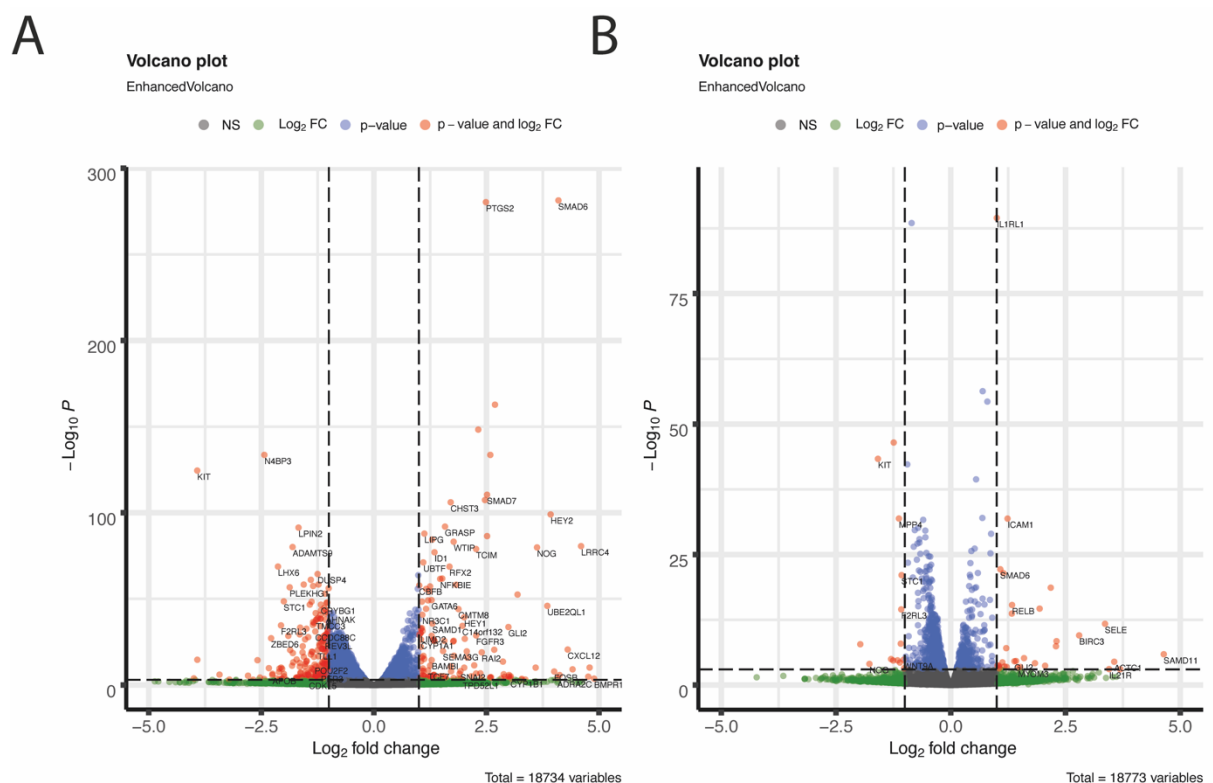


Figure 26. Relative expression changes of genes in HUVECs with MPO2hr or MPO8hr treatment detected in RNA-seq showed in volcano plots. (A) gene expression changes in cells with MPO2hr treatment. (B) gene expression changes in cells with MPO8hr treatment. Expression levels were normalized to housekeeping transcript YWHAZ. Red: significantly regulated genes ($p\text{-value} \leq 0,05$ and absolute $\log_2$ fold change ≥ 1); Green: non-significantly regulated genes (absolute $\log_2$ fold change ≥ 1); Blue: non-significantly regulated genes ($p\text{-value} \leq 0,05$); Grey: non-significantly regulated genes ($p\text{-value} \geq 0,05$ and absolute $\log_2$ fold change ≤ 1).

Compared to 8 hours, the expression of genes with 2 hours MPO treatment was more significantly altered (Figure 26).

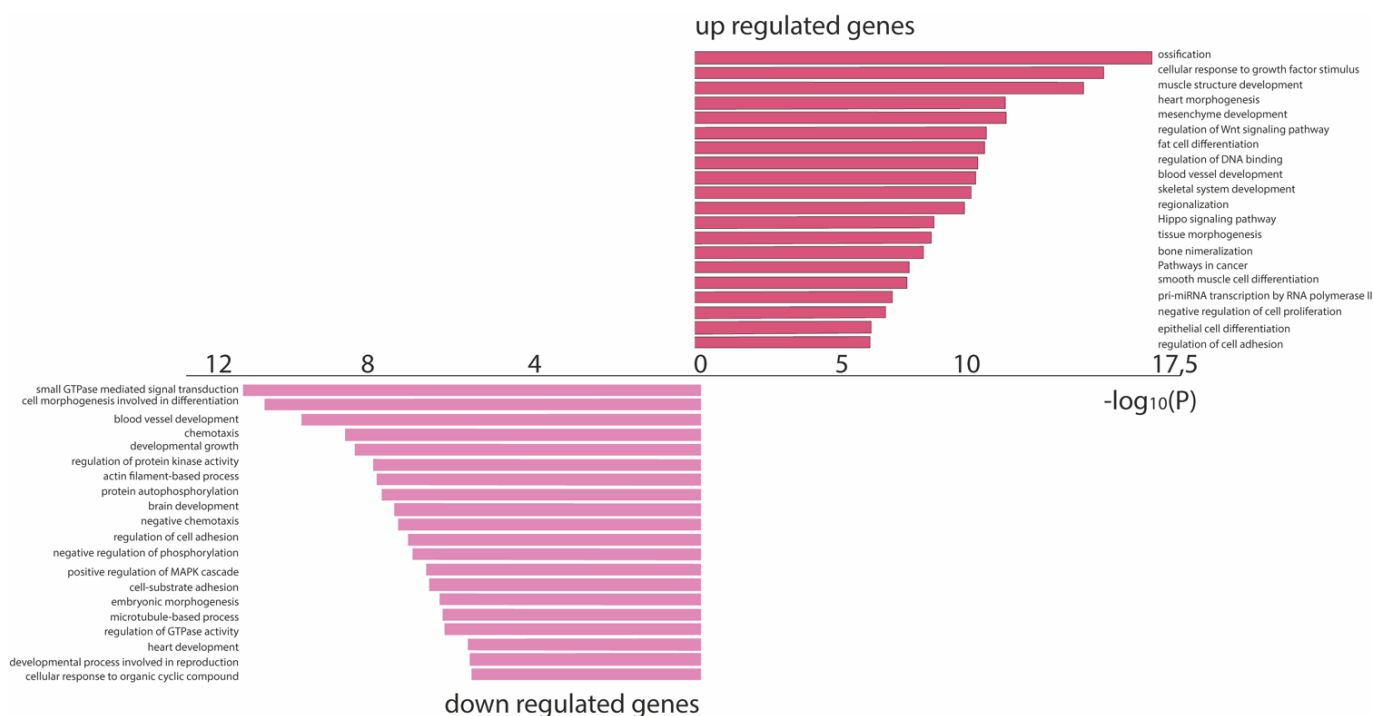


Figure 27. Gene Oncology analysis (GO) of HUVECs with MPO2hr treatment RNA-seq achieved by using Metascape online annotation tool[132].

By annotating regulated genes, they were clustered into different terms. The best 40 terms for each treatment are displayed with bar plots (Figure 27, 28). In 2 hours MPO treatment RNA-seq, 11/40 terms were found to be related to the process of endothelial to mesenchymal transition (endMT) (Figure 27, Table 3-1). In 8 hours MPO treatment RNA-seq, 11/40 other GO terms were found to be related to endMT (Figure 28, Table 3-1), making this biological process the most relevant transcriptional alteration trend upon MPO treatment.



Figure 28. Gene Ontology analysis (GO) of HUVECs with MPO2hr treatment RNA-seq.

There are three endMT related GO terms shared by 2 and 8 hours MPO treatment driven transcriptional remodelling: epithelial cell differentiation, blood vessel development, and regulation of cell adhesion. Besides, signalling pathways involved in endMT were found to be regulated upon MPO treatments (Figure 27, 28).

End-MT related GO terms

2 hours up regulated	ossification
	cellular response to growth factor stimulus
	muscle structure development
	mesenchyme development
	regulation of Wnt signalling pathway
	blood vessel development
	smooth muscle cell differentiation
	epithelial cell differentiation
	regulation of cell adhesion
2 hours down regulated	cell morphogenesis involved in differentiation
	blood vessel development
8 hours up regulated	NOTCH1 regulation of endothelial cell calcification
	epithelial cell differentiation
	TGF-beta receptor signalling in skeletal dysplasias
8 hours down regulated	cell-cell adhesion via plasma-membrane adhesion molecules
	MAPK cascade
	blood vessel development
	Burn wound healing
	regulation of cell adhesion
	PI3K-Akt signalling pathway

Table 3- 1. Summary of EndMT related GO terms annotated from 2 and 8 hours MPO treatment RNA-seq. Terms appear commonly in both RNA-seqs were marked in red.

Additionally, RNA-seq using HUVECs treated with a mutated form of MPO that lacks enzymatical activity, termed mMPO, for 8 hours was performed. Expression of endothelial marker CD34 was found to be down-regulated in all MPO and mMPO treated cells. The expression of ICAM is usually found to decrease in late endMT, and in this RNA-seq it was increased with 2 hours MPO and mMPO treatments, while decreased with 8 hours MPO treatment (Figure 29).

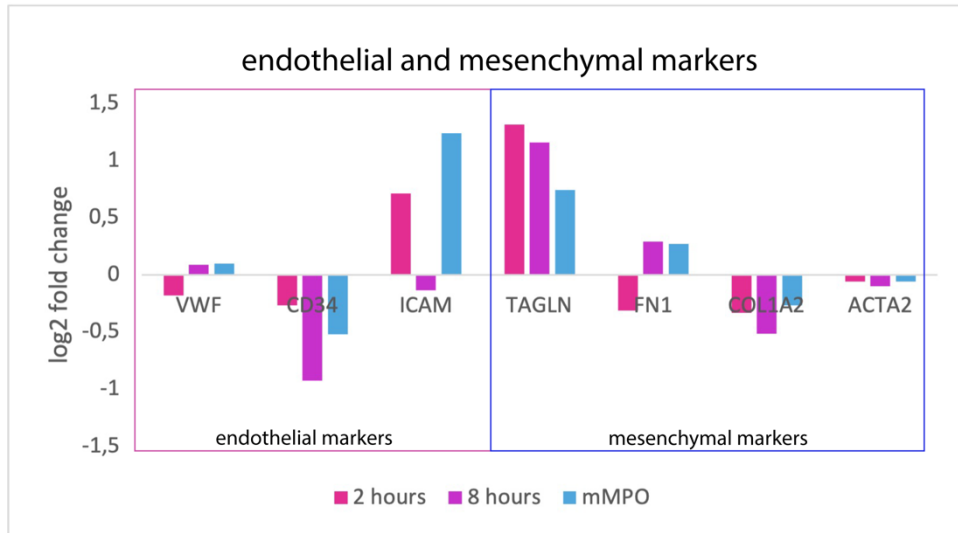


Figure 29. Relative expression of genes involved in endMT identified via RNA-seq, normalized to housekeeping transcript YWHAZ.

The expression of mesenchymal marker TAGLN increased with all three treatments, while the expression of FN1 slightly ($\log_2$ fold change $<0,5$) decreased with 2 hours and slightly increased with 8 hours MPO and mMPO treatments. The expression of COL1A2 slightly decreased in all treatments. The expression of ACTA2 nearly didn't change (Figure 29).

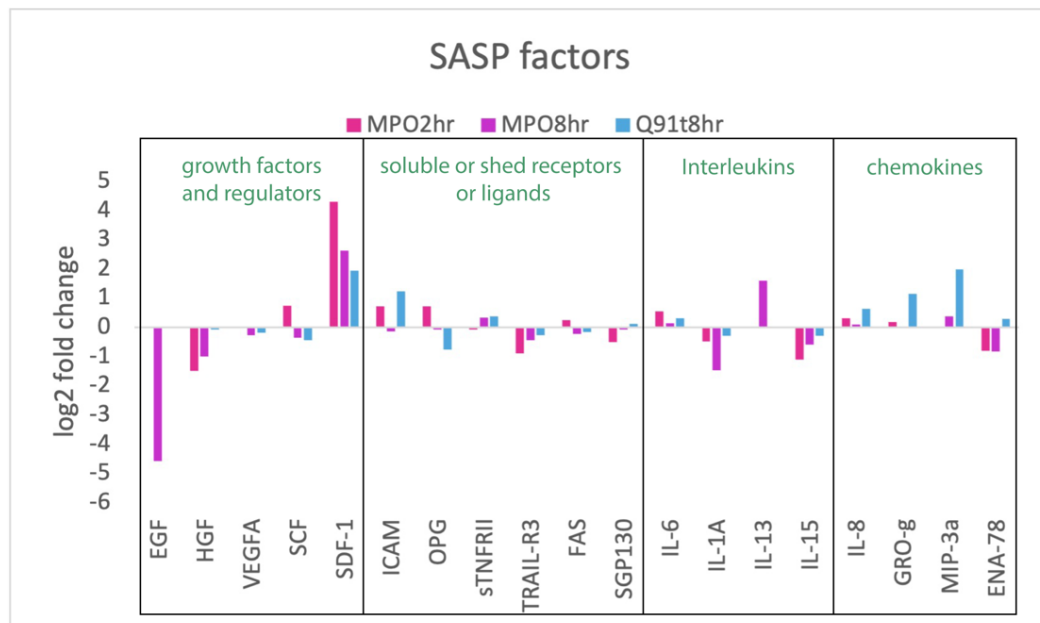


Figure 30. Relative expression of genes involved in SASP identified via RNA-seq, normalized to housekeeping transcript YWHAZ.

The expression of SASP factors were altered (Figure 30). The expression of EGF decreased with 8 hours MPO treatment; the level of SDF-1 increased with all three treatments; the level of IL-1A and the level of ILF13 decreased with 8 hours MPO treatment; the level of IL-15 decreased with 2 hours MPO treatment. The level of IL-8, GRO-8, and MIP-3a increased with 8 hours mMPO treatment.

3.3.2 MPO regulated endothelial to mesenchymal transition

In this session, RNA-seq result was partially verified in order to find out how HUVECs were remodelled upon MPO uptake.

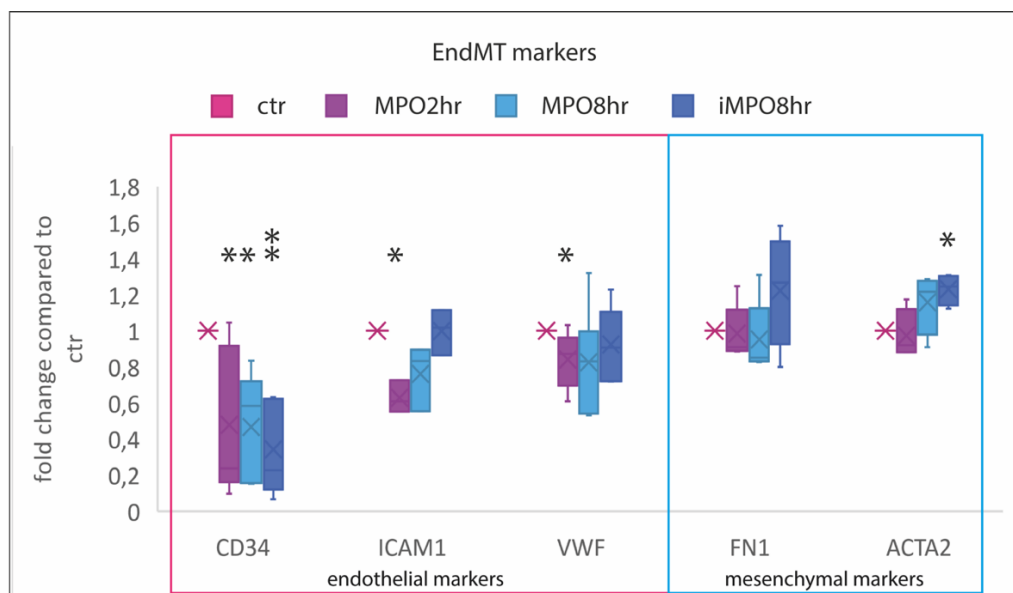
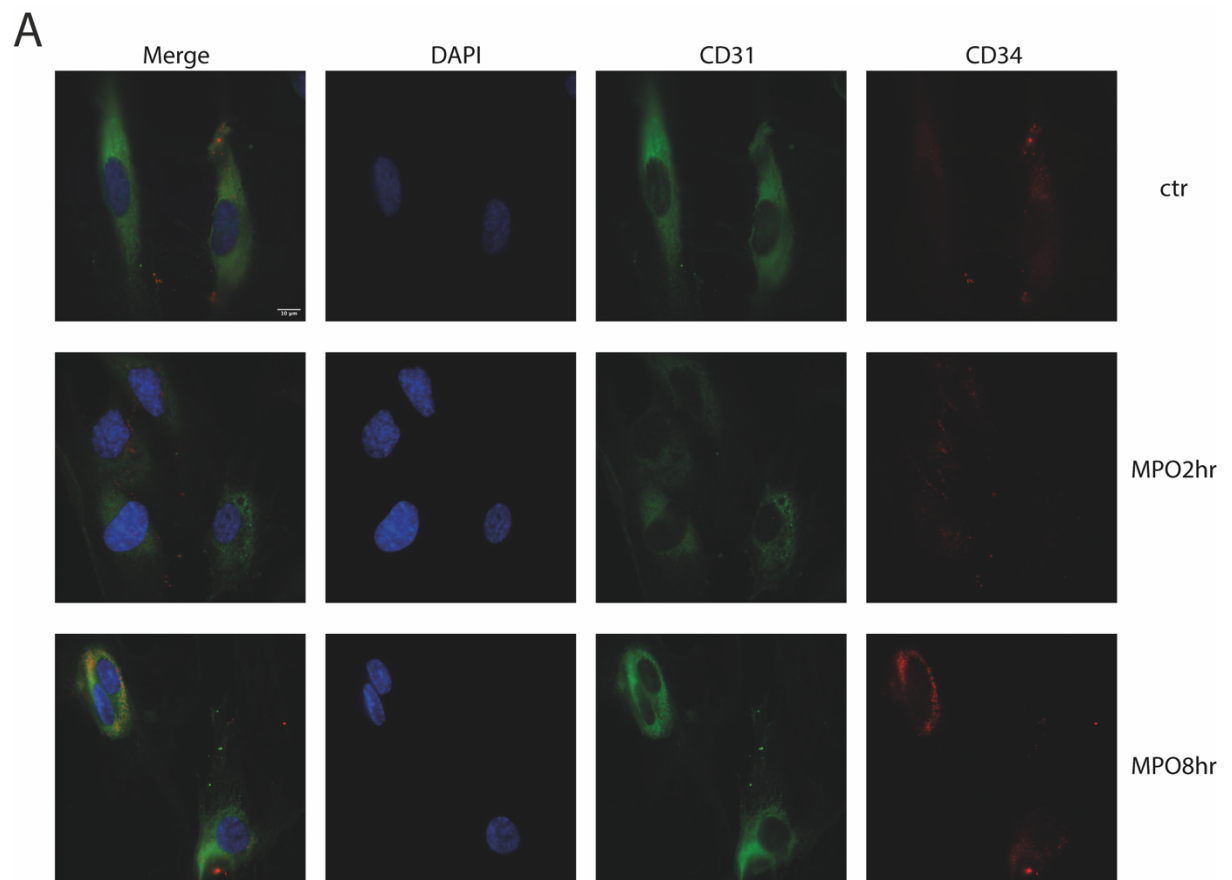


Figure 31. Relative expression of endothelial markers and mesenchymal markers detected via RT-qPCR showed in box plots, normalized to housekeeping transcript YWHAZ. Endothelial markers framed in pink box and mesenchymal markers in blue box. $p < 0,05$ (*), $p < 0,005$ (**).

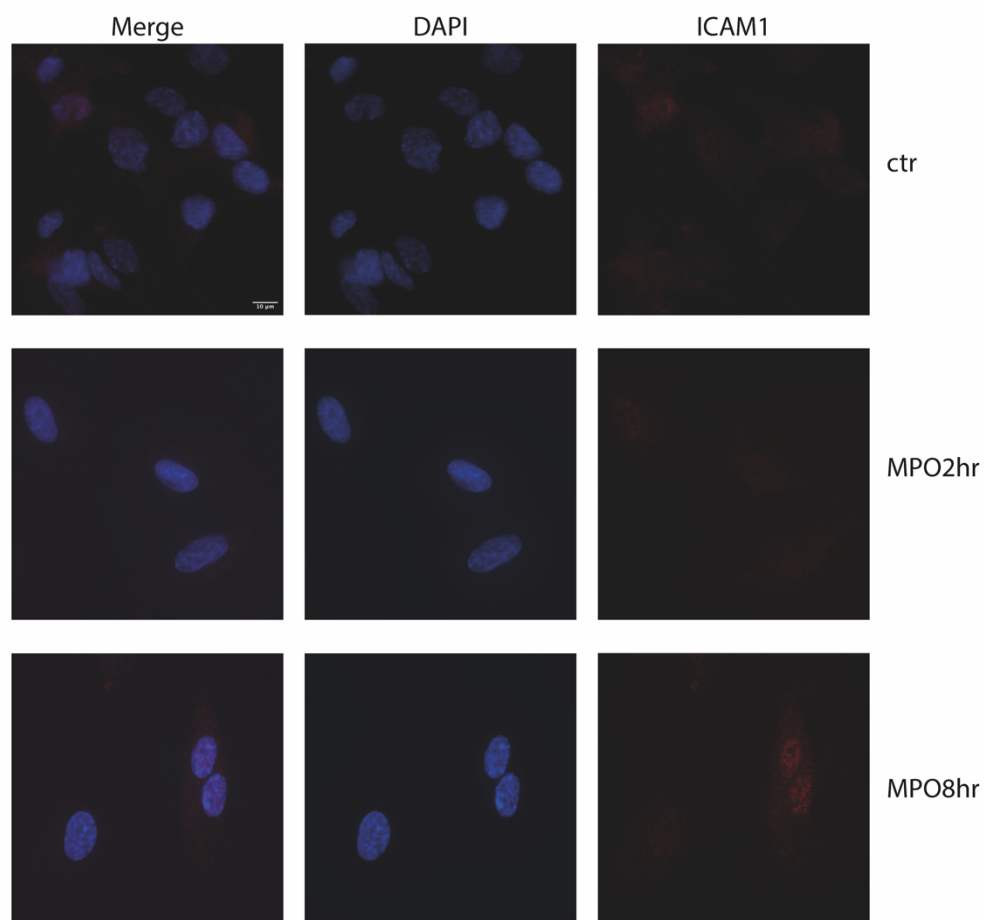
The expressional changes of endothelial and mesenchymal markers upon MPO treatments were verified via RT-qPCR. Instead of mMPO, iMPO was used to study peroxidase activity independent expressional changes in this experiment.

Compared to control, the level of endothelial marker CD34 significantly decreased in HUVECs with 2 hours MPO, 8 hours MPO and 8 hours iMPO. The level of ICAM and VWF significantly decreased in HUVECs with 2 hours MPO treatment (Figure 31).

The level of mesenchymal marker FN1 did not alter significantly with any MPO/iMPO treatment, besides a trend to increase in cells with 8 hours iMPO treatment. The level of ACTA2 did not significantly change in 2 and 8 hours MPO treatment groups, but have a trend to increase in 8 hours MPO group and significantly increased with 8 hours iMPO group (Figure 31).



B



C

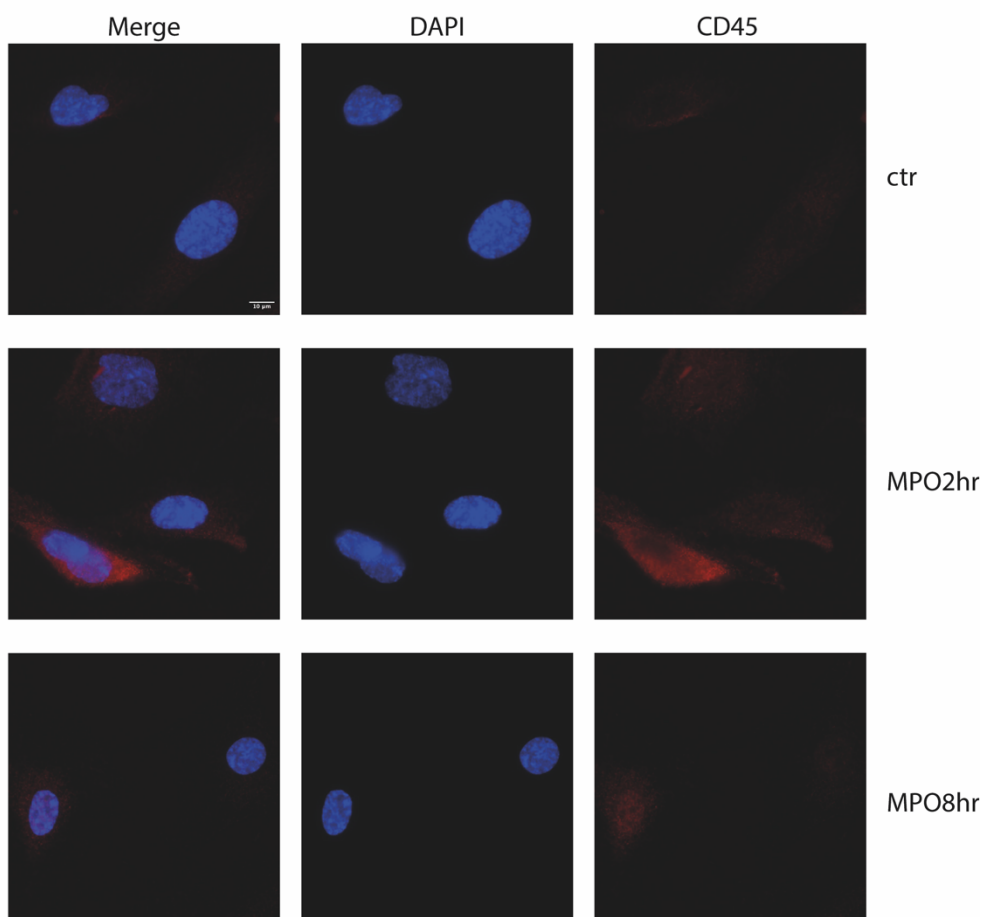


Figure 32. Representative microscopic images visualizing endothelial markers (CD31 and ICAM1 and mesenchymal markers (CD34 and CD45) level changes in HUVECs after 0 (ctr), 2(MPO2hr), and 8 (MPO8hr) hours of treatment. (A) Representative microscopic images of CD31 (Green) and CD34 (Red) staining merged with DAPI (Blue). (B) Representative microscopic images of ICAM1 (Red) staining merged with DAPI (Blue). (C) Representative microscopic images of CD45 (Red) staining merged with DAPI (Blue). Scale bar = 10 μ m.

Expression levels of endothelial markers were further verified with IF, uptake of MPO in HUVECs used for IF was verified (Figure 32, Figure 33 A). Compared to non-treated HUVECs, the level of endothelial marker CD31 did not change in HUVECs with 2 or 8 hours MPO treatment, with a subgroup of MPO up-taken cells in each treatments containing higher expression level. The level of ICAM1 significantly increased in HUVECs with 8 hours MPO treatment (Figure 33 B). The level of CD34 significantly decreased in cells with 2 hours MPO treatment, and significantly increased 8 hours MPO treatment group. The level of mesenchymal marker CD45 significantly increased in 2 hours MPO treatment group (Figure 33 B).

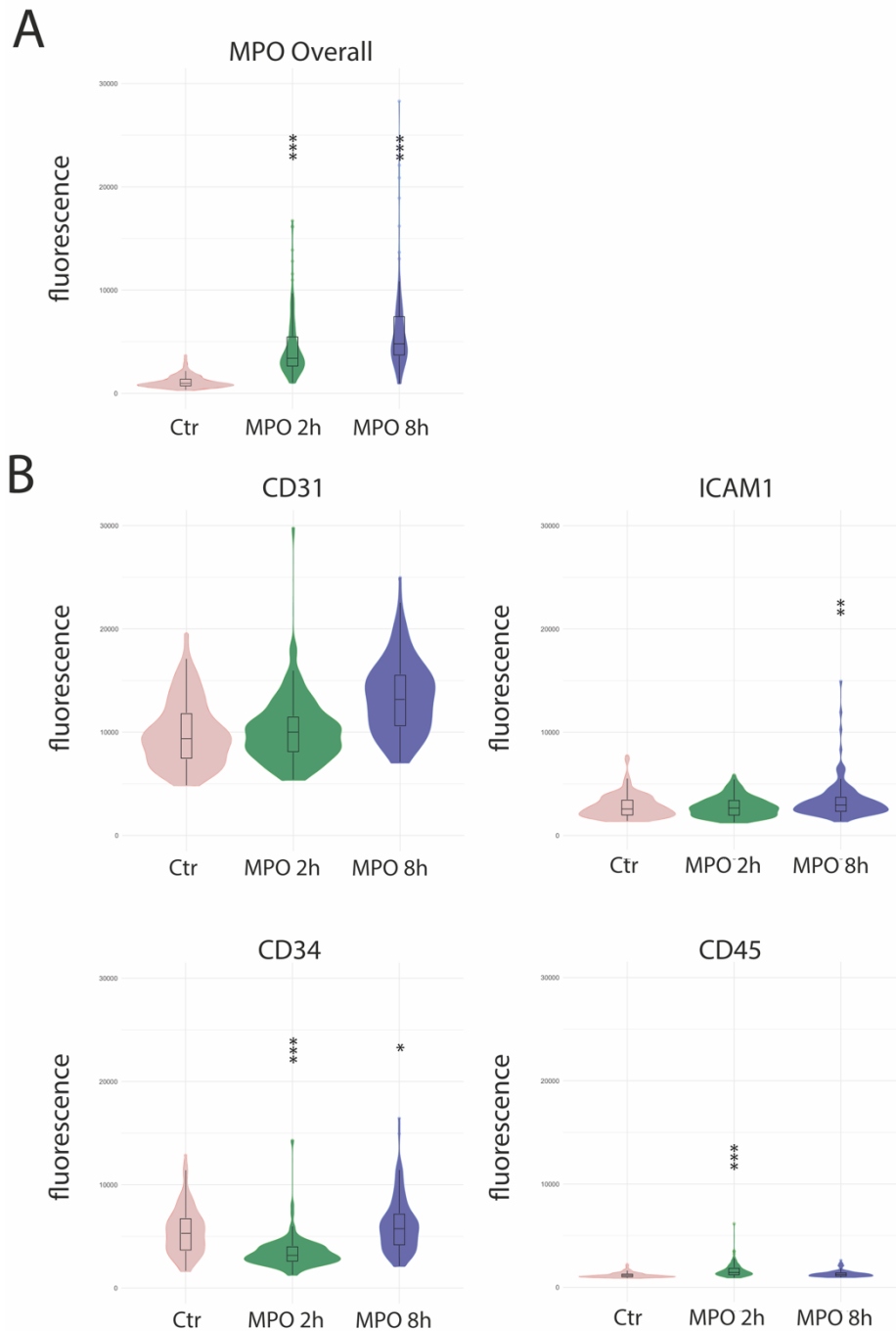


Figure 33. Analysis of endothelial and mesenchymal marker immunofluorescence reflects their level changes in HUVECs upon MPO treatments. (A) Violin plot shows whole cell (overall) MPO signal. (B) Violin plots show endothelial markers signals and mesenchymal marker CD45 signal changes in HUVECs with 0, 2, and 8 hours MPO treatments. Marker signals was used as masks for their analysis. $p < 0,05$ (*), $p < 0,005$ (**), $p < 0,0005$ (***)

Agreeing with the endMT trend observed with multiple methods, MPO resulted HUVECs morphology changes were observed repetitively in cell culture. In order to demonstrate microscopic view, light microscopy facilitated with camera was used to take pictures of same batches of HUVECs before and after 8 hours MPO treatment.

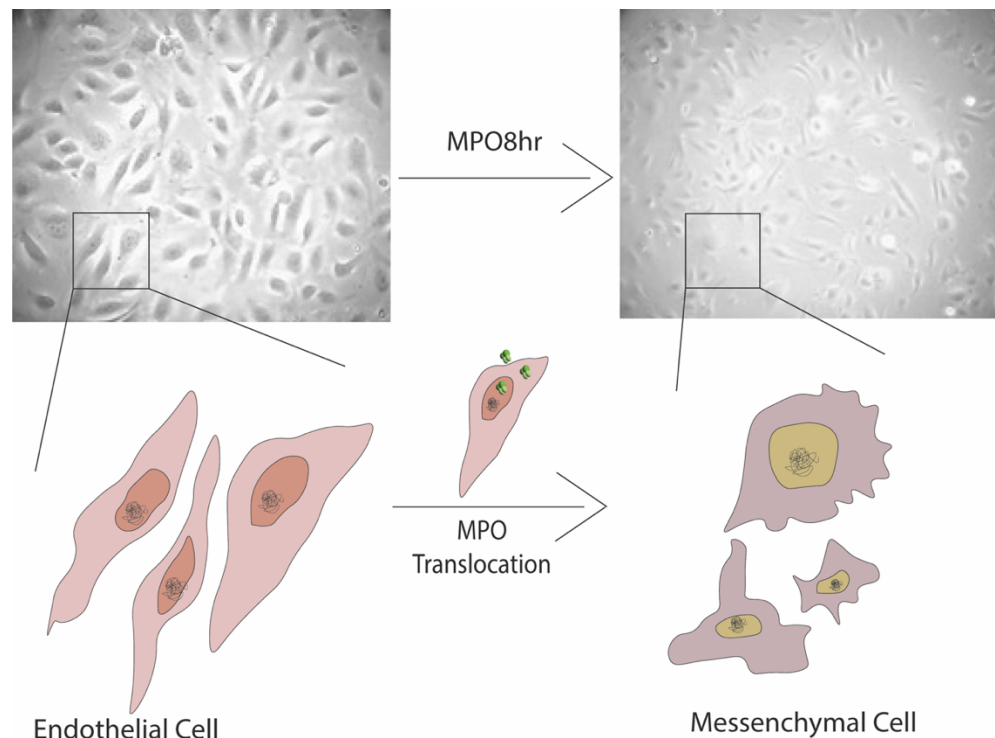
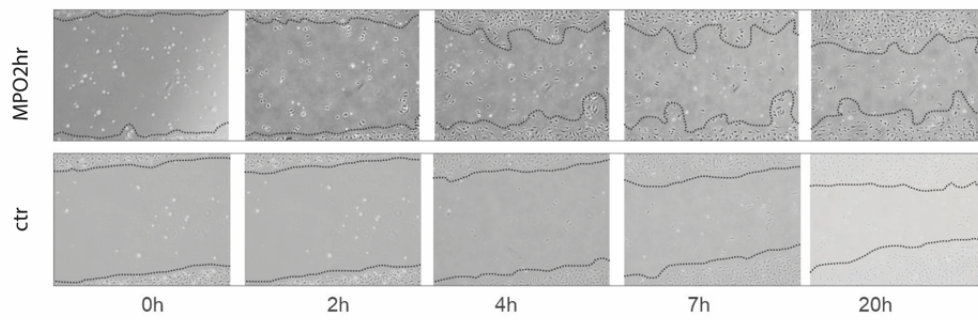


Figure 34. Microscopic images show morphological changes of HUVECs 8 hours upon MPO treatment.

Representative pictures of HUVECs before MPO treatment showed that most cells were with relatively regular and unified shapes. However, after 8 hours MPO treatment and on the same spot, dysregulated morphology was observed. The treated cells lost their shapes and unity, distributed more randomly on the plate (Figure 34).

A



B

Healed Area = Wound Area at 0hr - Wound Area at individual time points

C

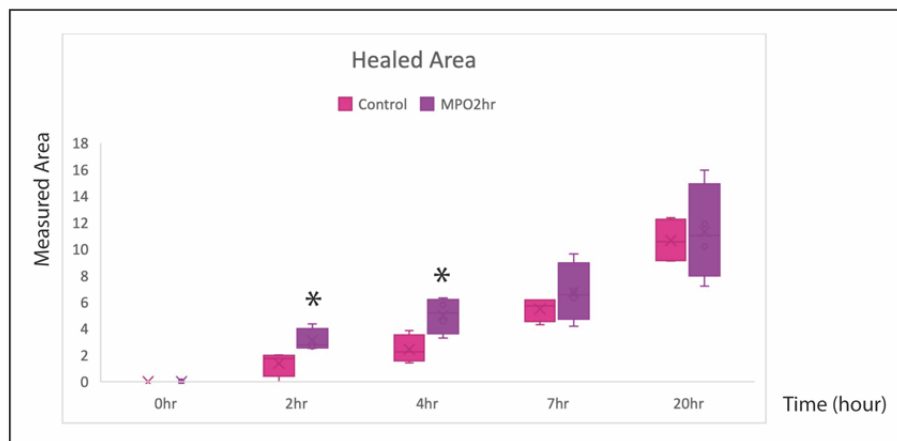


Figure 35. HUVECs with MPO2hr treatment migrates faster than non-treated (ctr) cells showed by wound healing assay. (A) Microscopic image with cell surface outlined with dashed lines via imageJ. (B) Healed Area calculation formula. (C) Analysis of healed area achieved with imageJ showed in box plot.

De-differentiating into mesenchymal cells usually indicates of higher migration rate due to decreased expression of adhesion proteins. To verify the migration rate of MPO treated HUVECs, wound healing assay was performed.

Compared to non-treated HUVECs, HUVECs treated with MPO for 2 hours before the wound was made migrated significantly faster at 2 and 4 hours post wounding (Figure 35).

3.3.3 Co-players of MPO in endothelial cytosol and nucleus

In previous chapters, MPO transcytosis dependent regulations to HUVECs chromatin structure and gene expression were elucidated. In this session, potential MPO co-players in HUVECs cytosol and nucleus were identified and studied.

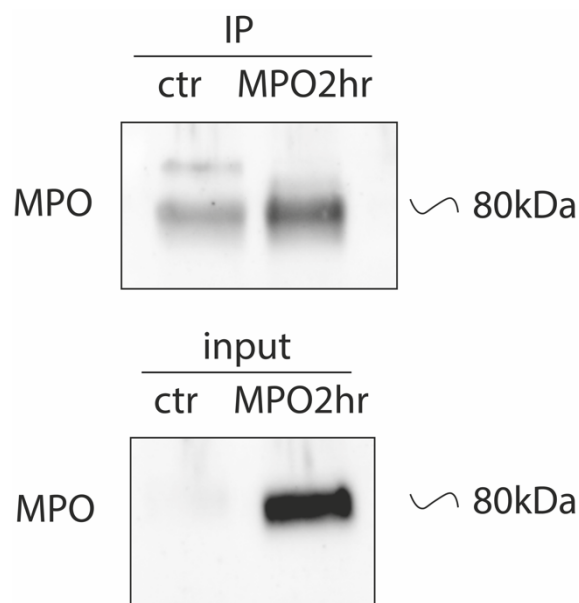


Figure 36. whole cell IP verification.

HUVECs were treated with MPO for 2 hours before immunoprecipitation with whole cell lysis, non-treated cells immunoprecipitated with the same MPO antibody were used as negative control. MPO was detected in both input and IP fractions (Figure 36).

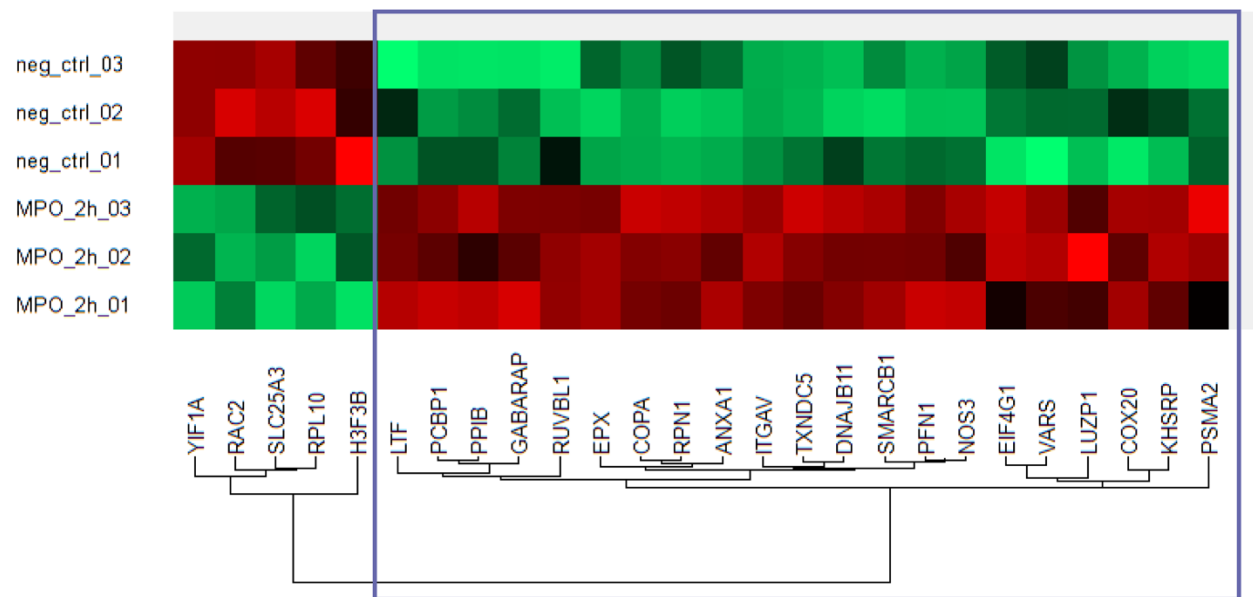


Figure 37. Gene names of MPO binding proteins in HUVECs detected in IP showed with a heatmap. Enriched proteins in MPO2hr treatment lysate are framed with a box.

In total, 21 potential MPO interacting partner candidates were found (Figure 37). Sub-cellular localization of these candidates is listed (Table 3-2) By performing annotation after mass spectrometry using metascape ver.4.4, the candidates can be best described to be involved in two biological processes: VEGFA-VEGFR2 signalling pathway and regulation of mRNA stability (Figure 38). Relevant candidates are listed in Table 3.

<i>GENE NAME</i>	<i>SUB-CELLULAR LOCALISATION</i>
ITGAV	cell membrane
PFN1	cytoskeleton
VAR3	cytosol
COPA	cytosol
RPN1	cytosol, ER
NOS3	cytosol, Golgi body, cytoskeleton
GABARAP	cytosol, lysosome
TXNDC5	ER
DNAJB11	ER
EPX	extracellular
COX20	mitochondrion
SMARCB1	nucleus
LUZP1	nucleus
KHSRP	nucleus
PSMA2	nucleus, cytosol
PCBP1	nucleus, cytosol

RUVBL1	nucleus, cytosol
ANXA1	nucleus, cytosol, endosome
LTF	nucleus, endosome
PPIB	nucleus, ER
EIF4G1	stress granule

Table 3- 2. Subcellular localization of proteins enriched in whole cell immunoprecipitation. Protein information collected from genecards.org.

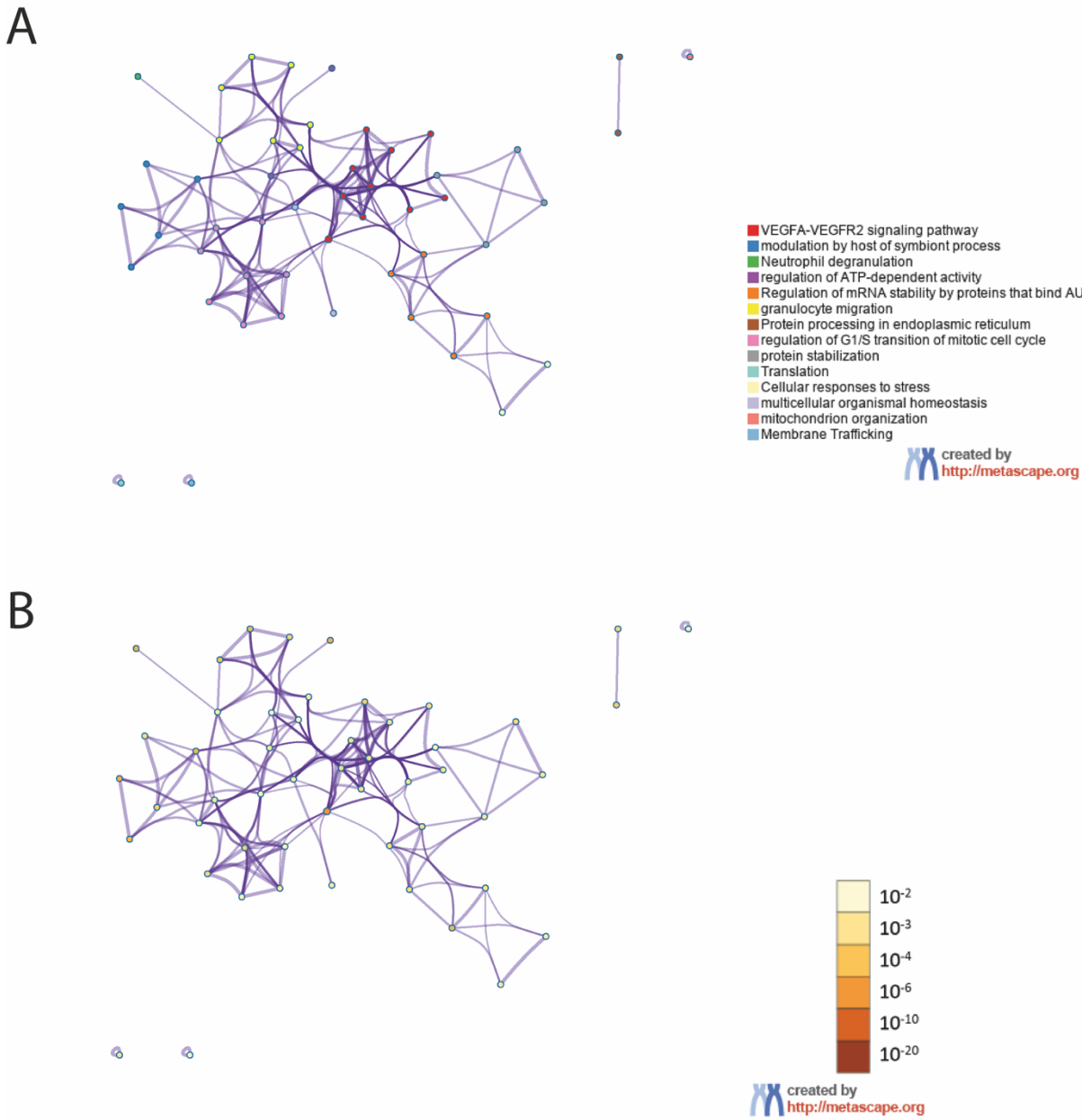


Figure 38. Whole cell HUVECs MPO IP global annotations via metascape. (A) annotations colored by cluster (B) annotations colored by p-value.

<i>VEGFA-VEGFR2 PATHWAY</i>	<i>SIGNALLING</i>	<i>REGULATION OF MRNA STABILITY</i>
ANXA1		PCBP1
ITGAV		ITGAV
TXNDC5		PFN1
SMARCB1		EIF4G1
NOS3		KHSRP
EIF4G1		PSMA2
LUZP1		
PFN1		

Table 3- 3. List of proteins enriched in whole cell IP that are clustered into VEGFA-VEGFR2 signaling pathway and regulation of mRNA stability.

In previous described IP with whole cell lysate, 9/21 proteins were identified locating in HUVECs nucleus (Table 3-2). Taken that nucleus protein was predicted to occupy only 10-20% of total protein [133], while MPO may have more co-plyers in HUVECs nucleus upon its translocation into, an additional IP using isolated nucleus was performed. HUVECs were treated with MPO for 0 or 8 hours before nucleus isolation followed by IP.

In total, 141 candidates were enriched in nucleus IP. Annotations following mass spectrometry revealed that the candidates are best related to biological processes showed in Figure 39.

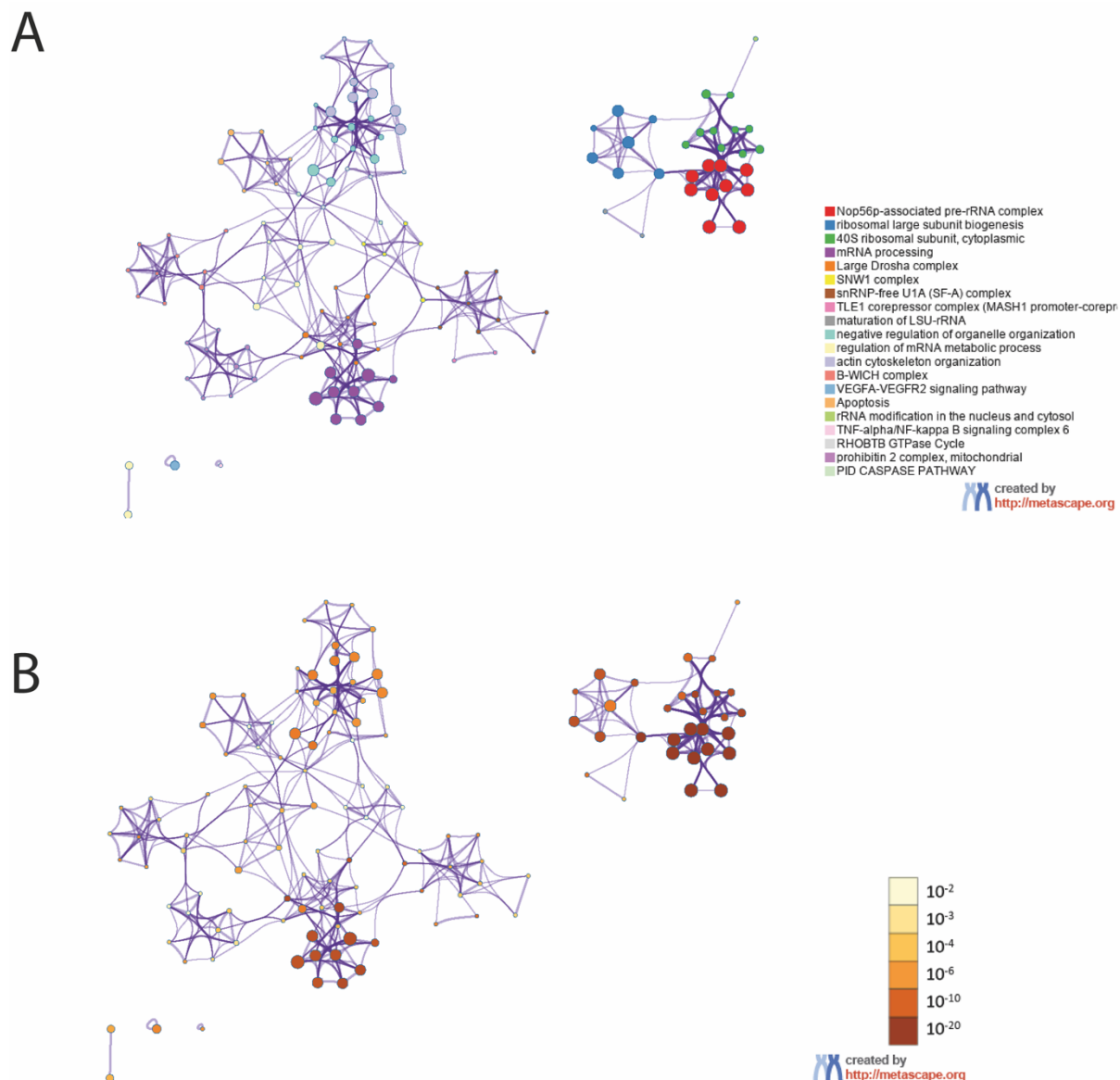


Figure 39. Nucleus HUVECs MPO IP global annotations via metascape. (A) annotations colored by cluster (B) annotations colored by p-value. n= 141.

Combining the term enrichment panel coloured by cluster (Figure 39 A) and by p-value (Figure 39 B), the best relevant terms can be summarized as: Nop56p-associated pre rRNA complex, large drosha complex, and mRNA processing (Figure 39). Candidates enriched in the first two terms are listed (Table 3-4, 3-5).

Nop56p-associated pre-rRNA complex

HNRNPU	RPL5	RPL17	RPL27A	RPS11
ILF2	RPL6	RPL18	RPL28	RPS14
ILF3	RPL7	RPL18A	RPL29	RPS17
HNRNPM	RPL7A	RPL21	RPL31	RPS23
NCL	RPL11	RPL23A	RPL32	RPS24
RPL10A	RPL12	RPL24	RPL35A	TOP1
RPL3	RPL13	RPL27	RPS6	RPL14
RPL4	RPL15	RPL30	RPS8	DDX21
RPL23	RPS19	NOP2	HNRNPR	ACTG1
MYBBP1A	RPS25	PCBP1	PRPF8	MYH10
NOP56	RPS27	PSMD1	SF3B2	SPTBN1
RPL35	DDX1	SNRPA	PES1	ARPC3
NOP58	DDX5	SNRPD2	SRRM2	ACTR2
RPL34	HNRNPC	SART1	NAT10	TUBB4B
RPL38	HNRNPD	PRPF3	PARP1	RRBP1
RPS4X	HNRNPL	EFTUD2	SLC25A6	CAPZA1
CAPZB	H1-4	HMGA1	HSPA5	LMNA
DDX17				

Table 3- 4. List of proteins enriched in nucleus IP that are clustered into Nop56p-associated pre-rRNA complex.

Large Drosha complex

DDX1	HNRNPM	NCL	SNRPA	PARP1
DDX5	TAF15	HNRNPR	SFPQ	RPL27
HNRNPU	HNRNPDL	HNRNPC	PHB1	PLEC
ILF2	DDX17	HNRNPD	ARID1A	PRPF8
ILF3	HSPA5	HNRNPL	ZNF326	PHB2
FLNA	ACTR2	MYO5A	RBM14	FN1
VIM				

Table 3- 5. List of proteins enriched in nucleus MPO IP that are clustered into Large Drosha complex.

Amongst other MPO interacting partners, ILF3 was identified to involve in both top relevant candidates lists. Besides, by cooperating with Kyle Moynahan, Homer motif analysis of previous ChIP was performed. It was found that MPO binds to ILF3-like upstream motives (figure 40).

HOMER Motif Analysis

Motif	p-value	Matching protein / motif description
	1e-94	downstream motif 5 (GCW-triplet)
	1e-46	PCBP1 - like upstream motif
	1e-41	SRSF1 - like RRM upstream motif
	1e-36	ILF3 - like upstream motif
	1e-16	ILF3 - like upstream motif

Figure 40. Homer motif analysis showing MPO associates ILF3-like upstream motif in ChIP-seq.

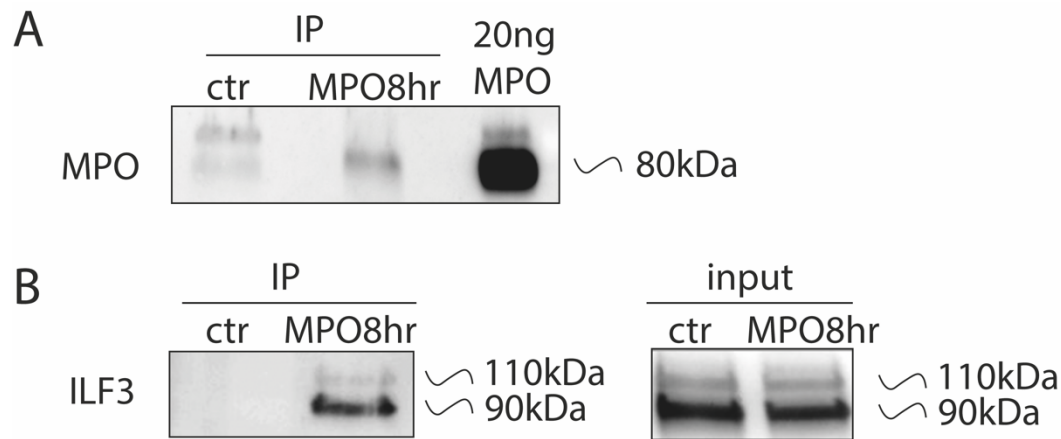


Figure 41. ILF3 co-immunoprecipitated with MPO showed by western blot. (A) MPO detected in MPO immunoprecipitation fraction with correct size. (B) ILF3 detected in MPO immunoprecipitation fraction and input, not in control (MPO antibody) immunoprecipitation.

Further verifications by western blot confirmed MPO and ILF3 co-immunoprecipitation in nucleus IP (Figure 41).

3.3.4 MPO-ILF3 synergistic effects

ILF3 has two isoforms due to alternative splicing and adenylation [134], their molecular weights are 90 and 110 kDa, individually. The 2 isoforms are also termed DRBP76/NF90 and ILF3/NF110. Previous study verified the expression of DRB76 in human Coronary Artery Endothelial cells (hCAECs), while the expression of ILF3 was not detectable [135]. In this session, the expression of ILF3 was determined. The antibody from Abclonal was used which detects both isoforms, verified by western blot in nucleus IP (Figure 41).

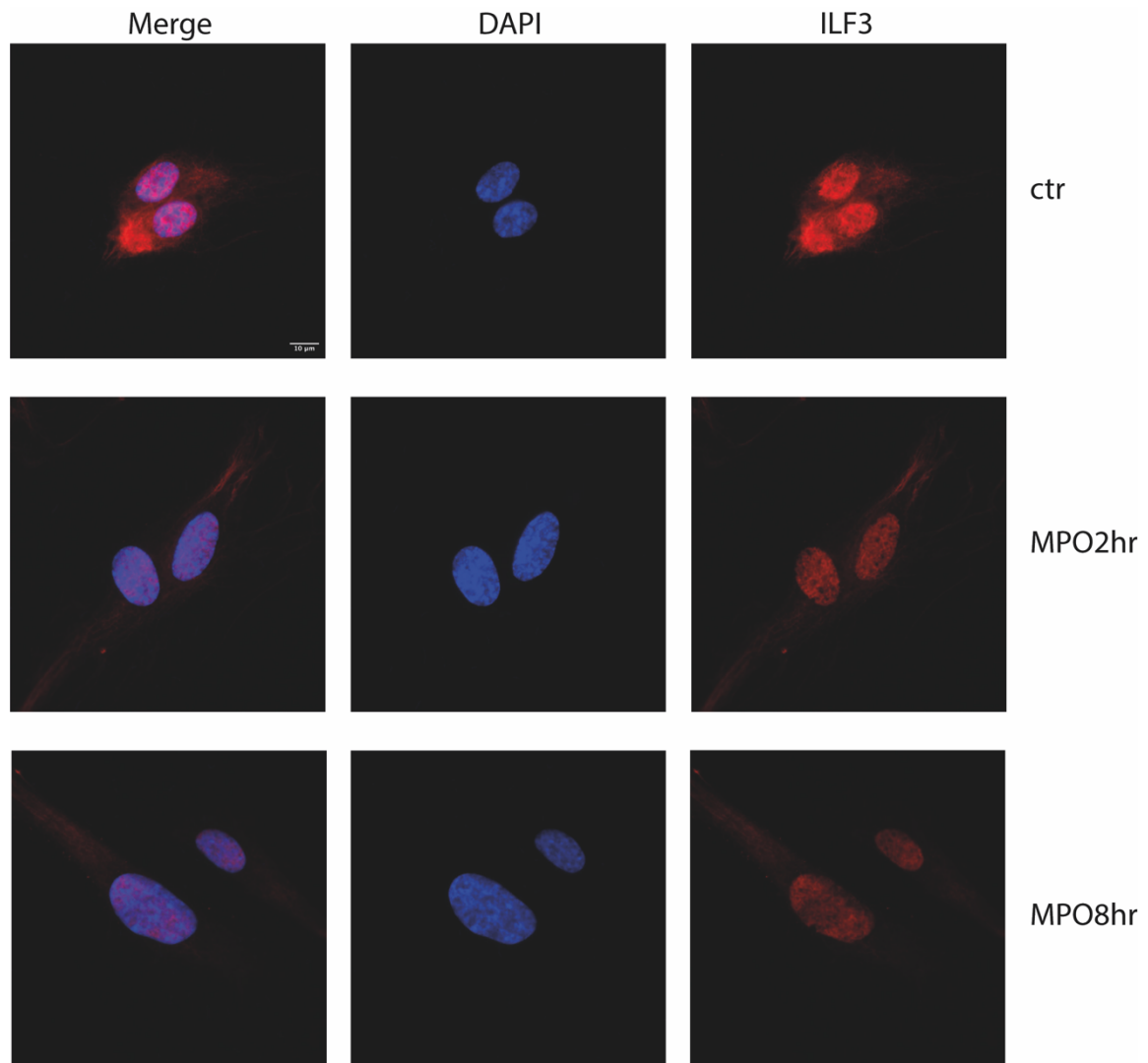
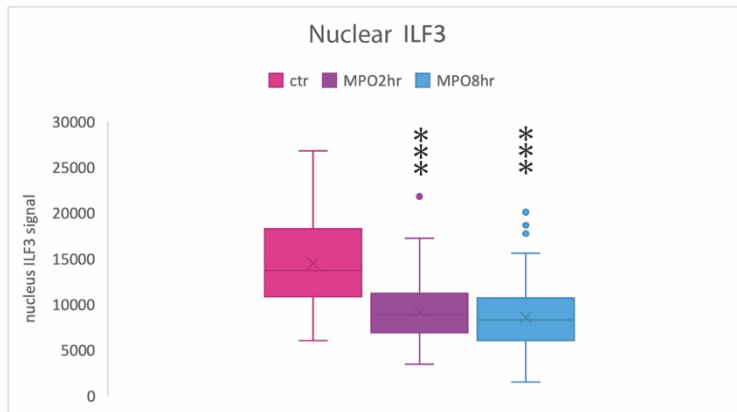


Figure 42. Representative immunofluorescence images of ILF3 staining in HUVECs after 0, 2, and 8 hours of MPO treatment. Scale bar = 10 μ m.

IF staining was performed on HUVECs with 2 or 8 hours MPO treatment to study sub-cellular localization of ILF3, non-treated HUVECs were stained as control. Its distribution in both cytosol and nucleus were detected (Figure 42). By analyzing, a significant decrease of both cytoplasmic and nuclear ILF3 was observed in treated HUVECs (Figure 42, 43 A, B). However, an increased nuclear/cytoplasmic ILF3 ratio was observed, significantly in 2 hours treatment group and non-significantly in 8 hours treatment group compared to control (Figure 43 B).

A



B

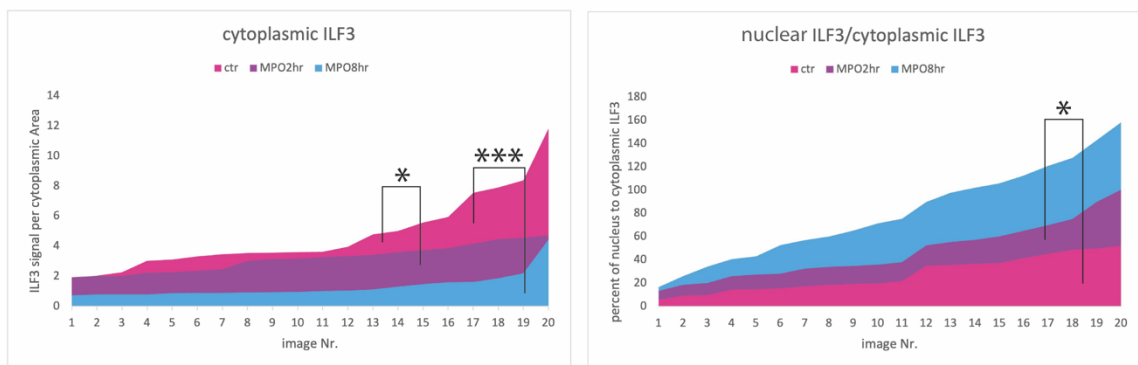


Figure 43. Immunofluorescence analysis revealed a whole cell ILF3 decrease in HUVECs upon MPO treatment and an increase of the ratio of nucleus ILF3. (A) Nucleus ILF3 significantly decreased after both 2 and 8 hours MPO treatments. (B) The Nucleus/Cytoplasmic ILF3 ratio significantly decreased with 2 hours MPO treatment and has a decreasing trend with 8 hours MPO treatment. Y axis marks image number, and in total 20 images containing around 120 cells per treatment were used for analysis.

In previous sessions, MPO dependent endothelial cell remodelling was described, and the MPO-ILF3 co-immunoprecipitation was verified. In the next experiments, the question of whether MPO mediated endothelial cell remodelling is dependent on ILF3 presentation was addressed. A scheme of working can be found in figure 44. In order to identify the ILF3 dependent transcriptional regulation, HUVECs ILF3 knock down using siRNA was performed.

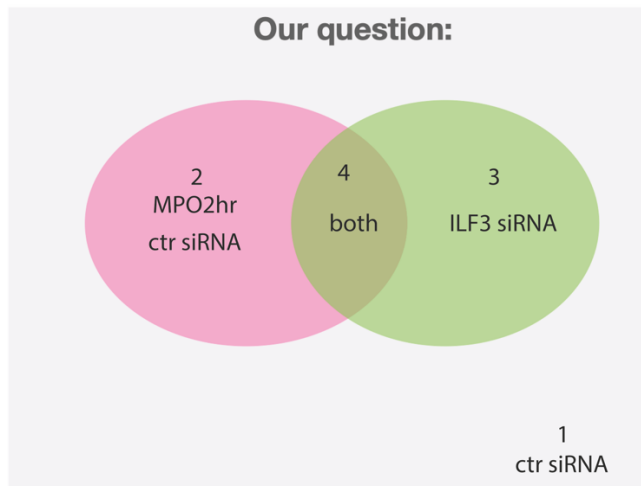


Figure 44. treatments of 3'-end sequencing showed with Venn diagram. Numbers mark for treatments. Eg. ctr siRNA = treatment Nr.1.

Firstly, the knock down rate of HUVECs ILF3 by two individual siRNAs against ILF3 (ILF3 siRNA 1 and 2) were tested using RT-qPCR and western blot. ILF3 expression significantly decreased with both ILF3 siRNAs (Figure 45 A). Thus, siRNA1 was selected randomly for further knock down experiments. After trying siRNA incubations lasting for various periods of time, it was found that ILF3 decreased the most at 72 hours post siRNA treatment. There was 30-40% ILF3 protein remain detected upon knock down (Figure 45 B).

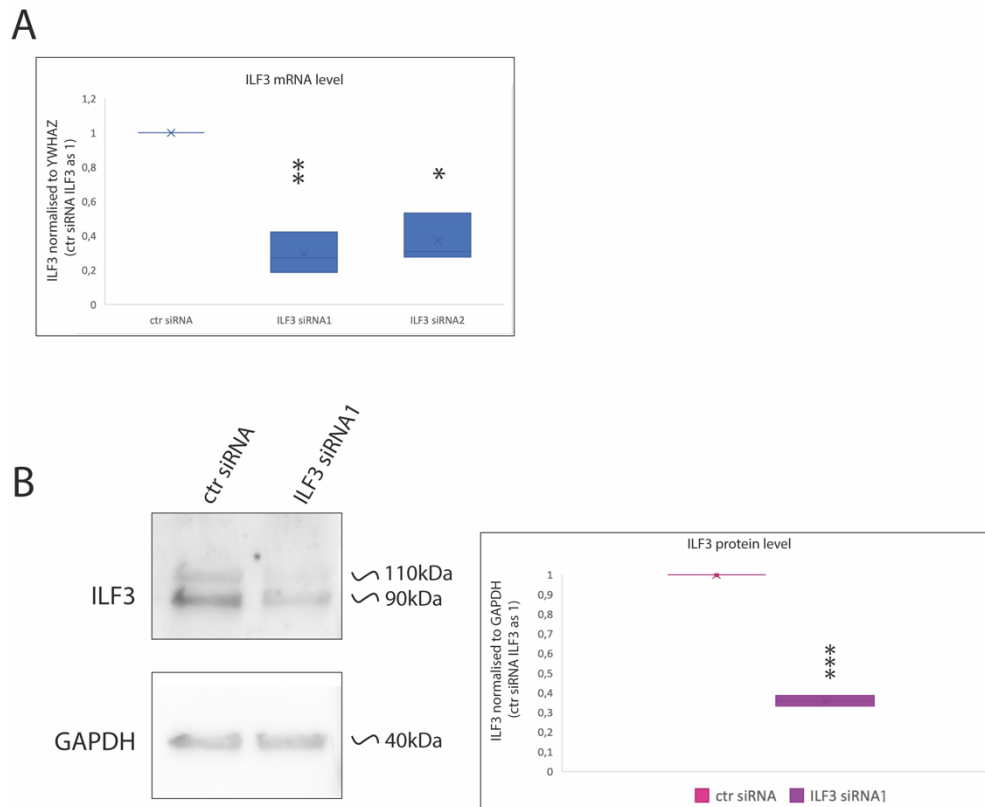


Figure 45. HUVECs ILF3 knock down via siRNA was verified on mRNA level and on protein level. (A) RT-qPCR analysis shows expression level of ILF3 decreased by more than 50% with both ILF3 siRNA1 and ILF3 siRNA2 treatments compared to ctr siRNA treatment, normalized to housekeeping transcript YWHAZ. (B) Western blot analysis shows a 30-40% ILF3 protein remain in HUVECs treated with ILF3 siRNA1 compared to that treated with ctr siRNA.

RNA was isolated from HUVECs with 2 hours MPO treatment under control or ILF3 partial loss backgrounds, and 3'-end RNA sequencing was performed. Analysis revealed slight (less than 0,5-fold) ILF3 dependent VEGFB down regulation counteracted by MPO uptake, and a slight MPO driven VEGFA up regulation counteracted by ILF3 partial loss (Figure 46 A, B). Both genes were found occupied by MPO via ChIP-seq (Figure 46 C).

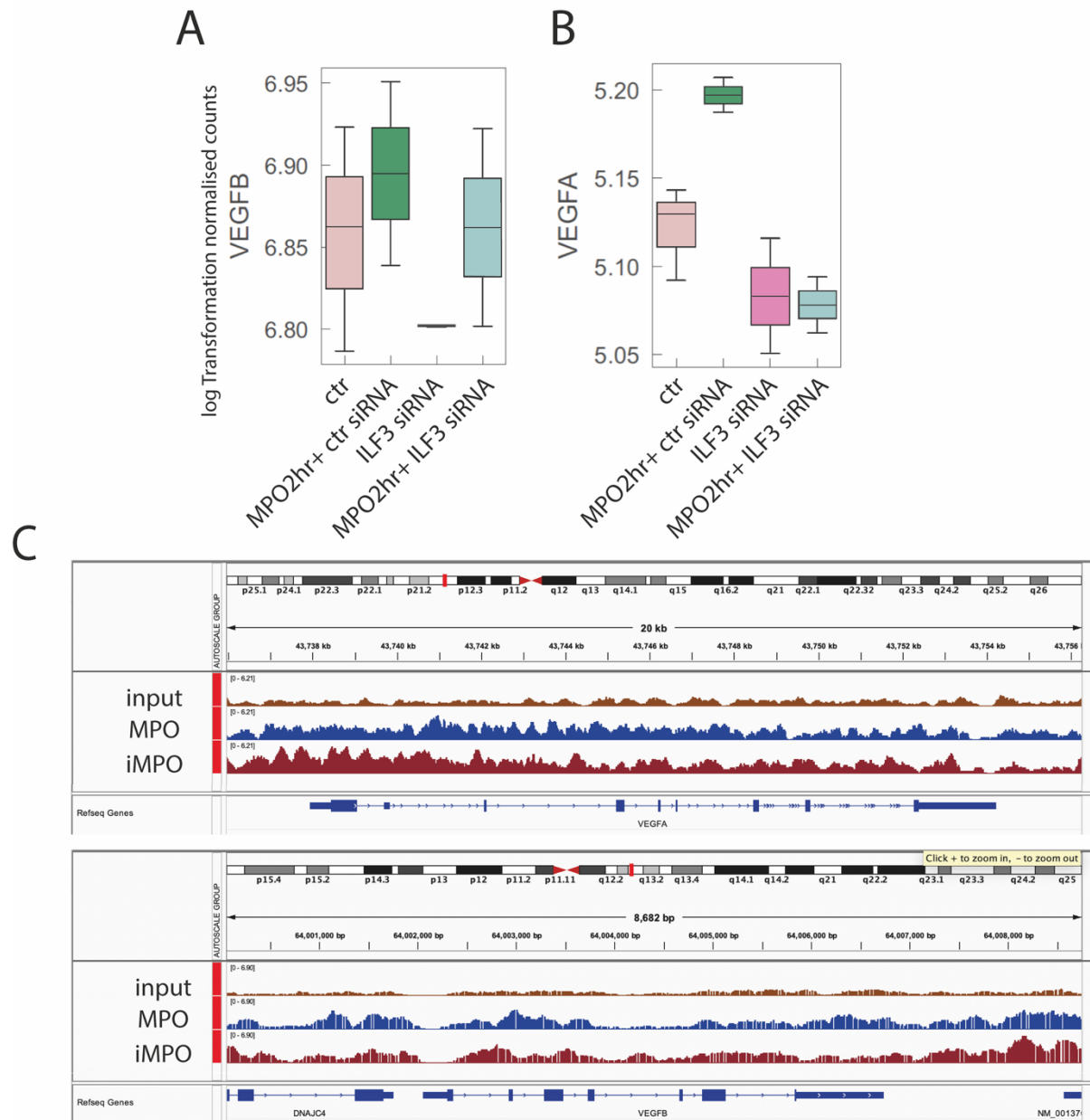


Figure 46. 3'-end RNA sequencing revealed incomplete loss of ILF3 counteracted MPO treatment resulted expressional changes of genes with MPO occupancy. (A) (B) relative expression of VEGFB and VEGFA upon MPO treatment under control and ILF3 knock down backgrounds, normalized to housekeeping transcript YWHAZ. (C) MPO occupancy over VEGFA and VEGFB genes.

Apart from expressional changes of MPO occupied genes, MPO unbound genes were also regulated. Examples like CXCL8 and 1 that are both slightly down regulated upon MPO uptake under ILF3 KD background (Figure 47 A, B).

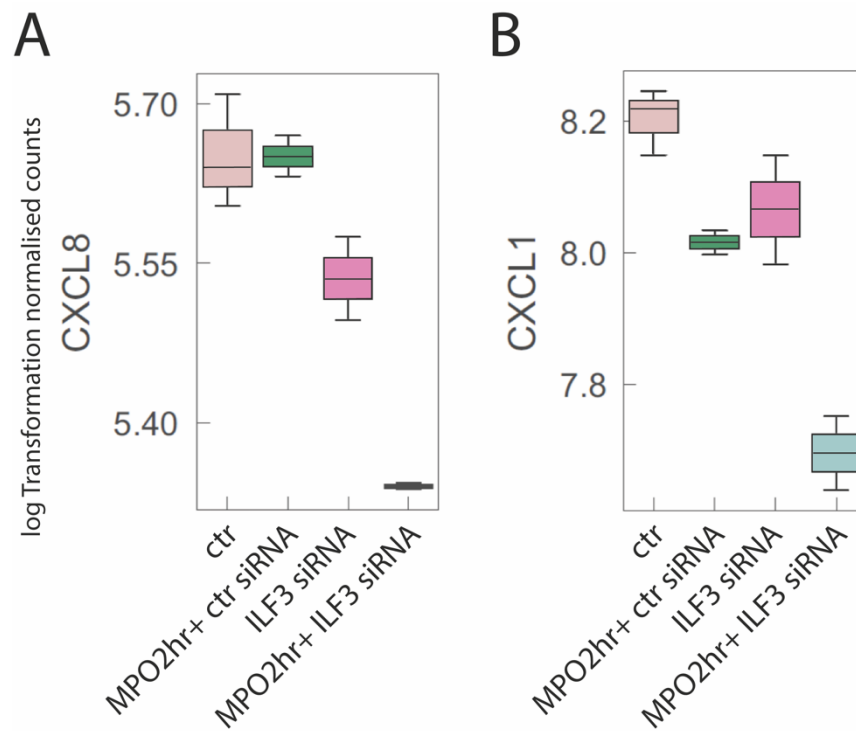


Figure 47. 3'-end RNA sequencing revealed incomplete loss of ILF3 counteracted MPO treatment resulted expressional changes of genes with no MPO occupancy. (A) (B) relative expression of CXCL8 and CXCL1 upon MPO treatment under control and ILF3 knock down backgrounds, normalized to housekeeping transcript YWHAZ.

ILF3 is known to stabilize mRNA in various cancer cell lines and in endothelial cells. In order to further elucidate MPO and ILF3 synergistical effects, RNA stability assay was carried out using transcription inhibitor DRB. The scheme is shown in Figure 48. In the case when mRNA stability is important for MPO-ILF3 driven cell regulation, the regulation should be recapitalized by ILF3 partial loss.

The stability of CXCL8 and PDDPF mRNA were tested under the four treatments and visualized via RT-qPCR. DRB treatments did not result in transcription inhibition in all replica due to technical problems. Compared to MPO treatment alone, a mild trend of mRNA stabilization could be observed upon ILF3 loss for both genes (Figure 49 A, B).

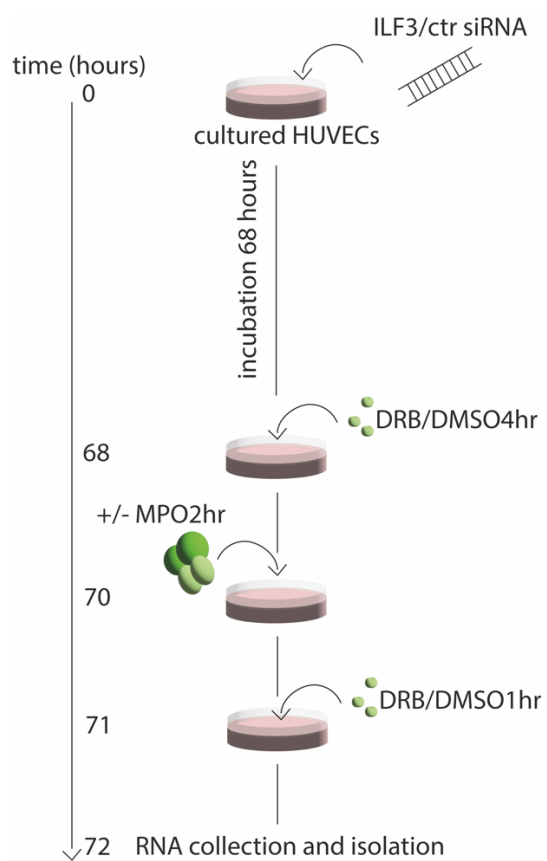
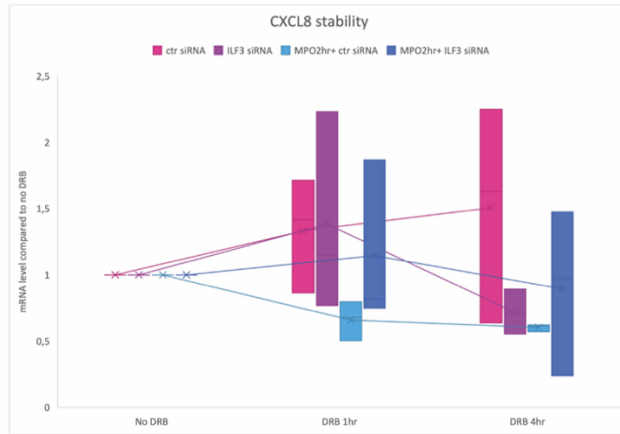


Figure 48. Scheme of RNA stability assay indicating of time points for treatments.

	MPO-/ctr siRNA	MPO-/ILF3 siRNA	MPO+/ctr siRNA	MPO+/ILF3 siRNA
DRB 0	0-ctr	0-KD	0+ctr	0+KD
DRB 1hr	1-ctr	1-KD	1+ctr	1+KD
DRB 4hr	4-ctr	4-KD	4+ctr	4+KD

Table 3- 6 List of abbreviations of treatments in RNA stability assay.

A



B



Figure 49. RNA stability assay verified via RT-qPCR. (A) (B) Relative expression of CXCL8 and PDDPF after 0, 1, or 4 hours DRB treatment upon MPO treatment under control and ILF3 knock down backgrounds, normalized to housekeeping transcript YWHAZ.

Discussion

Since its first description back in 1941, there have been hundreds of studies disclosing the diverse biological functions of MPO [1]. As a member in the peroxidase family, MPO exhibits distinctive features, including exclusive transcytosis capability and DNA binding activity. Accumulating evidence substantiated that the enzyme participate actively in the development of a number of chronic diseases and the focus of MPO studies is shifting from its bactericidal role in the host defence system towards the tissue remodelling roles in inflammation microenvironments. The majority of observations made so far pointed out that the tissue remodelling effect of MPO is dependent on its peroxidase activity and facilitated via MPO and its derivatives modifying proteins and lipids. Here, this research aims at unravelling the peroxidase activity independent endothelial cell-remodelling role of MPO, especially its chromatin structure altering and transcription regulating potential.

3.1 Determining MPO subcellular localization upon its translocation into HUVECs cytosol

Studies by Baldus et al. revealed that MPO and its derivatives give production to NO₂Tyr, meanwhile deprive of endothelial NO bioavailability, as a result disrupting endothelial cell ECM, causing endothelium dysfunction and vasomotor impairment in vascular inflammation [84], [98], [123]. Moreover, the same study discovered MPO transcytosis into Bovine Aortic Endothelial Cells (BAECs) *in vitro*, and showed that this process is dependent on its interacting with heparin-containing glycosaminoglycans. It was observed that MPO co-localizes with Tubulin in cytosol with either 2 minutes or 2 hours MPO treatment [123]. Based on previously gained knowledges, experiments were designed to start from verifying MPO transcytosis into HUVECs *in vitro*. In order to investigate the enzymatic activity independent roles of this protein, inactivation of MPO via H₂O₂ saturation was carried out and iMPO was generated (Figure 9). IF images taken with confocal microscopy demonstrated that MPO localized both in HUVECs cytosol and in nucleus (Figure 10, 11). Limited by material, iMPO was not detectable with the antibody against MPO manufactured by Invitrogen in IF, leaving its subcellular localization unsubstantiated. Observation with IF was further verified by chromatin fractionation coupled with western blot. The result showed MPO association with HUVECs chromatin in an enzymatic activity independent manner (Figure 12, 13). Additionally, comparison between 2 and 8 hours MPO treatments on HUVECs suggested a time line of the subcellular localization of the enzyme. Both IF and chromatin fractionation data showed a peak of MPO gathering in the nucleus at 2 hours after treatment, and a gradual leave within 8 hours

after treatment. Interestingly, analysis to total cellular MPO revealed equal otherwise increased amounts of total MPO with 2-, or 8-hours treatment, indicating that the enzyme stays in rather than leaves the cell after exiting from the nucleus (Figure 10, 14).

MPO is a positively charged protein consisting with high percentage of positively charged amino acids, particularly arginine [136]. Its DNA binding activity was reported earliest in the 1980s, while the binding specificity has not been fully understood [137]. One known significance of MPO-DNA association can be found in the host defense system, where MPO associates with neutrophil chromatin together with NE to facilitate the formation of NETs [138]. Following the investigation of MPO subcellular localization, its association with DNA was studied. With the help of EMSA, the enzymatical activity independent association of MPO with reconstituted nucleosome in a dose-dependent manner was determined, and the binding preference to nucleosome with long linker DNA was realized (Figure 15-18). Although the nucleosome association activity appeared to be not requiring MPO enzymatic activity, iMPO exhibited a weaker association compared to the holoenzyme. Furthermore, and for the first time on MPO research history, the occupied regions of both MPO and iMPO on HUVECs genome was mapped with ChIP-seq. Annotations to ChIP-seq revealed that both MPO and iMPO bound preferentially to TSSs (Figure 19, 20), and their occupied regions showed high consistency (Figure 21). Observation to the range of the peaks indicated a transient nature of MPO- and iMPO-DNA association (Figure 19, 20), unlike CTCF or other transcription factors which bind to DNA in a more stable and tighter way. This data provided a landscape view of MPO-DNA association and a data source for further researches.

3.2 HUVECs chromatin conformational changes following MPO uptake

Physical associations of MPO and HUVECs chromatin potentially will lead to chromatin conformational changes. Moreover, the nuclear accumulation of MPO at 2 hours post treatment seems to consume much nuclear space and may further limit chromatin dynamic within the nucleus. In order to appreciate the global chromatin dynamics upon MPO transcytosis, MNase assay was performed and visualized as DNA ladders on agarose gels. Assessment to nucleosomes of different hierarchies generated by 2 min MNase treatment revealed that both 2- and 8-hours MPO treatments resulted in more condensed chromosomes, while 2 hours treatment exhibited stronger chromosomal condensing effect reflected by more nucleosomes with higher hierarchies and less mononucleosomes (Figure 22). The condensation of chromosomes upon MPO transcytosis was best reflected in the 2 min MNase treatment

experiment, and can be observed in the 4 min MNase treatment experiment with a less extent (Figure 23 A). The chromosome condensation effect of 8 hours MPO treatment was not detectable in the 6 min MNase treatment experiment (Figure 23 B), supporting the conclusion that the condensation is due to MPO gathering in the HUVECs nucleus. To gain a closer view on the local chromatin dynamics, ATAC-seq was carried out with same MPO treatments. Previous determined MPO binding sites by ChIP-seq provided a chance for alignment. Analysis to ATAC-seq revealed a local relaxing surrounding MPO occupied regions upon both 2- and 8-hours MPO treatments (Figure 24). Treatment for 8 hours resulted in chromatin de-condensation in a larger scale (Figure 24 A).

3.3 MPO modulated HUVECs transcriptional regulations

Chromatin structural changes observed upon MPO treatments suggest for transcriptional alterations. To explore the transcriptomic regulation facilitated by MPO, RNA was isolated from MPO treated HUVECs, and RNA-seq was conducted. Comparison of numbers of regulated genes with 2- and 8-hours MPO treatments manifestly showed that more genes were regulated upon 2 hours treatment (Figure 25), consisting with IF and chromatin fractionation result that showed MPO gathering in nucleus at 2 hours after treatment. The same can be observed with volcano plot, where it is also showed that the gene expression upon 2 hours MPO treatment was altered to a larger extent (Figure 26). GO term enrichment revealed a trend of endMT over MPO uptake in HUVECs driven by transcription regulations. GO terms enriched with 2 hours treatment revealed that the positively regulated genes best associate with ossification, cellular response to growth factor stimulation, and muscle structure development (Figure 27), while 8 hours treatment positively regulated genes best associate with NOTCH1 regulation of endothelial cell calcification, bone morphogenic protein (BMP) signalling and regulation, and epithelial cell differentiation (Figure 28). Both treatments positively regulated genes involved in endMT. In addition, a dysfunctional mutant MPO (mMPO) was applied in parallel to understand whether the transcription remodelling is dependent on enzymatic activity. However, in later studies it was found that the actual content of mMPO is not clear (extra protein bands were detected with MPO antibody, data not shown), thus the Gene ontology (GO) term enrichment for this treatment was not shown, but the regulations were shown in bar plots for reference. The expressional regulation of selective endMT associated genes were shown on bar plot (Figure 29). Down regulation of endothelial marker CD34 was observed upon both MPO treatments, while ICAM1 upregulation was shown. Despite itself as an adhesion

molecule expressed in endothelial cells, ICAM1 is reported to express actively when endothelial cells are activated or stimulated with proinflammatory factors [139]. To better clarify the transcriptional remodelling trend observed in RNA-seq, RT-qPCR and IF was performed using HUVECs with the same MPO treatments. In line with the RNA-seq result, downregulation of CD 34 and upregulation of ICAM1 upon MPO treatment was verified with both methods (Figure 31-33). CD34 expression in cultured HUVECs was found to decrease as cells passages [140] and indicates of a de-differentiating trend towards endothelial progenitor[141]. In general, it is considered as an endothelial cell marker. However, the explanation to different CD34 expression can indicate of cell condition as well, which may explain its upregulation in IF using cells with 8 hours MPO treatment (Figure 32, 33). On contrary, the upregulation of the mesenchymal cell marker ACTA2 was observed with qPCR alone and CD45 with IF alone (Figure 31-33). A general down regulation of endothelial markers whilst up regulation of mesenchymal, and endMT markers was verified with multiple methods (Figure 29 and 31-33). Previous study revealed an increase of interleukins and chemokines in HUVECs upon MPO treatment [142]. By RNA-seq analysis, the generally altered SASP factors was shown without further verifications (Figure 30).

An early study on euthanized rat revealed that angiogenesis is initiated by endothelial cell migration [143], and wound healing assay has been regarded as a robust method for endothelial cell remodelling studies. Morphological observation made on MPO treated cells revealed that their edges became more rigid, their shapes stretched, and their organization lost (Figure 34). Wound healing assay comparing control and treated cells revealed a faster healing exhibited by the treated cells 2 and 4 hours after the wound was made (Figure 35).

3.4 MPO and ILF3 synergistically mediated HUVECs remodelling

To further dissect the mechanism of MPO mediated endothelial cell remodelling, the binding partners in HUVECs cytosol and nucleus were hunted for, separately. Mass spectrometry identified in total 21 MPO interacting candidates in whole cell, half in the cytosol and half in the nucleus (Figure 36 and 37, table 3-2). Among these candidates, 8 are involved in VEGFA-VEGFR2 pathway, one of the major pathways in angiogenesis (Figure 38) [144]. Mass spectrometry of nucleus isolated from MPO treated HUVECs identified 141 MPO-interacting candidates (Figure 39). The difference in number reflects previous subcellular MPO localization IF which showed that MPO gathers in the nucleus soon after treatment start. Annotations to nucleus candidates identified them belonging to 3 major clusters: Nop56p-

associated pre rRNA complex, large droscha complex, and mRNA processing (Figure 39). Taken together, the MPO interactions in nucleus is highly possible to relate to regulations of mRNA stability. Amongst the other candidates, ILF3 gained the most attention for its presents in ChIP analysis (Figure 40, analyzed by Kyle Moynahan). The MPO-ILF3 interaction in IP was verified via western blot (Figure 41).

ILF3 is known to locate in both endothelial cytosol and nucleus, and possess diverse and cell type specific biological functions that have not been fully unraveled. On one hand its mRNA stabilizing role in cytosol was identified in endothelial cells, on the other hand its association with DNA and working as a transcription factor was defined in erythroleukemia cells [135], [145]. To explore the potential ILF3 dynamics upon MPO transcytosis in HUVECs, IF using ILF3 antibody was performed. Both cytosolic and nucleus ILF3 were detected in HUVECs with or without MPO treatment (Figure 42, 43). Interestingly, cytosolic ILF3 decreases upon MPO treatment while the nucleus ILF3 increases, indicating of potential translocation into nucleus (Figure 42, 43).

ILF3 KD was carried out to answer the question of whether MPO mediated transcriptional remodelling is ILF3 dependent (Figure 44 and 45). By performing 3'-end sequencing using HUVECs treated with MPO under control or ILF3 deficient background, it was realized that the expressional alterations upon MPO treatment in VEGFA and VEGFB, which were both occupied by MPO and altered, were both counteracted by ILF3 partial loss (Figure 46). Additionally, some MPO unoccupied genes were also regulated with MPO treatment upon ILF3 partial loss (Figure 47). In general, the transcriptional regulations identified in 3'-end sequencing were mild (less than one cycle), indicating that the synergistical regulation is weak.

In order to verify if ILF3 dependent RNA stability maintenance was disturbed in MPO treated cells, transcription inhibition with DRB was carried out in HUVECs with MPO treatment and ILF3 KD (Figure 48, Table 3-6). The stabilization of CXCL8 and PDDPF mRNAs were found disturbed with both MPO treatments and ILF3 KD in 4hr DRB treatments, compared to control (Figure 49 A and B). However, it can be deduced comparing DRB0 and DRB 4hr groups that DRB treatment did not robustly result in transcription inhibition as supposed. Thus, the RNA stability assay supported but imperfectly the hypothesis that mRNA stability is disturbed upon MPO-ILF3 interaction.

3.5 Summary

In this study, MPO transcytosis into cytosol and translocation into nucleus was verified in HUVECs with 2 individual methods, enhancing the conclusions made by Baldus et al. in 2001 using BCAECs and further showed that both processes are independent of MPO enzymatic activity. The association of MPO with nucleosome was visualized and the preference association to linker DNA was realized. Again, the enzymatic activity was not required for the association, with an association aiding potential. The specific MPO association to endothelial cell genome was proofed, the MPO occupied regions were determined, and MPO association with TSS independent of enzymatic activity was revealed.

Innovatively, the impact of MPO transcytosis on endothelial cell chromosome was studied *in vitro*. An overall chromosomal condensing effect was observed upon MPO treatment, and a local chromatin relaxing effect surrounding MPO occupied regions was found.

The transcriptional remodelling of HUVECs upon MPO treatment was examined systematically. A transcriptional remodelling trend of endMT was identified and verified with multiple methods.

In the end, MPO interacting partners in endothelial cells were assessed, and a promising interactor was verified and investigated. Finally, we propose that upon transcytosis, MPO interact with cytosolic proteins and hijack ILF3 into the nucleus on its way. In the nucleus, highly positively charged MPO was attracted by negatively DNA. This attraction facilitated their association and made space for MPO-ILF3 synergistically mediated endothelial remodelling. The remodelled endothelial cells were partially dysfunctional with an increased mobility and increased pro-inflammatory factor expression. A schematic illustration of hypothesis can be found in Figure 50.

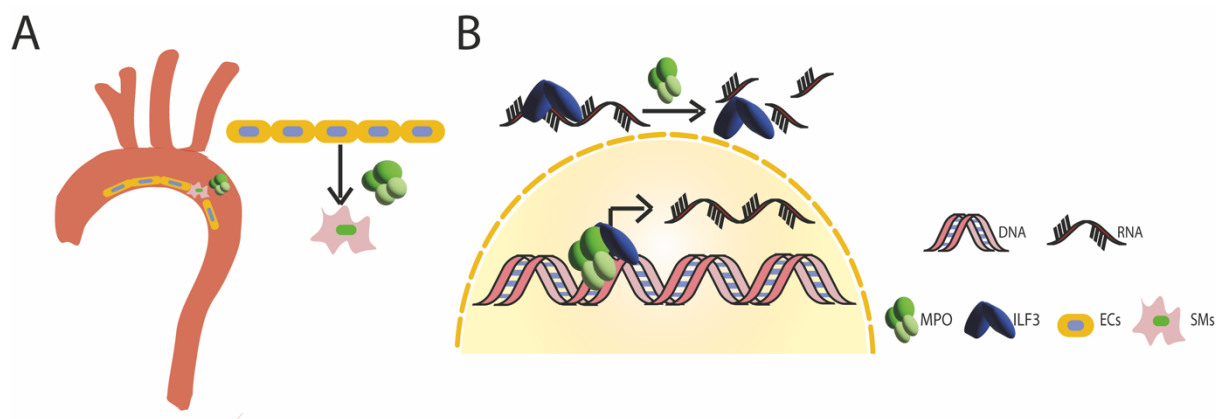


Figure 50. Hypothesis of MPO regulates HUVECs transcriptome synergistically with ILF3. (A) Illustration shows MPO being released into human aorta where it is attracted by negatively charged endothelial cell surface and remodelling endothelial cells. (B) Illustrations shows MPO hijacking ILF3 into endothelial nucleus where it occupies specific genome region and regulates gene expression together with ILF3.

3.5 Limitations and outlooks

Firstly, the experiments using iMPO can be improved to better separate the enzymatic activity from other features of MPO. At the beginning of the project, Calbiochem MPO antibody that was later found not suitable for IF was used for both IF and chromatin fractionation, resulting in completely different conclusions that does not agree with other observations. This could be avoided by closer checking the unusual fluorescence signal. Future IFs with suitable MPO antibodies can be planned to better verify iMPO translocation into HUVECs nucleus. The EMSA experiment can be improved by using nucleosomes collected in one isolation to avoid degradation. In the shown data, nucleosomes used for iMPO EMSA was prepared separately and earlier than the nucleosomes used in MPO EMSA, adding up to difficulties when comparing the results side by side. The result of the wound healing assay added up evidence to the MPO induced HUVECs endMT. However, limited by time, wound healing assay using iMPO was not performed to verify if the observed migration acceleration was peroxidase activity dependent.

Secondly, the described study on endothelial cell remodelling role of MPO was conducted completely in vitro, leaving a margin for future in vivo studies. For instance, it is worth to study if additional MPO or iMPO supply to MPO KO cardiology diseased mice would cause local endothelium dysregulation, and if the proposed MPO-dependent regulating hypothesis compensates for the cause.

Thirdly and due to a limit of time and material, whether the trend of endMT upon MPO treatment is dependent on ILF3 was not fully verified. To date, the ILF3 occupied regions on HUVECs genome has not been determined. It will be helpful to align ILF3 occupied regions to that of MPO, and annotate for common occupied regions. Potentially, the common occupied regions annotation can provide a hint on the gene set that are regulated by both proteins synergistically. Current result gained with 3'-end sequencing revealed that the synergistical MPO-ILF3 mediated endothelial remodelling is mild. Further wound-healing assay using HUVECs with MPO treatment under ILF3 KD background, and RNA-stability assay with shorter incubation time or with another transcription inhibitor can be carried out for more solid conclusions.

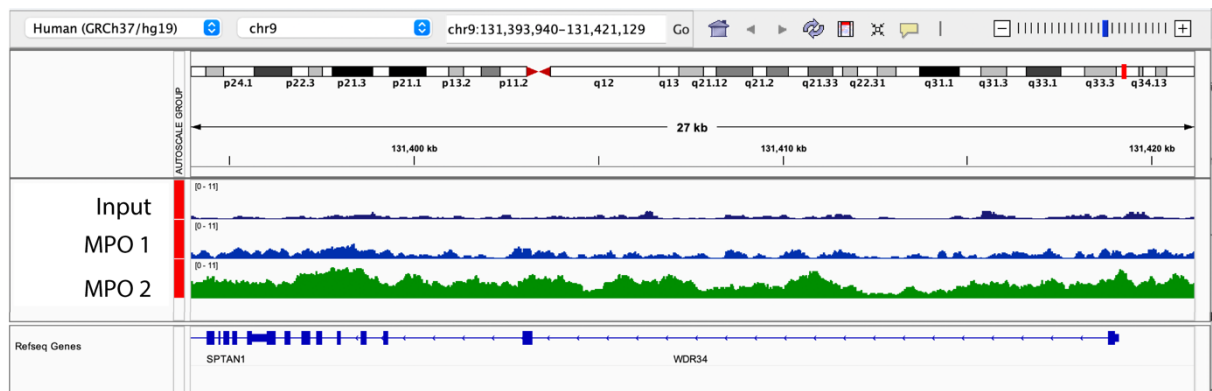


Figure 51. representative MPO binding peaks from ChIP, including two individual bio-replica with MPO-treated HUVECs (MPO 1 and MPO 2) and one with non-treated HUVECs (Input).

Finally, the DNA binding property of MPO remains unraveled. Although the observed MPO-DNA association is comparably weak and has a transient feature, the target sequences showed consistency between biological replica (Figure 51). Thus, the existence of a DNA binding motif is highly possible. Future experiments can be planned to locate the potential DNA binding domain of MPO by introducing incomplete forms of the protein followed by DNA-binding activity test via EMSA.

The DNA binding strategies and specificities of DNA binding proteins (DBPs) varies and thus enables various biological regulations. Some DBPs have well determined DNA binding properties such as zinc finger, and bind to specific sequences [146], [147]. Some DBPs are able to recognize and associate to specific DNA structures such as supercoiling, examples are DNA

topoisomerases [148]. Besides, there are DBPs or other molecules that bind to DNA with weak affinity and little realized specificities. However, the consequences of such bindings can be pronounced. In a study simulating *in vitro* DBPs binding to DNAs, researchers found that clustering of proteins and compaction of DNAs was driven by an entropic bridging-force that [149]. Such DNA compaction can be found in this research reflected by the MPO dependent local chromatin de-condensation. Overall, this study substantiated the specificity of MPO-DNA association which previously believed to linked to protein size, shape, and distribution of positively charged amino acids (AAs), and opens up a possibility to study such DBPs with weak DNA binding affinity, but have chromatin conformation altering and transcriptional remodelling potentials when teaming up with other DBPs.

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Curriculum Vitae

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Education History

Doctoral Education |2022| University of Cologne

- Starting date in the DFG-funded RTG (Number GRK2407): 15-12-2018, Ending date: 14-06-22
- Major: Cardiology and Chromosome Biology
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Master of Science | 2018 | University of Cologne

- Major: Biological Sciences
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Bachelor | 2014 | Capital Normal University of China

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Secondary Education | 2010 | Senior High School Attached to Beijing Technological University

- Majoring in Sciences

Posters and Talks

Poster | CaVaD Symposium | 02-06-2021

- The Cardiovascular Disease Symposium “CaVaD” is an event organized by doctoral researchers from the Heinrich Heine University Düsseldorf. It aims to provide a platform for young scientists from all around the world who are involved in cardiovascular research.

Talk | IPMM Symposium | 16-06-2021

- The “IPMM Days” is an annual internal symposium for IPMM (Interdisciplinary Program Molecular Medicine at the University of Cologne) students organized by IPMM students. It offers IPMM students the opportunity to present, discuss and develop their work in inspirational scientific talk and posters sessions in the area of molecular medicine.

Poster | IPMM Symposium | 22-06-2022

- IPMM symposium 2022 in the session: Cardiovascular Research.

Trainings and Workshops

- Ethics in Science and Good Scientific Practice
- Getting funded: Writing an individual fellowship
- Statistics for Experimental Life Scientists

IT Skills and Softwares

- Utilization of Adobe Photoshop, Illustrator, imageJ for data analysis and display.

Memberships

- Member of Mito club Cologne
- Member of Epi club Cologne
- Member of the Deutsche Gesellschaft für Kardiologie- Herz- und Kreislaufforschung.

Volunteer Work and Social Contribution

Volunteer Chinese-English interpreter in the event and talk” Experience Sharing of Medical Treatment in ICU of 2019 Corona Virus in China” given by MD. Jingxiao Zhang of The second hospital of Jilin University (ICU), team leader of critical illness in the Zhongfaxincheng campus of Tongji hospital to fight against the COVID-19 outbreak in Wuhan, and held by We Doctor team, China.

Publications

- Kargapolova Y, Geißen S, Zheng R, Baldus S, Winkels H, Adam M. The Enzymatic and Non-Enzymatic Function of Myeloperoxidase (MPO) in Inflammatory Communication. *Antioxidants*. 2021 Apr 5;10(4):562. Available from: <http://dx.doi.org/10.3390/antiox10040562>
- Zheng R, Moynahan K, Georgomanolis T, Pavlenko E, Geissen S, Mizi A, et al. Remodelling of the endothelial cell transcriptional program via paracrine and DNA-binding activities of MPO. *iScience*. 2024 Jan;108898. Available from: <http://dx.doi.org/10.1016/j.isci.2024.108898>

Declarations

Ich versichere, dass ich die von mir vorgelegte Dissertation selbstständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit - einschließlich Tabellen, Karten und Abbildungen -, die anderen Werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie - abgesehen von unten angegebenen Teilpublikationen -noch nicht veröffentlicht worden ist sowie, dass ich eine solche Veröffentlichung vor Abschluss des Promotionsverfahrens nicht vornehmen werde. Die Bestimmungen dieser Promotionsordnung sind mir bekannt. Die von mir vorgelegte Dissertation ist von Prof. Stephan Baldus betreut worden.

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