Influence of mitogen-activated protein kinase (MAPK) signaling on mast cell differentiation and histone acetylation modifiers

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**Abstract:**

**Introduction:** Mechanisms directing mast cell differentiation are incompletely defined. Epigenetic modifications by promoter methylation have been identified as key modulators of locus-specific chromatin accessibility during mast cell differentiation, but the role of histone acetylation (HA) has not been explored. Resultant changes in gene accessibility support a trajectory of lineage-specific gene expression as unique cell types mature from pluripotent progenitor hematopoietic stem cells. The MAPK signaling pathway contributes to regulating differentiation and proliferation and directly influences HA modifiers in a host of contexts. We aim to measure how the MAPK signaling pathway influences histone modifiers during mast cell differentiation *in vitro* toward the identification of potential key contributors.

**Methods:** Mast cell differentiation was initiated from cultures of isolated murine bone marrow and samples were collected throughout differentiation. Quantitative polymerase chain reaction (qPCR) measurement of the RNA level, western blotting assessment of the protein level, and flow cytometry assessment of cell-surface receptor phenotype were conducted for HDACs, HATs and key mast cell-specific markers and transcription factors.

**Results:** The MAPK signaling pathway significantly affected the expression of histone acetyltransferases, histone deacetylases and histone H3 post-translational modification. The inhibition of ERK (SCH772984) differently influenced the differentiation of mast cells through increased HDAC4 and increased PCAF gene expression. This was accompanied with changes in Histone H3 acetylation (K9) and phosphorylation (S10) and increased FceRIα surface receptor presence with ERK inhibition. The inhibition of p38 (Losmapimod) showed a reduction in mast cell differentiation through decreased mast
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cell specific transcription factors and enzymes, MITF, Tpsb2, Cpa3 and Cma1, while decreasing FccR1α receptor presence on the cell surface. The inhibition of JNK (JNK-IN-8) had no effect on mast cell differentiation.

**Conclusion:** This work demonstrates the differential and dynamic importance of the ERK and p38 MAPK signaling pathways in mast cell differentiation and suggests links between the mast cell lineage program and epigenetic modifications via HA. Activation of the p38 MAPK signaling pathway is shown to drive mast cell differentiation, while ERK activation hinders HSC mast cell differentiation.
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1. Introduction:
1.i. Gene expression, Epigenetics & Histone Acetylation
   Every living organism’s genome is composed of long deoxyribonucleic acid (DNA) sequences providing a complete set of hereditary information. The human genome contains large regions of DNA that do not code for proteins, consisting of introns and DNA of unknown function (Figure 1). The other regions, known as exons, are transcribed to messenger ribonucleic acid (mRNA) (1). The double stranded DNA is separated into sense and antisense strands. Pre-mRNA is formed when RNA polymerase enzymes are recruited to single strands of DNA. The RNA polymerase enzymes then bind to the sense strand to allow a complementary strand to form (Figure 1). This new complementary strand is released to create the pre-mRNA. The sequence of the individual bases is one very important factor that helps contribute to the fate and function of the cell (1).

   The 3-dimensional structure of the protein is also involved in the cell fate regulation. After transcription of DNA to RNA, mRNA undergoes translation with the help of transfer RNA (tRNA) (1). Translation begins within the cytoplasmic ribosomes so that the tRNA and mRNA encounter each other. Three initiation factors (IF1, IF2, and IF3) bind to the small subunit of the ribosome (1). The binding of the preinitiation complex and tRNA near the AUG start codon follows this to complete the formation of the initiation complex. This is accompanied with the release and linearization of amino acids to form the polypeptide chain (1). This 3D structure of amino acids determines the fate of the protein and is one such mechanism that is important in the regulation of cellular function.
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Figure 1: Transcription components present on DNA. Upstream promoter regions specify to other molecules where transcription is to begin. Introns are removed, while exons remain for RNA transcription. Core promoter primers span +1 bp, while non-promoter primers are downstream from transcription start site. Figure created by DD.

The 3D structure of DNA is similarly important to the base pair sequence in the regulation of transcription (2). The DNA in eukaryotic cells is tightly bound to proteins known as histones, which package the DNA in the cell nucleus. These complexes termed chromatin, typically contain twice as much protein to DNA (3). There are five types of histones: H1, H2A, H2B, H3, and H4. Histones H2A, H2B, H3, and H4 exist in duplicate forming an octamer complex that associates with DNA (Figure 2). Histones, specifically histone tails, are modified through substances such as acetyl groups, microRNAs, and enzymes altering chromatin structure and influencing gene expression (3). H1 is known as the linker histone, which organizes the nucleosomes (DNA and histones) into a more compacted chromatin fiber (4). Chromatin exists in two conformational states: as euchromatin or heterochromatin. Euchromatin is associated with chromatin remodeling through an open conformation, leading to an increase in gene expression and transcription at its specific gene loci (5). Heterochromatin is the reverse state, associated with a closed chromatin conformation and is related with inactive gene expression. Heterochromatin is
highly condensed, blocking transcription factors from binding to their respective promoter regions thus preventing transcription (5).

**Figure 2:** Chromatin conformation and organization of histones and DNA.
Chromatin contains histones and DNA tightly bound within the nucleus of a cell. Tightly folded chromatin is associated with non-expression. Nucleosomes are a subunit of chromatin and are the structural unit for genomic organization. Ac = acetylated lysine residues. Figure created by DD.

Epigenetics is the regulation of gene expression by post-translational factors other than the individual DNA sequence. These changes either turn on genes or turn off genes depending on the epigenetic process implemented (3). Functionally, epigenetic modification dictates gene activity based on chromatin state or structure (Figure 2). The interactions between the histones position the positively charged R groups of lysine so that it interacts with the negatively charged DNA backbone phosphates (6). When the positive R group is neutralized by acetylation, the histones and DNA binding force is reduced. The histone core octamer is then able to bind more tightly around itself allowing the DNA to be opened and accessible between the nucleosomes, resulting in an upregulation of transcription (Figure 2) (4). When this neutralization is prevented by deacetylation, the binding force existing between the histones and DNA is increased allowing the chromatin core to release its tight structure, decreasing transcription.

Transcription factors and RNA polymerase II (Pol II) can bind more readily to the DNA when in an open chromatin conformation (7).
Histone modification at acetyl sites is influenced by the presence or absence of histone acetyltransferases (HATs) and histone deacetylases (HDACs). HATs enzymatically add an acetyl group (COCH$_3$) from acetyl coenzyme A to the lysine residues present on the histone tails (Figure 3) (8). The process of histone acetylation is involved in the regulation of many cellular processes such as transcription, cell cycle progression, differentiation and DNA replication (9). Specific HATs have been shown as the critical enzymes regulating the overall chromatin conformation through histone H3 and H4 acetylation, with histone H3 being the preferential target for both HAT and HDACs (9). HDACs hydrolytically remove the acetyl groups from the histone lysine residues (Figure 3) with imbalances associated with tumorigenesis and cancer progression (10).
HDACs are further divided into four classes, separated based on sequence similarities. In humans, there are 18 HDACs enzymes that are separated into four classes: Class I Rpd3-like proteins (HDAC1-3, HDAC8), Class II Hda-like proteins (Class IIa HDAC4, 5, 7, and 9; Class IIb HDAC6 and 10), Class III Sir2-like proteins (SIRT1-7) and Class IV protein (HDAC11) (10,11). Class I and II HDACs are related to yeast Hos protein, while class III HDACs have sequence similarity to yeast Sir2 protein. Class IV HDACs sequence is similar to class I and II proteins, with the difference due to NAD⁺-dependency within the deacetylase domain (10).

Class I HDACs are ubiquitously expressed and located predominantly in the nucleus. HDAC1 and HDAC2 are generally found bound together as a repressive complex with sin3, NuRD, and CoREST, while HDAC3 complexes with N-CoR-SMRT (11). Class IIa HDACs are found predominantly within the cytoplasm and contains conserved binding sites for the transcription factor myocyte enhancer factor 2 (MEF2) and chaperone protein 14-3-3 (11). Class IIb HDAC6 is found in the cytoplasm and targets cytoskeletal proteins. The function of class IV HDAC11 is not well known, but does contain small N and C-terminal extensions (10,11). Therefore, the four HDAC classes have conserved mechanisms of action in their histone deacetylase activity, while having similar targets of lysine acetyl groups to regulate the rate of transcription and the conformation of chromatin.

The other members that regulate histone acetylation are histone acetyltransferases, (HATs) which facilitates the addition of an acetyl group to the lysine residues present on histone tails. The HAT enzymes can be separated into four different families that are based on sequence homology within their HAT domain. The first family is termed CBP/p300, with one such member being CBP (CREB-binding protein) which acts as a
transcriptional adaptor for the recruitment of transcription factors and contains an intrinsic HAT activity domain (12). The other member of the CBP/p300 family is p300 HAT which has been shown to be essential in the regulation of cellular growth and division, while also regulating maturation and differentiation. HAT p300 carries out its function by directly interacting with transcription factors and the transcription machinery (13). HAT p300 also has a conserved HAT activity domain, like CBP, which can modify histones and other chromosomal proteins by catalyzing the transfer of an acetyl group from acetyl-coenzyme A (acetyl-CoA) to the lysine residues found on histone tails (14).

The second family of HATs is the GNAT (Gcn5-related N-acetyltransferase) superfamily which contains members PCAF and KAT2A. PCAF (p300/CBP binding protein) contains both a HAT domain and a conserved bromodomain located directly beside the HAT domain. PCAF acetylates specific lysine residues found on the N-terminal tails of Histone H3 and H4 (15). As the proteins are within PCAF’s name, PCAF can directly associate with p300 and CBP to help contribute to their HAT activity and overall histone acetylation status (15). The second member of the GNAT family is KAT2A (Lysine acetyltransferase 2A), which functions primarily as a transcriptional activator through its preferential acetylation activity with histone H3 and H4 (16). Both these GNAT members PCAF and KAT2A act to catalyze the transfer of the acetyl group bound to acetyl-CoA to their targeted lysine residues located on the histone tails.

The other two HAT families are known as the Rtt109 and MYST families. The Rtt109 HAT family specifically targets lysine residues found on Histone H3 and regulates cellular survival in the presence of DNA damage. Rtt109 has also been shown to physically interact with Vps75, a Nap1 family histone chaperone protein, to propagate Rtt109’s histone acetyltransferase activity (17). The MYST family, termed based on its
four members MOZ, Ybf2, Sas2 and Tip60 all contain zinc fingers and chromodomains and preferentially acetylate lysine residues on histones H2A, H3, and H4. HAT activity stems from a cysteine-rich region located in the N terminus of the HAT domain (18). Like the other families of HATs, MYST HAT members catalyze acetylation by the transfer of the acetyl group from acetyl-CoA to its lysine residue (18,19).

1.ii. MAPK Signaling Pathways ERK/JNK/p38

The mitogen-activated protein kinase (MAPK) signaling pathway is a highly conserved serine/threonine protein kinase family that is involved in the majority of cellular processes including differentiation, proliferation, apoptosis and longevity (20). The MAPK signaling pathway splits into three different paths that are activated based on the presence or absence of upstream signaling molecules. Depending on the stimulus present, the MAPK signaling pathway diverges to follow the extracellular signal-regulated kinase (ERK1/2), Jun N-terminal kinases (JNK1/2/3) or the p38α/β/γ/δ pathways (Figure 4) (21).

Ligand-receptor binding activates the ERK1/2 signaling pathway. Binding of the ligand causes dual phosphorylation of the receptor (20). Shc binds to the intrinsic arm of the receptor recruiting Grb2 (growth factor receptor-bound protein 2) and SOS (son of sevenless). The activation of these receptors leads to downstream activation of cytosolic kinase proteins Ras and Raf (20). These kinases communicate within the cells leading to a cascade of phosphorylation events, ultimately leading to the phosphorylation of ERK1/2 (Figure 4). ERK activation couples cell surface signaling to transcription factor activation regulating gene expression. This pathway is also physiologically relevant in cell cycle progress, cellular apoptosis, and differentiation (21). Abnormal regulation is associated
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with cancer development and irregular cellular apoptosis to name a few. In the articles by Servant et al. (1996), and Morley et al. (1997), ERK1/2 inhibitor PD98059 (30 μM) blocked the stimulation of protein synthesis and reduced the insulin- and serum-induced phosphorylation of translation initiation factor eIF4E (22,23). This supports the role of ERK1/2 in cellular growth and translational control.

The JNK signaling pathway can be activated by several different stimuli including growth and stress factors and inflammatory cytokines. JNK signaling follows a similar trend as described in the ERK signaling pathway, with a cascade of phosphorylations occurring to activate JNK1/2/3 signaling (24). Important kinase proteins Ras and Rac phosphorylate upstream MAPK kinases. These kinases continue to phosphorylate additional kinases before the phosphorylation of direct upstream kinases of JNK, MEK4 and MEK7 (Figure 4). The JNK signaling pathway has been shown to be a key-signaling pathway involved in cellular apoptosis (24). Tournier et al. (2000), showed that in knockout mice models for JNK1 and JNK2, embryonic fibroblasts maintained a resistance to apoptosis in response to UV irradiation, while their native counterparts showed increased apoptosis (25). In addition, Zhao et al. (2016), showed the involvement of JNK signaling in pro-inflammatory cytokines (IL-6) and transcription factor (TNF-α) expression as JNK inhibitor SP600125 significantly decreased expression compared to control cells (26).

The third arm of the MAPK signaling pathway leads to the activation of p38. The activation of p38 isoforms is preferentially activated directly by MKK3 and MKK6 kinases, which in turn are activated by upstream kinases and proteins (Figure 4) (27). The activation of p38 is linked to the activation and regulation of inflammation, cellular apoptosis, cell cycle progression and differentiation (27). Zhang et al. (2017), indicated
that p38 signaling plays an important role in DANCR-regulated osteogenic differentiation. The inhibition of p38 by SB203580 significantly induced increased expression of DANCR, which translates to decreased mRNA expression of osteogenic marker genes (28). Deacon et al. (2003) showed that p38 inhibitors SB203580 (20 μM) and SB202190 (10 μM) reduced the number of apoptotic cells by 46 and 42%, respectively, indicating the involvement of p38 MAPK signaling in the regulation of cellular apoptosis (29).

**Figure 4: MAPK signaling pathway illustrated within a mast cell.** Once Ras is phosphorylated and activated, the MAPK signaling cascade leads to three different pathways ultimately leading to the phosphorylation of ERK, JNK, or p38. These kinases then lead to the activation of transcription by regulating nuclear transcription factors. Figure created by DD.
1.iii. MAPK signaling influence with HDACs and HATs

MAPKs can interact and bind with numerous proteins including kinases, phosphatases and transcriptional proteins. In addition to their enzymatic role, MAPKs can directly recruit proteins into transcription factor complexes found on gene promoters. This is accomplished by MAPKs remaining in the nucleus by their interactions with transcription factors (30). HDACs and HATs as well, are associated with multi-molecule complexes containing enzymatic activity. These complexes are controlled by the activity of transcription factors, i.e. HAT complex CBP-p300 is activated by C/EBP-α and p-Elk1, while Hox proteins complexed with Twist block activity (31,32).

In *S. cerevisiae*, in response to osmotic stress MAPK Hog1 recruits Rpd3-Sin3 HDAC complexes to their respective promoter genes (33). Knockdown of Rpd3 in the presence of osmotic stress significantly reduced the expression of various osmostress-inducible genes, HSP12, STL1, ALD3, to name a few. Thus, Rpd3 (HDAC) facilitates Hog1-mediated gene expression. Rpd3 deacetylates the acetyl groups specifically in histones H3 and H4 (33). A recruitment of the Rpd3-Sin3 HDAC complex causes a decrease in histone H4 acetylation at the promoter region of Hsp12 only in the wild-type strains, while no difference occurred in the Hog1-knockout strain. Gene activation involves the entry of Pol II at the targeted promoter region. RNA Pol II recruitment was markedly reduced at genes whose expression was Rpd3-dependent in the Rpd3-knockdown strain (33). This indicates that Rpd3 has a positive role in gene expression, unlike what is associated with histone deacetylation complexes (33). Histone deacetylation is normally associated with the repression of gene expression, however there are many examples of particular genes that shows an association of histone
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Deacetylation with transcription induction (34–36). Therefore, in response to osmotic stress, MAPK Hog1 is initially recruited to facilitate Rpd3 HDAC activity.

ETS-domain transcription factor Elk-1 forms a complex with fellow transcription factor SRF and is promptly activated by ERK MAPK. Once activated, a rapid induction of gene expression is initiated including c-fos, srf and egr-1 (37). Elk-1 transcriptional activity is decreased when bound to HDAC2 co-repressor complexes. Elk-1 and p300 associate in an inactive conformation bound at the enhancer elements located upstream of the regulatory region (38). However, ERK activation of Elk-1 leads to the conformation of complex between Elk-1 and p300 to result in an increase in histone acetylation and overall gene transcription (37,38). ERK is also shown to form a complex with HDAC4. HDAC4 is primarily located in the cytoplasm of the cell, however, the activation of the ERK complexed with HDAC4 facilitates the translocation of HDAC4 from the cytoplasm into the nucleus, where its specific targets are located (39). Therefore, ERK signaling has multiple complexes with histone modifiers regulating the level of histone acetylation and rate of transcription.

Transcriptional effector AP-1 is a direct target of JNK phosphorylation. AP-1 consists of Fos and Jun proteins that bind to DNA through conserved bZIP domains. Once AP-1 is phosphorylated by JNK, dFos results in an increase in associated HAT Chameau (Chm) activity (40). Specific histone target H4K16 level of acetylation is increased when Chm is recruited by dFos, while H3K9 and K14 level of acetylation remains unchanged (40). De-phosphorylation, however, of dFos results to the recruitment of Rpd3-HDAC complex decreasing histone modification and reducing gene transcription. Rpd3 physically outcompetes Chm at the ZIP domain, reducing Chm-dFos complex and increasing Rpd3-dFos complex formation (40). This new complex reduces
H4K16 and H3K4 acetylation mediated by Chm restoring to a more repressive transcriptional environment (40). Therefore, JNK signaling regulates the dueling actions of Chm and Rpd3, ultimately influencing the level of chromatin acetylation to regulate the rate of gene transcription.

Transcription factor ATF-2 is a DNA-binding protein that interacts with cyclic AMP-response elements. Unlike other transcription factors, ATF-2 is also shown to have intrinsic histone acetyltransferase activity (41). Once phosphorylated by JNK, ATF-2 specifically acetylates both free and bound histones H2B and H4 in vitro. As well, once phosphorylated ATF-2 recruits other sequence-specific transcription factors p300 and c-Jun to further recruit various HATs to these promoter regions to upregulate transcription (41). Therefore, the phosphorylation of transcription factor ATF-2 by JNK MAPK signaling leads to intrinsic HAT activity and further recruitment of other HATs to enhance transcription.

ATF-2 has also been shown to be involved in the p38 MAPK signaling pathway. The p38 pathway, as stated earlier, involves multiple kinases, including p38β (42). p38β activates downstream transcription factors including ATF-2 and c-Jun. This also leads to p300 acetylation activity and ATF-2 intrinsic HAT activity. p38β also interacts with HDAC3 to inhibit downstream kinase activity (42). The interaction between HDAC3-p38β led to reduced ATF-2 activity and protein expression. Therefore, ATF-2 positively and negatively influences transcription regulation through independent binding with HDACs and HATs.

MAPK signaling diverges into the ERK, JNK and p38 pathways depending on the upstream kinases that are activated. Through a cascade of phosphorylations all three pathways directly recruit proteins to transcription factor complexes at gene promoter
regions to regulate transcription. The regulation of gene transcription also occurs through chromatin modifications. Histone acetylation (HATs) and deacetylation (HDACs) regulate the conformation of chromatin. The octamer of histones within the chromatin is bound to the DNA helix preventing proteins and transcription factors from binding while in a closed form. When chromatin is in an open conformation, the DNA is not as tightly bound to the histones allowing transcription factors and proteins to bind to the DNA initiating transcription. Both histone acetylation and MAPK signaling is shown to influence gene expression, therefore an association between the processes may occur. It has been shown that the ERK and JNK signaling pathways play a role in the activation of p300-mediated histone acetylation, leading to an increase in gene expression. Meanwhile the p38 MAPK signaling pathway is involved in HDAC3-p38β complex formation, which reduces downstream signaling and transcription. Therefore, a more complex understanding on how MAPK signaling, and histone acetylation regulated by HDACs and HATs work together to influence gene expression is necessary.

1.iv. Hematopoietic Stem Cell Myeloid Differentiation

Cells of the immune system are derived from hematopoietic stem cells (HSCs). HSCs are undifferentiated cells that have the ability to either differentiate into other immunological cells or replicate themselves (Figure 5) (43). Specific markers and receptors that are found on the surface of HSCs used to differentiate them between other immunological cells are species specific. HSCs isolated from Mus musculus have a wide variety of surface markers that are used for their isolation, including Lin−, Sca-1+, CD34low, and c-Kit+ (44). HSCs are deemed multipotent stem cells as they differentiate into either the lymphoid or myeloid lineages (Figure 5) (43,44). The lymphoid lineage
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gives rise to important immune cells: T cells, B cells, and natural killer cells (44,45).
Importantly, the myeloid lineage further differentiates into additional immune cells. The
common myeloid cell differentiates into either granulocyte-macrophage or erythroid
progenitor cells (46). The erythroid progenitor cells produce important red blood cells and
platelets. The granulocyte-macrophage progenitor cell gives rise to macrophages,
dendritic cells, neutrophils, eosinophils, basophils, and mast cells (Figure 5). These cells
have prominent cytoplasmic granules that contain cytotoxic components, including but
not limited to cytokines and chemokines, that can kill microorganisms and cause
inflammation (46).

Figure 5: Hematopoietic stem cell myeloid cell fates. Myeloid cells split into either
erythroid or granulocyte-macrophage progenitor cells. Mast cells are derived from
granulocytes, however the direct precursor cell is unknown. Figure created by DD.
Mast cells are one of the cell fates of HSCs and begin development within the bone marrow circulating in an immature form. In its immature form, mast cells lack the high-affinity IgE receptor, FcεRI, which is present on mature mast cells (47). Full maturation occurs when progenitors move out of circulation and into the various tissues in which they function, particularly close environmental contact tissues including but not limited to the skin and mucosal regions (47). A mature mast cell begins maturation from its native status through the interaction of the c-kit receptor with stem cell factor (SCF). Additional mast cell cytokines including but limited to interleukin-3 (IL-3) and interleukin-4 (IL-4) help to promote the differentiation and proliferation of mast cells (48).

The mast cell lineage travels through the myeloid differentiation pathway before becoming an unknown direct precursor situated directly before the formation of an immature mast cell (49). The appearance of the unknown precursor mast cell appears less granulated compared to later immature mast cells. This lack of granules leads to difficulty identifying the cells through traditional histochemical staining techniques (47). This results in using cell surface receptor presence on the developing mast cells as an indicator, specifically CD34 and FcεRI expression (50). The mast cell precursor is highly regulated by transcription factors while circulating in its immature form (47). HSC lineage differentiation has been well established through both the myeloid and lymphoid lineages, however the presence of the unknown direct precursor cell presents the necessary for continued research on how the important inflammatory mast cell differentiates.
2. Literature Review:
I. JNK MAPK Signaling Pathway Influence on HDAC/HATs
   a. JNK Expression
   The JNK signaling pathway mediates important responses including growth and cellular apoptosis. JNK is also involved in the development of insulin resistance in diabetic and obese mice (51). Wang, et al., 2015, demonstrated that the inhibition of JNK by the inhibitor SP600125 (5 mg/kg) reduced the increase of phosphorylated JNK induced by streptozotocin STZ at 3 months (51). At 6 months, SP600125 was unable to reduce the JNK phosphorylation, as the relative level of expression for JNK was equivalent. At the mRNA level of expression, SP600125 significantly reduced relative p300 and CBP HAT expression to the level of control at both 3 and 6 months (51). Therefore, in the diabetic mice model used by Wang, et al., 2015, the JNK signaling pathway is involved in the regulation of the mRNA expression of p300 and CBP HAT.

   In the study by Choi, et al., 2017, the presence of JNK inhibitor SP600125 (10 μM) partially recovered the Fluoxetine (FLX)-induced cell death and showed a significant reduction in caspase-4 cleavage, an important molecule in apoptosis (52). As well, apoptotic cell death is associated with an increase in histone hyperacetylation through the up regulation of HATs and a downregulation of HDACs. In the presence of FLX, an increase in histone H3 and H4 acetylation protein levels occurred (52). When treated with SP600125, the relative level of protein p300 HAT expression was significantly reduced, as well as the level of histone H3 and H4 hyperacetylation. In terms of HDAC expression, the presence of SP600125 increased protein expression of HDAC1-4, reversing the downregulation induced by FLX (52). Therefore, the JNK MAPK signaling pathway is involved in apoptotic cell death as well as the regulation of HAT and HDAC expression, dictating expression.
The JNK MAPK signaling pathway is shown to impact HDAC and HAT RNA and protein expression. JNK inhibition by SP600125 reduced the level of histone H3 acetylation by 52% and 65% (52). SP600125 further decreased the expression of important molecules of apoptosis, caspase-4, 8, 9 and 3 (52). This decrease in histone acetylation was accompanied with decreased HATs, p300 and CBP mRNA expression (51,52). HDAC expression was also increased in the presence of SP600125 (52).

Therefore, JNK MAPK signaling influences the expression HDAC and HAT protein and mRNA to upregulate gene expression.

### Table 1: JNK Inhibition Influence on Histone Modifier Expression.

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>MAPK Inhibitor</th>
<th>Effects</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6 kidney</td>
<td>SP600125 (5 mg/kg)</td>
<td>SP = \downarrow p300/CBP mRNA DM-induced expression</td>
<td>(51)</td>
</tr>
<tr>
<td>SK-N-BE(2)-M17 and SH-SY5Y neuroblastoma cells</td>
<td>SP600125 (10 μM)</td>
<td>SP = \downarrow H3 &amp; H4 hyperacetylation SP = \downarrow p300 expression SP = \uparrow HDAC expression</td>
<td>(52)</td>
</tr>
</tbody>
</table>

### II. JNK Activity

Ethanol is known to influence various signal transduction processes including the MAPK signaling pathway, as well as their transcription factors, NFκB, CREB and AP-1 (53–55). In a study by Park, et al., the modulation of HAT and MAPK signaling pathways during ethanol-induced histone H3 acetylation was investigated. Ethanol treatment in hepatocytes increased the acetylation of histone H3 at lysine 9 by nine fold, but was unable to significantly increase acetylation of H3 at lysine 14, 18 and 23 (53). In the presence of JNK inhibitor SP600125 (30 μM), however, this increase in acetylation at lysine 9 was reduced by 52% and 65% when treated with 50 mM and 100 mM ethanol,
respectively (53). SP600125 did not affect H3 acetylation when it was co-treated with acetate or significantly affect peptide acetylation when nuclear extracts were incubated with H3-AcK14 (53). Therefore, the JNK signaling pathway specifically regulates the level of histone acetylation of position H3K9, and is not involved in K14, K19 and K23 acetylation regulation.

In the study by Lee, et al. (2013), JNK inhibitor SP600125 (20 μM) before quercetin exposure, significantly abolished the protein expression of JNK, and significantly reduced the activation of caspase-8, caspase-9 and caspase-3 (56). In respect to AP-1 activation, quercetin was only able to increase c-Jun protein expression. SP600125 significantly decrease the quercetin increase of c-Jun at 6 hours, indicating that quercetin mediates AP-1 apoptosis through JNK signaling (56). The presence of JNK inhibitor SP600125 significantly reduced HAT activity while also directly decreasing histone H3 acetylation (56). Finally, ChIP analysis showed that quercetin-induced histone H3-acetylation resulted in an up-regulation in FasL, with SP600125 decreasing FasL expression (56). Therefore, the JNK signaling pathway is involved in the regulation of quercetin-induced apoptosis through the regulation of AP-1 activation, FasL mRNA expression and histone H3 acetylation.

The JNK MAPK signaling pathway has many roles in HDAC and HAT RNA expression, protein expression, and activity. JNK inhibition by SP600125 reduced the level of histone H3 acetylation by 52% and 65% (52,53,56,57). SP600125 further decreased the expression of important molecules of apoptosis, caspase-4, 8, 9 and 3 (52,56). This decrease in histone acetylation was accompanied with decreased HATs, p300 and CBP mRNA expression (51,52). HAT activity and c-Jun protein expression was also reduced in the presence of JNK inhibitor. This decrease in HAT was accompanied
with an increase in HDAC, with HDAC expression increased in the presence of SP600125 (52). Therefore, JNK MAPK signaling positively regulates transcription through an increase in HAT and decrease in HDAC expression.

Table 2: JNK Inhibition Influence on Histone Modifier Activity.

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>MAPK Inhibitor</th>
<th>Effects</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepatocytes</td>
<td>SP600125 (30 μM)</td>
<td>SP = ↓ H3 acetylation 52-65%</td>
<td>(53)</td>
</tr>
<tr>
<td>HL-60 cells</td>
<td>SP600125 (20 μM)</td>
<td>SP = ↓ H3 acetylation</td>
<td>(56)</td>
</tr>
</tbody>
</table>

II. ERK MAPK Signaling Pathway Influence on HDAC/HATs

II.i. ERK Expression

The prolactin gene can be regulated through its promoter region. However, in vitro studies reviewed in an article by Urnov & Wolffe, 2001, indicated that the hormonal regulation of the prolactin promoter is not through transcription factors, but is instead through epigenetic changes at the nucleosomal histones (58). Therefore, in a study by Liu, et al., 2005, the levels of acetylation were measured at the prolactin promoter region. The level of acetylation of histone H3 and H4 were elevated at the prolactin promoter region under normal conditions (59). When these cells were treated with ERK inhibitor U0126 (10 μM) the level of acetylation expression of histone H3 were decreased by 60%. The relative level of acetylation found at the prolactin promoter was also significantly reduced by 50-60% in the presence of U0126 (59). Therefore, ERK signaling is shown to influence acetylation at the prolactin promoter region, with ERK inhibition associated with histone deacetylation.
Influence of MAPK on mast cell differentiation and histone acetylation modifiers D. Den Hartogh, 20

In a study by Ozaki, et al., 2006, the presence of ERK inhibitor PD184352 (10 μM) significantly induced G1 cell cycle arrest and complete growth suppression of HT-29 cells (60). When HDAC inhibitor HC-toxin (5 μM) was introduced to HT-29 cells, the proportion of cells in the sub-G1 phase increased by 90% (60). PD184352 (ERK inhibitor) co-administration with HC-toxin (HDAC inhibitor) further enhanced the ability of HC-toxin to induce apoptosis, even when HC-toxin was administered at low concentrations (0.05 μM) (60). This indicates that the ERK signaling pathway links HT-29 cell death with increased acetylation through HDAC inhibition. Apoptotic caspases cleavage was also increased in the co-administration of PD184352 and HC-toxin (0.1 μM), but not alone indicating that ERK signaling and acetylation interact to induce autophagic cell death (60). Therefore, the ERK signaling pathway interacts with HDAC expression to induce cellular apoptosis and mediate pro-apoptotic caspase cleavage.

Table 3: ERK Inhibition Influence on Histone Modifier Expression.

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>MAPK Inhibitor</th>
<th>Effects</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>GH4ZR7, L6, NIH3T3 and Jurkat Cells</td>
<td>U0126 (10 μM)</td>
<td>MEK1 = ↓ 50-60% prolactin-promoter acetylation</td>
<td>(59)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MEK1 = ↓ histone H3 acetylation by 60%</td>
<td></td>
</tr>
<tr>
<td>HT-29 cells, A431 cells, PC-9 cells</td>
<td>PD184352 (10 μM); U0126 (20 μM)</td>
<td>PD + HDACi = ↑↑↑ G1 cell cycle arrest</td>
<td>(60)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PD + HDACi = ↑ caspase-8, 9 &amp; 3 cleavage</td>
<td></td>
</tr>
</tbody>
</table>
II.ii. ERK Activity

As the JNK signaling pathway was not the only MAPK pathway shown to be involved in the regulation of quercetin-induced apoptosis and HAT activity, the ERK signaling pathway is also believed to be involved due to similar upstream kinases. In the study by Lee, et al., (2013), HL-60 cells pretreated with ERK inhibitor PD98059 (50 μM) before quercetin exposure showed a complete abolishment in ERK activation and a significant reduction in the activation of caspase-8, caspase-9 and caspase-3 (56). PD98059 further decreased quercetin’s increase of c-Jun at 6 hours, indicating that quercetin also mediates AP-1 apoptosis through ERK signaling (56). ERK signaling effects on histone H3 acetylation was also determined. PD89059 significantly reduced HAT activity while also directly decreasing histone H3 acetylation and decreased FasL expression similar to JNK inhibition (56). Therefore, the ERK signaling pathway is also involved in the regulation of quercetin-induced apoptosis through the regulation of AP-1 activation, FasL mRNA expression and histone H3 acetylation.

The study by Sakamoto et al., 2013, continued the study by Ozaki, et al., 2006, measuring the effects of ERK signaling inhibition and HDAC inhibition on subcutaneous HT-29 and H1650 tumors in vivo. Mice were treated orally with ERK inhibitor AZD6244 (50 mg/kg) and/or HDAC inhibitor MS-275 (20 or 40 mg/kg) to measure ERK signaling effects (61). The co-administration of the inhibitors did not interfere with the respective phosphorylation inhibition and increased acetylation of histone H3. The combination of AZD6244 and MS-275 significantly increased the number of cells undergoing apoptosis (61). Therefore, the co-activation of the ERK signaling pathway and histone acetylation of H3 is involved in the regulation of tumor growth and oxidative stress and indicates that a direct interaction occurs between ERK MAPK signaling and histone acetylation.
In the study by *Swank & Sweatt, 2001*, male mice injected with LiCl developed conditioned taste aversion (62). MEK inhibitor SL327 attenuated the LiCl developed aversion, suggesting that MAPK is involved in creating taste memory in the insular cortex. The kinase activity of MAPK has been shown to regulate the lysine acetyltransferase activity within the insular cortex (62). MEK inhibitor U0126 blocked the acetylation of p42AcK indicating that p42AcK is MAP-kinase dependent. However, another target, p55AcK, showed an increase in acetylation in the presence of U0126 (62). Therefore, ERK MAPK cell signaling negatively influences the lysine acetyltransferase activity that is involved in long and novel memory of taste stimuli and links the activation of the ERK signaling pathway to histone modification.

In the study by *Wang et al., 2010*, epithelial cells treated with PAR1 and PAR2 agonists TFLLR-NH2 (50 μM) and SLIGRL-NH2 (50 μM) significantly increased the mean protein and mRNA level of IL-8 (63). IL-8 is also involved in angiogenesis, however the molecular mechanism by how PARs induced IL-8 expression in epithelial cells was not known. MEK inhibitor PD98059 (20 μM) completely reduced the PAR-induced protein expression of IL-8, while also significantly reducing IL-8 mRNA. HDAC2 binds to p65 in epithelial cells to reduce the expression of IL-8 protein and mRNA. When PD98059 was introduced to the HT-29 cells, there was no effect on the PAR-induced dissociation of HDAC2 and p65 (63). Therefore, the ERK signaling pathway is not involved in the removal of HDAC2 from p65, but instead directly phosphorylates p65 at Ser-276 to directly recruit p300 to the NF-κB complex for IL-8 regulation.
The ERK MAPK signaling pathway regulates histone acetylation through the modulation of HDAC and HAT protein and RNA expression. ERK inhibitor U0126 significantly reduced the acetylation of the prolactin promoter region by 50-60%, while reducing histone H3 acetylation (56,59). p42 lysine acetylation was reduced, and p55 lysine acetylation was increased with U0126 (62). Another inhibitor of ERK, PD98059 further decreased HAT p300 recruitment at the p50 promoter, and decreased HAT activity (56,63). This was accompanied with an increased G1 cell cycle arrest, which was further propagated with an HDAC inhibitor MS-275 (60). Therefore, ERK MAPK signaling increases histone acetylation and activity to positively regulate transcription through the regulation of decreased HDAC and increased HAT expression and activity.

**Table 4: ERK Inhibition Influence on Histone Modifier Activity.**

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>MAPK Inhibitor</th>
<th>Effects</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>HL-60 cells</td>
<td>PD98059 (50 μM)</td>
<td>PD = (\downarrow) H3 acetylation</td>
<td>(56)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PD = (\downarrow) HAT activity</td>
<td></td>
</tr>
<tr>
<td>HT-29 and H1650 tumors (~200 mm(^3))</td>
<td>AZD6244 (50 mg/kg)</td>
<td>AZ + HDACi = (\uparrow) TUNEL-positive cells</td>
<td>(61)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AZ + HDACi = (\uparrow) 8-OHdG</td>
<td></td>
</tr>
<tr>
<td>Male Swiss Webster mice (30-35 gm)</td>
<td>SL327 (100 mg/kg s.c.); U0126</td>
<td>U = (\downarrow) p42AcK</td>
<td>(62)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>U = (\uparrow) p55AcK</td>
<td></td>
</tr>
<tr>
<td>HT-29 cells</td>
<td>PD98059 (20 μM); U0126 (10 μM)</td>
<td>PAR = (\uparrow) p300 to p50 promoter</td>
<td>(63)</td>
</tr>
</tbody>
</table>
III. p38 MAPK Signaling Pathway Influence on HDAC/HATs

III.i. p38 Expression

Zinc-finger containing transcription factor Kruppel-like factor (KLF)-4 interacts with GC-rich or CACCC TGF-β1 control element to regulate TGF-β1 induced proliferation and differentiation of vascular smooth muscle cells. In a study by He et al., 2015, H3 acetylation occurred most predominantly in the regions between -645 and +57 bp of the p21 promoter (64). The knockdown of p300 dramatically reduced the acetylation of H3 at the p21 promoter region, indicating the role of p300 in H3 acetylation during TGF-β1 KLF4-mediated recruitment. KLF4 is phosphorylated at serine-470 by p38 signaling, and when serine is substituted with alanine, a markedly reduced interaction between KLF4 and p300 occurs (64). The p38 inhibitor SB203580 (20 μM) blocked TGF-β1 KLF4-p300 acetylation. As well, SB203580 significantly decreased PTEN phosphorylation (64). Therefore, the p38 MAPK signaling pathway mediates KLF4 phosphorylation to p300 acetylation regulating gene transcription and links p38 MAPK signaling to p300 HAT histone modification.

Dichlorodiphenyltrichloroethane (DDT) has been shown to stimulate p38 phosphorylation and activation, through transcription factors AP-1 and the estrogen receptor (ER) activation (65). The transcriptional coactivator p300 is also able to interact with AP-1. Selective upstream p38 MAPK kinase MKK6, significantly stimulated p300 when HEK 293 cells were treated with GAL6-coactivator of MKK6 (65). Mutants of p38α which lack p38 phosphorylation ability, showed significant inhibition of DDT-induced p300 activity by 70-80%. As well, the p38 inhibitor SB203580 significantly inhibited the DDT-induction of p300 (65). Therefore, the expression of p300 is mediated through the p38 MAPK signaling pathway when treated with DDT.
Table 5: p38 Inhibition Influence on Histone Modifier Expression.

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>MAPK Inhibitor</th>
<th>Effects</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vascular smooth muscle cells (VSMCs)</td>
<td>SB203580 (20 μM)</td>
<td>SB = TGF-β1-p300 acetylation, SB = p-PTEN</td>
<td>(64)</td>
</tr>
<tr>
<td>HEK293 cells</td>
<td>SB203580</td>
<td>CA-MKK6 = p300 activity, SB = p300 activity</td>
<td>(65)</td>
</tr>
</tbody>
</table>

III.ii. p38 Activity

In the study by Tsai et al., 2015, p38 MAPK signaling activation mediates the activity and expression of p300 HAT to regulate the expression of pro-survival molecules following PDT (66). p300 HAT mRNA expression was significantly increased in human melanoma cells following PDT treatment. This increased p300 HAT mRNA expression also correlated with an increase in HAT enzyme activity induced by PDT. The presence of p38 MAPK inhibitor SB203580 (40 μM) significantly reduced the expression of both COX-2 mRNA expression and protein levels (66). SB230580 also significantly reduced the level of PDT-induced p300 HAT expression and enzymatic activity to that of untreated cells. The increased level of acetyl-H3 induced by PDT was also significantly reduced in the presence of SB203580. Therefore, the activation of p38MAPK by PDT enhances the expression of p300HAT and mediates the expression of pro-survival molecule COX-2.

In the study by Schmeck et al., 2008, epithelial cells infected with L. pneumophila showed that increases of H4 and H3K14 acetylation occurred time-dependently (67). This was accompanied by the recruitment of NF-κB and p65/RelA, critical components for IL-8 expression. Preincubation of cells with HDAC inhibitor TSA (1 ng/ml) followed by L. pneumophila significantly increased the release of IL-8, while preincubation of cells with
HAT inhibitor anacardiac acid (10 μM) significantly reduced IL-8 expression (67). At the IL-8 gene promoter, HATs CBP and p300 recruitment was significantly increased, while HDAC1 and HDAC5 recruitment was reduced during infection. Epithelial cells were preincubated with p38 MAPK inhibitor SB201290 (25 μM) before L. pneumophila infection with a significant reduction in IL-8 release and recruitment of p65/RelA to IL-8 promoter occurring. This reduction in p65/RelA was accompanied with a significant reduction in histone H3 acetylation (67). Therefore, p38 MAPK signaling is shown to be involved in the regulation of HAT and HDAC activity at the promoter region of IL-8 during L. pneumophila infection of epithelial cells, however further studies into the recruitment inhibition needs to be clarified.

Intracellular bacteria Listeria monocytogenes can activate transcription factor NF-κB and induce endothelial expression of cytokines and adhesion molecules. The recruitment of these cytokines is essential for the clearance of L. monocytogenes infection (68). In the study by Schmeck et al., 2005, HUVEC cells infected with L. monocytogenes showed a significant increase in p-H3Ser10, ac-H3Lys14 and ac-H4Lys8 expression (68). This increase in phosphorylation and acetylation of histone H3 was further shown to occur in respect to the IL-8 gene promoter. HUVECs pretreated with SB202190 (10 μM) blocked the induced phosphorylation and acetylation of histone H3 and the acetylation of histone H4 (68).

SB202190 also significantly reduced the binding of RNA Pol II at the IL-8 gene promoter and subsequent gene transcription. Infection with L. monocytogenes enhanced the binding of HAT CBP to the IL-8 promoter region, with SB202190 returning the binding potential of CBP to that of uninfected cells. HDAC1 binding to IL-8 promoter was abolished with L. monocytogenes infection. SB202190 induced recruitment of
HDAC1 to reduce IL-8 gene transcription (68). Therefore, activation of p38 MAPK contributes to the regulation of IL-8 expression and histone acetylation modifications during *L. monocytogenes* infection.

In the study by Hanna et al., 2009, SMCs treated with p38 MAPK inhibitor SB203580 (10 μM) significantly decreased CCN1 promoter activity by 40% (69). This was accompanied with increased HAT CBP and p300 activity at CCN1 promoter activity. The myocardin-related transcription factor-A (MRTF-A) complex, a major transcription co-activator of p67, also showed increased CBP HAT activity when under mechanical stress (69). The pretreatment of cells with SB203580 showed a significant reduction in HAT activity when associated with the MRTF-A complex. Thus, p38 MAPK is upstream of CBP activation (69). Therefore, p38 MAPK signaling is necessary for the activation of the CCN1 promoter and is required for CBP MRTF-A complex activity activation.

HDAC inhibitor apicidin effectively induces fetal hemoglobin γ (HbF) to interfere with the polymerization of deoxygenated sickle hemoglobin in SCD. This induction of γ-globin is believed to involve transactivation by transcription factors CREB and ATF-2 through the p38 MAPK signaling pathway (70). In the study by Wei et al., 2007, pretreatment with p38 inhibitor SB203580 (10 μM) significantly reduced the induction of γ-globin by apicidin (70). Apicidin causes H3 hyperacetylation and unexpectedly specific hypermethylation of histone H3K14 in K562 cells. The presence of SB203580 blocked the apicidin increase in H3 acetylation, while histone H3 Lys14 was unaffected (70). Therefore, HDAC inhibition by apicidin is dependent on p38 MAPK signaling, linking p38 MAPK signaling to histone acetylation.
p38 MAPK signaling pathway regulates histone acetylation through HAT activity and expression. The inhibition of p38 by SB203580 significantly decreased the acetylation and activity of p300 (64–66,69,70). The overexpression of upstream p38 kinase MKK6 increased the activity of p300, supporting the inhibitory effect of SB203580. Another inhibitor of p38 MAPK, SB202190 also showed significant decreases in histone H3 and H4 acetylation. This was accompanied with a decrease in polymerase II recruitment indicating an overall decrease in transcription (67,68). Therefore, the modulation of histone acetylation by p38 MAPK signaling is due the regulation of HAT activity and expression. An increase in p38 MAPK is associated with an increase in gene transcription.

**Table 6:** p38 Inhibition Influence on Histone Modifier Activity.

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>MAPK Inhibitor</th>
<th>Effects</th>
<th>Reference</th>
</tr>
</thead>
</table>
| A375 cells                 | SB203580 (40 μM) | SB = ⬇ p300HAT expression  
SB = ⬇ acetyl-H3 | (66)       |
| A549 cells                 | SB201290 (25 μM) | SB = ⬇ p65 and H3 acetylation at IL-8 promoter | (67)       |
| HUVEC cells                | SB202190 (10 μM) | SB = ⬇ H3 & H4 acetylation  
SB = ⬇ RNA Pol II | (68)       |
| Primary human bladder SMCs | SB203580 (10 μM) | SB = ⬇ CCN1 promoter activation  
SB = ⬇ HAT activity | (69)       |
| K562 cells                 | SB203580 (10 μM) | SB = ⬇ acetyl-H3                           | (70)       |
IV. MAPK Influence on HDAC/HAT Interactions  
IV.i. MAPK Influence with Transcription Factors on Histone Modifiers  
The loss of adhesion to the cell-extracellular matrix (ECM) induces epidermal stem cell to undergo terminal differentiation (71). In the study by Connelly et al., 2011, primary human keratinocytes treated in the presence of p38 inhibitors PD169316 (10 μM) and SB202190 (2 μM) significantly reduced the expression of terminal differentiation in positive cells (71). SB202190 also significantly inhibited SRF reporter activity and reduced SRF binding to target genes, FOS and JunB (71). Changes in histone acetylation influence terminal differentiation of epidermal stem cells (71). When human keratinocytes were treated with HDAC inhibitor TSA, enhanced SRF transcriptional activity occurred for FOS, EGR1 and JunB. The presence of p38 inhibitor SB202190, however, had no effect on global histone acetylation, but did specifically reduce the acetylation at FOS and JunB promoters (71). Therefore, p38 MAPK signaling mediates histone acetylation and SRF transcriptional activity during epidermal stem cell differentiation but does not affect stem cell marker gene expression.

Aberrantly regulated HDACs lead to the suppression or progression of tumor cells, such as hepatocellular carcinomas (HCCs), depending on the specific HDAC being activated. HDAC6 is associated with tumor transformation and formation through its interaction with α-tubulin (72). In the study by Bae et al., 2015, hepatoma-derived cell lines, PLC/PRF/5 and SNU182 exhibited low HDAC6 protein expression while having high miR-221 gene expression. JNK activator, anisomycin significantly increased miR-221 expression in PLC/PRF/5 cells, while suppressing HDAC6 expression (72). The treatment with JNK inhibitor SP600125 further supported this result, as SP600125 significantly reduced miR-221 while increasing HDAC6 expression in HCC cells (72).
Therefore, the JNK/c-Jun/miR-221 axis is involved in the regulation of HDAC6 expression and the progression of HCC cancer cells.

Adenosine triphosphate (ATP) is released from damaged neurons during spinal cord injuries (SCI). This extracellular ATP regulates multiple biological functions through its ionotropic P2X receptor (P2XR) and G-protein coupled metabotropic P2Y receptor (P2YR) subfamily (73). P2X4R is specifically involved in the regulation of tactile allodynia after SCI. In the study by Lu et al., 2013, the mRNA levels of P2X4R were significantly increased in the injured spinal cord tissue after SCI (73). When treated with HDAC inhibitor VPA, the level of P2X4R protein in the microglia was significantly decreased by 7 days after the SCI (73). P2X4R mRNA levels were also significantly reduced when treated with VPA for 18 hours. This was accompanied with an increase in histone H3 acetylation in the microglia (73). When LPS-simulated microglia were stimulated with p38 inhibitor SB203580 (1 μM), SB203580 significantly increased the mRNA expression of P2X4R in the microglia (73). Therefore, p38 inhibition inhibits the action of VPA on the microglia during SCI and regulates the expression of the P2X4R receptor.

The luteinizing hormone receptor (LHR) gene transcription is regulated through the activation and repression of epigenetic modifiers, acetylation and methylation. Sp1/Sp3 binding domains and the inhibitory nuclear orphan receptor-binding motif mediates the basal activity of the LHR gene promoter (74). In the study by Liao et al., 2008, the HDAC1/2/mSin3A repressor complex binds to the transcriptional activators Sp1 and Sp3 within the LHR gene promoter regulating its transcriptional activation (74). The repression of ERK upstream kinase PKC by inhibitor G06976 significantly reduced the basal activity of the LHR gene promoter (74). Direct ERK inhibitor U0126 (10 μM)
also significantly blocked the activation of the LHR promoter by PMA. PMA treatment alone reduced HDAC1 and mSin3A association with the LHR promoter, however HDAC2’s binding remained unchanged (74). The level of histone H3 acetylation was increased at the LHR promoter with the treatment of PMA, with U0126 completely blocking the increased acetylation and association of Pol II and TFIIB with the LHR promoter, thus reducing gene transcription (74). Therefore, the ERK MAPK signaling pathway mediates the phosphorylation of Sp1 and the release of HDAC1/mSin3A complex at the LHR promoter to regulate LHR transcription.

The myeloid differentiation primary response gene 88 (MyD88)-dependent pathway activates the NF-κB and MAPK signaling pathways through TNF receptor-associated factor (TRAF)-6, interleukin-1 receptor-associated kinase (IRAK) 4 and IRAK-1 signaling molecules in macrophages (75). ERK MAPK signaling induces the expression of MyD88 negative regulator IRAK M by disrupting the interaction between IRAK-4 and IRAK-1. In the study by Banerjee et al., 2011, LPS and poly (I:C) treatment induced higher ERK1/2 phosphorylation in tumor-associated macrophages (TAM) compared to control, while p38 MAPK phosphorylation was unaffected (75). The presence of ERK inhibitor PD98059 (10 μM) slightly increased the level of IL-12 and significantly reduced the level of IL-10 in TAMs activated with TLR. At the IL-10 promoter, PD98059 significantly reduced H3 phosphorylation for both the control and TAM macrophages (75). Therefore, the activation of ERK phosphorylates specifically at the IL-10 promoter, while the IL-12 promoter is unaffected to affect gene transcription.

Transcription factors influence the overall rate of transcription at gene promoters. The interactions between the MAPK signaling pathway and transcription factors modify histone acetylation levels to ultimately affect gene transcription. The inhibition of p38 by
SB202190 or PD098059 reduced the binding of transcription factors IRAK/1, IRAK/M and transglutaminase I at promoter regions, leading to decreased expression (71,75). The inhibition of ERK by U0126 reduced the activation of the hLHR promoter and significantly reduced the acetylation of histone H3 to reduce transcription (74). JNK inhibition by SB203580 increased TNF-α and P2X₄R expression (73). Another JNK inhibitor SP600125 increased the expression of HDAC6 to negatively regulate histone acetylation, while also decreasing miR-221 expression (72). Therefore, the MAPK signaling pathways act differently on genes and acetylation to influence the rate of transcription.

Table 7: MAPK Inhibition Influence with TFs on Histone Modifiers.

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>MAPK Inhibitor</th>
<th>Effects</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary human keratinocytes</td>
<td>SB202190 (2 μM); PD169316 (10 μM); PD098059 (10 μM)</td>
<td>SB = JUNB activation</td>
<td>(71)</td>
</tr>
<tr>
<td>Hep3B, SNU368, MiHA, HLK3, SNU182, PLC/PRF/5</td>
<td>SP600125</td>
<td>SP = miR-221 expression</td>
<td>(72)</td>
</tr>
<tr>
<td>Sprague-Dawley microglia</td>
<td>SB203580 (1 μM)</td>
<td>SB = VPA</td>
<td>(73)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SB = TNF-α mRNA expression</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>SB = P2X₄R mRNA expression</td>
<td></td>
</tr>
<tr>
<td>HeLa cells</td>
<td>U0126 (10 μM)</td>
<td>PMA = ERK activity</td>
<td>(74)</td>
</tr>
<tr>
<td>C57BL6 TAM and peritoneal</td>
<td>PD098059 (10 μM)</td>
<td>PD = histone phosphorylation at IL-12p40 promoter</td>
<td>(75)</td>
</tr>
</tbody>
</table>
IV.ii. MAPK Influence with Genes on Histone Modifiers

Focal adhesions (FAK) function as mechanical sensors that link fluid flow shear stress (FFSS) with the MAPK signaling pathway. RUNX2 transcription factor activation is necessary for osteoblast-specific gene expression and mesenchymal progenitor cell differentiation (76). In the study by Li et al., 2012, ERK1/2 stimulated RUNX2-dependent transcriptional activity through the direct phosphorylation of residues serine43, 301, 319 and 510. This was further shown in the study by Li et al., 2017, with ERK phosphorylation of RUNX2 specifically at S319 resulting in increased in osteoblast marker genes *Bglap2* and *Ibsp* mRNA expression (77). The pretreatment of cells with ERK inhibitor U0126 (10 μM) before FFSS completely blocked FFSS induction of *Bglap2*, *Ibsp* and *SPP-1* mRNA and showed increased levels of H3K9 and H4K5 acetylation, as well as, increased activation-associated methylation mark H4K5m (76,77). As well, U0126 significantly inhibited the acetylation of histone H3 and H4 and the phosphorylation of Histone H3S10. However, U0126 did not affect chromatin-bound RUNX2, indicating that RUNX2 does not need to be phosphorylated to remain bound to chromatin (76,77). Therefore, ERK signaling initiated by FAK and RUNX2 influence the state of the chromatin through acetylation and phosphorylation modifications to ultimately influence mesenchymal progenitor cell differentiation.

Interleukin-6 (IL-6) is produced during inflammation responses along with other proinflammatory cytokines. The IL-6 promoter sequence contains consensus-binding sites for NF-κB and cAMP response element binding protein (CREB). Mutations at these binding sites inhibit Ang II-induced IL-6 gene expression (78). In the study by Sahar et al., 2007, rat vascular smooth muscle cells (RVSMCs) were pretreated with ERK inhibitor U0126 (10 μM) and p38 MAPK inhibitor SB202190 (10 μM), with U0126
completely inhibited Ang II-induced IL-6 gene expression and SB202190 having no effect (78). U0126 inhibited the recruitment of HAT SRC-1 and significantly inhibited histone H3-K9/14 acetylation at the IL-6 promoter (78). Therefore, ERK signaling regulates HAT recruitment and histone acetylation at the IL-6 promoter.

Nuclear factor of activated T-cells (NFAT) family members are downstream targets for Ca\(^{2+}\)-dependent regulation during skeletal muscle fiber type transformation. Calcineurin and NFAT are essential in the upregulation of MyHCl/β promoter activity and mRNA. In the study by Meissner et al., 2011, ERK inhibitor U0126 (10 μM) significantly decreased MyHCl/β promoter activity (79). Transcriptional coactivator HAT p300 increased MyHCl/β promoter activity (79). NFATc1ΔSRR and p300 both upregulate promoter activity individually, leading to the hypothesis that they may interact within the same site (79). U0126 inhibition significantly augments this interaction of NFATc1ΔSRR and p300. Therefore, MyHCl/β promoter activity is regulated by the binding of NFAT and p300, which in turn is regulated through ERK signaling.

Granulocyte differentiation of leukemic cells is regulated by numerous factors including circulating granulocyte colony-stimulating factor (G-CSF) and retinoic acid (RA) transcription factor nuclear retinoic acid receptors (RARs) (80). In the study by Cassinat et al., 2011, RA and G-CSF treatment increased histone H3 and H4 acetylation at the RARα2 promoter and significantly increased the binding of HAT CBP/p300 to the promoter region (80). Treatment with ERK inhibitor U0126 (10 μM) completely blocked the differentiation induced by RA and G-CSF in UF-1 cells. As well, U0126 significantly reduced the acetylation of histone H3 and H4 and recruitment of CBP/p300 to the RARα2 gene promoter (80). Therefore, the ERK MAPK signaling pathway increases the
availability of the RAR promoter through the regulation of histone phosphorylation, HAT protein and transcription factor recruitment and histone acetylation.

TNF-α is involved in inflammation-associated cancers and exerts its biological functions through TNFR1 and TNFR2. The association between HAT p300, NFκB and AP-1 mediates the formation of short-range DNA loops to regulate transcription. NFκB and AP-1 are spatially located at the distal ends of the MMP-9 promoter yet is crucial in the regulation of the MMP-9 gene (81). In the study by Chen & Chang, 2015, treatment with JNK inhibitor SP600125 (10 μM) significantly reduced the upregulation of MMP-9 mRNA and activity induced by TNF-α in UF-1 cells (81). TNF-α regulation at the MMP-9 promoter reduced the recruitment of HDAC1 and increased the recruitment of p300 at the AP-1 and NFκB sites. This also resulted in increased binding of transcription factors p65 and c-Jun to the MMP-9 promoter (81). Treatment with SP600125 significantly reduced the TNF-α induced increase of histone H3 acetylation and the levels of DNA looping (81). Therefore, suppression of the JNK signaling pathway disrupts the formation of DNA looping and MMP-9 upregulation through the regulation of transcription factors p65 and c-Jun.

The ERK MAPK signaling pathway is responsible for the modifications of acetylation at genes and gene promoter regions. The inhibition of ERK by U0126 significantly reduces the level of histone H3 and H4 acetylation at specific promoter regions to influence its level of expression (76–78,80,81). HATs p300 and CBP recruitment of gene promoter regions were also significantly reduced in the presence of ERK inhibition (77,80). U0126 also negatively influences the acetylation of important transcription factor NFATc1 and RNA polymerase II (77,79). JNK inhibitor SP600125 also negatively influenced the level of acetylation at gene promoter regions. SP600125
significantly reduced MMP-9 promoter acetylation and reduced DNA looping preventing enhancer region and promoter region binding (81). Therefore, the ERK MAPK pathway influences promoter region acetylation to regulate gene transcription.

**Table 8: MAPK Inhibition Influence with Genes on Histone Modifiers.**

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>MAPK Inhibitor</th>
<th>Effects</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>K562 cells</td>
<td>SB203580</td>
<td>SB = (\downarrow) acH3, acH4 and ph/acH3 expression</td>
<td>(82)</td>
</tr>
<tr>
<td>MC3T3 subclone 42 preosteoblast cells</td>
<td>U0126 (10 μM)</td>
<td>U = (\downarrow) p-RUNX2-S319</td>
<td>(76)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>U = (\downarrow) Histone H3/4 acetylation</td>
<td></td>
</tr>
<tr>
<td>Rat VSMCs (RVSMCs)</td>
<td>U0126 (10 μM)</td>
<td>U = (\downarrow) IL-6 mRNA Ang II = (\uparrow) H3-K9/14 acetylation</td>
<td>(78)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>U = (\downarrow) H3-K9/14 acetylation</td>
<td></td>
</tr>
<tr>
<td>C2C12 cells; HEK293 cells</td>
<td>U0126 (10 μM)</td>
<td>U = (\downarrow) NFATc1 acetylation</td>
<td>(79)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>U = (\uparrow) p300 binding with NFATc1ΔSRR</td>
<td></td>
</tr>
<tr>
<td>MC3T3-Elc4 cells</td>
<td>U0126 (20 μM)</td>
<td>ERK = (\uparrow) p-RUNX2-S319</td>
<td>(77)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>U = (\downarrow) p-RUNX2, Pol II, p300</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>U = (\downarrow) H3K4m, AcH3K9 &amp; AcH4K4</td>
<td></td>
</tr>
<tr>
<td>UF-1 cells; HB4 cells</td>
<td>U0126 (10 μM)</td>
<td>U = (\downarrow) H3 and H4 acetylation</td>
<td>(80)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>U = (\downarrow) CBP/p300 recruitment</td>
<td></td>
</tr>
<tr>
<td>U937 cells</td>
<td>SP600125 (10 μM)</td>
<td>SP = (\downarrow) MMP-9 promoter Ac-H3</td>
<td>(81)</td>
</tr>
</tbody>
</table>
**IV.iii. MAPK Influence with MAPKs on Histone Modifiers**

In the study by Wagley et al., 2013, MOR-negative P19 cells treated with JNK inhibitor SP600125 (25 μM) significantly increased MOR mRNA expression.

Knockdown of JNK1, also slightly increased MOR expression, while JNK2 knockdown failed to increase expression (83). SP600125 MAPK inhibitor increased MOR expression increasing the DNA content and stability of the mRNA. SP600125 treatment in P19 cells increased the levels of phosphorylated p38 and ERK1/2 (83). The presence of p38 inhibitor SB203580 (25 μM) significantly inhibited SP600125 MOR increase. Whereas, ERK inhibitor U0126 (10 μM) had no effect. The MOR promoter’s activity was also significantly increased upon SP600125 treatment and reduced by SB203580 pretreatment (83). Additionally, HAT CBP binding to the MOR promoter was increased upon SP600125 treatment. The increased CBP binding was also associated with increased H3-demethyl-K4 and decreased H3-trimethyl-K9, HDAC2 and MeCP2 (83). Therefore, JNK, and p38 signaling pathways are involved in the regulation of MOR expression and activity through histone modifications.

Nuclear factor of activated T cells (NFAT) is activated in response to calcium signals derived from stress to regulate genes required for β-cell function and proliferation. In the study by Lawrence et al., 2015, NFATc3 and ERK both co-occupy the 5’ flanking end of the insulin gene promoter when in the presence of GLP-1. Promoter activity was significantly reduced with NFATc1-c3 knock-down. The nuclear translocation of NFATc2 and ERK corresponded with increased association at the insulin gene promoter (84). ERK inhibitor U0126 (5 μM) significantly reduced the association of NFATc2 and ERK at the insulin promoter 20 minutes after exposure in MIN6 β cells. U0126 at the
TNF-α promoter resulted in enhanced association of p38 and JNK, while SB203580 and SP600125 resulted in enhanced association of ERK.

Glucose and IL-1β increased HAT p300 to the TNF-α promoter in the same manner as for NFATc2 and MARKs (84). HDAC3 was rapidly inhibited at the DNA-promoter complexes in response to the increase p300 association. This sustained p300 and reduced HDAC1/3 association requires p38 and JNK signaling activity on the TNF-α promoter in response to IL-1β (84). U0126 blocked NFAT/ERK/p300 occupancy at the insulin promoter and increased HDAC1 association (84). Therefore, p38 and JNK MAPK signaling is required to activate TNF-α gene expression, while ERK contributes to repression through the regulation of histone modifications.

Parasitic protozoan Leishmania donovani’s outcome of infection is determine by the Th1 and Th2 effector response, acting through Toll-like receptor 2 (TLR2). In the study by Bhattacharya et al., 2011, Ara-LAM increased phosphorylation of p38 in infected macrophages, while ERK phosphorylation was significantly reduced (85). The preincubation with ERK inhibitor PD098059 followed by Ara-LAM slightly increased IL-12 and NO production, along with a decrease in IL-10 at both the mRNA and protein level of expression. p38 MAPK inhibitor SB203580 significantly blocked Ara-LAM induced IL-12 and NO, while increasing IL-10 mRNA and protein levels. At the IL-10 promoter, Ara-LAM pretreatment significantly increase histone H3 phosphorylation and acetylation in parasitized macrophages (85). PD098059 pretreatment slightly reduced histone H3 phosphorylation and acetylation at the IL-10 promoter with SB203580 having the reverse effect (85). Therefore, ERK and p38 act in opposing manners to regulate the acetylation and phosphorylation status of both IL-10 and IL-12 promoters in Ara-LAM treated parasitic macrophages.
PIASxα regulates the differential responses of transcription factor Elk-1 in the context of the ERK and p38 MAPK signaling pathways. PIASxα is a coactivator protein for Elk-1 facilitating HDAC2 release and decreased SUMO conjugation during ERK signaling (86). In the study by Yang et al., 2006, inactive Elk-1 is maintained by the SUMO-mediated HDAC2 recruitment at the Elk-1 promoter. In the presence of PIASxα, HDAC2 recruitment at the Elk-1 promoter was maintained, while recruitment was slightly increased following anisomycin treatment (86). When PIASxα was depleted, HDAC2 and SUMO-1 recruitment to the Elk-1 promoter was abolished. SB203580 in the presence of PIASxα and anisomycin reduced the association of HDAC2 and SUMO-1 at the Elk-1 promoter, whereas SP600125 had no effect. The decreased association of HDAC2 was also accompanied with the increased level in local histone H4 acetylation (86). Therefore, the p38 MAPK signaling pathway mediates the recruitment of HDAC2 and SUMO-1 to the Elk-1 promoter during PIASxα and anisomycin stimulation.

The interactions between MAPKs balance the expression of HDACs and HATs to influence histone acetylation. The co-inhibition of the p38 and JNK signaling pathways, SP600125 and SB203580, significantly reduces the level of histone H3K9 acetylation, while also reducing the recruitment of HDAC2 and MeCP2 to the promoter region of genes (83,85,86). At the insulin promoter region, SP600125 and SB203580 significantly reduced the binding of NFATc2 and ERK, decreasing activation (84). While, the inhibition of ERK with U0126 significantly increased p38 and JNK association to the TNFα promoter, increasing activation. ERK signaling prevents the activity of HDAC2, while simultaneously increasing histone H4 acetylation (84). Therefore, p38 and JNK signaling negatively influence ERK signaling to regulate histone acetylation.
Table 9: MAPK Inhibition Influence with MAPKs on Histone Modifiers.

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>MAPK Inhibitor</th>
<th>Effects</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>P19 cells</td>
<td>SP600125 (25 μM); U0126 (10 μM); SB203580 (25 μM)</td>
<td>SP + SB = ↑ CREB expression SP + SB = ↓ H3-K9, HDAC2 &amp; MeCP2 binding</td>
<td>(83)</td>
</tr>
<tr>
<td>MIN6 β cells</td>
<td>U0126 (5 μM); SB203580 (5 μM); SP600125 (5 μM)</td>
<td>U = ↓ NFATc2/ERK association to insulin promoter U = ↑ p38 &amp; JNK association to TNFα promoter SB &amp; SP = ↑ TNFα = ↓ ERK1/2</td>
<td>(84)</td>
</tr>
<tr>
<td>BALB/c macrophages</td>
<td>SB203580; PD098059</td>
<td>PD = ↓ acetyl-H3 SB = ↑ acetyl-H3</td>
<td>(85)</td>
</tr>
<tr>
<td>HeLa cells</td>
<td>SP600125 (10 μM); SB203580 (10 μM); U0126 (10 μM)</td>
<td>SP = ↓ PIASxα ERK = ↓ HDAC2 ERK = ↑ acetyl-H4</td>
<td>(86)</td>
</tr>
</tbody>
</table>

V. MAPK and HDAC/HAT Modulation on Immune Cell Differentiation

V.i. T-Cell Differentiation

Multiple sclerosis (MS) induces a transfer of CD4+ T cells to react against myelin antigens. The infiltration of the reactive cells generates a cascade of immunological reactions that can be influenced by HDAC inhibitors (87). HDACis promote the activity and frequency of regulatory Foxp3+ Treg cells and Th1 cells. In the study by Castelo-Branco, et al., 2014, HDACi valproic acid (VPA) (200 mg/kg) treatment in DA/Kini rats alone or in combination with thyroid hormone (T3) strongly reduced T cell amount and proliferation of Th17 cells (CD4+Foxp3+IFN-γ/IL-17+) and IFN-γ/IL-17+ cells (87). VPA and T3 co-treatment also increased IFNg+/IL-17- cells. This resulted in increased levels of histone H3K9 acetylation for both conditions. A synergistic response occurs between the co-treatment of T cells with VPA and T3 leading to an increased expression of regulatory
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T cell transcription factor Foxp3 (87). In contrast, chemokine receptor Cxcr3 expressed in Th1 cells (CD4+Foxp3+IFN-γ+IL-17) was significantly downregulated with VPA (87). However, the presence of VPA significantly lowered the number of CD4+ T cells and Th17 cells, indicating that VPA shifts the profile of cells towards a Th1 phenotype. Therefore, HDAC inhibition by VPA interferes with the differentiation of T cells shifting the phenotype towards Th1 and Treg cells and away from CD4+ and Th17 cells.

In the study by Regna et al., 2014, another inhibitor of HDAC activity, ITF2357 (5 mg/kg and 10 mg/kg) significantly decreased the ratio of CD4:CD8 T cells through an increased cytotoxic T cell subset and decreased Th cell number. The percentage of Treg cells were significantly increased in the presence of ITF2357, while Th17 cells was decreased in a dose-dependent manner (88). Histone acetylation was only increased with ITF2357 treatment at 10 mg/kg for both NZW and NZB/W mice, while 5 mg/kg was unable. ITF2357 (5 μM) treatment increased the level of acetylation of Foxp3 in vitro (88). Therefore, HDAC inhibition increased Treg cell differentiation through increased Foxp3 expression and decreased Th17 cell differentiation.

TGFβ-inducible Kruppel-like factor (KLF10) recruits HAT p300/CBP-associated factor (PCAF) to Foxp3 promoter regions to regulate the induction of the gene. Also, KLF10 recruits histone deacetylase binding protein Sin3 to Foxp3 promoter regions to negatively regulate Foxp3 gene expression (89). In the study by Xiong et al., 2014, KLF10 is required for the activation of Foxp3 during the differentiation of Treg cells in vivo. The overexpression of KLF10 significantly increased KLF10’s binding to the Foxp3 core promoter region (89). KLF10-SIDmt was unable to repress Foxp3 core promoter activity, identifying Sin3 as a mediator of KLF10 coupling to the Foxp3 promoter through its SID. A loss of histone H4 acetylation was also observed at the Foxp3 core promoter
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when Sin3 was bound (89). This was propagated with HDAC1 binding with Sin3 at the promoter region. This indicates that Foxp3 repression by KLF10 is due to the Sin3 and HDAC1 complex at the promoter (89). KLF10 is necessary for the regulation of Foxp3 and Treg cell differentiation mediated by the presence of Sin3-HDAC or PCAF.

T lymphocytes are activated through the T-cell receptor (TCR) ultimately leading to the activation of cell signaling pathway ERK (90). In the study by Dayoub et al., 2017, delphinidin inhibited T cell proliferation via the ERK1/2 signaling pathway (90). Following 24h of PHA treatment, T-bet, GATA3, RORγt and Foxp3 expression levels were increased, Th1, Th2, Th17 and Treg transcription factors respectively (90).

Delphinidin significantly decreased the upregulation of expression induced by PHA for T-bet, RORγt and Foxp3, but not GATA3. Furthermore, delphinidin decreased the production of Th1, Th17 and Treg cell cytokines, IL-2, IFNγ, IL-17A and IL-10, but not IL-4 produced by Th2 subsets (90). Therefore, delphinidin inhibits T cell differentiation towards Th1, Th17 and Treg cells but not Th2 cells.

Commensal microorganisms produced short-chain fatty acids (SCFAs), butyrate and propionate during starch fermentation. In the study by Arpaia et al., 2013, butyrate (250 μM) and propionate promote the extrathymic differentiation of Treg cells (91). The increased extrathymic differentiation of Treg cells was believed to be due to increased binding of butyrate by G protein-coupled receptors. In addition to increased Treg cell number, intracellular Foxp3 protein levels were also increased in both CNS1-sufficient and deficient mice (91). Histone H3K27 was significantly acetylated at the Foxp3 promoter and at the CNS1 enhancer when Foxp3GFP mice were treated with TGF-β and butyrate (91). The inhibition of HDAC activity by TSA (10 μM) also increased the expression of Foxp3 like the treatment with butyrate. Therefore, SCFA butyrate increases
the differentiation of CNS1-dependent Treg cells through increased expression of Foxp3 and increased acetylation at the promoter region.

Systemic lupus erythematosus (SLE) induces CD4+ T helper cells while inhibiting Treg cells. In the study by Regna et al., 2016, selective HDAC6 inhibitor ACY-738 (5 and 20 mg/kg) significantly decreased CD4+CD8− T cells while increasing CD3+CD4+CD8+ T cells (92). During SLE the number and functionality of Treg cells is also significantly reduced. When NZB/W mice were treated with ACY-738 a significant increase in the percentage of Treg cells occurred (92). SLE diseased NZB/W mice are also associated with elevated production of anti-dsDNA and IgM and IgG isotypes. ACY-738 treatment prevented the increase of anti-dsDNA and significantly decreased the levels of IgG2a and total IgG. As well, specific SLE-associated cytokines, IL-6 and IL-10 mRNA expression levels were reduced to undetectable levels when treated with 20 mg/kg of ACY-738 (92). Taken together, HDAC6 inhibition through the inhibitor ACY-738 prevented the altered differentiation of T cells towards an anti-SLE phenotype.

General control nonrepressed-protein 5 (Gcn5) is a catalytic subunit of the transcriptional co-activation complex SAGA, shown in literature to be involved in the regulation of gene transcription in many different tissues and organs (93–95). In the study by Gao, et al. (2017), Gcn5-gene deletion mice showed that Gcn5 is necessary for proper T cell development and activation with NFAT (93). In Gcn5-knockout mice, CD25+CD44−CD4−CD8− T cells translation to CD25−CD44+CD4+CD8+ T cells was inhibited. As well, a significant increase in the percentage of CD25+Foxp3+ Treg cells was present in the Gcn5 gene knockout samples (93). Furthermore, Gcn5 positively regulated histone H3K9 acetylation, while only low levels of acetylation status were detected in naïve T cells. At the IL-2 promoter, Gcn5-knockdown significantly abolished
H3K9 acetylation indicating that Gcn5 is responsible for H3K9 acetylation at the promoter during T cell activation (93). Therefore, Gcn5 HAT regulates histone acetylation and is necessary for the regulation of Treg cell differentiation.

The differentiation and activation of T cells is regulated through the modification of acetylation status. Histone H3K9 acetylation was significantly increased by both the treatment of HDAC inhibitor ITF2357 and the overexpression of HAT Gcn5 (88,93). Histone H4 level of acetylation was significantly decreased with HDAC1 complexed to Sin3, leading to decreased Foxp3 expression and Treg cell differentiation (89). HDAC inhibitors such as ITF2357, VPA, ACY-738, delphinidin, as well as KLF10-knockdown also negatively affect Treg cell differentiation (87–90,92). Treg cell transcription factor Foxp3 expression was also influenced with HDAC inhibitors TSA, ITF2357, along with butyrate, PCAF\(^\text{\gamma}\) and KLF10 overexpression (88,89,91,92). Another T cell subset, Th17 cell differentiation was also influenced by HDAC inhibition, indicating the role of histone acetylation in different populations of T cells. ITF2357, VPA and delphinidin significantly reduced the number and expression of Th17 cells, while also decreased the expression of Th17 transcription factors IL-17 and ROR\(\alpha\) (87,88,90). Finally, Th1 cell phenotype was increased when treated with VPA, TSA and acetate, linking all of T cell subset differentiation to histone acetylation (87,91). T cell differentiation and activation is linked to the modification of histone acetylation through the overexpression of HATs and the inhibition of HDACs.
Table 10: MAPK and HDAC Inhibition on T cell Differentiation.

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>HDAC/HAT Inhibition</th>
<th>Effects</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>DA/Kini rats</td>
<td>Valproic acid (200 mg/kg)</td>
<td>VPA = Th17 cells</td>
<td>(87)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>VPA = Th1 cells</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>VPA = H3K9 acetylation</td>
<td></td>
</tr>
<tr>
<td>NZB/W and NZW mice</td>
<td>ITF2357 (5 mg/kg or 10 mg/kg)</td>
<td>IT = CD4:CD8</td>
<td>(88)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IT = Th cells</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>IT = Treg cells</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>IT = Ac-Histone H3</td>
<td></td>
</tr>
<tr>
<td>FLP Jurkat cells</td>
<td>KLF10 and KLF10-SIDmt (FOXP3 promoter mutant); PCAF knockdown</td>
<td>KLF10 = p300/CREB to Foxp3 promoter</td>
<td>(89)</td>
</tr>
<tr>
<td>Human T lymphocytes, C57BL/6 WT and ERαKO mice</td>
<td>Delphinidin (0.01 g/L); TSA (100 nM); SKF96365 (10 μM)</td>
<td>Del = T cell proliferation</td>
<td>(90)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Del = HDAC activity</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Del = NFAT activation</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Del = Th1, Th17 and Treg cells</td>
<td></td>
</tr>
<tr>
<td>Foxp3ACN31, Foxp3GFP, Foxp3Thy1 and Gpr109α−/− mice</td>
<td>Butyrate (250 μM), TSA (10 μM)</td>
<td>But = Foxp3 protein</td>
<td>(91)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>But = Treg cell</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>But = Foxp3 protein acetylation</td>
<td></td>
</tr>
<tr>
<td>C57BL/6, NZB/W F1 mice</td>
<td>ACY-738 (5 mg/kg and 20 mg/kg)</td>
<td>AC = CD4+CD8− T cells</td>
<td>(92)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AC = CD3+CD4+CD8+ T cells</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>AC = Treg cells</td>
<td></td>
</tr>
<tr>
<td>HEK293 cells, Gcn5-null mice</td>
<td>Gcn5 knockdown, CsA (Conc.)</td>
<td>Gcn5 = Treg cells</td>
<td>(93)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Gcn5 = CD8+</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Gcn5 = Th1 cells</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Gcn5 = Th17 cells</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>CsA = Gcn5 to IL-2 promoter</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>Gcn5 = H3K9 acetylation</td>
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</tr>
</tbody>
</table>
3. Hypotheses:
The hypotheses corresponding to the proposed research are:

1. Over normal mast cell differentiation, specific HDACs (a) and HATs (b) will inversely change at both the RNA and protein level of expression ultimately influencing the overall state of histone acetylation.

2. The MAPK signaling pathway (JNK, ERK, and p38) will differentially influence normal mast cell differentiation.

3. The MAPK signaling pathway branches will differentially influence epigenetic regulator expression, indirectly influencing mast cell differentiation.
   a. The MAPK pathway will differentially influence mast cell specific marker and mediator expression.
   b. The MAPK pathway will differentially influence key mast cell specific transcription factors.
   c. The MAPK signaling pathway branches will have different influences HDACs and HATs acetylation expression and activity.

4. The structure of chromatin will dictate the accessibility of targeted MAPK loci; therefore the state and structural organization of chromatin is necessary for the activation of MAPK targeted DNA loci.
4. Objectives:
The objectives of the proposed research corresponding to the proposed hypotheses are to determine:

1. How expression of histone acetylation modifiers change over mast cell differentiation.
   a. Histone deacetylases (HDACs)
   b. Histone acetyltransferases (HATs)
2. The role of the MAPK pathway (JNK, ERK, and p38) in mast cell differentiation under normal differentiation conditions.
   a. Mast cell-specific mediators and markers
   b. Mast cell-specific transcription factors
3. The effects of the MAPK signaling pathway on epigenetic regulator expression and mast cell differentiation.
   a. HDACs and HATs
4. How chromatin accessibility changes at loci identified as potentially modulated by the MAPK pathway in mast cell differentiation
5. Methodology:
Animals and bone marrow isolation:
All animal protocols were approved by the Animal Care Committee (ACC) and adhere to the Canadian Council of Animal Care (CCAC) guidelines. The isolation of bone marrow was conducted within a biosafety cabinet (Class II A2 BSC, 100660-2888) to minimize the contamination of the cells. Bone marrow extraction media was prepared using 500 mL of RPMI 1640 (Life Technologies Inc., 11875119) with 1% penicillin/streptomycin (Gibco, 15140-122; 5.62 mL) and 10% Fetal Bovine Serum (FBS; Sigma, F2442; 56.2 mL). The lower half of each wild-type C57BL6 mouse (Charles River Laboratories) was wet with 70% ethanol completely removing the skin and muscle at the hip and leg. Individual mice leg bones were prepared with ~20 mL of RPMI in 1.5 mL tubes.

Twenty mL of the extraction media was inserted into the syringe with the needle (BD 302832, 305106) locked into position and inserted into each bone to flush the marrow until the bone appeared completely white (red marrow removed). The solution was pipetted up and down to break up the marrow and centrifuged at 1300 rpm for 10 minutes at 4°C.

Five mL of 1x red blood cell (RBC) lysis buffer (100 mL ddH2O, 8.02 g NH4Cl (Bio Basic Canada Inc., ADB0034), 0.84 g NaHCO3 (Bio Basic Canada Inc., SB0482), 0.37 g EDTA (Bio Basic Canada Inc., EB0185)) was added to resuspend the pellet for 5 minutes at room temperature (RT). The lysis was neutralized when the solution volume was returned to 40 mL with phosphate-buffered saline (PBS pH 7.4, Gibco, 10010-023). The solution was then centrifuged for 10 minutes at 4°C at 1300 rpm. The supernatant was removed and the pellet was resuspended with 10 mL 10% FBS pen/strep RPMI thoroughly. The media and cells were filtered through a 20 µm filter. The number of cells was counted (Life
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Technologies™ Countess II FL, AMQAF1000) with 23 μL cell sample and 2 μL of NucBlue (Life Technologies, R37605) on the hemocytometer (Figure 6, 7).

![Image](image.jpg)

**Figure 6: BMMCs differentiation with or without MAPK inhibitors.** HSCs are isolated from C57BL6/J bone marrow and differentiated in the absence or presence of an inhibitor of the MAPK signaling pathway. Non-differentiated HSCs were taken prior to the addition of BMMC complete media. Figure created by DD.

**Initiation of mast cell differentiation in the presence or absence of MAPK inhibitors:**

Mast cell differentiation was initiated by resuspending bone marrow cells in BMMMC Complete Media (500 mL RPMI (Life Technologies Inc., 11875119), 63 mL FBS (Sigma, F2442), 63 mL Wehi Supernatant (3B cell line), 6.3 mL 1% pen/strep (Gibco, 15140-122), 31.6 μL 1M β-mercaptoethanol (Sigma, M3148), 12.6 μL PGE-2 (Sigma, P5640)). Depending on the experimental condition, JNK (JNK-IN-8 (Calbiochem, 2891341, 1 μM)), ERK (SCH772984 (APExBIO, A3805, 1 μM)) or p38 (Losmapimod (Selleckchem, S7215, 10 μM)) MAPK inhibitors were introduced to the bone marrow cells at time of differentiation (Figure 6). At each time point, samples were taken for RNA, protein, and chromatin analysis (Figure 7).
ERK inhibitor SCH772984 specifically inhibits ERK1 and ERK2 activity with IC50 values of 4 and 1 nmol/L, respectively through the inhibition of ERK substrate p90 ribosomal S6 kinase and ERK activation loop phosphorylation. Importantly, SCH772984 inhibits both ERK enzymatic activity and MEK phosphorylation in a variety of contexts (Table 11). JNK inhibitor JNK-IN-8 inhibits the JNK signaling pathway by targeting c-Jun phosphorylation by multiple studies (Table 13). p38α inhibitor losmapimod is a relatively new inhibitor that is currently undergoing Phase III clinic trials and has shown inhibition of TNFα, a p38α downstream proinflammatory cytokine as shown in Table 12.

### Table 11: ERK Inhibitor SCH772984 Effects on ERK Signaling.

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Cells</th>
<th>Potency/Effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 μM</td>
<td>PC12 cells</td>
<td>p-ERK = ↓ protein expression</td>
<td>(96)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p-ERK = ↓ 70.9%</td>
<td></td>
</tr>
<tr>
<td>10 μM</td>
<td>Rat cerebellar astrocytes</td>
<td>p-ERK = ↓ SCH = ↑ MEK1/2</td>
<td>(97)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>phosphorylation</td>
<td></td>
</tr>
<tr>
<td>1 μM</td>
<td>PC12 cells</td>
<td>TGF-B1 = ↓ TGF-B1 regulated</td>
<td>(98)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cdk5 and p35</td>
<td></td>
</tr>
<tr>
<td>1 μM</td>
<td>HCC cells</td>
<td>SCH = ↓ G2/M phase arrest</td>
<td>(99)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SCH = ↓ p-ERK</td>
<td></td>
</tr>
<tr>
<td>1 μM</td>
<td>WM989 and WM1799 cells</td>
<td>SCH = ↓ ERK pRSK</td>
<td>(100)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SCH = ↓ p-ribosomal protein S6</td>
<td></td>
</tr>
<tr>
<td>20 μM</td>
<td>BxPC3 and SW1990 cells</td>
<td>SCH = ↓ VEGF expression</td>
<td>(101)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SCH = ↓ p-ERK</td>
<td></td>
</tr>
</tbody>
</table>

### Table 12: p38 Inhibitor Losmapimod Effects on p38 Signaling.

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Cells</th>
<th>Potency/Effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 μM</td>
<td>UROtsa and T24 cells</td>
<td>Los = ↓ T24 basal proliferation</td>
<td>(102)</td>
</tr>
<tr>
<td>10 μM</td>
<td>Primary mice splenocytes</td>
<td>Los = ↓ TNF-a release</td>
<td>(103)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Los = ↓ p38a MAPK p-ATF-2</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>phosphorylation</td>
<td></td>
</tr>
<tr>
<td>10 μM</td>
<td>COPD PBMCs</td>
<td>GW = ↓ IL-6</td>
<td>(104)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GW = ↑ CXCL8 suppression</td>
<td></td>
</tr>
</tbody>
</table>
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Table 13: JNK Inhibitor JNK-IN-8 Effects on JNK Signaling.

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Cells</th>
<th>Potency/Effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 μM</td>
<td>HEK-293 cells</td>
<td>JNK = ↑ 100-fold IC₅₀ for inhibition of JNK activity</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>JNK = ↓ c-Jun activity</td>
<td>(105)</td>
</tr>
<tr>
<td>1 μM &amp; 2.5 μM</td>
<td>HCC70 and SUM149 cells</td>
<td>JNK = ↓ p-c-Jun</td>
<td>(106)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>JNK = ↓ proliferation</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>JNK = ↑ FZD1, FRAT1, HDAC1 and BMP3 mRNA</td>
<td></td>
</tr>
<tr>
<td>1 μM</td>
<td>A375 and 1205Lu cells</td>
<td>JNK = ↓ p-c-Jun</td>
<td>(107)</td>
</tr>
</tbody>
</table>

Figure 7: Experimental process derived from C57BL6 wild-type mice. Mast cell differentiation beginning with hematopoietic stem cells culture in IL-3 and BMMC complete media to ensure mast cell development. Mice represent different C57BL6/J mice wild-types used, experiments done in three biological and two technical replicates. Figure created by DD.

Quantitative polymerase chain reaction:
RNA was isolated from 1 x 10⁶ BMMCs using the RNeasy Plus kit (Qiagen, 74134). One μg of RNA was diluted to a final volume of 20 μl with molecular grade water (MgH₂O, Sigma, W4502) to generate cDNA using EcoDry RNA to cDNA (Clontech, #639549). The resultant cDNA was diluted to 1:20 in MgH₂O. qPCR reactions
were set up in a MicroAmp plate (Applied Biosystems, 4346907) using KAPA SYBR Fast (D-Mark Biosciences, KR0390, 352340) in a 10 µl final reaction volume (5 µl mastermix, 0.2 µl of each primer (Table 14) (10 µM stocks), and 3.6 µl of water) per well. One µl of cDNA from each time point was added per well. Reactions were conducted in a StepOnePlus qPCR instrument (Applied Biosystems, 272004476) using the following protocol: 3 min @95°C followed by 5 s @95°C and 30 s @ 60°C for 40 cycles, followed by a melting curve protocol (Figure 6, 9). The threshold value was changed to 0.05 prior to exporting the Ct values. ΔΔCt analysis was performed with correction to an average of various reference genes as indicated (Table 15).

Table 14: Primers of Interest Sequences. HDACs, HATs and mast cell specific cell marker’s primer sequences in both the forward and reverse direction.

<table>
<thead>
<tr>
<th>Gene of Interest</th>
<th>Sequence</th>
<th>NCBI Accession Number</th>
</tr>
</thead>
</table>
| HDAC1           | F - TGAAGCCTCACCAGAATCCGCA  
R - TCTGCGGCTAGAAAGCGAGGA  
| NM_008228       |
| HDAC2           | F - AGTCAAGGAGAGGGGCAAGA  
R - CTTAGCATGGAATTTGAGGG  
| NM_008229       |
| HDAC3           | F - AGGCCCAACAAATGCAGGCT  
R - CGCCTGTGAACCGGAGCAAA  
| NM_010411       |
| HDAC4           | F - ATGCTTTCTACAGGGTGGCCT  
R - TCCACAGCAGATGACCCGA  
| NM_207225       |
| HDAC6           | F - TGGCTTGTCATCTACATGGGCT  
R - GTCAGAATGTTGACCTACCTAC  
| NM_010413       |
| CBP             | F - TGACTGTCTGTGTCCTCTCCCT  
R - ATAGGCCCAGCTCTAGGACT  
| NM_001025432    |
| p300            | F - TGCCAACAGCTCAGCAGCAGT  
R - AGCAGGATCCGCGAGTGGGAAAT  
| NM_177821       |
| PCAF            | F - AGAGACTGGAGAAAATCGCGCGTG  
R - AGGGCATGGCTACAGCGCAGGAC  
| NM_020005       |
| KAT2A           | F - GGACCTCTACTCTACTACCTCTCCC  
R - CGAAGCTGGCAGCAGTGGGATGCC  
| NM_020004       |
| CD34            | F - ATGGGCTGTCATTGCTTCTCTTCTC  
R - TGGCTGGGCTACTTCCAGGGATGCT  
| NM_001111059.1  |
| Cma1            | F - TGAGAACTACTGGCTCCGGTCGCT  
R - GCTTTTACCTCAAGGCTCTGCT  
| NM_010780.3     |
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<table>
<thead>
<tr>
<th>Gene of Interest</th>
<th>Sequence</th>
<th>Reference Gene ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>FcεRIα</td>
<td>F - AGCACTGCTGTTCATGTCTCTTTGA R - CCATGTTGGGTCCAAGTTCAGT</td>
<td>NM_010184.2</td>
</tr>
<tr>
<td></td>
<td>FcεRIβ</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cpa3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HIF1α</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tpsb2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MITF</td>
<td></td>
</tr>
</tbody>
</table>

Table 15: Reference Gene Sequences. Housekeeping genes chosen as they remain at a constant threshold for all conditions within our samples. The average ΔΔCt of the genes was used, instead of individual ΔΔCt values.

<table>
<thead>
<tr>
<th>Gene of Interest</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPRT</td>
<td>F - CTTGCTGGTTGAAAAGGACCTCTCG R - CGCTCATACTTTGATTTGG</td>
</tr>
<tr>
<td>GAPDH</td>
<td>F - CGTGCTGTAGTGATGCTGAGTC R - GGGGCTAAGCAGTGGGTGTG</td>
</tr>
<tr>
<td>ACTB</td>
<td>F - CCTGACCCCTGAAGTACCCATG R - ATGGCCTGGGTTGTTGAAAGTC</td>
</tr>
<tr>
<td>PPIA</td>
<td>F - GAGCTGTGGGCGACAAAAGTTC R - CCCTGAGCAGATCGGTG</td>
</tr>
</tbody>
</table>

Gel electrophoresis:
Agarose DNA gel was prepared with 1.8 g of agarose (Sigma, 9012-36-6), to 100 mL of 1x sodium boric acid buffer (8 g NaOH, (Sigma, S2770), 47 g Boric Acid (Bio Basic Canada Inc., BB0044), millipore H₂O until a final volume of 1L, pH 8.0). The solution was heated for 2 minutes until all the agarose particles were dissolved. Once allowed to cool, the volume was returned to that of the starting volume with sterile water (Baxter, JF7624), as determined by pre-weighing the flask with contents. Five μL of 10 mg/mL ethidium bromide (EtBr, Bio Basic Canada Inc., D0197) was added to solution before being poured into the gel electrophoresis apparatus. Once the gel hardened the
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combs were removed and placed into the gel electrophoresis apparatus containing 1x sodium boric acid buffer. 1.1 μl of 10X DNA load dye (Biobasic, GM303) was added to each well of the 96-well plate following the StepOnePlus qPCR instrument (Applied Biosystems, 4346907) run and 6 μl of each sample and DNA ladder was loaded per lane on a preset 1.8% agarose gel. The gel was run for 18 minutes at 200V. Gels were imaged on an Alpha GelDoc instrument (FluChem, 5500). See appendix S15-31 regarding individual gel electrophoresis analyses of targets and housekeeping genes.

**Protein extraction:**

Cells were collected at the indicated times by harvesting 2-3 x 10^6 per sample of both unstimulated and inhibited cells into their appropriate labelled and pre-chilled sterile 15 mL tubes and centrifuged at 1300 rpm for 10 mins at 4°C. The supernatant was removed and the pellet was lysed with 30 μL of RIPA+Trichostatan A (TSA; Cayman Chemical; 89730) buffer (473 μL sterile water (Baxter, JF7624), 500 μL 2X RIPA buffer (BioBasic, #RB4477), 2 μL leupeptin (5 mg/mL, Bio Basic Canada Inc., LDJ691), 2 μL pepstatin (5 mg/mL, Bio Basic Canada Inc., PDJ694), 2 μL aprotinin (5 mg/mL, Bio Basic Canada Inc., AD0153), 4 μL iodoacetamide (0.5 M, Bio Basic Canada Inc., IB0539), 5μ L NaF (200 mM, Sigma, S-7929, 1:2000 dilution), 5 μL Na_3 VO_4 (200 mM, Sigma, S-6508, 1:2000 dilution), 5 μL PMSF (100 mM, Bio Basic Canada Inc, PB0425) and 2 μL TSA). The samples were incubated on ice for 20 minutes and then centrifuged at max speed for 10 minutes at 4°C in Thermo Electron Corporation Centrifuge (CentraCL3R, #3755-1851).
The protein samples were quantified using a 96-well Tissue Culture Plate Flat Bottom with Lid (Sarstedt, 83.1835) in duplicate. A 1 in 20 dilution of protein samples was created by adding 2 μL of each corresponding protein to 38 μL of sterile water in a newly labeled 1.5 mL tube. The dilute samples were then gently vortexed, with 10 μL added to the 96-well plate. BSA protein standards and 1x RIPA-TSA blank were also run in duplicate alongside the protein samples. One times protein assay dye reagent was prepared with sterile water from 5x dye reagent stock (BioRad, 5x Protein Assay Dye Reagent Concentrate, #500-0006) in a volume that allows 200 μL to be added to each well. The absorbance of the protein samples, BSA standards and blanks were measured through a spectrophotometer (BIO-TEK, Synergy HT-1, #191356) set at a wavelength of 595 nM. The absorbance values were then used to calculate the actual protein concentrations and the required volume of protein, RIPA-TSA and 4x SDS buffer needed to create a sample with 30 μg of protein, see appendix Figures S53-54 and Tables S3-4 regarding standard curves and quantification results. In order, in newly labeled 1.5 mL tubes the calculated RIPA-TSA volume, protein, and SDS buffer was added and placed in an 85°C water bath for ~25 minutes for protein denaturation. The samples were then stored at -20°C for future use (Figure 7).

**Western blot:**
Thirty μg samples (15 μL) denatured in SDS loading buffer (Bio Basic Canada Inc., SB0485) were run on a 10% or 12% TGX FastCast acrylamide gel (Bio-Rad, 161-0172) for ~22 mins at 300 V. Gels were then transferred to PVDF membrane (Bio-Rad, #162-0177) using a Transblot Turbo (Bio-Rad, #170-4274) for 7 mins (using the Mixed MW* setting) using Transfer stacks (RTA kit) and 1x Transfer buffer (600 mL Millipore...
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H2O, 200 mL 100% Ethanol, 200 mL 5x Transfer Buffer (4°C, Trans-Blot Turbo, Bio-Rad, #1704274)). Membranes were then blocked in 5% milk powder (Pasteurized skim milk powder, no nameR) in TBS-Tween (tris-buffered saline (Bio Basics Canada Inc., TB0194) with 0.1% Tween-20 (Bio Basic Canada Inc, 9005-64-5)) for 2 hours at room temperature (RT) on an orbital shaker. Blocking buffer was then washed away with millipore water and membranes were re-equilibrated in TBS-T before addition of primary antibody at a 1:1000 dilution in a solution of 5% BSA in TBS-T buffer with 0.2% sodium azide (Sigma, 26628-22-8) (Table 16).

Table 16: Primary Antibody Targets. HDAC targets HDAC2, 4 and 6. HAT targets CBP, KAT2A, and PCAF. MAPK targets ERK1/2, JNK/SAPK, and p38. Histone protein targets Acetyl-Histone H2A, H2B, H3 and H4 and Phospho-Histone H3. Actin used as a positive control.

<table>
<thead>
<tr>
<th>Antibody Target</th>
<th>Company</th>
<th>Code</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>HDAC2</td>
<td>Cell Signaling</td>
<td>#5113</td>
<td>1:1000</td>
</tr>
<tr>
<td>HDAC4</td>
<td>Cell Signaling</td>
<td>#7628</td>
<td>1:1000</td>
</tr>
<tr>
<td>HDAC6</td>
<td>Cell Signaling</td>
<td>#7558</td>
<td>1:1000</td>
</tr>
<tr>
<td>CBP</td>
<td>Cell Signaling</td>
<td>#7389</td>
<td>1:1000</td>
</tr>
<tr>
<td>KAT2A</td>
<td>Cell Signaling</td>
<td>#3305</td>
<td>1:1000</td>
</tr>
<tr>
<td>PCAF</td>
<td>Cell Signaling</td>
<td>#3378</td>
<td>1:1000</td>
</tr>
<tr>
<td>ERK1/2</td>
<td>Cell Signaling</td>
<td>#4695</td>
<td>1:1000</td>
</tr>
<tr>
<td>JNK/SAPK MAPK</td>
<td>Cell Signaling</td>
<td>#9252</td>
<td>1:500</td>
</tr>
<tr>
<td>p38 MAPK</td>
<td>Cell Signaling</td>
<td>#8690</td>
<td>1:1000</td>
</tr>
<tr>
<td>Actin</td>
<td>Santa Cruz</td>
<td>Sc-1616 (I-19)</td>
<td>1:1000</td>
</tr>
<tr>
<td>Acetyl-Histone H2A (Lys 5)</td>
<td>Cell Signaling</td>
<td>#2576</td>
<td>1:1000</td>
</tr>
<tr>
<td>Acetyl-Histone H2B (Lys 5)</td>
<td>Cell Signaling</td>
<td>#12799</td>
<td>1:1000</td>
</tr>
<tr>
<td>Acetyl-Histone H3 (Lys 9)</td>
<td>Cell Signaling</td>
<td>#9649</td>
<td>1:1000</td>
</tr>
<tr>
<td>Acetyl-Histone H4 (Lys 8)</td>
<td>Cell Signaling</td>
<td>#2594</td>
<td>1:1000</td>
</tr>
<tr>
<td>Phospho-Histone H3 (Ser28)</td>
<td>Santa Cruz</td>
<td>Sc-374669 (C-2)</td>
<td>1:1000</td>
</tr>
</tbody>
</table>
Primary antibody (Table 16) was applied overnight on a rocking platform at 4°C. The following day, membranes were washed with TBS-T three times for 15 mins, followed by application of secondary antibody (anti-mouse-HRP at 1:2000 in 5% milk powder in TBS-T buffer, Cell Signaling, #7076; anti-rabbit-HRP at 1:2000 in 5% milk powder in TBS-T buffer, Cell Signaling, #7074). Three more washes were performed as above and 2 mL of ECL substrate was prepared per membrane (Clarity, Bio-Rad, 170-5060) and applied to the membrane drop-wise to fully coat the membrane. A protein marker, WesternSure Pen (Li-Cor, 926-91000), was used to mark the ladder to directly insure that the proteins are presented at the correct molecular size. Excess substrate was removed by touching the edge of the membrane to paper towel with membranes placed face down on a C-Digit blot scanner (Li-Cor, CDG-00244) and scanned for 6 mins (standard setting). Images were exported as .tif files and cropped in Photoshop (Adobe) (Figure 9).

**Chromatin Accessibility:**

DNA was isolated from 1 x 10⁶ BMMCs using the EpiQuik™ Chromatin Accessibility Assay kit (Epigentek, #P-1047). Pelleted cells were re-suspended with 1X lysis buffer before split into two separate 1.5 mL tubes; one as sample and the other as a no-Nse control. Tubes were then incubated on ice, followed by centrifugation at 5500 rpm for 5 min. The remaining pellet was washed twice with 1X wash buffer. Nuclease digestion buffer and nuclease mix was added to the sample tube, while nuclease digestion buffer was only added to the no-Nse control. The tubes were incubated at 37°C for 4 min before the addition of reaction stop solution. Proteinase K was then added to each tube increasing the incubation temperature to 60°C. Samples were transferred to a new 1.5 mL
tube prepped with DNA binding solution. DNA was cleaned up three times with 1X DNA washing solution before an additional centrifugation to dry the columns. Elution solution was directly added to each filter membrane to elute the DNA for use. qPCR reactions were set up to the same specifications located under quantitative polymerase chain reaction (Figure 7).

Table 17: Chromatin Accessibility Assay Promoter Genes. RHO, HBB, Prm2, Smcp, Tnp1 and Alb are negative controls based on their closed chromatin conformation. GAPDH and PPIA are positive controls that have fully open chromatin conformation. CD34, FcεRIα and FcεRIβ are targeted genes of interest.

<table>
<thead>
<tr>
<th>Gene of Interest</th>
<th>Primer Sequence</th>
<th>Gene ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>RHO (Rhodopsin)</td>
<td>F - GATTTGCGTGGGCCACCCTAACT</td>
<td>212541</td>
</tr>
<tr>
<td></td>
<td>R - CCGCGGGTTTCTTTTATAACCCGGTTC</td>
<td></td>
</tr>
<tr>
<td>HBB (Hemoglobin Beta)</td>
<td>F - GAGTGGCACACGATCCAGGGAGAAA</td>
<td>15127</td>
</tr>
<tr>
<td></td>
<td>R - CCACAGGCCAGAGACAGGAGCTTTCC</td>
<td></td>
</tr>
<tr>
<td>Prm2 (Protamine 2)</td>
<td>F - TGGCATGGTGAGCTGGACT</td>
<td>19119</td>
</tr>
<tr>
<td></td>
<td>R - TTGGCAACAGGGGAACCAT</td>
<td></td>
</tr>
<tr>
<td>Smcp (Sperm mitochondria-associated cysteine rich protein)</td>
<td>F - AGTGACACACTTCTGAGGCAGC</td>
<td>17235</td>
</tr>
<tr>
<td></td>
<td>R - GCATGGGGAGACAATGACAATTC</td>
<td></td>
</tr>
<tr>
<td>Tnp1 (Transition protein 1)</td>
<td>F - ATGTGGTCCTCCGAAAGCAGGTG</td>
<td>21958</td>
</tr>
<tr>
<td></td>
<td>R - CCTTTCCCTGGACATGGCAACC</td>
<td></td>
</tr>
<tr>
<td>Alb (Albumin)</td>
<td>F - AGGGAACAGCTCCCCAGATGGCA</td>
<td>11657</td>
</tr>
<tr>
<td></td>
<td>R - TGTTTCTGGGAACACACC</td>
<td></td>
</tr>
<tr>
<td>GAPDH (Glyceraldehyde-3-p-dehydrogenase)</td>
<td>F - CAGCTCCCCCTCCCCCTATCAGTTCG</td>
<td>14433</td>
</tr>
<tr>
<td></td>
<td>R - ACCAGGGAGGCTGAGTCCGTATT</td>
<td></td>
</tr>
<tr>
<td>PPIA (Peptidylprolyl isomerase A)</td>
<td>F - GATTTGCGTGGCCACCCTCCACTAACT</td>
<td>268373</td>
</tr>
<tr>
<td></td>
<td>R - CCGCGGCTTCTTTTATACCCGGTTC</td>
<td></td>
</tr>
<tr>
<td>CD34</td>
<td>F - CCTCCAAGACGACGACGAGGA</td>
<td>12490</td>
</tr>
<tr>
<td></td>
<td>R - CACGCCAGCAGAGACTTTCAGA</td>
<td></td>
</tr>
<tr>
<td>FcεRIα</td>
<td>F - AGCCTCTGTAAAATGTTGCCCA</td>
<td>14125</td>
</tr>
<tr>
<td></td>
<td>R - TGCCAGGACAGACGCTAGCAATCA</td>
<td></td>
</tr>
<tr>
<td>FcεRIβ</td>
<td>F - AGCAGGCCACCAAGACGAGA</td>
<td>121426</td>
</tr>
<tr>
<td></td>
<td>R - GCTTTTCTCTTCCAGAGTGCA</td>
<td></td>
</tr>
</tbody>
</table>
Flow Cytometry:
Cells were collected at the indicated times by harvesting 1.5 x 10^6 per sample for both unstimulated and inhibited cells. Samples were washed in PBS (pH 7.4, Gibco, 10010-023), 10% FBS (Sigma, F2442), and 1% sodium azide (Sigma, 26628-22-8) prior to the addition of conjugated primary antibody at a concentration between 0.1-10 μg/mL. The samples were incubated for 1 hour in the dark and washed once with PBS, 10% FBS and 1% sodium azide. The samples were then resuspended in 1% formalin and remained in the dark at 4°C until analysis using the Sony Flow Cytometer (Sony Cell Sorter SH800Z) (Figure 7, 8).

Figure 8: Schematic of flow cytometry. Cells can be stained intracellular or at the surface before being introduced as a single cell suspension to the flow cytometry apparatus. Analysis is completed, and an output result is produced based on targets MFI. Figure created by DD.
Table 18: Flow cytometry antibody targets. Isotype controls are used as negative controls to control for background levels. Each of the antibody targets are used for the identification of hematopoietic stem cells from the conglomerate of cells isolated from the bone marrow.

<table>
<thead>
<tr>
<th>Antibody Target</th>
<th>Laser</th>
<th>µl/1 x 10^6 cells</th>
<th>Sony Catalog #</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-mouse Lineage Cocktail</td>
<td>FITC</td>
<td>20</td>
<td>1266510</td>
</tr>
<tr>
<td>Anti-mouse CD34</td>
<td>PE</td>
<td>0.25</td>
<td>1196540</td>
</tr>
<tr>
<td>Anti-mouse CD117 (c-kit)</td>
<td>PE/Cy7</td>
<td>0.25</td>
<td>1129070</td>
</tr>
<tr>
<td>Anti-mouse Ly-6A/E (Sca-1)</td>
<td>PerCP</td>
<td>0.25</td>
<td>1140610</td>
</tr>
<tr>
<td>Rat IgG2b, κ Isotype Ctrl</td>
<td>PE/Cy7</td>
<td>0.25</td>
<td>2603090</td>
</tr>
<tr>
<td>Rat IgG2a, κ Isotype Ctrl</td>
<td>PerCP</td>
<td>0.25</td>
<td>2602650</td>
</tr>
<tr>
<td>Rat IgG2a, κ Isotype Ctrl</td>
<td>PE</td>
<td>0.25</td>
<td>2602540</td>
</tr>
<tr>
<td>Hamster IgG Isotype Ctrl</td>
<td>PE</td>
<td>0.25</td>
<td>2604540</td>
</tr>
<tr>
<td>Anti-mouse FceR1a</td>
<td>PE</td>
<td>0.25</td>
<td>1271540</td>
</tr>
</tbody>
</table>

Statistics:
Quantitative polymerase chain reaction (qPCR) data was analyzed using ΔΔCt analysis and fold change from the NT average was determined for the non-treated samples, see appendix Tables S1-2 for sample calculation of ΔΔCt analysis. Comparison for the inhibitor samples occurred between NT and inhibitors at each respective timepoint. For comparisons of data from two groups, two tailed t-tests were used. Each time point was duplicated in series for 3 to 6 different wild-type mice, and their average of biological replicates determined. The replicates were also used to calculate the standard deviation and SEM. P < 0.05 was considered significant.
Experimental Design:

![Figure 9: Experiment Design Set-Up.](image)

Experimental timepoints throughout mast cell differentiation spanning from 0 hours to 576 hours were used for RNA and protein samples and repeated for both the non-inhibitor samples and inhibited samples. The same timepoints were repeated for each C57BL6/J wild-type mice in the presence or absence of the MAPK inhibitors. Figure created by DD.
6. Results:

I. Description of Experimental Groups

Each of the different trios of wild-types, taken from the same species of C57BL6/J mice, used different amounts of RNA to measure the change in RNA expression over differentiation for the desired targets. For most targets, three different groups of wild-types were used, however each group is being treated as separate experiments due to the differing RNA amounts. The group termed WT26-28’s RNA was isolated at 9 weeks of age and had a concentration set to 85 ng. The group termed WT35-37’s RNA was isolated at 29 weeks of age and had a concentration set to 36 ng. While, the group termed WT47-49’s RNA was isolated at 10 weeks of age and had a concentration set to 200 ng (Table 19). The older group of mice (29 weeks) significantly produced an overall less amount of RNA when compared to the other wild-type groups.

Another difference that existed between the wild-type groups is the concentration of the ERK inhibitor SCH772984. WT26-28 was grown in the presence of SCH772984 at a concentration of 10 μM, while WT35-37 and WT47-49 were grown at a concentration of 1 μM, as shown in Table 19. This discrepancy was due to cell death induced by the high concentration of SCH772984 for WT26-28, while the lower concentration did not produce the same cell death. The gel electrophoresis analysis, as shown in appendix Figures S15-S31, and the melt curve analysis, see appendix Figures S36-51 confirmed the specificity of the gene targets amplified through qPCR. The results of the gel electrophoresis and melt curve analysis shows that the results for each gene of interest is highly specific, and is not a byproduct of unintended experimental consequences, such as primer-primer dimerization and unspecific primer binding.
Table 19: qPCR Experimental Group Classifications. All RNA targets were repeated three times, with slight differences occurring between the experiments. The first experiment was tested with the ERK inhibitor SCH772984 at a concentration of 10 µM, which was reduced to 1 µM with the repeated experiments. The age of the mice and the volume of RNA in ng was different across experiments.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Wild Type Mice</th>
<th>Age</th>
<th>Volume RNA in RT</th>
<th>Concentration SCH772984</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>WT26, 27, &amp; 28</td>
<td>9 weeks</td>
<td>85 ng</td>
<td>10 µM</td>
</tr>
<tr>
<td>2</td>
<td>WT35, 36, &amp; 37</td>
<td>29 weeks</td>
<td>36 ng</td>
<td>1 µM</td>
</tr>
<tr>
<td>3</td>
<td>WT47, 48, &amp; 49</td>
<td>10 weeks</td>
<td>200 ng</td>
<td>1 µM</td>
</tr>
</tbody>
</table>

II. HDAC and HAT RNA Expression

The RNA expression of HDACs and HATs is regulated by the activation and the inhibition of the MAPK signaling pathway, however this has only been shown in other cell types, not in mast cell differentiation, as shown in literature review Tables 1-9. To assess the potential role of MAPK signaling on epigenetic modifier expression during mast cell differentiation, bone marrow was collected and induced to undergo mast cell differentiation for the collection of samples over time. Significance was compared in two different calculations. Normal gene expression without the presence of the MAPK inhibitors was compared back to Time 0 hours. Significance was also compared between the MAPK inhibitors to the non-inhibited sample within the same timepoint.

HDAC1’s relative expression during normal differentiation for both the WT26-28 and WT35-37 groups showed a significant increase in expression 6 hours after the initiation of differentiation (Figure 10). This was followed by a decrease in expression with the level of expression remaining constant for the remained for differentiation. The inhibition of JNK at 72h significantly increased the relative gene expression of HDAC1, however this was only shown in WT35-37 (Figure 10B) and was not repeated in WT26-28 (Figure 10A). The inhibition of the other branches of the MAPK signaling pathway...
(ERK and p38) did not have a significant influence on HDAC1 expression during mast cell differentiation (Figure 10).

Another member of Class I HDACs with HDAC1, HDAC2 also demonstrated significantly decreased 24 hours after the initiation of differentiation, with the trend in gene expression remaining constant for the remainder of differentiation for normal mast cell differentiation without the presence of MAPK inhibitors (Figure 11A&B). The inhibition of the p38 MAPK signaling pathway did not have a significant effect on HDAC2 gene expression during mast cell differentiation. The inhibition of JNK significantly decreased HDAC2 gene expression at 3 hours and increased the relative level of HDAC2 gene expression 72 hours after the initiation of differentiation for WT47-49, however this was not shown in the experiments. The inhibition of ERK significantly reduced the relative level of HDAC2 gene expression at 576 hours for WT35-37 (Figure 11).

The final member of the HDAC Class I group is HDAC3, which in pace with the other members of its group, demonstrated a significant increase in normal HDAC3 gene expression 6 hours, followed by a decrease 72 hours after the initiation of differentiation. Normal HDAC3 gene expression was only significantly reduced after 72 hours of differentiation, for the WT35-37 group (Figure 12). The inhibition of ERK with SCH772984 significantly affected HDAC3 gene expression during differentiation. SCH772984 increased the relative level of HDAC3 gene expression across differentiation; however only at timepoint 72 hours was the increase significant. The other members of the MAPK signaling family, JNK and p38, did not affect HDAC3 gene expression during differentiation (Figure 12).
Figure 10: HDAC1 RNA expression in the presence of MAPK inhibitors. 
A. WT26-28 9 weeks old aged C57BL6/J mice and B. WT35-37 29 weeks old aged C57BL6/J mice demonstrated a slight increase in gene expression at 6 hours before returning to base level set by time 0 hours. A slight decrease in gene expression also occurred 72 hours after the initiation of differentiation. The MAPK signaling pathway inhibitors did not have a significant effect on expression during differentiation. * p < 0.05
Figure 11: HDAC2 RNA expression in the presence of MAPK inhibitors. A. WT26-28 9 weeks B. WT35-37 29 weeks and C. WT47-49 10 weeks old aged C57BL6/J mice demonstrated a slight decrease in expression at 3 and 24 hours compared to the baseline established by time 0h over differentiation. The inhibition of ERK only significantly decreased expression at 576 hours for WT35-37, while the inhibition of JNK had varying significant effects for WT47-49. * p < 0.05, ** p < 0.01
Figure 12: HDAC3 RNA expression in the presence of MAPK inhibitors.  
A. WT26-28 9 weeks old aged C57BL6/J mice and B. WT35-37 29 weeks old aged C57BL6/J mice demonstrated that HDAC3 gene expression is increased at 6 hours, while being significantly decreased at 72 hours when compared to the initiation of differentiation at 0 hours. Only the inhibition of ERK significantly influenced expression, with SCH772984 increasing relative expression over time.  
* p < 0.05, ** p < 0.01
The only member of HDAC Class IIa that was of interest was HDAC4, which showed highly dynamic expression over normal differentiation. HDAC Class IIa contains three members, HDAC4, HDAC5 and HDAC7. All three of these members are involved in the deacetylation of acetyl residues found on histones, however HDAC4 is the most active and prominent member (11). As well, we showed that across mast cell differentiation, these members are activated at the same timepoints, however significance was greater with HDAC4, see appendix S52. After 3 hours of induced differentiation, the relative level of HDAC4 gene expression was significantly increased. This remained until 72 hours, where the level of expression returned to baseline set by 0 hours, where it remained (Figure 13A, C). WT35-37 showed HDAC4 expression increase slightly after 3 hours of differentiation, however this immediately returned to baseline established by 0 hours (Figure 13B). The MAPK signaling pathway appears to be influential in the expression of HDAC4. The inhibition of ERK by SCH772984 significantly increased the relative level of HDAC4 gene expression from 3 hours to 24 hours for WT26-28 and WT47-49 (Figure 13A, C). The inhibition of p38 by Losmapimod also significantly increased the relative level of HDAC4 gene expression across differentiation, with significance reached for all wild-type groups (Figure 13).

The final Class of HDACs, Class IIb, only contains one member, HDAC6, which demonstrated constant gene expression across differentiation. The inhibition of ERK for WT35-37, showed a significant increase in HDAC6 expression 3 hours after the initiation of differentiation (Figure 14B). The other MAPK inhibitions, JNK-IN-8 and Losmapimod did not influence HDAC6 gene expression during mast cell differentiation (Figure 14).
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Figure 13: HDAC4 RNA expression in the presence of MAPK inhibitors.

A. WT26-28 9 weeks old and C. WT47-49 10 weeks old aged C57BL6/J mice demonstrated significant increase in gene expression following 3 hours of differentiation, remaining elevated until 72 hours where expression returns to the baseline established by time 0 hours. The inhibition of ERK and p38 significantly increased the level of expression over differentiation, while JNK-IN-8 had no effect. B. WT35-37 29 weeks old aged C57BL6/J mice had constant relative level of expression over differentiation with only the inhibition of p38 positively influencing expression. * p < 0.05, ** p < 0.01, *** p < 0.001
Figure 14: HDAC6 RNA expression in the presence of MAPK inhibitors. 
A. WT26-28 9 weeks old aged C57BL6/J mice and B. WT35-37 29 weeks old aged C57BL6/J mice demonstrated that HDAC6 gene expression was constant over mast cell differentiation. * p < 0.05
Important HAT member CBP (CREB-binding protein) produced differing results between the different wild-type groups (Figure 15). Normal differentiation for WT26-28 showed a significant increase 3 hours after the initiation of differentiation. This remained elevated until 72 hours, where the relative level of CBP gene expression returned to baseline set by 0 hours, where it remained until 576 hours where it was slightly elevated (Figure 15). Normal differentiation for WT35-37 and WT47-49 showed constant CBP gene expression across differentiation, much different from what was shown by WT26-28 (Figure 15B, C). However, the inhibition of the MAPK signaling pathway had no effect on CBP gene expression for any of the wild-type groups, maintaining levels of expression like that determined by the non-inhibited samples (Figure 15).

Another HAT this is important in the regulation of histone acetylation is HAT p300, which showed a significant increase in gene expression 3 hours after differentiation was induced for WT26-28 (Figure 16A). This increase remained elevated until 72 hours, where the relative level of expression remained slightly elevated above the baseline set by 0 hours. The other wild-type group produced slightly different results, showing constant p300 gene expression across differentiation, instead of the significant increase (Figure 16B). However, the presence of the MAPK inhibitors did not have any effect on normal p300 gene expression across differentiation for either wild-type groups (Figure 16).

KAT2A (Lysine acetyltransferase 2A) is another HAT that is important in histone acetylation regulation, however KAT2A gene expression demonstrated a constant trend across differentiation (Figure 17). The inhibition of ERK significantly decreased the relative level of KAT2A gene expression by 24 hours for WT26-28 (Figure 17A), however this was not observed for WT35-37 (Figure 17B). The inhibition of JNK and p38 did not influence KAT2A gene expression during mast cell differentiation.
PCAF (Lysine acetyltransferase 2B) is the final HAT that is necessary in the regulation of histone acetylation and balance with HDACs that is measured. WT26-28 PCAF gene expression was significantly increased after 3 hours of differentiation before returning to the baseline established by 0 hours at 72 hours (Figure 18A). Afterwards, PCAF gene expression begins to increase until significance is reached again at 576 hours. The inhibition of p38 significantly increased this trend across differentiation, while the inhibition of ERK and JNK did not have any influence on normal gene expression. WT35-37 and WT47-49 PCAF uninhibited gene expression decreased over differentiation until 72 hours and 144 hours which resulted in significance, before increasing and returning to the baseline set by 0 hours (Figure 18B, C). The inhibition of p38 increased the relative level of PCAF expression across differentiation, however significance was not reached. The inhibition of ERK increased the relative level of PCAF expression at 72 hours, however this was only observed in WT47-49 (Figure 18C) and with the other experimental groups. The inhibition of JNK did not have any effect on PCAF gene expression for either wild-type groups across differentiation.
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Figure 15: CBP RNA expression in the presence of MAPK inhibitors.
A. WT26-28 9 weeks old aged C57BL6/J mice CBP gene expression was significantly increased following 3 hours of differentiation until 72 hours when expression returned to baseline set by 0 hours. B. WT35-37 29 weeks and C. WT47-49 10 weeks old aged C57BL6/J mice had no relative change in expression over differentiation. The MAPK signaling pathway did not influence gene expression. * p < 0.05, ** p < 0.01
Figure 16: p300 RNA expression in the presence of MAPK inhibitors.
A. WT26-28 9 weeks old aged C57BL6/J mice relative p300 expression was significantly elevated after 3 hours of induced differentiation remaining elevated over time. B. WT35-37 29 weeks old aged C57BL6/J mice demonstrated no change in relative expression over differentiation. The MAPK signaling pathway did not influence gene expression over mast cell differentiation. * p < 0.05, ** p < 0.01, *** p < 0.001
Figure 17: KAT2A RNA expression in the presence of MAPK inhibitors. 
A. WT26-28 9 weeks old aged C57BL6/J mice and B. WT35-37 29 weeks old aged C57BL6/J mice KAT2A gene expression did not significantly change over differentiation. ERK inhibition only significantly decreased expression in WT26-28 mice at 24 hours, while the older mice had no significant effects by MAPK inhibition. * p < 0.05, ** p < 0.01
Influence of MAPK on mast cell differentiation and histone acetylation modifiers

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Figure 18: PCAF RNA expression in the presence of MAPK inhibitors.

A. WT26-28 9 weeks old aged C57BL6/J mice demonstrated an increase in expression at 3 hours, returning to baseline at 72 hours before increasing over differentiation. The inhibition of p38 significantly increased relative expression across all differentiation timepoints.

B. WT35-37 29 weeks old aged mice showed decreased expression over differentiation, reaching significance at 72 hours, before returning to the baseline set by 0 hours.

C. WT47-49 10 weeks old aged mice had constant expression across differentiation, with the inhibition of ERK significantly increasing PCAF expression at 72 hours. * p < 0.05, ** p < 0.01
III. Mast Cell Specific Marker Gene Expression

Cell surface marker expression is used as a phenotypic indicator for specific cells and can be used to separate the specific cell out of a larger population of cells. In humans, CD34^med/high expression is indicative of HSC populations and is a method of isolating them from the other white blood cells from within the bone marrow, whereas lin^−, c-kit^+, Sca-1^+, and CD34^low is typical in mice. CD34 RNA expression demonstrated that the relative level of CD34 gene expression increased across mast cell differentiation with the highest level of expression occurring after 576 hours (Figure 19). The inhibition of JNK significantly reduced the relative level of CD34 gene expression both early (3 and 24 hours) and late in differentiation (144 and 576 hours), while p38 inhibition significantly reduced CD34 gene expression late in differentiation at 144 and 576 hours. The inhibition of ERK had no effect on CD34 gene expression across mast cell differentiation.
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Figure 19: CD34 RNA expression in the presence of MAPK inhibitors. A. WT26-28 9 weeks old aged C57BL6/J mice and B. WT35-37 29 weeks old aged C57BL6/J mice demonstrated moderate levels of expression gradually increasing over differentiation, until high levels of expression was reached at 576 hours. The inhibition of p38 and JNK individually reduced the level of expression significantly over differentiation for A., while no significance was reached in B. * p < 0.05, ** p < 0.01, *** p < 0.001
An important enzyme that is required for mast cell function is Cpa3 (mast cell carboxypeptidase A). Normal Cpa3 RNA expression during mast cell differentiation demonstrated low relative levels of expression early in differentiation. However, 72 hours after differentiation, a significant increase in Cpa3 gene expression occurred, and continued to increase along with differentiation (Figure 20). The inhibition of the MAPK signaling pathway produced differing results depending on the inhibited branch. The inhibition of ERK significantly increased the relative level of Cpa3 gene expression throughout differentiation (Figure 20B, C). Whereas the inhibition of p38 decreased the relative level of Cpa3 gene expression with significance only being met by WT26-28 (Figure 20A). The inhibition of JNK significantly increased the relative level of Cpa3 expression 72 and 144 hours after the initiation of differentiation (Figure 20A, B).

Another enzyme that is required for adequate mast cell development is Cma1 (Chymase 1). Uninhibited Cma1 gene expression during mast cell differentiation showed low relative levels of expression early into differentiation; with a significant 1000 (Figure 21B) to 5000 (Figure 21A) fold increase in expression occurring by 576 hours after initiation. The inhibition of JNK and p38 significantly reduced the relative level of Cma1 expression throughout differentiation, individually. The inhibition of ERK also reduced the relative level of expression, with significance reached at 72 and 144 hours (Figure 21B, C).
The final enzyme of interest that is necessary for appropriate mast cell function is Tpsb2 (Tryptase beta 2). During mast cell differentiation, early timepoints demonstrated low levels of Tpsb2 gene expression, which significantly increased by the end of differentiation at 576 hours (Figure 22). The relative level of expression reached a 3000 (B) to 70000 (A) fold increase by 576 hours. The inhibition of all three branches of the MAPK signaling pathway (JNK, ERK and p38) significantly reduced the expression of Tpsb2 late in mast cell differentiation, individually (Figure 22). The inhibition of JNK significant decreased the relative level of Tpsb2 gene expression at 576 hours (Figure 22A). The inhibition of ERK by SCH772984 significantly reduced the relative level of Tpsb2 expression late in mast cell differentiation at 144 and 576 hours. The inhibition of p38 also significantly reduced the relative level of expression 72 hours to 576 hours for all the experimental groups (Figure 22).
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**Figure 20: Cpa3 RNA expression in the presence of MAPK inhibitors.**

A. WT26-28 9 weeks, B. WT35-37 29 weeks and C. WT47-49 10 weeks old aged C57BL6/J mice normal Cpa3 gene expression significantly increased over differentiation. The inhibition of p38 only reached significance in A., significantly decreasing expression over time. ERK inhibition significantly increased Cpa3 expression for WT35-37 and WT47-49. * p < 0.05, ** p < 0.01, *** p < 0.001
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Figure 21: Cma1 RNA expression in the presence of MAPK inhibitors. 
A. WT26-28 9 weeks, B. WT35-37 29 weeks and C. WT47-49 10 weeks old aged C57BL6/J mice demonstrated a gradual increase in expression over differentiation, until 576 hours when expression significantly increased. The inhibition of p38 and JNK significantly reduced Cma1 gene expression across all differentiation time points (A, C). ERK inhibition significantly reduced the relative level of Cma1 expression (C).

* p < 0.05, ** p < 0.01, *** p < 0.001
Figure 22: Tpsb2 RNA expression in the presence of MAPK inhibitors.
A. WT26-28 9 weeks, B. WT35-37 29 weeks C. WT47-49 10 weeks old aged C57BL6/J mice normal Tpsb2 gene expression significantly increased over differentiation. The inhibition of p38 and ERK significantly reduced the level of expression at the later timepoints, individually. The inhibition of JNK did not have an influence on Tpsb2 expression. * p < 0.05, ** p < 0.01, *** p < 0.001
One of the receptors that are indicative of a mature mast cell is the FcεRIα (Fc fragment of IgE receptor 1α). Normal RNA expression of FcεRIα demonstrated very low levels of expression early in differentiation, with expression significantly increasing as differentiation continues (Figure 23). The inhibition of ERK increased the relative level of FcεRIα gene expression over differentiation, with significance reached at 144 hours (Figure 23C) and 576 hours (Figure 23B). The inhibition of JNK and p38 did not have a significance effect on FcεRIα expression during mast cell differentiation (Figure 23).

The other receptor that is indicative of a mature mast cell is FcεRIβ (Fc fragment of IgE receptor 1β). Normal RNA expression of FcεRIβ showed low levels of expression early in differentiation, with expression significantly increasing through the later timepoints, beginning at 72 hours after the initiation of differentiation (Figure 24). The inhibition of ERK increased the relative level of FcεRIβ gene expression across FcεRIβ gene expression. Significance was only reached, however at 144 hours (Figure 24B). The inhibition of p38 affected FcεRIβ gene expression inversely to ERK inhibition, with the inhibition of p38 significantly decreasing FcεRIβ gene expression late into mast cell differentiation (Figure 24A). However, this was not shown for WT35-37, as the inhibition of p38 did not influence FcεRIβ gene expression (Figure 24B). The inhibition of JNK did not have any effect on FcεRIβ gene expression during differentiation.
Figure 23: FceRIα RNA expression in the presence of MAPK inhibitors. A. WT26-28 9 weeks, B. WT35-37 29 weeks and C. WT47-49 10 weeks old aged C57BL6/J mice demonstrated significantly increased gene expression over differentiation. The inhibition of ERK significantly influenced expression by positively increasing the relative level of expression late into differentiation (B and C). The inhibition of p38 and JNK did not influence FceRIα relative gene expression. * p < 0.05, ** p < 0.01, *** p < 0.01
Figure 24: FcεRIβ RNA expression in the presence of MAPK inhibitors. A. WT26-28 9 weeks old aged C57BL6/J mice and B. WT35-37 29 weeks old aged C57BL6/J mice showed significant increase in gene expression over differentiation. The inhibition of p38 only significantly decreased the relative level of expression in A., while no significant influence was shown in B. The inhibition of ERK only slightly increased expression, with no significance reached. JNK inhibition had no effect on FcεRIβ gene expression. * p < 0.05, ** p < 0.01, *** p < 0.001
An important transcription factor that is involved in the regulation of metabolic adaptations is HIF1α (Hypoxia-inducible factor 1 alpha). Normal HIF1α RNA expression during mast cell differentiation demonstrated a significant increase in gene expression 3 hours after differentiation was initiated (Figure 25). This increased until 24 hours, when HIF1α expression reached its peaked relative level of expression. After this, the relative level of HIF1α gene expression decreased through differentiation, remaining slightly above the baseline set by 0 hours (Figure 25). The inhibition of p38 significantly reduced the relative level of HIF1α gene expression only for the early timepoints, while having no influence on the later timepoints (Figure 25A). The inhibition of ERK did not affect the relative level of HIF1α gene expression across differentiation. The inhibition of JNK only significantly affected the relative level of HIF1α gene expression at 576 hours, with the relative level decreased. For WT35-37 no impact was demonstrated by the inhibition of the MAPK signaling pathway on HIF1α gene expression (Figure 25B).

Another important transcription factor that is involved in the lineage commitment of mast cells is MITF (Melanogenesis associated transcription factor). Normal MITF RNA expression during mast cell differentiation demonstrates low levels of expression early in differentiation, which slightly increases after 24 hours. This increase is followed by a decrease in relative MITF expression until 576 hours when a significant increase in gene expression occurred (Figure 26). The inhibition of JNK did not influence MITF gene expression during differentiation. The inhibition of p38 showed a decrease in the relative level of MITF gene expression (Figure 26A, C). However, significance was only reached at 24 hours (Figure 26A). ERK inhibition increased MITF gene expression, with significance only being reached at 144 hours (Figure 26B).
**Figure 25: HIF1α RNA expression in the presence of MAPK inhibitors.**

A. WT26-28 9 weeks old aged C57BL6/J mice and B. WT35-37 29 weeks old aged C57BL6/J mice showed a normal distribution trend. The inhibition of p38 delayed the increase in expression, while also slightly preventing maximal expression. * p < 0.05, ** p < 0.01, *** p < 0.001
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Figure 26: MITF RNA expression in the presence of MAPK inhibitors.

A. WT26-28 9 weeks old aged C57BL6/J mice and B. WT35-37 29 weeks old aged C57BL6/J mice demonstrated a slight increase in expression at 24 hours, returning to baseline set by the 0-hour timepoint, before a significant increase in expression at 576 hours. C. WT47-49 10 weeks old aged mice showed a significant increase in relative gene expression occurring 72 hours after the initiation of differentiation remaining elevated by 144 hours. The inhibition of p38 only significantly reduced expression (A), while ERK inhibition only significantly increased expression at the early timepoints (B). The inhibition of JNK had no effect on expression over differentiation. * p < 0.05, ** p < 0.01, *** p < 0.001
IV. HDAC and HAT Protein Expression

The level of protein expression for HDAC2 was measured at both early timepoints and later timepoints to determine how protein expression is affected during differentiation and to assess whether the trends that were observed at the RNA level of expression were translated into the protein level of expression. The western blot images depict the intensity of the bands with WT32 on top, WT33 in the middle and WT34 on the bottom (Figure 27A). Normal protein expression for HDAC2 demonstrated a trend in protein expression with the relative intensity of HDAC2 protein expression decreasing across mast cell differentiation (Figure 27). The inhibition of ERK had a trend of decreased HDAC2 protein expression across differentiation, with a significant decrease occurring at 144 hours. The inhibition of p38 also had a trend of decreased HDAC2 protein expression with a significant decrease observed 144 hours after the initiation of differentiation (Figure 27B). Coomassie staining was used as total protein to normalize the protein results (Figure 27A).

HDAC4 was further measured at both early timepoints and later timepoints to determine if the effects observed at the RNA level of expression were translated into the protein level of expression. The western blot images depict the intensity of the bands with WT32 on the top, WT32 in the middle and WT34 on the bottom (Figure 28A). Normal HDAC4 protein expression demonstrated a trend of decreasing HDAC4 protein expression over mast cell differentiation. The inhibition of p38 significantly reduced the level of HDAC4 protein expression at 24 hours, however this was not seen for the other timepoints (6 and 144 hours) (Figure 28B). The inhibition of the MAPK signaling pathway had no effect on HDAC4 protein expression. Protein results was normalized through Coomassie staining (Figure 28A).
Figure 27: HDAC2 protein expression in the presence of MAPK inhibitors. Normal protein expression of HDAC2 has decreased protein expression at 24 hours, which returns to baseline level by 144 hours. The inhibition of ERK significantly decreased the level of protein expression at 144 hours. The inhibition of p38 also decreased protein expression across all timepoints, however significance was not reached (A, B). A third attempt occurred, however no bands were visible. Protein was normalized by Coomassie staining. N = 2; * p < 0.05
Figure 28: HDAC4 protein expression in the presence of MAPK inhibitors. Normal HDAC4 protein expression decreases over differentiation, with significance reached at 144 hours compared to 0 hours. The inhibition of the MAPK signaling pathway had no effect on HDAC4 protein expression (A, B). Protein was normalized by Coomassie staining. N = 2; * p < 0.05, ** p < 0.01
Important HAT PCAF was measured at both early and late timepoints to determine how HAT protein expression changes during differentiation and to measure if the trends that were observed at the RNA level of expression were translated into the protein level of expression. The western blot images depict the intensity of the bands with WT32 on top, followed by WT33 on the bottom (Figure 29A). Normal protein expression for PCAF demonstrates no change in protein expression across differentiation. The presence of MAPK inhibitors did not have an impact on PCAF protein expression, with only the inhibition of ERK showing an increase in protein expression at 6 hours. However, the error bar for this condition deems the result untrusting and may be due to an outlier (Figure 29B). Protein results were normalized by Coomassie staining, which showed total protein loaded per sample (Figure 29A). A third attempted was performed, however, the blot did not show any bands.

Another HAT that was measured at the protein level of expression was CBP to determine the change that occurs during mast cell differentiation and how the MAPK inhibitors influence its expression. The western blot images depict the intensity of the bands with WT32 on top, followed by WT33 in the middle and WT34 on the bottom (Figure 30A). Normal protein expression for CBP demonstrated a constant level of CBP protein expression across mast cell differentiation. The inhibition of ERK increased the intensity of CBP protein expression at both 6 and 24 hours, however due to the size of the error bars this increase did not reach significance (Figure 30B). The inhibition of JNK and p38 did not have any influence on CBP protein expression across differentiation. Protein results were normalized by Coomassie staining, which fluorescently labels total protein for each sample (Figure 30A).
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**Figure 29:** PCAF protein expression in the presence of MAPK inhibitors. Normal level of PCAF protein expression does not change over mast cell differentiation. The presence of MAPK inhibitors did not significantly influence protein expression (A, B). A third attempt occurred, however no bands were visible. Protein was normalized by Coomassie staining. N = 2.
Figure 30: CBP protein expression in the presence of MAPK inhibitors. Normal CBP protein expression does not change over differentiation. The inhibition of ERK increased early protein expression, however significance was not reached. The other MAPK inhibitors (p38 and JNK) had no effect on protein expression (A, B). Protein was normalized by Coomassie staining. N = 3.
V. Histone H3 Protein Expression

Histone H3 has many highly targeted locations that significantly influences the status of gene transcription. Acetylated Histone H3 (Lys 9) protein expression without the presence of MAPK inhibitors demonstrated low levels of protein at 0 hours, which significantly increases 6 hours after the initiation of differentiation. This was followed by decreased protein expression over differentiation with protein expression shown below the baseline set by 0 hours at 144 hours (Figure 31B). The western blot images depict the intensity of the bands with WT32 on top, followed by WT33 in the middle and WT34 on the bottom (Figure 31A). The inhibition of p38 decreased the relative level of protein expression at 24 hours, however significance was not reached. The inhibition of JNK and ERK did not affect the level of Ac-Histone H3 protein expression across mast cell differentiation (Figure 31B). Each sample was normalized by Coomassie staining (Figure 31A).

Histone H3 is not only acetylated at specific lysine residues but is also phosphorylated at serine residues. Phosphorylated Histone H3 (Ser 10) protein expression without the presence of MAPK inhibitors demonstrated that the level of p-Histone H3 protein decreased across differentiation as the highest level of expression occurring before differentiation was initiated at 0 hours (Figure 32B). The western blot images depict the intensity of the bands with WT32 on top, followed by WT33 in the middle and WT34 on the bottom (Figure 32A). The inhibition of p38 decreased the level of p-Histone H3 protein expression at 24 and 144 hours, however significance was not reached. The inhibition of JNK and ERK did not influence p-Histone H3 protein expression across mast cell differentiation (Figure 32B). Total protein was determined by Coomassie staining and was used to normalize the results (Figure 32A).
Figure 31: Ac-Histone H3 protein expression in the presence of MAPK inhibitors. Normal acetyl-histone H3 protein expression significantly increased at 6 hours, while decreasing over differentiation with a significant decrease occurring at 144 hours. The inhibition of the MAPK signaling pathway (JNK, ERK and p38) did not influence Ac-Histone H3 protein activity (A, B). Protein was normalized by Coomassie staining. N = 3; * p < 0.05
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#### Figure 32: Phospho-Histone H3 protein expression in the presence of MAPK inhibitors

Normal phospho-histone H3 protein expression decreased over differentiation, reaching significance at 144 hours. The inhibition of p38 decreased p-Histone H3 protein at 24 and 144 hours, however, no significance was reached. The inhibition of JNK and ERK did not influence phospho-histone H3 protein expression (A, B). Protein was normalized by Coomassie staining. \( N = 2 \)

**A.**

<table>
<thead>
<tr>
<th>Time (Hours)</th>
<th>6</th>
<th>24</th>
<th>144</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>NT</td>
<td>JNK</td>
<td>ERK</td>
</tr>
<tr>
<td>24</td>
<td>NT</td>
<td>JNK</td>
<td>ERK</td>
</tr>
<tr>
<td>144</td>
<td>NT</td>
<td>JNK</td>
<td>ERK</td>
</tr>
</tbody>
</table>

**B.**

![Graph showing phospho-Histone H3 protein expression over time](image)

- **WT32**
- **WT33**
- **Coomassie**
VI. Chromatin Accessibility Through Epigentek’s Chromatin Accessibility Assay Kit

Eukaryotic chromatin is packed in tight complexes containing a histone octamer core and 147 bp of DNA wrapped around, separated by linker DNA. The relative positioning of these nucleosomes regulates in vivo availability of binding sites to transcription factors and the general transcriptional machinery (108). The measurement of chromatin accessibility allows to determine the direct effect that the chromatin structure has on gene transcription. Epigentek’s Chromatin accessibility assay kit aims to measure the level of accessibility that is present for each sample tested (109). As per the protocol suggested by the kit, each sample was prepared alongside a non-digested sample so that a direct comparison could be made on the degree of accessibility. However, the effectiveness of the enzyme was compromised, and optimization was required.

Optimization of the kit was completed with the use of negative controls, RHO, HBB, Tnp1, Alb, Smcp and Prm2, which are all known to have completely closed chromatin conformations that restricts the binding of exogenous nuclease (110–112), see appendix Tables S5-6 and Figures S55-56. Exogenous nuclease bind to open regions of DNAs found within the nucleosome and cut, separating the nucleosome into its individual histone octamer cores (113). These specific genes were chosen because of their shown closed chromatin conformation, so that a complete and adequate comparison between unknown chromatin conformation genes could be determined.

However, the Epigentek’s chromatin kit produced results suggesting that they are in an open conformation, unlike what is shown in literature. Further optimization began with the dilution of the enzyme, fearing that the enzyme was over digesting the samples which is causing the negative controls to appear with an open conformation. The enzyme was diluted up to a factor of 10,000, with results since showing over digestion of the
negative controls. The only condition that produced a closed chromatin conformation occurred at an enzyme dilution of 10,000 for RHO. This was then used to determine completely open chromatin conformation for the positive controls, GAPDH and PPIA. However, neither positive control produced results that indicated completely open chromatin, as shown in appendix Figure S57 and Table S7. Therefore, the Epigentek’s chromatin accessibility assay kit failed to adequate digest chromatin so that the negative controls remain closed and the positive controls are in a completely open chromatin conformation.

VII. Flow Cytometry Expression

The FcεRIα receptor is primarily found on the surface of mast cells, and not on the surface of HSCs. This allows for FcεRIα receptor expression to be an identification on the maturation and differentiation process of mast cells. The mean fluorescent intensity (MFI) at 2.5 weeks of FcεRIα showed that FcεRIα was significantly expressed in the non-inhibited samples when compared to the isotype control, which depicts no expression (Figure 33). The MAPK inhibitors influence on FcεRIα receptor expression was compared to non-inhibitor sample FcεRIα receptor intensity. When compared, the inhibition of JNK and ERK, independently, significantly shifted the histogram to the right indicating an increase in FcεRIα receptor expression (Figure 34), indicating an increase in mast cell differentiation. The inhibition of JNK (JNK-IN-8, 1 µM) had an MFI value of 33,557 compared to a NT MFI value of 23,148, indicating a 10,000 MFI increase (Figure 37). The inhibition of ERK (SCH772984, 1 µM) had an MFI value of 44,294 compared to NT’s MFI value of 23,148, demonstrating a 20,000 MFI increase (Figure 37). The inhibition of p38 significantly shifted the histogram to the left indicating a decrease in
FcεRIα receptor expression (Figure 34). The inhibition of p38 (Losmapimod, 10 µM) had an MFI value of 19,531, indicating a 4,000 MFI decrease (Figure 37). Therefore, the inhibition of JNK and ERK significantly increased the presence of FcεRIα receptors, while p38 inhibition decreased the presence of FcεRIα receptor presence 2.5 weeks after the initiation of mast cell differentiation.

By 3.5 weeks the MFI of FcεRIα under normal differentiation conditions continued to increase, indicating FcεRIα receptor expression was continually expressed on the cells surface (Figure 35). The inhibition of the MAPK signaling pathways had very different results on the MFI of FcεRIα. The inhibition of JNK (JNK-IN-8, 1 µM) did not significantly influence the MFI of FcεRIα, as NT had an average value of 20,924 and JNKi had an average value of 25,829 (Figure 36, 37). The inhibition of ERK (SCH772984, 1 µM) significantly increased the MFI of FcεRIα, 3.5 weeks into mast cell differentiation (Figure 36, 37). The MFI value increased from 20,924 for the NT to 36,067 with the inhibition of ERK (Figure 36). The inhibition of p38 MAPK (Losmapimod, 10 µM) significantly decreased the MFI of FcεRIα, 3.5 weeks into mast cell differentiation (Figure 36, 37). The MFI value decreased from 20,924 for the NT sample to 11,824 with the inhibition of p38 MAPK (Figure 36, 37). Therefore, the ERK and p38 MAPK signaling pathways is influential in the expression of the FcεRIα receptor during mast cell differentiation.
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Figure 33: Mean fluorescent intensity of FceRIα for isotype control cells compared to NT control. Isotype control (PE Armenian Hamster IgG) is shown in yellow, while NT samples are shown in red. The histogram depicts the effects of the fluorescent target compared to isotype control by MFI, 2.5 weeks after the initiation of mast cell differentiation.

Figure 34: Mean fluorescent intensity of FceRIα in the presence or absence of MAPK inhibitors at 2 and a half weeks. Non-treated samples compared to MAPK inhibited samples by MFI, 2½ weeks after the initiation of mast cell differentiation. The inhibition of JNK slightly shifted FceRIα intensity left, indicating an increase. ERK inhibition shifted the plot to the right, increasing FceRIα intensity. The inhibition of p38 shifted the plot to the left, decreasing FceRIα intensity.
Figure 35: Mean fluorescent intensity of FcεRIα for isotype control cells compared to NT control. Isotype control (PE Armenian Hamster IgG) is shown in yellow, while NT samples are shown in red. The histogram depicts the effects of the fluorescent target compared to isotype control by MFI, 3.5 weeks after the initiation of mast cell differentiation.

Figure 36: Mean fluorescent intensity of FcεRIα in the presence or absence of MAPK inhibitors at 3 and a half weeks. Non-treated samples compared to MAPK inhibited samples by MFI, 3 ½ weeks after the initiation of mast cell differentiation. The inhibition of JNK did not influence FcεRIα intensity. ERK inhibition significantly shifted the plot to the right, increasing FcεRIα intensity. The inhibition of p38 significantly shifted the plot to the left, decreasing FcεRIα intensity.
Figure 37: The mean fluorescent intensity of FcεRIα in the presence of MAPK inhibitors across differentiation. The inhibition of JNK and ERK significantly increased the MFI of FcεRIα. While, the inhibition of p38 significantly reduced the MFI of FcεRIα. N = 3; * p < 0.05, ** p < 0.01, *** p < 0.001
7. Discussion:
7.i. MAPK Inhibitor Conformation

This research was designed to investigate the role of the MAPK signaling pathway on histone modifiers at multiple levels of expression, including RNA, protein and cellular phenotype expression. Literature has shown that the MAPK signaling pathway is dynamically involved in influencing histone modifier expression, including HATs and HDACs. As indicated in the literature review section, each of the different branches of the MAPK signaling pathway differentially influences HAT and HDAC expression and activity. As well, important transcription factors, enzymes and receptors necessary for mast cell differentiation and function were also differentially influenced by the branches of the MAPK signaling pathway. This led us to the hypothesis that the MAPK signaling pathway is directly and/or indirectly involved in the regulation of mast cell differentiation through the modulation of histone modifiers.

The ERK signaling pathway branch is phosphorylated by MEKs, which in turn is activated by Raf and Ras phosphorylation. ERK inhibitor SCH772984 has been shown to inhibit ERK1 and ERK2 activity with IC50 values of 4 and 1 nmol/L, respectively, through the inhibition of p90 ribosomal S6 kinase and ERK activation loop residue phosphorylation (114). Importantly, SCH772984 inhibits both ERK enzymatic activity and MEK phosphorylation (114). We further showed that SCH772984 (10 µM) selectively inhibited ERK1/2 phosphorylation activity and did not inhibit the other targets of the MAPK signaling pathway, see appendix Figure S1. However, when the cells were treated with 10 µM of SCH772984, cell death was observed. This prevented the collection of RNA for those cells at the later timepoints. Therefore, 1 µM of SCH772984
was used for the remainder of experiments as that concentration did not cause cellular death.

The JNK signaling pathway is primarily activated by cytokines and environmental stress and is directly activated by the phosphorylation of MEK4/7. JNK then activates activator protein-1 (AP-1) transcription factor through the phosphorylation of c-Jun, allowing c-Jun to translocate into the nucleus upregulating transcription (115). JNK inhibitor JNK-IN-8 has been shown to inhibit the JNK signaling pathway preventing the phosphorylation of c-Jun (105). Due to experimental limitations and the non-presence of protein bands, we were unable to show that JNK-IN-8 (1 µM) selectively inhibits the JNK signaling pathway, see appendix Figure S3. This inconclusive inhibitory effect could, however, just be due to antibody specificity issues or the weak expression of JNK by the C57BL6/J differentiating mast cells. Despite this, the inhibition of JNK by JNK-IN-8 has been shown in literature regarding its mechanism of action and high specificity, allowing us the confidence that JNK-IN-8 is inhibiting JNK phosphorylation (105–107).

The p38α MAPK signaling pathway is rapidly phosphorylated in response to lipopolysaccharides and stress. Once activated by the phosphorylation of MEK3/6, p38α regulates cell survival and differentiation through the activation of its downstream signaling molecules (116). p38α inhibitor Losmapimod is a relatively new inhibitor that is currently undergoing Phase III clinic trials and has shown inhibition of TNFα, a p38α downstream proinflammatory cytokine (103). We have shown that Losmapimod (10 µM) selectively inhibited the relative level of p-p38 protein, while not inhibiting the other branches of the MAPK signaling pathway, see appendix Figure S2. Therefore, the use of Losmapimod selectively inhibited only the p38 branch and not the other branches of the MAPK signaling pathway.
The inhibition of the different branches of the MAPK signaling pathway, JNK, ERK and p38 by JNK-IN-8, SCH772984 and Losmapimod, respectively, is highly specific and we have confidence that they are being inhibited specifically with minimal crosstalk in our experiments, from both our experimental results and the results shown in literature. The results caused by the inhibition is due to the specific branch being inhibited and not due to the other branches being unintentionally inhibited.

7.ii. JNK MAPK Inhibition During Mast Cell Differentiation

JNK is specifically activated through the MAPK signaling pathway, leading to a cascade of phosphorylations until MEK4/5, which directly phosphorylates JNK1/2/3 (115). The inhibitor JNK-IN-8 specifically targets JNK1/2/3, preventing the signal propagating through this pathway to be continued. The JNK inhibitor was continually used at a concentration of 1 µM, for RNA, protein and cellular phenotype, and stated earlier, is designed to only inhibit its specific target with little unintended inhibitions.

The inhibition of JNK by JNK-IN-8 had no effect on any of the HDAC and HAT RNA targets, including HDAC1-4, HDAC6, CBP, p300, KAT2A and PCAF. This indicates that the JNK signaling pathway does not directly interact with the histone acetyl regulatory proteins and does not influence the histone structure during mast cell differentiation. As well, the inhibition of JNK did not have an influence on important transcription factors HIF1α and MITF, indicating that these transcription factors are activated through other signaling pathways. However, as the original conformation on JNK-IN-8 inhibition on JNK did not prove any results, the reason that these targets were not influenced may be due instead because JNK is weakly expressed in our cell model.
The inhibition of JNK significantly reduced the relative level of expression of HSC cell marker CD34 during mast cell differentiation (Figure 19). Normal expression of CD34 showed an increase in relative RNA expression across differentiation, with peak expression occurring 576 hours after the initiation. Therefore, the activation of the JNK signaling pathway increases the relative mRNA expression of CD34 and suggests a more HSC phenotype due to increased receptors expression.

The inhibition of JNK had varying effects on important mast cell enzymes during mast cell differentiation. JNK-IN-8 had no effect on Cpa3 mRNA expression, while slightly decreasing Tpsb2 and Cma1 mRNA expression. This decrease in Tpsb2 and Cma1 indicates that the activation of the JNK signaling pathway increases the transcription of these genes. Therefore, at the RNA level of expression the JNK signaling pathway had no effect on HDACs, HATs, transcription factors and mast cell-specific enzymes.

This translated to the protein level of expression as well, as the inhibition of the JNK signaling pathway had no effect on the protein level of expression of key HDACs and HATs. JNK-IN-8 (1 µM) had no effect on HDAC2 and HDAC4 protein expression across mast cell differentiation. This also occurred for PCAF and CBP, which depicted protein expression like that of the non-inhibited samples. This suggests that the JNK signaling pathway does not likely influence histone acetylation through the modulation of HDACs and HATs RNA and protein level of expression during mast cell differentiation.

Overall, these results suggest that the JNK signaling pathway has no influence on the chromatin accessibility of key mast cell specific genes and histone modifier gene expression during mast cell differentiation. The only effect that JNK had, was on Tpsb2 and Cma1 mRNA expression, with the activation of JNK slightly increasing their relative
RNA expression. This suggests that JNK has some influence on the differentiation events that lead to the expression of these two important mast cell enzymes.

Figure 38: JNK MAPK signaling effects on histone modifiers and mast cell specific mediator and markers expression. The inhibition of JNK by JNK-IN-8 had no effect on histone modifier expression, however JNK-IN-8 did decrease specific mast cell enzyme Cma1 and receptor FceRIβ expression. JNK inhibition only increased the expression of Cpa3. Figure created by DD.

7.iii. ERK MAPK Inhibition During Mast Cell Differentiation

ERK is the second branch of the MAPK signaling pathway and is activated through a cascade of phosphorylations until MEK1/2, which directly phosphorylates ERK1/2 (117). The inhibitor SCH772984 (1 µM) significantly inhibited ERK1/2, preventing the continuation of the phosphorylation cascade. Generally, the inhibition of the ERK signaling cascade acts as a positive regulator on HDAC gene expression, while inhibiting the expression of key HAT gene expression. This suggests that the ERK signaling pathway may work to change the balance of acetyl events toward looser
chromatin (increased acetylation), overall, working to attenuate, slow, or control mast cell differentiation, potentially through histone acetylation-dependent decreases in chromatin accessibility at specific genes.

The inhibition of ERK had varying effects on the relative level of HDAC RNA expression. SCH772984 had no effect on the relative expression of HDAC1, HDAC2 and HDAC6 mRNA expression during differentiation, with the trend in expression remaining the same as the non-treated samples. HDAC3 and HDAC4 mRNA expression was significantly increased across differentiation, indicating that the inhibition of ERK shifted the chromatin conformation towards a heterochromatin conformation, through reduced histone acetylation. Little is known in literature regarding the interactions of the ERK signaling pathway and HDAC3, however, HDAC4 and ERK are known to interact and cause HDAC4 to translocate from the cytoplasm to the nucleus upon binding.

In a study by Zhou, et al., ERK1/2 associates with HDAC4 to increase the number of cells expressing HDAC4 in the nucleus, and provides evidence linking ERK to chromatin remodeling through histone deacetylation (39). When ERK signaling protein, Ras is co-expressed with HDAC4, an increase in intracellular localization as nuclear-HDAC4 was observed. This also occurred with ERK1/2 activating kinase MEK1 resulting in an increase in nuclear-HDAC4 (39). This translocation of HDAC4 into the nucleus increasing histone deacetylase activity, and causes a conformational change forming a heterochromatin conformation.

These results are parallel to what is shown with our results, as an inhibition of ERK causes an increase in HDAC4 mRNA expression. This inhibition could cause HDAC4 to remain in the cytoplasm and outside of the nucleus, with HDAC4 requiring ERK to present as a complex to allow translocation. Therefore, our results show that the
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ERK signaling pathway negatively influences HDAC4 gene expression, possibly through a nuclear translocation mechanism. However, further research would be required to confirm this hypothesis. As well, in terms of histone modification, the activation of the ERK signaling pathway favors a heterochromatin conformation across mast cell differentiation, preventing the full maturation to a mast cell.

The inhibition of ERK had varying effects on HATs across mast cell differentiation, as it influenced some of the targeted HATs while having no effect on others. SCH772984 had no effect on CBP, p300 and KAT2A gene expression across mast cell differentiation. Instead, SCH772984 inhibition significantly upregulated the expression of PCAF RNA expression during early mast cell differentiation. The inhibition of ERK significantly increased PCAF gene expression, while having no influence late into differentiation. This was further shown at the protein level of expression, as the inhibition of ERK increased the relative level of PCAF protein. In a study by Puttagunta, et al., a decrease in p-ERK resulted in an increase in nuclear PCAF translocation and acetylation of H3K9 in primary neuron cultures (118). Therefore, the activation of the ERK signaling pathway reduces histone acetylation through decreased PCAF translocation, favoring a heterochromatin conformation and decreased mast cell differentiation.

The inhibition of ERK had a significant impact on mast cell enzymes Cpa3, Cma1 and Tpsb2 gene expression across mast cell differentiation. SCH772984 significantly increased Cpa3 RNA expression across mast cell differentiation. This increase in Cpa3 gene expression indicates that the activation of ERK acts as an inhibitor for mast cell marker Cpa3 gene expression, indicating a defective mast cell maturation. This decrease in Cpa3 gene expression could be due to the decreased PCAF and HDAC4, suggesting an
overall decrease in histone acetylation, preventing the promoter region of Cpa3 from being accessible for the transcriptional machinery. A direct influence may also occur, as ERK directly decreased the relative level of Cpa3 gene expression, without the influence of histone modifications. However, this decrease in Cpa3 gene expression still favors less mast cell maturation and reduces the process of mast cell differentiation.

SCH772984 had the opposite influence on Tpsb2 and Cma1 gene expression, as the inhibition of ERK significantly reduced their relative gene expression across mast cell differentiation. This indicates that the activation of the ERK signaling pathway increases key mast cell tryptase and chymase mRNA and leads to increased mast cell phenotype. Therefore, the ERK signaling pathway is directly involved in the production of key mast cell tryptase and chymase, Tpsb2 and Cma1, however this may not be through direct histone modifications, due to ERK not significantly influencing HDAC and HAT expression during differentiation. The inhibition of ERK by SCH772984 had no influence on MITF and HIF1α transcription factor RNA expression, indicating that ERK does not influence key mast cell transcriptional machinery.

This activation in mast cell differentiation was further seen in the increase in FcεRIα receptor on the surface of developing mast cells. The inhibition of ERK increased the MFI of FcεRIα during differentiation, indicating that ERK inhibition causes an increase in FcεRIα receptor presence on the surface of the cells. This would also indicate that the ERK signaling pathway acts to repress FcεRIα receptor presence on the developing mast cell. Therefore, the activation of the ERK signaling pathway represses mast cell differentiation due to the reduced presence of key mast cell receptor, FcεRIα.

The ERK signaling pathway is shown to be involved in the differentiation of mast cells both through histone modifications and direct mast cell specific marker expression.
The activation of the ERK signaling pathway may lead to the formation of either ERK-HDAC4, facilitating its translocation from the cytoplasm to the nucleus, while also preventing PCAF from acetylating lysine residues. This leads to an impairment in mast cell differentiation through a restriction of chromatin accessibility at specific genes, including important mast cell marker Cpa3. As well, ERK signaling may also directly influence mast cell specific tryptase and chymase RNA expression, leading to an increase in key mast cell enzymes. The cellular phenotype of the mast cell was also significantly impaired with the activation of ERK signaling, as the presence of FceRIα receptor on the cell surface was reduced during mast cell differentiation. Therefore, ERK signaling may be a positive regulator of HDAC4, while being a negative regulator of PCAF, to ultimately impair mast cell differentiation, shown through a decrease in mast cell surface receptors presence.

Figure 39: ERK MAPK signaling effects on histone modifiers and mast cell specific mediator and markers expression. The inhibition of ERK by SCH772984 led to the decreased expression of key mast cell enzymes and HDAC2, while increasing HAT and mast cell transcription factor and receptor expression. Figure created by DD.
7.iv. p38 MAPK Inhibition During Mast Cell Differentiation

p38 is the third branch of the MAPK signaling pathway, initially activated through ligand-receptor interactions and a cascade of phosphorylations before MEK3/6 which directly phosphorylates p38α/β/γ (116). The inhibitor Losmapimod (10 µM) significantly inhibited p38α/β preventing the cascade of phosphorylations from continuing. The activation of the p38 MAPK signaling cascade acts as a negative regulator for both HDACs and HATs and post-transcriptionally drives the expression of key mast cell markers. This indicates that p38 MAPK signaling may act on mast cell differentiation through histone modifications or possibly other mechanisms as well.

The inhibition of p38 had no effect on Class I HDACs, HDAC1, HDAC2 and HDAC3 and Class IIb HDAC6 gene expression. However, the inhibition of p38 significantly increased the relative level of HDAC4 gene expression across mast cell differentiation. In the study by Zhou, et al., the activation of p38 causes a significant increase in caspase activities leading to an increase degradation in HDAC4 and drives the continued differentiation of chondrocyte differentiation (119). Therefore, during mast cell differentiation, the activation of p38 through decreased HDAC4 expression drives the chromatin to form an euchromatin conformation for increased accessibility of key mast cell genes.

The inhibition of p38 MAPK had no influence on any of the tested HATs, including CBP, p300, KAT2A and PCAF. This indicates that the p38 MAPK signaling pathway is not involved in the regulation of histone acetylation through HAT mechanisms, but only involves histone modifications through the regulation of HDAC expressions. This was also translated to the protein level of expression, as the inhibition
of p38 did not have any influence of HDAC2, HDAC4, CBP and PCAF protein activity across mast cell differentiation.

The inhibition of p38 by Losmapimod did have a significant impact on mast cell marker and transcription factor expression across differentiation. The inhibition of p38 had a significant decrease on important transcription factor MITF RNA expression late into differentiation, indicating that p38 MAPK activation is required for its transcriptional activation. This is important in terms of mast cell differentiation, as MITF targets c-kit, a necessary receptor found on mature mast cells (120). In the study by Mansky et al., MITF was required for the terminal differentiation of osteoclasts. The expression of MITF was negatively impacted with the inhibition of p38 in RAW264.7 cells (121). The phosphorylation of MITF by p38 MAPK led to an increase in osteoclast-specific gene expression and differentiation (121). Therefore, the activation of the p38 MAPK signaling pathway leads to increased differentiation through increased MITF, like the manner shown in Mansky et al., however the exact target residue is still currently unknown.

The inhibition of p38 MAPK also significantly impacted the relative expression of key mast cell enzymes, Cpa3, Cma1 and Tpsb2 across mast cell differentiation. The reduced RNA expression of mast cell protease Cpa3 may be due to similar mechanisms that reduced the expression of other MMPs. It was shown by Huang et al. and Xu et al., that the activation of p38 MAPK up-regulated the expression of MMPs in invasive cancer cell models, however the exact regulation of this is not known, but is believed to occur through translational and maturation influences (122,123). The reduced RNA expression of mast cell tryptase Tpsb2 and chymase Cma1 by the inhibition of p38 further indicates that p38 MAPK signaling is necessary for the expression of key mast cell mediators.
Therefore, the activation of the p38 MAPK signaling pathway is shown to positively influence mast cell specific protease Cpa3 RNA expression leading to an increased mast cell profile.

The increases in mast cell markers were further shown with the phenotype of the mast cells during mast cell differentiation. The inhibition of p38 MAPK decreased the MFI of FcεRIα during differentiation, indicating that p38 inhibition causes a decrease in FcεRIα receptor activity or presence on the surface of the cells. This would also indicate that the p38 MAPK signaling pathway acts to drive FcεRIα receptor presence on the developing mast cell. Therefore, the activation of the p38 MAPK signaling pathway drives mast cell differentiation, with the increased presence of key mast cell receptor, FcεRIα.

The p38 MAPK signaling pathway mediates the expression of histone deacetylase HDAC4, and the expressions of important mast cell specific markers, MITF, Cpa3, Tpsb2, and Cma1 during mast cell differentiation. The exact mechanisms behind the influence is still not confirmed, however these results suggest that p38 MAPK influences both transcription factors and histone modifiers to ultimately influence the rate of transcription. MITF is an important transcription factor involved in the regulation of c-kit during mast cell differentiation and is shown to be driven by the p38 MAPK signaling pathway. The activation of the p38 MAPK signaling pathway is necessary for c-kit and MITF expression and is required for mast cell differentiation to occur. The activation of p38 MAPK also further increases the presence of the other key mast cell receptor, FcεRIα, present on the surface of mature mast cells. Furthermore, p38 MAPK signaling propagates the degradation of HDAC4, preventing HDAC4 from interacting with its targeted histones and cause a decrease in accessibility. Therefore, p38 MAPK signaling,
through multiple avenues, acts to drive the differentiation of mast cells from their precursor HSCs and increases the surface expression of mature mast cell markers, c-kit and FcεRIα.

Figure 40: p38 MAPK signaling effects on histone modifiers and mast cell specific mediator and markers expression. The inhibition of p38 by Losmapimod leads to the decreased expression of key mast cell mediators, markers and HATs, while increasing Cpa3 and HDAC4 expression. Figure created by DD.

7.v. MAPK Inhibition on Histone H3 Modifications

The histone octamer is composed of pairs of H2a, H2b, H3, and H4, each providing sites in varying degrees of accessibility for post-translational modifications. Histone H3 is the most extensively modified of the 4 histones found in the octamer and has been shown to be an important protein in epigenetics, due to its high involvement in the regulation of genes (124). Histone H3 is primarily acetylated at K9, K14, K18, K23 and K56, methylated at R2, K4, K9, K27, K36, and K70, and phosphorylated at S10, S28,
T3 and T11, to name a few (125). The influence of the MAPK signaling pathway during mast cell differentiation on two of these modification sites, AcK9 and pS10, was tested in this study.

Acetylated histone H3 protein at position K9 under normal differentiation conditions depicted a significant increase in protein expression 6 hours after initiation of mast cell differentiation. This was followed by a decrease in protein expression, until 144 hours, with significance ending just below the level of protein expression established at the start of differentiation (0 hours). This indicates that early in mast cell differentiation, the genome requires a more open chromatin conformation, due to the increased acetylation. As the relative level of Ac-histone H3 protein decreases across differentiation, the chromatin changes from the open conformation to a more closed conformation. This could indicate that changes to the genome are not occurring and in terms of differentiation, that the process has been completed.

This change in acetylation over differentiation is like what is shown by Meshorer et al. (2006), which showed that when embryonic stem cells are undergoing differentiation, the initial process occurs with a large increase in global acetylation, including the acetylation of Histone H3 at K9 (126). This increase in acetylation was then accompanied with a gradual decrease as the differentiation process progressed (126). Therefore, at the initiation of mast cell differentiation, an overall increase in chromatin acetylation, including Histone H3K9 may be necessary to drive the process, as this opens the chromatin and increases the accessibility for transcriptional genes.

The inhibition of the MAPK signaling pathway had varying effects on the level of Ac-Histone H3 protein. Both the inhibition of JNK by JNK-IN-8 and p38 by Losmapimod had no effect on Ac-Histone H3 protein expression across mast cell
differentiation. This shows that neither the JNK or p38 signaling pathway directly influences the process of acetylation at Histone H3K9. However, these pathways may still influence total histone H3 acetylation, however this would be through a different lysine residue(s). The ERK inhibitor SCH772984 significantly increased the relative level of Ac-Histone H3, thus the ERK signaling pathway acts to reduce Histone H3K9 during differentiation, reducing the formation of euchromatin and favoring a more heterochromatin conformation. Therefore, the decreased process of acetylation that occurs with the activation of the ERK signaling pathway suggests that the ERK signaling pathway reduces the progression of mast cell differentiation epigenetically, due to an increase in acetylation being associated with differentiation.

Epigenetic phosphorylation at histone H3 is associated with being an intermediate step in chromosome condensation and transcriptional regulation (127). Also, unlike acetylation, histone phosphorylation functions by establishing interaction between other histone modifiers and effector proteins leading to a cascade of events. Specifically, the phosphorylation at histone H3S10 is a known mitotic markers involved in chromatin compaction and regulation of structure (128).

Our results show a reduction in p-Histone H3 protein over mast cell differentiation. This shows that the initial induction of mast cell differentiation requires the phosphorylation of Histone H3S10 and is not necessary during differentiation. This may be due to the notion that histone phosphorylation is necessary to facilitate the binding of important histone acetyltransferases to their respective targets (129). Histone H3S10 would then need to be phosphorylated early into mast cell differentiation to propagate the acetylation of Histone H3 targets, such as H3K9. This is supported with the results of p-Histone H3S10 and Ac-Histone H3K9, with p-H3S10 having highest protein
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expression at the initiation of mast cell differentiation, and Ac-H3K9 having highest protein expression 6 hours after the initiation of differentiation. This suggests that Ac-H3K9 and p-H3S10 cooperate to facilitate the upregulation of transcription and the formation of euchromatin.

The MAPK signaling pathway had varying effects on the activity of p-H3S10 protein during mast cell differentiation. The inhibition of JNK by JNK-IN-8 and p38 by Losmapimod had no effect on p-H3S10 protein level during mast cell differentiation. This indicates that these branches of the MAPK signaling pathway are not involved in H3S10 phosphorylation. Instead, the inhibition of ERK by SCH772984 (1 µM) increased p-H3S10 protein activity across differentiation. Hence, the ERK signaling pathway acts to reduce the level of p-H3S10 protein during mast cell differentiation, favoring a more heterochromatin conformation. Therefore, the decrease process of phosphorylation that occurs with the activation of the ERK signaling pathway, reduces the affinity associated with histone phosphorylation and acetylation, ultimately reducing the progression of mast cell differentiation epigenetically.

Histone H3 post-translational modifications are important in the regulation of cellular differentiation. Both histone acetylation and phosphorylation are associated with increased gene transcription and the formation of euchromatin. During mast cell differentiation, an increase in p-Histone H3S10 occurs immediately at the initiation, while Ac-Histone H3K9 increases 6 hours after. This supports the notion that H3S10 and H3K9 cooperate to favor cellular differentiation, due to the phosphorylation of H3S10 being required before the acetylation of H3K9. As well, the ERK signaling pathway negatively affects this cooperation, acting to reduce overall p-H3S10 and ac-H3K9. Therefore, the
regulation of p-H3S10 and ac-H3K9 occurs with ERK signaling to ultimately influence the progression of mast cell differentiation.

7.vi. Isolation of HSCs and Identification of Mast cells Through Flow Cytometry Sorting

The initial experiments required isolation of HSCs directly from the bone marrow of C57BL6/J mice. However, due to the natural biology, HSCs are not the only cell type that can be found within the bone marrow of mice. Instead, the bone marrow contains a conglomeration of white and red blood cells, all in varying degrees of differentiation and maturation. Therefore, bone marrow cells were sorted based on cell surface proteins and receptors. Mice bone marrow HSCs are classified as being Lin−c-kit+Sca-1+CD34low-med, meaning that it is lineage negative for differentiated cell markers, such as CD4 and CD3, while containing Sca-1, c-kit and CD34 markers (130). These parameters were used to sort the HSCs out of the bone marrow using flow cytometry cell sorting (Sony Cell Sorter SH800S). Isolation and identification of HSCs began first with a lineage cocktail FITC flow antibody, which contains CD3, Ly-6G/Ly-6C, CD11b, CD45R/B220 and TER-119 markers.

CD3 is a T cell co-receptor that leads to the activation of both cytotoxic and helper T cells (CD8 and CD4). Furthermore, CD3 is initially expressed in the cytoplasm of pro-thymocytes, stem cells that give rise to T cells in the thymus (131). Ly-6G/Ly-6C markers are used for the identification of neutrophils, eosinophils, and a subset of monocytes/macrophages, with cells expressing either one or the other on their surface. Theses markers are also expressed on myeloid-derived suppressor cells, such as Ly6G+Ly6Clow granulocytic and Ly6G−Ly6Chigh monocytic cells (132). CD11b is an integrin family member that pairs with CD18 to form the CR3 heterodimer (133). The
CR3 heterodimer is expressed on the surface of many leukocytes including monocytes, neutrophils, natural killer cells, granulocytes and macrophages. The CR3 heterodimer is also expressed by memory B cells (134). B220 is the mouse CD45 isoform CD45R, and is predominantly expressed on all B lymphocytes including, pro-, mature and activated B cells. CD45R is also found expressed on T cells and natural killer cells (135). Finally, TER-119 is expressed by erythroid cells found from the early pro-erythroblast stage to fully mature erythrocytes. TER-119 is used to identify erythrocytes and cells in the erythroid lineage (136). The identification of these markers is useful to find the lineage negative HSCs, as the population that does not express these lineage markers were gated.

These lineage negative gated cells were also fluorescently labelled for Sca-1 and CD34. Further gating occurred, see appendix Figure S7, which partitioned the population of cells that were both Sca-1+ and c-kit+ for HSC sorting. The sorted Lin-c-kit+ Sca-1+ HSCs were grown in BMMC Complete media to induce mast cell differentiation with or without the presence of MAPK inhibitors (JNK-IN-8, SCH772984 or Losmapimod).

These cells were further used in a preliminary analysis of feasibility for differentiating the sorted progenitors to mature mast cells and to view the morphological changes that occurs when HSC undergo mast cell differentiation. Microscopic images of these cells, at 3 weeks and 4 weeks were taken to view how the effects of the MAPK pathway inhibitors affected the cells while growing, see appendix Figure S4. The presence of the ERK inhibitor, greatly reduced the growth of the cells and caused some morphological changes, as those cells appeared much smaller and highly grouped. The JNK and p38 inhibited samples when compared to the non-inhibited group, did not appear morphologically different, however, WT53 p38 inhibited cells did appear to be highly
grouped, appendix *Figure S4*. These HSC differentiated cells by 4 weeks appeared more morphologically like mast cells, due to high granularity, see appendix *Figure S5*.

HSCs isolated from the bone marrow of C57BL6/J mice are also known as Lin- Sca-1+ c-kit+ cells. This specific receptor presence does provide high specificity to HSCs; however, this does provide limitations as it does not include cells that are naturally undergoing differentiation through the myeloid lineage parallel to mast cell differentiation. Furthermore, HSCs when introduced to BMMC Completed media are undergoing mast cell differentiation, as an increase in FcεRIα mast cell receptor and granularity occurred.

7.vii. Future Scientific Directions
These results on how the MAPK signaling pathway influences key epigenetic modifiers during mast cell differentiation provides directions for future epigenetic targets at other levels of expression. These results depict that histone modifiers are influenced at both the RNA and protein level of expression. Furthermore, key mast cell receptors and markers are influenced during differentiation by the inhibition of the MAPK signaling pathway.

One additional method that will provide additional insights into the effects of MAPK inhibition on histone modifiers is chromatin immunoprecipitation (ChIP). ChIP is a technique that measures protein-DNA interactions within the cell and can be used to identify complexes of proteins associated with a specific region of native DNA (137). This will allow us to measure the relative amount of histone modifications that occur across the gene leading either to gene activation or inhibition. Furthermore, histone
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proteins, transcription factors and transcription co-factors can be analyzed to determine their binding efficiency (137).

This method begins with the fixation of cells, so that the proteins that are currently interacting with regions of DNA remain secure. A micrococcal nuclease (MNase) enzyme or sonication process follows to fragment the chromatin as MNases preferentially cleaves the DNA at locations rich in adenylate, deoxyadenylate or thymidylate to 3’ phosphomononucleotides and dinucleotides (138). Therefore, MNase enzymes can only bind and digest open regions of euchromatin, providing spatial and temporal dynamics on the interactions between associated proteins and chromatin. The remaining nuclear pellet incubates with target immunoprecipitation antibody, which binds to the protein that is currently fixed to the DNA (137). After antibody binding, magnetic beads bind to the antibody, so that this composite bound to the DNA can be separated using a magnetic separation rack. The remaining protein-DNA complex cross-linking is reversed so that the magnetic beads unbind to the antibody and the antibody is unbound to the protein that separates from the chromatin DNA (137). This resulting DNA contains the regions of the genome that the protein of interest associates with in vivo so that it can be measured in vitro.

Multiple transcription factors, and enzymes that are necessary for the differentiation of HSCs to mast cells were shown to be significantly influenced by the inhibition of the MAPK signaling pathway. Important transcription factor MITF was significantly influenced by the inhibition of ERK and p38, which increased and decreased MITF RNA expression respectively. The use of ChIP would provide further insights into the locational binding regions of MITF during differentiation, and if the inhibition of the different branches of the MAPK signaling pathway influences this binding and not just
the RNA. Multiple mast cell enzymes, Cpa3, Cma1 and Tpsb2 were also shown to be influenced at the RNA level of expression by the inhibition of the MAPK signaling pathway, specifically ERK and p38 inhibition. The inhibition of ERK significantly increased the relative level of RNA expression for Cpa3 and Cma1, while significantly decreasing Tpsb2 expression. The inhibition of p38 significantly decreased Cpa3 and Tpsb2 RNA expression. These significantly different responses by the MAPK signaling pathway can be further determined at the chromatin level, measuring how these enzymes directly influence DNA with ChIP. Therefore, ChIP is an important technique that provides information pertaining to physiological interactions that occur at the chromatin level.

Further flow cytometry looking more closely at both intracellular and extracellular targets would further advance the results that were shown at the RNA and protein level of expression. As we saw significant impact with MAPK inhibition on FcεRIα MFI, it would be beneficial to examine other receptors that are known to be present on mature mast cells. One such receptor that is known to be present on mature mast cells is c-kit, which helps in the development of mast cells, through enhancing maturation and regulating the survival of precursor mast cells (139). Another receptor that is also important in the regulation of mast cell differentiation is IL-3R (140). Further insight to how this receptor is regulated and activated during mast cell differentiation and how the inhibition of the MAPK signaling pathway influences IL-3R MFI, could provide significant information on overall mast cell receptor effects. Another reason to measure how IL-3R is influenced by MAPK inhibition during mast cell differentiation is due to the media used to induced mast cell differentiation. The BMMC Complete Media activates
mast cell differentiation through the IL-3R dimerization, and if this is influenced by MAPK inhibition, the differentiation and maturation of mast cells may be compromised.

Intracellular targets include Cpa3, Cma1, Tpsb2, MITF, HDAC2 and HDAC4, all due based on the effects of MAPK inhibition seen at the RNA level of expression. The enzymes, Cpa3, Cma1, and Tpsb2, as state earlier showed a significant effect with the inhibition of the MAPK signaling pathway on mast cell differentiation at the RNA level of expression. The use of flow cytometry will provide additional insights physiologically within the cell. Intracellular targets, such as these, require the cells to be punctured in a way that will allow the flow antibodies to transverse the cell membrane and bind to the desired targets (141). Therefore, the use of flow cytometry will measure the changes that are occurring at both extra and intracellular targets by the inhibition of the MAPK signaling pathway, ultimately influencing mast cell differentiation.

Another technique that would provide invaluable insights into histone acetylation and how the MAPK signaling pathway influences histone modifications is through histone modifier activity assays. Three different assays, each measuring different targets for histone acetylation include the histone acetyltransferase activity assay, HDAC2/HDAC4 activity assay and histone H3 modification multiplex assay.

The histone acetyltransferase activity assay detects HAT activity, specifically the acetylation of a peptide substrate through the release of CoA. CoA serves as a coenzyme to produce NADH, which can be detected spectrophotometrically (142–144). This assay will provide additional information on overall acetylation that occurs during mast cell differentiation. As well, MAPK signaling pathway inhibition could influence overall acetylation modification during mast cell differentiation, as it was shown that histone H3 acetylation and phosphorylation was influenced by the inhibition of ERK and p38. The
overall level of acetylation may then also be influenced by these inhibitions which can be detected through this assay experiment.

The second assay that measures histone acetylation specifically measures HDAC2 or HDAC4 histone deacetylase activity. This assay is a colorimetric assay that measures the total amount of HDAC2 or HDAC4 from the cells (145–147). This occurs by binding the nuclear proteins that contain either HDAC2 or HDAC4 to the bottom of strip wells before being recognized with a high-affinity specific antibody. This is then quantified using an HRP-conjugated secondary antibody that is fluorescently labelled for detection (145,146). HDAC2 and HDAC4 were both significantly changed across mast cell differentiation and significantly influenced by the inhibition of the MAPK signaling pathway at the mRNA level of expression. This assay would provide additional support to how the MAPK signaling pathway influences HDAC2 and HDAC4 activity, as HDAC2 protein activity was not conclusive with Western blotting and requires additional results on overall activity. This assay measures the specific HDACs activity in a different manner, so that the level of effect on HDAC activity can be determined.

The final assay measures post-translational modifications occurring on Histone H3 through a multiplex assay. This assay simultaneously measures 21 different histone H3 modifications, including phosphorylation, methylation and acetylation (148–150). Total histone H3 is also measured alongside these post-translational modifications to normalize for relative comparison of histone H3 between the different conditions. Each histone H3 modification is captured by a capture antibody that is coated to the bottom of strip wells. The bound histone modification is then detected with a detection antibody that changes colour when introduced to a colour development reagent (148,150). This assay will then provide additional information and targets on histone H3 post-translational
modifications, as both p-histone H3 (Ser28) and ac-histone H3 (Lys9) were influenced by the inhibition of the MAPK signaling pathway during differentiation. The 19 additional targets will provide a more physiological representation of what occurs within the cell while undergoing differentiation. Additionally, there is notions that some post-translational modifications are temporal in their modifications, requiring other post-translational modifications to occur before. This assay will provide a greater overview on the epigenetics that occur for histone H3 and will provide additional modification targets that can be influenced during mast cell differentiation by the inhibition of the MAPK signaling pathway.

All these future directions provide additional information that would further benefit to the results already seen. The use of ChIP will measure the protein-DNA interactions that occur to native DNA, continuing the results that were shown to transcription factors and enzymes at the RNA level of expression. The additional targets of flow cytometry measuring other receptors and intracellular enzymes important to the differentiation of mast cells will translate the effects of MAPK inhibition seen at the RNA level and protein level of expression. Finally, the different histone activity assays will measure specific and overall histone post-translational activity during mast cell differentiation, advancing the results shown specifically to HDAC2/4 and histone H3. The results shown in this document supply the basis for continued research, with these suggested future directions only being some of the different routes that could be continued.
8. Conclusion:
Inhibition of the MAPK signaling pathway produced dynamic results influencing mast cell differentiation. Each of the three branches, JNK, ERK and p38, had opposing results indicating that each pathway is separately activated for individual conditions. The JNK signaling pathway had no influence on histone modifier and mast cell specific marker expression during mast cell differentiation. As well, the inhibition of JNK by JNK-IN-8 (1 µM) had no effect on FcεRIα MFI when compared to normal differentiation conditions, indicating that it does not have any influence at multiple levels of expression on mast cell differentiation (Figure 41, 42).

The ERK signaling pathway had significant influence on histone modifier expression, at both the RNA and protein level of expression. HDAC4 RNA expression is decreased with the activation of ERK, suggesting that HDAC4 expression may be due to HDAC4-ERK complex formation, which is associated with a translocation from the cytoplasm to the nucleus. This would need to be further confirmed, either through measuring HDAC4’s activity or through fluorescent labelling of HDAC4, which would should if HDAC4 does move into the cell nucleus. This complex allows HDAC4 to transverse the nuclear membrane and remove acetyl groups bound to histone tails. This causes the binding force between the histones and DNA to be increased and causes a conformational change, favoring a heterochromatin conformation. As well, ERK activation causes a decrease in PCAF RNA expression, further strengthening the heterochromatin conformation and reduced gene accessibility. Two specific targets for histone modifications, Histone H3K9 and Histone H3S10, were also significantly affected by the activation of ERK. Both residues were temporally affected by the activation of ERK, being as Ac-H3K9 is linked to the phosphorylation of H3S10. p-H3S10 protein was
significantly decreased at the initiation of mast cell differentiation, while Ac-H3K9 protein decreased at 6 hours following initiation. The phenotype of the cells is also influenced by ERK signaling, with FcεRIα cell surface expression being increased by SCH772984. This indicates that the activation of ERK represses the formation of FcεRIα on the cell surface, preventing the differentiation process from occurring into a mature mast cell. Therefore, the balance of histone modifications and genome accessibility with the activation of ERK signaling, significantly shifts to a heterochromatin conformation and restricted accessibility, thereby preventing or delaying mast cell differentiation (Figure 41, 42).

The p38 signaling pathway has a different effect on mast cell differentiation than what is seen with ERK activation. p38 MAPK activation significantly increased MITF gene expression, a transcription factor that is involved in the transcription of mast cell receptor c-kit. The increase in MITF gene expression with p38 MAPK signaling activation could help drive the phenotype of the cell towards a mast cell commitment through increased c-kit receptor transcription. Another mast cell surface receptor, FcεRIα, is also influenced by the p38 MAPK signaling pathway. Therefore, both receptors found on the surface of mast cells are influenced by the activation of p38 MAPK signaling pathway, indicating a drive in the differentiation process. Furthermore, the activation of p38 MAPK increases the RNA expression of mast cell specific tryptase, chymase and protease enzymes. The activation of p38 MAPK significantly reduced the level of RNA expression of HDAC4, while increasing KAT2A gene expression, which favors a more euchromatin conformation and upregulation in transcription. Therefore, the activation of p38 MAPK helps to drive mast cell differentiation through increased chromatin
accessibility, by inhibiting HDAC4 deacetylase activity, and via the activation of mast cell specific receptors and mediators (Figure 41, 42).

**Figure 41: Summary of the effects each branch of the MAPK signaling pathway had on gene expression.** The activation of the JNK signaling pathway generally has little effect on mast cell differentiation through mediator and modifier expression. The ERK signaling pathway favors a more heterochromatin conformation through decreased HAT and increased HDAC expression, while also decreasing key mast cell marker and mediator expression. The activation of the p38 signaling pathway favors an euchromatin conformation through increased HAT and decreased HDAC expression, while increasing mast cell mediator and marker expressions to drive mast cell differentiation.
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Figure 42: Potential mechanisms of action of the MAPK signaling pathway during mast cell differentiation. The p38 signaling pathway influences key mast cell markers, transcription factors, and histone modifiers. The JNK signaling pathway does not influence mast cell differentiation. The ERK signaling pathway potentially influences through histone modifiers HATs and HDACs, and marker expressions. Figure created by DD.

This work will contribute significantly to the scientific community and advances our knowledge of the epigenetic and signaling mechanisms that may regulate how mast cells differentiate. The ERK signaling pathway favors a more heterochromatin conformation through increased HDAC expression, while also restricting important mast cell receptor FcεRIα and transcription factor MITF. The p38 MAPK signaling pathway favors an euchromatin conformation, with decreased HDAC and increased KAT2A HAT expression and drives the accessibility of key mast cell specific genes, including FcεRIα and MITF. Therefore, ERK and p38 MAPK signaling act as opposing drivers of mast cell differentiation both epigenetically and transcriptionally. This research provides further insights into how the MAPK signaling pathway influences key histone modifiers that ultimately impacts transcriptional gene expression during mast cell differentiation.
9. References:


66. Tsai Y-J, Tsai T, Peng P-C, Li P-T, Chen C-T. Histone acetyltransferase p300 is induced by p38MAPK after photodynamic therapy: the therapeutic response is increased by the p300HAT inhibitor anacardic acid. Free Radic Biol Med. 2015 Sep;86:118–32.


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10. Appendices:

10.i. MAPK Inhibitor Specificity:

Figure S1: Phospho-ERK protein activity in the presence of MAPK inhibitors. p-ERK protein increases across time, with a significant increase occurring at 6 hours. The inhibition of ERK by SCH772984 significantly reduced the relative level of p-ERK protein, while the other MAPK inhibitors had no effect. N = 3; * p > 0.05

Figure S2: Phospho-p38 protein activity in the presence of MAPK inhibitors. The presence of p38 Losmapimod significantly decreased the relative intensity of p-38. The inhibition of the other MAPK pathways did not affect p-p38 protein. N = 3; * p > 0.05, ** p > 0.01
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**Figure S3**: Phospho-JNK protein activity in the presence of MAPK inhibitors. Both the p-JNK and t-JNK antibodies used to test the specificity of JNK-IN-8 failed to show bands. This does not allow a definitive conclusion to be made regarding the specificity of the inhibitor on the JNK signaling pathway. N = 3

10.ii. HSC Appearance Following FCM Sorting

**Figure S4**: 3-week-old HSCs undergoing mast cell differentiation. HSCs isolated from the bone marrow of C57BL6/J mice were sorted through the Sony FACs instrument based on Lin−c-kit+Sca-1+ marker expression. Lin−c-kit+Sca-1+ HSCs were then grown in BMMC complete media to initiate mast cell differentiation and cultured with or without MAPK inhibitors JNK-IN-8 (1µM), SCH772984 (1 µM) or Losmapimod (10µM).
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**Figure S5: 4-week-old HSCs undergoing mast cell differentiation.** HSCs isolated from the bone marrow of C57BL6/J mice were sorted through the Sony FACs instrument based on Lin⁻c-kit⁺Sca-1⁺ marker expression. Lin⁻c-kit⁺Sca-1⁺ HSCs were then grown in BMMC complete media to initiate mast cell differentiation and cultured with or without MAPK inhibitors JNK-IN-8 (1µM), SCH772984 (1 µM) or Losmapimod (10µM).

10.iii. Flow Sorting Gates

**Figure S6: HSC sorting gates from the BM of C57BL6/J mice.** Original gate contains all alive immune cells. These cells were then sorted to exclude any doublet cells. Remaining cells are separated based on Lin⁻ expression and further separated based on being c-kit⁺ and Sca-1⁺.
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10.iv. ENCODE Database Chromatin Accessibility for HSCs.

**Figure S7: CD34 Chromatin Accessibility in Hematopoietic stem cells.** The CD34 gene is located on chromosome 1, band qH6. CD34 ATAC-seq data depicts both low and highly expressed regions of open chromatin. This indicates that the gene is not accessible to transcriptional modifications.

**Figure S8: Cpa3 Chromatin Accessibility in Hematopoietic stem cells.** The Cpa3 gene is located on chromosome 3, band qA2. Cpa3 ATAC-seq data depicts segmented regions of open chromatin that are accessible to transcriptional modifications. However, these segmented regions indicate that the overall gene is then closed, as it cannot be fully transcribed.
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Figure S9: Cma1 Chromatin Accessibility in Hematopoietic stem cells. The Cma1 gene is located on chromosome 14, band qC3. Cma1 ATAC-seq data depicts spatial regions of open chromatin, indicating that the genome is not accessible.

Figure S10: Tpsb2 Chromatin Accessibility in Hematopoietic stem cells. The Tpsb2 gene is located on chromosome 17, band qA3.3. Tpsb2 ATAC-seq data depicts sparse regions of open chromatin, indicating that Tpsb2 is slightly accessible to transcriptional modifications. However, the conformation is still considered closed, as transcription would not be continued.
Figure S11: FceRIα Chromatin Accessibility in Hematopoietic stem cells. The FceRIα gene is located on chromosome 1, band qH3. FceRIα ATAC-seq data depicts small regions of open chromatin across the genome. This indicates that FceRIα is not accessible to transcriptional modifications.

Figure S12: FceRIβ Chromatin Accessibility in Hematopoietic stem cells. The FceRIβ gene is located on chromosome 19, band qA. FceRIβ ATAC-seq data depicts segmental regions of accessible chromatin for transcriptional modifications. This indicates that it is therefore in a closed conformation.
Figure S13: HIF1α Chromatin Accessibility in Hematopoietic stem cells. The HIF1α gene is located on chromosome 12, band qC3. HIF1α ATAC-seq data depicts open regions of slightly accessible chromatin, indicating that the gene is in a closed conformation.

Figure S14: MITF Chromatin Accessibility in Hematopoietic stem cells. The MITF gene is located on chromosome 6, band qD3. MITF ATAC-seq data depicts a closed conformation, due to the absence of bands.
10.v. Gel Electrophoresis Images Separated by Target.

Figure S15: HDAC1 gel electrophoresis images for WT26-28 and WT35-37. A. WT26-28 B. WT35-37 both depict constant bands at the specific size of 156bp. Each timepoint and condition is indicated above the respective band. The absence of bands is due to no RNA isolated.
Figure S16: HDAC2 gel electrophoresis images for WT26-28, WT35-37 and WT47-49. A. WT26-28 B. WT35-37 and C. WT47-49 depict constant bands at the specific size of 80bp. Each timepoint and condition is indicated above the respective band. The absence of bands is due to no RNA isolated.
Figure S17: HDAC3 gel electrophoresis images for WT26-28 and WT35-37.
A. WT26-28 B. WT35-37 both depicts constant bands at the specific size of 108bp. Each timepoint and condition is indicated above the respective band. The absence of bands is due to no RNA isolated.
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Figure S18: HDAC4 gel electrophoresis images for WT26-28, WT35-37 and WT47-49. A. WT26-28 B. WT35-37 and C. WT47-49 depict constant bands at the specific size of 136bp. Each timepoint and condition is indicated above the respective band. The absence of bands is due to no RNA isolated.
Figure S19: HDAC6 gel electrophoresis images for WT26-28 and WT35-37.
A. WT26-28 B. WT35-37 both depict constant bands at the specific size of 138bp. Each timepoint and condition is indicated above the respective band. The absence of bands is due to no RNA isolated.
Figure S20: CBP gel electrophoresis images for WT26-28, WT35-37 and WT47-49. A. WT26-28 B. WT35-37 and C. WT47-49 depict constant bands at the specific size of 150bp. Each timepoint and condition is indicated above the respective band. The absence of bands is due to no RNA isolated.
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Figure S21: p300 gel electrophoresis images for WT26-28 and WT35-37.
A. WT26-28 B. WT35-37 both depicts constant bands at the specific size of 127bp. Each timepoint and condition is indicated above the respective band. The absence of bands is due to no RNA isolated.
Figure S22: KAT2A gel electrophoresis images for WT26-28 and WT35-37.
A. WT26-28. B. WT35-37 both depicts constant bands at the specific size of 106bp. Each timepoint and condition is indicated above the respective band. The absence of bands is due to no RNA isolated.
Figure S23: PCAF gel electrophoresis images for WT26-28, WT35-37 and WT47-49. A. WT26-28, B. WT35-37 and C. WT47-49 depict constant bands at the specific size of 162bp. Each timepoint and condition is indicated above the respective band. The absence of bands is due to no RNA isolated.
Figure S24: CD34 gel electrophoresis images for WT26-28 and WT35-37. A. WT26-28 B. WT35-37 both depicts constant bands at the specific size of 158bp. Each timepoint and condition is indicated above the respective band. The absence of bands is due to no RNA isolated.
Figure S25: FcεRIα gel electrophoresis images for WT26-28, WT35-37 and WT47-49. A. WT26-28 B. WT35-37 and C. WT47-49 depict constant bands at the specific size of 75bp. Each timepoint and condition is indicated above the respective band. The absence of bands is due to no RNA isolated.
Figure S26: FcεRIβ gel electrophoresis images for WT26-28 and WT35-37.
A. WT26-28 B. WT35-37 both depicts constant bands at the specific size of 115bp. Each timepoint and condition is indicated above the respective band. The absence of bands is due to no RNA isolated.
Figure S27: Cpa3 gel electrophoresis images for WT26-28, WT35-37 and WT47-49. A. WT26-28, B. WT35-37 and C. WT47-49 depict constant bands at the specific size of 167bp. Each timepoint and condition is indicated above the respective band. The absence of bands is due to no RNA isolated.
Figure S28: Tpsb2 gel electrophoresis images for WT35-37 and WT47-49. 
A. WT35-37 B. WT47-49 both depicts constant bands at the specific size of 105bp. Each timepoint and condition is indicated above the respective band. The absence of bands is due to no RNA isolated.
Figure S29: Cma1 gel electrophoresis images for WT26-28, WT35-37 and WT47-49. A. WT26-28 B. WT35-37 and C. WT47-49 depict constant bands at the specific size of 154bp. Each timepoint and condition is indicated above the respective band. The absence of bands is due to no RNA isolated.
Figure S30: HIF1α gel electrophoresis images for WT35-37. A. WT35-37 depicts constant bands at the specific size of 147bp. Each timepoint and condition is indicated above the respective band. The absence of bands is due to no RNA isolated.
Figure S31: MITF gel electrophoresis images for WT26-28, WT35-37 and WT47-49. 
A. WT26-28 B. WT35-37 and C. WT47-49 depict constant bands at the specific size of 181bp. Each timepoint and condition is indicated above the respective band. The absence of bands is due to no RNA isolated. Multiple bands indicate unspecific binding.
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10.vi. Gel Electrophoresis Images for Housekeeping Genes

Figure S32: HPRT gel electrophoresis images for WT26-28, WT35-37 and WT47-49. A. WT26-28 B. WT35-37 and C. WT47-49 depict constant bands at the specific size of bp. Each timepoint and condition is indicated above the respective band. The absence of bands is due to no RNA isolated.
Figure S33: GAPDH gel electrophoresis images for WT26-28 and WT35-37. A. WT26-28 and B. WT35-37 depicts constant bands at the specific size of bp. Each timepoint and condition is indicated above the respective band. The absence of bands is due to no RNA isolated.
Figure S34: ACTB gel electrophoresis images for WT26-28, WT35-37 and WT47-49. 
A. WT26-28, B. WT35-37 and C. WT47-49 depict constant bands at the specific size of bp. Each timepoint and condition is indicated above the respective band. The absence of bands is due to no RNA isolated.
Figure S35: PPIA gel electrophoresis images for WT35-37 and WT47-49. A. WT35-37 B. WT47-49 depict constant bands at the specific size of bp. Each timepoint and condition is indicated above the respective band. The absence of bands is due to no RNA isolated.
10.vii. Melt Curves Separated by Target.

Figure S36: HDAC1 Melt Curves for WT26-28 and WT35-37. A. WT26-28 and B. WT35-37 melt curves show one peak indicating the amplification of only one target. The top melt curve is the early time points, while the bottom melt curve is the late time points.
Figure S37: HDAC2 Melt Curves for WT26-28, WT35-37 and WT47-49. A. WT26-28, B. WT35-37 and C. WT47-49 melt curves show one peak indicating the amplification of only one target. The top melt curve is the early time points, while the bottom melt curve is the late time points.
Figure S38: HDAC3 Melt Curves for WT26-28 and WT35-37. A. WT26-28 and B. WT35-37 melt curves show one peak indicating the amplification of only one target. The top melt curve is the early time points, while the bottom melt curve is the late time points.
Figure S39: HDAC4 Melt Curves for WT26-28, WT35-37 and WT47-49. A. WT26-28, B. WT35-37 and C. WT47-49 melt curves show one peak indicating the amplification of only one target. The additional peaks indicate unspecific binding and amplification. The top melt curve is the early time points, while the bottom melt curve is the late time points.
Figure S40: HDAC6 Melt Curves for WT26-28 and WT35-37. A. WT26-28 melt curves show one peak indicating the amplification of only one target. B. WT35-37 melt curves has multiple bands and flat lines indicating unspecific binding or no binding, respectively. The top melt curve is the early time points, while the bottom melt curve is the late time points.
Figure S41: CBP Melt Curves for WT26-28, WT35-37 and WT47-49. A. WT26-28, B. WT35-37 and C. WT47-49 melt curves show one peak indicating the amplification of only one target. The top melt curve is the early time points, while the bottom melt curve is the late time points.
Figure S42: p300 Melt Curves for WT26-28 and WT35-37. A. WT26-28 and B. WT35-37 melt curves show one peak indicating the amplification of only one target. The top melt curve is the early time points, while the bottom melt curve is the late time points.
Figure S43: KAT2A Melt Curves for WT26-28 and WT35-37. A. WT26-28 melt curves only contain one specific peak, indicating specificity. B. WT35-37 melt curves show multiple peaks indicating the amplification of multiple targets and non-specificity. The top melt curve is the early time points, while the bottom melt curve is the late time points.
Figure S44: PCAF Melt Curves for WT26-28, WT35-37 and WT47-49. A. WT26-28, B. WT35-37 and C. WT47-49 melt curves show one peak indicating the amplification of only one target. The top melt curve is the early time points, while the bottom melt curve is the late time points.
Figure S45: CD34 Melt Curves for WT26-28 and WT35-37. A. WT26-28 and B. WT35-37 melt curves show one peak indicating the amplification of only one target. The top melt curve is the early time points, while the bottom melt curve is the late time points.
Figure S46: FcεRIα Melt Curves for WT26-28, WT35-37 and WT47-49. A. WT26-28, B. WT35-37 and C. WT47-49 melt curves show one peak indicating the amplification of only one target. The top melt curve is the early time points, while the bottom melt curve is the late time points.
Figure S47: FcεRIβ Melt Curves for WT26-28 and WT35-37. A. WT26-28 melt curves show one peak indicating the amplification of only one target. B. WT35-37 melt curves show one peak at the early time points, while multiple peaks for the late timepoints, indicating nonspecific binding and amplification. The top melt curve is the early time points, while the bottom melt curve is the late time points.
Figure S48: Cpa3 Melt Curves for WT26-28, WT35-37 and WT47-49. A. WT26-28, B. WT35-37 and C. WT47-49 melt curves show one peak indicating the amplification of only one target. The top melt curve is the early time points, while the bottom melt curve is the late time points.
Figure S49: Tpsb2 Melt Curves for WT26-28, WT35-37 and WT47-49. A. WT26-28, B. WT35-37 and C. WT47-49 melt curves depict multiple peaks at the early timepoints indicating the amplification of multiple targets and non-specificity. The later timepoints show better specificity and the amplification of only one target. The top melt curve is the early time points, while the bottom melt curve is the late time points.
Figure S50: Cma1 Melt Curves for WT26-28, WT35-37 and WT47-49. A. WT26-28, B. WT35-37 and C. WT47-49 melt curves for the early timepoints depicts multiple bands indicating non-specific binding and amplification. The later timepoints show one peak indicating the amplification of only one target. The top melt curve is the early time points, while the bottom melt curve is the late time points.
Figure S51: HIF1α Melt Curves for WT26-28 and WT35-37. A. WT26-28 and B. WT35-37 melt curves show one peak indicating the amplification of only one target. The top melt curve is the early time points, while the bottom melt curve is the late time points.

10.viii. ΔΔCT Analysis Sample Calculation

Table S1: Sample threshold cycle values for different conditions. NT indicates no inhibitor added to the cells while undergoing differentiation, while ERK indicates the ERK inhibition (SCH772984, 1 μM) was added to the cells.
Table S2: Housekeeping genes replicate CT values. The average of the four housekeeping genes used, HPRT, GAPDH, PPIA, and ACTB were used for each replicate, with the average taken of Rep1 and Rep2.

<table>
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<th>TIME</th>
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<th>REP2</th>
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<td></td>
<td>WT37</td>
<td>23.6</td>
<td>23.8</td>
</tr>
</tbody>
</table>

Equations:
- AVG Tech Reps = AVERAGE(Rep1:Rep2)
- ΔCT = AVG Tech Reps – Housekeeping Gene AVG
- Ref AVG = AVERAGE(WT35 ΔCT:WT37 ΔCT)
- ΔΔCT = ΔCT – Ref AVG
- Fold Change = $2^{-(ΔΔCT)}$
- AVG Bio Reps = AVERAGE(WT35 Fold Change:WT37 Fold Change)
- ST DEV = STDEV(WT35 Fold Change:WT37 Fold Change)
- SEM = STDEV/SQRT(N)
- T-Test = TTEST(WT35 Fold Change:WT37 Fold Change, WT35’ Fold Change:WT37’ Fold Change, 2, 2)

Sample Calculation:

**NT 0h**
- AVG Tech Reps WT35 = AVERAGE(27.5:28.2) = 27.9
- AVG Tech Reps WT36 = AVERAGE(27.2:26.8) = 27.0
- AVG Tech Reps WT37 = AVERAGE(27.4:28.0) = 27.7

- ΔCT WT35 = 27.9 – 21.0 = 6.85
- ΔCT WT36 = 27.0 – 21.4 = 5.55
- ΔCT WT37 = 27.7 – 21.3 = 6.43

- Ref AVG = AVERAGE(6.85:5.55:6.43) = 6.28

- ΔΔCT WT35 = 6.85 – 6.28 = 0.58
- ΔΔCT WT36 = 5.55 – 6.28 = -0.73
- ΔΔCT WT37 = 6.43 – 6.28 = 0.16

- Fold Change WT35 = $2^{(-0.58)} = 0.67$
- Fold Change WT36 = $2^{(-0.73)} = 1.66$
- Fold Change WT37 = $2^{(-0.16)} = 0.90$

- AVG Bio Reps = AVERAGE(0.67:1.66:0.90) = 1.08

- ST DEV = STDEV(0.67:1.66:0.90) = 0.52
- SEM = 0.52/SQRT(3) = 0.30
**NT 0h – 576h**
AVG Tech Reps WT35 = AVERAGE(22.6:22.4) = 22.5
AVG Tech Reps WT36 = AVERAGE(21.4:22.8) = 22.1

ΔCT WT35 = 22.5 – 23.0 = -0.45
ΔCT WT36 = 22.1 – 22.0 = 0.23

ΔΔCT WT35 = -0.45 – 6.28 = -6.73
ΔΔCT WT36 = 0.23 – 6.28 = -6.05

Fold Change WT35 = $2^{(-6.73)} = 106.14$
Fold Change WT36 = $2^{(-6.05)} = 66.14$

AVG Bio Reps = AVERAGE(106.14:66.14) = 86.14

ST DEV = STDEV(106.14:66.14) = 28.29
SEM = 28.29/SQRT(2) = 20.0

T-Test = TTEST(0.67:1.66:0.90, 106.14:66.14, 2, 2) = 0.011

**NT 576h – ERK 576h**
AVG Tech Reps ERK WT35 = AVERAGE(21.3:21.1) = 21.2
AVG Tech Reps ERK WT36 = AVERAGE(20.5:19.8) = 20.2
AVG Tech Reps ERK WT37 = AVERAGE(23.0:22.5) = 22.7

ΔCT ERK WT35 = 21.2 – 22.0 = -0.74
ΔCT ERK WT36 = 20.2 – 21.2 = -1.02
ΔCT ERK WT37 = 22.7 – 23.7 = -0.96

ΔΔCT ERK WT35 = -0.74 – 6.28 = -7.01
ΔΔCT ERK WT36 = -1.02 – 6.28 = -7.29
ΔΔCT ERK WT37 = -0.96 – 6.28 = -7.24

Fold Change ERK WT35 = $2^{(-7.01)} = 129.17$
Fold Change ERK WT36 = $2^{(-7.29)} = 156.95$
Fold Change ERK WT37 = $2^{(-7.24)} = 151.28$

AVG Bio Reps = AVERAGE(129.17:156.95:151.28) = 145.80

ST DEV = STDEV(129.17:156.95:151.28) = 14.67
SEM = 14.67/SQRT(3) = 8.47

T-Test = TTEST(106.14:66.14, 129.17:156.95:151.28, 2, 2) = 0.048
10.ix. Class IIa Gene Expression without the Influence of MAPK Signaling

**Figure S52:** Class IIa HDAC expression across mast cell differentiation. Mast cell differentiation was induced at time 0 and analyzed by qPCR for expression kinetics. HDAC4 and HDAC5 were significantly increased 6 hours after the initiation of differentiation, remaining elevated until 144 hours. HDAC7 was increased also at 6 hours, however significance was never reached. Significance is compared per HDAC to time 0. N = 5; ** p < 0.01, *** p < 0.001

10.x. Protein Standard Curves and Quantifications

**Figure S53:** WT29-31 protein standard curve. BSA standards used to determine the basis for the protein concentrations based on their absorbances. A positive correlation occurs between the absorbance and the protein concentrations. The trendline equation of the standard curve is \( y = 0.8344x + 0.0082 \).
y = 0.5926x + 0.0134
R² = 0.9992

**Figure S54: WT32-34 standard curve for protein quantification.** BSA standards used to determine the basis for the protein concentrations based on their absorbances. A positive correlation occurs between the absorbance and the protein concentrations. The trendline equation of the standard curve is \( y = 0.5926x + 0.0134 \).

**Table S3: WT29-31 sample protein quantification calculations.** The volumes of each solution necessary for 30 ug of protein per blot. The volume for 30 ug was calculated from the BSA standard curve for WT29-31. RIPA had trichostatan A (TSA) added to the solution as an HDAC inhibitor.

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<th></th>
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<th>WT31</th>
<th>WT29</th>
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<td>NT</td>
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**Table S4: WT32-34 sample protein quantification calculations.** The volumes of each solution necessary for 30 ug of protein per blot. The volume for 30 ug was calculated from the BSA standard curve for WT32-34. RIPA had trichostatan A (TSA) added to the solution as an HDAC inhibitor.

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<tr>
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</table>
Another approach on the determination of the level of chromatin accessibility was attempted. Chromatin was determined to be in an open conformation with a fold enrichment value above 750 was achieved, while closed conformation was determined with a fold enrichment value below 500. Tested genes CD34, FcεRIβ and FcεRIα for multiple wild-types showed that this method of determining the level of chromatin accessibility failed to determine the exact level of open chromatin or closed conformation, see appendix Figures S58-60. Fold enrichment values that were in the hundreds of thousands were considered to have the same degree of accessibility to values that were in the thousands, however this is significantly different. Due to the highly specific values that are used for open and closed chromatin, this fold enrichment method does not account for partial open or closed conformations. Therefore, further classification of what is open and closed chromatin is necessary.
Table S5: qPCR Negative Chromatin Controls AVG ΔCT. BMMCs cultured until full maturity WT4 and WT5 cells were used to determine the efficiency of the Assay Kit. Negative controls should have an AVG ΔCT less than 1.5 to be deemed a closed conformation.

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</table>
Influence of MAPK on mast cell differentiation and histone acetylation modifiers D. Den Hartogh, 197

Figure S55: qPCR Negative Chromatin Controls Gel Electrophoresis. RHO, Tnp1, Alb, HBB, Smcp and Prm2 are all known negative controls determined by their heterochromatin conformation. The presence of bands indicates accessible DNA and that the chromatin is not bound in a closed conformation.

Table S6: qPCR Negative Chromatin Controls AVG ΔCT DF 10,000. BMMCs digestion enzyme was further diluted to test enzymes effect. Negative controls should have an AVG ΔCT less than 1.5 to be deemed a closed conformation.

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<th>Gene</th>
<th>Concentration</th>
<th>AVG ΔCT</th>
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</thead>
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</table>
Figure S56: qPCR Negative Chromatin Controls DF 10,000 Gel Electrophoresis. RHO and HBB are known negative controls as their genome are bound in a heterochromatin conformation. The presence of bands indicates that the chromatin was digested.

Figure S57: qPCR Positive Chromatin Controls DF 10,000 Gel Electrophoresis. PPIA and GAPDH are known positive controls. Digested chromatin DNA produces a band, while undigested (closed chromatin) does not produce a band. The digestion enzyme was diluted up to 1:10,000 to limit the over-digestion that was occurring.

Table S7: Indication of Chromatin Accessibility. Accessibility is calculated by comparing a negative (closed) control to a positive control (open). The gene of interest is then placed between this scale to determine the level of accessibility.

<table>
<thead>
<tr>
<th>Accessibility</th>
<th>Chromatin Structure</th>
<th>Potential of Epigenetic Silencing</th>
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<tr>
<td>95 – 100%</td>
<td>Fully accessible</td>
<td>Not silenced</td>
</tr>
<tr>
<td>65 – 95%</td>
<td>Mostly accessible</td>
<td>Low level of silencing</td>
</tr>
<tr>
<td>20 – 65%</td>
<td>Low accessible</td>
<td>Moderately silenced</td>
</tr>
<tr>
<td>0 – 20%</td>
<td>Non-accessible</td>
<td>Completely silenced</td>
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</table>
Figure S58: qPCR CD34 Fold Enrichment Chromatin Accessibility. Chromatin is deemed to be in an open conformation when the fold enrichment is greater than 750 and closed when less than 500. CD34 promoter region shows open chromatin across differentiation, with 3 and 6 hours being slightly accessible for WT21. WT22 shows less accessibility at the CD34 promoter region.

Figure S59: qPCR FcεRIβ Fold Enrichment Chromatin Accessibility. Chromatin is deemed to be in an open conformation when the fold enrichment is greater than 750 and closed when less than 500. FcεRIβ promoter regions depicts open chromatin across differentiation, with the chromatin being more accessible at the beginning of differentiation.
Figure S60: qPCR FceRIα Fold Enrichment Chromatin Accessibility. Chromatin is determined as being in an open conformation when the fold enrichment is greater than 750 and closed when less than 500. WT21 promoter region had very accessible chromatin structure at the beginning of differentiation, by 3 hours chromatin remained partially open. WT22 had constant partially closed chromatin conformation across differentiation.