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Effect of non-muscle myosin II inhibition on epigenetics during oligodendrocyte development

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Introduction:

Oligodendrocytes: the myelinating glia of the CNS:

Oligodendrocytes (OL) branch and wrap multiple axonal targets in myelin in a process termed myelination. Regularly spaced polarized domains along the axons are wrapped concentrically by Schwann cells (SC) in the peripheral nervous system (PNS) and by OLs in the central nervous system (CNS). These domains include the node, paranode, juxtaparanode and internode.

Each myelin segment is broken up by 1 μm wide nodes of Ranvier, where action potentials are regenerated by Na+ gated voltage channels. Initially understood to allow for faster impulse propagation in axons, myelination has also been implicated in supporting long-term integrity of myelinated axons (Nave, 2010). Whether an axon becomes myelinated has functional consequences on how neurons transmit signals. Myelination plays a central role in modulating network activity in the brain (Fields, 2008).

The paranode is a region in myelinated nerve fibers where the terminal myelin loops form specialized septate-like junctions with the axolemma, the cell membrane of an axon (Thaxton and Bhat, 2009). These junctions are adjacent to the nodes of Ranvier and are thought to hinder free diffusion between the nodal space and the juxtaparanodal space (Pedraza et al. 2001). The formation of the paranodal region is critical to delineation and segmentation of axonal domains in nerve fibers that allow for proper conduction of the nerve impulse.

The juxtaparanode is a potassium channel-rich region that lies underneath the compact myelin sheath, proximal to the paranodes. The significance of localizing potassium channels in

this domain is thought to counteract instability of excitability as well as repolarizing the action potential (Thaxton and Bhat, 2009).

The portion of nerve fiber between two nodes is called the internodal region and it comprises 99% of total segment length. It is relatively free of specialized structures despite this it is an active site of axon-glial interactions mediated by Myelin-associated glycoprotein (MAG) as it localizes to the inner glial membrane (Salzer, 2003).

A transgenic mouse line displaying sparsely labeled OLs illustrate that a single OL can produce anywhere between 20-100 internodes with lengths that vary from 40-400 um. Variations in numbers and lengths of myelin internodes produced by OLs suggest that endogenous cues exist to achieve the precise and efficient myelination of axons during development (Chong et al., 2012). Even though subpopulations of oligodendrocyte progenitor cells (OPCs) are specified from different domains in a temporal-specific manner, the majority of OPCs differentiate and myelinate axons regardless of origin. When specific OPC populations are eliminated, OPCs from different regions can replace the lost OPCs, resulting in a normal phenotype (Kessaris et al., 2006).

Oligodendrocytes origin and early development:

OLs originate from multipotent neural progenitor cells (NPCs) residing in the pMN domain of ventral spinal cord (Emery and Lu, 2015). Neural progenitors from this domain will give rise to motor neurons and then to oligodendrocytes progenitors cells (OPCs) (Masahira et al., 2006).

The developmental progression of NPC to fully mature OL is as follows: primitive or uncommitted OPCs (OLIG1/2 +, A2B5 +, PDGFRα-low), committed OPCs (PDGFRα-high, NG2+), differentiating immature oligodendrocytes (O4+/CNP+), differentiated oligodendrocytes

(CC1+), and mature myelinating oligodendrocytes (MBP+, PLP+, MOG+). The differentiation process requires precise coordination of extensive extracellular and intracellular factors (Gregath and Lu, 2018).

Epigenetics and Oligodendrocyte Development:

A term coined by British developmental biologist Conrad Waddington in 1942 means 'above genetics' was conceived to describe the developmental processes connecting genotype and phenotype. Waddington understood that there appeared to be a "concatenation(s) of processes linked together in a network, so that a disturbance at an early stage may gradually cause more and more far reaching abnormalities in many different organs and tissues" (Waddington, 1942). His developmental studies revealed that embryo fruit flies could be persuaded to express different thorax and wing structures by changing the environmental temperature or through a chemical stimulus (Waddington, 1957). Since then, a variety of biological phenomena have been labelled as epigenetic including, paramutation, imprinting, X inactivation, histone modifications and cellular differentiation. Because normal development depends on communication between cells, a hormone, morphogen or growth factor that can initiate an epigenetic change that may be heritable. The environment of a cell is particularly important in determining its properties and fate (Holliday, 2006). Currently, epigenetics is understood as "the study of any potentially stable and, ideally, heritable change in gene expression or cellular phenotype that occurs without changes in Watson-Crick base-pairing of DNA" (Goldberg, Allis and Bernstein, 2007).

Chromatin can be in the open form (euchromatin), allowing transcriptional machinery access, or a closed form (heterochromatin), which does not allow for transcription. Modification of histones plays a critical role in development. The basic unit of chromatin is the nucleosome, composed of a core octamer of histones (H2A, H2B, H3 & H4) surrounded by 146 base pairs of

DNA. Post-translational modifications of specific amino acid residues on the tails of the histones transform the transcriptional activity of genes and the access of the transcriptional machinery to DNA. Chromatin modification can be separated into 2 groups of enzymatic activities. The first is responsible for secondary modification of histones, the second includes enzymes using ATP to unwind nucleosomes and affect DNA/histone interactions. (Liu and Casaccia, 2010). Understanding which genomic regions are characterized by heterochromatin and euchromatin within each cell allows for the definition of cell-specific patterns of gene expression. The oligodendrocyte lineage, progenitor nuclei are characterized by euchromatin while myelinated oligodendrocyte nuclei are characterized by heterochromatin (Tsai and Casaccia, 2019).

Acetylation, deacetylation, methylation and sumoylation are some of the modifications that can occur. Addition or removal of chemical groups on specific residues of histones contributes to transcriptional activation or repression. Interestingly, the same modification on a different residue produces opposing results. Deposition of a methyl group on lysine 4 of histone H3 is associated with gene activation, while methylation of lysine 27 (H3K27me3) results in repression. Removal of the methyl groups from these residues has the opposite effect, activation (Liu and Casaccia, 2010).

H3K27me3, is associated with downregulation of nearby genes it is catalyzed by a family of histone methyltransferases (HMTs), and methylated lysine residues are bound by specific domains called chromodomains. Oligodendrocyte lineage-specific ablation of the DNA methyltransferase (Dnmt1) in mice severely impaired developmental myelination and resulted in aberrant splicing and loss of immature oligodendrocytes, due to activation of an endoplasmic reticulum stress response (Moyon et al., 2016 & Tsai and Casaccia, 2019). The undifferentiated state of ESCs is a result of interactions between activating events and repressive mechanisms

involving transcription factors Oct4, Sox2, Nanog and deposition of repressive histone marks (H3K27me3) (Boyer et al., 2006). Neural precursors remove the H3K4me3 activation mark but keep the repressive H3K27me3 on mesodermal or endodermal genes resulting in silencing of genes associated with either lineage choice. Simultaneously, in regions containing neural genes H3K27me3 are removed while H3K4me3 are retained (Bernstein et al., 2006). The enzyme responsible for the deposition of H3K27me3, EZH2 (enhancer of Zeste Homolog2), was also identified as an important mediator of OPC specification in neural stem cells (NSC) by repressing neuronal and astrocytic genes as well as genes regulating pluripotency (Sher, Boddeke, Olah, & Copray, 2012; Tsai and Casaccia, 2019).

Among the post-translational modifications, acetylation of lysine residues in the N-terminal tail of nucleosomal histones mediated by histone acetyl transferases (HATs) is associated with transcriptionally competent chromatin. The acetylation of histone H3 lysine 9 (H3K9Ac) marks active promoters. Acetylated lysine residues are bound by α-helical structural motifs called bromodomains, which recruit transcription factors, coactivators, and components of the transcription initiation complex (Tsai and Casaccia, 2019).

Histone deacetylation by contrast is catalyzed by histone deacetylases (HDACs) and is functionally associated with transcriptional repression (Liu et al., 2007). HDACs are separated into 4 classes based on sequence homology, subcellular localization, and tissue distribution. Class III includes nuclear and cytosolic enzymes called Sirtuins, of which Sirt2 has been shown to regulate oligodendrocyte differentiation.

OLs from younger mice remyelinate more efficiently than those from old mice, recent evidence attributes the difference to epigenetic changes linked to recruitment of histone deacetylases (HDACs). Efficient recruitment of HDACs in young mice corresponds to the

silencing of transcriptional repressors in OLs. Reduced recruitment of HDACs in older animals leads to the accumulation of repressors, and hampers differentiation and remyelination. Old and young OLs were exposed to similar microenvironments, an ideal way to test the role of their intrinsic capacity to remyelinate. Surprisingly, when old OLs are exposed to a youthful systemic environment, their ability to differentiate and remyelinate is revitalized to levels found in younger cells. Findings suggest that environmental signals dominate intrinsic mechanisms to modulate the differentiation and myelination of oligodendroglia (Shen et al., 2008).

It is also established that actin plays important roles in the cell nucleus. In vitro studies show nuclear actin associates with all three RNA polymerases, suggesting it plays a critical role in communication between the cytoplasm and the nuclear expression of protein encoding genes (Hofmann et al. 2004; Hu, Wu and Hernandez, 2004; Kukalev, Nord, Palmberg, Bergman, & Percipalle, 2005; Philimonenko et al., 2004). Nuclear actin associates with and inhibits the activity of HDAC1 and HDAC2 (Serebryannyy, Cruz & de Lanerolle, 2016), thereby supporting an overall function for the integrity and activity of the BRG1-containing SWI/SNF chromatin remodeling complex (Kapoor & Shen, 2014). Nuclear actin appears to act as a regulator of histone acetylation and transcriptional activation. Actin was shown to co-immunoprecipitate with both LINC complex (a protein complex that acts a direct physical connection between the plasma membrane and nuclear chromatin) and repressive H3K9me3 histone mark in protein extracts from white matter tracts (Hernandez et al., 2016).

Epigenetic Marker	Function
HDAC2	Closed chromatin– transcriptional incompetence
H3K9Ac	Open chromatin- transcriptional activation
H3K27me3	Closed chromatin- transcriptional incompetence

Table 1. Epigenetic markers of interest in OL development and their effects on transcription.

Mechanotransduction affects the epigenetic state of cells in the CNS

It is well established that forces exerted at the cell surface or cytoskeleton can induce rapid, subnuclear deformations by propagating through the cytoskeleton and the LINC complex (Maniotis et al., 1997). The decision of NSCs to differentiate into either neurons, oligodendrocytes, or astrocytes is influenced by the stiffness of their environment. NSCs were directed toward the neuronal lineage when plated on soft gels (0.1-0.5kPa) but when plated on slightly stiffer gels (1-10kPa) they could be directed toward glial lineages (Tsai and Casaccia, 2019), while increased extracellular matrix rigidity results in significant reduction in OL branching complexity (Urbanski et al. 2016). *In vitro* studies have also shown that increased matrix stiffness promotes a reactive phenotype in astrocytes and other glia (Lu et al., 2010; Janmey and Miller, 2010). OPCs from the CNS are mechanosensitive. Cell survival, proliferation, migration, and differentiation vary with mechanical stiffness of the environment these are maximized at an intermediate gel stiffness over the physiological range (Jagielska et al. 2012).

Non-muscle myosin II: a cytoskeletal protein with multiple cellular functions

NMII in the cytosol:

Non-muscle myosin II (NMII) is a major contributor to cell organization, polarity, and differentiation, with altered NMII activity linked to several pathologies (Newell-Litwa, Horwitz and Lamers, 2015). For these reasons NMII is an important target for study and therapeutics.

NMII crosslinks actin filaments to form actomyosin filament bundles. These actomyosin bundles generate contractile forces capable of deforming the cell membrane, affecting many cellular functions including migration and neuronal synaptogenesis (Vicente-Manzanares, Ma, Adelstein and Horwitz, 2009). These forces also generate mechanical signals in a process called mechanotransduction which can alter gene expression. (Humphrey, Dufresne and Schwartz, 2014).

NMII consists of a heavy chain, including a globular head domain that binds both actin and ATP; a neck region, which binds both essential and regulatory light chains (ELC and RLC, respectively); and a tail region, which homodimerizes in a helical formation, and a non-helical tail region that directs subcellular localization of NMII isoform. Serine/threonine kinases regulate NMII activity by phosphorylation of myosin RLC. NMII filaments associate with each other in an anti-parallel formation allowing them to crosslink and slide actin filaments past each other (Newell-Litwa, Horwitz and Lamers, 2015).

NMII in the nucleus:

Because of its ability to regulate NMII through direct RLC phosphorylation and indirectly through inhibition of myosin light chain phosphatase (MLCP) RhoA-associated kinase (ROCK) is considered a master regulator of NMII activity. In the GTP-bound state, Rho GTPases promote downstream signaling through kinases like ROCK (Vicente-Manzanares, Ma, Adelstein and Horwitz, 2009). Guanine nucleotide exchange factors (GEFs) activate Rho GTPases by loading

GTP onto them, whereas GTPase-activating proteins (GAPs) stimulate GTP hydrolysis, to inactivate Rho GTPase signaling. RhoA activates NMII and promotes the formation of actomyosin filament bundles through ROCK (Katoh et al., 2001).

RhoA/ROCK-mediated NMII activation functions downstream of inhibitory chondroitin sulfate proteoglycans (CSPGs), and the ROCK inhibitor Y-27632 partially rescued axon regrowth (Borisoff et al., 2003). blebbistatin, an NMII inhibitor, restored control axon growth on inhibitory substrates *in vitro* (Hur et al., 2011). These findings make NMII an interesting target for axonal regeneration therapies while other studies also show that blebbistatin and Y-inhibitor can also be used to affect OL branching and myelination.

Non-Muscle myosin II is a negative regulator of OL branching and myelination

Our laboratory has previously reported that NMII regulates myelination in both the CNS and PNS (Wang et al., 2008). High levels of NMII activity prevents oligodendrocyte differentiation, while NMII inhibition promotes oligodendrocyte maturation and myelination (Wang et al., 2008,2012). In addition, NMII inhibition promotes re-myelination following brain lesions in mice (Rusielewicz et al. 2014).

Levels of NMII decrease as oligodendrocytes differentiate and inhibition of NMII activity increases branching and myelination, suggesting that NMII is a negative regulator of oligodendrocyte differentiation (Wang et al, 2012). Overexpression of NMII prevents oligodendrocyte branching and differentiation. OPC maturation is accelerated in NMII knockout mice shown by a significant increase in the percentage of mature MBP+ cells. Inhibition of branching induced by overexpression of constitutively active RhoA can be reversed by treatment with Y27632 (Y-inhibitor) or blebbistatin. In non-muscle cells NMII is activated by

phosphorylation of myosin light chain (MLC), several kinases can phosphorylate MLC, including Rho-associated kinase (ROCK), a major downstream effector of RhoGTPase. In the CNS, activation of ROCK by RhoA has been implicated in myelin-mediated inhibition of axonal outgrowth and OPC differentiation following nerve injury. Activation of Fyn kinase has also been shown to inhibit RhoA activity, thus promoting oligodendrocyte branching (Wang et al. 2012).

siRNA experiments were conducted to determine the responsible NMII isoforms and found that silencing of NMII isoforms A or B reproduces the increase in branching and myelination observed after total NMII inhibition. Both isoforms are expressed by oligodendrocytes, NMII-B being predominant. Overexpression of NMII-A or NMII-B results in decreased morphological complexity of OPC. Treatment with specific NMII inhibitor blebbistatin rescues the inhibition of process extension observed in cells overexpressing wild-type (WT) or constitutively active (CA) RhoA. (Wang et al., 2012).

Inhibition of myosin II activity in OL precursors does not interfere with their differentiation or their ability to form myelin but instead potentiates myelin formation in culture. Inhibition of myosin II activity by blebbistatin resulted in increase in the amount of myelin formed in DRG (dorsal root ganglia)-OL co-cultures. Also, in blebbistatin treated cultures, individual myelinating OL give rise to more internodes than OL in control cultures. Mean number of myelin segments made by a single OL in blebbistatin-treated cultures was 3-4 times more than OL in control cultures. Myosin II activity is not required for OL differentiation and myelination, and in the presence of blebbistatin, individual OLs are more branched and make more myelin. By controlling the spatial and localized activation of actin polymerization, myosin II regulates SC polarization along the axon and OL branching, and thereby their ability to form myelin (Wang et al., 2008).

Epigenetics states can be affected by changes in NMII activity

NMII inhibition in adult mouse fibroblasts using blebbistatin revealed that actin-myosin contractility is critical in mechano-modulation of epigenetic state and cell programming (Downing et al., 2013).While deleting NMIIA in mouse epidermis leads to attenuated H3K27me3 mediated silencing and lineage commitment, nullifying morphogenesis (Le et al., 2016). Exposure of OPCs to mechanical stimulus was associated with upregulation of the two upstream activators of NMII RhoA and ROCK-II (Jagielska et al. 2017). In vivo, ablation of this myosin removed inhibitory effects of rigid substrates on branching complexity of oligodendrocytes (Urbanski et al., 2016). Taken together it is clear that mechanosensitive proteins can affect lineage commitment of cells, specifically NMII inhibition has been shown to do just that by affecting the epigenetic marker deposition.

Considering its role as a mechano-sensitive motor protein capable of regulating OL differentiation and repair, the necessity of HDAC2 for OL development and that in the presence of blebbistatin individual OLs are more branched and make more myelin we chose to study if there were any epigenetic changes brought on by NMII inhibition in OL cultures. We expected NMII inhibited cultures to exhibit more transcriptional silencing markers (HDAC2 and 3K27me3) compared to control and a decrease in transcriptional activator mark H3K9Ac was also expected as HDACs and H3K27me3 begin to condense the chromatin structure allowing for only essential genes to be expressed.

Materials and Methods:

Glial Cell purification:

Methods for purification of primary rat OPC from P2 rat cortices have been previously described (Wang, et al. 2008). Briefly, primary OPC were purified by Ran2- and A2B5+ immuno-panning from mixed glial cultures of postnatal day 1 rat cerebral cortices. Purified OPC were seeded onto poly-lysine coated glass coverslips and maintained in Sato media (DMEM,100 μ g/ml transferrin, 100 μ g/ml BSA, 20 nM progesterone, 100 μ M putrescine, 30 nM sodium selenite, 2 mM L- glutamine, 5 μ g/ml insulin, 60 μ g/ml N-acetyl cysteine, and 10 μ M forskolin) with 10 ng/ml PDGF and 10 ng/ml bFGF for proliferation for three days. After this time, cultures were induced to differentiate in Sato containing T3 for 2-3 days (30 ng/ml). To look at the effects of myosin activity on differentiation and epigenetic markers expression cultures were treated with 10 μM of either ROCK inhibitor KD25 (Calbiochem) or blebbistatin (Calbiochem).

Immunofluorescence and imaging:

Cultures were fixed in 4% PFA and processed for immunocytochemistry as described previously (Wang, et al., 2008). Briefly, cells were blocked with 1% donkey serum and 5% BSA in PBS for 30 min, then incubated with primary antibody as described previously (Zhang et al., 2006). Cultures were examined by epifluorescence or confocal microscopy. Epifluorescence images were acquired using a microscope (DMI 4000B; Leica) with LAS 1.7.0 software equipped with a digital camera (DFC350FX; Leica) and the following objectives: N PLAN 10 × /0.25 NA and N PLAN L40 × /0.55 NA (all from Leica). Confocal images were acquired with a laser scanning microscope (LSM510; Carl Zeiss, Inc.) using Plan-Apochromat 20 × /0.75 NA or Neoflour 40 × /1.3 NA oil objectives and LSM software (all from Carl Zeiss, Inc.).

Antibodies:

For this study the following antibodies were used: H3K9Ac (Abcam, ab4441) , H3K9Me3 (Abcam, ab8898) and HDAC2 (Abcam, ab12169); MBP (SMI-94; Covance); TOPRO; Actin (acti-stain 670 phalloidin, Cytoskeleton Inc.). Secondary antibodies were purchased from Jackson Immuno-Research Laboratories Inc.

Morphological Analysis:

The numbers of OL (+T3 48 hours and 72 hours) cultures were counted in micrographs from 9 random low-power fields/coverslip in ImageJ 1.38v (total of 18 fields per condition per experiment; total of two experiments). Cells were classified according to their branching complexity as follows: 1) low complexity: cells with at least one or two branches extending directly from the cell body; 2) medium complexity: cells with processes extending from primary branches; and 3) high complexity: cells with processes extending from secondary branches (Table 3). Mature OL were also identified by MBP staining. In addition, type 1 and type 2 were MBP-, while type 3 cells were MBP+.

For quantitation of epigenetic markers nuclear staining, we used ImageJ 1.52p. Area, mean gray value (MGV) and integrated density values (INTDEN) (Table 2) were collected for each epigenetic marker in individual cells. When measuring area, a dim and bright object are considered equivalent if they are the same size. While measuring MGV, a large and small object are considered equivalent if they have the same brightness. Integrated density is the product of area and MGV so a small bright object and a large dim object would be considered equivalent. Because it takes into consideration both the size and concentration of the stain INTDEN was used to examine the changes in epigenetic markers staining. Statistical analyses (non-parametric Kruskal Wallis t-test, when comparing changes in marker intensity at 48 vs. 72 hours in each group, 2-way

ANOVA when comparing changes in intensity across different groups and treatments and Chi-squared when comparing changes in the distribution of cell types across different treatments) were performed using GraphPad Prism.

Measurement	Real-world correlate	Equivalent objects
Area	Cross-sectional or projected area	
Mean intensity	Concentration of probe	
Integrated density	Amount of probe	

Table 2. Epigenetic markers were measured by calculating the area, mean gray value, and integrated density of the stain. https://forum.image.sc/t/intden-vs-rawintden/5147/2

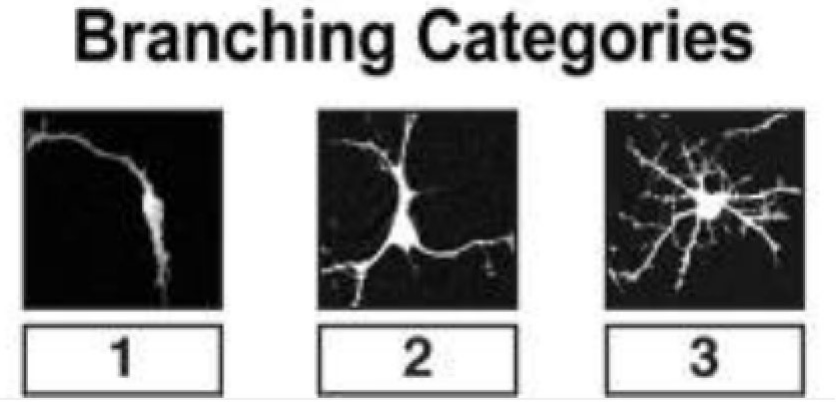

Table 3. Classification of cells by branching complexity with MBP staining. 1) low complexity: cells with at least one or two branches extending directly from the cell body (MBP-); 2) medium complexity: cells with processes extending from primary branches (MBP-); and 3) high complexity: cells with processes extending from secondary branches (MBP+). (Wang et al., 2012).

Results:

Exposure to T3 induces increased oligodendrocyte branching and MBP expression

It is known that immature OPC maintained in media containing PDGF cells exhibit a simple bipolar morphology. After addition of triiodothyronine (T3), a thyroid hormone known to induce OPC differentiation, OPCs become more branched and eventually mature into fully mature MBP+ OL.

As expected, cultures treated with T3 for 72h yielded more cells with mature morphology (primary and secondary branching), compared to less mature OLs which are MBP- and exhibit just primary branching or immature OLs which are characterized as having bipolar process branching (Figures 1 and 2). In control cultures treated with T3 for 48h, a total of 201 cells (average 67±3.5 cells per field; mean±SEM) were scored as exhibiting mature morphology, while 880 OLs (247.7±41.6) were scored in control cultures exposed to T3 for 72h (Figure 2 and 3). The same trend was observed in cultures treated with drugs affecting NMII activity, i.e. 226 (75±16.6) vs. 961 (320.3±46.6) mature OLs in blebbistatin treated cultures; and 163 (54.0±11.1) vs. 788 (262.7±53.7) mature OL in cultures treated with the ROCK inhibitor, KD025 (Figure 2 and 3).

This increase in branching complexity was also accompanied by increased expression of MBP. We found that control OPC cultures exposed to T3 for 48h had 9.56%±1.14 (mean±SEM) MBP+ cells compared to 58.7%±3.53 MBP+ after 72h. The same trend was observed in blebbistatin 13.6%±4.5 vs 69.3%±4.45 and KD95-treated cultures 9.35%±.15 vs. 46.63%±6.7 (Figure 4).

Collectively, these results indicated that longer exposure to T3 leads to extensive branching and increased MBP expression, and that this trend is maintained following NMII inhibition.

Nuclear HDAC2 staining increases as OLs mature

We next examined the expression of HDAC2 which is known to interact with other transcriptional regulators to form repressive complexes and to inhibit expression of OPC differentiation inhibitors or block the activation of neuronal differentiation genes (Ye et al. 2009). Histone deacetylation by HDAC2 plays a critical role in OPC differentiation (Liu and Casaccia, 2010).

We performed a 2-way ANOVA statistical test to determine whether the average INTDEN of categorized cell types were significantly different than the average INTDEN of other categorized cell types within the same culture. We found that the INTDEN of HDAC2 staining increases significantly ($p<0.0001$, non-parametric t-test, 2-way ANOVA) as cells mature (from type 1 to type 3) in T3 (Figure 6). Also, the INTDEN values overall were higher in 48-hour T3 treatment than 72-hour T3 treatment. (Figure 5).

Next, we examined the effect of NMII inhibition on HDAC2 staining using 2 different approaches blebbistatin a direct inhibitor of NMII activity and KD25 a ROCK inhibitor. We found the same trend observed in control cultures, that is, as cells mature from type 1 to type 3 the INTDEN levels increase for control (320.3±1330, INTDEN±SD vs 4014±1717), blebbistatin (2454±1215, vs 4165±1771) and KD25 (2401±1330, vs 4014±1717) treatments (Figure 5). We found no significant differences on the average INTDEN of HDAC2 staining when comparing control to myosin inhibited cultures after 48h or 72h of T3 treatment, suggesting that myosin inhibition has no effect on the levels of HDAC2 (Figure 5).

These results are in accordance with recent studies that found the effects of HDAC inhibition on OL development appear to be temporally specific. HDAC inhibitors impair the early phase of OPC differentiation, but do not affect myelinating gene expression after the onset of

myelination (Shen et al. 2005). Jointly, our results are consistent with previous work showing increased histone deacetylation by HDAC as a critical epigenetic regulator of OL differentiation.

Levels of nuclear H3K9Ac were unchanged as OLs mature

When H3K9Ac mark is deposited genes are turned on, it is associated with active promoters and has been shown to correlate with OL differentiation by promoting Sox2 binding (Lyssiotis et al., 2007) (Du et al., 2017). Unexpectedly, we found no remarkable change in H3K9Ac INTDEN across cell types in control or cultures treated with myosin inhibitors after 48-hours in T3 (Figure 7 and 8). Prolonged T3 treatment significantly increased the H3k9Ac INTDEN (values are higher at 72 hours than at 48 hours of T3), across all conditions tested (Figure 7). Although the magnitude of the change is comparable between control and cultures treated with blebbistatin (Figure 7). Cultures treated with ROCK inhibitor KD25, showed a significant increase in H3K9Ac INTDEN, as cells mature from type 1 (7078±2373, mean±SD) to type 3 (8262±1958) (Figure 8) after 72 hours of T3 treatment (Figure 7 and 8).

Thus, H3K9Ac levels increased after prolonged treatment with T3 in all conditions tested (control and myosin-inhibited), but surprisingly this increase is comparable across all cell types and not related to their differentiation, with the exception of cultures treated with KD25, where H3K9Ac levels were significantly increased after 72 hours in type 3 cells.

Nuclear H3K27me3 staining increases as OLs mature

Increased activity in repressive marks is expected as the cell begins to repress any unessential genes or any inhibitors of OL development as it differentiates and only promotes essential and OL specific genes.

The INTDEN of H3K27me3 stain increases significantly ($p<0.0001$, non-parametric t-test) as OLs mature in control and cultures treated with myosin inhibitors in 48-hour T3 (Figure 10). Interestingly, this change is not observed after 72 hours in T3, when the INTDEN of H3K27me3 were significantly decreased in all conditions tested (Figure 9 and 10).

When comparing the effects of myosin II inhibition, we found treatment with KD25 results in a decrease of H3K27me3 INTDEN in type 1 (2254±1108 vs 2864±1502), type 2 (2554±1035 vs 3683±1614), and type 3 (3973±2109 vs 4708±1495) cells compared to controls after 48-hours in T3. Treatment with blebbistatin has the opposite effect i.e. increased H3K27me3 INTDEN in type 3 cells when compared to controls (5837±1982 vs 4708±1495) (Figure 9 and 10).

Collectively, our data shows that after 48-hour T3 treatment NMII inhibition appears to have different appreciable effects on this repressive epigenetic mark compared to the control. blebbistatin appears to increase INTDEN while KD25 appears to decrease INTDEN in corresponding cell types compared to control. These differences are observed in the 72-hour cultures when comparing the NMII treatments with one another but not when comparing with control.

Conclusion:

Collectively our data suggests that despite being implicated in increased OL differentiation, NMII inhibition does not affect HDAC2 and H3K9Ac epigenetic modifications but appears to affect methylation marker H3K27me3.

A unique feature of the cytoskeleton is its ability to remodel in response to force. As force bearing cell to cell and cell to matrix contacts through the actomyosin cytoskeleton directly link to the nuclear lamina and to chromatin, it has been proposed that extrinsic forces could impact chromatin remodeling, but any mechanisms of force-dependent chromatin remodeling remain elusive (Le et al., 2016).

The epigenetic programming of stem cells into oligodendrocytes involves three sequential stages of lineage progression. First, pluripotent stem cells transition to neural precursors by repressing pluripotency genes and restricting the lineage potential to neural fate. Second, multipotential neural precursors to oligodendrocyte progenitors is associated with the progressive loss of plasticity and the repression of neuronal and astrocytic genes. Lastly, differentiation from oligodendrocyte progenitor into myelin-forming cells by activation of myelin genes (Liu and Casaccia, 2010). The ability of oligodendrocyte progenitor cells (OPCs) to acquire identity of myelin expressing cells or alternative fates depends on HDAC activity. According to our findings, this marker does not appear to be affected by NMII activity. In agreement with the current understanding that as OLs mature they begin to compact chromatin to silence any unessential genes and genes that inhibit OL development, we found that the levels of repressive marks HDAC2 increased as OLs mature.

The methylation mark H3K27me3 appears to be affected differently by blebbistatin and KD25 treatment when compared to controls. Our lab has previously shown that blebbistatin a

direct NMII-inhibitor promotes OL differentiation and myelin formation (Wang et al. 2008) which is in line with the H3K27me3 48-hour T3 data showing an increased INTDEN in blebbistatin cultures compared to control. Meanwhile, there is decreased INTDEN of H3K27me3 in the KD25 ROCK-inhibitor cultures compared to control in 48-hour T3 for all cell types thus the H3K27me3 methylation mark appears to be the most sensitive to changes in myosin activity.

NMII is a downstream target of RhoA through Rho-kinase (ROCK), which phosphorylates the myosin light chain (MLC) and activates NMII. KD25 a ROCK inhibitor prevents phosphorylation of the myosin light chain which prevents NMII activation and promotes differentiation. OPCs maintained in proliferating conditions (+PDGF) have higher levels of pMLC compared to mature oligodendrocytes (T3+). This could explain why no difference was found in H3K27me3 72-hour T3 cultures as most of the cells in this culture are mature and would exhibit low levels of pMLC anyway, ROCK inhibitor would be more effective in proliferating conditions. Our previous work also showed that while inactivation of RhoA/ROCK signaling promotes oligodendrocyte process extension via downregulation of MLC phosphorylation, MBP expression was not affected by this treatment (Wang et al. 2012). Because KD25 is an indirect NMII inhibitor it could affect other functions of ROCK other than NMII activation. Thus, while KD25 will initially promote OPC branching, once cells begin to mature and express MBP, ROCK activity might be required for other functions such as methylation. By comparison, blebbistatin, a direct inhibitor of NMII motor activity, was shown to promote OL branching expression, changes that are compatible with enhanced differentiation and methylation.

Future experiments should consider comparing OPCs cultured in proliferating conditions (+PDGF) to T3 treated cultures to better appreciate the change in epigenetic levels from immature progenitor cells to T3 treated cells. This experiment compares cells cultured in different T3

treatments, progenitors cultured in PDGF-α and bFGF were not included. This could account for why we could not find any significant differences as these cells are already differentiating. More differentiation marker staining to label progenitors (PDGFRα+, NG2+), newly formed oligodendrocytes (O4+, CNPase+), differentiated oligodendrocytes (CC1+), and mature myelinating oligodendrocytes (MBP+) would be more effective at identifying different cell types without relying on morphology, especially since ROCK inhibition has been shown to affect morphology and not MBP expression. Also, in this study, we evaluate three markers, HDAC2, H3K27me3 and H3K9Ac, the latter two are specific to H3 histones. Future studies may want to consider other histone modifying epigenetic markers such as phosphorylation or ubiquitylation. Finally, chromatin immuno-precipitation (ChIP) could provide specific location in the genome that various histone modifications are associated with, revealing which genes are being silenced and which are active during different stages of developments.

Although there is evidence linking physical stimulation to functional effects in OLs, the molecules responsible for transducing external force into a program of differentiation remain elusive.

Figures:

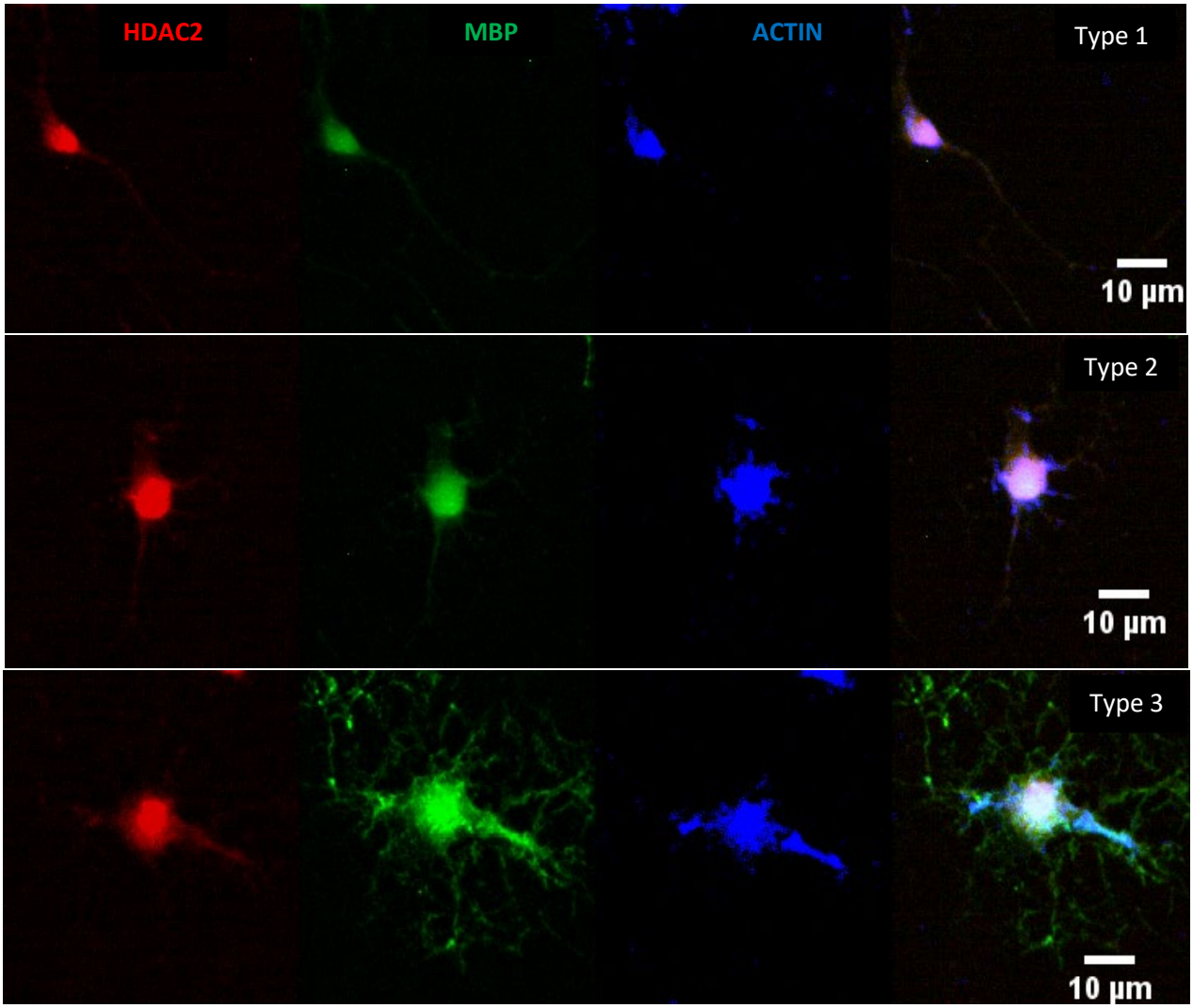

Figure 1. Representative field of HDAC2 staining in control cultures after 48 hours T3. Cells stained with HDAC (red), MBP (Green), Actin (Blue), Composite (right column); cells were sorted into 3 categories type 1 (MBP-), bipolar branching (top row), type 2 (MBP-); primary branching (center row), and type 3 (MBP+); primary and secondary branching (bottom row). Nuclear MBP staining seen in type 1 and type 2 is not considered specific, cells are considered MBP+ when staining is observed in the processes and cytoplasm. The same classification was applied for all epigenetic markers used for this study.

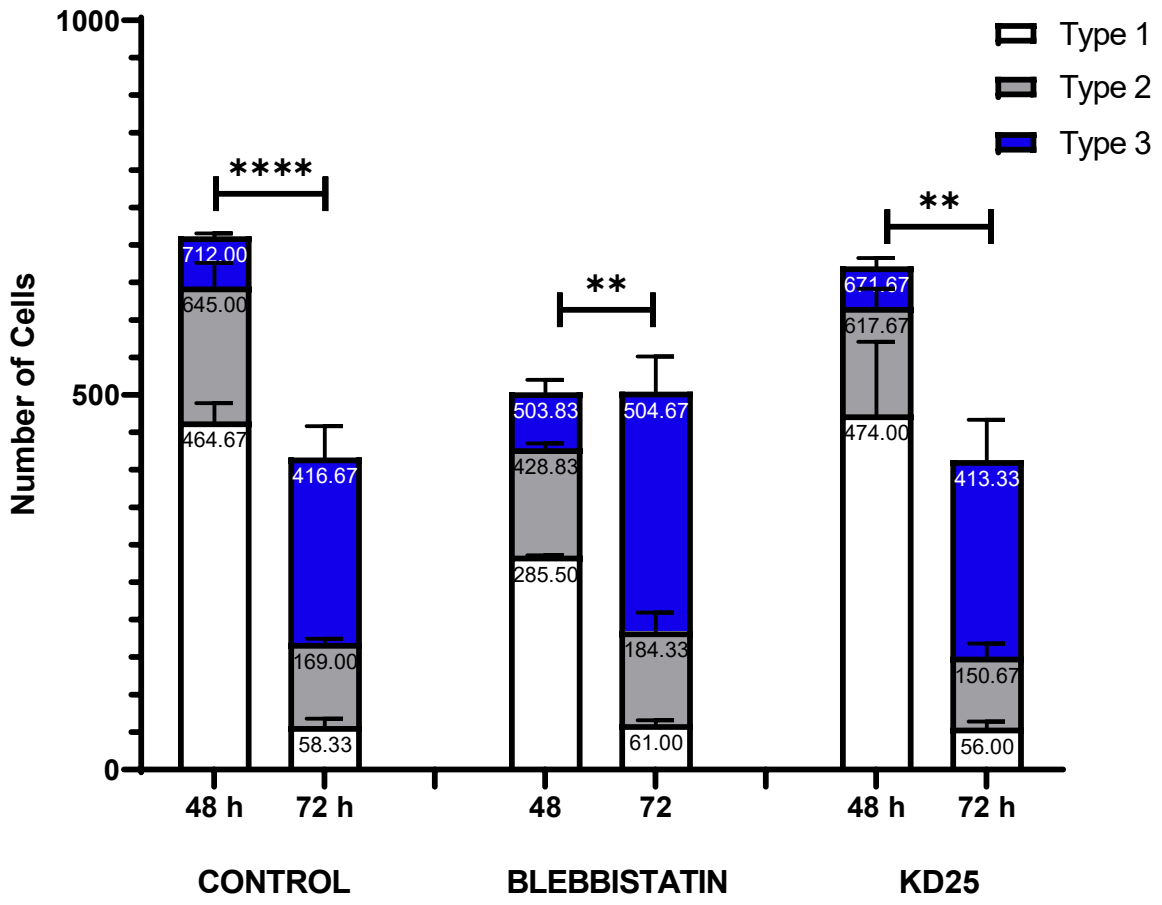

Figure 2. Average cell counts of OPC cultures treated with T3 for 48 hour and 72 hours in control (left), blebbistatin treated (middle) and KD025 treated (right) cultures separated by morphology; type1 or bipolar branching (white); type 2 or primary branching (gray); and type 3 or primary and secondary branching (blue). Graph show the mean number of cells in each category per field±SEM. (2 cultures per condition/experiment) An increase in type 3 cell at the expense of type1 cells was observed in all conditions tested. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, Kruskal-Wallis t-test.

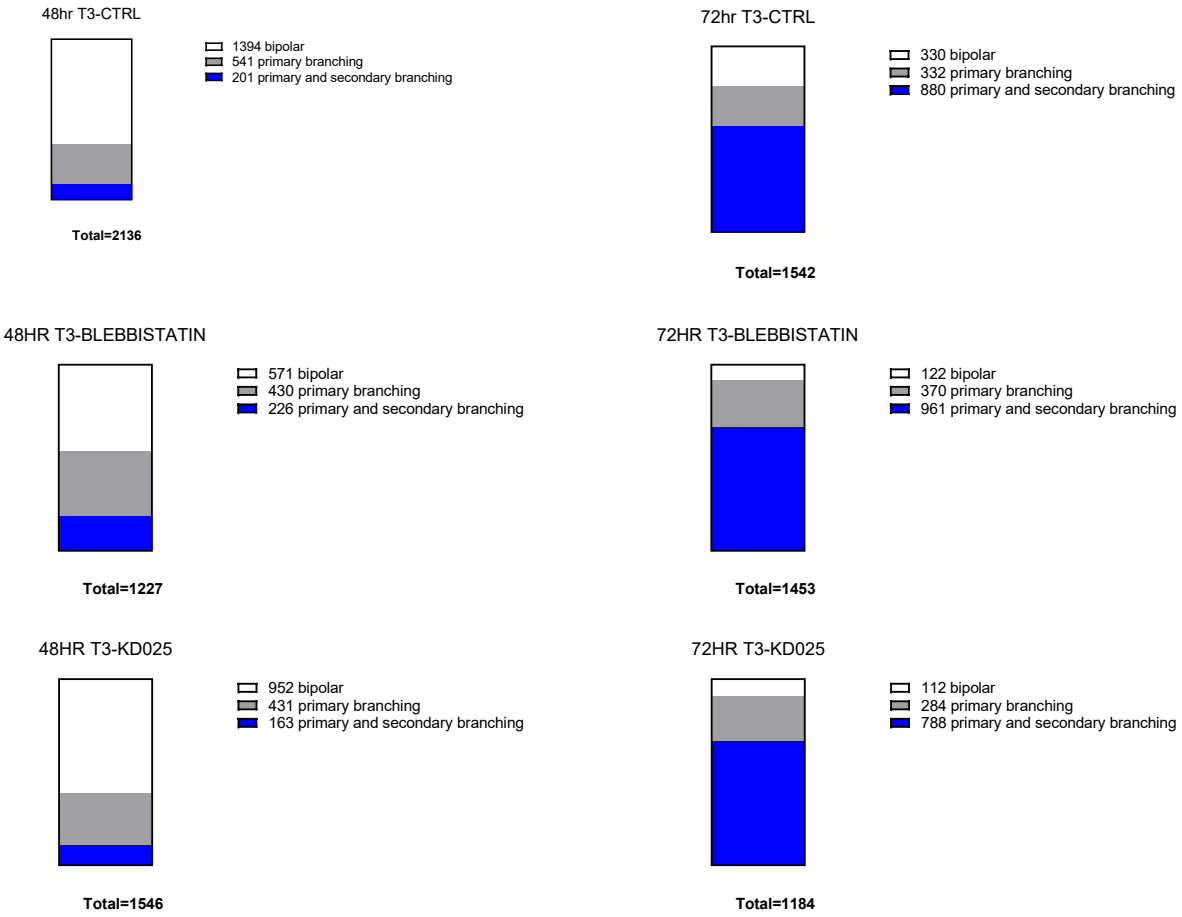

Figure 3. Total cell counts of OPC cultures treated with T3 for 48 hours (left column) and 72 hours (right column) for all the epigenetic markers, in control (top row), blebbistatin treated (middle row) or KD025 treated (bottom row) cultures. (6 cultures per condition)

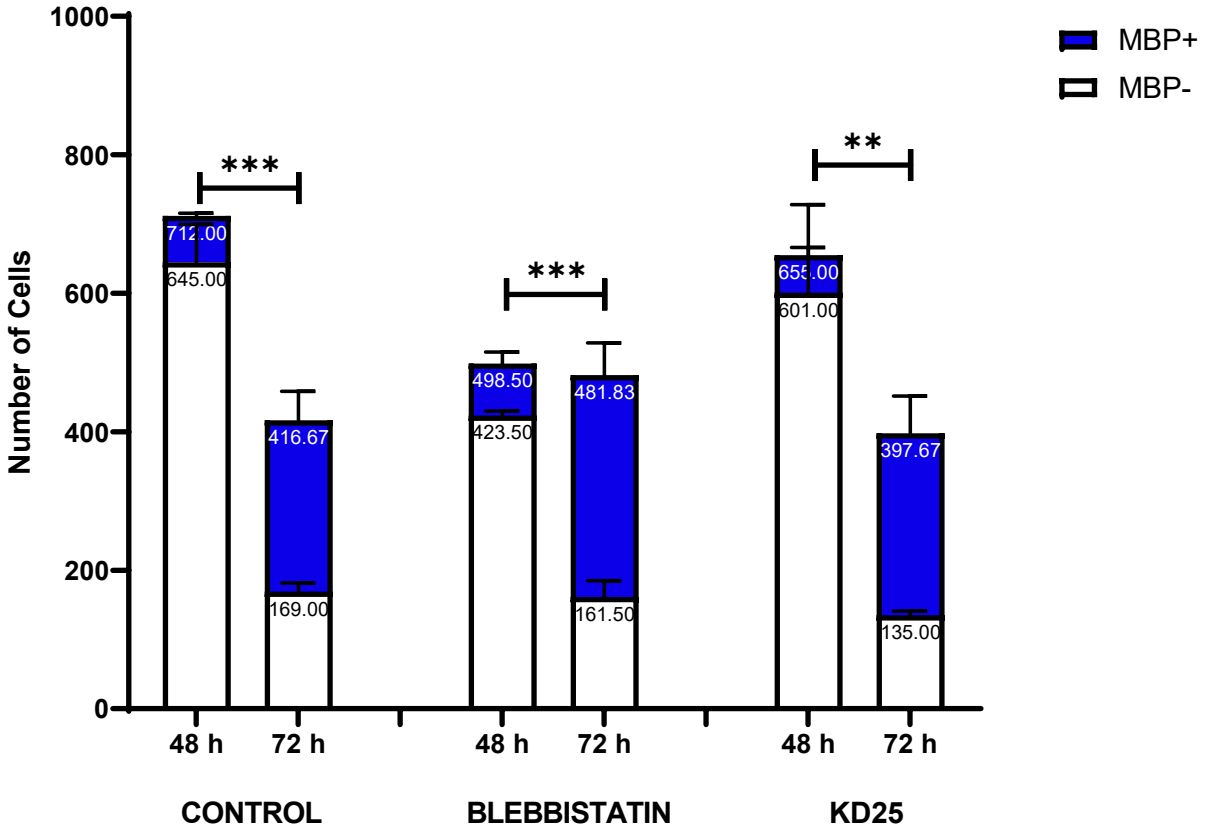

Figure 4. MBP expression of OPC cultures treated with T3 for 48 hours and 72 hours in control (left), blebbistatin treated (center) and KD025 treated (right) cultures. Numbers inside bars represent the mean number of cells per field±SEM (2 cultures per condition/experiment) *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, Kruskal-Wallis t-test.

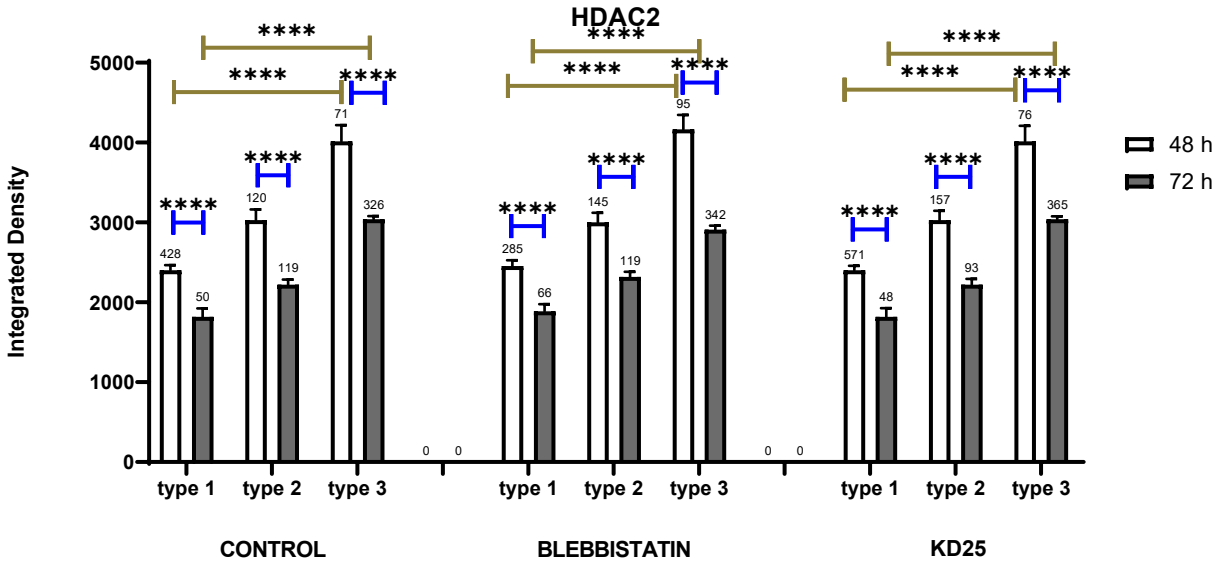

Figure 5. Mean Integrated Density of HDAC2 stain in control (left), blebbistatin (middle), and KD25 (right) stained OPC cultures of different branching complexity treated in T3 for 48 hours (white) and 72 hours (gray). Values above bar represent the total number of cells counted per condition. (2 cultures per condition/experiment) A significant increase in intensity is observed as cells mature in each condition in 48-hour and 72-hour T3, ****p<0.0001, 2-way ANOVA (red). A significant decrease in overall HDAC2 intensity as cells differentiate after 72 hours in T3 was observed in all conditions tested compared to 48 hours in T3 ****p<0.0001, Kruskal-Wallis test (blue).

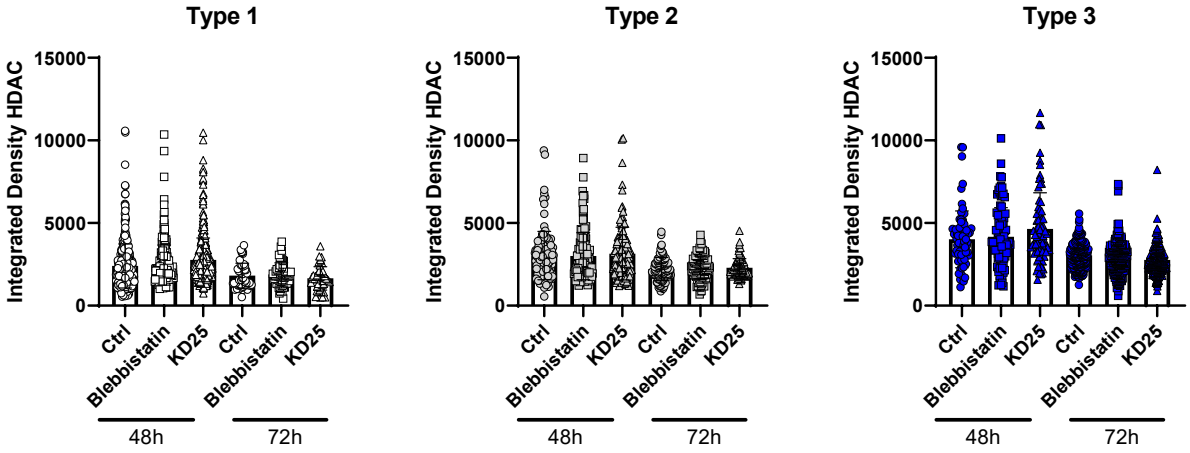

Figure 6. Integrated Densities for HDAC2 stain arranged by cell type. Points represent individual cells; black bar represents mean and SD (2 cultures per condition/experiment). No statistically significant differences were observed when comparing the same cell types across different treatments, 2-way ANOVA.

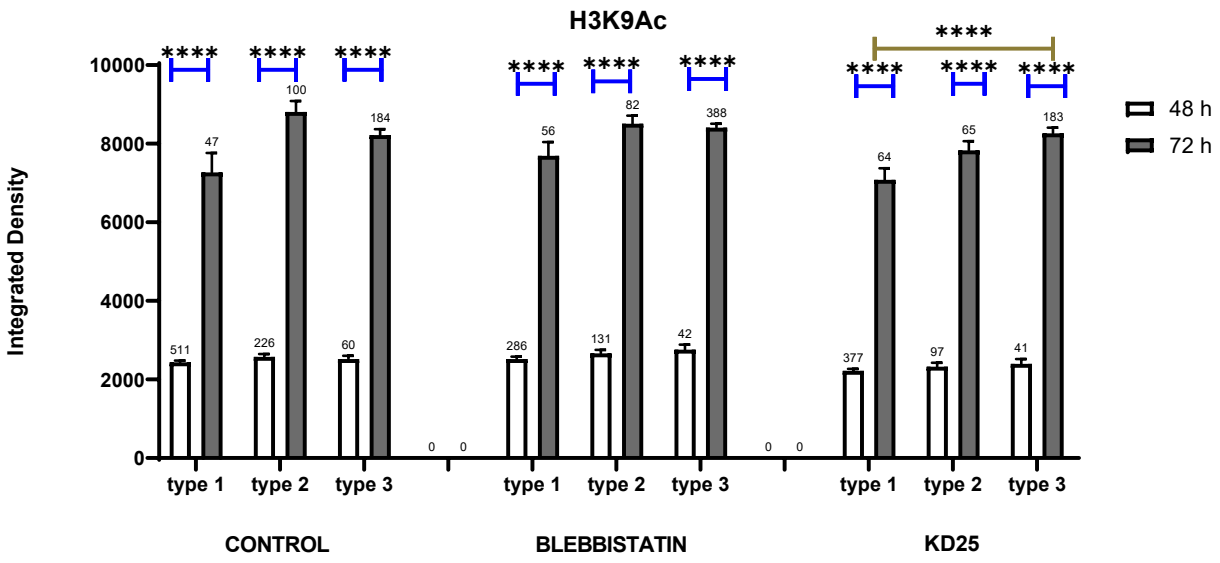

Figure 7. Mean Integrated Density of H3k9Ac stain in control (left), blebbistatin (middle), and KD25 (right) stained OPC cultures of different branching complexity treated in T3 for 48 hours (white) and 72 hours (gray). Values above bar represent sample size (2 cultures per condition/experiment). A significant increase in intensity in KD25 treated cultures as they mature at 72 hours was observed, ****$p<0.0001$, 2-way ANOVA (red). A significant increase in overall H3k9Ac intensity after 72 hours in T3 was observed in all conditions tested compared to 48 hours in T3, ****$p<0.0001$, Kruskal Wallis test (blue).

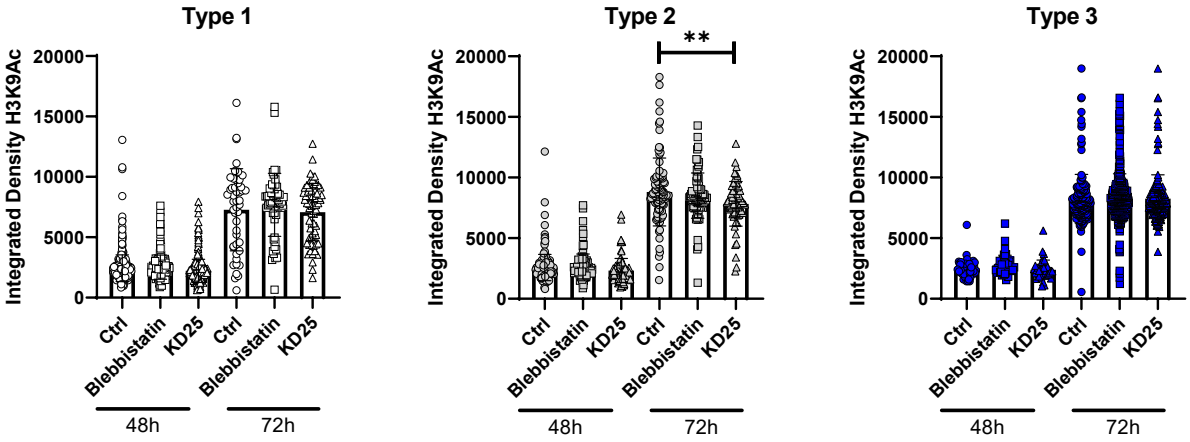

Figure 8. Integrated Densities of each H3K9Ac stain arranged by cell type. Points represent value from 1 cell, black bar represents mean and SD (2 cultures per condition/experiment) A decrease in intensity between type 2 control and KD25 cells in 72 hours T3 was observed, **p<0.01, 2-way ANOVA.

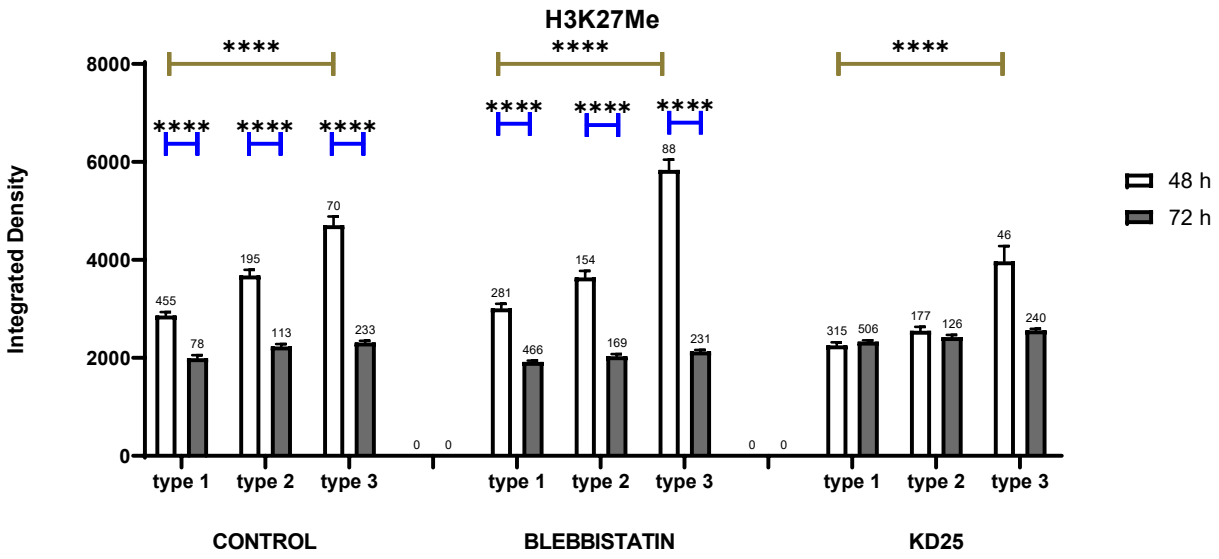

Figure 9. Mean Integrated Density of H3K27me3 stain in control (left), blebbistatin (middle), and KD25 (right) stained OPC cultures of different branching complexity treated in T3 for 48 hours (white) and 72 hours (gray). Values above bar represent the total number of cell counter per condition (2 cultures per condition/experiment). An increase in intensity is observed as cells mature in 48 hours T3 for all conditions, ****$p<0.0001$, 2-way ANOVA (red). A significant decrease in overall H3K27me3 intensity after 72 hours in T3 was observed in control and blebbistatin conditions compared to 48 hours in T3, ****$p<0.00001$, Kruskal-Wallis test (blue).

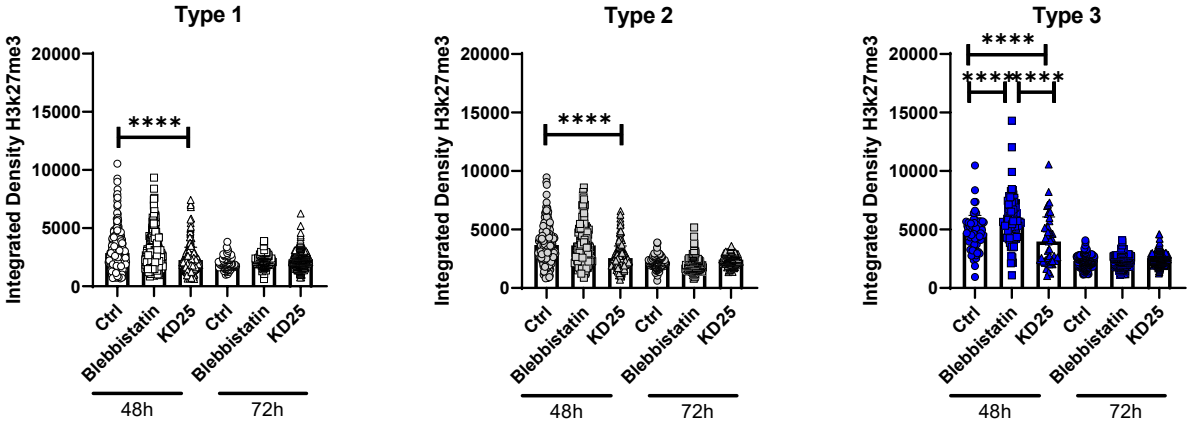

Figure 10. Integrated Densities of each H3K27me3 stain arranged by cell type. Points represent value from 1 cell, black bar represents mean and SD (2 cultures per condition/experiment). A significant difference in intensity is observed between control and NMII inhibited type 3 cells cultured in T3 for 48 hours. ***p<0.001, ****p<0.0001, 2-way ANOVA.

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