CHARACTERIZING A ROLE FOR NOS1AP IN CELLULAR MECHANOTRANSDUCTION

by

Ibrahim Shahin

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Dalhousie University is located in Mi'kma'ki, the ancestral and unceded territory of the Mi'kmaq. We are all Treaty people.

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ABSTRACT

In a screen for proteins that interact with the phosphotyrosine binding (PTB) domain of NOS1AP we identified the β-integrin family of proteins. Fibronectin stimulated mouse embryonic fibroblasts (MEFs) from NOS1AP$^{-/-}$ mice showed a significant increase in nuclear blebbing compared to controls. This defect could be rescued with the addition of the Rho Kinase inhibitor Y27632. Further characterization of MEFs lacking NOS1AP revealed a decrease in E-cadherin expression and an increase in N-cadherin expression. To further dissection of the role for NOS1AP in integrin dependent signalling, we turned to the RAW264.7 osteoclast-like cell line. He we found differentiated osteoclast-like cells showed an increase in NOS1AP expression. Using NOS1AP isoform specific antibodies revealed differential subcellular localization in differentiated osteoclast-like cells. Taken together, our data demonstrates that NOS1AP functions in integrin dependent signalling and that the different NOS1AP isoforms may contribute to the differentiation of osteoclasts.

LIST OF ABBREVIATIONS AND SYMBOLS USED

Arp2/3…….……Actin Related Protein 2/3 complex

ATP…….…….Adenosine triphosphate

AD…….Alzheimer's Disease

β-Pix……..β-p21-activated protein kinase exchange factor alpha

CAPON…….…….…….Carboxy-terminal PDZ ligand of nNOS

Cdc42…..……..Cell division control protein 42 homolog

c-Src……..…Proto-oncogene tyrosine-protein kinase Src

DexRas……...……..Dexamethasone induced Ras

Dlg…….Discs large homolog 1

DSM……..……..Diagnostic and Statistical Manual of Mental Disorders

E……...……..Extracellular matrix

FA……...……..Focal Adhesion

FAC……..…….Focal Adhesion Complex

FAK……..Focal Adhesion Kinase

Git1……...……..ARF GTPase-activating protein GIT1

GST……..……Glutathione S-transferases

KASH……..……Klarsicht/ANC-1/Syne Homology

LATS……..…….…….……Large tumor suppressor kinase

Lgl..…….. ……Lethal giant larvae

LINC..…… ……Linker of nucleoskeleton and cytoskeleton

LPA…….....Lysophosphatidic acid

LQTS……..…...……Long-QT Syndrome

M-CSF..……..……..Macrophage colony stimulating factor

MEF……..…….Mouse embryonic fibroblasts

MMPs….…….Matrix metalloproteases

MST…..……..…….……Macrophage stimulating

Nepsrin…….Nuclear envelope with spectrin repeats

NFTs……..……Neurofibrillary tangles

NMDA……..…....N-Methyl-D-aspartic acid

iNOS…….Inducible nitric oxide synthase

nNOS…...……..Neuronal nitric oxide synthase

NO……..…….Nitric oxide

NOS1AP……...……...Nitric oxide synthase 1 adaptor protein

NPxY……Asparagine-Proline-x-Tyrosine

NS….…….Nephrotic syndrome

nTSGs……...……..……Neoplastic tumor suppressor genes

p130cas…….…….p130 Crk-associated substrate

P2RX3…....……Purinergic Receptor P2X, ligand gated ion channel, 3

PAK……..serine/threonine p21-activated kinases

PBS…….. Phosphate-buffered saline

PDZ……PSD95, Drosophila disc large tumor suppressor, and zonula occludens-1

PFA…….…….……Paraformaldehyde

PI….……...Propidium Iodide

PMSF….……..……..Phenylmethylsulfonyl fluoride

PSD….……..…..……..Post-synaptic density

pSIVA-IANBD……...…….Polarity Sensitive Indicator of Viability & Apoptosis-IANBD

PTB…….……..Phosphotyrosine binding domain

Rho…….…..Ras homolog protein

RhoGAPs…….…….Rho guanine activating protein

RhoGDIs….……..Rho dissociation inhibitor

RhoGEFs……...……...Rho homolog guanine exchange factor

Rac……...……..Ras-related C3 botulinum toxin substrate 1

RANKL……...…….Receptor activator of nuclear factor-κB ligand

ROCK……Rho-associated protein kinase

SB……..…….Sample Buffer

Scrib…….…….Scribble

SEM…….…….…….……Standard error of the mean

SUN……..Sad1p, UNC-84

T2D……..Type 2 Diabetes

TAZ…….……...……..Tafazzin

TGF-β…..…….……Transforming growth factor beta

TNFR 19L……...Tumor necrosis factor receptor superfamily member 19L

TRAIL-R…..…….……TNF-related apoptosis-inducing ligand-receptor

TRAP……..……Tartrate resistant acid phosphatase

YAP……...…….Yes-associated protein 1

YFP…..……...Yellow fluorescent protein

ZO-1……..Zonula occludens-1 ZO-1

STATEMENT

Polymerase Chain Reaction test of Mouse Embryonic Fibroblasts was performed by Maggie Qi of Dr. Fawcett's Lab (Dalhousie University) (Figure 6A). Culturing, differentiating, phalloidin and TRAP staining of RAW264.7 cells were performed by Nichole McMullen of Dr. Chris Sinal's lab (Dalhousie University) (Figure 12A-D). All other work presented herein was performed by the author.

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CHAPTER 1 INTRODUCTION

1.1 NOS1AP

Nitric oxide synthase 1 adaptor protein (NOS1AP) is a protein that was first identified as a binding partner for neuronal nitric oxide synthase (nNOS) by Jaffrey et al. (1998). Initially, it was termed carboxyl-terminal (C-terminal) PSD95-Dlg-ZO1 (PDZ) ligand of nitric oxide synthase (CAPON), as it contains a class II C-terminal PDZ domain that was shown to bind to nNOS. In addition to NOS1AP containing a C-terminal nNOS binding motif, it also contains an N-terminal phosphotyrosine binding domain (PTB). NOS1AP is expressed throughout many tissues including the central nervous system, with the highest levels of expression occurring in the olfactory bulb, cerebellum, hippocampus, and cortex (Jaffrey et al. 1998, Clattenburg et al., 2015.).

The *NOS1AP* gene is spliced giving rise to various isoforms of with different molecular weights and are likely to contribute to different signalling complexes. The original NOS1AP isoform that was shown to bind nNOS has a molecular weight of approximately 70 kDa protein. This isoform has been identified as NOS1APa (herein NOS1APa). This isoform is the predominant isoform that is expressed in the brain and across the nervous system (Clattenburg et al., 2015). In addition to the NOS1APa isoform, an approximately 100 kDa isoform of NOS1AP was identified (Richier et al., 2011. and Clattenburg et al., 2015). This novel isoform was termed NOS1APc. Interestingly, NOS1APc does not interact with nNOS, as the splicing to generate this isoform lacks the PDZ binding motif found in NOS1APa, indicating a unique interactome for this isoform. In addition to

NOS1APa and NOS1APc, several other isoforms have been identified including NOS1APd, NOS1APe, and NOS1APf. NOS1APd and NOS1Ape, are similar to NOS1APc; however, they have exon 10 deleted from NOS1APc (Figure 1). NOS1APe differs from NOS1APd in that it contains a five amino insertion - leucine, leucine, leucine, leucine, glutamine (LLLLQ) insert in its PTB domain. NOS1APf contains exons 11 and 12 of the NOS1APc transcript, but also contains a unique 5'exon that lacks any sequence homology with any of the other NOS1AP isoforms (Clattenburg et al., 2015). Interestingly, Clattenburg et al. (2015) showed that each of the different isoforms, when expressed as a fusion with Yellow Fluorescent Protein (YFP) have different subcellular localizations when overexpressed in different cell types, with NOS1APa and NOS1APe showing a membrane localization and all other isoforms localizing to the cytoplasm (Clattenburg et al., 2015). The differential localization suggests that these different isoforms likely contribute to different signaling interactomes within cells.

1.2 DOMAINS OF NOS1AP

As mentioned, the predominant signaling domain in all identified NOS1AP isoforms except for NOS1APb and NOS1APf is the N-terminal phosphotyrosine binding (PTB) domain. PTB domains are conserved regions of scaffolding proteins that function to form protein complexes (Smith et al., 2006). As the name suggests, they preferentially bind regions of other proteins with phosphorylated tyrosine residues. Typically, they recognize regions of proteins containing Asparagine-Proline-X-phosphoTyrosine (N-P-X-pY), where X can be any amino acid residue (Margolis et al., 1999). There are three classes of

PTB domains, IRS1/Dok-like, Shc-like and Dab-like. Although the structure of each class of PTB domains is highly similar, there are differences in their binding specificities (Smith

et al, 2006). Domains in the Shc-like and IRS-1/Dok -like families bind to phosphorylated motifs with a higher affinity than non-phosphorylated motifs. On the other hand, Dab-like domains tend to bind motifs that are either phosphorylated or unphosphorylated, and some show higher preference to unphosphorylated ligands (Smith et al, 2006). All PTB domains share the same basic structure, a central β-sandwich comprised of seven antiparallel βstrands, and an α-helix cap found at the C-terminal region of the domain between β-sheets 1 and 2. NOS1AP contains a Shc-like PTB domain (Jaffrey et al., 1998).

In addition to the PTB domain, NOS1APa contains a C-terminal PDZ binding motif. PDZ domains are abundant in the genome, and are important motifs in many biological functions, such as cell signaling transduction (Lee & Zheng, 2010). They are small, modular, motifs, consisting of five or six β-strands and two or three α-helices (Lee & Zheng, 2010). PDZ domains are arranged in three classes. Class I domains associate with Serine/Threonine-X- Φ-COO (S/T-X- Φ -COO) motifs, where X is any amino acid and Φ denotes a hydrophobic residue (e.g. valine, leucine, etc.). Class II domains associate with Φ-X-Φ motifs and class III domains associate with Aspartate/Glutamate-X- Φ (D/E-X-Φ). NOS1AP contains PDZ binding motif that associates with class-II PDZ domains (Courtney et al., 2014).

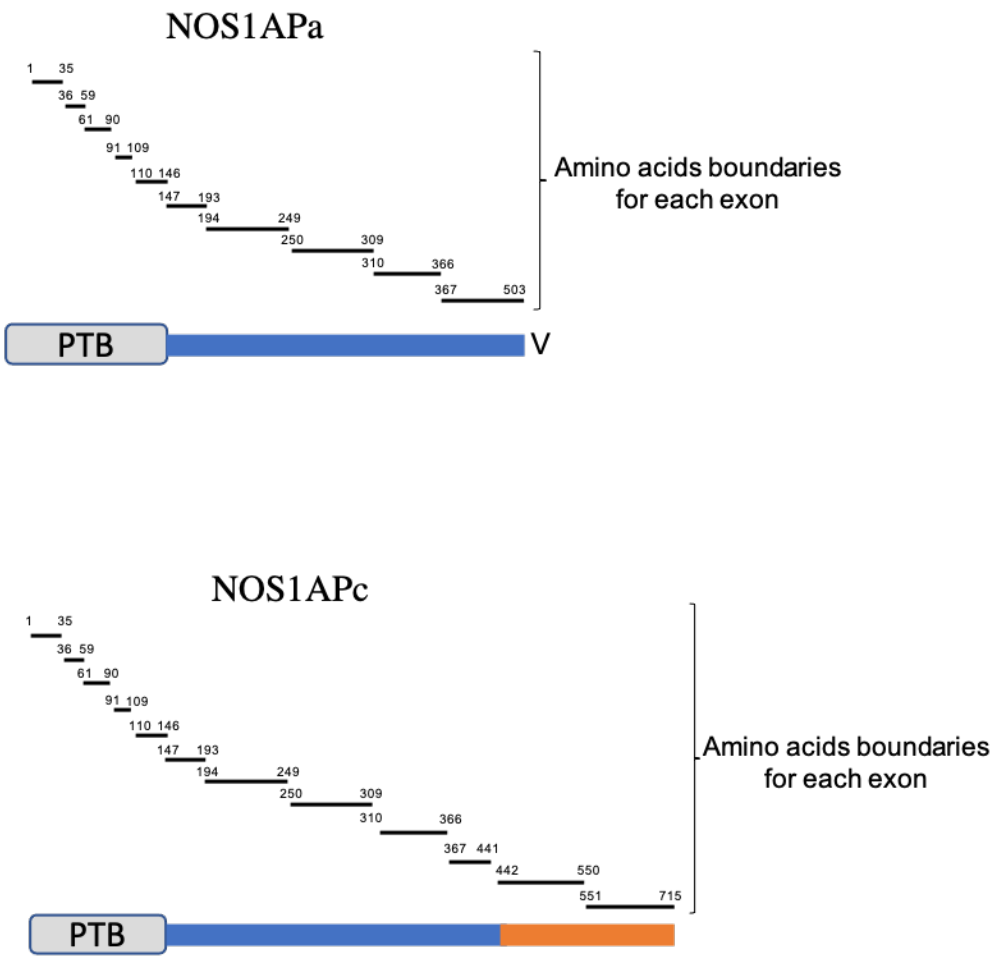

Figure 1. Diagram of the major signaling domains in NOS1APa and NOS1APc. Amino acids for each of the exon boundaries identified are annotated. Note the difference in the c-terminus between NOS1APa and NOS1APc.

1.3 NOS1AP INTERACTING PROTEINS

1.3.1 NOS1AP INTERACTS WITH nNOS

Jaffrey et al. (1998) showed that NOS1AP interacts with neuronal nitric oxide synthase (nNOS) through a direct interaction between the C-terminal region of NOS1AP and the PDZ domain of nNOS. Interestingly, although there are other NOS isoforms, including iNOS and eNOS, NOS1AP was shown to only associate with the neuronal NOS isoform (Jaffrey et al., 1998). The significance of this remains to be determined. Nonetheless, it was shown that the interaction between NOS1APa and nNOS could be competitively inhibited by overexpressing another nNOS binding protein post-synaptic density 95 (PSD 95) and post synaptic density 93 (PSD 93) as both NOS1AP and PSD93/95 bind to the PDZ domain of nNOS (Jaffrey et al. 1998). nNOS is the primary source of nitric oxide (NO) in the central nervous system. Animal studies suggest that abnormalities in the nitrogenic system are linked to behavioural abnormalities (Freudenberg et al., 2015). In fact, post-mortem analysis of brain tissue found that individuals with schizophrenia had dysregulated NO, whether NOS1AP contributes to this remains to be better defined. However, it is interesting to note that in patients diagnosed with schizophrenia and bipolar disorder there is an elevated expression of a unique NOS1AP isoform (Brzustowicz et al., 2000). This isoform, named NOS1APshort or NOS1APb (Clattenburg et al. 2015), termed NOS1APb (Xu et al., 2005) lacks the PTB binding domain, but contains the C-terminal PDZ binding motif (Xu et al., 2005). As this isoform can associate with nNOS, it was hypothesized that this isoform functions in a dominant negative manner; bind nNOS thus reducing its association with

PSD-95 at NMDA receptor complexes. The net effect is to reduce the overall synthesis of NO and thus reduce NMDA signaling effects at excitatory synapses. Together, this suggests that inhibiting the binding between nNOS and PSD-93/-95 may have a role in schizophrenia (Freudenberg et al., 2015).

1.3.2 NOS1AP INTERACTS WITH SCRIBBLE

In addition to nNOS, another protein that has been shown to associate with NOS1AP is the polarity protein Scribble (Scrib) (Richier et al., 2010, Anastas et al., 2012). Scrib is a large scaffolding protein that contains 16 N-terminal leucine rich repeats and four C-terminal PDZ domains (Bryant & Huwe, 2000). Work performed in Drosophila animal models have shown how Scrib is involved in regulating apicobasal cell polarity. Epithelial cells exhibit a polarized apicobasal axis which is important for the formation of tight junctions in mammalian cells (Humbert et al., 2008). To establish this polarized axis, Scrib interacts with Discs Large (Dlg) and Lethal Giant Larvae (Lgl), two proteins involved in establishing basolateral polarity (review). In Drosophila genetic interactions between *scrib*, *dgl* and *lgl*, show each is involved in tumor growth. Collectively, these genes are termed the neoplastic tumor suppressor genes (nTSGs). Mutations in either of the nTSGs caused extensive proliferation of epithelial cells, which was shown to be in result of a disruption in polarity (Bilder 2004). Furthermore, nTSG mutant cells are also exhibit a lack of proliferative control and differentiation, phenotypes typical of malignant tumors (Bilder 2004). In addition to neoplastic growth in these mutants, the apicobasal axis is lost and components of the basolateral and apical regions are disorganized (Humbert et al., 2008, Bilder 2004).). The loss of this apicobasal polarity disrupts epithelial integrity because of the disruption of adherens junctions and impaired cell to cell communication (Humbert et al. 2008). Much

of the work showing the importance of these genes in epithelial polarity in the Drosophila system, have been replicated in mammalian cells, showing that these genes retain evolutionary conservation with respect to their core function in cellular homeostasis.

In a screen to identify Scrib interactors, Richier et al. (2010) identified a novel interaction between Scrib and NOS1AP. Structure-function studies revealed that the PTB domain of NOS1AP associated with the PDZ domains of Scrib. Specifically, they identified the importance of the fourth PDZ domain of Scrib (Richier et al., 2010). Whether this interaction is direct or mediated by an intermediatory protein remains unknown, as others have not been able to show a direct interaction (Anastas et al., 2012). Nonetheless, the PTB domain of NOS1AP seems to be important for the association with Scrib, as both NOS1APa and NOS1APc isoforms associate with Scrib (Clattenburg et al., 2015).

The interaction between NOS1AP and Scrib was shown to be important for the bridging of the other proteins, including β-Pix, Git1 and PAK. These three proteins form a complex and are involved in regulating actin dynamics and the RhoGTPases Rac1 and cdc42 (Richier et al., 2010). Richier et al. (2010) showed that NOS1APa influenced the activation of Rac, leading to changes in neuronal architecture (Richier et al., 2010). Although Richier showed that NOS1AP and Scribble formed a complex with β-Pix, Git1 and PAK, another group confirmed a Scrib and NOS1AP interaction; however, they showed that the polarity protein Vangl1 was in a complex with Scribble and NOS1AP independent of the β-Pix,

Git1 and Pak complex (Anastas et al., 2012). Whether this difference is due to different cellular context, or whether the different NOS1AP isoforms that contain the PTB domain associate with these different complexes remains to be determined.

1.3.3 NOS1AP ASSOCIATES WITH SYNAPSIN AND DEXRAS

In addition to the PTB domain showing an association with Scrib, others have previously shown an association of NOS1AP with synapsin1, and the Ras homologue protein known as Dexamethasone induced Ras (DexRas) (Fang et al., 2000). Synapsin1 has been shown to regulate the release of synaptic vesicles, and Richier et al., showed that NOS1AP could be found in presynaptic regions as it colocalized with Scrib and synaptophysin (Richier et al., 2010). Whether NOS1AP functions in synaptic vesicle release or dynamics remains to be tested. In addition to synapsin1, others have shown that the PTB domain of NOS1AP is important for the association with DexRas. DexRas has been shown to be an important mediator of sleep, as it is highly expressed in the superchiasmatic nucleus, and mice lacking DexRas have defects in their entrainment to light/dark cycles (Cheng et al., 2006). It has been proposed that NOS1AP, through its association with DexRas, may be important for the light/dark entrainment, although this remains to be determined.

1.3.3 NOS1AP ASSOCIATES WITH CARBOXYPEPTIDASE E

In addition to the PTB and C-terminal PDZ binding motif, other regions of NOS1APa have been shown to be important for associating with protein complexes. This includes a region of NOS1APa between the two main signaling motifs that associate with a protein known as carboxypeptidase E (Carrel et al., 2009). Here loss of NOS1AP or overexpression of

NOS1AP was shown to affect dendritic patterning, and this was dependent on a small region in NOS1AP that was shown to associate with Carboxypeptidase E. Carboxypeptidase E is an enkephalin convertase enzyme that is involved in the cleavage of several precursor peptide proteins including insulin, vasopressin, and oxytocin (Jung et al., 1991). CPE cleaves C-terminal arginine or lysine residues from the precursor polypeptides to generate bioactive compounds (Carrel et al., 2009). Mechanistically, how CPE functions with NOS1AP to regulate dendritic patterning remains to be determined.

1.3.4 NOS1AP FUNCTIONALLY ASSOCIATES WITH YES ASSOCIATED PROTEIN

Yes associated protein (YAP) is a central component of the HIPPO pathway. YAP is a transcriptional activator, and along with transcriptional co-activator with PDZ-binding motif (TAZ) functions to regulate the HIPPO pathway. Recent work has demonstrated that YAP/TAZ are important for cancer initiation and have linked their signalling to EMT (Zanconato et al., 2016, Piccolo et al., 2014). As it is part of HIPPO pathway, YAP is important in cellular proliferation and mechanotransduction (Piccolo et al., 2014). Briefly, YAP is phosphorylated on serine 127 by LATS1/2 (Sugihara et al., 2018), two serine threonine kinases that are activated in response to changes in cell polarity and mechanical tension (Dupont et al., 2011). Another serine threonine kinases important to HIPPO signaling are macrophage stimulating protein 1 and 2 (MST1/2) which form a kinase signalling cascade along with LATS1/2 to control the phosphorylation of YAP and TAZ. When YAP is phosphorylated at these conserved serine residues it localizes to the cytosol, preventing the transcriptional activation of target genes.

It is known that Scrib associates with and regulates the MST, LATS1/2 and TAZ complex (Piccolo et al., 2014). Loss of Scrib inactivates the HIPPO cascade which leads to t the retention of YAP and TAZ to the nucleus, leading to an increase in cell proliferation (Piccolo et al., 2014). Since Scrib associates with NOS1AP, Clattenburg et al. (2015) tested whether NOS1AP functions in HIPPO signaling. They were able to show that NOS1AP functionally associates with YAP and modulates its phosphorylation. Using a panNOS1AP antibody, they were able to show that NOS1AP was able to co-precipitate with
YAP, and that overexpressing NOS1APa increased the phosphorylation of YAP on serine 127, implicating the activation of the core HIPPO cascade. Consistent with this, overexpression of NOS1APa also showed a reduction in the nuclear accumulation of YAP and affected cellular proliferation implicating NOS1AP in HIPPO dependent signaling (Clattenburg et al., 2015).

1.4 NOS1AP IN DISEASE

NOS1AP has been linked to several human disorders. As mentioned earlier, previous findings by Brzustowicz et al. (2000) and Xu et al. (2005) have linked NOS1AP expression in humans to Schizophrenia and bipolar disorder. Work by Crotti el al. (2009) found that a mutation in NOS1AP predisposes individuals to congenital long-QT syndrome.
Furthermore, roles for NOS1AP have been identified in muscular dystrophy (Segalat et al., 2005), chronic inflammation (Shao et al., 2011), Alzheimer's disease and more recently nephrotic syndrome.

1.4.1 SCHIZOPHRENIA AND BIOPLAR DISORDERS

Schizophrenia is a psychiatric disorder that effects 1% of the global population. Based on twin adoption studies, current findings suggest that that schizophrenia is an interplay between genetics and environmental factors, although it thought that genetics play a greater role than environmental factors (Fang et al., 2008). Schizophrenia is characterized by psychotic symptoms, such as delusions, altered perceptions, disordered thinking, deficits in motivation. The Diagnostic and Statistical Manual of mental disorders (DSM) V requires two or more of these symptoms to be present for a diagnosis (Keshavan et al., 2020). Schizophrenia is also characterized by distinct neural abnormalities. In fact, it has been proposed that abnormalities in early brain development as well as later into adolescence often predate the first onset of psychosis (Glantz et al., 2000, Keshavan et al., 2020). Glantz et al. (2000) found that deep layer 3 pyramidal neurons in the prefrontal cortex (PFC) had significantly lower spine density, while Pierri et al. (2000) found that deep layer 3 pyramidal neurons had significantly smaller somal volume. Neurological changes associated with schizophrenia also effect the morphology and molecular composition of specific neuronal, synaptic, and glial populations in the hippocampus and the dorsal thalamus (Harrison and Weinberger, 2005). Changes affecting synaptic plasticity, and in particular those affecting NMDA receptor-mediated glutamatergic neurotransmission, have been implicated in schizophrenia. (Harrison and Weinberger, 2005). NOS1AP was first implicated in schizophrenia by Brzustowicz et al. (2000).

NOS1AP is located on chromosome 1q22 and linkage disequilibrium studies from several groups have identified 6 single nucleotide polymorphisms (SNP's) within the genomic region of the NOS1AP gene (Brzustowicz et al., 2000). It is known that nitric oxide

synthase 1 (NOS1) is involved in NMDA based neurotransmission. nNOS is activated as a result of the calcium influx through the NMDA receptor (Weber et al., 2014). nNOS is coupled to the NMDA receptor region through the postsynaptic density, a scaffold of proteins such as PSD-95, SHANK, and DLGAP. Weber et al. (2014) propose a role for the interaction between NOS1AP and NOS-1 in schizophrenia. They found that single nucleotide polymorphisms (SNPs) in the genes of both NOS1AP and nNOS correlated significantly with the incidence of schizophrenia when analyzing from post-mortem human brain tissue (Weber et al., 2014). In another study by Xu et al. (2005) the NOS1APb isoform, 211aa protein composed of exons 9 and 10 that retains the capacity to bind nNOS was shown to be important in the pathophysiology of Schizophrenia (Xu et al., 2005). Since NOS1APb lacks the N-terminal PTB domain it is predicted that it would not interact with synapsin1, DexRas1 or scribble. Xu et al. (2005) show that mRNA levels of NOS1APb are increased in the dorsolateral prefrontal cortex in patients with schizophrenia and bipolar disorders (Xu et al., 2005). Since PSD-95 couples nNOS with the NMDA receptor, the competitive binding of NOS1APb to nNOS would prevent the localization of nNOS near NMDA receptor complexes thereby altering the levels of nitric oxide within the synapse (Xu et al., 2005).

Schizophrenia and bipolar disorder share a number of susceptibility genes that increase one's risk of developing either disorder (Carter, 2007). Many of these genes are linked to the NMDA receptor signalling axis as well as metabotropic glutamate receptors, including their downstream signalling partners. NOS1AP is implicated in this signalling as it is involved in downstream signalling of NMDA receptors, as mentioned in its competitive

binding to NOS1. The association of a truncated form of NOS1AP in bipolar disorder further solidifies the notion that NOS1AP is involved in regulating these processes neuronally through its interaction with NOS1 (Carter, 2007).

1.4.2 LONG-QT SYNDROME

Long-QT syndrome (LQTS) is a condition that is characterized by irregular heart rhythms. The QT interval is a characteristic feature of an echocardiogram (ECG), and it spans from the beginning of the Q-wave to the end of the T-wave, the period where the ventricles of the heart are repolarizing (Lu et al., 2010). In LQTS, the QT interval duration is prolonged, potentially leading to arrhythmias and sudden cardiac death (Crotti et al., 2009, Lu et al., 2010). The most common form of LQTS is caused by a mutation in KCNQ1, a gene encoding a pore-forming subunit of potassium channels that are important in maintaining normal rhythmicity (Crotti et al., 2009). NOS1AP has been implicated in LQTS. It is known that NOS1AP is involved in cardiac repolarization (Arking et al., 2006). Tomas et al. (2010) found that there was a correlation between a SNP near the 5' region of NOS1AP and a prolonged QT interval. It was later identified by Crotti, et al. (2009) that NOS1AP genetic variation is associated with duration of the QT interval in the general population. Based on their data, Crotti, et al., (2009) also concluded that NOS1AP variants modify the clinical severity of LQTS, and mutations are associated with an increased chance of a prolonged QT interval (Crotti et al., 2009.

1.4.3 ALZHEIMER'S DISEASE

Alzheimer's disease (AD) is a progressive neurodegenerative disease, and the most common form of dementia, comprising 50-70% of dementia cases (Winblad et al., 2016). The hallmark neuropathological component of AD is the deposits of amyloid-β (Aβ) plaques (Hashimoto et al., 2019). These commonly form senile plaques, and neurofibrillary tangles (NFTs). NFTs also contain high amounts of hyperphosphorylated tau protein. Although tauopathy is commonly observed in other neurodegenerative diseases, it is greatly enhanced following Aβ amyloidosis (Hashimoto et al., 2019). Using a human tau knock-in murine model Hashimoto et al. (2019) found that as a result of Aβ pathology NOS1AP expression is increased in the brain of AD mice. They show that this leads to tau pathology and neuronal cell death. Furthermore, they showed that NOS1AP overexpression in tau-KO mice led to significant neurodegeneration (Hashimoto et al., 2019). To confirm the link between NOS1AP and tauopathy, Hashimoto et al. (2019) used the murine tauopathy model P301S-Tau-Tg and found that these mice were deficient in NOS1AP. Based on their findings they concluded that NOS1AP deficiency in tauopathy attenuates tau pathology. Their finding is one of the first of its kind, describing a potential mediator between the Aβ pathology and tauopathy observed in patients with AD.

1.4.4 NEPHROTIC SYNDROME

Nephrotic syndrome (NS) is the second leading cause of kidney disease (Majmundar et al., 2021). It is characterized by proteinuria, lower amounts of blood albumin, and unusually high levels of blood cholesterol (Shin et al., 2018). These changes manifest as a result of impaired glomerular filtration as a result of a disruption in the podocyte foot processes

(Majmundar et al., 2021). Majmundar et al. (2021) found that patients with early onset NS were homozygous to a recessive variant of NOS1AP which has mutation changing a cysteine residue for a tyrosine (C143Y). Furthermore, Majmundar et al. (2021) showed that NOS1AP was expressed in mammalian glomeruli. Interestingly, they noted that NOS1AP localized to actin rich podosomes. Thus, they aimed to determine if the mutant NOS1AP from NS patients would promote filopodia and podosome formation. It was found that unlike WT NOS1AP, the recessive NOS1AP from NS individuals did not promote filopodia and podosome formation when overexpressed in cells. This process was shown to depend on the activity of Cdc42 (Majmundar et al., 2021). Additionally, Majmundar et al. (2021) constructed organoids that were homozygous to the recessive variant of NOS1AP and showed that they exhibited aberrant glomeruli formation.

1.4.5 MUSCULAR DYSTROPHY

NOS1AP has been shown to play a role in the physiology of skeletal muscle. There are three forms of NOS in skeletal muscle, the aforementioned nNOS, endothelial NOS (eNOS) and inducible NOS (iNOS) (Chen et al., 2008). The interaction between NOS1AP and NOS has been implicated in neuromuscular transmission, muscle contractility, and carbohydrate metabolism since NO levels in muscle are a critical parameter (Chen et al., 2008). NOS1AP expression has been localized to several regions in the muscle, including satellite cells, myotubes, myoblasts, dystrophic muscle and in growing muscle fibers (Segalat et al., 2005). Chen et al. (2008) showed that levels of NOS1AP and nNOS-μ decreased substantially two weeks following muscle injury. It is important to note that nNOS-μ is normally localized to the subsarcolemmal cytoskeleton, where it plays a key

role in providing the skeletal muscle with NO through its interaction with the dystrophindystroglycan complex (DGC) (Segalat et al., 2005). It is known that dystrophin and DGC mutations destabilize nNOS-μ in the skeletal muscle. As such, it has been proposed that NOS1AP acts to prevent instability of nNOS-μ, and an increase of NOS1AP expression could further stabilize nNOS, preventing aberrant destabilization (Segalat et al., 2005). This interaction has an important role in muscular dystrophy since NO is known to effect muscle growth and regeneration (Segalat et al., 2005).

1.4.6 NOS1AP AND HEPATIC INSULIN SENSITIVITY

Insulin insensitivity is a defining feature of type two diabetes (T2D). Hu et al. (2010) have shown that a single nucleotide polymorphism rs12742393 of NOS1AP was associated with T2D. As such, Mu et al. (2019) aimed to build on this work and characterize the role of NOS1AP in diabetes. They found that NOS1AP was highly expressed in the liver of mice. It is important to note that only the NOS1APa isoform was reported, it is unclear if other isoforms were expressed. In their study, Mu et al. (2019) used a liver specific Cre conditional knockout, which floxed the NOS1AP gene at exons 3-5. Both groups were fed a high-fat diet. The conditional knockout mice had impaired glucose and insulin tolerance. Hepatic overexpression of NOS1AP using adenovirus improved both glucose and insulin sensitivity, thereby demonstrating that NOS1AP is necessary for normal glucose homeostasis in the liver of obese mice (Mu et al. 2019). Furthermore, Mu et al. (2019) showed that NOS1AP's involvement in glucose homeostasis was dependent on its C-terminal PDZ motif. Interestingly, Zhao et al (2021) have shown that nNOS is important in

modulating insulin sensitivity in obese mice. The role of nNOS in insulin sensitivity was shown to be dependent on p38 MAPK activation.

1.5 CELL MIGRATION

Cell migration is an important feature of various processes in the human body, such as embryonic development and tissue repair. The directionality of migration, and the speed at which at the cells migrate at depend on external cues from the extracellular matrix (ECM) (Seetharaman & Etienne-Manneville, 2020). Mesenchymal cell migration occurs in five steps. First, there is an establishment of rear-front polarity within the cell. Second, the leading edge extends to form an actin-based protrusion known as the lamellipodium.

This process is mediated by actin polymerization (Seetharaman & Etienne-Manneville, 2020). After the lamellipodium has extended, step three is to form adhesions to extracellular matrix, known as nascent adhesions, which then mature into actin-linked focal adhesion. Once they have formed, the focal adhesions can permit the fourth step, which is generating the actomyosin contractility required for cell movement. Once the cell has started to move, the fifth and last step is for focal adhesions at the rear of the cell to release from the ECM and permit the rear of the cell to move along in the direction of migration (Seetharaman & Etienne-Manneville, 2020). Each step of cell migration will be highlighted in greater detail to elucidate the steps necessary for each step to occur.

1.5.1 CELL POLARITY

Animal cells have the capacity to orient themselves in several ways which polarize them to have a specific directionality. These include apicobasal polarity (up/down) and frontrear polarity (side to side) and planar cell polarity where cells organize themselves in the plane

of a tissue (Nelson, 2009). With respect to directional cell migration, front-rear cell polarity is of particular importance. This phenomenon is also fundamental for the function of cells in a variety of other processes, such as tissue development, cell differentiation, and cell division (Ladoux et al., 2016). Front-rear cell polarization is achieved in response to external cues from the environmental cues that are taken up from the ECM and surrounding structures (Ladoux et al., 2016). For these cues to translate into directional cell polarity there are host of cytoskeletal and molecular changes that must occur. Cell polarity, whether apical/basal or planar cell polarity are defined by protein-protein complexes, and these complexes are conserved across many species (Genevet & Tapon, 2011, Xu et al., 2019,). These complexes are beyond the scope of the current thesis; however, it is important to know that many of the core protein complexes defined to date converge on the actin and microtubule cytoskeletons. Thus, changes in the conserved polarity complexes have important regulatory connections to the actin cytoskeleton and understanding the connections has been an important area of research in understanding cellular polarity.

One area of focus has been on the small Ras homolog (Rho) GTPases family of proteins. These are a family of signalling proteins that are important for generating polarity in living cells (Nelson, 2009). Rho GTPases are a family of intracellular signalling proteins that belong to the Ras superfamily. There 20 proteins in the Rho family, however they three predominant proteins are RhoA, Rac1 and Cdc42 (Lawson & Burridge, 2014). When these proteins are bound to GTP they are activated, and they are capable of signalling to a host of downstream effectors that regulate cellular functions such migration, spreading, polarity and adhesion (Lawson & Burridge, 2014). In general, Rac1 and Cdc42 are localized to the

front end of polarized cells, where they function to promote the signalling environment necessary for front end cell polarity (Nelson, 2009). Once activated they function to promote the rapid assembly of actin filaments, a process that is mediated by the Arp2/3 complex (Pollard, 2007). They also function to extend the microtubule network to the leading edge of the cell by polarizing and stabilizing microtubule formation (Nelson, 2009). RhoA is localized to the rear end of the cell, where it functions to regulate the contractility of actin at the rear of cell, which is important to promote detachment when the cell is migrating away from its current location (Nelson, 2009). Xu et al. (2003) have demonstrated that inhibiting either Rac1/Cdc42 of RhoA leads to a loss of front-rear polarity, and disrupts the cytoskeletal organization, which was shown to hinder directional migration in neutrophils.

Rho GTPases are regulated by three classes of enzymes, guanine nucleotide exchange factors (RhoGEFs), GTPase-activating proteins (RhoGAPs) and guanine nucleotide dissociation inhibitors (RhoGDIs). RhoGEF's activate Rho proteins by stimulating the exchange GDP for GTP. RhoGAPs activate Rho GTPases' intrinsic GTPase activity, which in turn returns them to a GDP-bound, inactive state (van Buul et al., 2014). RhoGDIs have a role in regulating the cytoplasmic pool of RhoGTPases. By binding to RhoGTPases, RhoGDIs hold them in a passive state as well as protect them from degradation (Boulter et al., 2010). RhoGTPases have a fundamental role in cellular adhesion. They are important secondary messengers downstream of integrin activation since they regulate actin dynamics. (Lawson & Burridge, 2014).

1.5.2 ACTIN DYNAMICS IN CELL MIGRATION

The actin cytoskeleton is an important mediator of cell migration (Innocenti, 2018). Protrusions of the cell membrane, known as lamellipodium, are formed by the formation of branched actin filaments that extend the plasma membrane in the direction of migration. They are generally long protrusions (1-5 um), that adhere to the matrix on which the cell is migrating (Innocenti, 2018). The process of forming these branched actin structures in the lamellipodium is mediated by Rac1 and the Arp2/3 complex (Innocenti, 2018). During migration, the lamellipodium is anchored to the extracellular matrix through the development of integrin mediated adhesions. Like lamellipodia, cells also form filopodia at their front end. Filopodia are thin actin protrusions composed of 20-30 bundles of actin. Generally, they are more slender and shorter than lamellipodia, about one micrometer long (Innocenti, 2018). Cells use filopodia to probe the extracellular environment as they are the first part of the cell to associate with the extracellular signals (Bornschlögl, 2013). The polarity of the actomyosin cytoskeleton is an important factor. Cdc42 is known to influence the polarity of actin networks in migrating cells (Etienne-Manneville & Hall 2001, Cau & Hall, 2005) Filopodia play an important role in cell migration, where their attachment to the extracellular matrix pulls the cell in the direction of migration (Heidemann et al., 1990).

1.5.3 THE EXTRACELLULAR MATRIX

The extracellular matrix (ECM) is a complex structure that provides biochemical and mechanical signals to the cells it supports. It is secreted by cells in early development and provides crucial cues for cellular function (Rozario & DeSimone, 2010). Commonly, the ECM is composed of fibrous glycoproteins, such as fibronectin and vitronectin, proteoglycans and glycosaminoglycans (Rozario & DeSimone, 2010). Through its

influence on the surrounding cells, it helps maintain tissue development and homeostasis. It is a crucial component of tissues as it provides structural integrity, regulation of signalling molecules, such as cytokines and growth factors, and maintains a viable microenvironment (Pickup et al., 2014). Cells can either be embedded in the ECM or rest on top of it. ECM's on which cells rest are referred to as basement membranes, and they function to give cues about tissue organization and cell localization (Frantz et al., 2010). The composition of ECM's varies between different tissues (Lawson & Burridge, 2014). The tension that the ECM exerts onto cells is a crucial mechanical signal that triggers responses from the cell, such as migration or proliferation. Changes that dictate cellular adhesion, such as stiffing of the ECM in a developing tumor, are hallmarks of disease progression (Lawson & Burridge, 2014).

1.5.4 THE EXTRACELLULAR MATRIX IN DISEASE

The remodelling of the ECM has been established as a marker of disease development. Under most conditions, the ECM is continuously being remodelled, a process that is mediated by enzymes that degrade the ECM known as metalloproteases (Bonnans et al., 2014). Abnormal remodelling of the ECM can lead to variety of different pathologies depending on the nature of the remodelling as well as the tissue the error occurs in. For example, Kim et al. (2000) showed that transgenic mice that express human collagenase in the heart lead to abnormal heart physiology. By 12 months of age, the mouse hearts exhibited significantly impaired systolic and diastolic function. The characteristics of the ECM also have an effect in the progression of cancer. Burnier et al. (2011) showed that overexpression of collagen IV improves the survival of lung cancer cells in the liver. The link between ECM structure and cancer progression has led to some findings that stratify

ECM characteristics with subclasses of cancer. Using microarrays, Bergamaschi et al. (2008) found that patients with different ECM profiles could be classified into groups representing different clinical outcomes. In one set of patients, a tumor type termed *ECM4* showed significant overexpression of protease inhibitors, and these had a favourable clinical outcome. Another set of patients presented with a tumor type which showed a high expression of integrins and metallopeptidases. Patients with this type of ECM identity had poor outcomes, pointing to the importance of ECM and cell interactions in the health and normal function of cells, specifically in cancer (Bergamaschi et al., 2008).

1.5.5 INTEGRINS

Cellular adhesions are mechanical contacts between cells and the extracellular matrix. They are essential for the maintenance of cell migration and morphogenesis (Kechagia et al., 2019). Integrins are important part of adhesions as they facilitate the formation of a link between cells and the extracellular environment. Through their signalling they can transduce signals bidirectionally across the cell and ECM (Kechagia et al., 2019). This is achieved by the ability of integrins to bind to ECM components, such as fibronectin, collagen and laminin (Pickup et al., 2014). In vertebrates, there are 18 different integrin alpha subunits and 8 different integrin-beta subunits, which together can combine to form 24 different heterodimers (Barczyk et al., 2010). It is important to note there is a level of selectivity on which alpha and beta subunits can form dimers. For example, Integrin β3 can only form heterodimers with the alphaIIb and alphaV subunits, whereas Integrin β1 can form a heterodimer with 11 different alpha subunits (alpha1-11) (Barczyk et al., 2010). The alpha subunit is responsible for ligand binding, which causes the beta subunit to undergo a conformational change leading to downstream signalling through proteins such as focal

adhesion kinase (FAK), p130cas (for Crk-associated substrate) and paxillin. These in turn are important to mediate signals from the integrins to activate the small of RhoGTPases (Hotchin & Hall, 1995), Rac, Rho and cdc42 to affect changes in the actin cytoskeleton (Figure 2). The multiprotein scaffolds that occur at the cytoplasmic domain of integrins are known as focal complexes (Burridge, 2017).

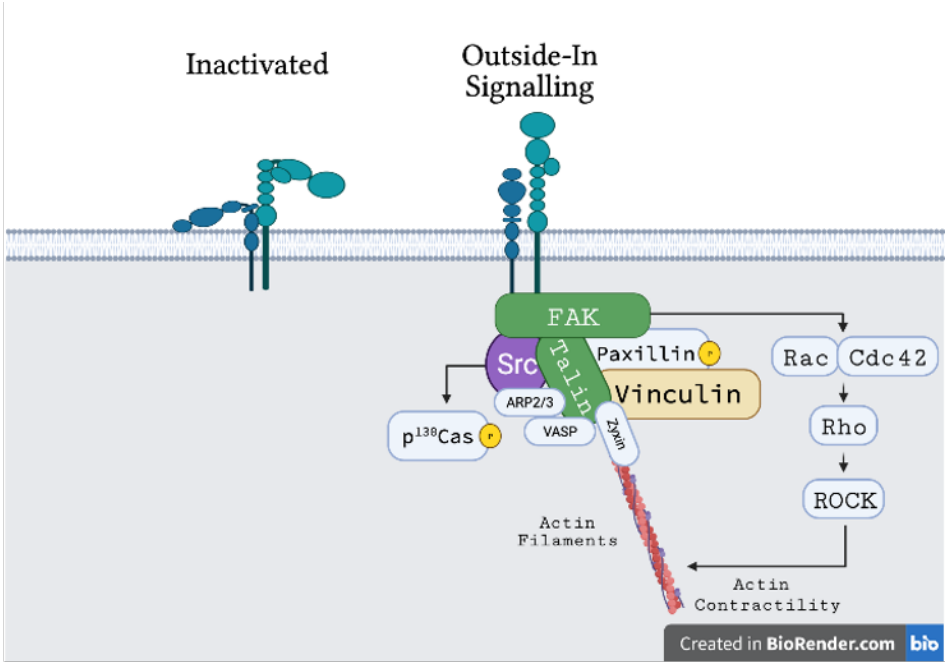

Figure 2. Diagram of major signaling molecules in integrin dependent signaling. Collectively, the scaffold of proteins is known as the focal adhesion complex (FAC). Note the activation of Src kinase to phosphorylate p130Cas upon binding of integrins to ligand. Modified from Yang et al (2017) and Boppart & Mahmassani (2019).

1.5.6 NASCENT ADHESIONS

Prior to assembly of the scaffolding proteins necessary for integrin-actin connection, these cellular adhesions are known as nascent adhesions. Nascent adhesions are integrinbased adhesions that play a key role in cell migration. They are formed at the leading edge of the lamellipodium. Nascent adhesions form in three consecutive steps. First integrins are activated in one of two ways, either by binding to an extracellular ligand in the extracellular matrix or as a result of binding to Talin. Second, the activated integrins cluster into nascent adhesions. Finally, these nascent adhesions can either mature into focal adhesions or disassemble (Henning et al., 2020). Initial maturation of nascent adhesions into focal adhesions is triggered by the tyrosine phosphorylation of paxillin and the crosslinking of actin through the actions of α-actinin (Zaidel-Bar et al., 2007, VicenteManzanares & Horwitz, 2011). Work in *Drosophila melanogaster* has identified that Talin, integrin-linked kinase (ILK), PINCH, Tensin, and Wech are necessary for the formation for the recruitment of actin to the focal adhesions (Geiger et al., 2009).

1.5.7 FOCAL ADHESIONS

Focal adhesions (FAs) are intracellular protein scaffolds that connect the incoming signals transduced from integrins to the actin cytoskeleton. These scaffolds are crucial to functionally link actin filaments to integrins (Geiger et al., 2009). Molecular analysis has determined that there are over 160 components of integrin-dependent adhesomes (the proteome network of adhesions), each with a specific regulatory or associative role in forming the focal adhesion (Geiger et al., 2009). Among them are talin, tensin, and vinculin, three proteins that provide a mechanical link between integrins

and the cytoskeleton (Stutchbury et al., 2017). Talin contains an N-terminal FERM-domain which binds to integrins, PIP2 and F-actin (Atherton et al., 2015). The binding of talin to the cytoplasmic domain of integrins has been shown to regulate their affinity for the ECM (Kim C et al., 2011). In fact, it has been shown that cells that have been depleted of talin are unable to spread and migrate, processes that depend on the proper assembly of FAs (Atherton et al., 2015). Interestingly, vinculin has been shown to mediate talin engagement with the cytoskeleton. Cells without vinculin have smaller FAs that are partially uncoupled to the cytoskeleton, and in particular, F-actin. This is thought to occur in consequence to vinculin's binding to talin. Vinculin binds to talin via its N-terminal region and binds to Factin via its C-terminal tail. (Atherton et al., 2015). Carisey et al. (2013) show that vinculin can regulate the transmission of mechanical signals from the ECM to the cytoskeleton due to its intimate link with talin and by extension integrins. Even though vinculin does not directly associate with integrins, it is able to exert a regulatory effect onto the mechanical signals that are relayed across them, a process that is dependent on its binding to talin (Carisey et al. 2013). Furthermore, Carisey et al. (2013) demonstrate that the interaction between talin and vinculin is responsible for stabilizing FAs. Vinculin was shown to highly colocalize with talin, paxillin, p130cas, tensin, FAK, all of which play a crucial role in mediating mechanotransduction (Carisey et al. 2013).

1.6 MECHANOTRANSDUCTION

Once cells have associated with a substrate through ECM and integrin dependent signaling, there is a signaling event sensed by cells that determines the stiffness of the substrate. Recent work has shown that different stiffness of substrates can have a profound influence on cellular migration and proliferation (Najafi et al., 2019). The *transduction* of these

mechanical signals across the plasma membrane is known as Mechanotransduction (Wang et al. 1993). These mechanical signals are influenced by the rigidity and density of the ECM, as well as cell-cell contacts. Changes in these mechanical forces cause changes in integrin-based cell-ECM adhesions as well cadherin based cell-cell contacts (Geiger & Yamada, 2011). These processes lead to downstream signalling pathways that transfer the signal on to the cytoskeleton. The cytoskeleton then transmits the mechanical signals to various organelles and compartments in the cell, among which is the nucleus.

1.6.1 NUCLEAR DYNAMICS IN MECHANOTRANSDUCTION

The largest organelle cell in the is the nucleus. It performs a variety of important functions, such as genome organization, gene regulation and other forms of signalling (Wu et al., 2014). The correct orientation and positioning of the nucleus is a crucial step in cell polarization, migration and division. As the cell is maneuvering through the 3D environment of the ECM, the nucleus must be repositioned and often experiences confining environments (Mistriotis et al., 2019). As such, it is crucial that the cell can communicate positioning cues to the nucleus. The mechanisms that underlie the precise positioning of the nucleus in within a migrating depend on the contraction of the actomyosin cytoskeleton and nuclear-linked microtubule motors (Wu et al., 2014). The cytoskeleton is connected to the nucleus through the Linker of Nucleoskeleton and Cytoskeleton (LINC) complex (Bouzid et al., 2019). The nucleoskeleton is composed of lamins, inner nuclear membrane proteins and chormosomes and is separated from the cytoskeleton by the nuclear envelope.

Forces from the cytoplasm are transferred across the membrane and into the nucleus to engage signaling within the nucleus. The LINC complex is responsible for this event.

1.6.2 LINC COMPLEX

The LINC complex is composed two proteins known as SUN (for Sad1 and Unc-84) and nepsrin (for nuclear envelope with spectrin repeats). In humans, there are at least five SUN proteins that can form multimers and interact with lamins. SUN proteins are components of the inner nuclear membrane with conserved, C-terminal SUN signaling domains. SUN domains consist of approximately 175 amino acids and are unique signalling domains. Nesprin proteins contain a C-terminal Klarsicht/ANC-1/Syne Homology (KASH domain). The SUN domain spans the inner nuclear membrane, whereas the nesprins span the outer nuclear membrane (Bouzid et al., 2019). The C-terminal KASH domain of the nesprin protein interacts with SUN domain of the SUN protein in the perinuclear space in order to connect the two proteins, thereby forming the LINC complex. Different nesprin proteins interact with either the actin cytoskeleton, intermediate filaments or microtubules thus linking the nucleus to the rest of the cell (Bouzid et al., 2019). Nesprin-1 and nesprin-2 interact directly with filamentous actin (Factin) through an N-terminal calponin homology domain. Nesprin-3 contains an Nterminal domain that interacts with plectin, a protein that links nesprin-3 to intermediate filaments, while nesprin-4 interacts directly with microtubules (Bouzid et al., 2019).

The shape and movement of the nucleus during polarization and cell migration are thus regulated by the LINC complex (Wu et al., 2014). Using a dominant negative construct of the LINC complex, Luxton et al. (2010) demonstrated that nuclear movement was

significantly reduced in cells lacking nesprin-2G (Luxton et al., 2010). Their investigation used NIH3T3 fibroblasts exposed to lysophosphatidic acid (LPA), a compound that stimulates motility. As such, they were able to directly examine the parameters of nuclear migration in the absence of LINC proteins in migratory cells (Luxton et al., 2010). Their findings showed that in the absence of LINC proteins, the nuclei of NIH3T3 fibroblasts were unable to orient themselves properly during the cell migration in response to the signals LPA (Luxton et al., 2010). Thus, LINC complex proteins have been shown to be central to linking the nucleus to the movement of the rest of the cell, a message that is relayed through the cytoskeleton (Figure 3).

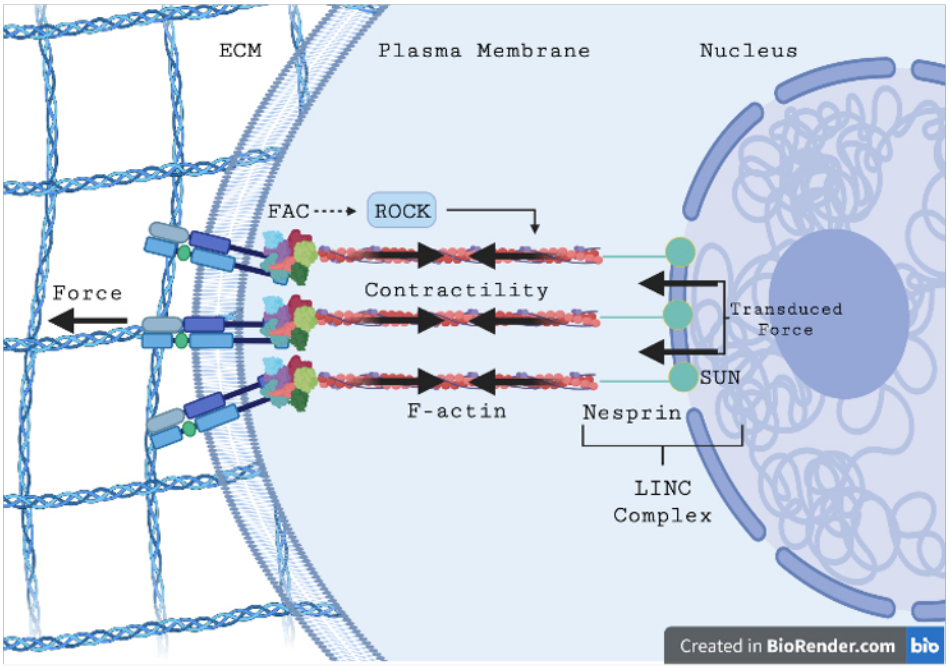

Figure 3. Diagram of major signaling molecules connecting the cytoskeleton to the nuclear membrane. Integrins bind to the ECM and recruit the FAC. Signaling through Rho GTPases activates ROCK which promotes actin contractility. The contraction of the cytoskeleton pulls on the nuclear membrane through the LINC complex. Modified from Bouzid et al. (2019).

1.6.3 NUCLEAR CONFINEMNT AND BLEBBING

Cell migration is a crucial step of metastasizing cancers. When migrating through the ECM, cells are prone to confined spaces approximately 1 to 20 μm in diameter (Mistriotis et al., 2019). As such, the cell must be able to effectively move itself and all its contents through this space. The nucleus is the largest and stiffest organelle, and as such it has a rate-limiting role in the process of confined cell migration (Lammerding, 2011). The cell can communicate to the nucleus via LINC complex in order to achieve nuclear translocation, thereby permitting cell migration. When the nucleus is confined, the nuclear envelope experiences substantial mechanical stress, and this can lead to an increase in pressure within the nucleus. This ultimately causes blebbing in the nuclear envelope, which may lead to rupture and DNA damage (Denais et al., 2016). The process of nuclear blebbing has been shown to depend on the activity of RhoA/myosin-II and the LINC complex (Mistriotis et al., 2019). Mistriotis et al. (2019) show that confinement leads to a polarization of RhoA/myosin-II within the cell, and along with LINC-complex dependent nuclear anchoring gives rise to a posterior/anterior cell polarization with respect to the nucleus. RhoA/myosin-II activity leads to an increase of nuclear influx from the posterior regions of the cell. This causes the nucleus to undergo volume expansion, leading to nuclear bleb formation, and ultimately nuclear envelope rupture (Mistriotis et al., 2019).

Mistriotis et al. (2019) also demonstrate that inhibition of Rho-associated protein kinase (ROCK) using Y27632, which effectively eliminates actomyosin contractility, significantly decreased the amount of nuclear blebbing (Mistriotis et al., 2019). Briefly, Y27632 is a ROCK inhibitor that inhibits by competing for the ATP binding site (Cite).

Ishizaki et al. (2000) have determined that the K_i values of Y-27632 are 0.22 and 0.30 μM for ROCK-I and ROCK-II, respectively. As such, confinement appears to place a significant stress on the nucleus, which leads to nuclear envelope deformation and blebbing. This process is mediated by ROCK and is linked to the cytoskeleton via its connection to nucleus through the LINC complex (Mistriotis et al., 2019).

1.8 EPITHELIAL TO MESENCHYMAL TRANSITION

Under normal conditions, epithelial cells form layers of apicobasal polar cells. These cells form cell-cell contacts, such as adherens junctions or tight junctions, which tightly hold adjacent cells in close contact (Ribatti et al., 2020). Epithelial to mesenchymal transition was first correlated to cancer in the early 1980s. It was found that benign tumors become metastatic because of EMT (Thiery et al., 2009). As result of this, cells begin to exhibit migratory and invasive characteristics, traits associated with mesenchymal cells. EMT has been shown to play a role in a variety of cancers, including lung, prostate, breast and pancreatic cancers (Ribatti et al., 2020). Xu et al. (2016) showed that the transforming growth factor β (TGF-β)/Smads pathway is an inducer of the EMT pathway, through its ability to upregulate the transcription of EMT-related
proteins. Following the entry into EMT, cells lose their polarity and the cell-cell adhesions they form with surrounding cells are compromised.

Adherens junctions, which are composed of E-cadherin in epithelial cells, connect the cytoskeletons of adjacent cells together. E-cadherin is a transmembrane protein that spans the intercellular domain and engages with the adherens junctions of neighbouring cells. In the cytoplasm, it is linked to β-Catenin, which interacts with α-catenin, which in turn

associates with the cytoskeleton through vinculin and α -actinin (Loh et al., 2019). The loss of E-cadherin is a hallmark of EMT, which leads to cytoskeletal rearrangement and altered cell signalling. While the loss of E-cadherin is a key event in EMT, it is important to note that loss of E-Cadherin alone is not sufficient to cause EMT to occur, nor is overexpression of E-Cadherin capable of restoring epithelial phenotype (Ribatti et al., 2020). The loss of E-cadherin is commonly accompanied by the increase of N-Cadherin expression. NCadherin is usually absent in epithelial tissues, however it is common in other cells, such as neural, endothelial, and stromal cells (Loh et al., 2019). In EMT, an increase of NCadherin expression is another key indicator (along with loss of E-Cadherin) of the transition of cells from epithelial to mesenchymal. This change over from E-Cadherin to N-Cadherin is termed "cadherin switching" and leads to changes in Rho-GTPase signalling. RhoA and Rac1, two members of the Rho GTPase family, play a key role in changing the apicobasal polarity of cells into front-back polarity, a characteristic polarity of migratory cells. Rac1 is important in triggering cytoskeletal changes to allow for enhanced directional migration, while RhoA stabilizes N-Cadherin adherens junctions and enhances the formation of stress fibers (Loh et al., 2019). Aside from promoting cell migration, EMT also inhibits apoptosis. This has been shown to be the result of inhibiting the TNF-related apoptosis-inducing ligand-receptor 1 (TRAIL-R1) pathway, as well as TRAIL-R2. E-Cadherin promotes apoptosis through the TRAIL-R1/R2 pathway, as well as through caspase-8, and as such loss of E-Cadherin prevents this signalling (Lu et al., 2019).

Like adherens junctions, tight junctions are also compromised in EMT. Tight junctions are like adherens junctions in that they consist of transmembrane proteins that link two adjacent cells together. However, their components and specific functions differ. Tight junctions are

composed of transmembrane proteins include members of occludin, claudin, and junctional adhesion molecule classes. They function to tightly hold cells together, forming an impermeable seal. Among the proteins that make up tight junctions is Zonula occludens-1 (ZO-1), a tight junction protein that has been studied with respect to EMT (Polette et al., 2007). It has been observed that ZO-1 is delocalized from the membrane in epithelial cell migration (Gottardi et al., 1996). Nagai et al. (2016) found that mRNA samples from hepatocellular carcinoma tumors had lower levels of ZO-1. They went on to conclude that lower levels of ZO-1 could be a predictor of poor prognosis of hepatocellular carcinoma, as well as potential markers for treatment. ZO-1 and

Cadherin signalling is connected through the intracellular protein β-Catenin. Normally, ZO-1 and β-Catenin are found at the cell membrane in tight junction complexes. However, during EMT they have been observed to delocalize from the cell membrane and exhibit diffuse cytoplasmic as well as nuclear localization (Polette et al., 2007). It is important to note levels of β-Catenin have been shown to be significantly higher in tumor cells.

As such, E-Cadherin, N-Cadherin, ZO-1 and β-Catenin are representative proteins in the process of EMT. Changes in the expression levels in each of these proteins have been linked to the change over from stationary epithelial to migratory mesenchymal cells commonly seen cancer. As outlined above, these changes accompany the loss of apicobasal cell polarity, in a changeover to front-back polarity. These processes lead to intracellular signalling changes, among which is the altered RhoA and Rac1 signalling. Together, these processes lead to a mesenchymal cell fate that is migratory and invasive, unlike its epithelial origin.

1.9 INTEGRINS IN BONE

Integrins are widely expressed in bone cells. They can be found in osteocytes, the primary mechanosensitive cell in bone, as well as osteoclasts. In osteocytes, integrins have been shown to mediate mechanotransduction. Similarly, integrins play a crucial role in mediating osteoclast adhesion to the bone matrix, where they form tight connections between the osteoclast membrane and the matrix (Geoghegan et al., 2019). Both integrin β1 and β3 have been shown to play a key role in facilitating mechanotransduction. Integrins play a key role in sensing the mechanical forces placed on the bone. Mechanical loading has been shown to regulate bone remodeling. If the bone is placed under consistent loading, it will result in bone deposition. However, if little load is experienced, such as with limited physical activity, mechanical stimuli at the cellular level will result in bone resorption. This is because mechanical stimuli at the organ level are transferred to the bone matrix, and they result in shear stress that ultimately stimulates the cell membrane of osteocytes. This is achieved through the mechanosensing ability of integrins and serves as the signal for bone remodelling (Geoghegan et al., 2019).

1.9.1 BONE REMODELLING

Bones are metabolically active parts of the skeleton that help provide the body its structural framework as well as maintain hematopoiesis and mineral homeostasis (Ono & Nakashima, 2018). Like all tissues, bone is primarily composed of living cells, however what is unique to bone is the deposition of hydroxyapatite, a type of calcium phosphate crystal. The calcium phosphate crystals exist in the extracellular matrix, which along with the surrounding cells provides the bone with

its structural support. Here, the ECM is referred to as the bone matrix. It makes up most of the organic matter of the bone (Hadjidakis & Androulakis, 2007). Bone matrix is composed of 90% type I collagen, with the rest consisting of other proteins such as proteoglycans and fibronectin and osteopontin (Xu et al., 1998, Marie, 2009). These matrix proteins play an important role in bone remodelling. The surrounding cells that are responsible for carrying out the bone remodelling process communicate with the matrix primarily via integrins. It is important that this process occurs properly because bones need to continuously undergo remodelling to stay healthy (Marie, 2009). The bone remodelling cycle begins with resorption, where cells known as osteoclasts digest old bone and form pits in the surface. This is followed by reversal, where mononuclear cells appear on the bone surface. Following this step, osteoblasts replace the resorbed bone until it is fully replaced (Hadjidakis & Androulakis, 2007).

1.9.2 OSTEOCLASTS

An osteoclast is a large multinucleated that is primarily responsible for bone resorption. It is derived from the monocyte-macrophage lineage. Osteoclast differentiation depends on macrophage colony stimulating factor (M-CSF) and receptor activator of nuclear factorκB ligand (RANKL) (Ono & Nakashima, 2018). It is known that osteocytes express the RANKL and M-CSF that is needed to differentiate osteoclast progenitors into osteoclasts (Feng et al., 2013). Interestingly, Geoghegan et al., (2019b) show that integrin αvβ3 expression levels influenced RANKL levels in osteocytes. They found that MLO-Y4 cells (osteocyte-like cells) that were deprived of oestrogen had higher ratio of RANKL to osteoprotegerin. Given this finding, Geoghegan et al., (2019b) then tested what occurred

when MLO-Y4 cells were deprived of oestrogen in the presence of an integrin αvβ3 blocker. They found that in the absence of integrin αvβ3 signalling the ratio of RANKL to osteoprotegerin did not change when compared to control cells with only αvβ3 blocker (Geoghegan et al., 2019b). This is important because post-menopausal osteoporosis is characterized lower levels of oestrogen in circulation. Given the findings of Geoghegan et al., (2019b), it would be expected that an increase of RANKL would cause excess differentiation of osteoclasts. In mice, it is known that excess osteoclast differentiation leads to osteoporosis, a condition where mice exhibit lower bone density (Miyamoto, 2011). If the opposite occurs, and there is a net loss of osteoclast differentiation, mice often experience osteopetrosis, a condition where the bone becomes too dense as result of the lack of bone resorption. (Miyamoto, 2011). Osteoclast differentiation requires both MCSF and RANKL to achieve their cell fate. Signalling from M-CSF alone on osteoclast progenitor cells leads to the formation macrophages, which unlike osteoclasts are mononuclear (Miyamoto, 2011).

To resorb bone, osteoclasts must attach to the bone matrix. This is achieved through a link between integrins in the osteoclast membrane and matrix proteins. Osteoclasts may attach to the matrix through integrin heterodimers αvβ1, α2β1 and αvβ3. Among them, αvβ3 is known to form the adhesion structures that give rise to the sealing zone (Geoghegan et al., 2019). Integrin based adhesomes form a macrostructure known as a podosome, which is an actin-rich ring of integrin-matrix adhesion complexes that forms a tight connection between the osteoclast membrane and the bone matrix, providing an isolated region of the bone surface. It is in these segregated areas that bone resorption occurs (Edwards & Mundy, 2011). Resorption is carried out by the secretion of H+, Cl, cathepsin K and matrix

metalloproteases (MMPs) into the resorption area, also known as the resorption pit (Ono & Nakashima, 2018). Osteoclasts secrete H+ via V-ATPases located on the ruffled border, a highly permeable folded region of the membrane that is unique to osteoclasts. The secretion of H+ lowers the pH of the pit to approximately 4.5, which provides the environment under which resorption takes place. The ruffled border is also rich in chlorine anti-porter CIC-1. The secretion of Cl- anions into the pit helps achieve the low pH of the resorption pit (Ono & Nakashima, 2018). Acidification of the pit activates cathepsin K, and along with the MMPs they function to degrade the organic material in the pit, such as collagen (Ono & Nakashima, 2018). It is crucial that these processes occur within a sealed zone as to prevent aberrant digestion of the bone surface.

1.9.3 OSTEOCLAST CELL LINE MODEL

In vitro cell line models are important tools that help investigators determine the molecular intricacies of cells. One example of this is murine macrophage RAW 264.7 cells, a precursor cell line to osteoclasts. These cells can be stimulated with RANKL to differentiate them into osteoclast-like cells, exhibiting podosomes among other hallmarks of osteoclasts (Collin-Osdoby & Osdoby, 2012). There are several advantages to using RANKL differentiated RAW 264.7 cells instead of generating primary cell cultures of osteoclasts. First, RANKL differentiated RAW 264.7 cells can be generated in several days, making them more practical for the researcher when planning for experiments. Furthermore, the sheer number of cells that can be generated using this method is greater than that of a primary cell line. This is advantageous because researchers can investigate

on a larger sample size, as well have greater amounts of RNA and protein for biochemical analysis (Collin-Osdoby & Osdoby, 2012).

While RAW264.7 cells offer a practical model to investigate osteoclasts, it is important that the differentiated cells have high resemblance to their naturally occurring counterparts, both biochemically and morphologically. Song et al. (2018) evaluated the efficacy of RANKL induced osteoclasts from RAW 264.7 cells. Their findings show that RAW264.7 cells incubated with RANKL at concentrations of between 30 and 100 ng/ml show a number of similarities to osteoclasts. First, differentiated RAW264.7 cells were able to form resorption pits, exhibited high amounts of F-actin rings, and had a two-fold increase in Cathepsin K production, all only occurred at ≥ 30 ng/ml (Song et al., 2018). Interestingly, Song et al. (2018) demonstrated that in each of these markers, there were no statistical differences between 30, 50, 75 and 100 ng/ml RANKL, hinting that this phenomenon could be dependent on a threshold of RANKL. Although the marker of osteoclasts was not significantly changed at concentrations of over 30ng/ml, Song et al. (2018) did note that cells in the 100ng/ml dose group did exhibit a larger size than the rest of the groups. As such, RANKL differentiated RAW264.7 cells offer researchers with the ability to investigate the parameters of osteoclast signalling and the molecular interactions that occur to give rise to their unique structures. Among these are resorption pits and podosomes, both of which are present in RANKL differentiated RAW264.7 cells.

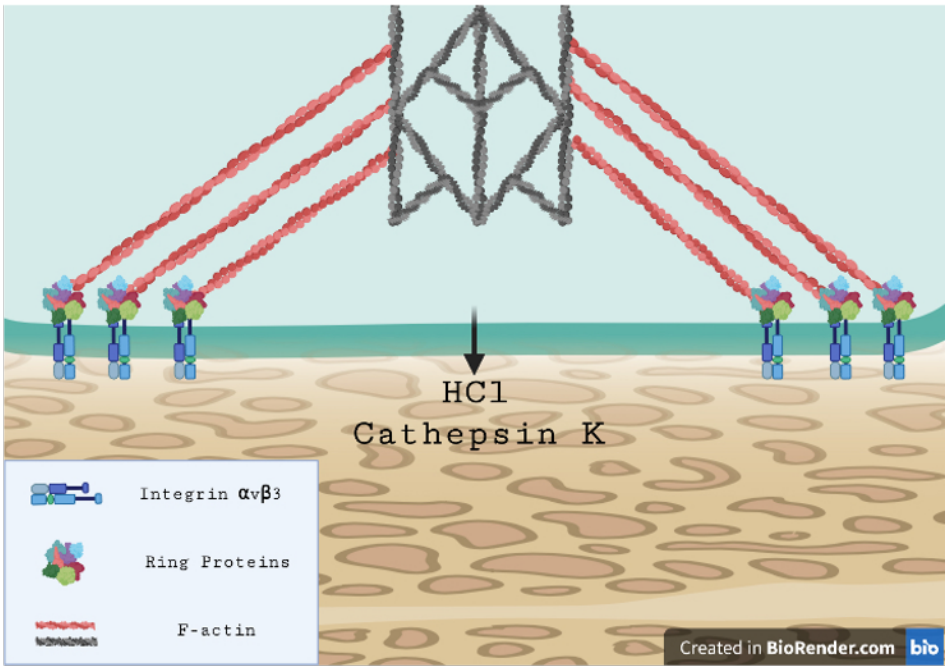

Figure 4. Diagram of major signaling molecules in osteoclast podosomes. Integrins bound to the bone matrix recruit ring proteins. Ring protein complex contains proteins such as p130Cas, Src, and Talin, among others. This arrangement of integrins and actin forms a sealing zone. Within the sealing zone, the osteoclast secretes HCl and Cathepsin K to degrade the bone matrix. Modified from Mecahnobio.info (1,2) (2018).

1.9.4 PODOSOMES

The formation of a sealed zone for the resorption of bone is dependent on the proper assembly of podosomes. Podosomes are actin rich adhesion structures that are found in monocytes and osteoclasts. They are composed of an F-actin core which is surrounded by various scaffolding proteins, such paxillin and talin (van den dries et al., 2019). Around the F-actin core is a ring of integrin-based adhesion complexes. Although there are a variety of integrins expressed in osteoclasts, expression of integrin β3 is the highest (Kong et al., 2020). The formation of rings is unique to podosomes, a feature that differentiates them from other integrin-based adhesions, such as focal adhesions. Podosomes are found in a variety of cell types and in each cell type they form unique assemblies. They form clusters in macrophage and dendritic cells, rosettes in smooth muscle cells, and rings in osteoclasts (van den dries et al., 2019).

During the differentiation of osteoclasts, the individual podosomes arrange into rings. In the final stage, they form a sealing zone on the bone surface (Jurdic et al., 2006). The formation of this ring structure is crucial for the degradation of bone. It has been shown that individual podosomes are not capable of excreting degrading proteases alone and must be in a ring macrostructure for resorption to occur. As such, the podosome ring has been thought to act as a hub for vesicles containing proteases to reach the sealing zone and aid in degrading the bone (Saltel et al., 2004). Interestingly, it has been shown that the podosome stability has been linked to RhoA activity. Saltel et al. (2004) showed that the inhibition of Rho using C3 exoenzyme prevents the formation of the sealing zone.

For podosome formation to occur, as well as the coordinated regulation of its cytoskeletal elements, there must be a line of communication between the extracellular environment and within the osteoclast. This form of signalling is termed 'outside-in signalling and is dependent on the signalling of integrins and the receptors they work in tandem with. These signalling cascades are initiated by ECM molecules which interact with the extracellular domain of integrins. This causes an internalization of the signal, which relays the signal through proteins such as p130cas, c-Src and c-Cbl (Kong et al., 2020). P130cas is a scaffold protein that plays a key role in regulating the cytoskeletal reorganization that occurs because of integrin activation. It has previously been reported that p130cas is involved in actin ring formation, however until recently the direct effect of loss of p130cas in osteoclasts *in vivo* has been unknown, since silencing it is embryonically lethal (Nagai et al., 2013). Using an osteoclast specific p130Cas knockout, Nagai et al.

(2013) showed that these mice exhibit a higher bone mass phenotype. Furthermore, analysis of the individual osteoclasts showed that actin ring formation was inhibited in the absence of p130Cas. Although it was previously known that p130Cas regulates integrin signalling towards the cytoskeleton, Nagai et al. (2013) confirmed this in osteoclasts. Interestingly, Nagai et al. (2013) demonstrated that p130cas regulated activity of Rac1, as well as its distribution, in osteoclasts.

1.10 SUMMARY AND RATIONALE

The regulation of cell function requires the integration of numerous biological and mechanical signals from the surrounding cells and the extracellular matrix (ECM). Transduction of stimuli from the extracellular matrix is an important process for cells in facilitating migration and adaption to environment. This concept is known as cellular mechanotransduction. Mechanical signals from the ECM are ultimately relayed intracellularly to remodel the actin cytoskeleton, a process which is known to be regulated by RhoGTPases. Nitric oxide synthase 1 adaptor protein (NOS1AP) is implicated in this process due to three key findings: 1) it is known that NOS1AP influences RhoGTPase activity (Richier et al., 2010), 2) NOS1AP has been linked to the Hippo signaling pathway which is involved in mechanotransduction (Clattenburg et al., 2015) 3) NOS1AP associates with Scrib, a protein implicated in EMT transition and cellular migration (Richier et al., 2010).

1.11 HYPOTHESIS AND AIMS

I hypothesize that ***NOS1AP functions in integrin dependent signaling to regulate Rho dependent signaling and that loss of NOS1AP contributes to an EMT phenotype***.

To test this hypothesis, I have the following aims:

1. Characterize Mouse Embryonic Fibroblasts (MEFs) generated from wild type and NOS1AP$^{-/-}$ mice.
2. Identify and characterize novel PTB associating proteins
3. Determine if NOS1AP is necessary for mechanosensory signaling
4. Characterize the expression of NOS1AP in osteoclast cell line model

CHAPTER 2 METHODS

2.1 ANTIBODIES

Antibodies used in this study include GST-NOS1AP – rabbit polyclonal GST fusion antibody (Clattenburg et al., 2015), used at 1:200 for IP, 1:1000 for Western blot, 1:500 for immunocytochemistry. PPIT-NOS1APc – rabbit polyclonal antibody specific to a region in NOS1APc (Clattenburg et al., 2015), used at 1:200 for IP, 1:1000 for Western Blot, 1:500 for immunocytochemistry. Pre-Immune NOS1AP – rabbit polyclonal antibody (Clattenburg et al., 2015) used at 1:200 dilution for IP. Integrin β3 – mouse monoclonal antibody (Santa Cruz Biotechnology; Cat# sc-46655), used at 1:1000 for Western blot, 1:1000 for immunocytochemistry. Flag M2 – mouse monoclonal antibody from (Sigma; Cat# F3165) used at 1:5000 for Western blot. Anti-GFP – rabbit polyclonal antibody (Abcam; Cat# ab290) used at 1:200 for IP, 1:5000 for Western blot. Blotting Grade Protein A-Horseradish Peroxidase Conjugate – Secondary antibody (Bio-Rad; Cat# 170-6522) used at a dilution of 1:10,000 for Western blot. Blotting Grade Affinity Purified Goat Anti-Mouse IgG (H+L) Horseradish Peroxidase Conjugate – secondary antibody (Bio-Rad Cat# 170-6516) used at a dilution of 1:10,000 for Western blot.

2.2 PREPARATION OF MOUSE EMBRYONIC FIBROBLASTS CELL LINES

Pregnant mouse was sacrificed at E13.5. The belly region of the pregnant mouse was sprayed with 70% ethanol then the uterus was dissected out using sterile instruments. The uterus was placed in a 100mm petri dish on ice. Each embryo was processed separately in a 60mm dish, on ice. The head and all internal organs were removed, and a piece of the tail was kept for genotyping. Following the dissection of each embryo, each was transferred to a 15 ml tube containing 12 ml of cold HBSS on ice and rinsed twice to remove any remaining blood. Following this step, the HBSS was decanted into a beaker and the carcass was transferred to a 60 mm dish. The remaining tissue was minced into approximately 1mm diameter pieces with sterilized forceps and scissors. All tissue pieces were transferred to a 15 ml tube containing 2 ml of 0.125% Trypsin/EDTA + Pen/Strep. The tubes were then incubated at 37°C water bath for 30 min, mixing the tube every 10 min. After 30 mins, another 2 ml of Trypsin/EDTA + Pen/Strep was added to each tube and incubated at 37°C for another 30 min. Cells from individual embryo was then briefly triturated through a flamed pasture pipette and then plated in 2 separate T-75 flasks containing 5ml of DMEM + 10% FBS+ Pen/Strep each. Flasks were left to sit in incubator at 37°C and 5% CO_2 for 3 hours. After 3 hours the cells were refed and looked to be about 30% confluent. After a few days, when cultures reached about 70% confluency, cells from one flask were frozen in 2 vials and labeled as primary cells. The other flask was refed and left to grow for another 3 days. Once they reached 90-95% confluency, the cells were then transformed with SV40.

2.3 SV40 TRANSFORMATION

100,000 cells/well were plated in a 6-well plate and left overnight to attach and spread. The following day, cells were transfected using Lipofectamine 2000. Here,120ul Lipofectamine (ThermoFisher; Cat# 11668019) in 80ul Serum Free Dulbecco's Modified Eagle Medium (DMEM) (Wisent; Cat# 319-005-CL) DMEM, containing no antibiotics. The solution was vortexed solution and incubated for 5 min. 40ug of a SV40 cDNA (addgene plasmid #21826, a gift from David Ron (SV40 1:pBSSVD2005) solution was added to the Lipofectamine Solution. This mixture was vortexed and incubated for 15 min. Cells were then washed twice with 3ml/well of antibiotic free DMEM+ 10% fetal bovine serum (FBS) (Wisent; Cat# 080450) FBS and re-fed with 3 ml of same media. 100ul of DNA complex was added to each well in a dropwise fashion. Following 48h, each well was subcultured in a T75 flask. Once the cultures reached about 95% confluency, cells were again subcultures 1:10. The rest of the cells were frozen at -80°C. The cells were split at 1:10 until they reached about 95% confluency. Once the cell cultures reached about 95% confluency, cells were split at 1:20. This step was repeated twice. When cells reached approximately 95% confluency, they were then sub cultured at 1:40 a further 2 times prior to testing by PCR analysis for genotyping and used for experiments. In addition, all cells were tested for mycoplasma at least twice in the immortalization stage using Lonza MycoAlertTM kit (Cat #). Note, most experiments performed were done on cells that had not been passaged more than 5 times from the final immortalization stage.

2.4 CELL CULTURE AND TRANSFECTIONS

Mouse Embryonic Fibroblasts (MEFs), generated from NOS1AP$^{-/-}$ and control mice, were grown and maintained at 37ºC, 5% carbon dioxide in Dulbecco's Modified Eagle Medium (DMEM) (Wisent; Cat# 319-005-CL) supplemented with 10% heat inactivated fetal bovine serum (FBS) (Wisent; Cat# 080450), 100 U/ml penicillin and 100 ug/ml streptomycin.

For transfection, MEFs were grown in a 12-well plate (Sarstedt; Cat# 83.3921.005) and allowed to reach 50% confluency. 2ug of cDNA was added to 100ul of serum free media (SFM) DMEM and vortexed and left to stand for 5 min. The cDNA solution was then added to a solution containing 4ul of polyethyleneimine in 100ul of SFM. After the tubes were combined, the resulting cDNA-PEI solution was vortexed and then left to incubate at room temperature for 30 minutes, vortexed once more at 10 minutes after mixing. The solution was then added dropwise directly into the well containing the MEFs grown as outlined above. The following day, the cells were rinsed three times with 1ml of DMEM with 5% FBS.

2.5 IMMUNOPRECIPITATION

For immunoprecipitation, MEFs were washed two times with room temperature phosphate buffered saline (PBS). MEFs were lysed using NP40 lysis buffer (10% glycerol, 1% NP40, 20mM tris pH 8.0, 37.5mM NaCl) with 1mM phenylmethylsulphonyl fluoride (PMSF), 10ug/ml aprotinin, 10 ug/ml leupeptin, and 1:1000 sodium pervanadate. The cells were incubated in NP40 on ice for 10 minutes. The cell lysate slurry was scraped and transferred into an Eppendorf tube. Once collected, the lysate was centrifuged at 14000 rpm for 15

minutes. The supernatant was then removed and collected in new Eppendorf tubes. Following the transfer of the supernatant, appropriate primary antibodies were added to each tube (see 2.1 for antibody dilutions). After the addition of a primary antibody, the cell lysate was left to mix overnight on a nutator at 4°C. The following day, 100 ul of 10% protein A Sepharose beads (GE Healthcare; Cat# 17-0780-01) was added to each tube. The tubes were placed back on a nutator for 1hr. The lysate-bead mixture was then centrifuged for 5 min at 5000 rpm. Following each centrifugation, the supernatant was aspirated, and the bead pellet was resuspended in 1ml of ice-cold NP40 lysis buffer. This was repeated between 3-5 times. Following a final spin, the supernatant was removed, and the beads were resuspended in 30ul of 2x laemmli sample buffer (SB), containing 4% SDS, 10% beta-mercaeptoethanol, 20% glycerol, 0.1 M Tris pH 6.8 and 0.005% bromophenol blue.

2.6 WHOLE CELL LYSATE

For detection of protein in whole cell lysate from the NOS1AP$^{-/-}$ or control MEFs, cells were washed two times with room temperature phosphate buffered saline (PBS). MEFs were lysed using NP40 lysis buffer (10% glycerol, 1% NP40, 20mM tris pH 8.0, 37.5mM NaCl) with 1mM phenylmethylsulphonyl fluoride (PMSF), 10ug/ml aprotinin, 10 ug/ml leupeptin, and 1:1000 sodium pervanadate. The cells were incubated in NP40 on ice for 10 minutes. The cell lysate slurry was scraped and transferred into an Eppendorf tube. Once collected, the lysate was centrifuged at 14000 rpm for 15 minutes. The supernatant was then removed and collected in new Eppendorf tubes. Following the transfer of the supernatant, protein concentrations were determined using a modified Bradford protein assay. Once protein concentrations were determined, 30ug of protein samples from either

control or NOS1AP-/- MEFs, were added to 2XSB and brought up to a 30ul volume with PBS, and either run on SDS-PAGE or stored, frozen at -80ºC, until subjected to SDSPAGE.

2.7 WESTERN BLOTTING

For Western Blot analysis, 7.5% and 10% SDS-PAGE gels were used. Samples, either lysate or immunoprecipitations that were isolated in 2xSB were boiled at 95ºC for 5minutes, then loaded into the gel. Once loaded, they were electrophoresed using a BioRad Mini-Protein Gel apparatus. All blots were run between 50-90 constant Voltage (V) through the stack then 150V through the separation gel. Following electrophoresis, gels were transferred onto a polyvinyl difluoride (PVDF) membrane (Millipore; Cat# ISEQ00010) overnight at 40V. Membranes were then rinsed in distilled water, washed in either phosphate buffered saline (PBS) or Tris-buffered saline (TBS) and then blocked for an hour in a 5% non-fat milk in Tris-buffered saline 0.01% Tween (TBST) solution. Membranes were then incubated on a nutator, in appropriate primary antibody diluted in a solution of 5% milk-TBST overnight at 4ºC. Following overnight incubation membranes were then washed 3 times in TBST for 10 minutes each, and then incubated in appropriate HRP (horseradish peroxide conjugated secondary antibody for one hour at 1:10,000 dilution in TBST. Following incubation in secondary antibody, membranes were washed three times in TBST for 10 minutes. Once washed, Enhanced chemiluminescence (ECL) Clarity reagent (BioRad Cat#1705061) was added according to manufacturer's guidelines and blots were visualized using a ChemiDocTM MP Imaging System, (BioRad).

2.8 IMMUNOCYTOCHEMISTRY

Cells were grown as outlined previously. To activate integrin signaling, MEFs were grown on fibronectin-coated (Sigma; Cat# F0895). Here fibronectin was diluted 1:100 dilution in sterile PBS and added, drop wise, onto 18mm cover slips that had been placed into 12-well plates (Sarstedt; Cat# 83.3921.005). The fibronectin was left to dry in the incubator at 37ºC for 45 minutes. Once polymerized, the MEFs were seeded at an appropriate concentration into the wells containing the fibronectin coated coverslips containing 1ml of DMEM. Once the MEFs had reached the desired confluency, the cover slips were washed twice with 2ml of PBS then fixed with 1ml of 4% paraformaldehyde (PFA) in PBS for 10 minutes at room temperature. Following fixation, coverslips were washed twice with 2ml of PBS, then permeabilized in a PBS solution containing 0.1% Triton-X100 (Sigma; Cat# X100) for 10 min. Cells were then washed in PBS and blocked in 3% BSA (Cat#) for 1hour. Following blocking, MEFs were incubated overnight in primary antibody in 3% BSA solution at 4°C. The following day, the coverslips were washed three times for 10 minutes prior to adding the appropriate secondary antibody in 3% BSA solution for 1h. If co-staining for actin, phalloidin (ThermoFisher; Cat# A12381) was added to the with the secondary antibody. To detect the nuclei of the MEFs, they were stained with 1:1000 Bisbenzimide (Hoechst – 33258; Sigma Cat#B1155) in PBS for 2 mins. Coverslips were then washed twice in PBS for 10 minutes. Following the final wash, the coverslips were mounted onto glass slides (Fisherbrand; Cat# 12-552-3) in 30ul of fluoromount mounting medium (Sigma; Cat# F4680).

2.9 QUANTIFICATION OF NUCLEAR BLEBBING

MEFs were plated at 50000 cells/well in 12-well plates (Sarstedt; Cat# 83.3921.005) on sterilized coverslips. After 16 hours, either 1:100 DMSO or 100μM Y27632 dihydrochloride Rho Kinase Inhibitor (Sigma; Cat# Y0503) in DMEM was added to each well and incubated for 6 hours at 37°C supplemented with 5% CO_2. After 6 hours, the cells were rinsed 3x with PBS and fixed with 4% PFA in PBS. Cells were then washed 3x for 10 min with PBS and stained with 1:1000 Bisbenzimide (Hoechst – 33258; Sigma Cat#B1155) in PBS for 2 min. Following the staining, the cells were washed 3x for 5 min, and the coverslips were mounted onto microscope slides (Fisher Scientific; Cat# 12-55015) using fluoromount aqueous mounting solution (Sigma; Cat# F4680). Nuclei were visualized on a Leica CTR6000 microscope with a Hamamatsu camera using a 20x objective using the DAPI filter. Images of ten fields of each condition were collected for data processing. To quantify the data, the number of nuclear blebs per field was divided by the total number of nuclei in the field to provide a ratio of the extent of nuclear blebbing in each condition. The experimenter was blinded to the genotypes during the acquisition of the nuclei images as well during the statistical analysis.

2.10 PEPTIDE SPOTS ARRAY SYNTHESIS

Peptide SPOTS arrays were performed by Smith et al. (2006) according to the SPOTSsynthesis method as outlined by Frank, R. (1992). In short, NPxY motifs from 126 proteins in the genome were spotted on a cellulose membrane. Each NPxY motif had two variants, one with a non-phosphorylated tyrosine, and an adjacent motif that had a phosphorylated tyrosine. The spotted membrane was then probed with a GST-tagged PTB

domain (amino acids x-x) of NOS1AP in Tris-buffered saline-Tween-20 for 2h at 4°C. Then, the membrane was washed three times and probed with anti-GST to determine which NPxY sequences the PTB domain bound successfully. An interaction was said to be selective if the PTB domain was able to bind to the NPxY motif in a phosphotyrosine dependent manner. Based on the amino acid sequence of the successful binding partners of NOS1AP's PTB in phosphotyrosine dependent manner a consensus motif was generated. The consensus motif was dependent on the amino acid character (hydrophobic/hydrophilic, acidic/basic) at each position from -8 to +3 relative to the tyrosine residue for all NPxY sequences that bound in a phosphotyrosine dependent manner. The proteins which matched this consensus motif the closest were selected as potential binding partners of NOS1AP.

2.11 REAL TIME APOPTOSIS ASSAY

To measure cell death, a pSIVA™ REAL-TIME Apoptosis Fluorescent Microscopy Kit (Bio-Rad; Cat# APO004) was used. MEFs grown as outlined previously were added to 1mL DMEM supplemented with 5% FBS in a 12 well plate. Cells were left to adhere for 6 hours. Following the adhering period, 10µl/ml of the pSIVA-IANBD probe was added drop wise to each well. The probe was mixed by gently moving the culture plate side to side, as pipetting to mix would damage the probe. To distinguish between apoptotic and necrotic cells, as recommended by the manufacturer's guidelines, 5µl/ml of propidium iodide was added to each well, and the plate was gently moved side to side to ensure even distribution. Cells were imaged at 0h, 24h, and 48h to measure the rate of cell death in each condition. For imaging, cells were visualized on an inverted Leica CTR6000 microscope with a Hamamatsu camera using a 20x objective on a 12-well plate stage.

2.12 STATISTICAL ANALYSIS

All data are expressed as means ± standard error of the mean (SEM) and all statistics were analyzed using Prism 6 (GraphPad Software, inc). When comparing more than two groups for the nuclear blebbing data, ANOVA One-way multiple comparisons tests were used.

CHAPTER 3 RESULTS

3.1 IDENTIFYING AN NPxY CONSENSUS BINDING MOTIF FOR NOS1AP'S PHOSPHOTYROSINE BINDING DOMAIN

Previously, Richier at al. (2010) showed that overexpression of NOS1APa influenced the activation of Rac. Furthermore, structure-function analysis of the NOS1AP protein domains revealed that the phosphotyrosine binding (PTB) domain of NOS1AP was sufficient to increase the GTP-loading of Rac, suggesting the importance of this domain in influencing Rac activation (Richier et al., 2010). As well, Clattenburg et al. (2015) have shown the PTB domain to be important for membrane targeting and association with phospholipids. Furthermore, the PTB domain of NOS1AP, has been shown to have a role in the development of podocyte foot processes, structures that are dependent on integrin-based adhesions (Majmundar et al., 2021). PTB domains are protein signaling domains that can associate with target motifs in associating proteins (Smith et al., 2007). Importantly, PTB domains associate with motifs containing Asparagine (N), Proline (P), any amino acid (x), Tyrosine (Y), or NPxY motifs. PTB domain containing proteins can associate with these motifs in both a phosphotyrosine or non-phosphotyrosine dependent manner (Smith et al., 2006). To determine if the PTB domain of NOS1AP associates with any conical NPxY or NPxpY motifs, a bioinformatic screen as outlined in Smith et al., (2007), where all receptors containing an NPxY motif were identified. 12mer peptides surrounding the core of each NPxY regions from each motifs of the identified proteins were synthesized as either phosphotyrosine or non-phosphotyrosine peptides and then spotted onto nitrocellulose membranes (Figure 5A, B).

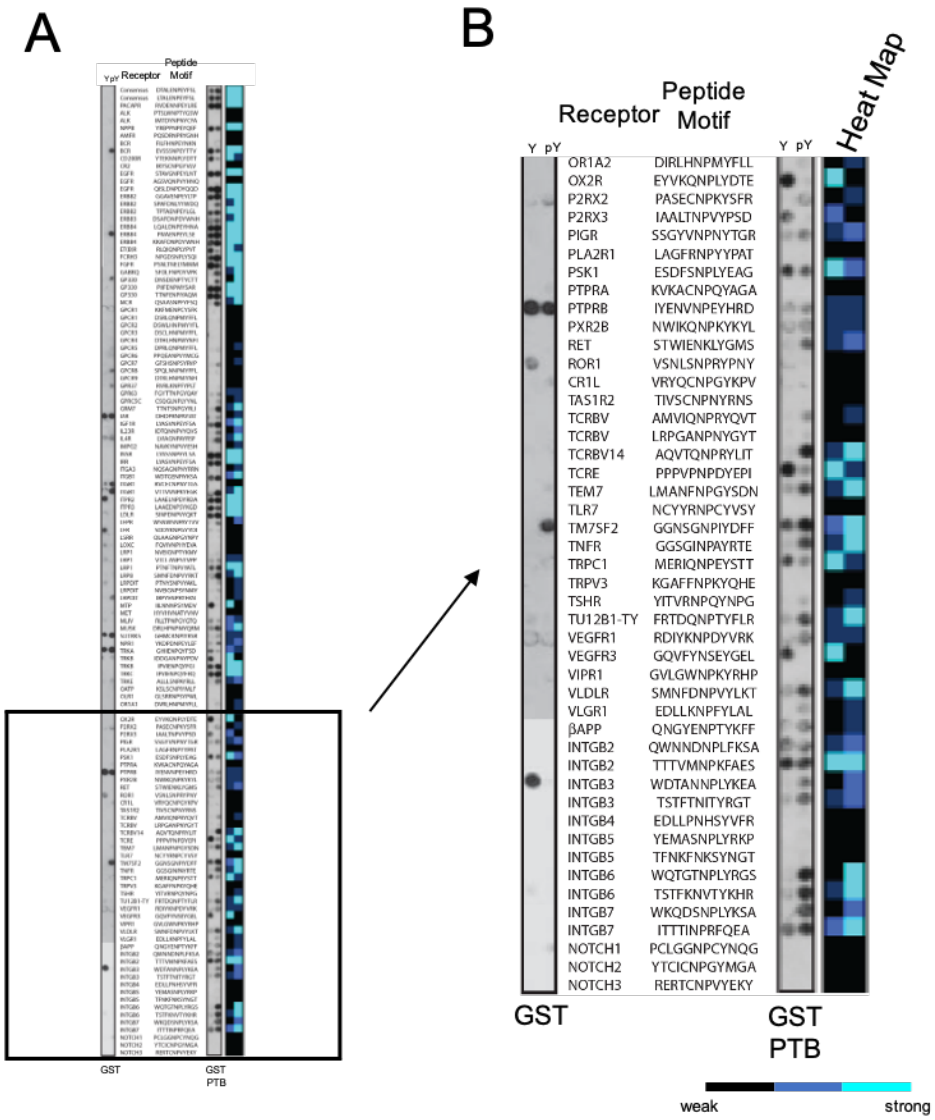

Figure 5. Spots blot showing major non-phosphorylated (Y) and phospho dependent (pY) NPxY motifs identified in bioinformatic screen of receptors containing and NPxY motif from the human genome. **(A)** Major NPxY motifs were screened with either GST alone or GST fusion with the PTB of NOS1AP as indicated. **(B)** Blow up of boxed region in (A). Colour indicates a heat map of binding strength.

The resulting 'spots blot' was then incubated in a solution containing 50ug of either GST or GST-NOS1AP-PTB (Figure 5A, B). The peptides that bound to the GST-NOS1AP-PTB domain in a phosphotyrosine dependent or non-phosphotyrosine dependent manner were then aligned to reveal the identity of the amino acid residues at each position relative to the NPxY motif. The consensus motif of peptides from phosphorylated NPxY motif was "Φ - ζ Φ Φ N P Φ Y - - -," where Φ represents a hydrophobic residue; ζ, hydrophilic; -, nonspecific (Table 1). A list of proteins that contain this consensus motif are listed in Table 1. Those peptides that associated in a non-phosphotyrosine dependent manner had an ideal binding consensus motif that contained "- - Φ Φ ζ N P - Y - ζ -," where Φ represents a hydrophobic residue; ζ, hydrophilic; -, non-specific (Table 2).

Analysis of peptides that match the consensus motif for association with the PTB domain of NOS1AP in a non-phosphodependent manner revealed the ideal candidate to be Purinergic Receptor P2X, ligand gated ion channel, 3 (P2RX3), a cation permeable ligand gated channel that opens in response to ATP and is involved in pain (Wirkner et al., 2007). Whether this interacts with NOS1AP will be interesting to test in relation to pain sensation as the purigenic receptor family of proteins are linked to ATP and pain (Wirkner et al., 2007). While examination of the ideal peptide phosphodependent NPxY motif revealed several proteins as potential ideal NOS1AP PTB binding proteins, including members of the integrin family of protein, such as integrin β3, β6, and Tumor necrosis factor receptor superfamily member 19L (TNFR 19L) (Table 1).

Table 1. List of NPXY motifs that bind to NOS1AP's PTB domain in a phosphotyrosine dependent manner (upper panel). Peptides from proteins that match the consensus motif identified (lower panel).

Protein	NPXY Sequence										
ERBB2	S	P	A	F	D	N	L	Y	Y	W	D	Q
ERBB3	D	S	A	F	D	N	P	D	Y	W	N	H
GABRQ	S	F	D	L	F	N	P	D	Y	V	P	K
GP330	D	N	S	D	E	N	P	T	Y	C	T	T
MCR	Q	S	A	A	S	N	P	F	Y	F	S	Q
GRM7	T	T	N	T	S	N	P	G	Y	R	L	I
IGF1R	L	Y	A	S	V	N	P	E	Y	F	S	A
IL23R	I	D	T	Q	N	N	P	V	Y	Q	V	S
IL4R	L	V	I	A	G	N	P	A	Y	R	S	F
LEPR	Q	L	A	A	G	N	P	G	Y	N	P	Y
LRP8	S	M	N	F	D	N	P	V	Y	R	K	T
MUSK	D	R	L	H	P	N	P	M	Y	Q	R	M
NPR1	Y	K	D	P	D	N	P	E	Y	L	E	F
TRKE	A	L	L	L	S	N	P	A	Y	R	L	L
PIGR	S	S	G	Y	V	N	P	N	Y	T	G	R
RET	S	T	W	I	E	N	K	L	Y	G	M	S
TCRBV14	A	Q	V	T	Q	N	P	R	Y	L	I	T
TEM7	L	M	A	N	F	N	P	G	Y	S	D	N
TNFR 19L	G	G	S	G	I	N	P	A	Y	R	T	E
TU12B1-TY	F	R	T	D	Q	N	P	T	Y	F	L	R
VLDLR	S	M	N	F	D	N	P	V	Y	L	K	T
INTGB3	W	D	T	A	N	N	P	L	Y	K	E	A
INTGB3	T	S	T	F	T	N	I	T	Y	R	G	T
INTGB6	W	Q	T	G	T	N	P	L	Y	R	G	S
INTGB6	T	S	T	F	K	N	V	T	Y	K	H	R
INTGB7	W	K	Q	D	S	N	P	L	Y	K	S	A
INTGB7	I	T	T	T	I	N	P	R	F	Q	E	A
Consensus	Φ	-	ζ	Φ	Φ	N	P	Φ	Y	-	-	-

Amino Acid Identity
Basic Θ – R, K, H
Acidic Λ – D, E
Hydrophobic Φ – G, A, V, I, L, M, F, W
Aromatic Ω – Y, W, F
Hydrophilic ζ – S, T, Y, C, N, Q
Aliphatic Ψ – V, I, L, M

TNFR 19L	G	G	S	G	I	N	P	A	Y	R	T	E
INTGB3	W	D	T	A	N	N	P	L	Y	K	E	A
INTGB6	W	Q	T	G	T	N	P	L	Y	R	G	S
Consensus	Φ	-	ζ	Φ	Φ	N	P	Φ	Y	-	-	-

Table 2. List of NPXY motifs that bind to NOS1AP's PTB domain in a non-phosphotyrosine dependent manner (upper panel). Peptides from proteins that match the consensus motif identified (lower panel).

Protein	NPXY Sequence										
CD200R	Y	T	E	K	N	N	P	L	Y	D	T	T
ET(B)R	R	L	Q	I	Q	N	P	L	Y	P	V	T
MTP	I	I	L	N	N	N	P	S	Y	M	D	V
MLIV	R	L	L	T	P	N	P	G	Y	G	T	Q
TRKA	G	H	I	I	E	N	P	Q	Y	F	S	D
TRKB	I	D	D	G	A	N	P	N	Y	P	D	V
OX2R	E	Y	V	K	Q	N	P	L	Y	D	T	E
P2RX3	**I**	**A**	**A**	**L**	**T**	**N**	**P**	**V**	**Y**	**P**	**S**	**D**
TCRE	P	P	P	V	P	N	P	D	Y	E	P	I
TRPC1	M	E	R	I	Q	N	P	E	Y	S	T	T
VEGFR3	G	Q	V	F	Y	N	S	E	Y	G	E	L
Consensus	-	-	Φ	Φ	ζ	N	P	-	Y	-	ζ	-

Amino Acid Identity
Basic Θ – R, K, H
Acidic Λ – D, E
Hydrophobic Φ – G, A, V, I, L, M, F, W
Aromatic Ω – Y, W, F
Hydrophilic ζ – S, T, Y, C, N, Q
Aliphatic Ψ – V, I, L, M

P2RX3	**I**	**A**	**A**	**L**	**T**	**N**	**P**	**V**	**Y**	**P**	**S**	**D**
Consensus	**-**	**-**	**Φ**	**Φ**	**ζ**	**N**	**P**	**-**	**Y**	**-**	**ζ**	**-**

3.2 IDENTIFYING AN ASSOCIATION BETWEEN NOS1AP AND INTEGRIN β3

Given that our PTB screen revealed the potential for an association between the PTB domain of NOS1AP and integrins, we first set out to determine if NOS1AP and integrins could associate. We choose integrin β3 as it had the ideal consensus motif, as it is more widely expressed than integrin β6. Briefly, integrin β6 is normally not expressed in resting epithelia, however it is induced during wound healing (Häkkinen et al., 2004). To test this, we generated mouse embryonic fibroblasts (MEFs) generated from E13.5 wild type or NOS1AP mutant mice. The two independent NOS1AP$^{/-}$ MEF lines were generated from two different mouse embryos from two different litters (herein KO1 and KO2). Confirmation of the genotypes of the different cell lines were confirmed both by PCR (Figure 6A) and by immunoprecipitation followed by Western blotting, using NOS1AP specific antibodies (Figure 6B).

As we were interested in whether integrin β3 associated with NOS1AP, we confirmed that all the lines of MEFs expressed equivalent levels of integrin β3 protein (Figure 7A). Early passage immortalized wild-type MEF lysate were immunoprecipitated with pre-immune or either a pan-NOS1AP from Sigma or a pan-NOS1AP antibody that the Fawcett lab had previously characterized (#2093, see Clattenburg et al., 2015) (Figure 7B and C). While the #2093 and Sigma anti-NOS1AP show a band at the correct molecular weight for integrin β3, the presence of a band in the pre-immune sample suggests that integrin associated non-specifically. Together these results remain inconclusive as to whether there is an interaction between integrin β3 and NOS1AP.

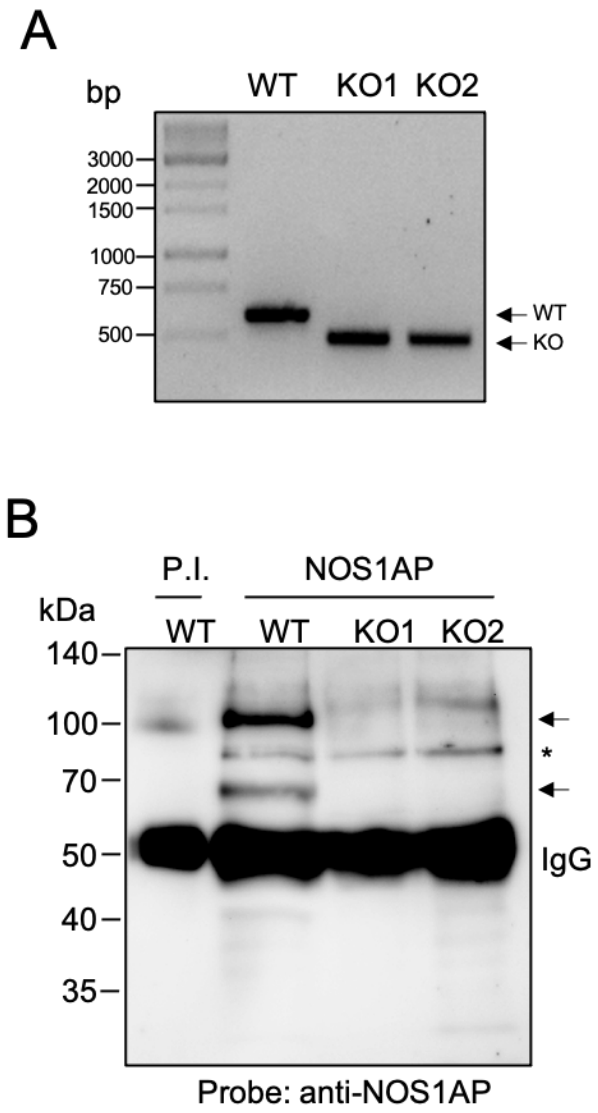

Figure 6. Characterization of NOS1AP mutant mouse embryonic fibroblasts. (**A**) PCR confirmation of wild type and two independent MEF lines lacking NOS1AP. (**B**) WT and NOS1AP mutant MEFs precipitated with either a non-specific IgG or NOS1AP antibodies and probed with a pan-NOS1AP antibody. Arrows indicate the NOS1APc and NOS1APa isoforms. Asterisks indicates an unknown band not seen in the pre-immune lane.

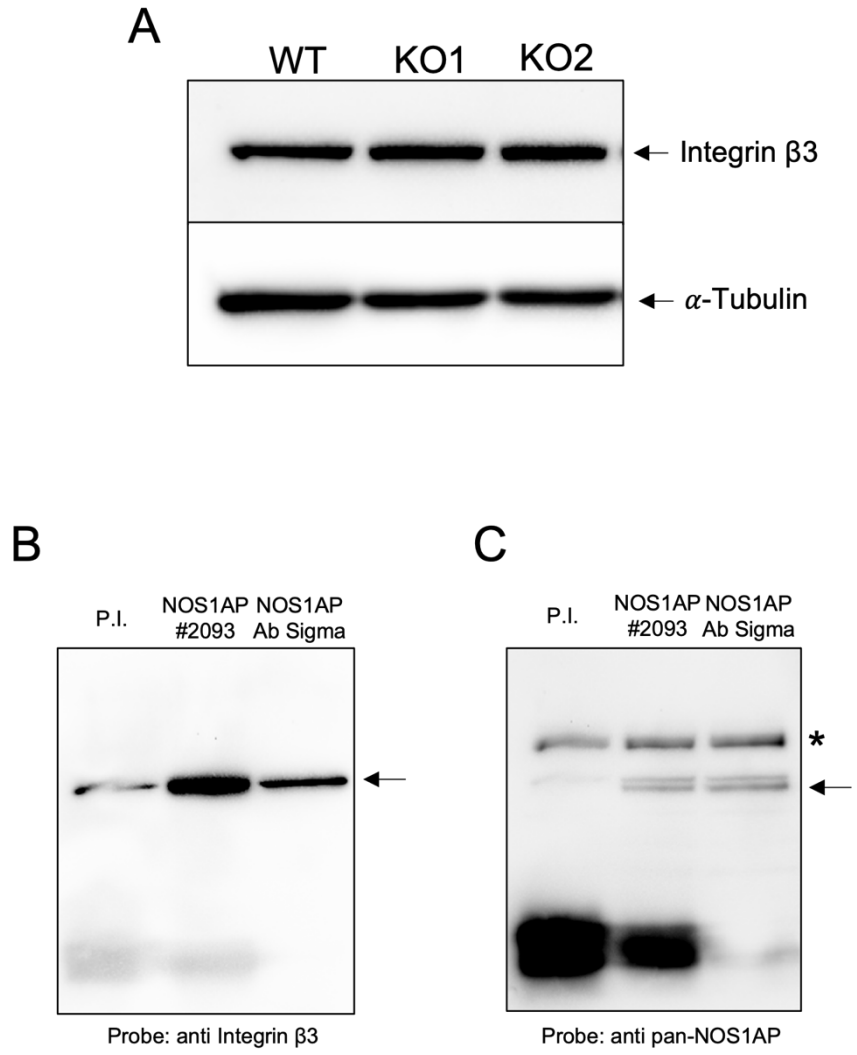

Figure 7. NOS1AP may associate with integrin β3. (**A**) Integrin β3 is expressed in both the control and NOS1AP-/- MEFs. (**B**) Control or NOS1AP-/- mutant cells were immunoprecipitated with NOS1AP antibodies or non-specific IgG as indicated and probed for integrin β3 (**B**) or a pan-NOS1AP antibody (**C**).

3.3 NOS1AP MUTANT MOUSE EMBRYONIC FIBROBLASTS EXHBIT NUCLEAR BLEBBING

Since we could not reliably show an association between NOS1AP and Integrin β3, we next set out to determine whether NOS1AP and integrin signaling are functionally linked. Here we grew control or the two NOS1AP mutant lines on coverslips that were either not coated or coated with fibronectin, an activator of integrin signaling (Ria et al., 2002). One of the more pronounced phenotypes we noted was that NOS1AP mutant lines showed increased nuclear blebbing when plated on fibronectin (Figure 8A-D). The NOS1AP mutant MEFs showing an increase of 37.9% and 46.5% over control, respectively (Figure 8E). Since integrin signaling has been linked to nuclear tension and blebbing in several cell lines (Mistriotis et al., 2019), these data suggest that NOS1AP plays a functional role in modulating integrin signaling. We confirm that NOS1AP KO MEFs do not exhibit significantly higher amounts of nuclear blebbing when plated on uncoated glass coverslips (Figure 8E). As such, we confirm that the nuclear blebbing phenotype observed (Figure 8F) is a result of integrin activation by plating on fibronectin.

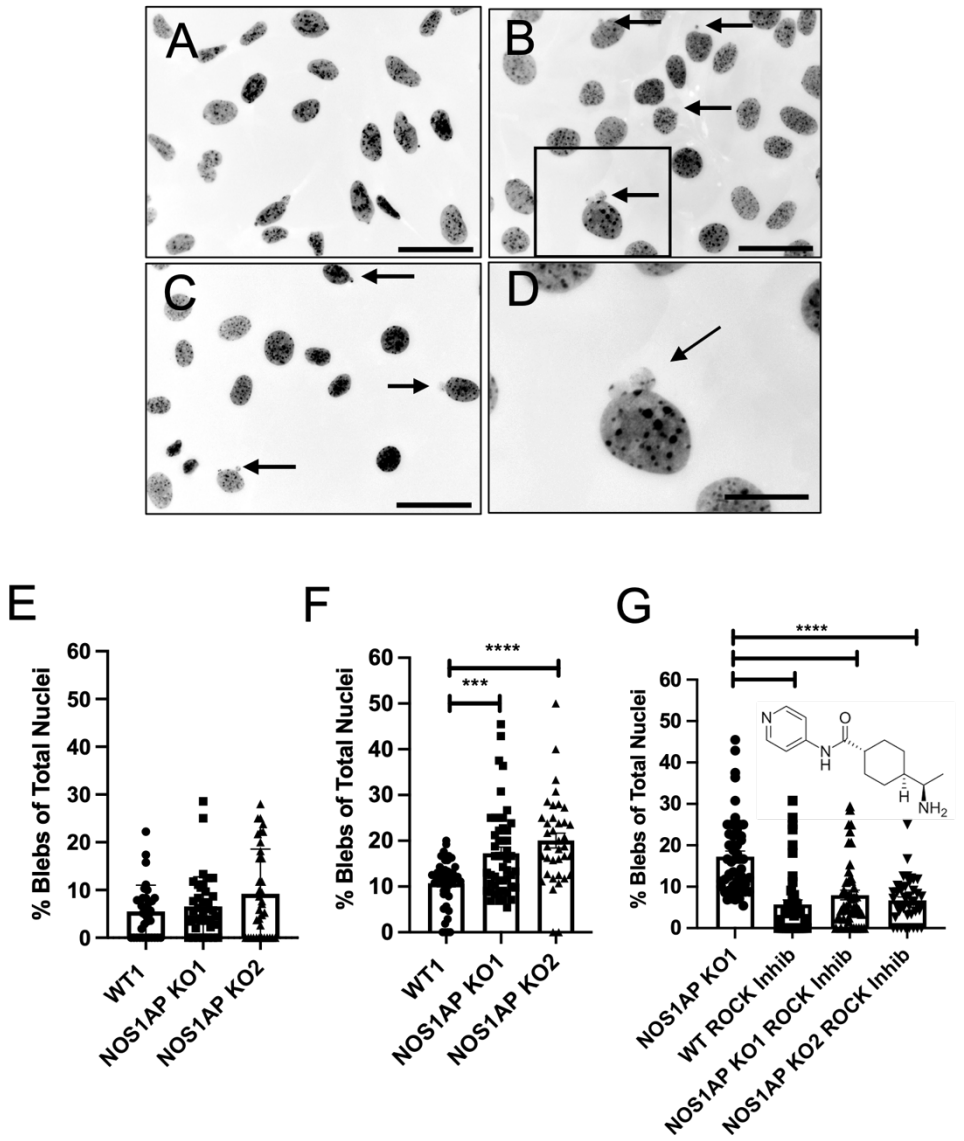

Figure 8. NOS1AP regulates nuclear membrane integrity. (**A-D**) Control (**A**) and NOS1AP$^{-/-}$ MEFs (**B** and **C**) were plated on fibronectin and nuclear blebbing was seen in both control and NOS1AP-/- cells. (**D**) One nuclei from (**B,** boxed area) showing blebbing phenotype (arrows). **(E)** Quantification of nuclear blebbing in cells plated on uncoated coverslips. (**F**) Quantification of nuclear Blebbing in cells from Control and NOS1AP$^{-/-}$ MEFs (**G**) Nuclear blebbing is rescued with the ROCK inhibitor Y27632 (structure shown). Data shown as mean ± SEM, $p<0.05$.

Nuclear blebbing has been shown to be dependent on Rho GTPase signaling (Mistriotis et al., 2019), and previous reports have demonstrated that NOS1AP influences Rho GTPase dependent signaling including Rac (Richier et al., 2010) and Cdc42 (Majmundar et al., 2021). Whether NOS1AP influences Rho activity remains unknown. Thus, to address whether the increase in nuclear blebbing in the absence of NOS1AP was dependent on Rho GTPase activity, we plated the control and NOS1AP mutant MEFs on fibronectin and then treated the cells with the Rho Kinase inhibitor Y27632. Consistent with previous studies, the Rho Kinase inhibitor reduced the blebbing in control cells, interestingly, in the NOS1AP mutant MEFs, the blebbing was reduced to control levels (Figure 8F). Taken together, these data implicate NOS1AP influencing Rho activity downstream of integrin activation.

Since membrane blebbing is a sign of cell death we next wanted to test whether the integrin stimulation was causing an increase rate of cell death in the MEFs lacking NOS1AP. To test this, we stained the control and NOS1AP mutant MEFs with propidium iodide (Figure 9A, left panel) and Polarity Sensitive Indicator of Viability & Apoptosis (pSIVA) (Figure 9A, middle panel), an Annexin XII based probe that detects phosphatidylserine externalization following apoptosis. As a positive control, we removed fetal bovine serum from the media of both control and NOS1AP mutant MEFs and noted that all cells showed increase in cell death (Figure 9B). Surprisingly, treatment of the control and NOS1AP mutant MEFs on fibronectin, in the same conditions that lead to an increase in nuclear blebbing showed no increase in cell death (Figure 9A). This suggests that the increase in integrin dependent nuclear blebbing did not lead to an increase in apoptosis in these cells.

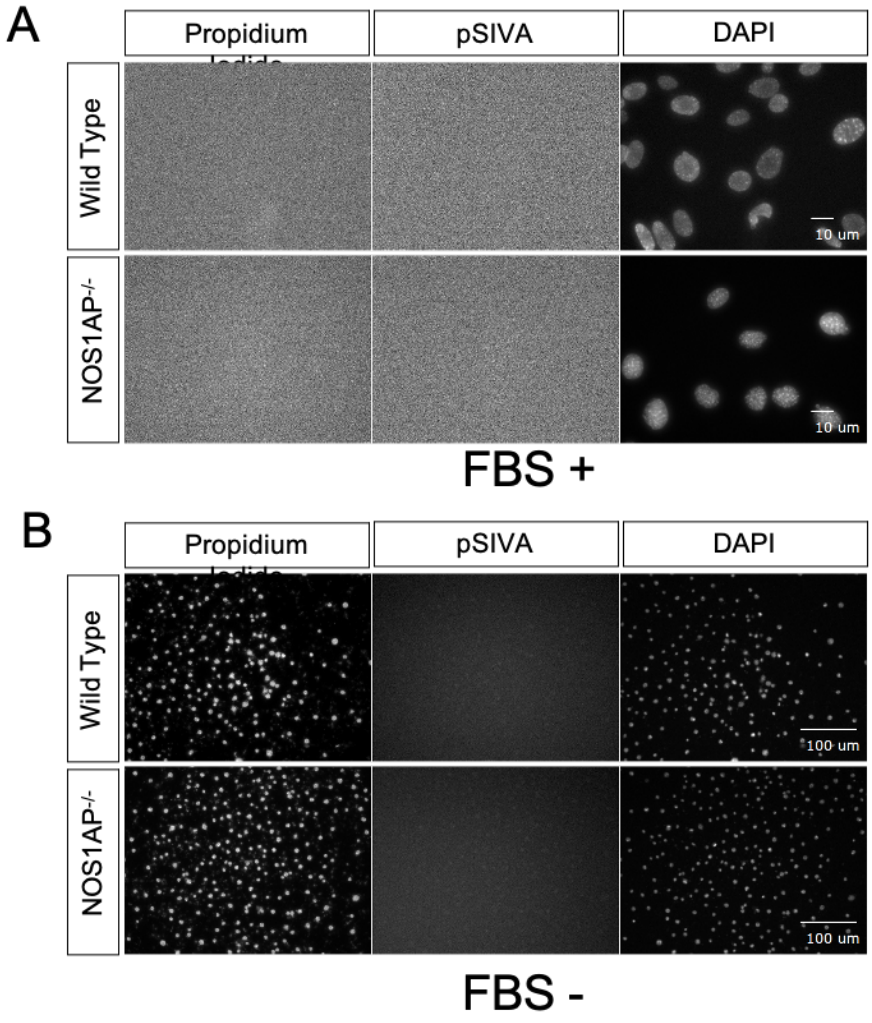

Figure 9. Loss of NOS1AP does not affect cell death in cells plated on fibronectin. Cells as indicated were plated on fibronectin and assessed for cell death/apoptosis in the presence (**A**) or absence (**B**) of FBS.

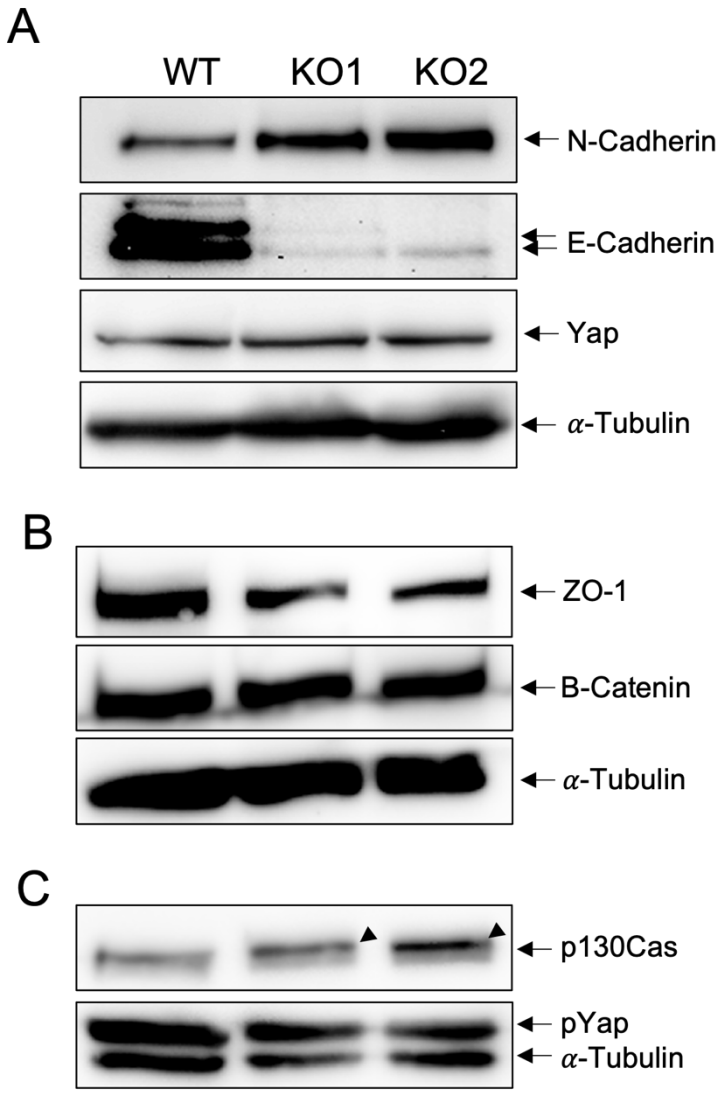

Figure 10. Loss of NOS1AP alters E-Cadherin and N-Cadherin expression. (**A**) Equal levels of lysate from Control and NOS1AP-/- MEFs were probed for expression of N-cadherin, E-cadherin and Yap as indicated. (**B**) Equal levels of lysate from control and NOS1AP-/- MEFs were probed for expression of ZO-1 and B-catenin as indicated. (**C**) Equal levels of lysate from Control and NOS1AP-/- MEFs were probed for expression of p130Cas and pYap as indicated. Note the slower migrating band in the p130Cas isoform in the NOS1AP-/- MEFs (arrowhead). Tubulin control **A-C** (lower panels) shows equal loading of protein in each condition.

3.4 LOSS OF NOS1AP EFFECTS CADHERIN EXPRESSION

Previously, Clattenburg et al. (2015), showed that NOS1AP was an important protein involved in Hippo signaling. Others have shown the NOS1AP binding protein Scribble functions in regulating Hippo signaling and functions as a tumor suppressor (Piccolo, 2014). As such we next wanted to determine if NOS1AP is important in EMT. A hallmark of EMT transition is that cells lose their polarity and begin to express proteins involved in mesenchymal transition, among which is an increase in the expression N-cadherin and loss of E cadherin (Wong et al., 2018). We first assessed whether there were any major morphological features of the MEFs. Consistent with EMT phenotype, the two NOS1AP$^{/-}$ cell lines showed cells with a more elongated cell shape compared to the control more cuboidal shape (Figure 11A vs 11B, left most panels). It will be important to stain these cells with phalloidin to better characterize and define this phenotype. Next, we probed membranes of clarified lysate from the control or mutant MEFs with antibodies against N-cadherin and E-cadherin (Figure 10A, upper two panels). We noted an apparent loss in expression of E-cadherin expression in the NOS1AP mutant line compared to the control line (Figure 10A) and an apparent increase in expression of N-cadherin in the NOS1AP mutant cells compared to the control cell line (Figure 10A), phenotypes consistent with the NOS1AP mutant cells undergoing an EMT transition (Loh et al., 2019).

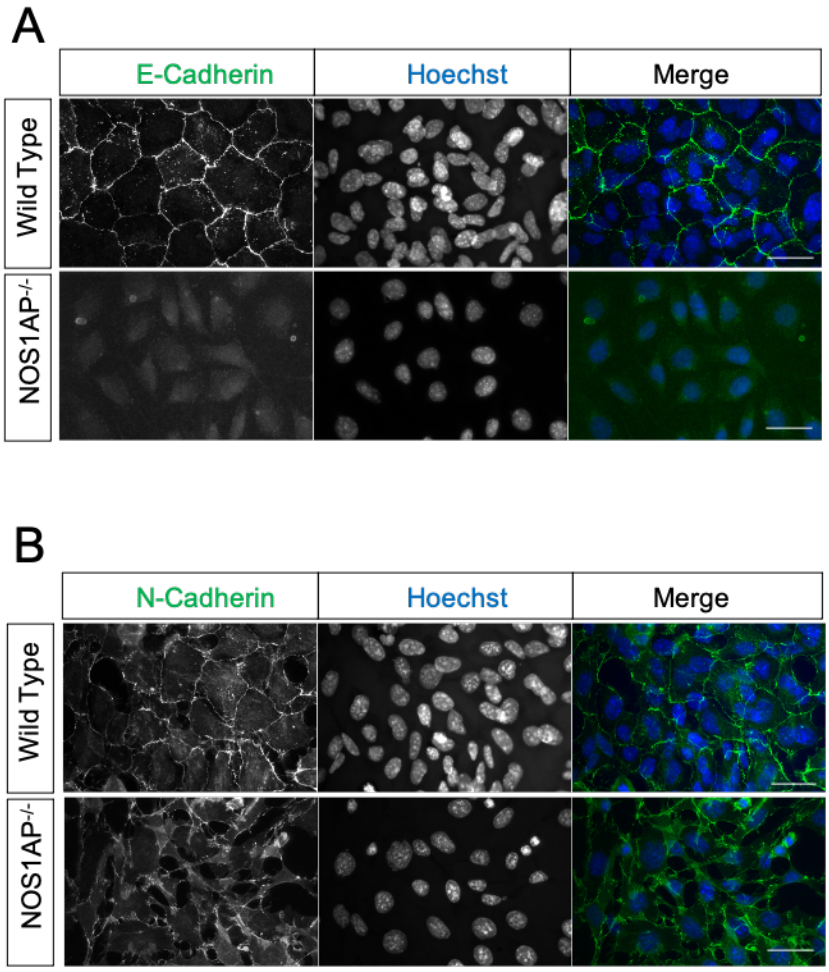

Figure 11. Loss of NOS1AP leads to the loss of E-cadherin expression. (**A**) Control and NOS1AP$^{-/-}$ MEFs, as indicated, were fixed with paraformaldehyde and stained with E-cadherin antibodies and Hoechst to label the nuclei. Note the loss of E-cadherin staining in the NOS1AP$^{-/-}$ mutant cells (lower panel). (**B**) Control and NOS1AP$^{-/-}$ MEFs, as indicated, were fixed with paraformaldehyde and stained with N-cadherin antibodies and Hoechst to label the nuclei.

Since NOS1AP is functionally connected to Hippo dependent signaling (Clattenburg et al., 2015), and Hippo signaling is linked with EMT transition, we also tested whether the levels of the downstream Hippo regulator Yap were affected. Qualitatively, we noted no changes in Yap (Figure 10A, third panel) or pYap (Figure 10C, second panel, upper band) levels were detected. In addition to loss of E-cadherin, the NOS1AP$^{-/-}$ MEFs had an apparent decrease in expression of the junctional protein ZO-1 (Figure 10B, upper panel) but showed no change in the levels of β-catenin (Figure 10B, middle panel). Finally, since we had shown that NOS1AP is important in integrin mediated signaling, and p130Cas is known to be important in the activation of focal adhesion complexes, we tested whether there were any changes in p130Cas levels. Although we detected no change in overall levels of p130Cas protein between control and NOS1AP$^{-/-}$ MEFs (Figure 10C, upper panel), we did note that in the NOS1AP-/- MEFs that p130Cas appeared to migrate slower than in the control cell line (Figure 10C, upper panel, arrowhead). Whether this shift is due to post translational modification such as phosphorylation remains to be tested. Nonetheless, together, these data are consistent with NOS1AP being an important protein in regulating the expression of N-cadherin and E-cadherin.

Since we had detected differences in the expression of N-cadherin and E-cadherin in the NOS1AP mutant MEFs, we next wanted to confirm these changes in expression. In confluent monolayers, we saw robust expression of E-cadherin in control cells (Figure 11A, upper panels), however, there was almost no expression of E-cadherin in the NOS1AP KO1 cell line (Figure 11A, lower panels). Note, only the NOS1AP KO1 mutant line was used in these studies, due to limited time as COVID restrictions prevented a full analysis of both

mutant cell lines. Next cells the control and the NOS1AP KO1 cell line were stained for N-cadherin (Figure 11B). Robust N-cadherin expression was found at cell-cell contacts in the control cells (Figure 11B, upper panels), while the N-cadherin expression in the mutant cell line was more diffuse, it was found at cell-cell contact (Figure 11B, lower panels). It is important to note in this cell line, that we did not see robust cell-cell contacts as these cells were more mesenchymal in shape and lacking cell-cell contacts as is found in mesenchymal cells. These data support the loss of E-cadherin found in Figure 10A, supporting the idea that NOS1AP plays an important role in maintaining cells in a differentiated state. Further work to define whether NOS1AP functions as a true tumor suppressor and if the loss of NOS1AP effects proliferation will be interesting considering these findings.

3.5 NOS1AP IS EXPRESSED IN RANKL DIFFERENTIATED RAW 264.7 CELLS

Since integrin β3 has been extensively linked to osteoclast function (Duong et al., 1998), we next asked whether NOS1AP might play a role in bone homeostasis. Here we utilized an *in vitro* model of osteoclast using RAW 264.7 cells differentiated with RANK-ligand. In collaboration with Dr. Chris Sinal's laboratory (Dalhousie University), we explored whether the differentiation of RAW 264.7 cells into osteoclasts affected the expression of NOS1AP. To confirm that these were osteoclasts, RAW264.7 cells were differentiated in RANKL (Figure 12B and D) or not (Figure 12A and C). Cells were then stained with Tartrate resistant acid phosphatase (TRAP) (Figure 12A and B), a staining procedure for marking osteoclasts or actin to identify podosomes (Figure 12C, and D). All RANKL differentiated cells were positive for TRAP confirming the differentiation protocol (Figure 12 A vs B). As well, the RANKL differentiated cells showed robust actin staining at cell-cell (Figure 12D), unlike the undifferentiated cells (Figure 12C). (Note: these cells were

cultured, differentiated, and stained by Nicole McMullen of Dr. Chris Sinal's Laboratory at Dalhousie University.)

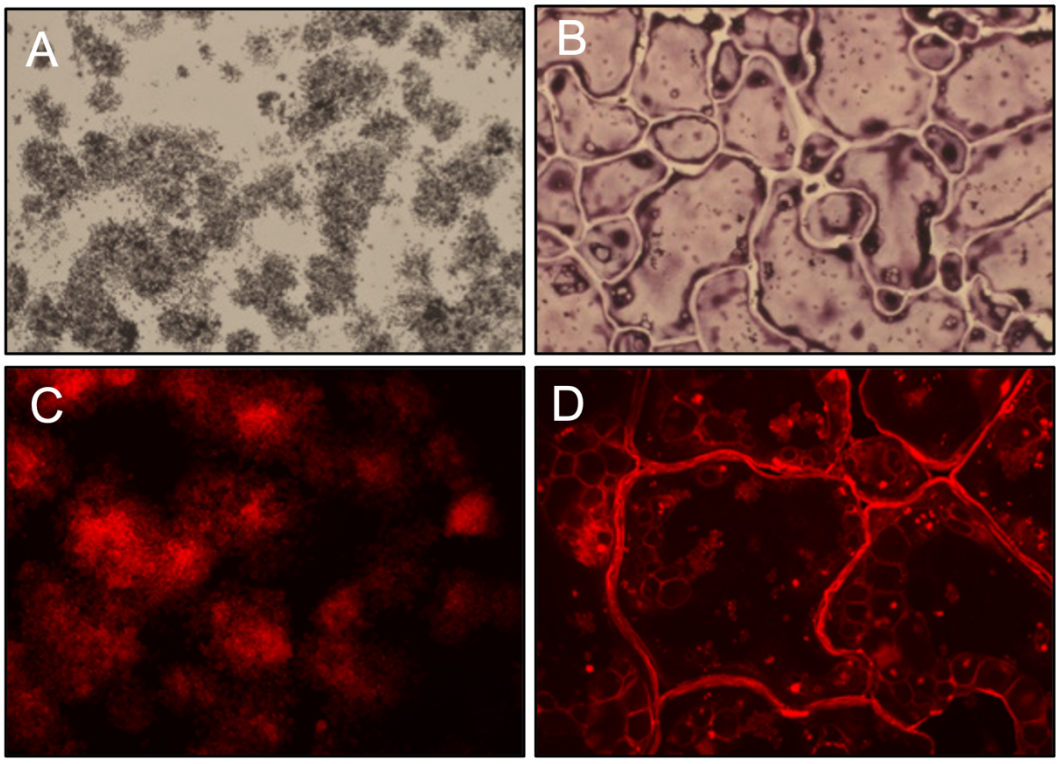

Figure 12. Characterization of RANKL Differentiated RAW 264.7 Cells . (**B**) RAW 264.7 cells were differentiated in RANKL (**B**), or not (**A**) and stained with TRAP to show the RAW cells have differentiated into an osteoclast lineage. (**C** and **D**) undifferentiated (**C**) and differentiated (**D**) RAW cells stained with phalloidin.

Next, we tested whether there were any changes in the expression of NOS1AP after differentiation of the RAW 264.7 cells with RANKL. Using a pan-NOS1AP antibody, we saw NOS1AP staining was primarily cytoplasmic in the multinucleated, differentiated RAW cells (Figure 13A and B, arrowhead points to a multinucleated cell). We also detected NOS1AP staining in the nuclei of the differentiated cells (Figure 13A and B, arrows). While there was NOS1AP staining in the undifferentiated cells, it was more diffuse in nature (Figure 13A and B, asterisks). This suggests that upon differentiation into an osteoclast linage, there is an increase expression of NOS1AP. It is important to note that the differentiation experiments were conducted with a pan-NOS1AP antibody. This antibody has been shown to detect several NOS1AP isoforms, including NOS1APa and NOS1APc (Richier et al., 2010 and Clattenburg et al., 2015). To better distinguish whether either or both NOS1AP isoforms are present in the differentiated osteoclast model, we stained the differentiated RAW 264.7 cells with a NOS1APc specific Antibody previously characterized by Clattenburg et al. (2015). Interestingly, we noted in some of the undifferentiated cells a small number of cells that were differentiated with RANKL had a more intense staining of NOS1APc in a subset of cells that had lost actin staining at cell-cell contacts, presumably indicating that these had recently differentiated and begun to fuse (Figure 154, arrows). We then focused on fully differentiated multinuclear cells and found a more intense staining of NOS1APc in the nuclei of these cells compared to undifferentiated neighbouring cells (Figure 14A-D, arrows). Confocal images of these cells showed that indeed NOS1APc was enriched in the nucleus (Figure 14E-G). The functional significance of this remains to be determined. Together this suggest that there may be a differential localization of NOS1AP isoforms in differentiated RAW cells, with

the NOS1APa isoform being more prevalent in the cytoplasm while NOS1APc seems to enrich in the nucleus of RANKL differentiated RAW cells. The functional significance of this remains to be determined. Given that we saw NOS1AP staining in the cytoplasm of differentiated RAW cells and that one of the hallmarks of osteoclasts is their ability to form tight associations with the bone matrix, a step that is necessary for them to properly resorb the bone, we next wanted to determine if NOS1AP was found in these regions. The tight connection between the osteoclast and the bone matrix is mediated by regions known as podosomes, that are actin enriched structures that are dependent on integrin β3 signaling (Cao et al., 2020). Consistent with this, in our differentiated RAW 264.7 cells we observed the development of podosomes, enriched with both actin and integrin (Figure 15 A-G).

In addition, we saw that the localization of endogenous NOS1AP in the podosomes (Figure 15A and B, arrows); however, there was no apparent colocalization of NOS1AP with the integrin staining (Figure 15D vs E and F). Whether NOS1AP is important for the formation of these podosomes remains to be tested. One could test this by knocking down NOS1AP in these cells and assessing if they form intact podosomes. It is important to note that in the kidney NOS1AP has been shown to be important for the formation of podocytes (Majmundar et al., 2021). Mice lacking or humans with mutations in the PTB domain have defects in glomerular formation and show proteinuria (Majmundar et al., 2021). Nonetheless, these data suggest that NOS1AP is present within the podosome structure; however there appears to be no co-localization with integrin β3 or actin in these structures.

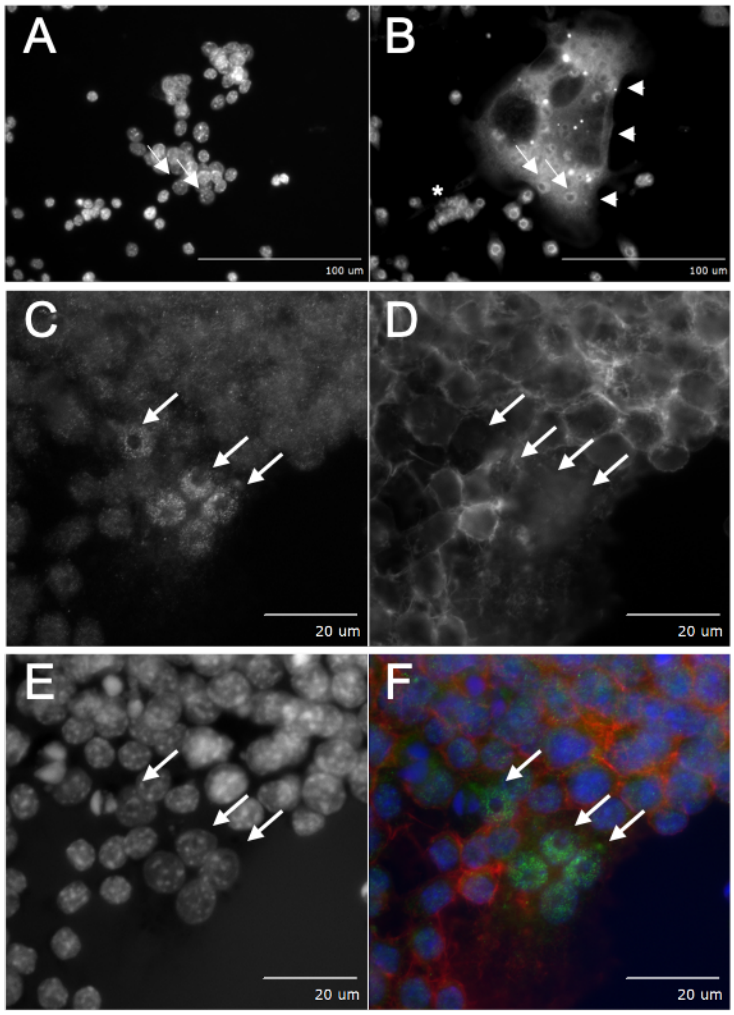

Figure 13. NOS1AP expression is increased in RAW cells after differentiating with RANKL. Increased expression of NOS1AP in RAW cells differentiated with RANKL **(A and B).** RAW cells stained with a pan-NOS1AP antibody (**B**) and Hoechst (**A**) to show multinucleated cell, outlined in arrowhead (**B**). Asterisk denotes mononuclear, undifferentiated RAW cells (**B**). Arrows indicate NOS1AP staining in nuclei of differentiated RAW cells (**B**). (**C-F**) RAW cells undergoing differentiation with RANKL show increased colocalization of NOS1AP (**C**) with Hoechst (**E,** arrows). Note the loss of actin at cell cell contact in the differentiating cells (**D**, arrows). (**F**) Represent panels **(C-D**) merged – NOS1AP (Green), Actin (Red) and Hoechst (Blue).

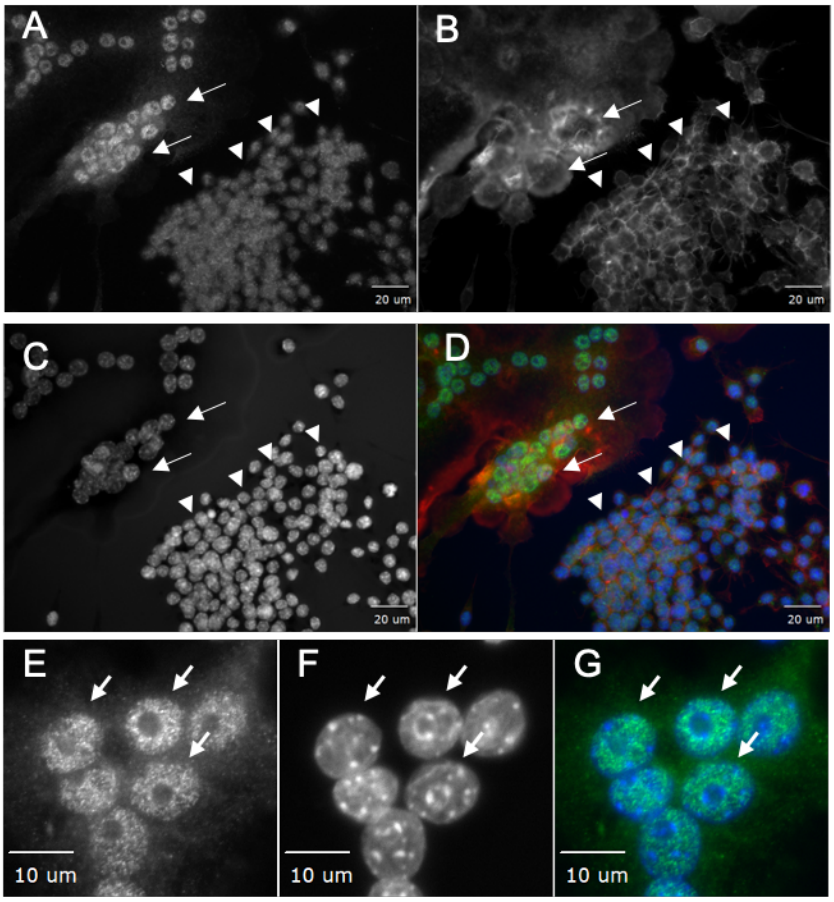

Figure 14. Increased expression of NOS1APc in the nuclei of RAW cells differentiated with RANKL. (**A-D**) RAW cells stained with a NOS1APc antibody (**A**), and actin (**B**) and Hoechst (**C**). Note the increased co-localization of NOS1APc to the nuclei of the differentiated RAW cells (**A-C**, arrows). (**D**) Panels A-C merged. (**E-G**) Confocal image of NOS1APc (**E**) and Hoechst (**F**) and merge (**G**) revealing colocalization of NOS1APc to the nuclei in differentiated RAW cells, arrows **E-G**.

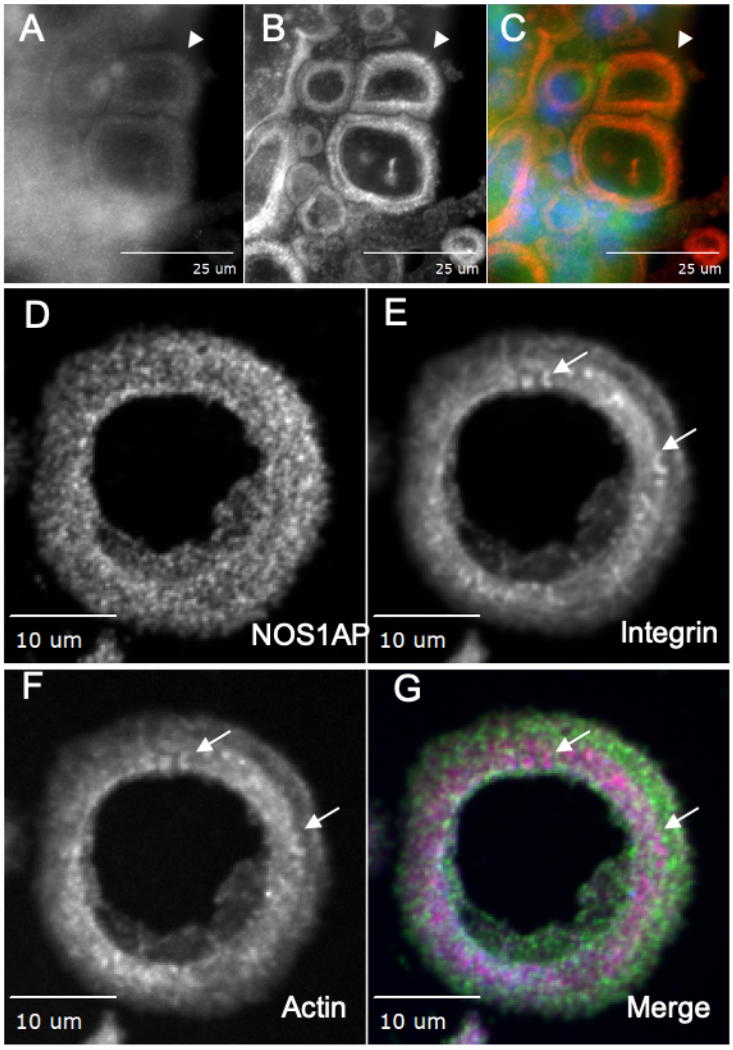

Figure 15. NOS1AP is found in podosomes of RAW cells differentiated with RANKL. (**A-C**) Differentiated RAW cells stained with a pan-NOS1AP antibody (**A**) and actin (**B**). Note the localization of NOS1AP in actin rich podosomes (**A-C**, arrowhead). (**D-G**) Confocal image of isolated podosome showing NOS1AP (**D**), integrin β3 (E and actin (F). Note NOS1AP puncta in a podosome; however, it does not colocalize with integrin β3 and actin (**D-F**, arrows).

CHAPTER 4 DISCUSSION

4.1 SUMMARY OF FINDINGS

The aim of the current study was to characterize the role of NOS1AP in cellular mechanotransduction. Previous findings have shown that NOS1AP is linked to mechanotransduction since it can associate with p130cas, a member of the FAC. Furthermore, NOS1AP has been linked to cytoskeleton dynamics since it has been shown that NOS1AP influences the activation of Rac1. Since these findings implicate NOS1AP in the process of mechanotransduction, I aimed to investigate the role of NOS1AP in mechanotransduction. First, in a bioinformatic screen I determined that NOS1AP may bind to integrin β3 and integrin β6 through an interaction between the PTB domain of NOS1AP and the NPxY motifs of integrin β3 and β6. Since integrin β3 has been extensively documented to be a crucial signaler in the process of mechanotransduction, I aimed to determine if NOS1AP can directly associate with integrin β3 through immunoprecipitation in MEFs. Unfortunately, no association was detected. Following this, I examined if NOS1AP has a functional role in regulating mechanotransduction signalling by plating MEFs on fibronectin and found that MEFs lacking NOS1AP had significantly higher amounts of nuclear blebbing. Next, I aimed to test if this nuclear blebbing in the absence of NOS1AP was mediated by ROCK, a kinase central to actomyosin contractility. I found that in the presence of Y-27632, a potent ROCK inhibitor, nuclear blebbing diminished significantly. Further examination of our NOS1AP$^{-/-}$ MEFs showed that cadherin expression was altered in the absence of NOS1AP. In short, through western blotting techniques I determined that our NOS1AP$^{-/-}$ MEFs had almost no E-Cadherin expression,

and dramatically higher amounts of N-Cadherin. This was confirmed through immunocytochemistry where I observed that the NOS1AP$^{-/-}$ MEFs had virtually no ECadherin staining, while the N-Cadherin staining was rich around that cell membrane. Following our findings in MEFs, I next aimed to test if NOS1AP has a role in integrin mediated cell processes. It is known that integrin β3 has an important role in osteoclast dynamics, cells known to degrade the bone matrix and influence bone mineralization. In short, we have confirmed that NOS1AP is expressed in RAW 264.7 cells, a cell line model for osteoclasts. Furthermore, we show that using a pan-NOS1AP antibody we can detect NOS1AP at podosomes, as well as in the cytoplasm. Using a NOS1APc specific antibody we show that NOS1APc can localize to the nuclei of RANKL differentiated RAW 264.7 cells.

4.2 BIOINFORMATIC SCREEN SHOWS NOS1AP MAY ASSOCIATE WITH INTEGRIN β3 THROUGH IT'S PTB DOMAIN

PTB domains are protein signaling domains that can associate with NPxY motifs in target protein receptors (Smith et al., 2006). These associations can occur if the tyrosine residue in the NPxY motif is either phosphorylated or nonphosphorylated. We find that NOS1AP's PTB domain preferentially binds to phosphotyrosine containing motifs with the sequence of "Φ - ζ Φ Φ N P Φ Y - - -," and binds to nonphosphorylated tyrosine residues in NPxY motifs with the sequence of "– – Φ Φ ζ N P – Y– ζ –," where Φ represents a hydrophobic residue; ζ, hydrophilic; -, non-specific. Interestingly, between both consensus motifs, NOS1AP's PTB domain appears to have an affinity for motifs containing hydrophobic and hydrophilic residues, as in both cases those are the conserved residues in the consensus motif. The consensus motif for phosphotyrosine binding contains four hydrophobic

residues and one hydrophilic, while the nonphosphorylated binding motif contains two hydrophobic and two hydrophilic residues. This may help our understanding of the nature of interactions between NOS1AP's PTB and target motifs as it guides our understanding of the type of motifs that NOS1AP has a high affinity for. Based on the consensus motifs, we can deduce which proteins match the amino acid identity at each position to predict a potential interaction. Based on the phosphotyrosine dependent binding consensus motif we find that sequences in integrins β3 and β6 match the consensus motifs. This is interesting as previous findings have shown that NOS1AP may play a role in integrin-based signalling. Previously a student in the Fawcett lab has shown that NOS1APc can associate with p130cas (data not shown), demonstrating that NOS1AP can functionally associate with a member of integrin signalling. Furthermore, Richier et al. (2011) show that NOS1AP's PTB domain has a direct effect on the activity of Rac. We know that modulation of Rho GTPase activity could play an important role downstream of integrin signalling since Rho GTPases have an important role in regulating actin contractility. As such, based on the results from the bioinformatic screening combined with what we know from previous studies we aimed to determine the role of NOS1AP in integrin signalling.

To determine if NOS1AP is important in integrin β3 signalling it is first important to verify if NOS1AP interact integrin β3 using co-immunoprecipitation methods. We were unable to definitively conclude that NOS1AP associates with integrin β3 since our pre-immune control showed a band when probed with anti-integrin β3 (Fig. 7B). A positive signal from the pre-immune sample indicates that a non-specific interaction that brings down integrin

β3 was occurring during the immunoprecipitation process. When reprobing for NOS1AP we see that the IP did work (Fig. 7C). Although we could not verify the interaction between NOS1AP and integrin β3 biochemically, we turned to cell culture methods to determine if NOS1AP is important in integrin signalling *in vitro*. Integrin β3 as it is known to be expressed in MEFs and have multiple crucial roles within cells (Schmidt et al., 2013). While integrin β6 has been less characterized than integrin β3, future experiments to determine co-immunoprecipitation with NOS1AP would be valuable as it is known to be involved in cancer cell metastasis (Eberlein et al., 2013). This is of particular interest as integrin β6 also binds to fibronectin via the RGD domains, like integrin β3 (Eberlein et al., 2013).

4.3 CHARACTERIZAITON OF MECHANOTRANSDUCTION IN MEFs LACKING NOS1AP

4.3.1 MEFs LACKING NOS1AP EXHIBIT HIGHER AMOUNTS OF NUCLEAR BLEBBING

To test if NOS1AP is important in integrin signalling, we first had to develop two cell lines of MEFs lacking NOS1AP and one control WT line. To activate integrin signalling we plated cells on fibronectin, which binds to integrins via it's Arginine-Glycine-Aspartic Acid (RGD) motifs. This assay lacks the specificity to directly determine if the signalling that occurs is strictly a result of integrin β3, since fibronectin can associate with integrins β1, β3 and β6 through their RGD domains (Johansson et al., 1997). Nonetheless, plating on fibronectin helped deduce that NOS1AP functionally plays a role in integrin dependent signaling. Indeed, we found that two independent lines of mutant MEFs lacking NOS1AP exhibited significantly higher amounts of nuclear blebbing when compared to WT control.

The NOS1AP mutant MEFs showing an increase of 37.9% and 46.5% over control, respectively (Figure 8E). This shows that NOS1AP has a potential role in regulating integrin signalling, however based solely on this finding it is unclear where in the signalling axis this occurs. Since increased nuclear blebbing has been linked ton increase mechanotransduction, these results suggest that NOS1AP is important to restrict or control mechanotransduction in normal cells. Following this, it will be interesting to determine if cells lacking NOS1AP, or animals, show any defects in genomic stability, as nuclear blebbing has been linked to genomic instability and development of susceptibility to cancers (Shah et al., 2017).

4.3.2 NUCLEAR BLEBBING IN ABSENCE OF NOS1AP IS ROCK MEDIATED

Mistriotis et al. (2019) have shown that nuclear blebbing is mediated by Rho associate protein Kinase - ROCK, a kinase that is downstream of Rho. ROCK phosphorylates and activates downstream targets including LIM kinase, which in turn phosphorylates cofilin in activating actin depolymerization. It also has a function on regulating myosin light chain kinase leading to increased stress fiber assembly and contraction. Thus, this kinase is important in regulating actin contractility (Yang et al., 2017). As such, we asked if the nuclear blebbing phenotype in the absence of NOS1AP was dependent on ROCK signalling. To test this, we added either DMSO or Y27632 (100 μm), a potent ROCK inhibitor, to the media after 16 hours after plating. Consistent with the findings of Mistriotis et al. (2019) we show that inhibiting ROCK signalling dramatically decreased the amount of nuclear blebbing observed across all three cell lines (Figure 8F). Based on these findings, we show that NOS1AP's role in integrin signalling is Rho dependent, since inhibiting its

downstream effectors decreased the amount of nuclear blebbing from 17.3% and 20.1%, for KO1 and KO2 respectively, to 8% and 6.7% (Figure 8F). This suggests a novel finding that NOS1AP is involved in regulating Rho activity as in its absence, Rho activity is increased. This finding now suggests a complex role for NOS1AP in RhoGTPase signaling as it has been linked to Rac (Richier et al., 2010), Cdc42 (Majmundar et al., 2021) and now Rho. Whether this is a function of the cell or tissue NOS1AP is expressed in, or a function of the different NOS1AP isoforms remains to be determined. Nonetheless, future studies carefully characterizing how NOS1AP contributes to RhoGTPase function will be an important area of research.

4.3.3 NUCLEAR BLEBBING DOES NOT LEAD TO APOPTOSIS

Since nuclear blebbing often leads to genomic instability and DNA damage, we next aimed to test if the MEFs were dying because of the increase in integrin stimulation on fibronectin. Our results show that both control and NOS1AP MEFs do not exhibit any appreciable levels of apoptosis (Figure 9A). This was confirmed using both propidium iodide and pSIVA, two forms of detecting cell death based on different markers. To confirm that our assay works, we plated cells on fibronectin and incubated them in the absence of FBS for 24h, a positive control that will lead to cell death. In the absence of FBS, both mutant and control cells showed nearly 100% apoptosis as marked by propidium iodide (Figure 9B). Interestingly, the pSIVA probe did not detect any cells, indicating that the form of cell death that occurs in the absence of FBS does not exhibit phosphatidylserine externalization following apoptosis. Despite there being an increase in cell death in our experimental conditions, nuclear membrane blebbing has been linked to genomic instability, as the

nuclear membrane complex is linked to chromosomal structure. Thus, it will be important to determine if loss of NOS1AP contributes in any way to genomic instability or defects in chromosome integrity.

4.3.3 LOSS OF NOS1AP INFLEUNCES CADHERIN EXPRESSION

Aberrant integrin signaling is known to play a crucial role in the progression of various cancers, such as breast, prostate and pancreatic (Pan et al., 2018). In its heterodimeric form, integrin αvβ3 is known to influence the growth, survival, invasiveness, and migratory potential of cancer cells (Pan et al., 2018). Interestingly, Monier-Gavelle & Duband (1997) have shown that integrin β1 and β3 influence the distribution of N-Cadherin. This is of particular interest because cadherin expression is known to be a hallmark of EMT, a process through which cancerous cells lose their cell-cell contacts and become migratory and invasive (Thiery et al., 2009). During EMT, cells lose the E-cadherin found at adherens junctions and begin to express N-cadherin (Loh et al., 2019). Cells undergoing EMT also show decreases in ZO-1 expression (Nagai et al., 2016). We then asked if the absence of NOS1AP would affect cadherin expression and distribution in our MEFs. We observed that levels of E-cadherin were virtually undetectable our NOS1AP KO MEFs. This finding was verified through western blotting and immunocytochemistry (Figure 10A, Figure 11A). Conversely, N-cadherin levels appear to be higher in the NOS1AP mutants (Figure 10A). Immunocytochemistry analysis of the mutant MEFs also shows that they exhibit a spindly phenotype and have minimal cell-cell contacts (Figure 11B). Additionally, we show that ZO-1 levels appear to be decreased across both NOS1AP KO lines (Figure 10B). Based on the changes in E- and N-cadherin, as well as the decrease in ZO-1 expression, our findings

suggest that NOS1AP could potentially play a role in regulating cell progression into EMT. This is an interesting finding considering what is known about scribble, a tumor suppressor protein that has been shown to associate with NOS1AP. Scribble functions to maintain the apico-basal epithelial cell polarity (Bilder, 2004). Based on our finding it is unclear if the loss of epithelial polarity in the NOS1AP mutants is dependent on altered signaling through scribble. These findings support previous studies that showed a role for NOS1AP in breast cancer (NOS1AP and Vangl1 paper). Further work in describing the activity of scribble in the absence of NOS1AP will help elucidate its role in the EMT changes observed in our mutant MEFs.

4.3.4 LOSS OF NOS1AP ALTERS P130CAS ACTIVITY

Previously members of the Fawcett lab have shown that NOS1APc is able to co-precipitate p130Cas (data not published). It is well characterized that p130Cas is an important protein downstream of integrins in mechanotransduction (Boppart & Mahmassani, 2019). Based on our findings that NOS1AP plays a role in integrin β3 signaling I aimed to investigate expression levels of p130Cas through western blotting to determine if the loss of NOS1AP in the mutant MEFs impacts p130Cas levels. Our data shows that there is a slight increase in p130Cas expression in the KO MEFs (Fig. 10C). Although the amount of p130Cas is not dramatically different among the different cell lines, it appears that it migrates slower in SDS-PAGE gel (Figure 10C). This shift is observed in both KO lines and is evidence of an increase in phosphorylation. An increase in phosphorylation entails that in the absence of NOS1AP p130Cas is more constitutively active than in the WT MEFs. This is interesting as it is known that p130Cas is phosphorylated downstream of integrin activation (Boppart

& Mahmassani, 2019) and important for linking focal adhesions with the actin cytoskeleton network (Braniš et al., 2017). It will be interesting to determine whether this shift is related to phosphorylation or some other post translational modification. Further investigation of the size and stability of the focal adhesions in these cells will be important and whether this dynamic regulation converts to a migratory phenotype will be an important area to explore.

4.4 NOS1AP IS EXPRESSED IN RANKL DIFFERENTIATED RAW 264.7 CELLS

4.4.1 NOS1AP EXPRESSION IS ALTERED AFTER DIFFERENTIATION WITH RANKL

Given the importance of integrin signalling in osteoclast signalling, we next aimed to determine if NOS1AP is expressed in osteoclasts. To achieve this, we used RAW 264.7 cells differentiated with RANKL (Figure 12 A-D). Our analysis shows that NOS1AP expression is altered after differentiation (Figure 13B). Before differentiation with RANKL NOS1AP appears to localize around the nuclei of undifferentiated cells. Post differentiation NOS1AP expression is altered, and enriched within the cytoplasm of the large, multinucleated osteoclast-like cells (Figure 13B). Furthermore, analysis using a NOS1APc specific antibody shows that NOS1APc localizes to the nuclei of differentiating cells. We observed that cells that are losing their individual cell membranes and are in the process of differentiating into osteoclast-like cells express NOS1APc in their nuclei (Fig. 13 C-F). Upon further analysis of a larger group of cells we see that in fully differentiated cells NOS1APc is greatly enriched in the nuclei of RANKL differentiated RAW 264.7 cells (Fig. 14 A). Furthermore, we asked if NOS1APc was localized within the nuclei or on the surface. Confocal microscopy showed that NOS1APc localizes to the surface of nuclei, indicating that it plays a potential role in the dynamic nuclei of osteoclasts (Figure 14E).

The functional significance of the nuclear localization remains to be determined. Others have noted that NOS1AP can localize to the nucleus, in different cell types (Michael O'Brien MSc Thesis, Dalhousie University) and overexpression of the PTB domain alone reveals a nuclear localization (James Fawcett, personal communication). How the PTB domain is directed to the nucleus and whether it functions to regulate transcription remains to be determined. It is important to note that the NOS1AP associating protein p130Cas, has also been localized in the nucleus. In pro-apoptotic signaling, p130Cas has been shown to be cleaved by caspase-3, creating a small C-terminal fragment that can enter the nucleus and contribute to cell death pathways. Whether NOS1APc functions in a similar manner remains to be determined. As well, it will be interesting to determine if NOS1APc enters the nucleus during a specific point in the cell cycle, or in differentiated post mitotic cells.

4.4.2 NOS1AP LOCALIZES TO PODOSOMES

Since we have shown that NOS1AP is expressed in RANKL differentiated RAW264.7 cells, we then asked if they are found at podosomes. In differentiated kidney cell line, NOS1AP overexpression has been shown to be found in podocytes (Majmundar et al., 2021), suggesting that NOS1AP can be localized in podosome structures. Briefly, podosomes are actin rich structures that are rich in integrin expression (Cao et al., 2020). Integrin β3 is known to be expressed at the podosomes in osteoclasts, where it links the osteoclast to the bone matrix (Cao et al., 2020). As such, we predicted that NOS1AP would be expressed at the podosomes. We show that is richly expressed at podosomes (Figure 15D). To confirm that our RAW264.7 cells did have properly assembled podosomes we first show that actin and integrin were expressed at the podosomes in our differentiated

RAW264.7 cells (Figure 15E-F). Here we show that actin and integrin β3 colocalize. However, our findings show that endogenous NOS1AP does not colocalize with actin and integrin β3 (Figure D-G). While they might not colocalize, it may be that NOS1AP is a downstream effector of integrin signalling. This is likely the case since we know that NOS1AP binds to p130cas, a downstream protein in integrin signalling. Additionally, we show that integrin signalling in the absence of NOS1AP is mediated by ROCK, which is also a downstream effector in integrin signalling. As such, the role of NOS1AP in podosome function requires additional experimentation to determine its role and spatiotemporal localization. Further, work to define whether endogenous NOS1AP is localized to podosomes, and which isoform will be interesting to determine. Finally, it will be important to determine in the NOS1AP mutant mice, whether the nature of the bone defect, if any. Characterizing whether these animals show an osteoporotic or osteopetrosis phenotype will be important, and whether this is a function of osteoclast dysfunction remains to be determined.

CHAPTER 5 CONCLUSION

In all, we characterize a novel role for NOS1AP in integrin signalling. By generating NOS1AP mutant MEFs we were able to test our hypothesis of the involvement of NOS1AP in integrin signalling, however we were unable to verify that NOS1AP does directly associate with integrin β3. We show that MEFs lacking NOS1AP have unusually high amounts of nuclear blebbing when plated on fibronectin, and that this phenomenon is rescued with the application of a ROCK inhibitor. Following these findings, we aimed to further characterize the NOS1AP mutant MEFs. Using western blotting techniques, we show that the absence of NOS1AP dramatically alters cadherin expression. These findings suggest that the absence of NOS1AP is sufficient to lead to an EMT-like cell fate. Furthermore, we show that NOS1AP expression is altered in RAW264.7 cells after differentiating with RANK ligand (RANKL). Briefly, we show that NOS1AP is expressed in RANKL differentiated RAW264.7 cells, NOS1APc localizes to nuclei, and that NOS1AP is expressed at podosomes. Further characterizing the role of NOS1AP downstream of integrin signalling. Future studies should focus on the link between NOS1AP and p130Cas to build on our findings. Second, determining the basis for the altered cadherin expression in the absence of NOS1AP would help expand the role of NOS1AP in tumor suppression, much like its interacting protein scribble. Furthermore, further analysis on the role of NOS1AP in osteoclasts will help elucidate its importance in osteoclast biology and bone development.

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