THE REGULATION OF NOTCH SIGNALING BY SRC KINASE AND POLYPHENOLIC COMPOUNDS

by

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DEDICATION

Dedicated to Jean, David, Charlie, and Cricket.

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ABSTRACT

Cellular signaling pathways provide cells with the means to sense their environment and communicate with other cells. The Notch signaling pathway is comprised of a set of protein machines which work in unison to coordinate cellular processes in response to stimuli coming from neighboring cells and changing microenvironmental conditions. Notch signaling is an important mode of cellular communication which is crucial to many processes involved in development and disease. During Notch activation, information about the extracellular environment is fed into the cell and relayed to the nucleus through a number of biochemical processes. The information-rich messages carried by Notch signaling is used to make genetic decisions through alteration of gene expression which ultimately controls cellular physiology. Critical to Notch function, are a series of regulatory steps which serve as points of integration where other sources of information are fed into the Notch pathway. In this dissertation, I describe five years of work, where I sought to discover new ways in which Notch signaling is regulated. Through this work, I have come to regard Notch signaling as a highly tunable mode of cellular signal transduction, which harmonizes extracellular cues in order to orchestrate cellular behavior. Here, I described a series of experiments, performed by myself and my collaborators, which have served to uncover novel regulatory mechanisms by which Notch signaling is controlled. Through bettering our understanding of this critical mode of cellular communication, we prime science with the knowledge which may one day fuel the development of new therapeutic strategies to combat Notch-related diseases.

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CHAPTER ONE

Introduction

Why We Study Cell Signaling Pathways

Within the body there are many levels of organization. Multicellular creatures are made up of organs, which are comprised of various tissues that are composed of cells and extracellular matrices. Organ function and organization requires tight coordination between cells within the organ's tissues. Critical to the success of the organism is the ability of cells to communicate, or signal, to one another. Cells within the body are highly specialized, meaning they have specific jobs which they need to perform. The ability of a cell to perform a given function depends on its capacity to perceive and react to an assortment of stimuli. Cells are stimulated in a variety of ways, and are highly tuned in order to facilitate an appropriate response to a specific set of stimuli amongst a cacophony of signals. Biochemical signals move through the body, from one tissue to the next, in order to regulate bodily functions. These signals are transmitted through cells by molecular machines, which are coordinated into cell signaling pathways. During signal transduction, an initial signal can be targeted to a particular cell type, which then converts the information into a different type of signal, which can be targeted to a different cell type. Cell signaling pathways allow cells to interpret a variety of signals in order to mount an appropriate response.

When components of cell signaling pathways act abnormally it causes aberrant signal transduction, and in turn disturbs tissue function. Improperly functioning proteins can cause the dissemination of inappropriate signals. Even the amplitude of a signal being too strong or too weak can interrupt tissue activity. This disruption to normal physiological activity is at the root of many diseases. Additionally, because signaling pathways are natural cellular control circuits, signaling proteins serve as druggable targets for therapeutic agents which fight disease. Considering that the human body is made up of approximately 37 trillion cells [1], all communicating and working together, it is imperative that science understands the principles behind cellular communication. In doing so, we equip ourselves with the knowledge and tools that aid medical advances which are used to treat and cure disease.

Notch Signaling

Multicellular creatures have evolved a multitude of diverse cell signaling pathways which coordinate tissue function. One such pathway, Notch signaling, is a highly regulated signal transduction system present in all animals. Being an ancient pathway, Notch signaling components first evolved in single-celled eukaryotes before the rise of multicellularity and are present in the closest living metazoan relatives [2]. Notch has played an important role in metazoan evolution as evidenced by the fact that proteins of the Notch pathway are highly conserved among animal species [3,4]. The Notch cascade is responsible for governing cell fate decisions, proliferation, and apoptosis and thus plays a vital role in development and disease [4]. Paramount to Notch's ability to regulate the aforementioned cellular processes is the dynamic regulatory nature of the Notch interactome.

One commonality amongst various cell signaling pathways is that they are comprised of a myriad of molecular machinery which responds to specific stimuli in order to elicit a cellular response. Generally speaking, signaling pathways employ a receptor protein whose job it is to react to an information containing stimuli, often times a ligand. This stimuli evokes a response from the receptor which passes biochemical information along to other proteins within its signaling network. Notch signaling is a prototypical pathway, in that it responds in exactly this manner to stimuli. Here, the Notch receptor responds to a touch-induced signal sent from a signal sending cell, which uses a transmembrane ligand to engage the transmembrane Notch receptor of an adjacent cell (figure 1.1A). This type of signaling mechanism, which responds to direct cell to cell contact, is known as juxtacrine signaling. Mammals have four different forms of Notch receptor protein (Notch1-4). The Notch receptor contains many different domains (figure 1.1B), which afford the protein specific functions [5]. In mammals, Notch ligand diversity consists of several transmembrane ligands, including two Jagged ligands (Jag1 and Jag2) and three Delta-like ligands (DLL1, DLL3, and DLL4). Upon ligation, the signal receiving cell's Notch receptor then undergoes a series of proteolytic cleavages, first by ADAM and then by γ-secretase, which releases the intracellular portion of the protein from the membrane [6–8]. Following proteolytic processing, the Notch ligand will be endocytosed back into the signal sending cell, taking the extracellular domain of the Notch receptor with it [9,10]. The Notch intracellular domain (NICD) is tasked with the job of relaying the signal to the nucleus.

Notch signaling affords one cell the capacity to alter gene expression in a neighboring cell through a physical interaction. Upon release from the cell membrane, the

NICD undergoes nuclear translocation [11]. After moving into the nucleus the NICD binds the transcriptional co-factors Recombining Binding Protein Suppressor of Hairless (RBPJ/CSL) [12] and Mastermind-Like (MAML) [13] forming a ternary complex which induces target gene transcription [14,15]. In addition, this ternary complex can recruit the histone acetyltransferase p300/CBP which facilitates chromatin acetylation and Notch target gene expression [16]. RBPJ acts as a transcriptional repressor in the absence of NICD, but is transformed into a transcriptional activator through NICD binding [17]. Mechanistically, assembly of this transcriptional complex is thought to take place in a step-wise manner. First, the RBPJ-associated module (RAM) domain of the NICD binds RBPJ, followed by NICD ankyrin domain adhesion to RBPJ [18]. Together, NICD and RBPJ create an interface with a long groove which facilitates MAML recruitment [19]. Bound to DNA, this ternary complex activates transcription of Notch target genes, such as hairy and enhancer of split (*HES*) genes and hairy/enhancer of split related with TYRPW motif (*HEY*) genes [20]. These genes code for proteins which act as basic helix-loop-helix transcriptional repressors that are well-known for their suppression of tissue specific transcriptional activators [21]. Through Notch target gene transcription, activation of Notch signaling can turn on or off genetic switches which directly affect cell-fate decisions [22].

Phosphoregulation of Notch Signaling

After a protein is produced, it can undergo a number of post-translational modifications, which alter its function. Phosphorylation events are one of the most common ways in which proteins are post-translationally modified. Kinases are enzymes which chemically attach phosphate groups to a substrate protein. Generally, phosphorylations occur at serine, threonine, or tyrosine amino acids. This type of

modification has regulatory implications for the phosphorylated protein, as phosphorylations can enhance or inhibit a protein's activity, or even cause it to take on a new function. Many interesting studies have demonstrated the phosphoregulation of Notch.

Perhaps the best studied way in which phosphoregulation alters Notch activity is by mediating the stability of the NICD. There have been several studies which have connected Notch phosphorylation with its proteasomal degradation, whereby phosphorylations act as a signal which prime the protein for destruction. Generally, it is the phosphorylation of the NICD PEST domain that regulates its stability. This is due to the recognition of phosphorylated residues by the E3 ubiquitin ligase F-box and WD repeat domain-containing 7 (FBW7/Sel-10) which ubiquitinates the phosphorylated NICD, thus marking it for proteasomal degradation [23]. The transcriptional co-activator MAML is thought to induce both the disassembly of the tripartite complex and proteolytic turnover of NICD immediately following transcriptional induction [24]. MAML facilitates NICD turnover by recruiting cyclin-dependent kinase 8 (CDK8) to the Notch transcriptional complex [25]. Serine and threonine phosphorylation of the trans-activation domain (TAD) and PEST domains of Notch1, by CDK8, acts to stimulate FBW7/Sel-10 induced NICD degradation [25]. Another serine/threonine kinase, known as integrin linked kinase, phosphorylates NICD and reduces its stability through FBW7 mediated proteasomal degradation [26]. While the phosphorylation-induced proteasomal degradation on NICD is well documented, the opposite mechanism has been observed, whereby phosphorylation protects the NICD from destruction. Here, Glycogen synthase kinase-3β phosphorylates threonine 2512 and this activity protects NICD from degradation [27].

As it pertains to Notch signaling, not all kinase activity has been linked to the degradation of the NICD. Some kinases phosphorylate Notch in order to perturb Notch transcriptional activity through disruption of the ternary complex. Whereas PEST domain phosphorylation regulates stability, here it is phosphorylation of the ankyrin domain which governs ternary complex formation. It has long been established that hyperphosphorylated NICD can be detected in the nucleus associated with RBPJ [28]. It wasn't until years later that mechanistic insight was uncovered pertaining to how the nuclear behavior of NICD was altered by its phosphorylation status. A serine/threonine kinase, Casein kinase 2 (CK2), phosphorylates the NICD in hierarchical fashion. First, CK2 phosphorylates serine 1901 within the ankyrin domain, and then produces a second phosphorylation at threonine 1898, which stimulates dissociation of the ternary complex from DNA [29]. This disruption of ternary complex association causes a reduction in Notch mediated gene transcription [29]. Similarly, a mitogen-activated protein kinase member, known as Nemo-like kinase (NLK), also phosphorylates the NICD at multiple sites. NLK processively phosphorylates the NICD on residues C-terminal to the ankyrin domain, in both its membrane-bound and liberated forms, in order to inhibit NICD-MAML binding and suppress Notch signaling [30].

While most of the research regarding phosphorylation of the Notch protein has focused on serine/threonine phosphorylation, there exists scant evidence that Notch is phosphorylated on tyrosine residues. Through broad spectrum phosphoproteomics studies, where the investigators purified all of the phosphotyrosine proteins from cells and identified them using mass spectrometry, Y2074 and Y2145 on the Notch protein was discovered to be phosphorylated [31,32]. Although, the kinase responsible for phosphorylation of these sites was unknown at the time. Two cytoplasmic tyrosine kinases have been shown

to interact with the Notch protein. Abelson kinase (ABL) is a tyrosine kinase which has been observed to tyrosine phosphorylate the Notch protein [33]. The activity of another tyrosine kinase, known as Src kinase, synergizes its activity with Notch signaling in order to coordinate fruit fly development [34]. Additional research has uncovered that Src kinase responds to extracellular factors which stimulate cell growth in order to regulate Notch receptor maturation in the golgi apparatus [35]. Yet another study, discovered that Src physically interacts with the Notch protein, and that Notch has phosphotyrosine residues which can be abated by disrupting Src activity [35]. Through these reports, tyrosine phosphorylation of Notch has been documented, but five reports are a tiny fraction of the more than 8,000 Notch-specific studies in the literature. Since the insight gained from the study of serine/threonine phosphorylation of the Notch receptor has advanced our understanding of Notch modulation during development and disease, more work should be done to investigate the implications of tyrosine phosphorylation of the Notch receptor.

Notch Crosstalk

Signaling pathways do not work in an autonomous fashion, rather, they coordinate with the machinery of other signaling mechanisms. In the same way that multiple circuits can be controlled by one switch, so too can multiple signaling pathways be controlled by a single stimuli source. Proteins which regulate Notch signaling components are shared amongst multiple signaling pathways. In this way, information from a variety of stimuli can be fed into Notch signaling. Notch is able to respond to signals emanating from Wnt, Hedgehog, TGFβ, VEGF, hypoxia, and integrin signaling pathways [36,37]. Thus, Notch responds to multiple signals and integrates this information to mount an appropriate cellular response.

What Can Notch Exploration Offer Medicine?

Notch signaling is instrumental for proper embryonic development as well as the progression of many diseases. A well-studied example of Notch functionality occurs during angiogenesis. Angiogenesis is the growth of new blood vessels from existing structures. Notch responds to proangiogenic stimuli to coordinate endothelial migration, growth, and differentiation [38]. Angiogenesis occurs during development, but also is stimulated in tumor growth. Angiogenesis can be induced through both stimulation [38] and blockade [39] of Notch signaling. It is thought that differential Notch ligand expression is the main mechanism by which blood vessel cells communicate in order to coordinate their growth [40]. This demonstrates that tight regulation of Notch signaling is essential for governing angiogenesis. During cancer treatment, anti-angiogenic therapies which target Notch signaling components are employed to disrupt the vascularization of tumors [41].

Mutations within Notch signaling proteins are responsible for causing many genetic disorders. Mutated proteins can function inappropriately either by disrupting or over-activating signaling output. Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) is a brain disorder which causes stroke and dementia. Patients with this disorder have been found to harbor mutations within the *Notch3* gene [42]. Additionally, Notch signaling has also been implicated for its regulation of cancer growth. One of the best known examples occurs in a type of leukemia called T-cell acute lymphoblastic leukemia (T-ALL), which has been heavily connected with aberrant Notch activation [43]. Over 50% of T-ALL patients harbor a Notch receptor mutation [44]. In order to combat the disease, several therapeutic strategies have been developed which directly inhibit Notch signaling machinery [45,46]. While mutations within

the Notch receptor have been linked with CADASIL and T-ALL, it is mutations found in the Notch ligand Jagged1 which causes Alagille syndrome [47]. Alagille syndrome is a genetic disease that affects many parts of the body, most notably the liver, where abnormally developed bile ducts cause liver damage [48]. However, in patients afflicted with Alagille syndrome who harbor no Jagged1 defects, it is mutations within Notch2 which causes the disease [49]. These various mutations of Notch machinery cause abnormal Notch signaling, and in turn disease. By furthering our understanding of the molecular events involved in Notch signaling, we lay the foundation on which new therapeutic strategies to treat these disorders will be built.

Integrin Signaling

Integrins are transmembrane proteins which serve as adhesion proteins and mechanoreceptors at the cell surface. These mechanosensors bind components of the extracellular matrix, inducing cytoskeletal rearrangements that causes the integrin complex to tug on matrix proteins [50]. This application of physical force, about 40 piconewtons, is required to fully activate the integrin [51]. Once activated, a multitude of intracellular components carry out integrin signal transduction. Integrins are heterodimeric proteins which are comprised of an α subunit and a β subunit. There are 18 α and 8 β subunits in vertebrates, which can combine to form 24 different αβ heterodimers [52]. Each integrin has the ability to bind multiple ligands, and integrin ligands are usually recognized by multiple integrins. This has led to the grouping of integrins based on their substrate specificity. αVβ3 integrin recognizes tripeptide Arg-Gly-Asp (RGD) motifs in extracellular matrix molecules such as vitronectin and fibronectin [53]. RGD motifs which are insolubilized within extracellular matrix proteins activate RGD binding integrins such as αVβ3 integrin,

whereas solubilized RGD peptides can be used to inactivate them [54]. αVβ3 integrin is upregulated in tumor vasculature and has been robustly implicated for its role in angiogenesis [55,56], a process which Notch is a known regulator [57]. The β3 integrin subunit is an activator of the intracellular tyrosine kinases Focal Adhesion Kinase (Fak) [58] and Src family kinases [59,60].

Src Family Kinases

Src kinases are thought to have appeared in metazoans over 500-600 mya [61] with their evolution being an important step in the rise of multicellularity [62,63]. However, new evidence suggests that Src kinase orthologs are present in choanoflagellates [64], thus their evolution predates multicellularity affording ample time for coevolution with other components of the integrin adhesion/signaling machinery. The Src family has nine members of non-receptor tyrosine kinases including c-Src, Blk, Fgr, Fyn, Hck, Lck, Lyn, Yes, and Yrk [65]. Src kinases share a conserved domain organization. From N- to C-terminus Src family members contain: a SH4 membrane targeting domain which contains a myristoylation site, a 50-70 amino acid unique domain which is divergent among Src family members, a SH3 domain that mediates ligand binding, a SH2 phosphotyrosine recognition domain which is important for both substrate recognition and autoinhibition, a SH1 tyrosine kinase domain, followed by a C-terminal regulatory segment [65,66]. These kinases interact with, and phosphorylate, a whole host of membrane bound [67], cytosolic [68], and nuclear proteins [69]. One study found that c-Src interacted with Notch1 and Notch1 had phosphotyrosine residues which could be abated with Src inhibitors [35]. This interaction was determined to occur between the kinase domain of c-Src and the ankyrin domain of Notch1 [35]. However, no specific tyrosine residues were implicated as c-Src substrates in this study.

The ankyrin domain of N1ICD is important for RBPJ trans-activation and transcriptional activity [70] suggesting that Src may be able to modulate Notch transcriptional output through its interaction with the N1ICD ankyrin domain.

Polyphenols as Nutraceuticals

There exists many compounds within foods which act as micronutrients providing the body with health benefits. Some of these molecules have been studied for their medicinal properties. The term nutraceutical has been given to these promising compounds, and an entire industry has begun producing and marketing these molecules as health supplements. It is thought that supplementing the diet with nutraceuticals may help prevent or treat disease. However, their mechanisms of action are often not well defined or completely uncharacterized. While these nutraceuticals escape regulation by the US Food and Drug Administration (FDA), it is up to science to scrutinize what impact these compounds have on human health.

Resveratrol is a polyphenolic compound produced by plants such as grapes and peanuts [71], where it acts to protect the plant from pathogens [72]. Besides plant scientists, nutritionists have known about resveratrol for decades. Since resveratrol is ingested through the intake of wine, peanuts, and many other plant-based products, what effects this molecule has on the human body has been the subject of investigation for decades. In 1992, an epidemiological study compared the diets of various populations worldwide and noted the incidence of heart disease within these groups [73]. This seminal paper coined the term ‘the French paradox’, which describes the paradoxical phenomenon in which French populations displayed a lower incidence of heart disease, even though they consumed equivalent amounts of saturated fats and alcohol compared to other western populations in

the study. In this paper, the authors suggest that high levels of wine consumption by the French people may be protective against heart disease. Since alcohol intake among these protected populations was no different than that of afflicted populations, researchers began to identify other compounds within wine in order to test their effects on heart disease.

Out of this work, resveratrol was isolated from wine and found to be highly active in human tissues by cellular biologists. The formation of atherosclerotic plaques is a major symptom of heart disease. Atherosclerotic plaques are formed when healthy, elastic arterial walls become rigid and swollen through an inflammatory process in which fats, calcium, and white blood cells become deposited in the vessel wall [74,75]. Resveratrol has been shown to be therapeutic for processes involved in atherosclerosis, including cholesterol [76] and white blood cell deposition [77,78] in the arterial wall. Thus, wine consumption is thought to be heart-healthy in part due to its high levels of resveratrol [79].

Besides its ability to inhibit atherosclerosis, resveratrol has been reported to promote cardiovascular health in general [80]. In addition, resveratrol has been identified as an anti-oxidant [81], anti-cancer [82,83], anti-angiogenic [84–87], anti-inflammatory [88], anti-aging [89], and neuroprotective [90–92] agent. This has led to over the counter sale of resveratrol, often times being marketed as a heart-healthy dietary supplement. Even the cosmetic industry has adopted resveratrol, marketing it as a key ingredient in anti-aging skin creams [93]. However, the molecular signaling pathways by which resveratrol promotes health are not well defined [74].

Resveratrol is not the only polyphenol which has been reported to provide health benefits. Attempts have been made to divide polyphenols into subclasses in order to characterize their health benefits [94]. Along with resveratrol, other stilbenes such as

piceatannol and pterostilbene are found in grapes [95] and blueberries [96]. Aside from stilbenes, another large class of polyphenols known as the flavonoids have been studied for their potential use as nutraceuticals [97]. The flavonoid class of compounds can be further subdivided into subclasses such as flavones, isoflavones, and flavonols. Apigenenin is a flavone found in several plants, but it is particularly abundant in chamomile flowers [98]. Chrysin is a flavone found in honey and many plants, and is commercially derived from passion flowers of the genus *Passiflora* [99]. Another flavone, luteolin, is one of the most common flavonoids known and is found in hundreds of species of edible plants [100]. The isoflavone, genistein, is found in soy and beer and is also known to acts as a phytoestrogen [101]. The flavonol, myricetin, in found in various plant-derived products such as wine and nuts [92,102]. Quercetin is another flavonol, which is abundantly produced in onions [103]. While all of these compounds have been characterized for their various disease fighting properties, it is rare to find reports comparing the potency of different polyphenols simultaneously. Furthermore, while many of these polyphenolic compounds have promising therapeutic potential, the molecular mechanisms responsible are not well understood.

Polyphenolic Regulation of Notch and Integrin Signaling

Several polyphenolic compounds with similar structures have been shown to control Notch signaling. Compared to other polyphenols, resveratrol has received the most attention for its role in regulating the Notch cell signaling pathway [104,105]. In a high-throughput screen of over 7,000 compounds attempting to find Notch activating molecules, resveratrol demonstrated the greatest capacity to stimulate Notch activity [106]. Chrysin has been shown to enhance NICD protein levels and turn on Notch target gene transcription

[107]. Genistein can regulate cellular migration through regulation of Notch [108]. Luteolin suppresses cancer development by regulating Notch [109,110]. Pterostilbene exerts anti-cancer activities through stimulation of Notch activity [111]. All of this evidence suggests that many dietary polyphenols are able to alter Notch signaling.

Aside from Notch signaling, polyphenols have also been connected with integrin function. Integrin αVβ3 bears a receptor site for resveratrol within the extracellular domain of the β3 subunit [112]. Resveratrol binding to αVβ3 integrin inhibits atherosclerosis [84]. Apigenin attenuates cancer cell migration through regulating integrin activity [113,114]. Chrysin inhibits αIIbβ3 integrin function, serving to disrupt platelet aggregation [115]. Luteolin inhibits integrin β3 function during cancer cell migration [116]. Quercetin alters integrin expression levels in order to decrease fibrotic wound healing and scar tissue formation [117]. Polyphenolic regulation of integrin signaling provides yet another mechanism of action in which these molecules may provide health benefits.

Aim and Scope

Aim

The aim of this project was to discover new regulatory mechanisms of Notch signaling. Little is known about the precise mechanisms by which Notch signaling responds to the cellular microenvironment. Our research group focuses on understanding how Notch signaling integrates messages coming from adjacent cells with the information contained within the cellular microenvironment such as hormones, cytokines, shear stress, hyperglycemia, hypoxia, and the extracellular matrix. Thus, we study Notch signaling with the view that it acts as a sensor of the cellular microenvironment. Previously, we had discovered that an extracellular matrix-associated protein, Microfibril-associated

glycoprotein 2 (MAGP2) is able to bind αVβ3 integrin, causing an inhibition of Notch signaling through an unknown mechanism [118]. While we had discovered how a MAGP2-αVβ3 integrin interaction at the cell surface was somehow able to inhibit Notch signaling, the cytosolic events leading to this inhibition were unknown. Much of the work in this dissertation was aimed at discovering what intracellular machinery linked integrin signaling with Notch activity. While Notch signaling is heavily involved in many developmental and disease processes, we know almost nothing about its regulation by tyrosine kinases. There exists only a couple of reports which have observed the tyrosine phosphorylation of the Notch receptor. Additionally, since resveratrol has been shown to activate Notch signaling, we sought to test whether other polyphenols act as Notch regulators.

Scope

In **chapter II**, we describe a novel mechanism by which Src kinase regulates Notch signaling activity. We began by examining the mechanism by which MAGP2 and αVβ3 integrin are capable of coordinating Notch activity. We discovered that Src, a cytosolic tyrosine kinase, responds to integrin activation and phosphorylates the Notch intracellular domain. To date, there exists only a couple of reports which have observed the tyrosine phosphorylation of the Notch receptor. In this chapter, we identify specific tyrosine residues on Notch which Src phosphorylates. We found that these sites of phosphorylation serve to decrease Notch transcriptional potency. This is carried out through interference of MAML recruitment by phosphorylated NICD. All of this reveals new insight into a little-known crosstalk mechanism between Src and Notch signaling.

In **chapter III**, we investigated the Notch activating ability of nine polyphenolic compounds. We then compared how these polyphenols affect endothelial cell biology. While studies have previously addressed how some of these molecules affect these processes, a direct comparison of their activity had never been performed. We found that resveratrol is a potent Notch activator, but also that other similar polyphenols are capable of Notch activation. Next, we discovered that several of these polyphenolic compounds are capable of inhibiting endothelial cell proliferation. We then characterized how these polyphenols affect endothelial migration during wound healing. In doing so, we discovered novel polyphenolic regulators of Notch signaling while also characterizing the potency of these molecules for their ability to mitigate angiogenic behaviors in endothelial cells.

Chapter IV is a summary of the studies and a general discussion about the results, including future perspectives. **Appendix A** is a review of the literature, which expands our view of Notch signaling beyond its classical job as a mediator of juxtacrine signaling. Here, we shed light on the role Notch signaling plays as a microenvironmental sensor, responding to a multitude of cues within the extracellular environment. **Appendix B** is a review of the literature covering non-cononical integrin ligation. Here, we discuss many of the ligands of integrins which are not components of the extracellular matrix, such as bacterial proteins, viruses, hormones, small molecules, polyphenols, venoms, and cell-cell adhesion proteins.

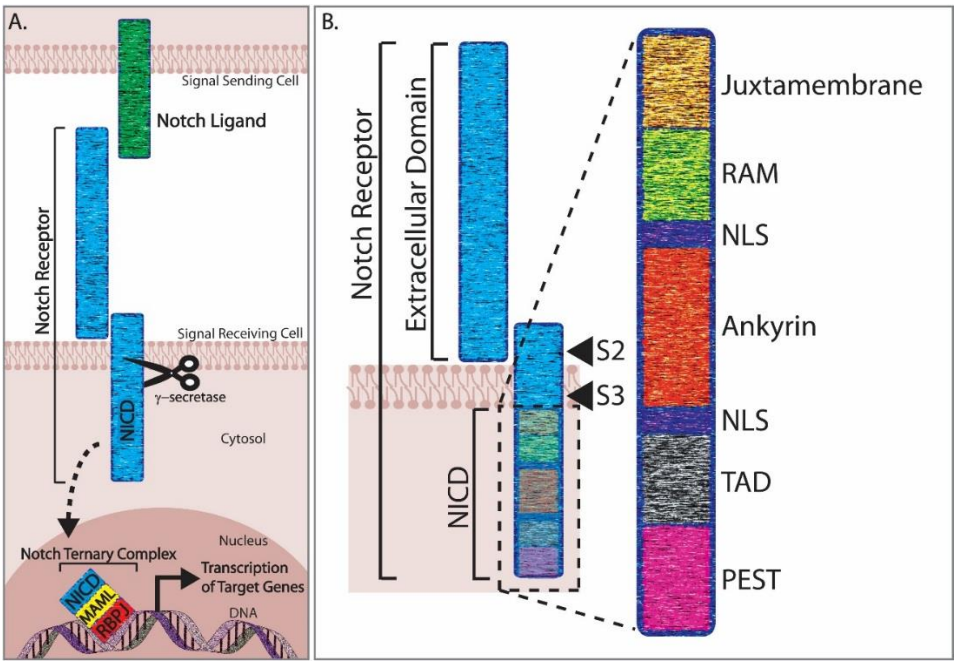

Figure 1.1: The Notch Signaling Pathway and the Domain Architecture of the Notch Receptor

A.) The Notch signaling pathway. Notch signaling facilitates cell to cell communication. Here, the signal sending cell uses a transmembrane ligand to engage the Notch receptor of the signal receiving cell. Upon ligation, γ-secretase cleaves the Notch receptor, releasing the Notch intracellular domain (NICD). The NICD travels to the nucleus and forms a ternary transcriptional complex with MAML and RBPJ to induce transcriptional expression of Notch target genes.

B.) Domain architecture of the Notch receptor. S2/S3= proteolytic cleavage sites, RAM = RBPJ Associated Module, NLS = Nuclear Localization Signal, Ankyrin = Ankyrin Repeat Domain, TAD = Transcriptional Activation Domain, PEST = Proline/Glutamic acid/Serine/Threonine rich domain.

CHAPTER TWO

Src Kinase Phosphorylates Notch1 to Inhibit MAML Binding

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Abstract

Notch signaling is a form of intercellular communication which plays pivotal roles at various stages in development and disease. Previous findings have hinted that integrins and extracellular matrix may regulate Notch signaling, although a mechanistic basis for this interaction had not been identified. Here, we reveal that the regulation of Notch by integrins and extracellular matrix is carried out by Src family kinases (SFKs) working downstream of integrins. We identify a physical interaction between the SFK member, c-Src, and the Notch intracellular domain (NICD) that is enhanced by β3 integrin and the integrin binding ECM protein, MAGP2. Moreover, we also find that SFK activity decreases NICD half-life. Mechanistically, our results demonstrate that c-Src directly phosphorylates the NICD at specific tyrosine residues and that mutation of these phosphorylation sites increases Notch responsive transcriptional activity. Furthermore, we also find that phosphorylation of the NICD by SFKs attenuates Notch mediated transcription by decreasing recruitment of MAML to the Notch co-transcriptional complex. Collectively, our results provide important mechanistic data that underlie the emerging role of Notch as a general sensor and responder to extracellular signals.

Introduction

Canonical Notch signaling is a form of juxtacrine cell communication that affords one cell the ability to induce changes in a neighboring cell's transcriptome via physical interaction. Notch signaling begins when a Notch ligand from one cell (i.e. the signal sending cell), binds to the transmembrane Notch receptor on an adjacent cell (i.e. the signal receiving cell). Mammals have four different Notch receptors (Notch1-4). Mammalian Notch ligand diversity consists of several transmembrane ligands, including three Delta-

like ligands (DLL1, DLL3, and DLL4) and two Jagged ligands (Jag1 and Jag2), as well as an assortment of soluble Notch ligands [119,120]. The force applied to the signal receiving cell's Notch receptor by a neighboring cell's ligand is critical for Notch activation [51], and induces a series of proteolytic cleavage events of the Notch receptor, first by ADAM (A Disintegrin and Metalloproteinase) and then by γ-secretase [4,6]. The later severance, known as the S3 cleavage [7], results in the liberation of the Notch intracellular domain called the NICD fragment. The emancipated NICD fragment translocates to the nucleus where it binds the co-transcription factors Recombining Binding Protein Suppressor of Hairless (RBPJ/CSL), Mastermind-Like (MAML), and the histone acetyltransferase p300/CBP inducing target gene transcription [121,122]. RBPJ associates with chromatin at specific promoter sites, known as CSL sites [11]. It is the Notch-RBPJ interface which forms a groove that recruits the basic domain of MAML, which settles into this furrow as a long α-helix [19]. Assembly of the RBPJ-NICD-MAML ternary complex at CSL sites activates transcription of genes such as, hairy and enhancer of split (*HES*) genes and hairy/enhancer of split related with TYRPW motif (*HEY*) genes [20].

Being highly versatile and tunable, Notch signaling does not orchestrate cell to cell stimuli exclusively, but rather harmonizes juxtacrine signals with extracellular cues. Recent research has revealed that Notch can respond to a multitude of components within the cellular microenvironment such as hypoxia, hyperglycemia, shear stress, crosstalk with other signaling pathways, and the composition of the extracellular matrix [36]. Previously, we identified a novel signaling axis consisting of the matricellular, integrin binding protein MAGP2 (aka MFAP5), αVβ3 integrin, and Notch1 [118]. We showed that ligation of αVβ3 integrin by the RGD containing MAGP2, soluble RGD peptide, and integrin blocking

antibodies all were able to regulate the accumulation of the Notch1 intracellular domain (N1ICD) and its transcriptional output [118]. Although we identified a novel mechanism of Notch regulation we were unable to report on the cytosolic events which αVβ3 integrin harnesses in order to coordinate Notch signaling. In this work, we have exposed Src family kinases (SFKs) as modulators of the Notch signaling axis. We reveal that the Notch-Src interaction is enhanced through β3 integrin and MAGP2. We show that interaction of a SFK member, c-Src, with the Notch intracellular domain leads to tyrosine phosphorylation of the NICD. Furthermore, we identify specific tyrosine residues on the NICD that are phosphorylated by c-Src and are important for RBPJ/NICD transcriptional activity. Finally, we reveal that these tyrosine residues on the NICD serve to decrease NICD-MAML binding. All of this combined reveals mechanistic insight on a novel phosphotyrosine regulatory mechanism of Notch signaling carried out by Src family kinases.

Results

Notch1 Intracellular Domain Interacts with c-Src

Our previous work outlined a connection between Notch signaling and integrin signaling in which integrin activation by MAGP2 serves to inhibit Notch. We wondered what cytosolic mechanism was responsible for this crosstalk between Notch and integrin signaling. A well-known mediator of integrin signaling, c-Src (a Src family kinase member), has been demonstrated to interact with Notch1, and Notch1 has phosphotyrosine residues which can be abated with Src inhibitors [35]. Based on this, we hypothesized that the MAGP2 and αVβ3 integrin enlisted c-Src to carry out Notch regulation. First, we sought to confirm that c-Src physically interacted with N1ICD through co-immunoprecipitation experiments. Due to a lack of commercially available antibodies

capable of immunoprecipitating endogenous NICD we were forced to use an overexpression model to test our hypothesis. 293T cells were transfected with cDNA encoding for a 3xFLAG tagged murine N1ICD +/- cDNA encoding c-Src, and immunoprecipitated with α-FLAG antibodies. Western blot analysis with anti-Src and anti-Notch1 antibodies confirmed a physical N1ICD-Src interaction (figure 2.1A). To determine if c-Src activation downstream of β3 integrin or MAGP2 could facilitate the NICD-Src interaction, we co-transfected 293T cells with combinations of 3xFLAG-N1ICD and cDNAs encoding β3 integrin or MAGP2. As shown in figure 2.1A, expression of either β3 integrin or MAGP2 drove a N1ICD-Src interaction even in the absence of overexpressed c-Src protein.

c-Src Phosphorylates the Ankyrin Region of N1ICD

Since Src family members are tyrosine kinases, we wondered whether the N1ICD was phosphorylated by c-Src. To this end, we turned to NetPhos 3.1, which predicts phosphorylation sites of eukaryotic proteins [123], in order to determine what tyrosine residues on the N1ICD might be phosphorylated by c-Src. Y2074 had the highest probability of c-Src phosphorylation, 0.5 out of 1 while Y2145 phosphorylation was predicted with a score of 0.3 out of 1. These findings are partially confirmed by previous studies involving whole cell immunoprecipitations using α-pY antibodies which were then analyzed by mass spectrometry. These studies identified phosphorylation at Y2074 and Y2145 on Notch1 [31,32], although the identity of the kinase responsible for phosphorylation of these residues was unknown. We sought to verify c-Src mediated phosphorylation using an *in vitro* Src kinase assay. We constructed a C-terminally FLAG tagged 103 amino acid fragment of N1ICD (amino acids 2058-2161) which contains both of the tyrosine sites of

interest (Y2074 & Y2145) (figure 2.1B). This peptide, which maps to the C-terminal of the ankyrin domain (ankyrin repeats 6a – 7b + EP region) was expressed in *E. coli,* purified using an anti-FLAG affinity column, and then incubated with purified c-Src kinase in an *in vitro* Src kinase assay. After a 15 minute incubation, samples were subjected to western blot analysis and probed with α-pY (phosphotyrosine) antibodies. We found that c-Src was able to phosphorylate this portion of the N1ICD *in vitro* in an ATP-dependent manner (figure 2.1C). Seeking to identify which residues (Y2074, Y2145, or both) became phosphorylated we created two more peptides in which we substituted tyrosine for phenylalanine (Y→F) at these sites and submitted these proteins to our *in vitro* c-Src kinase assay (figure 2.1D). We found that the Y2074F mutant was not as heavily tyrosine phosphorylated when compared to the wild type (WT) peptide, thus pointing to this being an important residue for c-Src phosphorylation. Although, the Y2074F mutant did display some tyrosine phosphorylation, suggesting that while Y2074 was still a likely candidate of phosphorylation, c-Src must be phosphorylating at least one other site on the peptide. Surprisingly, Y2145F mutant had enhanced tyrosine phosphorylation when compared to WT peptide. Subsequent densitometry analysis revealed that c-Src phosphorylated the WT N1ICD ~10 times greater than the Y2074F N1ICD, whereas the Y2145F N1ICD had ~four times more phosphorylation than the WT (figure 2.1E). These results confirm that c-Src can phosphorylate at least two sites on the N1ICD.

Upon confirmation that c-Src phosphorylates N1ICD, we next asked if the 2058-2161 amino acid region of Notch1 was sufficient for Src interaction in living cells. To test this, we made fusion proteins of the full-length N1ICD domain and the 2058-2161 fragment fused to HA tagged BirA biotin ligase, in order to perform proximity biotin

ligation (i.e. BioID [124]) experiments (figure 2.2B and 2.2D). In these experiments, the BirA enzyme biotinylates proteins which are within close proximity (20-30 nm) to the fusion protein (figure 2.2A and 2.2C). In addition, we co-expressed wild type Src (WT), constitutively active (CA), and dominant negative (DN) forms of Src kinase in combination with our fusion proteins. Streptavidin pulldowns were performed to affinity capture biotinylated proteins, and streptavidin-HRP was used to detect biotinylated species by western blot. Subsequent blotting with α-Src primary antibodies confirmed Src interacts with, or at least is within close proximity to both the N1ICD::BirA and 2058-2161::BirA fusions. Furthermore, upon overexpression of WT and CA Src, both fusions were found to be tyrosine phosphorylated. Additionally, overexpression of DN Src induced no such phosphorylation in the 2058-2161::BirA fusion. These results suggest the 2058-2161 region which maps to the ankyrin domain of N1ICD is sufficient for Src interaction.

N1ICD is Phosphorylated in a SFK-Dependent Manner

Having established that Src family kinases interact with and phosphorylate the intracellular domain of Notch, it was important to determine whether SFKs alone are responsible for NICD phosphorylation, or if other tyrosine kinases phosphorylate NICD. To this end, we expressed 3xFLAG N1ICD in 293T cells in the presence or absence of c-Src overexpression and immunoprecipitated the N1ICD using α-FLAG antibodies. Western blot analysis reveals that c-Src overexpression induces greater pY signal emanating from N1ICD compared to the no c-Src overexpression control (figure 2.2E). In the reciprocal experiment, N1ICD-Src pulldowns were performed in the presence of a highly specific inhibitor of SFKs (AZM475271) [125], and it was found that SFK activity is required for tyrosine phosphorylation of N1ICD (figure 2.2F). Taken together, this

evidence suggests that phosphorylation of tyrosine residues on the N1ICD occurs in a SFK-dependent fashion. Finally, we wanted to see whether this phenomenon is specific to Notch1. We expressed FLAG tagged N1ICD and N4ICD in the presence or absence of c-Src overexpression in 293T cells and immunoprecipitated the NICDs using α-FLAG antibodies (figure 2.2G). Western blot analysis of the immunoprecipitated material revealed that both NICD molecules immunoprecipitated with c-Src under conditions of c-Src overexpression. Furthermore, N4ICD was also tyrosine phosphorylated and this was enhanced through c-Src overexpression. Thus, the interaction between c-Src and the Notch intracellular domain is not specific to Notch1, but rather a broader mechanism which encompasses Notch4 as well.

It was important to map tyrosine residues that were phosphorylated by SFKs. To accomplish this, we first submitted our *in vitro* c-Src kinase assays samples (figure 2.1) to mass spectrometry analysis. No phosphotyrosine residues were detected in samples which were not incubated with c-Src. Only in c-Src incubated samples were phosphotyrosines detected at the Y2074 (Y2064 in mice) and Y2145 (Y2135 in mice) as predicted by our NetPhos 3.1 analysis. In addition, we were able to identify phosphotyrosine at Y2116 (Y2106 in mice) which had previously escaped our initial attention. To determine if these sites are phosphorylated in living cells, 3xFLAG N1ICD was transfected into 293T cells, then immunoprecipitated using α-FLAG antibodies. Mass spectrometry analysis of these samples verified phosphorylation at Y2074, Y2116, and Y2145 and also identified an additional phosphorylated residue, Y1938 (Y1928 in mice), which is not present on our 2058-2161 peptide. To examine the conservation of these sites, we performed a sequence alignment analysis of the identified phosphorylation sites across several species and across

all four Notch isoforms in humans and mice (figure 2.3A). For our analysis we also included the NetPhos 3.1 score which predicts the likelihood of c-Src induced tyrosine phosphorylation at each of these sites. This analysis revealed that Y1938 and Y2074 are highly conserved, Y2116 is moderately conserved, and Y2145 is somewhat conserved. It should be noted, that residues which lie within the highly structured ankyrin domain (Y1938, Y2074, and Y2116) are more conserved than Y2145 which lies proximal to the ankyrin domain. The position of residues Y1938, Y2074, and Y2116 within the N1ICD/RBPJ/MAML co-crystal (PDB# 3NBN) are indicated in figure 2.3B.

SFKs Regulate Notch1 Transcriptional Activity

Having established a physical interaction between N1ICD and SFKs, it was important to determine if SFKs control Notch transcriptional activity. To accomplish this, 293T cells were transfected with Notch responsive 4xCSL reporter which expresses luciferase in response to activation of Notch signaling. Cells were transfected with N1ICD in the presence or absence of co-expression of a constitutively active SFK, v-Src. Cells transfected with v-Src demonstrated decreased luciferase activity (figure 2.4A). This result suggests that v-Src activity downregulates Notch mediated transcription. Having found and confirmed sites of SFK mediated tyrosine phosphorylation on the N1ICD, it was important to determine if phosphorylation at these sites was important for Notch transcriptional activity. To this end, we introduced Y→F substitutions at Y1938, Y2074, Y2116, and Y2145 within our 3xFLAG N1ICD construct. We also constructed a "Quad" mutant N1ICD which has Y1938F, Y2074F, Y2116F, and Y2145F substitutions. We expressed these mutant forms of N1ICD in 293T cells and immunoprecipitated them using α-FLAG antibodies. To our surprise, we did not detect an obvious correlation between Y→F

mutation and anti-pY western blot signal (figure 2.4B). Nonetheless, all of the Y→F N1ICD mutants demonstrated enhanced transcriptional activity on the 4xCSL and Hes5 promoters as compared to WT N1ICD (figure 2.4C and 2.4D). To summarize these findings, enhanced SFK activity (i.e. v-Src expression) decreases Notch transcriptional output, while removal of sites of tyrosine phosphorylation increases Notch transcriptional output. Taken together, these results suggest that SFK activity impedes Notch target gene transcription.

SFK Phosphorylation Sites Decrease NICD-MAML Binding

Based on the crystal structure analysis of the Notch ternary complex, we sought to assess whether SFKs play a role in Notch ternary complex formation. To test this, we expressed FLAG tagged N1ICD Quad mutant or WT N1ICD in 293T cells in the presence or absence of transfected MAML1 cDNA and used anti-FLAG co-immunoprecipitation to monitor N1ICD-MAML interactions. As shown in figure 2.5A and 2.5B, the Quad mutant co-immunoprecipitated more MAML1 than the WT N1ICD. We then hypothesized that inhibition of SFK activity would equilibrate the amount of MAML interaction between WT and Quad N1ICD forms. To test this, we performed the same co-immunoprecipitation, but this time in the presence of SFK inhibitor. Under conditions of SFK inhibition both WT N1ICD and Quad N1ICD co-immunoprecipitate equal amounts of MAML1 (figure 2.5C and 2.5D). In figure 2.1A, we demonstrated that Src-N1ICD interaction was strengthened by overexpression of β3 integrin or MAGP2, therefore we sought to determine if β3 integrin or MAGP2 may also regulate N1ICD-MAML interaction. To test this, we examined the N1ICD-MAML interaction by co-immunoprecipitation in the presence of β3 integrin or MAGP2 expression. As shown in figure 2.5E, overexpression of

integrin machinery including c-Src, β3 integrin, or MAGP2 all decreased WT N1ICD-MAML interaction while the Quad N1ICD mutant was resistant to this effect. Taken together, these results suggest that SFK phosphorylation of N1ICD decreases N1ICD-MAML binding.

Having confirmed that SFKs are important for N1ICD-MAML interaction, we next sought to determine if the increased transcriptional potency of the N1ICD tyrosine mutants was due to enhanced MAML recruitment. We hypothesized that the enhanced transcriptional potency of the N1ICD tyrosine mutants was due to their enhanced ability to recruit MAML from a limited endogenous supply. We therefore reasoned that providing a surplus of MAML (i.e. overexpression) would elevate WT N1ICD transcriptional output to match that of the N1ICD tyrosine mutants. To test this, we compared the ability of the WT and tyrosine mutant N1ICD forms to drive transcription from the 4xCSL luciferase reporter assay in the presence or absence of MAML cDNA (figure 2.5F). In contrast to conditions of basal MAML expression where tyrosine mutant N1ICD forms display enhanced transcriptional potency, during MAML overexpression the WT and all six tyrosine mutant forms of N1ICD had equivalent transcriptional potency (figure 2.5F). These results suggest that MAML is the rate limiting factor during Notch target gene transcription in this system, because when MAML is in surplus (during overexpression), WT and tyrosine mutant N1ICDs have equilibrated transcriptional activity. Taken together, this evidence suggests SFK phosphorylation sites within the N1ICD serve to impede N1ICD recruitment of MAML, thus decreasing Notch transcriptional output.

SFK Activity Decreases N1ICD Half-Life.

Since Notch is heavily involved in many developmental processes such as angiogenesis [57,126], and MAGP2 coordinates Notch signaling in order to stimulate angiogenesis [39], we wanted to establish whether SFKs alters Notch activity in endothelium. To accomplish this, human HMEC-1 microvascular endothelial cells were transfected with Notch responsive Hes1 and Hes5 luciferase reporters and treated with the SFK inhibitor, AZM (AZM475271). As shown in figure 2.6A, both the Hes1 and Hes5 promoters demonstrated enhanced transcriptional activity under conditions of SFK inhibition compared to DMSO control. Since Notch activation leads to generation of the transcriptionally activity NICD, we sought to investigate what effect SFK activity has on endogenous N1ICD accumulation. To this end, we utilized AZM to inhibit SFK activity and analyzed endogenous N1ICD accumulation in HMEC-1 cells. N1ICD levels were determined by blotting with α-N1ICD antibodies that detect the newly formed Val1744 epitope at the N-terminal of N1ICD after γ-secretase cleavage of full length Notch1 receptor. As shown in figure 2.6B, N1ICD appeared as a doublet when blotting HMEC-1 cell lysates with α-N1ICD antibodies, and it was noticed that after SFK inhibition, a larger proportion of N1ICD migrated as a low molecular weight species. We estimated that the lower N1ICD band migrated ~9kDa faster than the upper band.

Ubiquitin is a 9kDa protein [127] that is attached to proteins marking them for proteasomal degradation [128]. Since the two N1ICD species migrated with a difference of ~9kDa, we hypothesized that the higher molecular weight species might be mono-ubiquitinated and that SFKs may regulate Notch signaling through manipulation of NICD half-life. In order to track N1ICD half-life, HMEC-1 cells were treated with DAPT (10

μM) to inhibit γ-secretase activity and remaining N1ICD was monitored by western blot 0, 45, 90, and 135 minutes after DAPT treatment. Interestingly, it was found that before DAPT treatment a N1ICD doublet was produced, but following DAPT treatment the lower molecular weight species disappeared with time before the higher molecular weight species. Performing this same experiment in the presence of SFK inhibition resulted in a majority of the N1ICD species to run as the lower molecular weight band (figure 2.6C) that did not accumulate as a higher molecular weight band. Densitometry of both higher and lower molecular weight species showed that N1ICD had a half-life of ~80 minutes which was lengthened to ~135 minutes in the presence of SFK inhibitor (figure 2.6D). Since N1ICD is degraded by the proteasome and accumulates with treatment of the proteasome inhibitor, MG132 [129], we hypothesized that SFKs normally decrease N1ICD stability by inducing ubiquitination of N1ICD. To test this, we treated HMEC-1 cells overnight with DMSO or SFK inhibitor then with MG132 for 0-2 hours and monitored N1ICD accumulation by western blot (figure 2.6E). In support of our hypothesis, it was found that the higher molecular weight species accumulated upon proteasomal disruption. We noted that the higher mobility fragment accumulates rapidly under control conditions, but this accumulation is delayed through inhibition of SFKs. Taken together, these results reveal that SFK inhibition reduced the accumulation, whereas proteasomal inhibition enhanced accumulation, of the higher molecular weight N1ICD in HMEC-1 cells.

Discussion

The Notch signaling mechanism has long been known to facilitate communication between adjacent cells thus allowing cells to sense, and respond, to their immediate neighbors in the cellular microenvironment [130]. However, sensing and responding to cell-

cell interactions appears to be only a part of the larger emerging function of Notch. Indeed, Notch has shown the ability to act as a general sensor for diverse signals in the cellular microenvironment including growth factors, extracellular matrix, hyperglycemia, hypoxia, and shear stress [36].

The understanding that extracellular matrix (ECM) is capable of regulating Notch has been known for some time and several mechanisms for this have been described. For instance, basement membrane laminins regulate expression of the Notch ligand DLL4 thereby restricting tip cell development in branching endothelial cells [131,132]. Other ECM proteins including collagen IV [133], CCN3 [134], and YB-1 [135] regulate Notch through direct interactions with Notch receptors. Finally, our previous work determined that the ECM proteins MAGP2 and EGFL7 control Notch through RGD-dependent integrin binding, but did not describe the molecular mechanism through which this was accomplished [39,118]. Our current results build upon our previous work by now describing a signaling mechanism that couples ECM proteins to Notch through an integrin/SFK signaling circuit. We observed that c-Src interacts with the intracellular domain of Notch1, and this interaction is enhanced through β3 integrin or MAGP2 expression (figure 2.1A). Furthermore, we have identified four tyrosine residues (Y1938, Y2074, Y2116, and Y2145) on the N1ICD which serve as SFK substrates (figure 2.1-2.3). We demonstrated that removal of these SFK phosphorylation sites leads to enhanced transcriptional activity and MAML binding (figure 2.4C, 2.4D, and 2.5).). We also found that N1ICD stability is regulated by SFK activity (figure 2.6C-E). Based on this evidence, we propose a regulatory mechanism whereby integrin activation of Src family kinases drives phosphorylation of N1ICD to impede the Notch signaling pathway by decreasing N1ICD-MAML interactions (figure 2.7). In

addition, Src family kinase induced phosphorylation serves to target the N1ICD for proteasomal degradation (figure 2.7). Interestingly, our results are not the first to link Notch to integrin signaling. Rather, Mo et al., previously determined that Integrin-linked kinase (ILK) directly phosphorylates NICD to recruit the ubiquitin ligase FBW7 and ultimately destabilize the N1ICD protein [26]. Collectively, our findings greatly enhance our molecular understanding of how Notch activity is modulated by extracellular matrix within the cellular microenvironment.

The tyrosine residues we analyzed in this study are found within or in close proximity to the Notch ankyrin domain, and their positions offer clues as to the biological function of tyrosine phosphorylation at these sites. Y1938 is located between alpha-helices 2a and 2b of the ankyrin domain [136] and is highly conserved in vertebrate Notch 1-3 proteins (figure 2.3A). The conservation of this site suggests an important role in Notch signaling which, given the projection towards RBPJ, may be to regulate RBPJ-NICD interactions (figure 2.3B). Y2074 is located in alpha-helix 6a, while Y2116 is located just C-terminal to alpha-helix 7b [136]. Y2074 is conserved in Notch1 of all species examined as well as human and mouse Notch 1-3 proteins. Y2116 is conserved in vertebrates as well as human and mouse Notch 1-2 proteins (figure 2.3A). These residues either project towards (Y2074) or are within (Y2116) the EP region of the transactivation (TAD) domain (figure 2.3B). The EP region of the TAD domain is required for recruitment of p300 acetyltransferase to the N1ICD transcriptional complex and thus phosphorylation at these sites may regulate p300 interaction [16,136]. In addition, the seventh ankyrin repeat has been implicated in controlling the stability and folding of the entire ankyrin domain [137] and it is tempting to speculate that phosphorylation of Y2074 and/or Y2116 may affect the overall structure of

the ankyrin domain. Finally, the Y2145 residue is also conserved in vertebrates, but is only found in Notch1. Y2145 is not located within the existing crystal structures of N1ICD, but maps to a position just N-terminal to the second nuclear localization signal. Our transcriptional data determined that Y→F mutation at any of these residues enhanced Notch transcriptional activity, suggesting that phosphorylation at these sites represses Notch mediated transcription (figure 2.4C and 2.4D). In support of this, Notch mediated transcription was enhanced in the presence of SFK inhibitors and repressed in the presence of constitutively active v-Src (figure 2.4A and 2.6A). We also observed that simultaneous Y→F mutation of all four tyrosine residues (i.e. Quad mutant) resulted in increased N1ICD binding to MAML, and that this was nullified by treatment with SFK inhibitor (figure 2.5A-D).

Several lines of evidence are now converging to suggest that the EP and TAD domains are important regulatory sites for controlling Notch activity. The EP region is located at the N-terminal region of the TAD domain, overlaps with ankyrin repeat 7b at the C-terminal of the ankyrin domain, and is defined as a region of NICD critical for p300 interaction [16]. This region of NICD has also been proposed to be at the N1ICD/MAML interface [19]. Our data demonstrate that MAML interaction with N1ICD is enhanced by Y→F mutations in this region and decreased by c-Src overexpression (figure 2.5A-E). Similarly, previous results also found that Nemo-like kinase (NLK) phosphorylates serine residues in this region (S2121 and S2141) resulting in reduced MAML interaction [30]. Together, these results suggest that tyrosine and/or serine phosphorylation within the EP or TAD domains may decrease MAML interaction with N1ICD, an idea that is supported by our data showing that overexpression of MAML equilibrates mutant N1ICD

transcriptional activity to WT levels (figure 2.5F). An additional point of NICD regulation that falls within the TAD domain is ILK mediated phosphorylation of human N1ICD at S2184 which recruits FBW7 and drives ubiquitination of NICD [26]. Finally, our results showing that Y2145F mutation resulted in increased phosphorylation *in vitro* (figure 2.1D and 2.1E) hint that phosphorylation at Y2145 may suppress phosphorylation at other NICD sites thus functioning as a regulatory site for Notch signaling. Collectively, the fact that SFKs, NLK, and ILK all influence Notch signaling through phosphorylation with the NICD EP and TAD domains, support a central role for these domains in controlling Notch signaling output.

Broader implications of our findings have yet to be determined. However, the idea that Notch signaling can be influenced by integrins and SFKs may have broad reaching implications for how Notch functions within cellular microenvironments. For instance, SFK signaling is not restricted to integrins but rather, SFKs are activated by a wide range of other signaling mechanisms such as RTK and GPCR receptors [138]. Therefore, an important research goal will be to determine if these signaling mechanisms engage in crosstalk with Notch through SFK mediated NICD phosphorylation. In addition, Notch and integrins are highly conserved and function in a wide variety of biological circumstances. It will be important to discover how the interplay between integrins and Notch function in this wide range of biological situations. Finally, based on our sequence comparison of N1-N4ICD (figure 2.3a), it is clear that not all of the tyrosine sites we identified are conserved in all Notch NICDs. Based on this observation, an interesting possibility is that SFKs may be able to differentially regulate various Notch isoforms and establish alternative Notch signaling activities thereby diversifying overall Notch output.

In summary, this work continues to build on the growing evidence that Notch is more than a cell-cell signaling mechanism, but rather, an integrator of multiple cues within the cellular microenvironment. This work adds an important piece to this puzzle by providing mechanistic insight about how extracellular matrix composition regulates Notch via integrins and Src family kinase signaling. Future work will continue to dissect additional molecular mechanisms by which Notch senses and responds to stimuli from the cellular microenvironment.

Materials and Methods

Antibodies

For western blotting primary antibodies against cleaved Notch1 (N1ICD) (Val1744, #2421), phosphotyrosine (pY) (P-Tyr-100, #9411), Src (32G6), and MAML1 (MAML1, #4608) were purchased from Cell Signaling Technology. Primary antibodies against β-actin (sc-47778), HA tag (sc-57592), Notch1 C-terminal domain (N1CTD) (sc-6014-R), and vinculin (sc-5573) were purchased from Santa Cruz Biotechnology. Primary antibodies against FLAG (DYKDDDDK) tag (A00170) were purchased from GenScript. Secondary antibodies consisted of horseradish peroxidase conjugated antibodies α-mouse (NA931V) and α-rabbit (NA934V) purchased from GE Healthcare Life Sciences.

Cell Culture

HMEC-1 cells were cultured in MCDB 131 media supplemented with 10% fetal bovine serum (FBS), 10 ng/ml epidermal growth factor, and 1 µg/ml hydrocortisone. 293T cells were cultured in Dulbecco's Modified Eagle's medium (DMEM) (Mediatech) supplemented with 10% FBS and 1x pen-strep. Cells were grown in 10 cm plates and passaged before reaching confluency.

Plasmids

The 3xFLAG N1ICD construct (Addgene #20183) was a gift from Raphael Kopan and contains amino acids Val1744 to Lys 2531 of the murine Notch1 intracellular domain with a 3xFLAG N-terminal tag [139]. The 3xFLAG N1ICD construct was subjected to site directed mutagenesis in order to create Y→F substitutions at Y1928 (Y1938 in humans), Y2064 (Y2074 in humans), Y2106 (Y2116 in humans), Y2135 (Y2145 in humans). This 3xFLAG N1ICD plasmid and a doxycycline inducible lenti viral destination vector, pCW57.1 (Addgene #41393, a gift from David Root) was used to construct a 3xFLAG N1ICD lentiviral expression vector. The integrin β3 construct (Addgene #27289) was a gift from Timothy Springer and contains human integrin β3 with a C-terminal myc-his tag [140]. The c-Src construct was a gift from William Schiemann and contains full length human c-Src with a C-terminal myc-his tag cloned into a pcDNA 3.1/myc-His B vector [141]. The constitutively active Src construct (Addgene #13660) was a gift from Joan Brugge and contains chicken Src with a Y527F mutation. The dominant negative Src construct (Addgene #13657) was a gift from Joan Brugge and Peter Howley and contain mouse Src K295R Y527F. The v-Src construct (Addgene #14578) was a gift from Joan Brugge and contains Src isolated from Rous sarcoma virus. The pcDNA 3.1 C-terminally myc-his tagged MAGP2 construct was previously described [142]. The Hes1 luciferase construct was a gift from Jan Jensen and consists of nucleotides -2553 to -201 relative to the murine Hes1 transcriptional start site while transcribing for firefly luciferase [143]. The Hes5 luciferase construct (Addgene #41724) was a gift from Ryoichiro Kageyama and Raphael Kopan and contains the murine Hes5 promoter (-800 to +73) relative to the Hes5 transcriptional start site while transcribing for firefly luciferase [143]. The 4xCSL luciferase construct (Addgene

#41726) was a gift from Raphael Kopan and consists of 4 tandem repeats of the high affinity CSL binding sites (5′CGTGGGAA3′) while transcribing for firefly luciferase [139]. The BirA(R118G)-Ha destination vector (Addgene #53581) was a gift from Karl Kramer and was used to construct the N1ICD::BirA fusion and 2058-2161::BirA fusion which contains 2048-2151 of murine Notch1 (amino acids 2058-2161 in the human protein). The 2058-2161 peptide use in the *in vitro* kinase assays was constructed through gateway cloning into pET-DEST42. The MAML1 plasmid was a gift from Brandon J. White and contains human MAML1 with a myc tag cloned into a pCS2 backbone.

N1ICD Half-Life Analysis

In order to track N1ICD half-life, steady-state populations of HMEC-1 cells were treated with the γ-secretase inhibitor DAPT (10 μM) to specifically block S3 N1ICD cleavage/synthesis and cells were sacrificed after 0, 45, 90, 135 minutes DAPT treatment and blotted for N1ICD. Using densitometry, N1ICD half-life was calculated under conditions of SFK inhibition and DMSO control by dividing the N1ICD amount after each time point by the starting amount.

Proximity Biotin Ligation Assays

In this BioID experiment, a mutated version of BirA biotin ligase (R118G BirA) was employed, which non-discriminately biotinylates proteins within close proximity allowing for streptavidin pull down of proximal proteins [144]. 293T cells were *Trans*IT®-LT1 (Mirus) lipid transfected with either 2058-2161::BirA or N1ICD::BirA fusion constructs. 48 hours after transfection, cells were incubated in serum free media supplemented with 50 μM biotin for 6 hours before harvesting. Streptavidin magnetic beads (10 μL, New England BioLabs) were used to precipitate biotinylated species on a

magnetic tube rack. Specific protein targets were detected using primary antibodies followed by membrane stripping before detection of overall biotinylated proteins. Biotinylated proteins were detected using horseradish peroxidase conjugated streptavidin (1:40,000) which was purchased from Thermo Scientific.

In Vitro c-Src Kinase Assays

A 14kDa FLAG tagged region (amino acids 2058-2161) of the N1ICD protein was produced in *E. coli* and purified using FLAG affinity resin and dissolved in a TBS solution. In addition to a wild type form, two mutant forms of the peptide were created, Y2074F and Y2145F. An *in vitro* c-Src kinase assay was performed with purified GST tagged human c-Src (Sigma-Aldrich) using the manufacturer's protocol. Briefly, the 14kDa peptide was mixed into a cocktail containing 2 mM MOPS (pH 7.2), 1 mM glycerol 2-phosphate, 1.6 mM $MgCl_2$, 1 mM $MnCl_2$, 0.4 mM EGTA, 0.16 mM EDTA, 0.02 mM DTT, 16 ng/μL BSA, +/- 0.05 mM ATP, +/- 200 ng purified Src. Reaction volumes totaled 25 μL and were incubated for 15 minutes at 30°C. Reactions were terminated by adding SDS page lysis buffer, vortexing, and boiling for 5 minutes and submitted to western blot analysis.

Luciferase Assays

HMEC-1 cells were seeded into 24-well plates at a density of 25,000 cells/well. 293T cells were seeded into 24-well plates at a density of 50,000 cells/well. The following day, cells were transfected using *Trans*IT®-LT1 liposomes (Mirus). Cells were transfected with 100 ng/well Hes1 luciferase, Hes5 luciferase, or 4xCSL luciferase plasmids which produce luciferase in response to Notch pathway activation and 30 ng/well CMV-β-gal plasmid. Co-transfection of a CMV-Beta-Galactosidase construct was used to normalize data for transfection efficiency and potential cell death/proliferation. For AZM treated

cells, cells were treated with 10 µM AZM for 24 h before being sacrificed on the next day. Cells were lysed 48 h after transfection using passive lysis buffer (Promega) and lysates were submitted to a luciferase reporter assay as per manufacturer's protocol and analyzed using a Promega© Glomax Multi Detection System luminometer. Luciferase activity was normalized to Beta-Galactosidase activity and values were reported as fold change to control. All conditions were performed in triplicate for each independent experiment.

Immunoprecipitations

Cells were washed twice with 1x PBS, scraped up on ice, and transferred to 1.7 ml tubes followed by pelleting by centrifugation whereupon supernatant was removed. Cells were lysed for 30 minutes in 500 µL of co-immunoprecipitation buffer (200 mM KCL, 25 mM Hepes, 1% NP-40, 20 mM NaF, 1 mM Na-orthovanadate, 0.2 mM EGTA, 1x protease arrest [G Biosciences], 1x phosphatase inhibitor cocktail II [Alfa Aesar], 20 µM nicotinamide, pH 7.5) and sonicated. Lysates were then subjected to centrifugation and whole cell lysate samples were generated using the 50 µL of the total lysate, to which 4x SDS page lysis buffer was added, followed by vortexing and boiling for 5 minutes. For immunoprecipitation of FLAG tagged proteins, the remaining 450 µL of lysate was incubated with 20 µL of anti-FLAG G1 affinity resin (GenScript) overnight on a tube rotator at 4°C. After incubation, washing was performed to remove non-specific binding contaminates. Briefly, samples were centrifuged, supernatant removed, washed with 500 µL of co-immunoprecipitation buffer, and allowed to rotate at 4°C on a tube rotator for 5 minutes. These steps were repeated three times, before a final centrifugation and removal of supernatant, after which 50 µL of 1x SDS page lysis buffer was added to the pellets before vortexing and boiling for 5 minutes.

Western Blotting

Cells were lysed in 1x SDS page lysis buffer and boiled for 5 minutes. Proteins were separated through SDS page on 6%-15% polyacrylamide gels and blotted onto nitrocellulose membranes. Membranes were blocked in TBS-T (140 mM NaCL, 25 mM Tris-HCL, pH 7.4, 0.1% Tween-20) with 5% bovine serum albumin for 1 hour at room temperature. Membranes were incubated with primary antibody (1:250, 1:500, or 1:1000) overnight on a rotator at 4°C. After incubation, membranes were washed 3 x 10 minutes in TBS-T before 1 hour incubation in secondary antibodies at room temperature. Horseradish peroxidase conjugated secondary antibodies were used at a concentration of 1:5000. After incubation with secondary antibodies, proteins were detected by enhanced chemiluminescence.

Acknowledgements

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Conflicts of Interest

The authors declare no conflicts of interest.

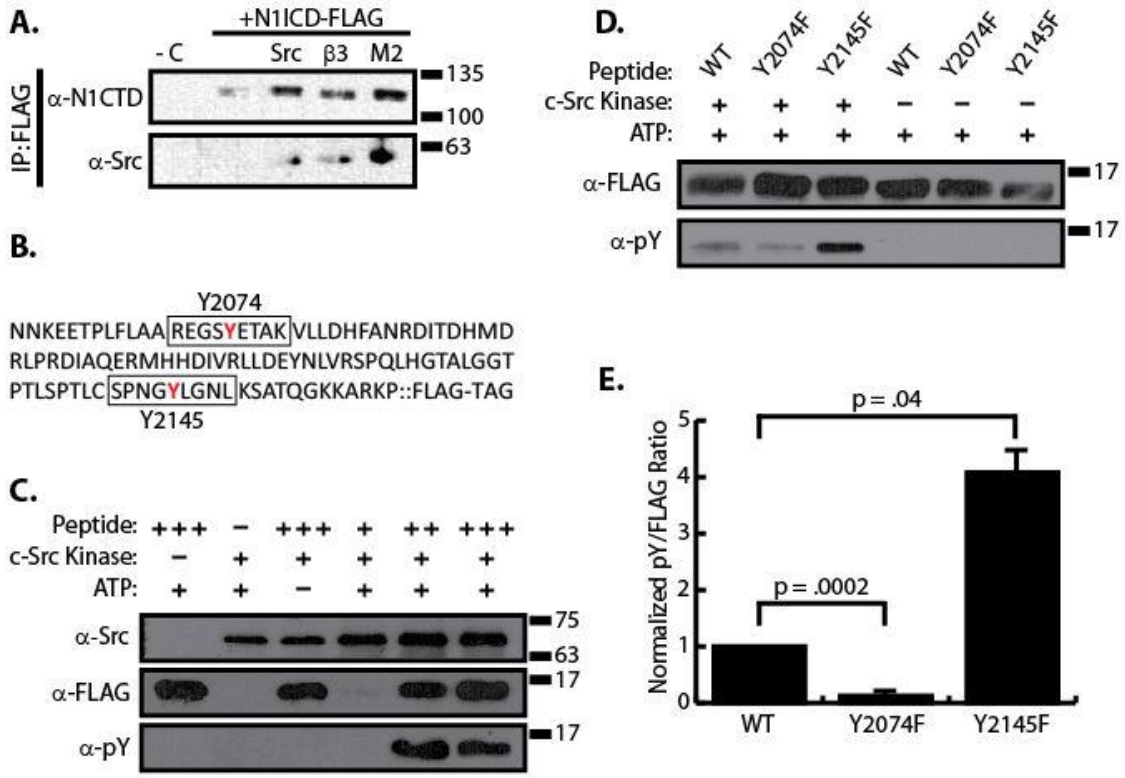

Figure 2.1: c-Src Phosphorylates the N1ICD Ankyrin Domain

(A) Western blot of N1ICD co-immunoprecipitation experiment. –C denotes a no N1ICD transfection control. Src represents c-Src overexpression. β3 represents β3 integrin overexpression. M2 denotes MAGP2 overexpression. (B) Sequence of amino acids 2058-2161 of N1ICD used in subsequent *in vitro* kinase assays. Y2074 and Y2145 are outlined. (C) *In vitro* c-Src kinase assay with FLAG tagged peptide containing amino acids 2058-2161 of N1ICD. (D) *In vitro c*-Src kinase assay comparing phosphorylation of Y2074F and Y2145F mutant forms of the 2058-2161 peptide. (E) Densitometry analysis comparing the level of phosphotyrosine signal from the +Src lanes of panel C combined with multiple identical experiments, n=4. Normalization was achieved by dividing α-pY by α-FLAG signal. Student's t-test was performed to determine statistical significance. P-values are reported as p=#. In all panels western blots depict representative images from experiments that were replicated at least three independent times.

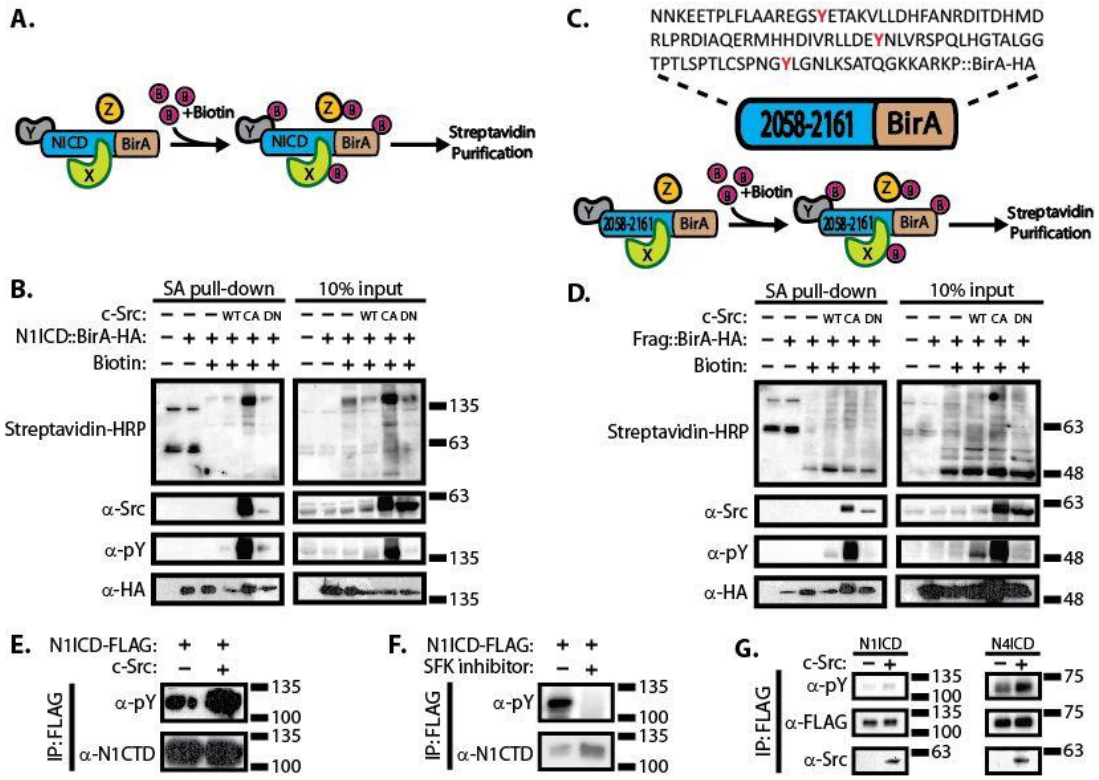

Figure 2.2: Phosphorylation of Tyrosine Residues on N1ICD are SFK Dependent

(A) Diagram of BioID experiment. In the presence of biotin, the NICD::BirA fusion protein biotinylates nearby proteins which can be affinity captured through streptavidin purification. (B) Western blot analysis of affinity captured material from BioID experiment using a N1ICD::BirA-HA fusion protein. Expression of Src variants is denoted as WT = wild-type, CA = constitutively active, and DN = dominant negative. (C) Amino acid sequence of 2058-2161::BirA-HA fusion protein containing a 104 amino acid fragment of N1ICD fused to a biotin ligase. (D) Western blot analysis of affinity captured material from BioID experiment using the 2058-2161::BirA-HA fusion. (E) Western blot analysis of immunoprecipitated N1ICD in the presence or absence of c-Src overexpression. (F) Western blot analysis of immunoprecipitated N1ICD under conditions SFK inhibition (AZM) and control. (G) Western blot analysis of immunoprecipitated N1ICD and N4ICD

in the presence or absence of c-Src overexpression. In all panels, western blots depict representative images from experiments that were replicated at least three independent times.

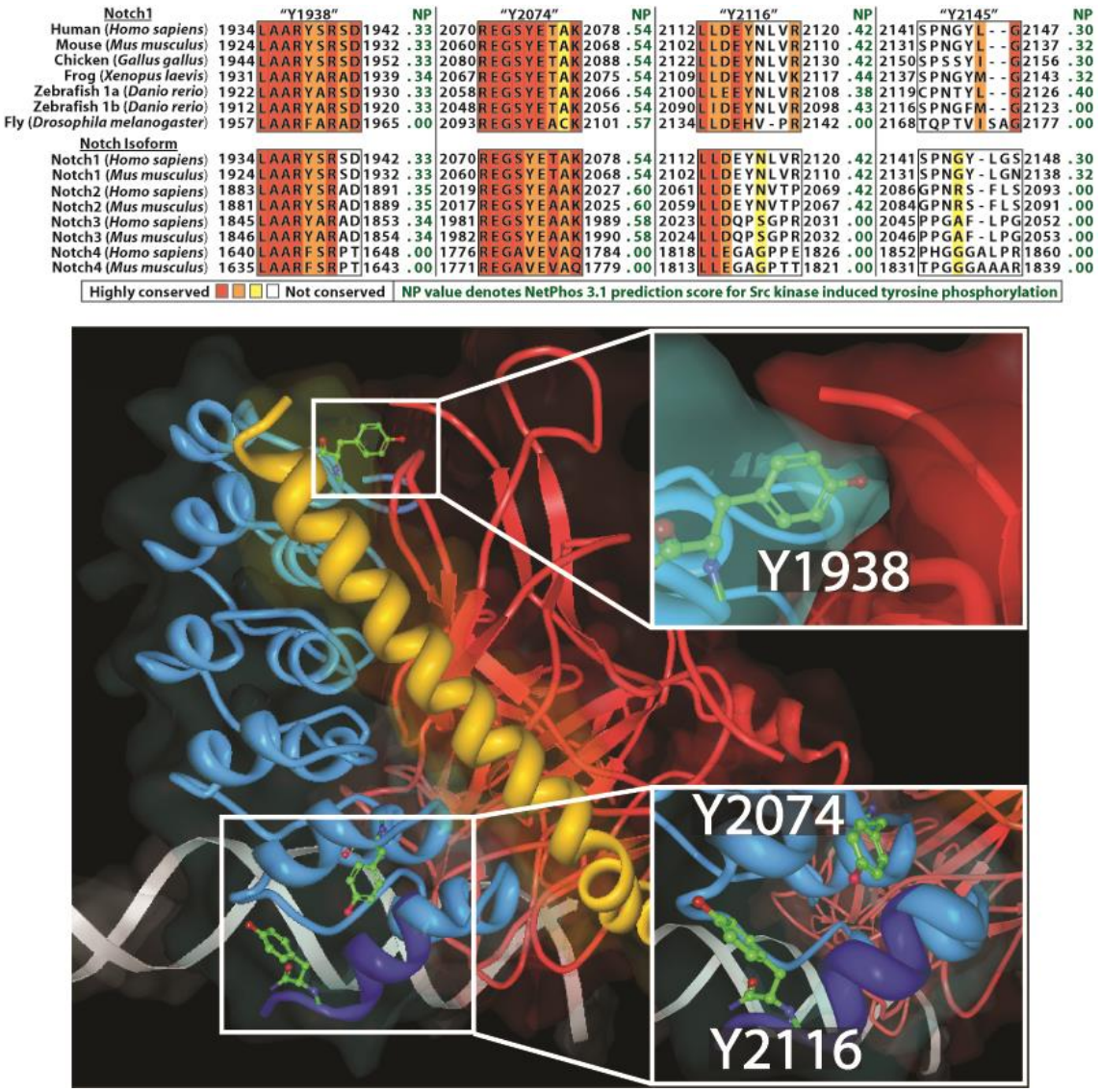

Figure 2.3: Sequence Alignment and Crystal Structure Analysis of NICD Phosphotyrosine Sites

(A) Sequence alignment of four tyrosine sites within the Notch intracellular domain comparing conservation of sites across different species and different Notch isoforms. Red = highly conserved, white = not conserved. NP denotes NetPhos 3.1 prediction scores for likelihood of c-Src phosphorylation of the sequence. (B) Partial crystal structure analysis of Notch ternary transcriptional complex. N1ICD ankyrin domain = blue (EP region = dark blue), MAML basic domain = yellow, RBPJ = red. Insets depict where Y1928, Y2074, and Y2116 are found in the N1ICD ankyrin domain.

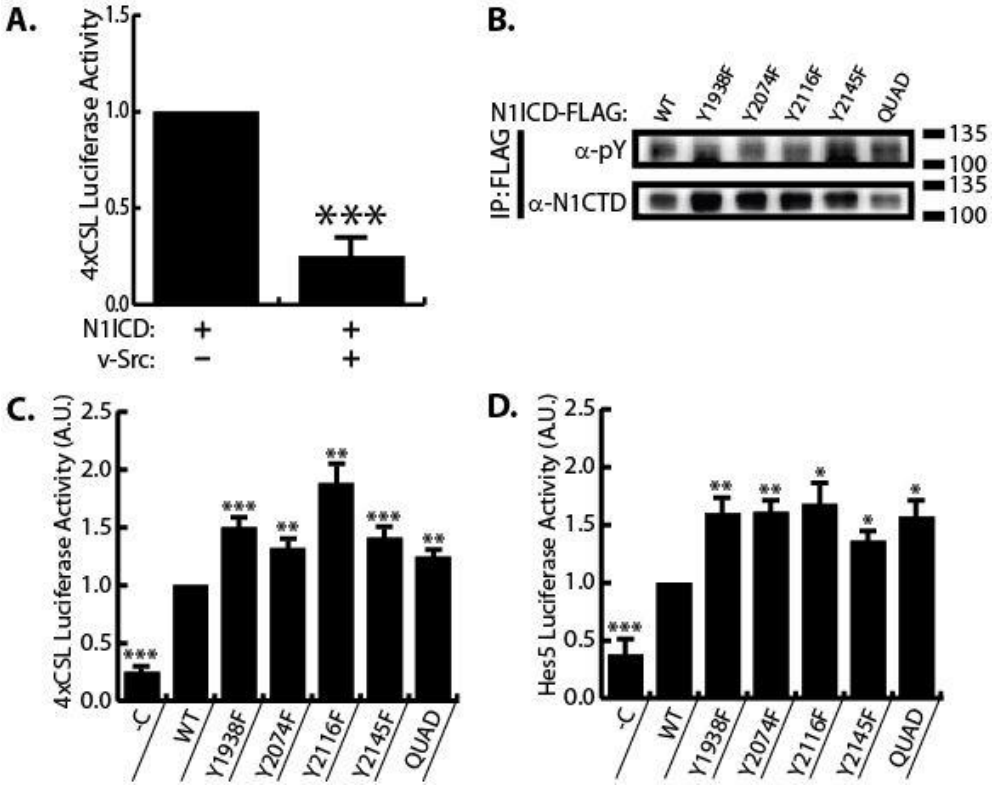

Figure 2.4: SFKs Reduces N1ICD Transcriptional Activity

(A) 4xCSL luciferase assay in 293T cells in the presence or absence of v-Src overexpression, n=6. (B) Anti-phosphotyrosine western blot analysis of immunoprecipitated N1ICD tyrosine mutants. "Quad" denotes a Y1938F, Y2074F, Y2116F, Y2145F mutant N1ICD. Image is a representative image from experiments that were replicated three independent times (C) 4xCSL luciferase assay in 293T cells expressing WT and mutant N1ICDs, n=16. –C denotes non-N1ICD transfected control. (D) Hes5 luciferase assay in 293T cells expressing WT and mutant N1ICDs, n=7. –C denotes non-N1ICD transfected control. For A-C, student's t-test was performed to determine statistical significance compared to DMSO control. For E-F, student's t-test was performed to determine statistical significance compared to WT control. P-values are reported as

*<.05, **<.01, ***<.001. In all panels, western blots depict representative images from experiments that were replicated at least three independent times.

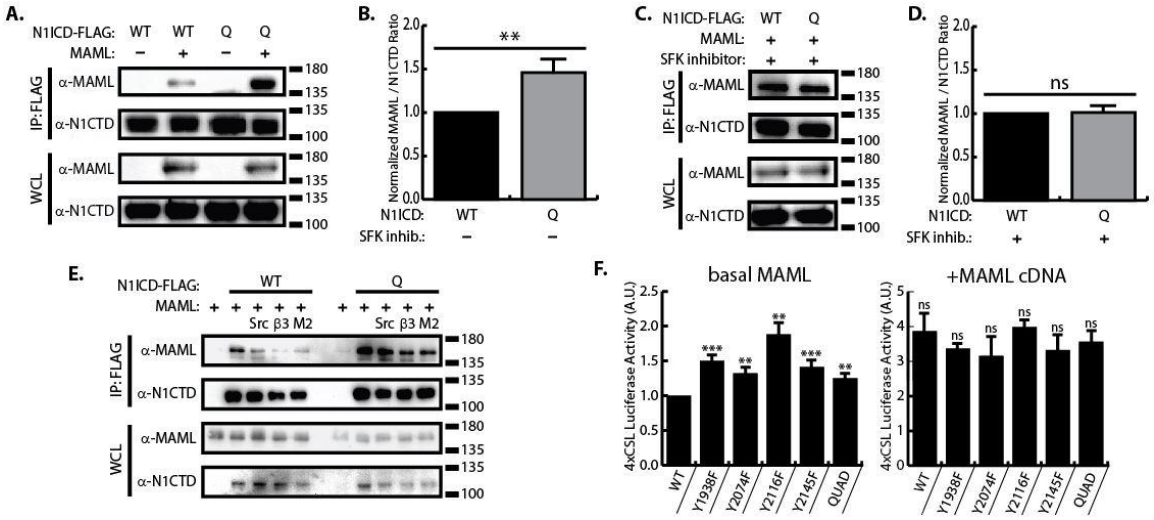

Figure 2.5: N1ICD Tyrosine Mutants Display Enhanced MAML Binding

(A) Western blot analysis of N1ICD/MAML co-immunoprecipitations comparing WT N1ICD and Quad N1ICD. (B) Western blot analysis of N1ICD/MAML co-immunoprecipitations comparing WT N1ICD and Quad N1ICD under condition of SFK inhibition (AZM). (C) Densitometry comparing levels of MAML co-immunoprecipitation with WT vs Quad N1ICD under conditions of normal SFK activity (n=4) and SFK inhibition (n=3). (D) Western blot analysis of N1ICD/MAML co-immunoprecipitations in the presence of c-Src, β3 integrin, or MAGP2 (M2), (n=2). (E) 4xCSL luciferase assay comparing transcriptional activity WT N1ICD and Quad N1ICD under conditions of RBPJ and MAML coexpression, n=5. (F) 4xCSL luciferase assay comparing transcriptional activity WT N1ICD and Quad N1ICD under conditions of RBPJ and MAML coexpression and basal RBPJ/MAML expression, n=5. (G) 4xCSL luciferase assay comparing transcriptional activity of N1ICD tyrosine mutants in the presence or absence of MAML overexpression, n=8. In all panels, western blots depict representative images from experiments that were replicated at least three independent times. Where applicable,

student’s t-test was performed to determine statistical significance. P-values are reported as *<.05, **<.01, ***<.001, ns>.05.

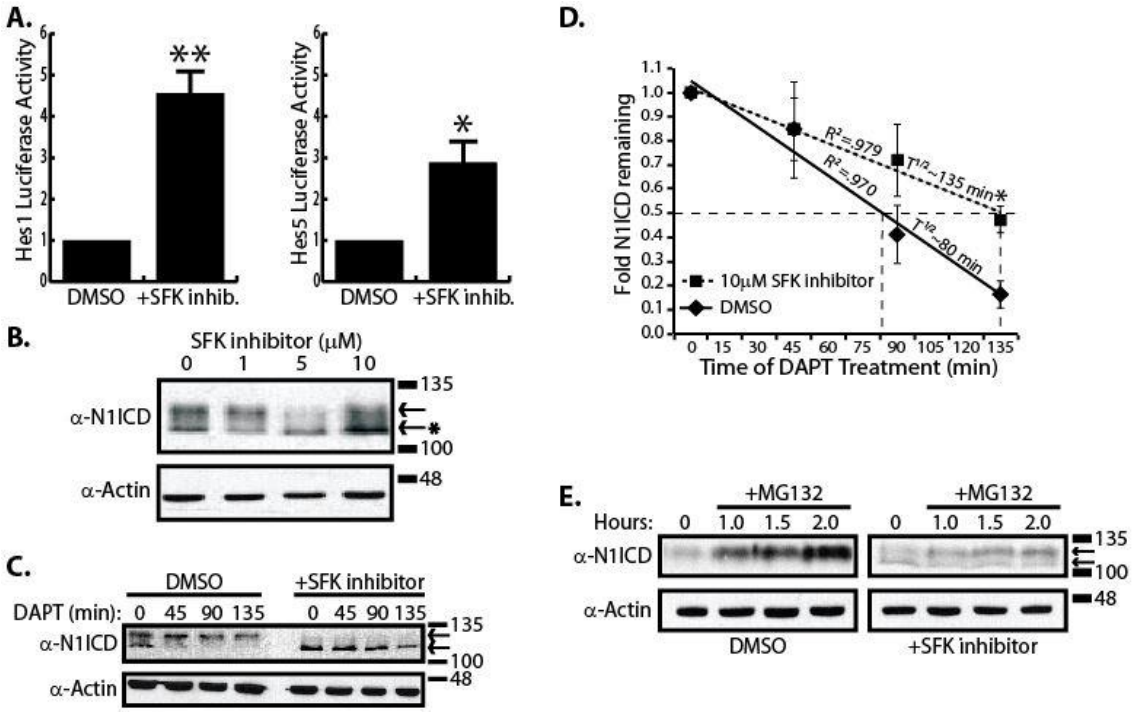

Figure 2.6: SFKs Decrease the N1ICD half-life

(A) Hes1 and Hes5 luciferase assay in HMEC-1 cells in the presence or absence of SFK inhibitor, n=4. (B) Western blot of HMEC-1 lysates under conditions of increasing concentrations of SFK inhibitor (AZM475271). The arrows denote two distinct N1ICD species. The asterisk points out that SFK inhibition leads to accumulation of a higher mobility N1ICD species. (C) Western blot analysis of N1ICD half-life under conditions of SFK inhibition and DMSO control. The arrows denote two distinct N1ICD species. (D) Densitometry and half-life analysis of western blot from panel C**.** Data represents n=4 independent experiments and * indicates $p<.05$, students t-test. (E) Western blot analysis of N1ICD accumulation under condition of proteasomal inhibition using MG132.

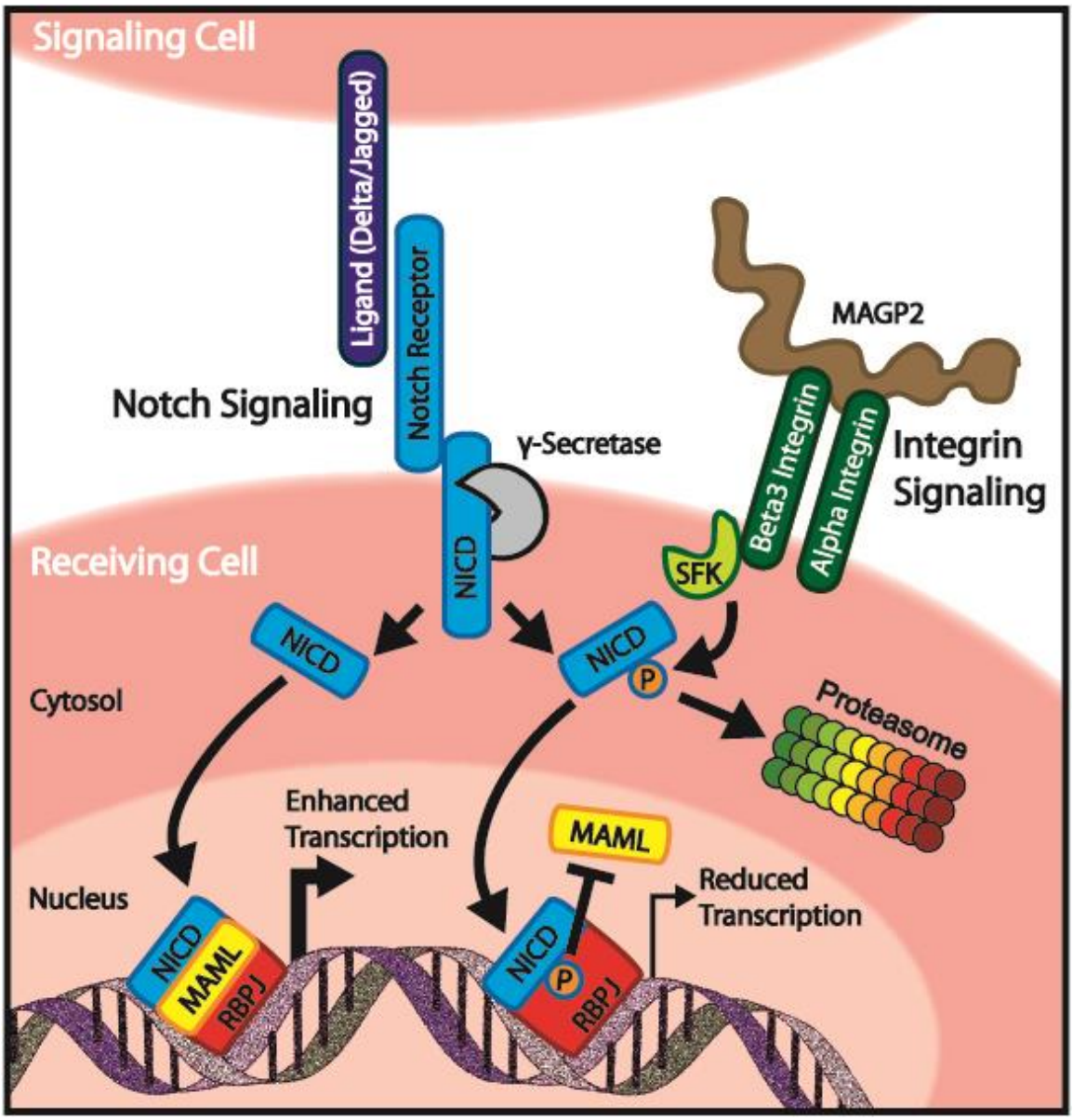

Figure 2.7: SFK Regulation of the Notch Signaling Pathway

Proposed mechanism of the regulation of Notch signaling by Src family kinases. Integrin-induced SFK activity serves to phosphorylate the Notch intracellular domain. This phosphorylation leads to decreased recruitment of MAML to the Notch transcriptional complex and subsequent reduction in target gene transcription. Phosphorylation of the Notch intracellular domain also leads to the proteasomal degradation of the protein.

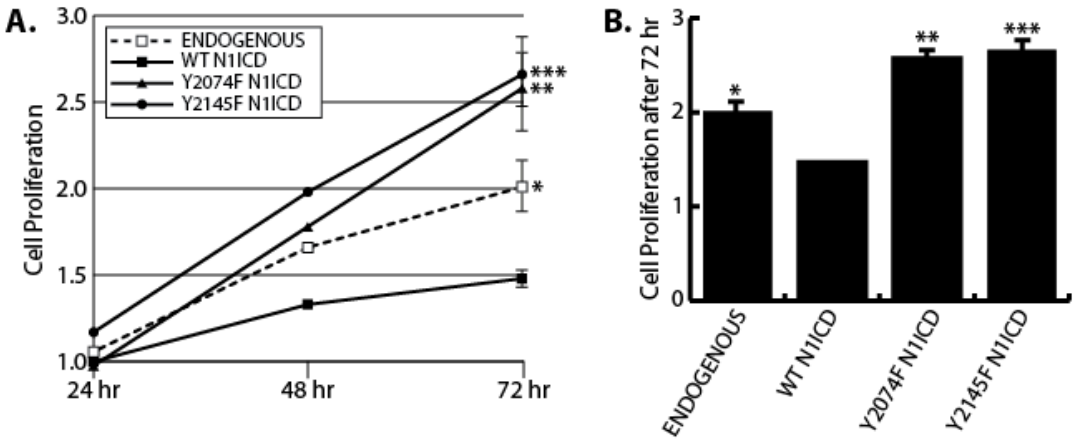

Figure 2.S.1: N1ICD Tyrosine Mutants Confer Enhanced Endothelial Cell Proliferation

(A) Proliferation of HMEC-1 cells overexpressing WT N1ICD, Y2074F N1ICD, and Y2145F N1ICD was tracked for 72 hours. HMEC-1 cells expressing endogenous N1ICD levels were also compared, n=5. (B) Bar graph of HMEC-1 proliferation after 72hours. A student's t-test was performed to determine statistical significance compared to WT N1ICD overexpressing cells. P-values are reported as *<.05, **<.01, ***<.001

CHAPTER THREE

A Comparison of Resveratrol and Other Polyphenolic Compounds on Notch Activation and Endothelial Cell Activity

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Abstract

Resveratrol is a polyphenolic compound produced by plants which makes its way into the human diet through plant-based foods. It has been shown to provide many health benefits, helping to ward off age-related diseases and promoting cardiovascular health. Additionally, resveratrol is a potent activator of the Notch signaling pathway. While resveratrol receives the most attention as a polyphenolic nutraceutical, other compounds with similar structures may be more potent regulators of specific cellular processes. Here, we compare resveratrol, apigenin, chrysin, genistein, luteolin, myricetin, piceatannol, pterostilbene, and quercetin for their ability to regulate Notch signaling. In addition, we compare the ability of these polyphenolic compounds to regulate endothelial cell proliferation and migration. Out of these compounds we found that resveratrol is the best activator of Notch signaling, however, other similar compounds are also capable of stimulating Notch. We also discovered that several of these polyphenols were able to inhibit endothelial cell proliferation. Finally, we found that many of these polyphenols are potent inhibitors of endothelial migration during wound healing assays. These findings provide the first side-by-side comparison of the regulation of Notch signaling, and endothelial cell proliferation and migration, by nine polyphenolic compounds.

Introduction

Our understanding of the role in which diet shapes human health is constantly evolving. A nutraceutical is a compound found naturally in food which has medicinal benefits. The use of nutraceuticals to combat disease and improve health is an ever-expanding area of research. One class of molecules, known as polyphenols, are derived from various plants and renowned for their health benefits. Major sources of dietary

polyphenols include tea, wine, coffee, chocolate, vegetables, and beer [145]. However, the molecular mechanisms by which these polyphenolic compounds affect human health are unclear.

Perhaps the best-studied polyphenol, resveratrol (RSVT), has been characterized for its anti-aging [89], anti-cancer [82,83], anti-oxidant [81], anti-inflammatory [88], and neuroprotective [90–92] properties. Trans-RSVT is a polyphenolic stilbene derived from plants, such as grapes and peanuts [71]. In plants, it acts as a phytoalexin, protecting plant tissues against pathogenic assault [72]. Once ingested by humans RSVT is thought to promote many favorable physiological processes such as the maintenance of vascular health, prevention of atherosclerosis [76,146], inhibition of tumor angiogenesis [84–87,147], and improvement of cardiovascular function [80,148,149]. In addition to RSVT, many other polyphenols have been described in the literature that have demonstrated similar activities to RSVT. While there exists a vast literature describing the molecular mechanisms by which RSVT governs endothelial cell behavior, little is known about how other polyphenols perform similar roles.

RSVT, has been heavily-linked with the Notch cell signaling pathway [104–106]. Despite the clear association between RSVT and Notch, conflicting results from different cell lines suggest that RSVT can enhance or suppress Notch in a cell type dependent manner. Being a form of juxtacrine cell communication, Notch signaling begins when the transmembrane Notch receptor of one cell (i.e. signal receiving cell) is bound by a transmembrane ligand on an adjacent cell (i.e. signal sending cell). A force of 4-12 pN [150] is applied to the Notch receptor through ligand endocytosis in the signal sending cell. This pulling force exposes cleavage sites and facilitates proteolytic processing of the Notch

receptor, first by ADAM (A Disintegrin and Metalloproteinase) and then by γ-secretase [4]. These cleavage events result in the release of the Notch intracellular domain (NICD), which then travels to the nucleus where it induces transcription of Notch target genes. Hairy and enhancer of split (HES) genes and Hairy/enhancer of split related with TYRPW motif (HEY) genes are well-known examples of Notch target genes [20].

Here, we compare RSVT and several other polyphenols for their ability to regulate Notch signaling and endothelial cell proliferation and migration. We chose to compare the effects of RSVT with apigenin, chrysin, genistein, luteolin, myricetin, piceatannol, pterostilbene, and quercetin in order to see if these molecules, which share similar structures, behave similarly to one another. We found that the majority of these polyphenols, but not all, enhanced Notch signaling to varying degrees. Similarly, the majority of tested polyphenols, but not all, inhibited cell proliferation and migration. These results should prove useful to other researchers seeking to harness the biochemical properties of polyphenols for therapeutic uses.

Results

Resveratrol Induces Notch Target Gene Transcription

A robust literature exists connecting RSVT with the Notch signaling pathway. The association between Notch and RSVT was first established when Pinchot et al. employed a high throughput chemical screening method to screen 7,264 compounds in order to identify Notch activating compounds [106]. Out of all the compounds screened in this study, RSVT was identified as the strongest Notch activator. RSVT has been shown to induce apoptosis of endothelial cells and it was therefore important to first determine a sub-apoptotic concentration of RSVT in which to examine Notch activation. HMEC cells were

cultured in 1 (.23μg/ml), 10 (2.3μg/ml), or 100 (23μg/ml) μM concentrations of RSVT and apoptosis was monitored by western blot analysis of cleaved caspase 3 (Figure 3.1A). Similar to previous studies [151], we found that 100μM solutions of RSVT induced caspase 3 cleavage, but 1-10μM RSVT showed no evidence of apoptosis. To determine the effect of RSVT on Notch signaling, we transfected Human Aortic Vascular Smooth Muscle Cells (HAVSMC) and Human Microvascular Endothelial Cells (HMEC-1) with Notch responsive Hes-1, Hes-5, and 4X-CSL-luciferase constructs and incubated these cells in the presence of 1-10μM RSVT. In both cell types, and across all three Notch-responsive reporters, RSVT activated Notch target gene transcription in a dose-dependent manner (Figure 3.1B and 3.1C). These results demonstrated that RSVT controls Notch independent of apoptosis and established a model on which we could examine additional polyphenols for Notch regulatory activity.

Other Polyphenols Induce Notch Target Gene Transcription

A robust literature exists connecting RSVT with the Notch signaling pathway. The association between Notch and RSVT was first established when Pinchot et al. employed a high throughput chemical screening method to screen 7264 compounds in order to identify Notch activating compounds [106]. Out of all the compounds screened in this study, RSVT was identified as the strongest Notch activator. RSVT has been shown to induce apoptosis of endothelial cells and it was therefore important to first determine a sub-apoptotic concentration of RSVT in which to examine Notch activation. HMEC-1 cells were cultured in 1 (.23 μg/ml), 10 (2.3 μg/ml), or 100 (23 μg/ml) μM concentrations of RSVT in the presence or absence of the Notch inhibitor DAPT and apoptosis was monitored by western blot analysis of cleaved caspase 3 (Figure 1A). Similar to previous

studies [151], we found that 100 μM solutions of RSVT induced caspase 3 cleavage, but 1-10 μM RSVT showed no evidence of apoptosis. Notch inhibition did not induce apoptosis at 0-10 μM RSVT concentration, and did not reverse or enhance the stimulation of apoptosis by 100 μM RSVT treatments. To determine the effect of RSVT on Notch signaling, we transfected Human Aortic Smooth Muscle Cells (HAVSMC) and Human Microvascular Endothelial Cells (HMEC-1) with Notch responsive Hes1, Hes5, and 4xCSL-luciferase constructs and incubated these cells in the presence of 1-10 μM RSVT. In both cell types, and across all three Notch-responsive reporters, RSVT activated Notch target gene transcription in a dose-dependent manner (Figure 1B and 1C). These results demonstrated that RSVT controls Notch independent of apoptosis and established a model on which we could examine additional polyphenols for Notch regulatory activity.

Polyphenolic Regulation of Endothelial Cell Proliferation

There are conflicting reports on the role RSVT plays in the regulation of cellular proliferation [83,152] and there has not been a head-to-head comparison of polyphenol effects on endothelial cell proliferation. Given our results that many polyphenols control Notch signaling, we next compared the effect of RSVT and the other polyphenols on endothelial cell proliferation in the presence or absence of elevated Notch signaling. For this study we used HMEC-1 cells which had been transduced with lentiviral particles encoding N1ICD under the control of a doxycycline inducible promoter (HMEC-1-N1ICD cells). HMEC-1-N1ICD cells were treated with polyphenolic compounds in the presence (i.e. high Notch activity) or absence (i.e. basal Notch activity) of doxycycline and cell proliferation was monitored daily by WST-1 over the course of 72 hours (Figure 3.3). In agreement with previous reports, overexpression of N1ICD reduced cell proliferation [153,154]. Under

conditions of basal Notch activity (i.e. no doxycycline induction) only luteolin and piceatannol were capable of inhibiting cellular proliferation. However, in the presence of elevated Notch activity, all of the polyphenols, except pterostilbene and quercetin, were demonstrated to cause a reduction in cellular proliferation compared to DMSO control under conditions of high Notch activity. Overall, RSVT reduced cellular proliferation by 22%, but luteolin and piceatannol were the best inhibitors of proliferation, displaying a 42% and 46% reduction in cellular proliferation respectively. From this evidence, we conclude Notch activity is required for the inhibition of proliferation by resveratrol, apigenin, chrysin, genistein, and myricetin. Whereas, high Notch activity potentiates, but is not required for the inhibition of proliferation by luteolin, and piceatannol. Finally, pterostilbene and quercetin did not significantly affect proliferation in our model system.

Polyphenolic Regulation of Endothelial Wound Healing

RSVT and several other polyphenols have been shown to decrease endothelial migration however a head-to-head comparison of how these polyphenols affect cell migration has not been reported. Therefore, we sought to compare the effect of various polyphenols on endothelial cell migration using a wound closure scratch assay. To this end, HMEC-1 cells were grown to confluency and treated with 10μM concentrations of various polyphenols for 24 hours prior to monolayer wounding. After wounding, cells were allotted 18 hours for migration, followed by subsequent wound healing quantification as a percent area of wound closure. As previously observed [155], we found that RSVT significantly decreased endothelial cell migration (Figure 3.4B). We also found that apigenin, chrysin, genistein, luteolin, myricetin, and piceatannol also significantly inhibited endothelial cell migration. Of these compounds, luteolin had the largest effect on migration, with only 30%

wound closure after 18 hours (Figure 3.4A). Pterostilbene and quercetin did not reduce cell migration in a statistically significant manner.

Discussion

Notch signaling, endothelial cell proliferation, and endothelial cell migration are collectively important for angiogenesis and many polyphenolic compounds have been identified as regulators of angiogenesis. However, there has been no direct comparison of polyphenols on these cellular activities. Therefore, the goal of this study was to compare several polyphenolic compounds for their ability to control Notch signaling, endothelial cell proliferation, and endothelial cell migration. Throughout this study, we performed several side-by-side comparisons of the biological potency of nine polyphenolic compounds. Despite the highly similar structures of these polyphenols, we have found that some, but not all, of these natural products are activators of Notch signaling or inhibitors of endothelial cell proliferation and migration. Two of the polyphenolics (pterostilbene and quercetin) failed to show biological activity in any of the experimental systems we examined.

Polyphenolic compounds fall into several categories according to their structure [156]. In this work, we examined several polyphenolics with similar structures including the stilbenes RSVT, piceatannol, and pterostilbene and the flavonoids apigenin, chrysin, genistein, luteolin, myricetin, and quercetin. Many polyphenolic compounds have been shown to control Notch signaling, however a direct comparison of the Notch regulating activities of these compounds has not been performed. Compared to other polyphenols, RSVT has received the most attention for its role in regulating the Notch cell signaling pathway [104,105]. Through our analysis, it is clear that RSVT warrants its attention as a robust

polyphenolic activator of Notch as it demonstrated the greatest Notch inducing activity. While RSVT was the most potent Notch activator out of the polyphenols we tested, apigenin, chrysin, genistein, and piceatannol were also able to regulate Notch to lesser degrees. Our results are consistent with previous findings showing that chrysin [107], and genistein [108] can control Notch, however the findings that apigenin and piceatannol can also control Notch is novel. In contrast, two other polyphenols which have been previously identified as Notch regulators, luteolin [109,110] and pterostilbene [111], did not act as Notch regulators in our hands. Finally, myricetin and quercetin have not been linked to Notch activity, and our results do not support a Notch regulatory role for these polyphenols. Taken together, our results show that polyphenolic compounds are a promising source of Notch regulators, but also provide a warning that cell-type specific responses to polyphenols may account for conflicting data concerning these molecules.

While we found that RSVT works synergistically with Notch signaling to suppress endothelial cell proliferation, apigenin, chrysin, genistein, luteolin, myricetin, and piceatannol also demonstrated similar activity. In fact, out of the compounds we tested, luteolin and piceatannol were the most potent inhibitors of endothelial cell proliferation. Since luteolin and piceatannol have previously been identified as an anti-angiogenic agents [157,158], our identification of these compounds as suppressors of endothelial cell proliferation may provide mechanistic insight into their anti-angiogenic properties. Additionally, the effects these molecules have on cellular proliferation may expand beyond endothelium. Future work should assess the effectiveness of these polyphenols for their ability to suppress tumor growth. Previous work has found that luteolin [109,159] suppresses cell migration. In accordance, luteolin was the most potent inhibitor of endothelial migration

we tested. While our identification of these molecules as inhibitors of endothelial migration is relevant to angiogenesis, future work should compare polyphenolic regulation of cancer cell migration for their potential use as anti-metastatic agents.

These results provide the first side-by-side comparison of nine polyphenolic compounds in their ability to regulate Notch signaling, and endothelial cell proliferation and migration. Angiogenic growth requires tight coordination of Notch signaling, endothelial cell proliferation, and migration in endothelial cells [160]. This study has demonstrated that polyphenols act as modulators of angiogenic processes (Summarized in Figure 3.5). Future work should expand upon this analysis, comparing how polyphenols behave in more sophisticated angiogenic models. Additionally, while angiogenesis is an essential step in tumor progression, performing a comparative analysis of polyphenolic treatment in the context of cancer cell behavior is necessary, and would complement this endothelial-based study, in order to gain a better scope of the use of polyphenols as anti-cancer agents. More broadly, our findings have laid the groundwork for the potential use of polyphenols as modulators of any developmental or disease process in which Notch signaling, cellular proliferation, and/or cellular migration are involved. While further exploration is necessary, we have shown that polyphenols are promising anti-angiogenic compounds which may facilitate natural product based cancer-related therapies in the future.

Materials and Methods

Cell Culture

293T cells were cultured in Dulbecco's Modified Eagle's medium (DMEM, Mediatech) supplemented with 10% fetal bovine serum (FBS) and 1x pen-strep. HAVSMC

cells were cultured in EBM2 basal media (Lonza) supplemented with EGM2 growth media and 10% FBS. HMEC-1 cells were cultured in MCDB131 supplemented with 10% fetal bovine serum (FBS), 10 ng/ml epidermal growth factor, and 1 µg/ml hydrocortisone. Cells were grown in 10 cm plates and passaged before reaching confluency.

Materials

Trans-RSVT was purchased from Caymen Chemicals. Apigenin, chrysin, luteolin, and quercetin were purchased from Alfa Aesar. Myricetin, piceatannol, and pterostilbene were purchased from Enzo Life Sciences. Genistein and doxycycline were purchased from Tokyo Chemical Industry. All drugs were dissolved in DMSO.

Plasmids

The N1ICD construct (Addgene #20183) was a gift from Raphael Kopan and contains amino acids Val1744 to Lys 2531 of the mouse Notch1 intracellular domain with a 3xFLAG N-terminal tag [139]. N1ICD was inserted into a doxycycline inducible lenti viral destination vector, pCW57.1 (Addgene #41393, a gift from David Root) in order to construct a N1ICD lenti viral expression vector. The 4xCSL luciferase construct (Addgene #41726) was a gift from Raphael Kopan and contains 4 tandem repeats of the high affinity CSL binding sites (5′CGTGGGAA3′) while transcribing for firefly luciferase [139]. The Hes1 luciferase construct was a gift from Jan Jensen and consists of nucleotides -2553 to -201 relative to the murine Hes1 transcriptional start site while transcribing for firefly luciferase. The Hes5 luciferase construct (Addgene #41724) was a gift from Ryoichiro Kageyama and Raphael Kopan and contains the murine Hes5 promoter (-800 to +73) relative to the Hes5 transcriptional start site while transcribing for firefly luciferase [143].

Apoptosis Assays

HMEC-1 cells were seeded into 6 well plates at a density of 150,000 cells/well and allowed to grow for 24 hours. Cells were then treated with 0-100 μM concentrations of RSVT and/or 10 μM DAPT and allowed to incubate for 24 hours. After incubation, cell culture media was collected and cells were lysed in SDS page lysis buffer. Cell culture media was pelleted and added to cell lysates. For a positive control for apoptosis, cells were exposed to 15 minutes of ultraviolet light before lysing. Apoptosis was monitored through western blotting for the presence of cleaved caspase 3.

Western Blotting

Cells were lysed in 1x SDS page lysis buffer and boiled for 5 minutes. Proteins were separated through SDS page on 6%-15% polyacrylamide gels and blotted onto nitrocellulose membranes. Membranes were blocked in TBS-T (140 mM NaCL, 25 mM Tris-HCL, pH 7.4, 0.1% Tween-20) with 5% bovine serum albumin for 1 hour at room temperature. Membranes were incubated with primary antibody (1:250, 1:500, or 1:1000) overnight on a rotator at 4°C. After incubation, membranes were washed 3 x 10 minutes in TBS-T before 1 hour incubation in secondary antibodies at room temperature. Horseradish peroxidase conjugated secondary antibodies were used at a concentration of 1:5000. After incubation with secondary antibodies, proteins were detected by enhanced chemiluminescence. Primary antibodies against β-actin (sc-47778) were purchased from Santa Cruz Biotechnology. Primary antibodies against caspase 3 (#9662) were purchased from Cell Signaling Technology.

Luciferase Assays

HMEC-1 cells were seeded into 24-well plates at a density of 25,000 cells/well. 293T cells were seeded into 24-well plates at a density of 50,000 cells/well. The following day, cells were transfected using LT-1 liposomes (Mirus). Cells were transfected with 100 ng/well Hes1 luc, Hes5 luc, or 4xCSL luc plasmids which produce luciferase in response to Notch pathway activation. Co-transfection of a 30 ng/well CMV-Beta-Galactosidase construct was used to normalize data for transfection efficiency and potential cell death/proliferation. Cells were lysed 48 hours after transfection using passive lysis buffer (Promega) and lysates were used to perform a luciferase reporter assay as per manufacturer's protocol and analyzed using a Promega© Glomax Multi Detection System luminometer. Luciferase activity was normalized to Beta-Galactosidase activity and values were reported as fold change to control. All conditions were performed in triplicate for each independent experiment.

Proliferation Assays

HMEC-1 cells were lenti viral transduced with doxycycline inducible constructs that contain WT N1ICD under the control of a CMV promoter. Cells were treated with 10 µM polyphenols and seeded into 96-well plates at a density of 2,500 cells/well. Doxycycline was added to appropriate wells in order to induce N1ICD overexpression. After 24, 48, and 72 hours, a triplicate of wells for each condition was analyzed for cell density using a WST-1 colorimetric assay. Absorbance spectra was measured at 410 nm using a BioTek Synergy Mx plate reader.

Scratch Assays

HMEC-1 cells were seeded into 24-well plates at a density of 25,000 cells/well. Upon reaching confluency cells were treated with 10 µM and incubated for 24 hours. Wounds were made using 200 µL pipette tips. Wells were washed 3 times with 1x PBS and media/polyphenol treatments were replaced. Cells were place in on on-stage incubator (5% CO_2, 37°C). Images were captured using an EVOS FL auto microscope which automatically captured images every 30 minutes for 18 hours. After 18 hours, images were analyzed and wound area was calculated using ImageJ. Percent area of wound closure was calculated using the following formula, (area of wound at 0h - area of wound at 18h) / (area of wound at 0h X 100).

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Conflicts of Interest

The authors declare no conflicts of interest.

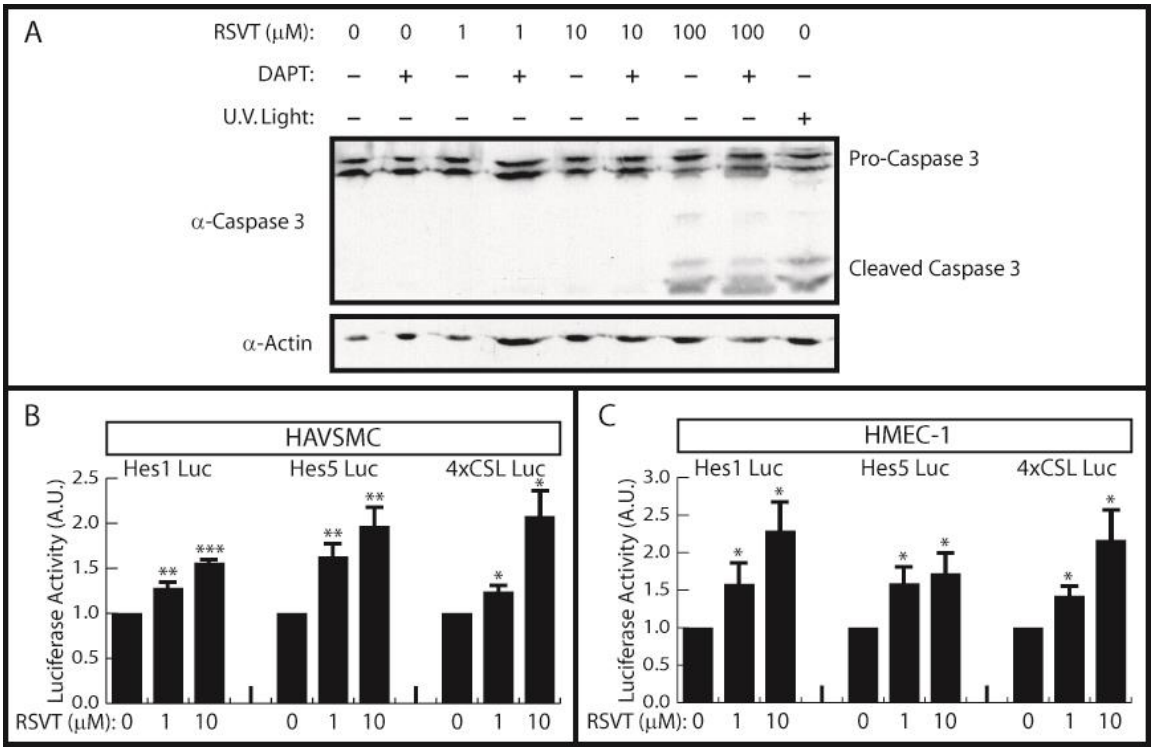

Figure 3.1: Resveratrol is a Potent Stimulator of Notch Signaling

(A) RSVT does not induce HMEC apoptosis at 1-10 μM. HMEC cells were treated with increasing concentrations of RSVT +/- DAPT. Cellular apoptosis was indirectly examined by monitoring cleavage of cleaved caspase 3 from pro-caspase 3 by western blot. HMEC cells were treated with UV as a positive apoptosis control and protein loading was monitored by western blotting for α-actin. (B) Notch activation measured by three luciferase reporter constructs in HAVSMC cells. Student's t-test was performed to determine statistical significance compared to 0 μM resveratrol control. P-values are reported as *<.05, **<.01, ***<.001. Data represents n≥5. (C) Notch activation measured by three luciferase reporter constructs in HMEC-1 cells. Student's t-test was performed to determine statistical significance compared to 0 μM resveratrol control. P-values are reported as *<.05, **<.01, ***<.001.

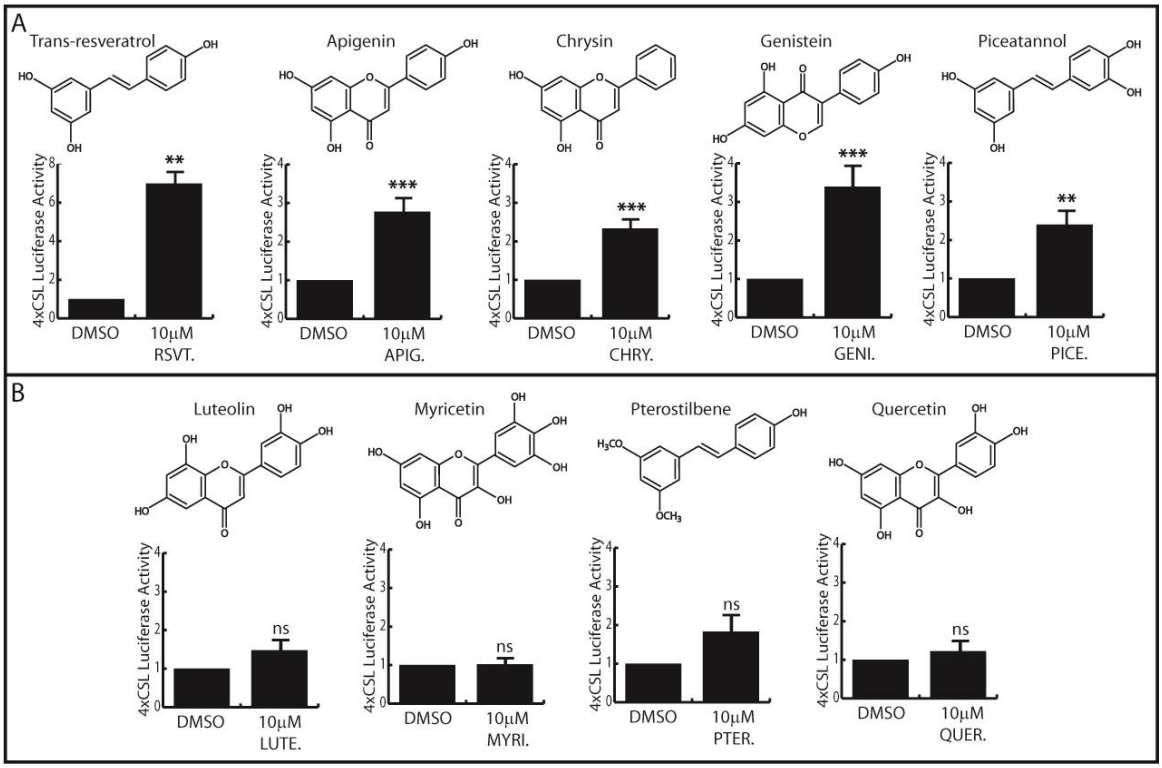

Figure 3.2: Other Polyphenolic Compounds are Notch Activators.

Notch activation measured by 4xCSL luciferase assays in 293T cells in the presence N1ICD overexpression. Student's t-test was performed to determine statistical significance. P-values are reported as *<.05, **<.01, ***<.001. Data represents n≥5. Structures of each polyphenol are shown above respective graph. (A) Polyphenols which stimulated Notch target gene transcription. (B) Polyphenols which did not stimulate Notch target gene transcription.

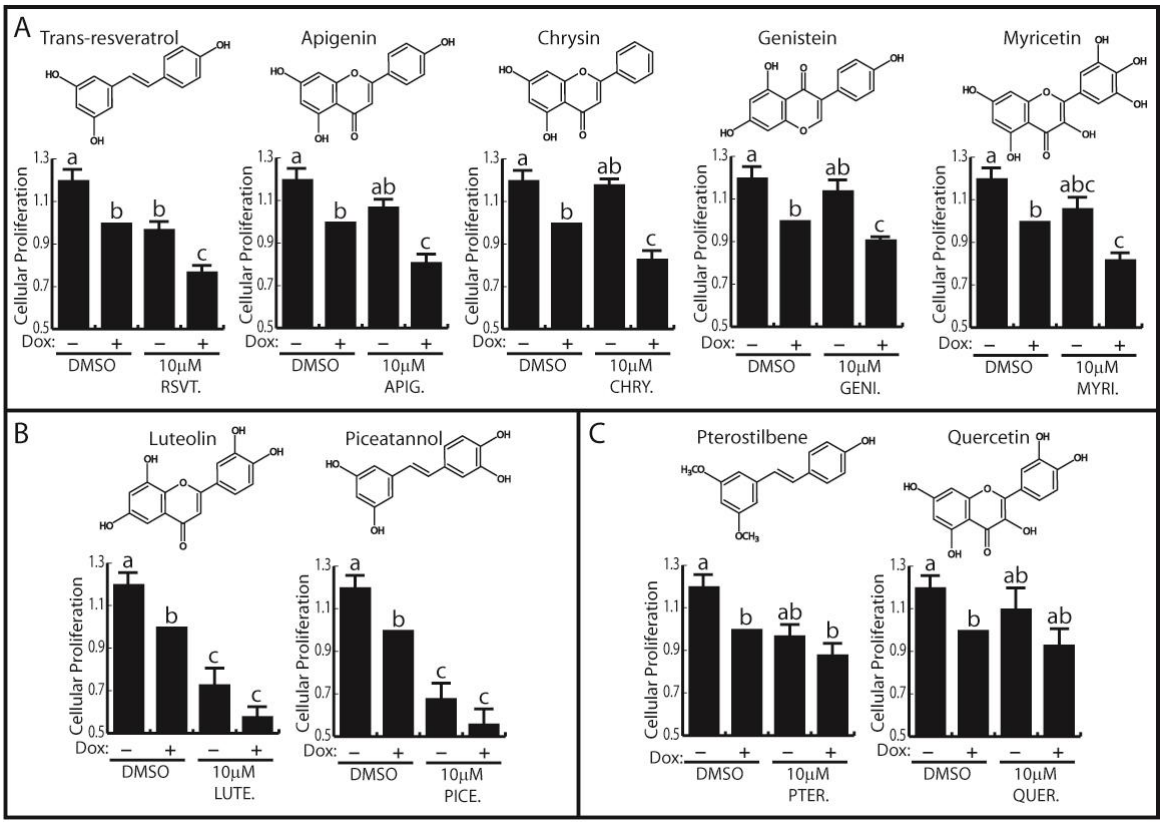

Figure 3.3: Polyphenolic Regulation of Endothelial Cell Proliferation

Proliferation of HMEC-1 cells measured by WST-1 proliferation assays. HMEC-1 cells which had been transduced with lentiviral particles encoding N1ICD under the control of a doxycycline inducible promoter were used. One-way anova followed by Bonferroni's post-hoc tests was performed to determine statistical significance. Data represents n=6. (A) Polyphenols which inhibit HMEC-1 proliferation in a Notch dependent manner. (B) Polyphenols which inhibit HMEC-1 proliferation during both basal and high Notch activity. (C) Polyphenols which do not alter HMEC-1 proliferation.

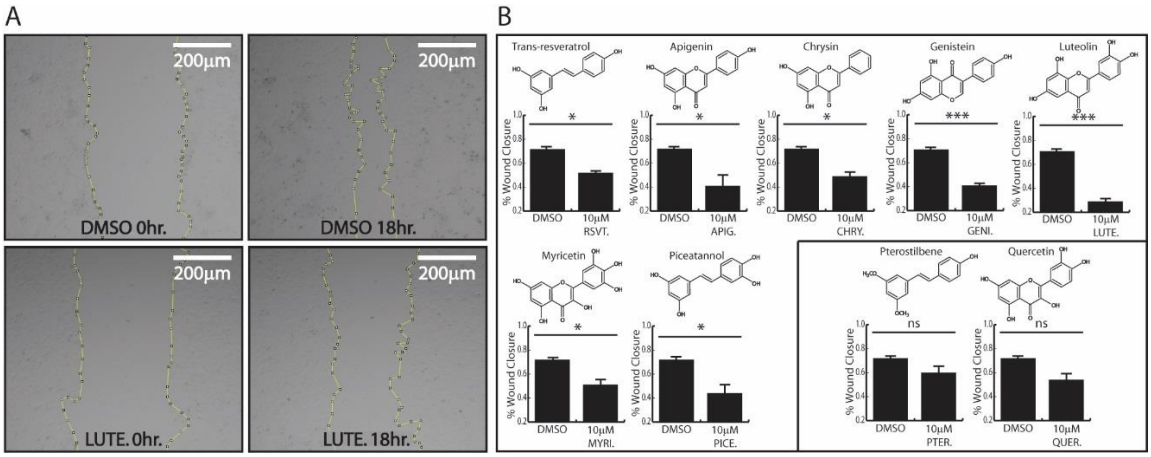

Figure 3.4: Polyphenolic Regulation of Endothelial Cell Migration

Migration of endothelial cells measured through scratch assay analysis. (A) Micrograph images of scratch assay in HMEC-1 cells for DMSO and luteolin. Area of wound is outlined. (B) Data depicts % wound closure after 18 hours. Student's t-test was performed to determine statistical significance. Polyphenols are grouped according to migration inhibitors (RSVT., APIG., CHRY., GENI., LUTE., MYRI., PICE.) and polyphenols which do not alter migration (PTER. and QUER.). P-values are reported as *<.05, **<.01, ***<.001. Data represents n=3.

E = enhances I = inhibits N = neutral	NOTCH ACTIVITY	PROLIFERATION	MIGRATION
RESVERATROL	E	I	I
APIGENIN	E	I	I
CHRYSIN	E	I	I
GENISTEIN	E	I	I
LUTEOLIN	N	I	I
MYRICETIN	N	I	I
PICEATANNOL	E	I	I
PTEROSTILBENE	N	N	N
QUERCETIN	N	N	N

Figure 3.5: Summary

The summarization of the ability of individual polyphenols to regulate Notch activity, endothelial cell proliferation, and endothelial cell migration. E=enhances, I=inhibits, N=neutral.

CHAPTER FOUR

Summary

Notch Signaling is Regulated by Src Kinase

In **Chapter II**, I discussed our findings which led to the discovery of the molecular mechanism whereby Notch signaling is regulated through activation of αVβ3 integrin. Integrin stimulation by the extracellular protein, MAGP2, activates the intracellular tyrosine kinase, Src. Src phosphorylates the intracellular domain of the Notch receptor at several tyrosine residues. These sites of phosphorylation serve to inhibit transcriptional expression of Notch target genes. This occurs through phosphotyrosine inhibition of MAML recruitment to the ternary transcriptional complex. All of this evidence reveals a novel regulatory mechanism whereby Notch signaling is controlled by extracellular cues through an integrin-based crosstalk mechanism.

This discovery has potentially far-reaching implications. Firstly, we have outlined a novel phosphotyrosine regulatory mechanism previously unknown to science. Since Src is controlled by other signaling mechanisms besides integrin signaling, future work should explore whether these pathways regulate Notch. Src is controlled by several major cell signaling mechanisms such as vascular endothelial growth factor (VEGF) [161], g-protein couple receptor (GPCR) [162], transforming growth factor beta (TGFβ) [163] and Wnt/β-catenin [164] signaling. Additionally, many of these pathways are known to synergize with Notch (as

reviewed in Appendix A). Investigating whether stimulation of these pathways governs Notch behavior may uncover new modes of Src-induced pathway crosstalk.

Additionally, based on our discovery of a phosphotyrosine regulatory mechanism of Notch signaling, we speculate that the Notch receptor protein may be phosphorylated by other tyrosine kinases besides Src. Since there exists many different tyrosine kinases within cells, future work should attempt to uncover if Notch is phosphorylated by other tyrosine kinases working under the control of other cell signaling pathways. For example, another tyrosine kinase, Abelson kinase (ABL), has been demonstrated to phosphorylate Notch [33]. Focal adhesion kinase (FAK), is another tyrosine kinase activated by integrin signaling [58]. FAK and Src activity are highly coordinated [165], so the possibility that FAK phosphorylates Notch, or that FAK is involved in the Src-Notch interaction, should be explored. In this way, we may be able to uncover novel ways in which Notch signaling is controlled by other tyrosine kinase activity.

Lastly, since we have characterized a crosstalk between αVβ3 integrin and Notch signaling, future work should explore whether other integrins coordinate Notch. Integrins are involved in many developmental and disease processes. Many chemotherapeutic strategies attempt to disrupt integrin activity in tumors [166,167]. However, many of these approaches fail in their effectiveness. Based on our findings that integrin signaling inhibits Notch, it is possible that abatement of integrin function leads to aberrant Notch activation which may in turn facilitate tumor progression. Moving forward, it may be prudent to use a co-targeting approach, whereby Notch and integrin signaling is simultaneously attenuated during cancer treatment. More broadly, this strategy may provide new

treatments for diseases where both integrin and Notch signaling have been implicated, such as fibrosis [168,169].

Polyphenolic Compounds Control Notch Signaling and Endothelial Cell Behavior

In **Chapter III**, I discussed our comparative study which analyzed how different polyphenolic compounds modulate Notch signaling. Out of the nine polyphenols we tested, we found resveratrol to be the most potent Notch activator, while identifying novel polyphenolic Notch activators. In addition, we tested the inhibition of endothelial cell proliferation by these polyphenols. Here, we found that most of the polyphenolic compounds we tested acted to mitigate endothelial cell proliferation. Additionally, we tested polyphenolic control of endothelial cell migration, and found genistein and luteolin to be highly active inhibitors of migration.

How dietary constituents alter cellular signaling pathways is an active area of research. This is particularly relevant during cancer treatment where chemotherapeutics target specific signaling mechanisms. Highly active nutraceuticals could potentially aid or hinder these treatment strategies. Without first establishing how dietary constituents affect the body at the cellular level, these interactions could be overlooked. Our findings that polyphenolic compounds are potent stimulators of Notch activity may help medical professionals prescribe an appropriate diet for patients battling specific diseases. As an example, a patient who suffers from T-cell acute lymphoblastic leukemia (T-ALL), a disease hallmarked by over-active Notch signaling [43], may potentially benefit from curtailing intake of polyphenolic-rich foods that might exacerbate the condition.

Since we identified some polyphenolic compounds as capable inhibitors of endothelial proliferation and migration, these compounds may serve as good anti-

angiogenic agents. Angiogenesis is characterized by enhanced endothelial proliferation and migration, so natural compounds which curtail these endothelial cell behaviors may be of medical interest. Patients undergoing conventional anti-angiogenic strategies during cancer treatment could benefit from foods rich in natural angiostatic compounds. Hybridization of traditional drug treatments with the use of polyphenolic nutraceuticals may become effective holistic anti-cancer approaches in the future.

Future Directions

Modern medicine relies heavily on the scientific advancements made through basic research. This dissertation has made strides in furthering our collective knowledge about Notch signaling, an important cellular communication pathway. Moving forward, it will prove necessary to engage in translational research in order to validate if our findings have clinical significance. The details we unearthed about these novel regulators of Notch signaling may prove key to combating Notch-related developmental disorders and disease. Understanding how discoveries made in the petri dish are relevant to human health is essential to advancement of modern medicine.

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APPENDIX A

Notch: A Multi-Functional Integrating System of Microenvironmental Signals

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Abstract

The Notch signaling cascade is an evolutionarily ancient system that allows cells to interact with their microenvironmental neighbors through direct cell-cell interactions, thereby directing a variety of developmental processes. Recent research is discovering that Notch signaling is also responsive to a broad variety of stimuli beyond cell-cell interactions, including: ECM composition, crosstalk with other signaling systems, shear stress, hypoxia, and hyperglycemia. Given this emerging understanding of Notch responsiveness to microenvironmental conditions, it appears that the classical view of Notch as a mechanism enabling cell-cell interactions, is only a part of a broader function to integrate microenvironmental cues. In this review, we summarize and discuss published data supporting the idea that the full function of Notch signaling is to serve as an integrator of microenvironmental signals thus allowing cells to sense and respond to a multitude of conditions around them.

Introduction

The Cellular Microenvironment

Conditions of the local environment in which a cell resides can vary widely depending on the species and its anatomical location within the organism. In recent years, cellular microenvironments have gained wide acceptance as major determinants influencing cellular physiology, especially as it pertains to the cancer microenvironment (Liotta and Kohn 2001), the stem cell niche (Morrison and Spradling 2008), the vascular system (Giordano and Johnson 2001), and wound healing/granulation tissue (Junker, Caterson et al. 2013). A multitude of components contribute to a cell's microenvironment. Extracellular matrices which surround and support cells contribute chemical and physical

properties to the microenvironment. Both the chemical composition and the physical stiffness of the matrix provide signaling cues that are actively monitored by cells. Neighboring epithelial cells, endothelial cells, leukocytes, and fibroblasts are all known to influence nearby cells chemically through cytokine and hormone secretions, and physically through cell-cell interactions. Other properties of the cellular microenvironment include concentrations of dissolved gases such as O_2 and CO_2, blood sugar concentrations, temperature, shear stress, oxidative stress, the presence/absence of foreign antigens, and osmolality. Moreover, cellular microenvironments can change rapidly and dramatically in response to situations such as wounding and subsequent healing, tumor development, hypoxia, glucose availability, and fibrosis. Due to the potentially dynamic nature of the cellular microenvironment, cellular responses to both static and changing microenvironments need to be calibrated to properly and rapidly respond to these situations. Understanding how cells respond to the incredible complexity of the microenvironment requires a systems biology approach to integrate the microenvironmental information, a task that is immensely complicated.

Notch

The Notch signaling mechanism is a highly conserved developmental pathway that is used during differentiation in numerous tissues in most, if not all, multicellular organisms. Evolutionary evidence for the emergence of the Notch receptors first appears in the choanoflagellates, unicellular flagellated free-living eukaryotic cells widely considered the closest extant protist relative to metazoans (King, Westbrook et al. 2008, Richter and King 2013). The genome of the choanoflagellate *Monosiga brevicollis* encodes three domains that show similarity to metazoan Notch receptors (figure A.1A). However,

these domains are split amongst three separate transmembrane proteins in the *M. brevicollis* genome including one gene that encodes 36 epidermal growth factor (EGF) like domains, a second gene that encodes two Lin-12-Notch repeats (LNR domains), and a third gene that encodes six ankyrin repeats (King, Westbrook et al. 2008). Presumably, these three ancestral partial Notch homologs were responsible for individual functions. This suggests that modern metazoan Notch receptors, which unify these domains in a single receptor, might represent an amalgamation of three independent proteins with independent ancestral functions and may help explain why Notch is capable of integrating a multitude of cellular microenvironmental signals and conditions as described in this review. Despite the lack of a bona fide Notch receptor or Notch ligands, *M. brevicollis* genome does encode several other components of the Notch system (Gazave, Lapebie et al. 2009) (figure A.1B). Therefore the origin of Notch domains (if not function) likely predates the rise of the metazoans. It has been postulated that these proto-Notch receptors might have served an adhesive function that was independent of Notch signaling activity and is conserved in modern Notch function (reviewed in (Murata and Hayashi 2016)). It was not until the rise of sponges however, that bona fide Notch receptors and ligands appeared and exhibited the developmental roles that are representative of the metazoan Notch mechanism (Richards and Degnan 2012). Thereafter, Notch receptors, ligands, and other Notch processing and/or modifying proteins are expressed throughout all metazoans examined to date (Gazave, Lapebie et al. 2009).

In mammals, the core of the Notch mechanism consists of five Notch ligands (Jagged1, 2 and Delta like (Dll) 1, 3, 4) present on the “signaling cell”. The Jagged and Dll 1 and 4 ligands directly interact with and activate a transmembrane Notch receptor (four

different isoforms in mammals) present on the “receiving cell” (Kopan and Ilagan 2009, Kopan 2010) (figure A.1B). The Dll3 ligand is a decoy receptor that interferes with Notch activation (Ladi, Nichols et al. 2005). Notch receptors undergo a maturation process involving three proteolytic cleavage events that ultimately result in Notch activation. The first cleavage is performed by a furin convertase during translocation through the Golgi complex on the way to the cell membrane (Logeat, Bessia et al. 1998). The resulting two Notch fragments remain non-covalently associated at the membrane where canonical Notch activation is initiated by interaction between Notch receptors and ligands. Canonical Notch activation at the membrane is commonly thought to be dependent on a physical tugging mechanism of ~ 4-12 pN (Gordon, Zimmerman et al. 2015, Chowdhury, Li et al. 2016) that is initiated by Notch ligand endocytosis in the signaling cell (Parks, Klueg et al. 2000). This pulling force sets up a second cleavage by an ADAM metalloprotease (α-secretase) producing the transient NEXT (Notch Extracellular Truncation) fragment (Brou, Logeat et al. 2000), and a third cleavage by γ-secretase (Mumm, Schroeter et al. 2000) thus releasing the intracellular NICD domain of Notch that translocates to the nucleus and functions as a co-transcription factor in association with the CSL transcription factor and other co-transcription factors including MAML and p300. In addition to this canonical mechanism, evidence for several non-canonical Notch activation mechanisms have also been gaining traction. In particular, Notch activation that is independent of canonical ligands (Palmer and Deng 2015), NICD cleavage and transcriptional activity (Guruharsha, Kankel et al. 2012), as well as several non-canonical ligands (D'Souza, Meloty-Kapella et al. 2010) have all been described in the literature. Finally, in addition to the core receptors and ligands, a wide variety of cellular and secreted proteins have been characterized that

modify Notch signaling either through direct interaction and/or modification of extracellular Notch receptor or ligand domains (Kadesch 2004) or via post-translational modification of intracellular NICD fragments (Fortini 2009). References (Kopan and Ilagan 2009, Kopan 2010) provide excellent in-depth reviews of the Notch signaling mechanism.

Notch as an Integrator of Cellular Microenvironments

While the traditional view of Notch activation focuses on Notch receptor – ligand interactions, it is becoming increasingly clear that Notch signaling is also influenced by a wide array of molecules and events in the cellular microenvironment. In particular, extracellular matrix (ECM) mediated Notch signaling is emerging as a new paradigm for controlling Notch signaling. Regulation of Notch by ECM occurs on several levels, including direct interaction between ECM and Notch receptor/ligands, transcriptional control of Notch receptors and/or ligands, and via cross-talk with other ECM stimulated signaling networks, such as integrins. In addition, Notch is engaged in crosstalk with a number of signaling pathways that are initiated by growth factors and cytokines commonly present in cellular microenvironments including, TGF-β, WNT/β-catenin, and VEGF. Finally, Notch can also be regulated by additional conditions such as shear stress, hypoxia, and hyperglycemia. These microenvironmental conditions are summarized in Table A.1. Taking into consideration the wide variety of cellular microenvironmental cues that regulate Notch signaling output, a new picture of Notch is emerging which depicts Notch as an integrating system for the cellular microenvironment, which enables cells to respond appropriately to changing ECM composition, growth factor secretions, oxygen tension, shear stress, and glucose levels. Importantly, this idea is not inconsistent with the classical

model of Notch receptor activation by Notch ligands on adjacent cells, but rather builds on this model since cellular neighbors are also an important part of a cell's microenvironment. The goal of this review is to summarize what is known about the role Notch signaling plays in responding to and integrating changing microenvironmental conditions, and to explore and develop the idea of Notch as a multi-functional integrating system of microenvironmental signals.

ECM-Notch Interactions

Extracellular matrices are a major component of a cell's microenvironment. In some instances ECM can be stable over decades. In other situations, ECM is rapidly turned over and remodeled. Therefore, cells need to be able to adjust to these stable or changing conditions. Notch responsiveness to the composition of the ECM has only recently begun to be characterized. The interactions between ECM and Notch can be summarized as either direct interactions between ECM and Notch receptors or ligands, indirect (transcriptional) responses of Notch receptors or ligands to ECM, and indirect (crosstalk) interactions between Notch and ECM stimulated signaling cascades (figure A.2). Below, we summarize and discuss these interactions between ECM and Notch signaling.

Direct ECM-Notch Interactions that Control Notch Signaling

Direct interactions between Notch receptors and several ECM proteins have been described in the literature. Below, we summarize the current data available for several ECM proteins including Microfibril Associated Glycoprotein-2 (MAGP-2), Epidermal Growth Factor–like 7 (EGFL7), Nephroblastoma Overexpressed (NOV, CCN3), Thrombospondin-2 (TSP-2), syndecans 2/3, collagens type I and IV, the Y-box binding protein (YB-1), and Galectin-3. An interesting observation is that while all these proteins

have been shown to regulate Notch signaling via direct interactions with Notch receptors or ligands, there is not a common thread of increased or decreased activity connecting these proteins. Thus, the Notch regulatory activities of these molecules most likely do not rely solely on mechanisms involving a simple steric hindrance model. Moreover, several of these molecules appear to control Notch via multiple mechanisms, suggesting that ECM control of Notch may be a highly regulated activity.

MAGP-2 is a component of elastic fibrils that are thought to help recruit tropoelastin to fibrillin containing microfibrils during the development of elastin networks (Gibson, Hatzinikolas et al. 1996). Since elastin is critical for Windkessel function and structural integrity of the aortic wall (Belz 1995), it is not surprising that loss of function of MAGP-2 is linked to aortic dilation in mice (Combs, Knutsen et al. 2013) and familiar thoracic aortic aneurisms in humans (Barbier, Gross et al. 2014). MAGP-2 may also serve other functions in the cardiovascular system since MAGP-2 contains an αvβ3 integrin binding RGD domain and has been shown to control angiogenesis (Albig, Roy et al. 2007) and vascular density in ovarian cancers (Mok, Bonome et al. 2009). The link between MAGP-2 and Notch was first made when MAGP-2 was identified as a Jagged1 interacting protein by yeast-two hybrid screening (Nehring, Miyamoto et al. 2005) and was shown to induce Jagged1 shedding from the cell surface (Miyamoto, Lau et al. 2006). Subsequent analysis determined that MAGP-2 and the related protein MAGP-1 both increased Notch signaling in COS cells (Miyamoto, Lau et al. 2006). Mechanistically, MAGP-2 mediated stimulation of Notch signaling was shown to involve direct binding between the MAGP-2 C-terminal domain, and the EGF-like domains of Notch1 and Jagged1 (Miyamoto, Lau et al. 2006). In addition, RGD→RGE mutation of the MAGP-2 integrin binding domain

converted MAGP-2 from a suppressor to an activator of Notch signaling in endothelial cells suggesting that MAGP-2 may also regulate Notch via interactions with integrins (Deford, Brown et al. 2016). This finding may help to explain the cell type dependent effects of MAGP-2 on Notch signaling previously observed (Albig, Becenti et al. 2008) and suggests that cell type specific control of Notch may be dependent on several factors including integrin and Notch ligand expression profiles.

EGFL7 is a secreted protein that is specifically expressed from endothelial cells during development (Fitch, Campagnolo et al. 2004, Parker, Schmidt et al. 2004). EGFL7 is predominantly found in the vascular microenvironment where it appears to be an important regulator of elastogenesis (Lelievre, Hinek et al. 2008) and angiogenesis (Nichol, Shawber et al. 2010, Nikolic, Stankovic et al. 2013). In particular, EGFL7 is important for the formation and maintenance of vascular lumen structures (Parker, Schmidt et al. 2004, Charpentier, Tandon et al. 2015) and suppressing angiogenic sprouting (Nichol, Shawber et al. 2010). The first observations that EGFL7 could control Notch signaling were made in neural stem cell cultures where it was found that the N-terminal half of EGFL7 specifically interacted with EGF domains in Notch1-4 and inhibited Notch signaling (Schmidt, Bicker et al. 2009). Subsequent work showed that EGFL7 control of Notch in endothelial and placental trophoblast cells was important for placenta development and that decreased EGFL7 expression may be linked to preeclampsia (Lacko, Massimiani et al. 2014, Massimiani, Vecchione et al. 2015). In addition to controlling Notch via direct interaction with Notch receptors, recent work showed that RGD→RGE mutation of the EGFL7 integrin-binding domain enhanced Notch signaling in endothelial cells (Deford, Brown et al. 2016). By regulating Notch via direct interactions with Notch receptors and

via RGD integrin binding, EGFL7 demonstrates similarities with MAGP-2 and suggest that dual control of Notch by ECM molecules is a common theme.

In addition to MAGP-2 and EGFL7, CCN3 (NOV) has also been implicated in the regulation of Notch signaling (Sakamoto, Yamaguchi et al. 2002). CCN3 belongs to the ECM CCN family of proteins (CCN1-6) that share a modular structure including of conserved cysteine knot C-terminal (CT) domain and are multi-functional regulators of diverse processes including development, osteogenesis, and angiogenesis (Katsube, Sakamoto et al. 2009). The Notch regulatory activity of CCN3 appears to be important for controlling a variety of activities including osteoblast differentiation (Minamizato, Sakamoto et al. 2007, Katsuki, Sakamoto et al. 2008) and trophoblast senescence (Wagener, Yang et al. 2013, Kipkeew, Kirsch et al. 2016). Regulation of Notch signaling may be a general feature of the CCN family since CCN2 (CTGF) suppresses Notch signaling (Smerdel-Ramoya, Zanotti et al. 2008) and CCN1 (Cyr61) is linked to suppression of Notch1 during the epithelial to mesenchymal transition (EMT) (Haque, De et al. 2012). Although, mechanistic details describing how CCN3 manipulates Notch signaling are lacking, the cysteine rich C-terminal tail of CCN3 binds to Notch1 (Sakamoto, Yamaguchi et al. 2002) and is required for Notch regulation (Katsuki, Sakamoto et al. 2008). Similar to MAGP-2 and EGFL7, CCN3 also interacts with several integrins (in an RGD independent manner) (Lin, Leu et al. 2003) although it is unknown if control of Notch by CCN3 involves integrin ligation. Interestingly however, CCN1 is highly expressed near developing blood vessels where it enhances Notch signaling in an integrin dependent manner (Chintala, Krupska et al. 2015).

Although MAGP-2, EGFL7, and CCN3 are the best characterized examples of ECM proteins known to regulate Notch activity via direct Notch receptor and/or ligand interactions, several other ECM molecules have also been implicated in the Notch pathway and appear to control Notch via direct interactions with Notch receptors and/or ligands. A common thread among these molecules is Notch3, which appears to be frequently targeted by ECM interactions compared to other Notch receptors. For instance, Thrombospondin-2 (TSP-2) and Syndecan-2 specifically interact with Notch3 and promote Notch3 – Jagged1 signaling (Meng, Zhang et al. 2009, Zhao, Liu et al. 2012). Conversely, collagen type I and IV also bind to Notch3 and Jagged1 but suppress downstream Notch signaling (Zhang, Meng et al. 2013). An additional example of Notch3 regulation by microenvironment is YB-1. The multi-functional YB-1 protein has widespread DNA/RNA binding activities (Kohno, Izumi et al. 2003) and has historically been thought of as a cold shock protein (Kohno, Izumi et al. 2003). Interestingly however, YB-1 can be secreted from mesangial and immune cells after cytokine stimulation via a non-classical mechanism that involves ubiquitin E3 ligase HACE-1 mediated K27 ubiquitination and association with the Tumor Susceptibility Gene 101 (TSG101) (Frye, Halfter et al. 2009, Palicharla and Maddika 2015). In turn, secreted YB-1 has been found to specifically interact with Notch3 EGF repeats and to control Notch3 downstream signaling, but not Notch1 signaling (Rauen, Raffetseder et al. 2009, Raffetseder, Rauen et al. 2011). More recently, non-secreted YB-1 was found to control Notch4 expression in triple negative breast cancer cells suggesting that YB-1 may control Notch on multiple levels (Reipas, Law et al. 2013). Finally, while Notch3 appears to be a common target for many ECM molecules, it is not the only target. For instance, while Syndecan-2 regulates Notch3 – Jagged1 signaling (Zhao, Liu et al.

2012), Syndecan-3 interacts with Notch1 receptor, regulates processing by ADAM17/TACE, and is required for Notch signaling activity in skeletal muscle progenitor cells (Pisconti, Cornelison et al. 2010). Finally, the sugar binding protein Galectin-3 has been reported to directly interact with Notch1 in a sugar-dependent manner and to activate downstream Notch signaling without affecting expression of Notch1 receptor (Nakajima, Kho et al. 2014).

Collectively, these examples demonstrate that a diverse array of ECM molecules can influence Notch utilizing a wide variety of mechanisms. Given that each of these molecules exhibits tissue and/or temporal specific expression patterns, these examples serve as a dynamic illustration of how Notch responds to changing ECM microenvironments. With this understanding, it will be interesting to see how future work refines our understanding of ECM – Notch interactions.

Indirect ECM-Notch Interactions that Control Notch (Transcriptional Mechanisms)

Each of the examples described thus far involve matricellular control of Notch that appears to be mediated at least in part by direct protein interactions with Notch receptors and/or Notch ligands. However, other matricellular proteins control Notch activity in a less direct manner by influencing the expression of Notch signaling components. For instance, the SPARC protein (Secreted Protein, Acidic, and Rich in Cysteine) stimulates differentiation of medulloblastoma cells by suppressing Notch signaling (Bhoopathi, Chetty et al. 2011). However, instead of direct interaction with Notch receptors or ligands, SPARC seems to transcriptionally control Notch signaling since SPARC null osteoblasts express increased Notch1 protein (Kessler and Delany 2007) and SPARC protein transcriptionally suppresses Notch1 expression (Bhoopathi, Chetty et al. 2011). In

comparison to SPARC, which seems to control Notch via direct manipulation of Notch1 expression, other ECM proteins such as Fibulin-3, basement membrane laminins, and MGP influence Notch signaling by controlling expression of Notch ligands. Fibulin-3 is a member of the fibulin family of extracellular matrix glycoproteins that are characterized by tandem repeats of calcium binding EGF sites and a C-terminus fibulin-type module (Timpl, Sasaki et al. 2003). Fibulin proteins are commonly misregulated during cancer and have emerged as important microenvironmental regulators of cancer and tumor angiogenesis (Albig, Neil et al. 2006). In particular, Fibulin-3 has emerged as a biomarker for pleural mesothelioma and malignant glioma where Fibulin-3 appears to enhance glioma malignancy by stimulating tumor cell motility and invasion (Hu, Thirtamara-Rajamani et al. 2009). Fibulin-3 also enhances tumor angiogenesis in glioma by increasing endothelial expression of the Notch ligand Dll-4 and simultaneously stimulating ADAM10/17 activity and downstream Notch signaling (Nandhu, Hu et al. 2014). An interesting observation however, is that Dll-4 has been extensively shown to limit branching angiogenesis by suppressing the endothelial tip cell phenotype (Hu, Lu et al. 2011). As an example of this idea, basement membrane laminins including Laminin-α4 and Laminin-111 promote Notch activation by increasing Dll-4 expression via interaction with integrins (Estrach, Cailleteau et al. 2011, Stenzel, Franco et al. 2011). As opposed to Fibulin-3 however, Dll-4 induction by these laminins appears to be an important mechanism to maintain endothelial quiescence by limiting tip cell behaviors. Thus, perhaps simultaneous regulation of ADAM10/17 and Dll-4 enables Fibulin-3 to behave as an angiogenic promoter in glioma, but to inhibit angiogenesis in other tumors as previously described (Albig, Neil et al. 2006). Finally, Matrix Gla Protein (MGP) is a well-known inhibitor of

vascular calcification (Luo, Ducy et al. 1997) that functions by binding to and suppressing the osteogenic programs initiated by BMP-2 and other BMPs (Zebboudj, Imura et al. 2002). In addition to suppressing vascular calcification, MGP has additional roles in the vasculature since MGP deficiency in mice leads to increased vascular densities, enhanced tumor angiogenesis (Sharma and Albig 2013), and the development of arteriovenous malformations (Yao, Jumabay et al. 2011). Mechanistically, MGP deletion results in increased Notch signaling via enhanced expression of the Notch ligand Jagged1 (Sharma and Albig 2013) and accordingly, deletion of a single Jagged1 or Jagged2 allele in MGP knockout animals suppresses arteriovenous malformations (Yao, Yao et al. 2013). Although it is not yet clear how MGP controls Jagged1 expression, it appears that MGP expression is also controlled by Notch in shear-stressed aortic valve endothelium, (White, Theodoris et al. 2015) suggesting that Notch and MGP are coordinated by a feedback regulation.

An important observation is that many of the ECM proteins discussed above not only control Notch signaling, but have also been implicated in the matricellular control of angiogenesis. Indeed, MAGP-2, EGFL7, the CCN family of proteins (CCN1, 2, 3), Thrombospondin-2, Syndecan-2, SPARC, collagens I and IV, Fibulin-3, MGP, and laminins have all been characterized as angiogenic regulators. Given that Notch has emerged as a major regulator in the cardiovascular system (discussed below), matricellular control of Notch activity may be a common mechanism whereby the vascular microenvironment exerts control over angiogenic activity. Hopefully, future research will be able to determine the relative contributions of these matrix molecules towards Notch

regulation during angiogenic processes and begin to understand how these multiple signals integrate to control Notch.

Direct ECM-Notch Interactions that Control Notch Signaling (Crosstalk Mechanisms)

Reelin is a secreted glycoprotein that is an important regulator of neuronal cell migration in the developing brain, (Dulabon, Olson et al. 2000, Yip, Yip et al. 2000) and provides one last mechanism to demonstrate how ECM molecules may control Notch. Deletion of Reelin in mice causes an abnormal "reeling" gait referred to as a *Reeler* phenotype (D'Arcangelo, Miao et al. 1995). Reelin has several cell surface receptors including the VLDLR and ApoER2 lipoprotein receptors on neuronal cells that have been described by several investigators (Hiesberger, Trommsdorff et al. 1999, Ballif, Arnaud et al. 2003, Bock and Herz 2003). In addition, Reelin has also been described to interact with integrins $\alpha3\beta1$ and $\alpha5\beta1$ (Dulabon, Olson et al. 2000, Sekine, Kawauchi et al. 2012). Downstream from these receptors, Reelin signaling typically propagates through Disabled-1 (DAB-1) phosphorylation and downstream PI-3K, AKT, and SRC signaling mechanisms (Ballif, Arnaud et al. 2003, Bock and Herz 2003, Hashimoto-Torii, Torii et al. 2008, Keilani and Sugaya 2008, Keilani, Healey et al. 2012, Sekine, Kawauchi et al. 2012). Early work by Keilani et al (Keilani and Sugaya 2008) and Hashimoto-Torii et al (Hashimoto-Torii, Torii et al. 2008) suggested that Notch may be important for activities downstream of Reelin. For example, Reelin induces a radial glial phenotype in human neural progenitor cells, and this effect is phenocopied by activation of the Notch signaling cascade (Keilani and Sugaya 2008). Moreover, the Notch NICD domain is sufficient to rescue abnormal migration in neurons from reeler mice (Hashimoto-Torii, Torii et al. 2008). Mechanistically, Reelin does not appear to directly interact with, or control the expression

of Notch receptors and/or ligands. Instead, Reelin appears to control Notch via manipulation of downstream signaling networks. For instance, it has been shown that the downstream Reelin signaling intermediate, DAB-1, physically associates with NICD (Keilani and Sugaya 2008), that Notch works through DAB-1 to regulate axon guidance in *Drosophila* (Le Gall, De Mattei et al. 2008), and that DAB-1 phosphorylation and SRC activity are essential for Notch1 activation by Reelin (Keilani, Healey et al. 2012). Taken together, these results suggest that Reelin may regulate Notch via a mechanism independent of Notch expression or Notch processing, but dependent on downstream DAB-1 and/or SRC kinase activities. Although further research is required to confirm this, the molecular interaction between Notch and SRC is further explored in the Integrin/Notch crosstalk section below.

Notch Crosstalk with other Signaling Networks: Integrins, TGF-β, WNT, and VEGF

An important distinction between Notch regulation by Reelin compared to other molecules mentioned in the previous section is that Reelin does not depend on direct Notch receptor/ligand interactions nor on transcriptional control of individual Notch signaling components. Instead, the evidence supports a mechanism whereby Reelin interaction with its cell surface receptors triggers downstream signaling (DAB-1 and SRC) that then regulates Notch via undefined mechanisms. Thus, Reelin serves as an example of how Notch signaling can be influenced by crosstalk with other signaling pathways. Similarly, crosstalk between Notch and several other signaling mechanisms initiated by molecules including integrins, TGF-β/BMP, VEGF, and WNTs in the cellular microenvironment (figure A.3) have been described and are discussed below.

Crosstalk between Notch and Integrins

The earliest evidence that integrins and Notch coordinate comes from studies which explored the effect of Notch on integrin activation. For instance, Leong et al. demonstrated that Notch4 activation in microvascular endothelium increased β1 integrin affinity for collagen (Leong, Hu et al. 2002). This was taken one step further by Hodkinson et al., who demonstrated that activation of the small GTPase R-Ras by Notch1 resulted in increased β1 affinity for collagen (Hodkinson, Elliott et al. 2007). Subsequent work on this topic began to uncover the reciprocal interaction wherein integrins also exert control over Notch. Initially, integrin control of Notch was focused on transcriptional regulation of Notch receptors or ligands. For example, work by Weijers et al. (Weijers, van Wijhe et al. 2010) described an effect of integrin blocking low molecular weight fibronectin fragments on the expression of the Notch ligand Dll-4 and subsequent Notch activation in endothelial cells. Similarly, Estrach et al. (Estrach, Cailleteau et al. 2011) and Stenzel et al. (Stenzel, Franco et al. 2011) demonstrated that Laminin 111 and Laminin α4 increase Dll-4 expression in endothelial cells via α2β1 and α6β1 integrins, and that disruption of this signaling system had dramatic complications for normal angiogenesis. While these studies suggested a functional coordination of ECM, integrins, and Notch they did little to dissect a molecular mechanism, beyond transcriptional control, through which coordination occurs. A handful of reports however have suggested that Notch control by integrins is not restricted to transcriptional regulation, but rather, may also engage Integrin Linked Kinase (ILK) and/or SRC signaling downstream from activated integrins. For instance, Mo et al. (Mo, Kim et al. 2007) observed that ILK decreased Notch signaling by stimulating ubiquitination and rapid degradation of the NICD fragment. Similarly, Suh et al. (Suh and Han 2011) showed

that collagen type I increased ILK signaling and NICD accumulation through interaction with α2β1 integrin. In addition to ILK, the non-receptor tyrosine kinase SRC which is commonly activated by integrin ligation, may also regulate Notch. As eluded to above, Reelin has been shown to control Notch in a DAB-1 and SRC dependent manner (Keilani, Healey et al. 2012). Although a molecular interaction between Notch and SRC was not explored in this study, the authors did show that SRC inhibitors did not affect expression of Notch1, suggesting a more direct Reelin-SRC-Notch interaction. In support of this, SRC was found to be an important regulator of Notch S1 processing by furin and that the kinase domain of SRC binds to and phosphorylates the ankyrin domain of active NICD (Ma, Shi et al. 2012). Moreover, a genetic interaction between SRC and Notch has been uncovered during *Drosophila* development that is critical for normal eye formation (Ho, Pallavi et al. 2015). Taken as a whole, these publications show that Notch can control integrin adhesion (*i.e.* inside out signaling), and that integrins can control Notch (*i.e.* outside in signaling). Therefore, these data suggest that integrins and Notch are coordinated into a cellular signaling network that involves feedback control between Notch and integrins and may involve ILK and/or SRC signaling.

The implications of integrin/Notch crosstalk are potentially quite numerous. In particular, one field of research that may be impacted by this crosstalk is the study of pathological tissue fibrosis. Fibrotic diseases are defined by excessive deposition of fibrotic ECM molecules, increased tissue stiffness, and can occur in most any tissue although fibrosis of the liver, lung, kidney, and heart represent the major impacts of fibrosis on human health. Given the increased ECM present in fibrotic tissues, it is not surprising that integrins figure predominantly in the pathology of fibrosis (Patsenker and Stickel 2011,

Pozzi and Zent 2013, Chen, Li et al. 2016). Adding to this, it has become apparent that Notch is also an important regulator of fibrosis in the lung, liver, kidney, and skin (Kavian, Servettaz et al. 2012, Hu and Phan 2016). For example, strong expression of Notch was observed in myofibroblasts, the pathological cells associated with the progression of fibrosis, in lung specimens from patients with idiopathic interstitial pneumonias and in bleomycin-induced pulmonary model of fibrosis (Aoyagi-Ikeda, Maeno et al. 2011). Moreover, in airway subepithelial fibrosis, the Notch pathway stimulated the promoter activity of collagen type I through a Hes1-dependent mechanism (Hu, Ou-Yang et al. 2014). In the kidney, Bielesz et al. showed that upregulation of Notch pathway components (Jag1/Notch1/HeyL) regulated the development of tubulointerstitial kidney fibrosis in mice and humans (Bielesz, Sirin et al. 2010). In the liver, the number of Notch1, Notch3 and Notch4 positive cells were highly upregulated in CCL4 induced fibrosis (Chen, Zheng et al. 2012). Moreover, activated hepatic stellate cells (HSC) showed an increased expression of Notch2, Notch3, Hey2 and HeyL (Zhang, Xu et al. 2015). However, after blocking with the γ-secretase inhibitor DAPT, activated HSC reversed back to quiescent HSC (Zhang, Xu et al. 2015) and attenuated hepatic fibrosis (Chen, Zheng et al. 2012). Collectively, these examples clearly illustrate the importance of Notch signaling during fibrotic responsis. Given the crosstalk between integrins and Notch, it will be interesting to determine if integrins have a strong impact on Notch mediated fibrosis.

Notch and TGF-β

The TGF-β superfamily encompasses more than 30 ligands including TGF-βs, BMPs, activins/inhibins, and Mullerian Inhibiting Substance (MIS) that specifically interact with at least seven ALK receptors. Activation of ALK receptors by TGF-β or other

ligands stimulates SMADs to translocate to the nucleus where they coordinate transcriptional responses (reviewed in (Gordon and Blobe 2008)). TGF-β and Notch signaling are both involved in several physiological and patho-physiological processes including embryonic development, wound healing, cancer, and fibrosis. Several lines of evidence indicate that TGF-β and Notch can engage in crosstalk (reviewed in (Kluppel and Wrana 2005, Tang, Urs et al. 2010)). The first molecular evidence for this interaction was revealed in a series of papers showing that Notch is synergistic with both TGF-β and BMP signaling. Specifically, Blokzijl et al. demonstrated that NICD can form a transcription factor complex with SMAD3, an intracellular transducer of TGF-β signaling, in chicken embryos and in mouse myoblast C2C12 cells (Blokzijl, Dahlqvist et al. 2003). In this study, it was also observed that TGF-β upregulated the expression of Hes-1, a Notch target gene, and the effect was abolished by using a dominant negative form of CSL (Blokzijl, Dahlqvist et al. 2003). A similar interaction was observed in mouse regulatory T cells in which NICD cooperates with activated SMAD3 and accelerates its nuclear translocation (Asano, Watanabe et al. 2008). The importance of TGF-β/Notch crosstalk is illustrated by several reports showing that Notch activity is required for some TGF-β effects such as TGFβ-induced EMT (Zavadil, Cermak et al. 2004) and the well-known pro-fibrotic activity of TGF-β (Kavian, Servettaz et al. 2010, Nyhan, Faherty et al. 2010, Chen, Zheng et al. 2012, Xiao, Zhang et al. 2014). Finally, although the majority of interactions between TGF-β and Notch appear to be synergistic, this may be an oversimplified view of the TGF-β/Notch interaction. In support of this, Fu et al (Fu, Chang et al. 2009), found that while Notch did enhance expression of some TGF-β responsive genes including PAI1, CTGF, and CYR61, other TGF-β responsive genes including ID1, and ID2, were decreased by

Notch activity. The authors also found that Notch enhanced expression of SMAD3 while decreasing expression of SMAD1, 2, and 6, suggesting that differential regulation of R-SMADs by Notch may be responsible for positive and negative TGF-β/Notch interactions. From this analysis, it appears that the interaction between TGF-β and Notch may be more complex than currently thought.

TGF-β however is not the only member of the TGF-β superfamily that engages in crosstalk with Notch. Early work observed that BMP and Notch signals synergistically reinforced one another during various developmental processes such as *Xenopus* tail bud formation (Beck, Whitman et al. 2001) and tooth morphogenesis (Mustonen, Tummers et al. 2002). Mechanistically, the BMP/Notch crosstalk involves the formation of a SMAD/NICD transcription factor complex, much like the TGF-β/Notch crosstalk mechanism. Formation of this complex was observed and found to be important for endothelial function and neuroepithelial cell differentiation (Mustonen, Tummers et al. 2002, Itoh, Itoh et al. 2004). Follow up work has now determined that crosstalk between Notch and BMP is important for a wide variety of cellular responses including osteoblastic differentiation (Nobta, Tsukazaki et al. 2005, Sharff, Song et al. 2009, Hill, Yuasa et al. 2014) and vascular biology/angiogenesis (Larrivee, Prahst et al. 2012, Moya, Umans et al. 2012). Finally, besides TGF-β and BMP, little is known regarding crosstalk between other TGF-β superfamily members and Notch. However, given that the majority of the other TGF-β superfamily members utilize ALK receptors and SMAD signaling intermediates, it seems likely that future research may uncover new crosstalk mechanisms between members of the TGF-β superfamily and Notch.

Notch and WNT

Like Notch, the WNT signaling network is evolutionarily ancient and heavily utilized during development. Consisting of ~19 ligands that can bind to ~10 frizzled receptors and their co-receptors (LRP5/6), the canonical WNT signaling pathway is mediated by ligand binding to receptor, stabilization and nuclear translocation of β-catenin, and subsequent association with LEF/TCF transcription factors to activate gene specific promoters. In the absence of WNT signaling, β-catenin is phosphorylated by GSK3β which triggers β-catenin ubiquitination and rapid protein turnover (reviewed in (Komiya and Habas 2008)). The first evidence suggesting a crosstalk between WNT and Notch signaling was uncovered in *Drosophila* where it was shown that Notch and WNT cooperate to control wing development (Couso and Martinez Arias 1994, Hing, Sun et al. 1994). The first molecular evidence supporting crosstalk between Notch and WNT was made by Ross and Kadesch (Ross and Kadesch 2001), when N1ICD was found to increase transcriptional activity of the LEF transcription factor independently of the canonical Notch transcription factor, CSL. Instead, it was found that the NICD/WNT crosstalk was mediated by a NICD/LEF transcriptional complex that regulated a unique subset of promoters compared to the β-catenin/LEF complex (Ross and Kadesch 2001). Similarly, NICD/β-catenin complexes have been identified and found to be important for suppression of neural precursor cells (Shimizu, Kagawa et al. 2008) and for inducing an arterial fate in vascular progenitors (Yamamizu, Matsunaga et al. 2010). Despite these results, this NICD/β-catenin complex does not appear to be required for all instances of Notch/WNT crosstalk. Instead, Hayward et al. demonstrated that membrane-bound Notch is capable of interacting with, and deactivating β-catenin at the cell membrane in a Notch ligand and cleavage

independent fashion (Hayward, Brennan et al. 2005). Subsequent reports reinforced this finding by showing that β-catenin's association with uncleaved Notch at the membrane is also important for β-catenin regulation in stem cells (Kwon, Cheng et al. 2011), and imaginal disc development in *Drosophila* (Sanders, Munoz-Descalzo et al. 2009). Thus, Notch signaling can alternatively increase or decrease β-catenin function, depending on the nature of Notch/β-catenin interaction. Finally, while these reports show that β-catenin is a shared point of overlap during crosstalk between WNT and Notch, other WNT signaling intermediates have also been shown to interact with the Notch mechanism. For instance, GSK3β, a serine/threonine kinase that is inactivated by WNT signaling (Komiya and Habas 2008), directly phosphorylates NICD resulting in decreased NICD stability and signaling output (Foltz, Santiago et al. 2002, Espinosa, Ingles-Esteve et al. 2003). In this way, inhibition of GSK3β by WNT signaling results in a positive interaction between the WNT and Notch signaling mechanisms. In contrast to this, WNT activation of the Dishevelled protein triggers an inhibitory interaction between WNT and Notch. It is not completely clear how this is accomplished however since Dishevelled has been shown to interact both with NICD itself, and with the NICD transcriptional factor CSL in the nucleus, where it inhibits NICD/CSL mediated transcription (Axelrod, Matsuno et al. 1996, Collu, Hidalgo-Sastre et al. 2012).

In summary, the interplay between WNT and Notch is very complex and involves at least four independent mechanisms. This extensive co-regulation may reflect the fact that both Notch and WNT are both heavily utilized during development, where WNT and Notch must cooperate for proper development (Collu, Hidalgo-Sastre et al. 2014). Future

studies will no doubt further dissect and define the relative contributions of these pathways to crosstalk between Notch and WNT signaling.

Notch and VEGF

The vascular endothelial growth factor (VEGF) signaling pathway coordinates vascular development through VEGF ligand binding to cell surface receptor tyrosine kinases. The core of VEGF signaling consists of six broadly expressed VEGF ligands and four VEGF receptors that are highly restricted to vascular and lymphatic tissues (reviewed in (Koch and Claesson-Welsh 2012)). A flurry of publishing activity in recent years now supports a strong crosstalk between the Notch and VEGF signaling mechanisms in the vascular system. The basis for Notch/VEGF crosstalk appears to be rooted in the reciprocal transcriptional control of Notch ligands by VEGF, and VEGF receptors by Notch. For instance, early work determined that VEGF was an important regulator of Notch receptors and ligands (Lawson, Vogel et al. 2002, Liu, Shirakawa et al. 2003). Around the same time, it was also becoming apparent that Notch activity was an important determinant of VEGF receptor expression (Taylor, Henderson et al. 2002, Holderfield, Henderson Anderson et al. 2006, Williams, Li et al. 2006). It was not until later, however, when a more complete picture of the interaction between Notch and VEGF began to come into focus. The prime example demonstrating reciprocal regulation between Notch and VEGF occurs during angiogenesis, wherein Notch/VEGF crosstalk has been implicated in the selection and differentiation of tip versus stalk cells on growing columns of endothelial cells (reviewed in (Blanco and Gerhardt 2013)). During tip cell selection, VEGF binding to VEGF Receptor 2 (VEGFR2) at the quiescent endothelial membrane causes a phenotypic switch into a motile cell state known as a tip cell, while also inducing the expression of Dll-4

(Hellstrom, Phng et al. 2007, Lobov, Renard et al. 2007, Suchting, Freitas et al. 2007). Dll-4 expression in tip cells and subsequent binding to Notch receptors on adjacent endothelial cells (stalk cells) reduces stalk cell sensitivity to VEGF through the down regulation of VEGFR2, thereby preventing stalk cells from taking on the tip cell phenotype and restricting the number of new vascular branches (Hellstrom, Phng et al. 2007, Leslie, Ariza-McNaughton et al. 2007). Dll-4 signaling in tip cells also increases Jagged1 expression in stalk cells which in a twist of understanding, inhibits Dll-4-Notch signaling in tip cells resulting in increased VEGFR2 expression and VEGF sensitivity (Benedito, Roca et al. 2009, Pedrosa, Trindade et al. 2015). In this way, VEGF first elevates Dll-4 expression, which then represses VEGF sensitivity in adjacent cells, thus demonstrating reciprocal regulation between Notch and VEGF. Beyond tip/stalk cell differentiation, crosstalk between Notch and VEGF has also been shown to be an important mechanism controlling other aspects of cardiovascular biology such as arteriovenous differentiation (Fish and Wythe 2015), differentiation of vascular progenitors from stem cells (Sahara, Hansson et al. 2014), heart valve development (van den Akker, Caolo et al. 2012), tumor angiogenesis (Liu, Fan et al. 2014), as well as neuronal development (Thomas, Baker et al. 2013).

Other Microenvironment Conditions that Control Notch (Shear Stress, Hypoxia, and Hyperglycemia)

As an integrator of cellular microenvironments, the crosstalk between Notch and other signaling pathways is fairly well described compared to crosstalk between Notch and other microenvironmental conditions. Nonetheless, compelling evidence has been emerging in recent years, that stimuli such as shear stress in the cardiovascular system, low oxygen levels (hypoxia), and even hyperglycemia all have significant impacts on Notch

signaling (figure A.4). Additionally, Notch has been reported to respond to other microenvironmental conditions including high salt in endothelial precursor cell media (Karcher, Hoffmann et al. 2015) and temperature flux in *Drosophila* (Shimizu, Woodcock et al. 2014, Ishio, Sasamura et al. 2015), however these responses will not be further discussed here. Below we summarize the data and provide mechanistic information (where possible) for interactions between Notch and shear stress, hypoxia, and hyperglycemia.

Notch and Shear Stress

Notch signaling has in recent years been shown to be an extremely important regulator in the development and function of vascular systems, and many excellent reviews have been published on the role of Notch in vascular development and function (Gridley 2010, Benedito and Hellstrom 2013). In addition, Notch has also been tightly linked to several vascular malfunctions including the development of atherosclerotic lesions (Rusanescu, Weissleder et al. 2008). Recently however, a previously unrecognized role for Notch in sensing shear stress in the vascular system has also begun to emerge. Shear stress in the vascular system is a mechanical force applied to endothelial cells by fluid flow and normally ranges from 1-5 Pa (10-50 dynes/cm^2) in arteries and capillaries, to 0.1-0.5 Pa (1-5 dynes/cm^2) in veins (Cohen, Wang et al. 1995). Shear stress is an important component of the endothelial cellular microenvironment that strongly influences endothelial cell biology. Laminar (undisturbed) shear stress provides an athero-protective signal to endothelium, while non-laminar, disturbed, or oscillatory shear stress provokes the development of endothelial dysfunction and atherogenesis (Glagov, Zarins et al. 1988). Several endothelial shear stress sensors have been identified and include a wide range of transmembrane proteins on both the apical and basolateral endothelial surfaces and the

intracellular kinases and signaling networks that are stimulated by these surface proteins (Zhou, Li et al. 2014). The first demonstration that Notch can serve as a sensor for shear stress was provided by Wang et al. who showed that Notch signaling targets were differentially regulated after exposure to shear stress for as little as 10 minutes (Wang, Fu et al. 2007). Masumura et al. subsequently showed that shear stress activates Notch signaling and that this signal is critical for embryonic stem cell differentiation to endothelium (Masumura, Yamamoto et al. 2009). Although protein expression of Notch1 and 4, as well as the Notch ligands Dll-4, Jagged1 and 2 increased after exposure to laminar flow, increased abundance of the active Notch NICD domain was observed prior to increased Notch receptor/ligand expression, suggesting that shear stress may regulate Notch signaling on both transcriptional and post-translational levels (Masumura, Yamamoto et al. 2009). Mechanotransduction by Notch signaling has since been demonstrated to be an important player in both vascular development (Jahnsen, Trindade et al. 2015) and dysfunction (Tu, Li et al. 2014). Interestingly, inhibition of Notch under atherogenic / low shear stress conditions was shown to inhibit several pro-inflammatory molecules, suggesting that inappropriate activation of Notch by low shear stress may also be linked to the early stages of atherogenesis (Qin, Zhang et al. 2016). While these findings clearly implicate Notch in endothelial shear stress responses, it is not currently understood how Notch signaling is activated by shear stress. It seems unlikely that activation of Notch is wholly dependent on transcriptional mechanisms since Notch activation is observed after as little as 10 minutes of shear stress (Wang, Fu et al. 2007). However, it has also been shown that inhibition of VEGFR2 signaling during shear stress blocks Notch activation (Masumura, Yamamoto et al. 2009), suggesting that if Notch receptors are flow sensors,

that they do not act alone during endothelial response to shear stress. Taken together, these findings illustrate the important role Notch plays in responding to shear stress in the endothelial microenvironment. Future work will hopefully further explore the mechanism by which Notch is activated by shear stress and continue to define the importance of Notch in endothelial/vascular response to shear stress.

Notch and Hypoxia

Notch signaling responds to oxygen content within the cellular microenvironment, showing differential activity under normoxic and hypoxic conditions. The first evidence suggesting that Notch might be involved in hypoxic responses came when researchers observed that Dll4 expression was increased in hypoxic tissues (Mailhos, Modlich et al. 2001). Soon after, Notch signaling was observed to be increased in hypoxic neuroblastoma cells (Jogi, Ora et al. 2002). Further investigation has discovered a physical interaction between NICD and HIF-1α (Hypoxia Inducible Factor α), which was promoted by hypoxia and lead to inhibition of cellular differentiation (Gustafsson, Zheng et al. 2005). A similar observation was also made in *Drosophila* when it was observed that Sima (the *Drosophila* HIF1-α homolog) could also activate Notch receptor in a ligand-independent manner (Mukherjee, Kim et al. 2011). HIF-1α binds to NICD, stabilizes it, and enhances the transcriptional activation of Notch downstream genes through an association with NICD transcriptional complexes (Gustafsson, Zheng et al. 2005). Subsequently, it was shown that hypoxia induced HIF-1α also serves to activate γ-secretase through a direct interaction, promoting invasiveness and metastasis in murine breast cancer cells (Villa, Chiu et al. 2014). Furthermore, HIF-1α can upregulate expression of the Notch ligand Dll-4 in endothelial progenitor cells (Diez, Fischer et al. 2007), and lymphatic vessels (Min, Lee et

al. 2016). Finally, further sophistication of this mechanism is achieved through FIH (Factor Inhibiting HIF-1α), an asparagine hydroxylase (Scholz, Rodriguez et al. 2016), which works to inhibit HIF-1α activity in an O_2 dependent mechanism (Mahon, Hirota et al. 2001). FIH also negatively regulates Notch target gene transcription (Gustafsson, Zheng et al. 2005), likely through its hydroxylation and destabilization of NICD under normoxic conditions (Zheng, Linke et al. 2008). Collectively, the multiple mechanisms by which hypoxia controls Notch including HIF-1α association with Notch transcriptional complexes, γ-secretase induction, promotion of ligand expression, and FIH activity provide at least four independent mechanisms by which Notch cooperates in hypoxic responses.

Notch and Hyperglycemia

Recent work has begun to dissect a molecular mechanism by which Notch signaling may respond to increased or decreased blood sugar and possibly play a role in diabetes and the vascular and renal complications associated with diabetes. For example, Notch signaling in hepatocytes is increased in response to high sugar concentrations (Valenti, Mendoza et al. 2013) and hyperglycemia induced Jagged1 expression in endothelium was proposed to be an important mediator of diabetic vasculopathy (Yoon, Choi et al. 2014). Moreover, several investigators have shown that hyperglycemia elevates Notch receptor expression/signaling in cultured podocytes (Gao, Yao et al. 2013, Wang, Yao et al. 2014, Liu, Zhang et al. 2015), and elevated Notch signaling has been linked to a loss of glomerular filtration due to a negative impact on podocyte function (Niranjan, Bielesz et al. 2008, Waters, Wu et al. 2008). Thus, hyperglycemic stimulation of Notch may be extremely important for understanding the pathology of diabetic nephropathy, especially since podocyte damage in diabetic kidneys has been proposed to be an early triggering

event leading to other downstream renal complications (Lin and Susztak 2016). In support of this, treatment of Streptozotocin induced diabetic mice with the angiotensin inhibitor Valsartan, simultaneously decreased renal damage and Notch activation (Gao, Yao et al. 2016). Mechanistically, the link between hyperglycemia and Notch has been elusive, however it is known that hyperglycemia (as well as hypoglycemia) induce VEGF secretion and signaling (Natarajan, Bai et al. 1997, Kemeny, Figueroa et al. 2013). Given the reciprocal transcriptional regulation between VEGF and Notch (as described above), it seems likely that VEGF and Notch may pathologically synergize in hyperglycemic conditions. In support of this, a recent report by Chiu et al. found that hyperglycemia increased endothelial secretion of heparinase leading to increased VEGF release from neighboring myocytes thus enhancing endothelial Notch activity (Chiu, Wan et al. 2016). Interestingly, anti-VEGF therapies have shown some success in reducing diabetic renal dysfunction (de Vriese, Tilton et al. 2001, Flyvbjerg, Dagnaes-Hansen et al. 2002), although it is unknown whether these anti-VEGF approaches also reciprocally decrease Notch signaling. It has also been shown that inhibition of Notch reduced the elevated VEGF secretions in podocytes cultured under hyperglycemic conditions, as well as diabetic nephropathy in diabetic rats (Lin, Wang et al. 2010). Finally, there may be other Notch regulatory mechanisms beyond VEGF that are operant during hyperglycemic conditions. For example, hyperglycemia induced Notch activity seems to be also be linked to decreased CARM1 methyltransferase activity (Kim, Lim et al. 2014), a recently discovered negative regulator of Notch signaling (Hein, Mittler et al. 2015). Hopefully future research will be able to more clearly define the molecular mechanism by which hyperglycemia controls Notch in order to more fully understand the downstream implications of Notch signaling

in diabetes.

Conclusions

Through the examples presented in this review, it is clear that Notch signaling is capable of responding to a range of changing microenvironmental conditions that go far beyond the basic model of receptor-ligand interaction for Notch activation. Instead, evidence is building that the basic model of Notch activation is manipulated by a variety of microenvironmental cues and that the basic cell-cell interaction model of Notch activation represents only a part of the broader function of Notch to sense and respond to a wide variety of microenvironmental conditions. Despite these findings, many of the results discussed here have been gained from simplified models of cellular microenvironment. In order to build a more complete understanding of how Notch serves its role as an integrator of cellular microenvironments, future studies will need to examine how Notch responds to these conditions in more complex in vivo models, an undoubtedly complex task. In addition, fleshing out the molecular underpinnings of how Notch responds to microenvironmental conditions is an important goal that should provide opportunities for pharmacological intervention in the many diseases and processes that are characterized by altered microenvironments. Lastly, efforts to therapeutically manipulate Notch are ongoing for the treatment of a wide variety of diseases (reviewed in (Andersson and Lendahl 2014)). Therefore, an understanding of the full spectrum of activities that Notch serves in the microenvironment is an important consideration in developmental biology and various Notch targeting strategies.

Acknowledgements

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Table A.1 Basic and Proposed Mechanism by which Notch Responds to Various Microenvironmental Signals

Table 1
Basic and proposed mechanisms by which Notch responds to various microenvironmental signals.

ECM – Notch (Direct interaction mechanism)		
Protein name	**Basic/Proposed mechanism (s)**	**Ref.**
MAGP-2	-Decrease Notch in RGD/integrin dependent manner	Deford et al., 2016
	-Interacts with EGF-like domains of Notch1 + Jagged1, activates cleavage	Nehring et al., 2005; Miyamoto et al., 2006
EGFL7	-RGD/integrin dependent Notch activation	Deford et al., 2016
	-Interaction with Notch EGF domains and resultant Notch inhibition	Schmidt et al., 2009
CCN3	-Binds Notch1 receptor and stimulates Notch receptor activation	Sakamoto et al., 2002; Katsuki et al., 2008
TSP-2	-Binds Notch3, activates Notch3/Jagged signaling	Meng et al., 2009
Syndecans	-Syndecan-2 binds Notch3, activates Notch3/Jagged signaling	Zhao et al., 2012
	-Syndecan-3 interacts with Notch1	Pisconti et al., 2010
Col IV	-Interacts with Notch3, blocks Jagged1 – Notch3 signaling	Zhang et al., 2013
Col I	-Interacts with Notch3, blocks Jagged1 – Notch3 signaling	Zhang et al., 2013
YB-1	-Binds Notch3 EGF-like repeats, blocks Notch3 but not Notch1 signaling	Rauen et al., 2009; Raffetseder et al., 2011
Galectin-3	-Binds Notch1 glycosylations, increases NICD processing	Nakajima et al., 2014
ECM – Notch (Indirect transcriptional mechanism)		
Protein name	**Basic/Proposed mechanism**	
SPARC	-Suppresses Notch by blocking Notch1 transcription	Bhoopathi et al., 2011; Kessler and Delany, 2007
Fibulin-3	-Enhances Dll-4 expression, ADAM10/17 activity, and Notch signaling	Nandhu et al., 2014
Laminin α4	-Increases Dll-4 expression, stimulates Notch	Stenzel et al., 2011
Laminin-111	-Increases Dll-4 expression, stimulates Notch	Estrach et al., 2011
MGP	-Decreases Jagged1 expression, suppresses Notch	Sharma and Albig, 2013; Yao et al., 2013
ECM – Notch (Indirect cross-talk mechanism)		
Protein name	**Basic/Proposed mechanism**	
Reelin	-Stimulates DAB-1 and SRC signaling. Increases Notch activity	Keilani and Sugaya, 2008; Keilani et al., 2012; Hashimoto-Torii et al., 2008
Cross-talk with other signaling pathways		
Pathway	**Basic/Proposed mechanism**	
Integrin	-RGD/RGE control of Notch activity. Possible SRC or ILK involvement	Deford et al., 2016; Mo et al., 2007; Ma et al., 2012
	-Notch4 / R-Ras mediate increase in β1 integrin affinity	Leong et al., 2002; Hodkinson et al., 2007
TGF-β/BMP	-Transcriptional complex between NICD and SMAD3	Blokzijl et al., 2003; Asano et al., 2008; Mustonen et al., 2002; Itoh et al., 2004
WNT	-Transcriptional complex between NICD and β-catenin	Ross and Kadesch, 2001; Shimizu et al., 2008; Yamamizu et al., 2010
	-β-Catenin interaction with undeaved Notch receptor	Hayward et al., 2005; Kwon et al., 2011; Sanders et al., 2009
	-GSK3β mediated phosphorylation of NICD, Notch inhibition	Foltz et al., 2002; Espinosa et al., 2003
	-Dishevelled interaction with CSL, Notch inhibition	Collu et al., 2012; Axelrod et al., 1996
VEGF	-Suppression of VEGFR2 receptors by Dll-4	Liu et al., 2003; Lawson et al., 2002
	-VEGF signaling increases Notch1 and Dll-4 expression	Williams et al., 2006; Taylor et al., 2002; Holderfield et al., 2006
Other microenvironmental conditions		
Condition	**Basic mechanism**	
Shear Stress	-Rapid NICD cleavage, transcriptional control of receptor/ligands, possible involvement of VEGFR2	Masumura et al., 2009; Jahnsen et al., 2015; Tu et al., 2014
Hypoxia	-Transcriptional complex between NICD and Hif1α	Gustafsson et al., 2005
	-Activation of γ-secretase	Villa et al., 2014
	-Increase of Dll-4 expression by Hif1α	Benedito et al., 2009; Min et al., 2016
	-FIH mediated hydroxylation and destabilization of NICD (Normoxia)	Mahon et al., 2001; Zheng et al., 2008
Hyperglycemia	-VEGF mediated increase in Notch receptor expression and NICD accumulation.	Wang et al., 2014; Liu et al., 2015; Gao et al., 2013
	-Possible involvement of NICD methylation by CARM1	Kim et al., 2014

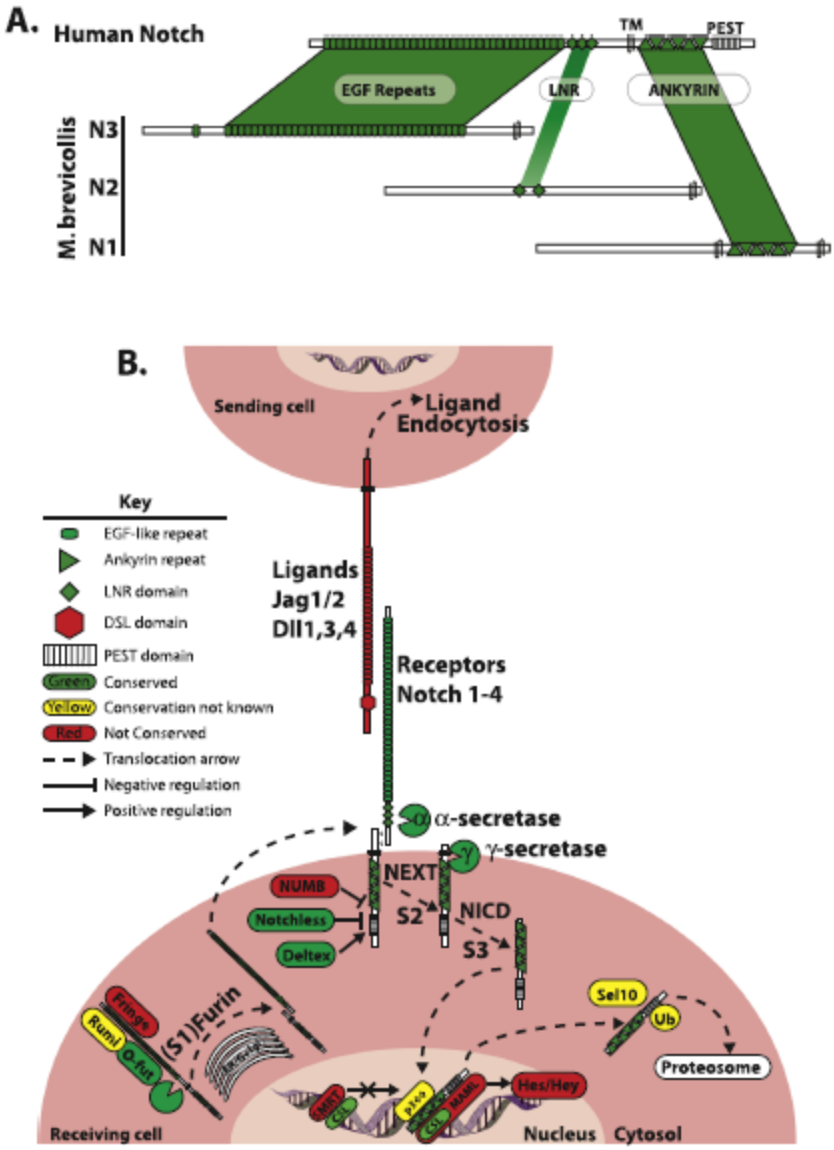

Figure A.1: Canonical Notch Signaling and Notch Conservation between Human and *Monosiga brevicollis*.

Throughout Figure A.1, conservation of Notch proteins or domains between human and *M. brevicollis* is indicated by green (positive), yellow (unknown), or red (negative) shading according to references (King, Westbrook et al. 2008) and (Gazave, Lapebie et al. 2009). (A) Conservation of mammalian Notch receptor domains in *M. brevicollis*. Mammalian (human) Notch receptors contain 36 EGF-like repeats and three LNR or NRR (Lin-12 Notch Repeats or Negative Regulatory Region) repeats in the extracellular domain. The intracellular portion of human Notch contains seven ankyrin domains and a PEST sequence at the C-terminal. For simplicity, the intracellular RAM (RBPj Association Module)

domain, two NLS (Nuclear Localization sequence) domains, and TAD (Transactivation Domain) are not shown in this figure. Please refer to references (Kopan and Ilagan 2009, Kopan 2010) for complete details. Three separate proteins (N1, N2, and N3) in *M. brevicollis* contain six Ankyrin domains, two LNR domains, and 36 EGF-like repeats respectively (King, Westbrook et al. 2008). (B) Model of canonical Notch activation mechanism. Notch receptors are modified in the secretory pathway (ER/golgi) by Furin cleavage (S1 cleavage) and glycosylation of EGF-like domains by O-fucosyltransferase (O-fut), Rumi/Poglut1 (Protein O-Glycosyltransferase 1), and fringe family glycosyltransferases. The Furin cleavage products remain non-covalently associated in the membrane where a pulling force initiated by Notch ligand endocytosis in sending cells enables further cleavage by α-secretase (S2 cleavage, NEXT fragment) at the LNR domain, and γ-secretase (S3 cleavage, NICD fragment) in the membrane of receiving cells. Several regulatory proteins including Numb, Notchless, and Deltex control Notch availability at the membrane. After γ-secretase cleavage, the NICD fragment translocates to the nucleus where it displaces the transcriptional co-repressor SMRT from CSL/RBP-jk. NICD participates in a transcriptional complex with CSL, MAML, and p300 to drive transcription of Notch targets such as Hes and Hey genes. NICD steady-state levels are controlled by nuclear export, ubiquitination (Ub) by Sel10, and subsequent degradation in the proteasome.

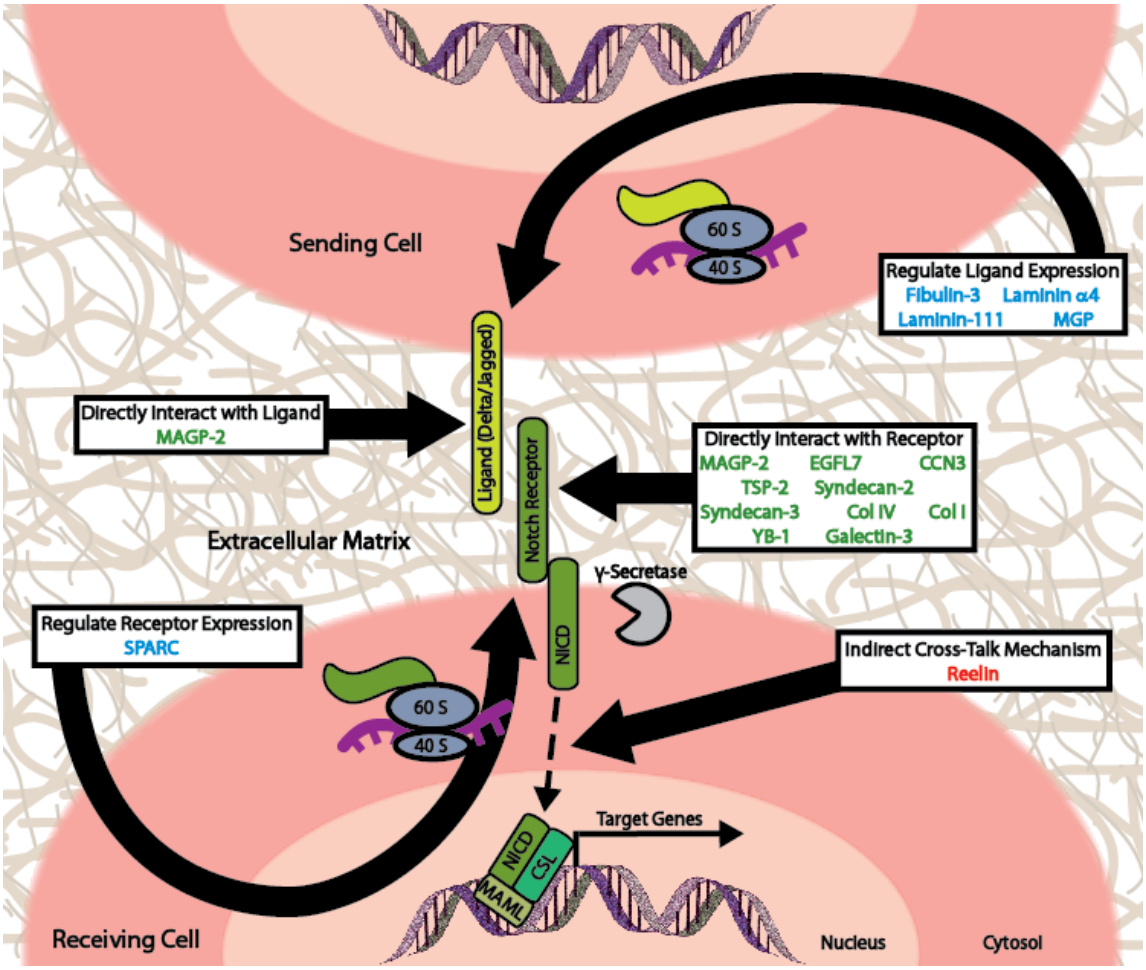

Figure A.2: Summary of ECM Control of Notch Signaling.

Canonical activation of Notch receptors by Notch ligands can be manipulated in three ways by cellular interactions with ECM. 1.) Direct interactions between Notch receptors or ligands and various ECM molecules can either inhibit or promote activation of Notch signaling. 2.) Indirect interactions between ECM and Notch are characterized by ECM mediated increased or decreased expression of Notch ligands on sending cells or Notch receptors on receiving cells. 3.) Indirect interactions between Notch and ECM are characterized by ECM mediated activation of signaling pathways that post-translationally intersect with Notch proteins or signaling intermediates.

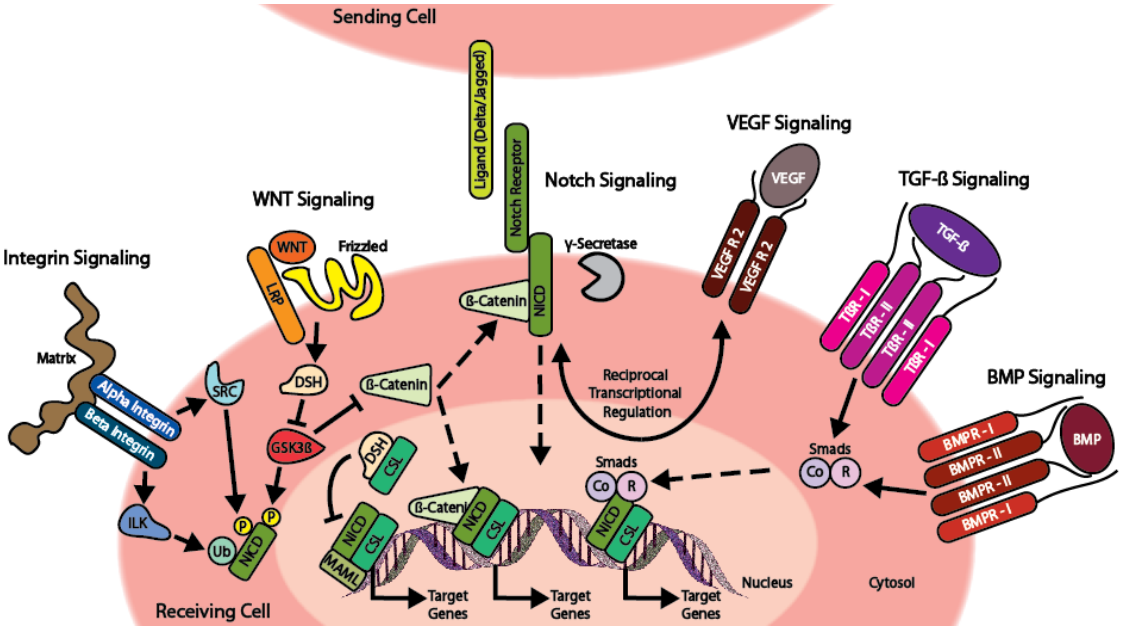

Figure A.3: Crosstalk between Notch and Other Signaling Pathways.

Crosstalk between WNT and Notch occurs on several levels including the formation of a β-catenin-NICD transcriptional complex, interaction between Notch receptors and β-catenin at the membrane, phosphorylation of NICD by GSK3β, and inhibitory interactions between Dishevelled and CSL. The mechanistic interaction between integrins and Notch is poorly characterized, but existing evidence suggests ubiquitination and/or phosphorylation of NICD by SRC and ILK kinases. Interaction between the Notch and VEGF pathways involves the reciprocal transcriptional regulation of Notch ligands by VEGF, and VEGFR2 by Notch. Notch/TGF-β, or Notch/BMP crosstalk occurs downstream of ALK (TBR1/TBR2 or BMPR1/BMPR2) receptors and is dependent on R-SMAD and Co-SMAD activation and subsequent formation of a SMAD/NICD transcriptional complex similar to the β-catenin/NICD complex.

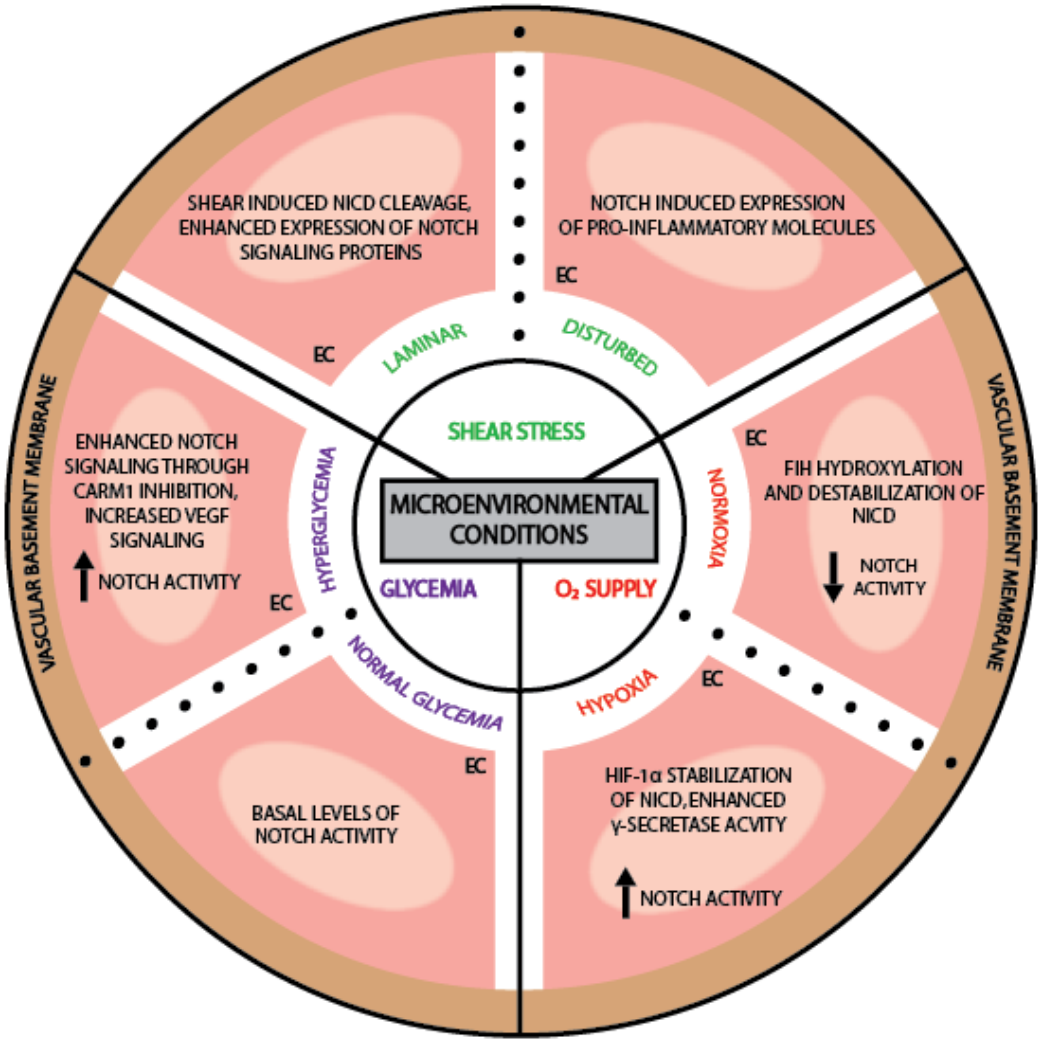

Figure A.4: Summary of Microenvironmental Conditions (Shear Stress, Hypoxia, and Hyperglycemia) that Control Notch.

Depicted is a cross-sectional view through a blood vessel showing endothelial cells (EC) and vascular basement membrane. Shear stress (laminar versus disturbed or non-laminar) controls Notch by largely undefined mechanisms that may include regulation of Notch receptors and/or ligands. Hypoxia controls Notch signaling by several mechanisms including the formation of HIF1α-NICD transcriptional complexes, HIF1α mediated stabilization of NICD, enhanced γ-secretase activity, and FIH mediated NICD destabilization. Hyperglycemia controls Notch by largely uncharacterized mechanisms that may include increased NICD stability due to decreased CARM1 expression and/or increased VEGF release from other cells in the vascular microenvironement.

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APPENDIX B

Beyond the Matrix: The many non-ECM ligands for Integrins

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Abstract

The traditional view of integrins portrays these highly conserved cell surface receptors as mediators of cellular attachment to extracellular matrix (ECM), and to a lesser degree, as coordinators of leukocyte adhesion to endothelium. These canonical activities are indispensable, however, there is also a wide variety of integrin functions mediated by non-ECM ligands that transcend the traditional roles of integrins. Some of these unorthodox roles involve cell-cell interactions and are engaged to support immune functions such as leukocyte transmigration, recognition of opsonization factors, and stimulation of neutrophil extracellular traps. Other cell-cell interactions mediated by integrins include hematopoietic stem cell and tumor cell homing to target tissues. Integrins also serve as cell-surface receptors for various growth factors, hormones, and small molecules. Interestingly, integrins have also been exploited by a wide variety of organisms including viruses and bacteria to support infectious activities such as cellular adhesion and/or cellular internalization. Additionally, disruption of integrin function through the use of soluble integrin ligands is a common strategy adopted by several parasites in order to inhibit blood clotting during hematophagy, or by venomous snakes to kill prey. In this review, we strive to go beyond the matrix and summarize non-ECM ligands that interact with integrins in order to highlight these non-traditional functions of integrins

Introduction

Adhesion of cells to extracellular matrices is a fundamental requirement for multicellular organisms, and animals employ many mechanisms to fulfill this demand. Amongst these mechanisms of adhesion, integrins are perhaps the most ubiquitous. Integrins are heterodimeric transmembrane proteins, made up of non-covalently paired α and β subunits,

which serve as adhesion and signaling hubs at the cell surface. In mammals, there are 18 α-integrin subunits and 8 β-integrin subunits that can combine to form as many as 24 unique heterodimeric receptor complexes [1]. Typically, ligand binding is carried out through integrin receptor recognition of small peptide sequences. Target sequences for integrins can be as simple as the RGD or LDV tri-peptides, or more complex as in the case of the GFOGER peptide [1]. Many classical extracellular matrix (ECM) proteins contain these short integrin recognition motifs. RGD sequences are found in both vitronectin and fibronectin, a LDV motif is present in fibronectin, GFOGER is found within collagen, and the target sequence within laminin has not yet been defined [1]. These sequences are not globally recognized by all integrins; therefore, integrin heterodimers are often grouped by the target sequences they specialize in recognizing (figure B.1). Once bound to its ligand, an integrin not only provides adhesion, but also initiates signaling mechanisms which allow cells to respond to the mechanical and chemical properties of the cellular microenvironment. The primary signaling mediators working downstream of integrins include focal adhesion kinase (FAK), Src-family protein tyrosine kinases, and integrin-linked kinase (ILK) [2]. Upon adhesion, cytoskeletal proteins are recruited to the cytoplasmic tails of integrins, forming a linkage between the ECM and cytoskeleton [2].

As a family of proteins, integrins and many of their downstream signaling intermediates, have a long evolutionary history. Beginning at the root of the metazoan lineage, sponges have been shown to express α- and β-integrin subunits [3, 4] that bind to peptides in a similar fashion as mammalian integrins [5]. Interestingly, integrin encoding genes have been found in the single-celled eukaryotic relatives of metazoans, thus the origin of integrins predates the emergence of metazoans [6]. Moreover, components of

integrin signaling machinery such as FAK, Src, and ILK, and integrin-interacting cytoskeletal proteins such as α-actinin, talin, vinculin, and paxillin, have pre-metazoan origins [6]. This suggests that integrins and their aforementioned signaling machinery may have played an important role in the evolution of multicellularity.

Beyond their traditional role as mediators of ECM attachment, a vast literature has developed that describes interactions between integrins and ligands that are not located in the classical extracellular matrix. For example, integrins have been shown to interact with various proteins on the surfaces of eukaryotic, prokaryotic, and fungal cells, as well as a range of viruses. Within eukaryotes specifically, integrin mediated cell-cell adhesion has been shown to coordinate a range of interactions and processes including leukocyte extravasation, stem cell homing, tumor cell migration, erythrocyte development, and interactions in the immune system. For infectious prokaryotes, integrins are exploited as cell surface adhesion receptors that mediate colonization and/or the bypassing of epithelial or endothelial barriers. Beyond mediating cellular interactions, integrins can also serve as cell surface receptors for hormones, growth factors, and polyphenols. Finally, integrins are also common targets for a class of small molecules called disintegrins, which are components of various snake venoms, and are also employed by hematophagous parasites. Collectively, the range of non-ECM molecules that interact with integrins is vast, making integrins indispensable mediators of cell biology at large. The goal of this review is to highlight some of the best understood non-ECM ligands of integrins, and discuss the diverse biological roles for these interactions.

Integrin-Mediated Cell-Cell Interactions

The first integrins discovered were isolated based on their ability to bind to fibronectin, which had itself, just recently been identified (reviewed in [7]). However, in the early days of integrin research, several groups studying cell-cell adhesion in the immune system were also on the forefront of integrin identification (reviewed in [8], [9]). In fact, integrins that mediate cell-cell adhesion in the immune system were among the first integrins to be characterized [8]. As more integrins were discovered, it became apparent that the majority of integrins established cell-ECM connections rather than cell-cell connections. Nonetheless, it is important to understand that integrins are important mediators of cell-cell adhesion. The term counterreceptor has often been used to describe membrane-bound, non-matrix integrin ligands which facilitate cell-cell contact, and will be used to differentiate them from the other non-matrix ligands in this review. While there are many types of counterreceptors, the best known examples include the immunoglobulin superfamily cell adhesion molecules (IgCAMs) and junctional adhesion molecules (JAMs). Collectively, interactions between integrins and these counterreceptors mediate a range of immune cell functions including leukocyte extravasation from the blood stream, immunological surveillance in the gut, and hematopoietic stem cell homing and mobilization. Additionally, non-ECM ligands enhance the interaction between pathogens and phagocytic immune cells, acting as phagocytic primers and inducers of neutrophil extracellular traps. Beyond the immune system, non-ECM based integrin interactions are important during the transmigration and metastasis of tumor cells, and during erythrocyte development. Integrins and the non-ECM ligands that mediate these cell-cell interactions are listed in Table B.1.

Integrin-Counterreceptor Interactions in Leukocyte Extravasation

Integrin-counterreceptor interactions play multiple roles during extravasation, a process in which white blood cells are recruited from the blood stream to a site of inflammation (depicted in figure B.2). Extravasation begins when glycoproteins on the leukocyte cell surface, such as PSGL-1, bind endothelial selectins which allows the leukocyte to slow down as it rolls along the vessel wall [33]. Next, local chemokines stimulate leukocyte integrins to adopt a high-affinity state causing them to bind specific immunoglobulin superfamily cell adhesion molecules (IgCAMs) on endothelial cells [34]. There are many integrin-IgCAM pairs involved in this process: αLβ2 (LFA-1) integrin binds to ICAM1, 2, or 3, αMβ2 (Mac-1) integrin binds to ICAM1, and α4β1 (VLA-4) integrin binds to VCAM1 or MAdCAM1 (reviewed in [11], [10]). Additionally, leukocyte integrins can bind a family of proteins known as junctional adhesion molecules (JAMs) found on endothelial cells. Similar to integrin-IgCAM interactions, integrins display specificity for particular JAM proteins: JAM-A binds to αLβ2, JAM-B binds to α4β1, and JAM-C binds to αMβ2 [11]. All of these integrin-counterreceptor binding events serve to tightly adhere the leukocyte to the endothelium, enabling the white blood cell to cross the endothelial layer (a process known as transendothelial migration) in order to reach the inflamed tissue.

Non-ECM Integrin Ligands as Primers for Phagocytosis

One of the best-characterized examples of non-ECM integrin binding ligands in the immune system involves the interplay of integrins with the complement system. Complement proteins aid in the immune system's clearance of pathogens by attaching to invaders and tagging them for destruction. Integrin β2 is essential for complement

recognition by the complement receptors αMβ2 (Mac-1, CR3) and αXβ2 (CR4) integrins [23]. αMβ2 and αXβ2 ligation with the iC3b component of complement induces phagocytosis of complement opsonized pathogens and particles by phagocytic immune cells (depicted in figure B.2) [24]. Despite high homology between both integrins, they bind the iC3b fragment of complement via distinctive receptor sites which may afford leukocytes greater diversity in opsonized target recognition modes [35]. This leads to the intriguing possibility of cooperativity between two integrins binding the same complement molecule [35].

Phagocytosis mediated by integrins is not strictly complement dependent. Human cathelicidin peptide LL-37, an antimicrobial peptide that binds to the prokaryotic cell wall, inserts itself into the membrane, and enhances phagocytosis by interacting with αMβ2 integrin present on neutrophils and macrophages [26, 27]. As an important part of innate defenses, LL-37 is expressed in various mammalian tissues and released upon contact with bacterial invaders [29]. For example, upon infection by *Helicobacter pylori*, gastric epithelial cells express and secrete LL-37, thus tagging the bacterial invaders for destruction by phagocytic immune cells (depicted in figure B.2) [28]. Interestingly, LL-37 binds αMβ2 with a comparable strength to complement C3d, a ligand with one of the strongest known affinities for αMβ2 [25].

Non-ECM Integrin Ligands as Triggers for NETosis

Another example of non-ECM integrin ligation at work in the innate immune system is the neutrophil extracellular trap (NET). In the process of NETosis, chromatin is ejected from neutrophils upon interaction with pathogens, thus entangling foreign invaders in a web of DNA and histones (depicted in figure B.2) [36]. This process is mediated through

pathogen recognition by neutrophil integrins. For example, the pathogen associated molecular pattern, β-glucan, found on *Candida albicans* is recognized by αMβ2 at a unique lectin-like domain and whose binding stimulates NETosis [21]. Once stimulated, anti-microbial peptides are integrated into NETs. These include defensins and the αMβ2 ligand LL-37 [22]. NETosis is not exclusively used to trap foreign invaders as it is also involved in wound healing and sterile inflammation [37]. For instance, during cell necrosis the chromatin protein high-mobility group box 1 (HMGB1 aka amphoterin) is released extracellularly and recruits neutrophils by binding integrin β2 [38]. HMGB1 has been demonstrated to be an inducer of NETosis when presented on platelets during thrombosis [30]. This evidence suggests that HMGB1 serves as a molecule that is capable of signaling to white blood cells the presence of tissue damage through leukocyte integrins. Although αMβ2 plays a starring role in the literature connecting NETosis and integrins, other integrins may be involved. Bacterial invasin proteins from *Yersinia pseudotuberculosis* interact with neutrophil integrin β1, stimulating phagocytosis while also causing the release of NETs [39]. In addition to trapping cells within a tangle of DNA and histones, fibronectin has been identified in NETs, which ligates to αVβ3 and α5β1 integrins found on neutrophils and cancer cells, thus potentially enhancing cancer cell-leukocyte interaction [40]. Collectively, this information demonstrates rich evidence for the importance of integrin engagement during NETosis.

Non-ECM Integrin Ligands in Immune Surveillance

The intestinal immune system must display tolerance towards commensal microbiota and food antigens, while still maintaining immunogenicity against pathogens. In the gut mucosa, resident antigen-presenting cells (APCs) have the job of sampling foreign

antigens. APCs then transport these antigens to specialized gut-associated lymphoid tissue where they can interact with naïve T cells to promote their maturation. Additionally, the APCs imprint intestinal homing properties on the T cells through inducing expression of α4β7 integrin and CCR9, a receptor for the gut-associated chemokine CCL25 [41]. Mature T effector cells then reenter the circulation and can be recruited back to the gastrointestinal tract during times of inflammation through gut endothelial expression of CCL25 and the α4β7 counterreceptor, MAdCAM1 [17]. There is also a role for α4β1-VCAM1 interactions in the gut; this pair mediates binding of effector T cells to inflamed gut epithelium [12].

Integrins in the gut also bind to cadherins to modulate the immune response. For instance, cadherin 26 binding to integrins αE and α4 can lead to a T cell immunosuppression phenotype [42]. Moreover, this study found that a similar phenotype is provoked through treatment of T cells with a soluble form of cadherin 26. So, unlike the integrin-mediated IgCAM interactions in the gut, cadherin binding appears to moderate the immune response. It has been suggested that this interaction may therefore be involved in resolving inflammation [42]. Cadherin-integrin interactions in the lungs have been shown to mediate the engagement of cytotoxic T lymphocytes (CTLs) with cancer cells. Here, CTLs employ αEβ7 integrin to engage E-cadherin on cancer cell surfaces in order to facilitate accurate targeting and release of cytotoxic granules [20].

Integrin-Mediated Stem Cell Homing

Homing and mobilization of hematopoietic stem cells (HSCs) to and from the bone marrow is also regulated by integrins (reviewed in [13]). After treatment of hematologic malignancies with large doses of radiation and/or chemotherapy, transplantation of HSCs is commonly performed. Success of the HSC engraftment within the bone marrow is

dependent upon proper HSC homing to a bone marrow niche where they can regenerate hematopoietic lineages. New evidence is revealing that integrin engagement of counterreceptors plays a critical role in this homing process. For example, Murakami et al. determined that a subpopulation of murine HSCs expressing integrin β7 have enhanced homing capabilities to bone marrow niches compared to their counterparts which do not express β7 [18]. Mechanistic insight was provided when it was revealed that α4β7 integrins on HSCs were binding MAdCAM1 present on endothelial cells within the bone marrow niche, and β7 knockout HSCs showed decreased CXCR4 homing receptor expression [18].

In addition to α4β7-MAdCAM1 interactions, α4β1-VCAM1 binding also mediates HSC retention in bone marrow niches. The importance of α4 integrin to this interaction is supported by the phenotypes of multiple α4 knockout mouse models that show elevated numbers of HSCs in the bloodstream relative to wild-type littermates (reviewed in [13]). Treatment of mice with Bortezomib, which inhibits the expression of VCAM1, also increases HSC mobilization [14]. Together, these results support a role for integrins in holding HSCs within the bone marrow and have raised great clinical interest in using Bortezomib-induced mobilization for the harvesting of HSCs from the peripheral blood of healthy individuals for use in transplantation.

Another integrin-targeting small molecule antagonist is the drug Firategrast; it inhibits α4β1 and α4β7 activity and can also be used to mobilize HSCs from the bone marrow to the circulation, making HSC harvesting much less invasive. There is particular interest in using Firategrast for *in utero* hematopoietic cell transplants (IUHCT). These transplants can be especially useful for diseases where a more mature immune system can thwart the therapeutic benefit of the transplanted cells (reviewed in [43]). Firategrast was tested in a

mouse model of IUHCT and found to increase long-term engraftment of HSCs; there was 15% engraftment at six months with Firategrast, compared to 3% with vehicle alone [44]. The current thinking is that mobilization of endogenous HSCs through disruption of integrin adhesion by Firategrast makes room in the bone marrow for transplanted HSCs to compete with endogenous cells for niche binding. Although still in preclinical studies, Firategrast is well-tolerated by adults but has not yet been tested in children (reviewed in [43]).

Some interesting new data on mesenchymal stem cell (MSC) homing demonstrates that the role of integrin αL (CD11a) in MSC transmigration across vessel endothelium differs from that of leukocyte extravasation [45]. Using zebrafish with GFP-labeled endothelium as a model system, mammalian leukocytes, cardiac stem cells, and MSCs were transplanted to determine their transmigration properties. As expected, leukocyte extravasation proceeded in an αL-dependent fashion, as αL-blocking antibodies inhibited leukocyte extravasation. However, the blocking antibodies did not inhibit the transmigration of cardiac stem cells or MSCs, indicating that these cells were traversing the endothelium in an αL-independent fashion that was found to rely on remodeling of the endothelium for vascular expulsion of these types of stem cells. Based on this evidence and additional phenotypic differences in the transmigration of cardiac stem cells and MSCs, the authors have named this alternate process angiopellosis [45].

Integrin-Counterreceptor Interactions in Tumor Cell Migration

Integrin binding to IgCAMs also mediates tumor cell binding to endothelial cells, influencing metastasis. Many of these interactions involve L1CAM (reviewed in [46]); this protein contains an RGD motif that binds to αVβ3 integrin [47, 48]. Expression of L1CAM

by various types of cancer cells is utilized to engage αVβ3 on endothelial cells. It has been demonstrated that L1CAM expression in glioma tumor cells serves to promote the motility of both cancer cells [31] and endothelial cells [32], thus having important implications for both metastasis and angiogenesis respectively. Other non-ECM integrin ligands have been implicated in tumor cell migration. For example, when expressed on cancer cells, VCAM1 has been identified as a driver of metastasis due to its ability to bind α4β1 integrin expressed on lymph node endothelium (reviewed in [16]). Additionally, metastatic breast cancer cells express the transmembrane glycoprotein NMB that contains an RGD motif and can bind to α5β1 integrin on adjacent tumor cells. This interaction activates Src and FAK signaling within the tumor and leads to increased growth and metastasis [19].

Integrin-Counterreceptor Interactions in Erythrocyte Development

Since integrins can bind to both ECM and other cells, it is perhaps not surprising that there are modulators that can push integrins towards either a cell-ECM or a cell-cell interaction. During erythrocyte differentiation in the bone marrow, immature erythroblasts cluster around a central macrophage forming what is known as erythroblastic islands. This cell-cell interaction is mediated by α4β1 on erythroblasts and VCAM1 on macrophages and is an essential part of the maturation process [15]. The same α4β1 integrin can bind to fibronectin in the ECM, and the modulation of α4β1 binding to either macrophages or ECM is in part due to the activity of erythrocyte tetraspanin proteins CD81, CD82, and CD151 [49]. These tetraspanins are co-expressed with α4β1 on human proerythroblasts, where they increase affinity and/or clustering of integrins to favor α4β1-VCAM1 interactions over α4β1-fibronectin interactions [49].

Non-ECM Integrin Ligands of Viruses

Although there is debate as to when viruses first emerged in the evolution of life, it is likely that viruses (in one form or another) have co-existed with cells for nearly as long as cells have existed [50]. It is also safe to assume that viruses have a long history of exploiting cell surface receptors to facilitate their infectious cycles. As already discussed, integrins are first present in evolutionary history at the root of the metazoan lineage, and perhaps predate metazoans [3, 4, 6]. Therefore, it is not surprising that many species of viruses have exploited (and continue to exploit) integrins as a major point of cell attachment, entry, and eventually infection of target cells. A common theme among many of the viruses discussed here is the display of RGD motifs on viral capsids to bind to integrins that are commonly found on either epithelial or endothelial surfaces [51, 52]. Presumably, the RGD motif serving as a minimal integrin binding unit accommodates the viral quest for genomic minimization. Additionally, RGD-recognizing integrins are common in tissues targeted by invading viruses. Although, RGD-based mechanisms are not the only means of integrin engagement by viruses, as some viruses employ other integrin targeting motifs. The virus-integrin interactions we have chosen to highlight are in no way an exhaustive list (for a more comprehensive review of the subject refer to [53, 54]. Integrins that participate in viral interactions that we discuss are listed in Table B.2 and depicted in figure B.3.

Non-ECM Integrin Ligands of *Picornaviridae*

Viruses of the *Picornaviridae* family cause a variety of human diseases including aseptic (viral) meningitis, paralysis, hepatitis, and poliomyelitis [87] and there are currently no approved treatments to minimize picornavirus infection. Picornaviruses are non-enveloped viruses with icosahedral capsids with each face of the 20-sided capsid consisting

of 3 capsid proteins (VP1-3) to form a protomer with 60 subunits. The VP4 protein is contained within the capsid and is thought to help package the single-stranded RNA genome (reviewed in [88]). Several picornaviruses have been shown to exploit integrins as cell surface receptors to facilitate cell invasion. In most cases, the non-ECM ligands that enable picornavirus binding to integrins are located on the VP1-3 capsid proteins.

Members of *Picornaviridae* include the enteric cytopathic human orphan (echo) viruses. Echovirus 1 (EV1) utilizes the α2I functional domain of α2β1 integrin as a docking receptor on the surface of a target cell [56, 57]. Although the precise peptide sequence of EV1 that binds to α2β1 integrin has not been discovered, it is known that EV1 binds to the α2I domain of α2β1 integrin 10 times more tightly than collagen [56, 89, 90]. The structure of the EV1 capsid provides a pentameric arrangement of binding sites for α2β1, which induces clustering of α2β1 integrins, and is thought to promote entry of the virus [56]. During infection, EV1 along with α2β1 integrin, are taken into the host cell via caveolar endocytosis and moved to a caveosome, where it is thought that the virus ejects its genome into the cytosol [91-94]. While EV1 utilizes a non-RGD signal to bind its integrin receptor, echovirus 9 (EV9) docks to integrin αVβ3 via an RGD domain located on the EV9 VP1 capsid protein [72]. RGD motifs are also thought to be critical in host cell attachment for echovirus 22 (EV22) to αVβ1 integrins [69, 70]. Another member of the *Picornaviridae* family, coxsackievirus A9 utilizes the coxsackievirus and adenovirus receptor (CAR) together with an RGD motif situated in the C-terminal of its VP1 to bind αVβ3 and αVβ6 integrins and gain cellular entry [73, 95]. Yet another member of the *Picornaviridae* family, foot-and-mouth disease virus (FMDV) is a major scourge of animal husbandry. The VP1 protein of FMDV has an exposed flexible loop, termed the GH loop, which

contains an RGD motif and mediates binding to host α5β1, αVβ3, and αVβ6 integrins [64, 74, 84, 85].

Non-ECM Integrin Ligands of *Flaviviridae*

Flaviviridae is a family of single-stranded, positive sense RNA viruses that are commonly transmitted to human hosts from arthropods such as ticks and mosquitos [96]. Japanese encephalitis virus (JEV), a mosquito-borne member of the genus *Flavivirus*, is a leading cause of viral encephalitis in humans and animals [97, 98]. JEV has an envelope protein, called E protein, which contains an RGD motif [99]. Data suggest that JEV utilizes this RGD motif to bind αVβ3 integrin to aid in cellular infection. Specifically, JEV infectivity is reduced by shRNA knockdown of integrin αV and β3 subunits, pretreatment of cells with soluble RGD peptides, or αV/β3 blocking antibodies. Conversely, expression of β3 integrin promotes infectivity in otherwise resistant cell lines [75]. Finally, utilization of integrin receptors appears to be a common infection strategy for the *Flaviviridae* family since other members such as West Nile virus [100-102], Murray Valley encephalitis virus [103], dengue virus [104], and yellow fever virus [105] have all been connected with integrin-mediated infection or have at least been demonstrated to possess RGD containing E proteins.

Non-ECM Integrin Ligands of *Herpesviridae*

Members of the *Herpesviridae* family of viruses also use integrins for cellular attachment and entry. Epstein-Barr virus (EBV) utilizes α5β1 integrin for infectivity in tongue and nasopharyngeal epithelium by binding host cell integrins with its RGD containing envelope glycoprotein, BMRF-2 [65]. In addition, engagement of EBV envelope glycoproteins gH and gL with αVβ5, αVβ6, and αVβ8 integrins induces a

conformation in these glycoproteins which facilitates fusion with the target cell membrane [83]. More mechanistic insight is provided by herpes simplex virus (HSV) which also uses gH and gL to dock αVβ6 and αVβ8 integrins, and this engagement routes HSV to acidic endosomes, thus promoting viral entry [86]. Another herpes virus, Kaposi's sarcoma-associated herpesvirus (KSHV) uses αVβ3 [76], αVβ5 [82], and α3β1 [61] integrins as entry receptors. Expression of the envelope protein, known as glycoprotein B (gB), which is highly conserved across *Herpesviridae* and contains an RGD sequence near its N-terminus, affords KSHV its integrin binding capacity. However, RGD mediated binding is not the only mechanism of KSHV-integrin interaction. KSHV gB also contains a disintegrin like domain (DLD) which is capable of binding integrin β1 in an RGD-independent fashion [58]. Walker et al. discovered that α9β1 is the integrin target of the gB DLD and plays a critical role in KSHV infection [67]. This mechanism is not unusual among *Herpesviridae* members, as human cytomegalovirus (HMCV) also uses gB to bind αVβ3, α2β1, and α6β1 through its DLD [58, 106].

Non-ECM Integrin Ligands of *Togaviridae*

Ross River fever is a mosquito borne disease caused by the Ross River virus (RRV), a member of the *Togaviridae* family. This disease induces arthritis by viral infection of macrophages within synovial joints [107]. It is believed that the spike protein, E2, of RRV contains two conserved domains which fold in a manner that mimics collagen IV [55]. This allows for infection of mammalian cells by docking α1β1 integrin in matrix binding adherent cell types [55].

Non-ECM Integrin Ligands of *Adenoviridae*

Human adenoviruses, known for causing respiratory, gastrointestinal, and ocular infections, are non-enveloped viruses with icosahedral capsids. At each capsid vertex, a penton base supports a fiber protein [108]. Many adenoviruses require two receptors for efficient infection of cells. The coxsackievirus and adenovirus receptor (CAR) is required for initial adhesion of adenoviral particles to target cells while subsequent integrin engagement is required for internalization of the viral particle [109]. It is the penton base that affords adenovirus a diverse array of integrin targets. RGD peptide sequences are located atop each monomer of the penton base, forming an RGD ring around the fiber protein [78]. The RGD peptides mediate docking to αVβ1, αVβ3, αVβ5, and α5β1 integrins for the purpose of internalization [66, 71, 110-112]. Mechanistically, it is thought that the pentameric structure of the base stimulates integrin clustering and downstream integrin signaling which further facilitates viral internalization [113-115]. Adenovirus also interacts with the laminin binding integrin, α3β1, via its penton base, but in an RGD-independent manner [62]. Additionally, αMβ2 integrin on myeloid cells can be targeted by adenovirus, but this interaction is dictated through a yet undetermined sequence within the penton base [68].

Non-ECM Integrin Ligands of *Hantaviridae*

As a member of the *Hantaviridae* family, the rodent-targeting Andes virus can spread to humans through inhalation of aerosolized excreted virus, targeting human endothelial cells and resulting in several fatal diseases such as hantavirus hemorrhagic fever with renal syndrome and hantavirus pulmonary syndrome [116]. Infection of αVβ3 integrin expressing endothelial cells occurs through viral targeting of the PSI domain within the β3

subunit [77]. Interestingly, a human polymorphism that has a leucine to proline substitution at position 33 of the integrin β3 PSI domain, was experimentally shown to abolish Andes virus infectivity [77]. Sin Nombre virus also utilizes β3 containing integrins, such as αIIbβ3 and αVβ3, for viral attachment [81]. Using atomic force microscopy (AFM) to study membrane dynamics upon Sin Nombre virus interaction, more mechanistic insight was provided for integrin dependent hantavirus infectivity. Bondu et al. used AFM data to propose a model in which viral docking to the β3 PSI domain of αIIbβ3, when the integrin is in a low affinity state, enhances integrin *cis* interaction with an RGD containing G-protein coupled receptor known as $P2Y_2R$ [117]. This *cis* interaction is thought to induce a switchblade-like conformational change within the integrin that ultimately leads to endocytosis of the viral bound integrin [117]. Other pathogenic hantaviruses also bind and cause the dysregulation of β3 integrins, resulting in blockade of endothelial cell migration [118], and enhancement of vascular endothelial growth factor (VEGF) mediated vascular permeability [119].

Non-ECM Integrin Ligands of *Birnaviridae*

Infectious bursal disease virus (IBDV) is an immunosuppressive avian pathogen in the *Birnaviridae* family that attacks the bursa of Fabricius (the site of hematopoiesis in birds) of young chickens, having a major negative impact on the poultry industry. The IBDV capsid is built by 260 trimers of the VP2 polypeptide arranged in an icosahedral lattice [120]. VP2 is the only component of the virus capsid, and contains a conserved, fibronectin mimicking, IDA peptide sequence that binds to α4β1 integrins present on target cell membranes [63]. IBDV binding to α4β1 integrin triggers c-Src tyrosine phosphorylation and actin rearrangement that creates membrane protrusions that internalize the virus [121].

Non-ECM Integrin Ligands of *Reoviridae*

The family *Reoviridae* includes the gastrointestinal pathogens, known as the rotaviruses, which are the leading etiological factor of diarrheal disease in young children worldwide [122]. The outer layer of the rotavirus capsid consists of 60 VP4 spike proteins protruding from a VP7 protein shell [123]. It is these outermost structures which mediate host cell binding and infectivity. The VP4 spike protein subunit, VP5, contains a DGE tripeptide sequence that serves to recognize α2β1 integrin on target cells [59, 60]. Rotavirus VP7 contains an αXβ2 recognizing GPR tripeptide, as well as an α4β1 ligating LDV motif, embedded in a disintegrin-like domain of the protein [60]. Additionally, rotaviruses can target αVβ3 integrin for the purpose of cellular entry, however, this binding does not occur within the RGD pocket [80]. Rather it is a novel αVβ3 targeting NEWLCNPDM amino acid sequence within the VP7 protein that is thought to mediate rotavirus-αVβ3 interaction [79]. It has been proposed that reoviruses employ a sequential binding mechanism to multiple receptors for the purpose of internalization. Initial binding to the counterreceptor JAM-A is thought to position the virus for subsequent binding to β1 containing integrins that facilitate internalization [124].

Non-ECM Integrin Ligands in Venoms

Selectively blocking integrins is a major therapeutic goal when combatting a number of pathologies, and a wide variety of approaches have been initiated. One rich source for anti-integrin compounds are venoms from various snake species [125, 126], and the study of venom derived integrin antagonists remains an active area of research. A venom is defined as a secreted toxin, produced by various types of animals, which is injected into another animal for the purpose of defense or predation. The Viperidae family of snakes

(collectively known as the vipers) produce a venom which causes local necrosis and blood coagulation within their prey. The discovery of small integrin targeting peptides found in the venom of these snakes initiated the study of disintegrins. These small molecular weight (40-100 amino acids in length), non-enzymatic proteins were originally characterized for their platelet disrupting properties through antagonistic targeting of αIIbβ3 integrin [127]. Since the identification of the first disintegrins, the field has grown with the discovery of many more examples. As discussed below, major families of venom-derived disintegrins include the RGD, MLD, PIII, and KTS/RTS disintegrins. While C-type lectin-like proteins are an example of non-disintegrin toxins which also disrupt integrin acitivity. Integrin targeting venomous compounds are summarized in Table B.3.

The RGD family of disintegrins is the largest family, although RGD sequences are not strictly required to be members in this family. Instead, disintegrins containing RGD or similar motifs, such as KGD, MGD, VGD, and WGD, are all capable of targeting RGD binding integrins serving to disrupt their physiological functions. Moreover, not all RGD disintegrins target RGD binding integrins exclusively either. For example, lebein1 and lebein2 are two RGD containing disintegrins found in the venom of *Macrovipera lebetina*, which have the unusual property of targeting the laminin binding integrins α3β1, α6β1, and α7β1 in an RGD-independent fashion [133]. They are thought to mimic the integrin-binding motif of laminin, thus allowing these molecules to disrupt cellular attachment to the laminin-rich basement membrane [133].

Other disintegrin families include the MLD, PIII, and KTS/RTS containing disintegrins. Whereas the RGD family of disintegrins possess an RGD (or similar motif) tripeptide within the integrin binding loop of the protein, the MLD motif is found at this

same position in MLD containing disintegrins [128]. These MLD disintegrins appear in heterodimeric complexes and are highly dependent on adjacent sequences to target the α4β1, α4β7, and α9β1 leukocyte specific receptor family of integrins [128]. PIII class disintegrins are large multi-domain toxins (60-100 kDa) which use an ECD integrin targeting motif and contain a metalloprotease domain which is a close homologue to cellular ADAMs [150]. The disintegrin known as alternagin uses an ECD tripeptide motif to target α2β1 integrin and disrupt matrix binding [151]. Once bound, alternagin uses its protease domain to cleave β1 causing integrin shedding and further disruption of collagen-induced platelet aggregation [152]. Finally, the KTS/RTS group of disintegrins found in Viperidae venom, are monomeric proteins which bind the collagen receptor α1β1 integrin [129]. This high level of specificity is not matched by RGD and MLD disintegrins as KTS/RTS disintegrins only target α1β1 integrin [128].

Another class of toxin found in Viperidae venom is the C-type lectin-like proteins (CLPs). These proteins do not exhibit the sugar binding capabilities of C-lectin proteins, but instead target collagen-binding integrins [153]. The viper species *Echis carinatus multisquamatus* produces EMS16 a potent and selective inhibitor of α2β1 integrin [130]. X-ray crystallography reveals that EMS16 spatially blocks collagen-integrin ligation through docking with the α2I domain of α2β1 integrin and stabilizing a low matrix affinity integrin conformation [131]. Several studies have shown that many viper derived CLPs target endothelium and block angiogenesis [130, 154, 155], while applying CLPs to cancer cells can inhibit cell-collagen binding [153] and metastasis [156]. Integrins that interact with CLPs are summarized in Table B.3.

Bacterial Use of Non-ECM Integrin Ligands

For many bacterial cells, successful adhesion to host cell surfaces is a prerequisite for successful colonization and/or infection. Many bacteria take advantage of the binding capabilities of integrins on cell membranes for infectious purposes. Some bacteria utilize specific integrin dimers for cellular binding while others exploit extracellular fibrous proteins that naturally bind to integrins for the purpose of translocating virulence factors. For this review, we will highlight three of the most commonly studied interactions between bacteria and integrins. There are other notable examples of bacterial cells using integrins as host cell receptors that we will not discuss: the intimin protein of *Escherichia coli* that binds to α4β1 and α5β1 integrins [157], the IpaB, C, and D proteins of *Shigella flexneri* that bind to α5β1 integrin [158], and the filamentous hemagglutinin protein of *Bordetella pertussis* that binds αMβ2 integrin [159]. Integrins that participate in bacterial interactions are listed in Table B.4.

Non-ECM Integrin Ligands of *Borrelia burgdorferi*

The spirochete *Borrelia burgdorferi* is the causative agent of Lyme disease, a devastating disease of the nervous system. The natural reservoir for *B. burgdorferi* includes mice, birds, and lizards [160]. These spirochetes are transmitted to humans via tick vectors of the *Ixodes* genus [160]. Once injected into the blood stream, *B. burgdorferi* spirochetes adhere to the microvasculature, transmigrate through the endothelium, and disseminate into various tissues [161]. Characterizing the proteins that enable this pathological mechanism illustrates several interesting examples of how microbes take advantage of host integrins.

A variety of screening techniques have identified at least 19 *B. burgdorferi* proteins that mediate or enhance adhesion to target cells [162]. The majority of these proteins

mediate indirect adhesion to mammalian cells via interactions with various ECM molecules. Three proteins however, P66, BBB07, and BB0172, have been shown to interact with integrins on platelets and a variety of cells such as endothelial cells. Prior to the discovery of the P66 protein, it had been known for some time that *B. burgdorferi* cells could adhere to β3 chain containing integrins [163, 164]. The P66 protein was later identified by phage display and shown to bind αVβ3 and αIIbβ3 integrins [165]. P66 displays no typical integrin binding sites [165], although adhesion of P66 to integrins can be blocked by soluble RGD peptides suggesting that P66 may bind into the RGD pocket of β3 integrins [166]. Moreover, a minimal seven amino acid sequence (QENDKDT) from P66 was found to bind integrins and deletion of the aspartic acid residues from this peptide eliminated P66 integrin binding [167]. Despite the integrin binding activity of P66, deletion of P66 does not appear to affect *B. burgdorferi* adhesion to microvasculature, a key step proceeding tissue invasion [168]. Instead, the P66 protein (presumably via its integrin binding activity) appears to be essential for the endothelial transmigration and dissemination of *B. burgdorferi* spirochetes into host tissues [167, 168]. Although P66 deletion did not affect microvascular adhesion, *B. burgdorferi* binding to various cells can be blocked by soluble RGD peptides [163], suggesting the presence of other integrin binding proteins. In support of this, two additional integrin-binding outer membrane surface proteins, BBB07 and BB0172, have been detected on *B. burgdorferi* [169, 170]. Although both BBB07 and BB0172 have been shown to interact with α3β1 integrins, only BBB07 contains an RGD motif [170]. Currently, there is little known about the function of α3β1 integrins in endothelial biology, although it has been proposed that α3β1 binding to Laminin 511 in the basal lamina may be linked to endothelial barrier function [171],

which could provide a link to the transendothelial migration of *B. burgdorferi* during infection.

Non-ECM Integrin Ligands of *Helicobacter pylori*

Helicobacter pylori infects roughly half of the world's human population and shares responsibility for gastric complications including stomach ulcers and gastric adenocarcinoma through its infection of gastric epithelial cells [172-174]. *H. pylori* utilizes a type IV protein secretion system (T4SS) involving the cytotoxin-associated gene L (CagL) adhesion tip protein, to infect target cells with the virulence factor, cytotoxin-associated gene A (CagA) [175, 176]. Efficiency of CagA injection is enhanced by an RGD domain present on the CagL protein [177]. CagL interacts primarily with α5β1 integrin, however αVβ3, αVβ5, and αVβ6 have also been implicated [177-180]. Interestingly, while the CagL RGD domain is necessary for CagA injection, additional CagL sequences have been identified that enhance integrin binding. For example, an RGD helper sequence, FEANE, is located in close proximity to the RGD domain of CagL and reinforces integrin engagement [177]. Additional domains on CagL that enhance RGD binding include, a TSPSA sequence [181], an LXXL sequence that is directly adjacent to the RGD domain [180], and a TASLI sequence located opposite the RGD domain in the CagL integrin binding domain [181]. CagL-α5β1 interaction leads to the activation of the kinases Src and FAK [179], followed by subsequent tyrosine phosphorylation of the CagA EPIYA motifs by Src and ABL kinases [182]. These phosphorylation events potentiate CagA pathogenicity (reviewed in [183]). Phospho-CagA interacts with Shp-2 while initiating mitogen-activated protein kinase (MAPK) signaling, and inducing cytoskeletal rearrangements which serve to cause an elongation of epithelial cells and enhance their

mobility. CagA also disrupts cell-cell junctions while triggering an inflammatory response including nuclear factor-kB (NF-κB) activation and chemokine production. Additionally, in a negative feedback loop phospho-CagA down-regulates Src activity, ensuring that a reservoir of nonphospho-CagA remain in the cell, which is necessary for a prolonged infection. As mentioned previously, CagL is capable of interacting with other integrins. Interestingly, a novel mechanism of CagL-αVβ5 induced production of gastrin has been uncovered. It was found that CagL ligation to αVβ5 on gastric epithelial cells, activates ILK, which in turn activates the epidermal growth factor receptor (EGFR) and subsequently MAPK pathways, serving to induce gastrin expression [184]. This mechanism may explain *H. pylori* induced hypergastrinemia, which is a major risk factor for gastric adenocarcinoma. The integrin dependent mechanisms of *H. pylori* infection discussed here are depicted in figure B.2.

Non-ECM Integrin Ligands of *Yersinia*

The gram negative bacteria, *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* commonly cause food borne illnesses. These *Yersinia* species express two adhesion proteins that facilitate cellular attachment and invasion of target cells in the small intestine. The *Yersinia* adhesion A (YadA) protein indirectly binds to integrins via interaction with various molecules of the ECM, but is dispensable for cellular invasion [185, 186]. However, the *Yersinia* invasin protein directly binds to a variety of β1 subunit containing integrins (α3, α4, α5, α6, αV) and is crucial for cellular adhesion and invasion [187, 188]. *Yersinia* species invading through the small intestine, target the apical membrane of Peyer's patch M-cells, which express integrin β1 [189, 190]. Invasins lack the typical RGD domain used to bind integrins, although RGD peptides prevent invasin binding to β1

integrins [191]. This suggests that invasin proteins interact with the RGD binding domain of β1 containing integrin heterodimers. In support of this, structural analysis of the invasin protein, and comparison to fibronectin, reveals similar structures with key conserved integrin-binding residues, suggesting the convergent evolution of invasins to match fibronectin [192].

Protists and Multicellular Parasites that Use Non-ECM Integrin Ligands

A broad array of examples of non-ECM ligands for integrins are employed by many parasitic organisms. Here we discuss just a few examples, including non-ECM ligands produced by the amoebozoa *Entamoeba histolytica* and a range of hematophagic (blood-sucking) organisms. These examples illustrate the importance of non-ECM ligands to parasitic infections. Although compared to bacteria and viruses, there is far less literature on the subject of non-ECM ligands as components of pathogenicity in protozoan and multicellular parasites. Non-ECM integrin ligands derived from parasitic organisms are summarized in Table B.4.

Non-ECM Integrin Ligands of *Entamoeba histolytica*

Entamoeba histolytica (Eh) causes amoebic dysentery and liver abscess [205] and is responsible for ~100,000 deaths/year [206]. Eh invasion into host tissues involves multiple integrin-mediated steps. The best characterized of these integrin-mediated steps involves the EhCP5 cysteine proteinase 5 protein (PCP5) binding to αVβ3 integrins [203, 204]. Binding of EhCP5 to αVβ3 integrins on colonic epithelial cells via an RGD domain triggers NF-κB mediated inflammation [203], and mucin exocytosis [204]. The EhCP5 protein has also been shown to interact with α5β1 integrins to mediate local inflammation which is crucial to Eh invasion into host tissues [201]. Additional involvement of integrins in Eh

invasion have been linked to β2 integrin activation and release of reactive oxygen species [207, 208] as well as an integrin β1-like receptor present on Eh trophocytes that mediates adhesion to host fibronectin [209].

Non-ECM Integrin Ligands of Hookworms

The hookworm platelet inhibitor (HPI) protein illustrates another fascinating example of non-ECM integrin ligands. Hookworms are blood-feeding intestinal parasites and a leading cause of iron deficiency in humans. HPI was isolated from the hookworm, *Ancylostoma caninum*, based on its ability to inhibit function of integrins αIIbβ3 and α2β1 [193, 194]. HPI appears to block platelet aggregation and blood clothing, thus enabling continued feeding. Interestingly, sequence and structural analysis has failed to identify any integrin binding domains in the HPI protein [210]. In addition to the HPI protein, *Ancylostoma caninum* also expresses the neutrophil inhibitor factor (NIF) that interacts with αMβ2 integrins present on neutrophils [202, 211]. NIF disrupts αMβ2 interaction with ICAM1 [202] which is necessary for stable neutrophil adhesion to endothelium and transendothelial migration thus suppressing local inflammation. Collectively, the combined actions of HPI and NIF help ensure hookworms are able to feed from their host for a prolonged period of time.

Non-ECM Integrin Ligands of Blood-Sucking Parasites

In addition to *Entamobea histolytica* and *Ancylostoma caninum*, several other examples of integrin inhibition by hematophagic (blood-sucking) arthropods have been described in the literature (reviewed in [212]). Many of these strategies involve non-ECM integrin ligands that interfere with various integrin-mediated steps that are essential for blood coagulation. The majority of these non-matrix ligands block platelet αIIbβ3 integrin

interactions with fibrin, von Willebrand factor, and vitronectin which are collectively essential for blood coagulation. Examples of these proteins include the decorsin protein from the leech *Macrobdella decora* [195], the vasotab TY and tablysin-15 proteins from the horsefly *Tabanus yao* [196, 197], and the disagregin (*Ornithodoros moubata*), YY-39 **(***Ixodes pacificus* and *Ixodes scapularis)*, and variabilin (*Dermacentor variabilis*) proteins from ticks ([198-200]. Many of these proteins contain RGD or similar integrin binding domains (KGD, VGD, MLD, KTS, RTS, WGD, or RED) which bind to and interfere with αIIbβ3 function on platelets. Additional RGD or RGD-like integrin antagonists have been identified *in silico* from other blood-sucking arthropods such as mosquitos and sand flies [212], but have yet to be explored.

Hormones, Small Molecules, and Growth Factors that Mimic Integrin Ligands

To this point, we have focused on the non-ECM integrin ligands utilized by various organisms to mediate adhesion to target cell membranes. However, as it turns out, a wide variety of small molecules (including hormones and growth factors) can also interact with integrins thus broadening the role for integrins in non-ECM interactions. As described in the examples below, integrins binding to small molecules serve a number of cellular functions ranging from cell surface receptor-signaling roles as in the case of thyroid hormone, DHT, ANGPTLs, and VEGF, to activation of growth factors as in the case of TGFβ. Integrins that interact with hormones, small molecules, or growth factors are summarized in Table B.3 and depicted in figure B.4.

Small Molecules and Hormones that Bind Integrins (Resveratrol, Thyroid Hormone, DHT)

Trans-resveratrol is a stilbenoid produced in plants such as grapevines that is well known for its anti-inflammatory activity [213], anti-angiogenic function [214] and anti-cancer properties [215-217]. Resveratrol binds the extracellular portion of the β3 monomer of αVβ3 integrin near the RGD pocket [137]. This binding inhibits αVβ3 integrin dependent endothelial cell adhesion to vitronectin-coated plates, while also exhibiting angiostatic function and inhibiting tumor growth [139]. Resveratrol binding to αVβ3 integrin induces extracellular signal-regulated kinase (ERK1/2) activation, which leads to p53 induced apoptosis in various cancer cell lines [137, 138]. This evidence implicates resveratrol binding αVβ3 integrin as being at least in part responsible for resveratrol's ability to mitigate angiogenesis and tumorigenesis.

Integrin αVβ3 bears a receptor site for the thyroid hormones T3 and T4 and thyroid hormone analogs (reviewed in [140]). Perhaps the first evidence of this interaction was uncovered when Hoffman et al. [218] used an αVβ3 inhibitor (SB-273005) to block T4 induced bone resorption in rats. Binding of T3 and T4 to αVβ3 integrin induces cell proliferation and angiogenesis through MAPK activation, and this effect is negated by a T4 derivative tetraiodothyroacetic acid (tetrac), RGD peptide, and αVβ3 integrin blocking antibodies suggesting that the thyroid hormone receptor site is at or near the RGD binding pocket [141-143]. Through radioligand binding experiments, it was shown that purified αVβ3 integrin binds T4 preferentially over T3, and binds T4 with high affinity, having a dissociation constant (K_d) of 333 pM and an EC_{50} of 371 pM [219]. Lin et al. have proposed a model for the thyroid hormone receptor activity of αVβ3 integrin that describes two

distinct thyroid hormone binding sites on αVβ3 [220]. The site known as ‘site 1’, appears to bind T3 but not T4, while another site called ‘site 2’ binds both T3 and T4 [220]. T3 binding at site 1 leads to Src and phosphatidylinositol 3-kinase (PI3K) activation that induces nuclear translocation of thyroid hormone receptor (TR) α1, and these effects can be disrupted through addition of RGD peptide [220]. Whereas, T3/T4 binding at site 2 induces ERK activation that causes nuclear translocation of TRβ1, and only T4 induced effects at this site are disrupted by RGD peptides [220]. This suggests that αVβ3-dependent thyroid hormone signaling acts as a complex, hierarchical system capable of mediating distinct site-specific activities. Since some of these activities are disrupted through RGD binding, this leads to the possibility that cells embedded in an RGD-rich matrix may respond differentially to thyroid hormone compared to those embedded in a RGD-deficient matrix. Perhaps this is a mechanism by which a ubiquitous receptor, such as αVβ3, can provide tissue specific responses to thyroid hormone.

In addition to thyroid hormones, αVβ3 integrin also interacts with the biologically active form of testosterone, DHT. Whether or not this interaction is involved in the normal physiological roles of DHT is unknown; however, DHT binding to αVβ3 has been implicated in cancer cell growth. For example, DHT binding to αVβ3 stimulates MDA-MB-231 breast cancer cell proliferation [143]. Additionally, DHT binding to αVβ3 integrin inhibits resveratrol induced p53 dependent apoptosis effects in MDA-MB-231 cells [144], thus highlighting the complexity of hormone signaling through αVβ3 integrin. Through these examples, it is clear that αVβ3 integrin has diverse receptor activity which affords hormones additional non-canonical signaling capacity.

Growth Factors that Bind Integrins (ANGPTLs, TGFβ, VEGF)

Many growth factors are capable of binding integrins. An interesting example is the angiopoietin-like proteins (ANGPTLs), also known as angiopoietin-related proteins (ARPs), which consist of a family of proteins that display structural similarity to the growth factor angiopoietin, although they do not bind classical angiopoietin receptors [221]. Instead, ANGPTLs have been demonstrated to bind various integrins through a C-terminal fibronectin like domain containing a conserved RGD sequence [222]. In human prostate cancer (LNCaP) cells, ANGPTL2 binds α5β1 integrin inducing migration and proliferation, and this effect can be negated by use of integrin-blocking antibodies [134]. Furthermore, ANGPTL2 binding α5β1 integrin on macrophages mediates pro-inflammatory responses in mice, and ANGPTL2 knockout mice have muted immune responses leaving them more susceptible to infections [135]. In the kidney, glomerular podocyte motility is enhanced through cytoskeletal rearrangement induced by ANGPTL3 binding podocyte αVβ3 integrin [145]. Deletion of ANGPTL3 can reduce proteinuria in mouse models of nephropathy, and ANGPTL3 activation of integrin β3 has been identified in patients with nephrotic syndrome [223]. The ANGPTL family also affects vascular integrity. In response to decreased albumin levels during peak proteinuria, podocytes and extrarenal tissues secrete ANGPTL4 into the blood, which binds glomerular endothelial αVβ5 integrin and serves to reduce proteinuria [147]. This effect may be explained by another study where surface plasmon resonance and proximity ligation assays were used to discover that ANGPTL4 also binds another endothelial integrin, αVβ3, which serves to recruit Src kinase and enhance endothelial junction stability, thereby reducing vascular permeability [146]. Taken collectively, these studies suggest that ANGPTL3 binding

podocyte integrins enhances proteinuria, whereas ANGPTL4 binding glomerular endothelial integrins decreases proteinuria. The ANGPTLs are a good example of a protein family that mimics a classical extracellular matrix protein in order to bind integrins and implement their cellular effects.

Integrins also play a critical role in the activation of TGFβ (reviewed in [148]). An inactive form of TGFβ (pro-TGFβ) is secreted from cells with an RGD containing latency associated peptide (LAP) non-covalently bound to TGFβ, which must be removed before TGFβ is biologically active. While the RGD binding αVβ6 integrin plays a key role in separating LAP from TGFβ, other αV containing integrins, including αVβ3, αVβ5, and αVβ8, have been implicated in this process. Mutation of the LAP integrin binding site in mice yields normal levels of pro-TGFβ, but results in a lethal phenotype which appears identical to TGFβ deletion [224]. LAP separation is mediated by a tensile force generated by a cell's cytoskeleton that is transmitted via αVβ6 integrin in order to reshape and activate the pro-TGFβ [149]. The dependence of pro-TGFβ on αVβ6 for activation, and the fact that TGFβ is a well-known master regulator of fibrosis [225], has led to the suggestion that inhibition of αVβ6 integrin binding may represent a clinical strategy to treat diseases characterized by fibrosis, such as scleroderma [226]. This idea is supported by observations showing that αVβ6 knockout mice [227] or treatment with αVβ6 blocking antibodies [228, 229] substantially decrease fibrosis in mouse models of lung fibrosis.

The vascular endothelial growth factors (VEGFs) comprise a group of cytokines which are important mediators of angiogenesis and lymphangiogenesis. VEGF signaling functions through VEGF binding to a group of receptor tyrosine kinases, known as VEGF receptors (VEGFRs). Since this pathway is an inducer of angiogenesis, it has been the

target of many anti-cancer therapies with the hope of inhibiting tumor vascularization. One therapeutic strategy involves inhibiting VEGF-VEGR binding through the targeting of VEGFRs with monoclonal antibodies [230]. However, this approach has not proven as effective as drug developers and clinicians envisioned [230, 231]. One reason for this failure may be that VEGFRs are not the only membrane-bound receptor of VEGFs, as these growth factors are also known to bind integrins. Some VEGF isoforms are integrated into the extracellular matrix, where they bind α3β1, αVβ3, and other αV integrins to promote endothelial cell adhesion [132]. Interestingly, the solubility of VEGF ligands greatly effects the integrin response. Vlahakis et al. found that when α9β1 integrin binds immobilized VEGF-A it induces the recruitment of VEGFR2 into macromolecular structures at the cell membrane [136]. This serves to permit endothelial cell adherence and migration on VEGF-A functionalized petri dishes, and stimulates the phosphorylation of the downstream effectors paxillin and ERK [136]. In contrast, when soluble VEGF binds α9β1 integrin, paxillin is phosphorylated, but neither the phosphorylation of ERK nor formation of VEGFR2 macromolecular complexes are induced [136]. Moreover, VEGF-A is not the only VEGF member to have these functions. VEGF-C and VEGF-D also bind α9β1 integrin, stimulating the phosphorylation of paxillin and ERK, while contributing to lymphangiogensis [232]. Taken together, these findings suggest a VEGF induced synergy between VEGFR and integrins. Therefore, it may be beneficial to co-target integrins when employing an anti-VEGF therapeutic strategy during cancer treatment.

Conclusions

Throughout this review, we have sought to venture beyond the matrix and highlight biological examples of integrin ligands that do not fit the classical model of ECM-mediated

integrin function. Given the strong conservation of integrins across much of the biological world, it is no surprise that there exists an extremely diverse array of these non-ECM integrin ligands. Consequently, interactions between integrins and non-ECM ligands are actively being exploited for a number of applications in the biotechnology realm. RGD peptides are being used to target liposomes and small molecules to specific tissues for various purposes, including the improvement of chemotherapeutic delivery to cancer cells [233-235]. Similarly, RGD peptides are also being used to target viral particles to various tissues. For instance, the new field of "chemical virology" seeks to load viral capsids with chemotherapeutics that in some instances, utilize RGD functionalization to deliver these nanoparticles to specific tissues [236]. In a related example, a plant virus known as the cowpea mosaic virus, which does not normally target mammalian cells, was functionalized with RGD peptides to successfully target cancer cell lines [237]. Demonstrating another example of applied integrin biotechnology, various artificial "extracellular matrices" are now being created and designed with incorporated RGD peptides to enable cell seeding and growth [238]. Two exciting examples include the development of graphene that has been functionalized with RGD peptides which is being used to detect nitric oxide release from living cells [239], and DNA origami tubes that have been tagged with RGD peptides and shown to bind neural stem cells and promote their differentiation [240]. These instances and many others provide fascinating examples of how the unique binding properties of integrins continue to be uncovered and utilized.

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Author Contributions

All authors contributed to literature research of the topic, writing individual sections, and proofreading the final manuscript. B. LaFoya designed and constructed all figures. B. LaFoya, J. Munroe, and A. Albig prepared the final manuscript.

Conflicts of Interest

The authors declare no conflicts of interest.

Table B.1 Selected Non-ECM Ligands which Mediate Cell-Cell Interactions

Table B.1: Selected non-ECM ligands which mediate cell-cell interactions.

Integrin dimers	Common name		non-ECM ligand	Function of interaction [key refs]
$\alpha 4\beta 1$	VLA-4	Very late antigen-4	MAdCAM1	Leukocyte adhesion [10-12]
			VCAM1	Leukocyte adhesion [10-12] Erythrocyte differentiation [13-15] Cancer cell metastasis [16]
			JAM-B	Leukocyte transmigration [11]
$\alpha 4\beta 7$	LPAM	Lymphocyte Peyer's patch adhesion molecule	MAdCAM1	T-lymphocyte homing [17] HSC homing to bone marrow [18]
$\alpha 5\beta 1$	Fibronectin receptor	Fibronectin receptor	NMB	Cancer cell growth, metastasis [19]
$\alpha E\beta 7$			E-cadherin	Cytotoxic T cell targetting of tumor cells [20]
$\alpha L\beta 2$	LFA-1	Lymphocyte function associated antigen-1	ICAM1, 2, 3	Leukocyte adhesion [10, 11]
			JAM-A	Leukocyte transmigration
$\alpha M\beta 2$	Mac-1 / CR3	Macrophage antigen-1 / Complement receptor-3	ICAM1	Leukocyte adhesion [10, 11]
			β-glucan	NETosis [21, 22]
			Complement C3	Phagocytosis [23, 24]
			LL-37	Bacterial opsonization [25-29]
			JAM-C	Leukocyte transmigration [11]
			HMGB1	NETosis [30]
$\alpha V\beta 3$	Vitronectin receptor	Vitronectin receptor	L1CAM	Cancer cell metastasis [31, 32]
$\alpha X\beta 2$	CR4 / CD11c /CD18	Complement receptor-4	Complement C3	Phagocytosis [23, 24]

Table B.2 Selected Integrin Binding By Viruses

Table B.2: Selected integrin binding by viruses	
Integrin	**Virus name [key refs]**
$\alpha 1\beta 1$	Ross River virus [55]
	Echovirus 1 [56, 57]
$\alpha 2\beta 1$	Cytomegalovirus [58]
	Rotavirus [59, 60]
$\alpha 3\beta 1$	Kaposi's sarcoma-associated herpesvirus [61]
	Adenovirus [62]
$\alpha 4\beta 1$	Infectious bursal disease virus [63]
	Rotatvirus [60]
	Foot-and-mouth disease virus [64]
$\alpha 5\beta 1$	Epstein-Barr virus [65]
	Adenovirus [66]
$\alpha 6\beta 1$	Cytomegalovirus [58]
$\alpha 9\beta 1$	Kaposi's sarcoma-associated herpesvirus [67]
$\alpha M\beta 2$	Adenovirus [68]
$\alpha V\beta 1$	Echovirus 22 [69, 70]
	Adenovirus [71]
	Echovirus 9 [72]
	Coxsackievirus A9 [73]
	Foot-and-mouth disease virus [74]
	Flaviviridae [75]
$\alpha V\beta 3$	Kaposi's sarcoma-associated herpesvirus [76]
	Cytomegalovirus [58]
	Andes virus [77]
	Adenovirus [78]
	Rotavirus [79, 80]
	Sin Nombre virus [81]
	Kaposi's sarcoma-associated herpesvirus [82]
$\alpha V\beta 5$	Adenovirus [78]
	Epstein-Barr virus [83]
	Coxsackievirus A9 [73]
$\alpha V\beta 6$	Foot-and-mouth disease virus [84, 85]
	Epstein-Barr virus [83]
	Herpes simplex virus [86]
$\alpha V\beta 8$	Epstein-Barr virus [83]
	Herpes simplex virus [86]
$\alpha X\beta 2$	Rotavirus [60]
$\alpha IIb\beta 3$	Sin Nombre virus [81]

Table B.3 Integrin Binding by Small Molecules, Hormones, Venoms, and CLPs

Table B.3: Integrin binding by small molecules, hormones, venoms, and CLPs		
Integrin	**Non-ECM ligand**	**Function [key refs]**
$\alpha 1\beta 1$	KTS/RTS disintegrins	Block cell adhesion [128, 129]
$\alpha 2\beta 1$	EMS16	Block adhesion to collagen [130, 131]
$\alpha 3\beta 1$	VEGF	Cell adhesion [132]
	Disintegrin Lebein 1/2	Block cell adhesion [133]
$\alpha 4\beta 1$	MLD disintegrins	Block cell adhesion [128]
$\alpha 4\beta 7$	MLD disintegrins	Block cell adhesion [128]
$\alpha 5\beta 1$	ANGPTL2	Cancer cell migration / proliferation [134]
		Macrophage pro-inflammatory response [135]
$\alpha 6\beta 1$	Disintegrin Lebein 1/2	Block cell adhesion [133]
$\alpha 7\beta 1$	Disintegrin Lebein 1/2	Block cell adhesion [133]
$\alpha 9\beta 1$	VEGF-A, -C, -D	Endothelial adhesion & lymphogenesis [136]
	MLD disintegrins	Block cell adhesion [128]
$\alpha V\beta 3$	Resveratrol	Anti-angiogenesis [137-139]
	Thyroid hormones (T3/T4)	Cell proliferation / Angiogenesis [140-142]
	DHT	Cancer cell proliferation [143, 144]
	ANGPTL3	Podocyte motility [145]
	ANGPTL4	Enhanced endothelial junctions [146]
	VEGF	Endothelial cell adhesion [132]
$\alpha V\beta 5$	ANGPTL4	Reduce proteinuria [147]
$\alpha V\beta 6$	Pro-TGFβ	TGFβ activation [148, 149]

Table B.4 Integrin Binding by Bacteria and Parasitic Organisms

Table B.4: Integrin binding by bacteria and parasitic organisms.		
Integrin	**Species**	**Binding protein [key refs]**
$\alpha 2\beta 1$	*Ancylostoma caninum*	Hookworm platelet inhibitor (HPI) [193, 194]
	Ancylostoma caninum	Hookworm platelet inhibitor (HPI) [193, 194]
	Macrobdella decora	Decorsin [195]
	Tabanus yao	Vasotab TY [196]
$\alpha IIb\beta 3$		Tablysin-15 [197]
	Ornithodoros moubata	Disagregin [198]
	Ixodes pacificus	YY-39 [199]
	Dermacentor variabilis	Variabilin [200]
$\alpha 3\beta 1$	*Borrelia burgdorfori*	BBB07, BB0172 [170]
	Yersinia	Invasin [187, 188]
$\alpha 4\beta 1$	*Escherichia coli*	Intimin [157]
	Yersinia	Invasin [187, 188]
	Helicobacter pylori	CagL [177, 179]
	Escherichia coli	Intimin [157]
$\alpha 5\beta 1$	*Shigella flexneri*	Ipa B, C, D [158]
	Entamoeba histolytica	EhCP5 [201]
	Yersinia	Invasin [187]
$\alpha 6\beta 1$	*Yersinia*	Invasin [187, 188]
$\alpha M\beta 2$	*Bordetella pertussis*	Filamentous hemagglutinin protein [159]
	Ancylostoma caninum	Neutrophil inhibitor factor (NIF) [202]
$\alpha V\beta 1$	*Yersinia*	Invasin [187]
	Borrelia burgdorfori	P66 [165]
$\alpha V\beta 3$	*Helicobacter pylori*	CagL [177]
	Entamoeba histolytica	EhCP5 [203, 204]
$\alpha V\beta 5$	*Helicobacter pylori*	CagL [177, 184]
$\alpha V\beta 6$	*Helicobacter pylori*	CagL [180]

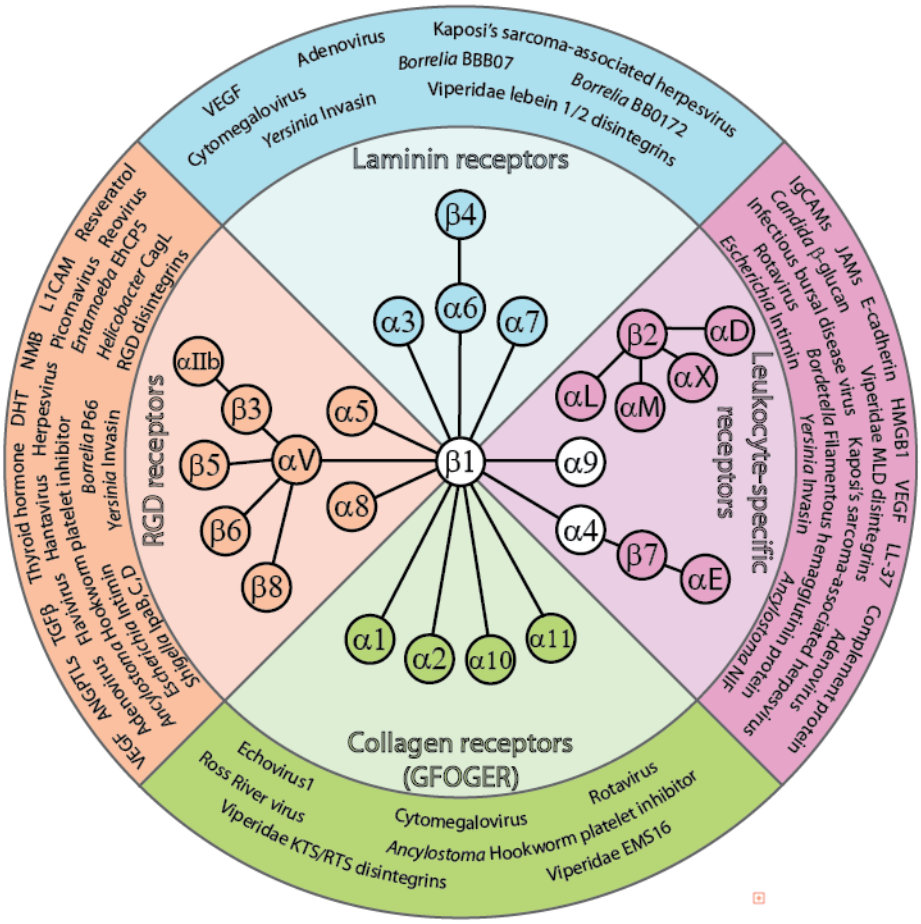

Figure B.1 Integrin Heterodimers and their Ligands.

Integrins are heterodimeric cell surface receptors that bind extracellular matrix (ECM) molecules. In addition to this role, integrins also bind many non-ECM ligands. Integrin subunits connected by a ray represent heterodimeric α/β binding partners. The inner ring depicts integrin heterodimers grouped into families based upon their classical binding profile. These families include RGD receptors, collagen (GFOGER) receptors, laminin receptors, or leukocyte-specific receptors. Within the outer ring, the non-ECM ligands of these families are listed. Non-ECM ligands include growth factors, hormones, venomous compounds, disintegrins, bacterial proteins, fungal polysaccharides, viruses, polyphenols, and counterreceptors.

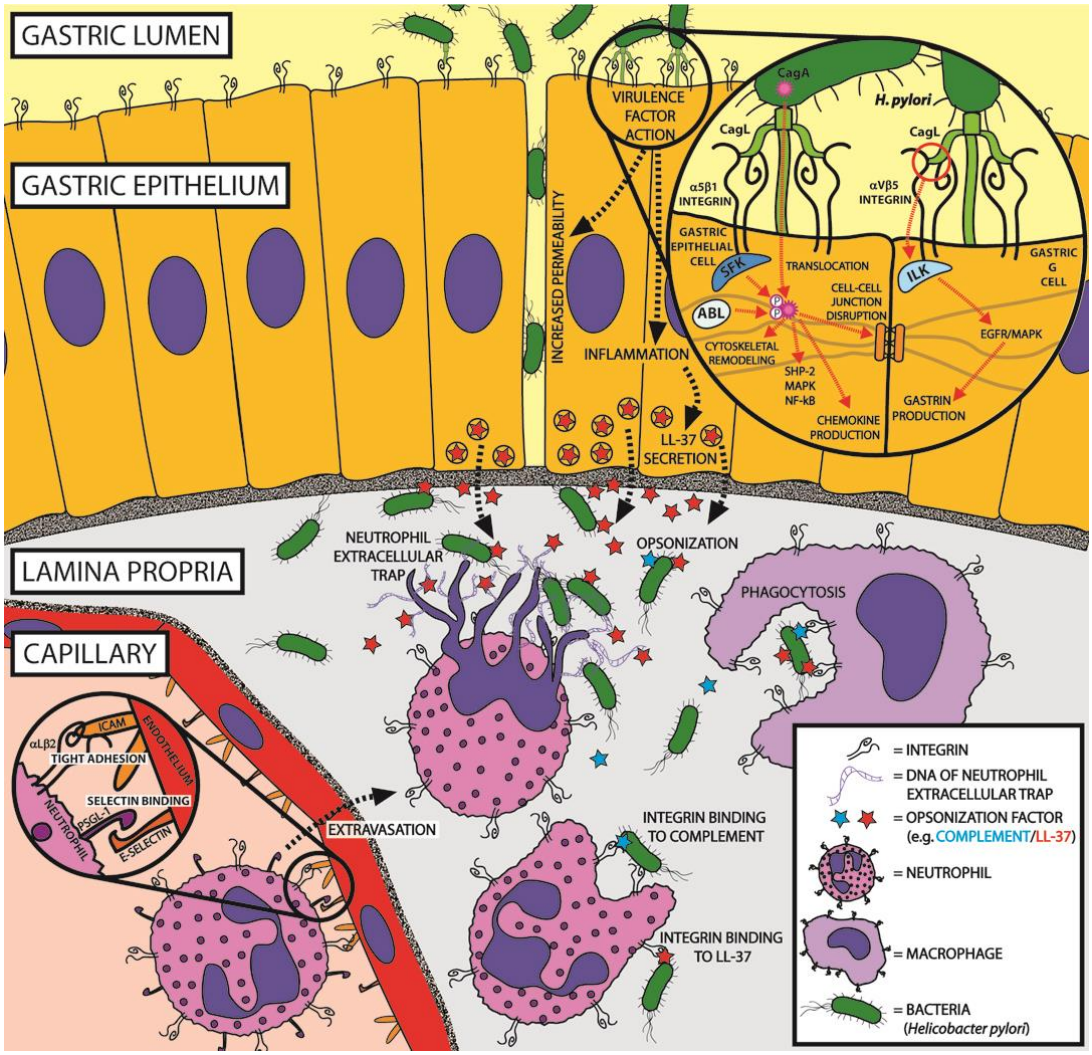

Figure B.2 Integrins Act as "Double Agents" During Infection, Serving to Potentiate Bacterial Pathogenicity while also Aiding in the Immune Response.

During *Helicobacter pylori* infection in the stomach, integrins play diverse roles. *H. pylori* bacteria in the gastric lumen are able to bind integrins on gastric epithelial cells and inject virulence factors. As seen in the magnified view of this process, docking of α5β1 integrin is achieved through integrin affinity for the RGD motif of the CagL protein component of the type IV secretion system (T4SS). Integrin α5β1 mediated stabilization of the T4SS facilitates the translocation of the virulence factor CagA and activation of intracellular kinases. Once in the cytosol, CagA is phosphorylated by Src family kinases (SFKs) and ABL kinases, which potentiates its virulence. Phospho-

CagA then activates SHP-2 and MAPK signaling, triggering cytoskeletal remodeling. CagA also disrupts cell-cell junctions, activates the NF-kB pathway, and stimulates cytokine production. Alternatively, CagL docking with αVβ5 integrin on gastric G cells activates ILK, which stimulates EGFR and MAPK activation, inducing gastrin production. These mechanisms increase the permeability of the gastric epithelium which aids *H. pylori* dissemination into the underlying lamina propria. This process initiates an inflammatory response that causes the release of the antimicrobial peptide LL-37 from gastric epithelial cells. The immune system responds by recruiting leukocytes from the blood stream. In the magnified view of the recruitment process, we see that leukocytes first stick to inflamed endothelial cells through selectin binding which facilitates integrin mediated tight adhesion. Tight adhesion leads to leukocyte transendothelial migration into the lamina propria where immune cells, such as neutrophils and macrophages, phagocytize bacteria. Phagocytosis is mediated through integrin recognition of the opsonization factors LL-37 and complement. Neutrophil extracellular traps (NETs) are stimulated through integrin interaction with pathogens.

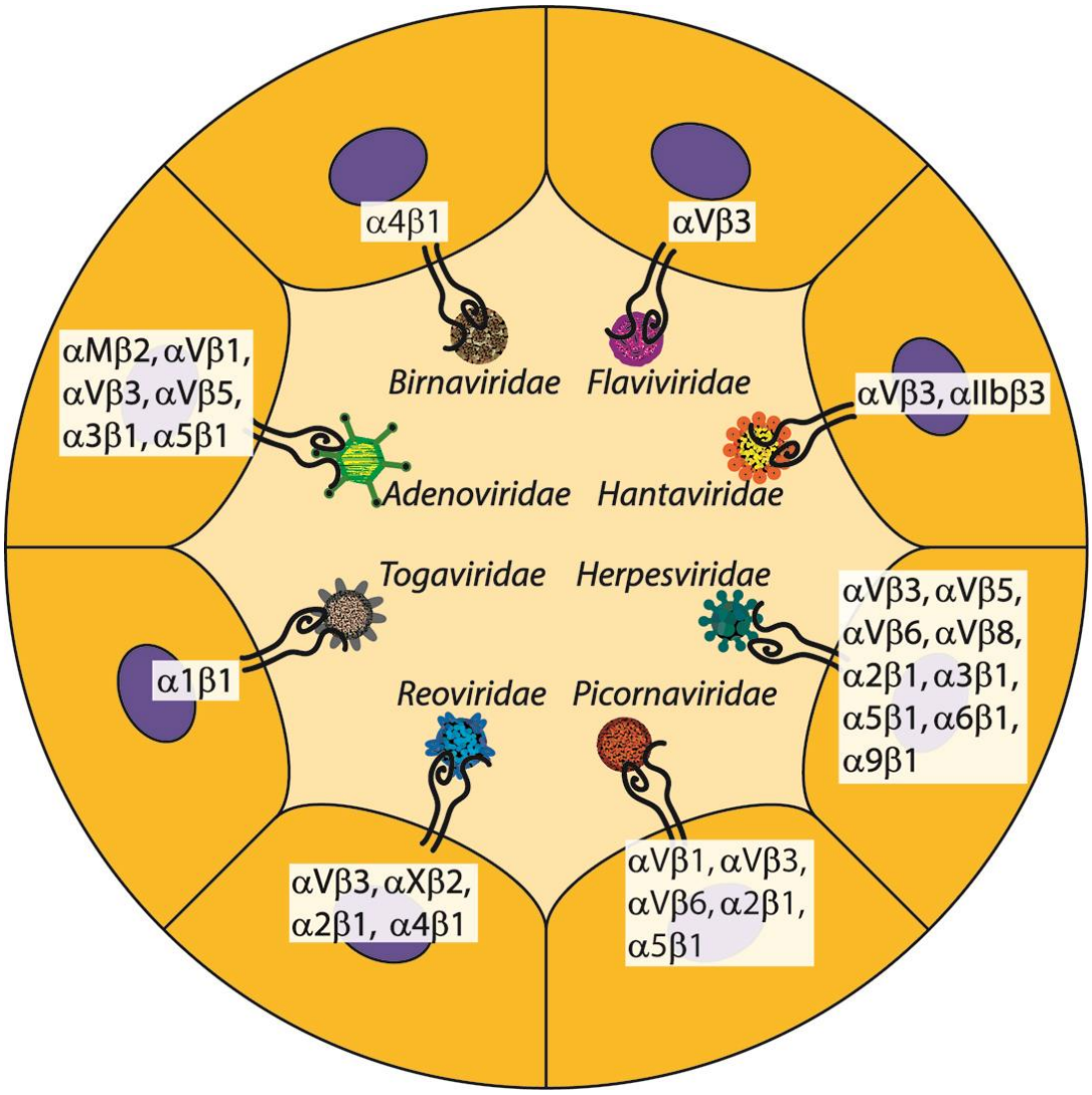

Figure B.3 Viruses Hijack Integrins for Adhesion and Infectivity.

Virus families use specific integrins in order to adhere to target cells for the purposes of internalization and infectivity. Members of the family *Adenoviridae* are non-enveloped viruses with icosahedral capsids that have penton base structures which facilitate RGD-dependent docking with αVβ1, αVβ3, αVβ5, and α5β1 integrins and RGD-independent engagement of α3β1. Adenoviruses also target αMβ2 integrin through an undetermined mechanism. *Birnaviridae* contains members who employ a fibronectin mimicking IDA peptide to bind α4β1 integrin. Members of the

Flaviviridae family have an RGD containing E-protein which binds αVβ3 integrin. Viruses in the family *Hantaviridae* target the PSI domain of αVβ3 and αIIbβ3 integrins. *Herpesviridae* has members that employ a few different mechanisms of integrin engagement for the purposes of viral entry. The envelope protein BMRF-2 contains an RGD sequence that docks α5β1 integrin. The envelope proteins gH and gL dock with αVβ5, αVβ6, and αVβ8. Whereas another envelope protein, known as gB, contains both an RGD motif and disintegrin-like domain which affords viral targeting of αVβ3, αVβ5, α2β1, α3β1, α6β1, and α9β1 integrins. Members of the *Picornaviridae* family use capsid proteins to target integrins. Targeting of α2β1 integrin proceeds in an RGD-independent manner, while αVβ1, αVβ3, αVβ6, and α5β1 integrins are bound in an RGD-dependent fashion. *Reoviridae* contains members which employ a DGE sequence within a VP4 capsid protein to engage α2β1. Additionally, the reovirus VP7 capsid protein has a GPR tripepetide which recognizes αXβ2, an LDV motif that ligates α4β1, and a novel NEWLCNPDM amino acid sequence that targets αVβ3. *Togaviridae* has members which have a collagen mimicking spike protein that docks α1β1 integrin.

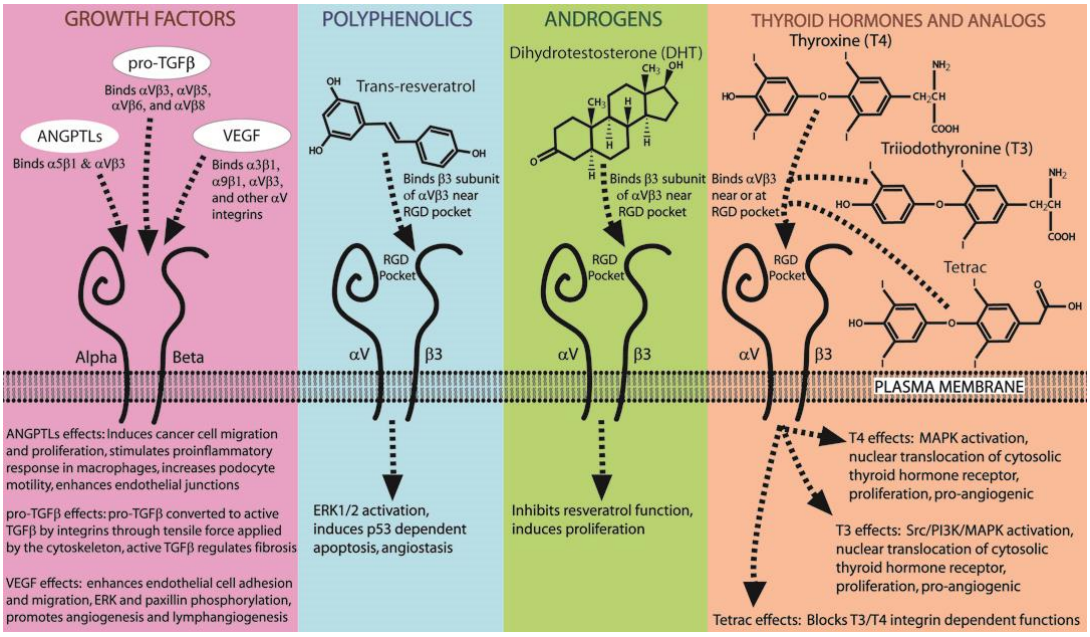

Figure B.4 Integrins Serve as Cell Surface Receptors for Growth Factors, Hormones, and Small Molecules.

Various growth factors use integrins as cell surface receptors. Angiopoietin-like proteins (ANGPTLs) bind α5β1 and αVβ3 integrins to facilitate a host of cellular effects. Pro-TGFβ is activated by αVβ3, αVβ5, αVβ6, and αVβ8, through integrin dependent dissociation of an RGD containing latency associated peptide (LAP), thus converting it to its active form. Activated TGFβ acts as a master regulator of fibrosis among other roles. Vascular endothelial growth factor (VEGF) ligates α3β1, α9β1, αVβ3, and other αV containing integrins, resulting in cellular effects that promote angiogenesis and lymphangiogenesis. The polyphenol trans-resveratrol, which is derived from grapevines, binds the β3 subunit of αVβ3 integrin near the RGD recognition pocket. This binding event induces ERK activation and p53 dependent apoptosis, while promoting angiostasis. Like trans-resveratrol, the active form of testosterone (DHT) also binds the β3 subunit of αVβ3 integrin near the RGD pocket. DHT-αVβ3 interaction inhibits trans-resveratrol induced effects and stimulates

cellular proliferation. The thyroid hormones, T3 and T4, utilize αVβ3 integrin as a cell surface receptor to activate a range of signaling molecules which induce angiogenesis. When binding to αVβ3 integrin, the thyroid hormone analog tetrac blocks T3/T4 integrin-induced effects.

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