

Structural analysis of integrin $\alpha 5 \beta 1$ and Tie2 activation and modulation

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Abstract

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The cardiovascular system constantly remodels to facilitate effective transport of nutrients, oxygen, and immune cells. Angiogenesis is the formation of new vasculature from preexisting vessels and is one of the major contributors to vessel expansion and homeostasis. Clinical and biochemical studies have significantly enhanced our understanding of angiogenic signaling pathways and the proteins involved in both healthy and diseased states such as chronic inflammation and cancer. This thesis investigated the structural mechanisms of integrin $\alpha 5 \beta 1$ and Tie2 receptors in angiogenic regulation. Integrins are heterodimeric receptors critical for cell adhesion and motility. Integrin $\alpha 5 \beta 1$ is involved in the migration of endothelial cells during angiogenesis. Dysregulation of integrin $\alpha 5 \beta 1$ is associated with tumor progression and metastasis. Despite numerous efforts, $\alpha 5 \beta 1$ -targeting therapeutics have been unsuccessful due to poor efficacy and off-target effects. A contributing factor is our limited understanding of how integrin conformation influences interactions with therapeutics. Using cutting-edge cryogenic electron microscopy (cryoEM) techniques in combination with cell-based assays, I analyzed the

impact of potential therapeutic molecules such as antibodies and de novo-designed proteins on integrin $\alpha 5\beta 1$'s conformation. These insights highlight how therapeutic binding influences integrin $\alpha 5\beta 1$ conformation and underscore the importance of considering these effects during the development of future integrin-targeted therapeutics. Tie2 is a receptor tyrosine kinase that facilitates tight cell-cell contacts, regulating vascular quiescence. It is regulated by a family of growth factors called angiopoietins, which cluster and activate Tie2. Though the angiogenic role of Tie2 is clear, little is known about the allosteric structural perturbations that Tie2 undergoes to propagate the angiopoietin-binding signal across the cell membrane. In addition, it has been demonstrated that integrin $\alpha 5\beta 1$ can modulate the activity of Tie2 through receptor crosstalk. However, there is no defined mechanism for this interaction. I took advantage of modern structural tools to overcome these gaps using full-length integrin $\alpha 5\beta 1$ and Tie2 proteins. These findings contribute to our understanding of the underlying mechanisms of receptor crosstalk between integrin $\alpha 5\beta 1$ and Tie2.

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Con cảm ơn bố, mẹ và em gái rất nhiều.

Chapter 1 Angiogenesis in health

The cardiovascular system is one of the largest organ systems and supports oxygen and nutrient delivery, waste removal, tissue homeostasis, and immune cell trafficking. In adulthood, much of the vasculature is stable, but the vascular network maintains the capacity to remodel quickly in response to developmental, physiological, and pathological stimuli. These dynamic changes are mediated by signaling initiated by multiple cell-surface receptors that coordinate endothelial cell proliferation, motility, survival, and vessel stabilization. Extensive biochemical and biomedical research has established many of the molecular players that govern this remodeling process. This knowledge has shaped our understanding of diseases marked by abnormal vasculature, including cancer. This chapter will provide an overview of how the vascular network is regulated, maintained, and expanded through angiogenesis.

1.1 Vascular organization and emergence of angiogenesis

The vasculature extends throughout the human body and is organized as a hierarchical branching system. Large arteries distribute oxygen-rich blood away from the heart, whereas veins return blood to the heart. These large vessels branch into smaller arterioles and capillary beds, increasing the surface area available for exchange with surrounding tissues. This hierarchical organization allows larger vessels to efficiently transport nutrients, oxygen, waste, and immune cells over long distances ¹. This design overcomes the limited diffusion range of oxygen needed for cellular metabolism, tissue growth, homeostasis, and repair.

The endothelium is a single layer of endothelial cells that lines the luminal surface of the blood and lymphatic vessels. This cell layer forms a selectively permeable barrier that prevents vessel leakage while allowing for immune cell extravasation and exchange of substrates ². The structure of a vessel consists of three layers: an endothelial cell monolayer, smooth muscle, and connective tissue containing extracellular matrix components (**Figure 1 - 1**). The endothelial cell monolayer is selectively permeable and forms the inner lining of a vessel. The endothelial monolayer of larger vessels is supported by smooth muscle cells to maintain vessel tone, which describes the degree of constriction ³. The subsequent layer consists of smooth muscle cells that can expand or constrict to regulate vessel diameter and maintain vascular pressure. In contrast, capillaries are supported by basement membranes and pericytes ⁴. Lastly, connective tissue

anchors the vessel to the surrounding tissues. Vascular function depends on the organization of endothelial cells and the broader vessel network, which is remodeling through expansion and pruning. Understanding how new vessels are formed became a central focus of biological and biomedical research, due to its implications for tumor progression.

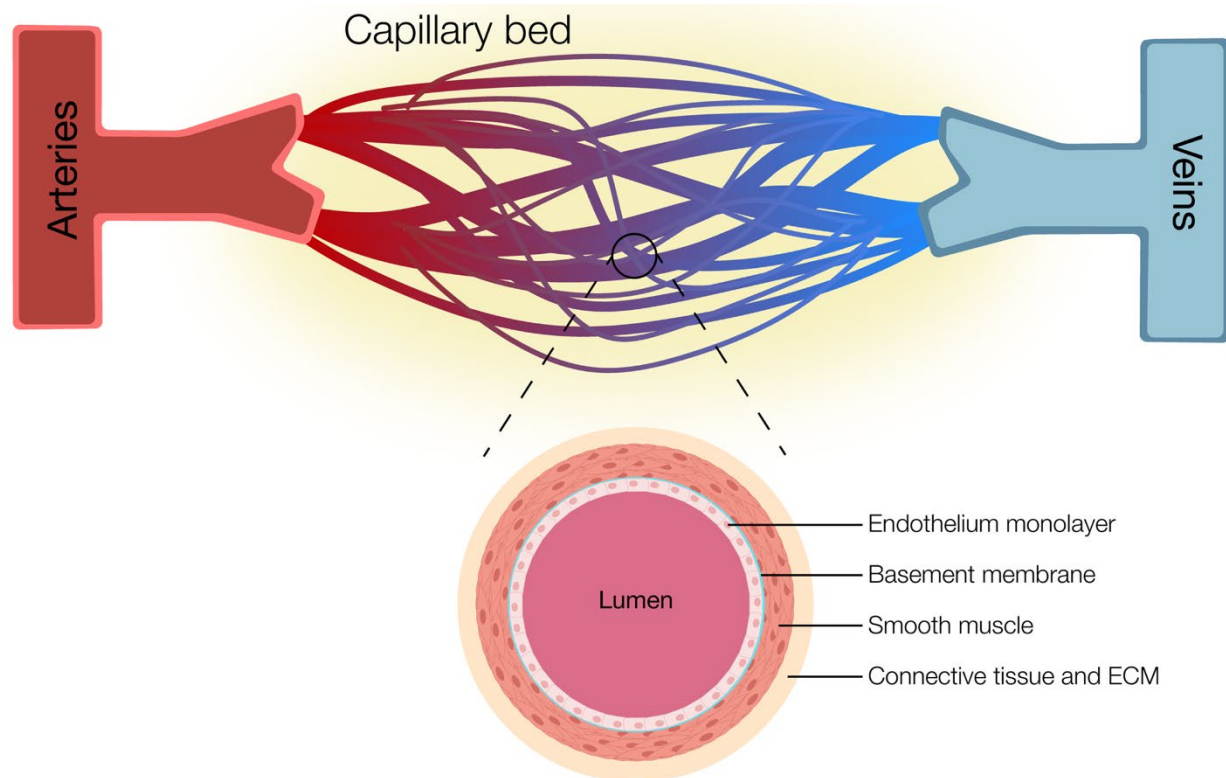


Figure 1 - 1 Hierarchical structure of the vascular system. The vascular network consists of arteries, which supply oxygen-rich blood to the rest of the body, and veins, which return blood to the heart. These structures function as conduit vessels, moving blood over long distances. Capillary beds are located at the interface between arteries and veins and increase the surface area available for gas exchange with surrounding tissues. All vessel types consist of a single layer of endothelial cells that line the luminal surface and create a selectively permeable barrier for exchange of gases and nutrients. This monolayer is structurally supported by smooth muscle cells or by pericytes for capillaries.

Milestones of angiogenesis research

Early descriptions of angiogenesis date back to the late 18th century with John Hunter, a British surgeon, who made observations of the blood vessel growth patterns during inflammation⁵⁻⁸. Unbeknownst at the time, Hunter described what was later termed “angiogenesis” by Joseph Flint, who was also describing vessel growth within the adrenal gland^{9,10}. Throughout the following centuries, angiogenesis remained largely descriptive as multiple groups observed vessel growth in different organisms and tumor models¹¹⁻¹³. These authors all noted that tumors

seemed to be correlated with the local vasculature. Following these early observations of the characteristics of blood vessel regulation, the field of angiogenesis experienced its first major advancement in the mid-20th century.

These observations continued with Judah Folkman's demonstration that tumor growth was linked to vascularity. The tumor microenvironment was enriched with a large number of immature blood vessels ¹⁴. He further noted that tumors lacking substantial vascularity were limited to 1-2 mm³. This led to his hypothesis that tumor growth was directly linked to angiogenesis and that inhibition of angiogenesis could be a therapeutic strategy for treating tumors ¹⁵. The following decades were marked by studies identifying factors responsible for tumor vascularization and angiogenesis. These efforts began with the isolation of a vessel promoting tumor angiogenesis factor using chick chorioallantoic membranes (CAM) ^{14,16}. One of the first purified angiogenic proteins was basic fibroblast growth factor, later identified as FGF2 (fibroblast growth factor 2) ^{17,18}. This factor stimulates fibroblast cell growth and proliferation, providing structural support for blood vessels and endothelial cells ¹⁹. To date, there are 22 members of the FGF family that signal through tyrosine kinase receptors ²⁰.

A major advancement was the identification of vascular permeability factor (VPF) followed by vascular endothelial growth factor (VEGF) ²¹⁻²⁴. VPF, as the name suggests, was a secreted protein that increased vessel permeability, leading to extravasation of plasma proteins to the tumor microenvironment ²⁵. When VPF was given to endothelial cells, they exhibited increased cellular proliferation and migration in vitro ²⁴. In parallel, VEGF was purified and subsequently cloned from conditioned media and found to induce angiogenesis in vivo ²⁶. VEGF demonstrated high specificity for endothelial cells, serving as a potent mitogen ^{27,28}. These proteins were later found to be identical and collectively known as VEGF, tying together the concept of vascular permeability and endothelial cell growth ^{21,29}.

While VEGF is essential for initiating blood vessel sprouting and endothelial cell proliferation, proper vascular structure must also be mature and stabilized. The identification of FGF and VEGF with their receptors established that receptor tyrosine kinases could be important regulators of angiogenic responses. The discovery of Tie receptors and their angiopoietin ligands added to this conceptual framework ^{30,31}. In brief, although this will be further discussed in Chapter 3, Tie receptors mediate endothelial cell-cell contact to ensure a tight but selective seal

of the vessel ^{32,33}. The loss of these receptors results in embryonic death, emphasizing the importance of vessel stabilization in addition to vessel growth ^{34,35}.

Collectively, Folkman's hypothesis is now widely accepted. The angiogenesis field has expanded from studying vessel growth to understanding the mechanisms of vessel maturation and homeostasis ^{6,36,37}. Starting at the turn of the century, a considerable effort has been devoted to targeting angiogenesis to treat cancer and other vascular diseases ^{38,39}. There have been some successes in targeting these proteins and their receptors as anti-angiogenic therapies ⁴⁰. Some of these therapeutics have demonstrated clinical success in limiting tumor angiogenesis. However, tumors can develop resistance to these drugs which other pro-angiogenic factors are also upregulated, compensating for the targeted pathway ⁴¹⁻⁴³.

1.2 Mechanisms of vessel network expansion

The discovery of angiogenic factors and their receptors established the molecular players of blood vessel homeostasis. These growth factors are secreted by surrounding tissues, fibroblasts, pericytes, and tumor cells to guide endothelial cell proliferation and migration toward angiogenic stimuli ³⁹. The two mechanisms that establish and remodel the vascular network to meet physiological and pathological demands are vasculogenesis and angiogenesis (**Figure 1 - 2**).

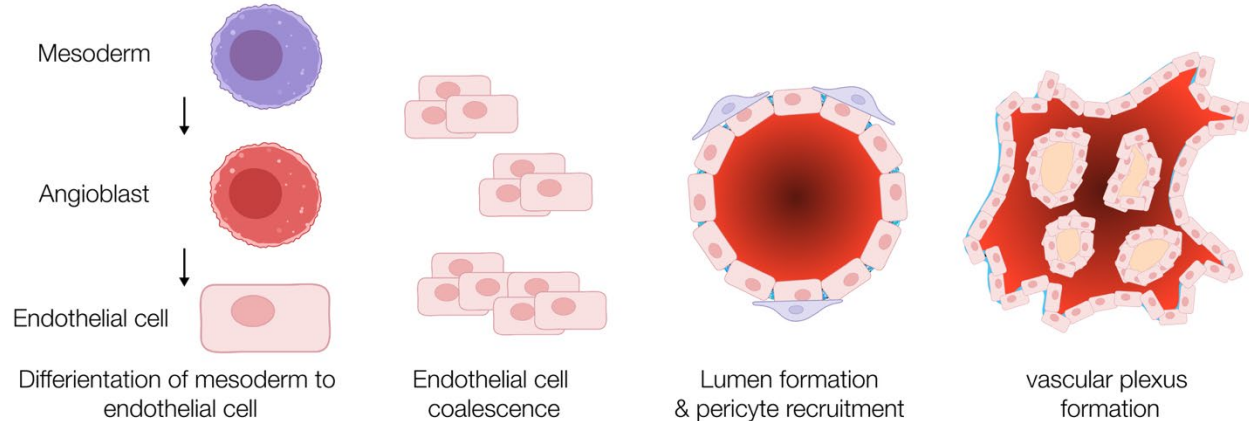
Vasculogenesis is the de novo formation of new blood vessels from endothelial progenitor cells that give rise to the vascular plexus ⁴⁴. This is one of the first functional organ systems to develop in vertebrate embryos ⁴⁵. During early embryogenesis, mesoderm-derived hemangioblasts undergo differentiation, giving rise to angioblasts, which are precursors to endothelial cells ^{46,47}. Endothelial cells then form the immature vascular plexus in their respective locations within the embryo ⁴⁸. Vascular development continues as these early vessels connect to each other and with the heart ^{46,49}. The newly established network is then remodeled through angiogenesis to form the tree-like hierarchical vessel structure. Supporting cells such as pericytes and smooth muscle cells are recruited for vessel stabilization. Any dysfunction in vasculogenesis can result in embryonic death ⁴⁴. After vasculogenesis establishes the initial network connecting the heart to vessels throughout the organism during embryogenesis, angiogenesis takes over as the primary vessel remodeling process. In the presence of an angiogenic cue, endothelial cells will exit quiescence and become activated, altering the

expression of genes associated with proliferation and motility⁵⁰. Angiogenesis is the process in which blood vessels form from existing vasculature^{51,52}. Within angiogenesis, there are two mechanisms defined by the manner in which new vessels are formed. Sprouting angiogenesis occurs when endothelial cells migrate outward to form a new stalk from the parent vessel. Intussusceptive angiogenesis occurs when blood vessels split into two through the formation of intraluminal pillars⁵³. Angiogenesis serves as the primary mechanism of vascular remodeling in response to metabolic demands.

Two modes of angiogenesis: sprouting and intussusceptive

Sprouting angiogenesis is defined by the formation of a new vessel from existing vasculature through endothelial sprouting. This process can be segmented into four phases: endothelial cell activation, sprouting extension, vessel morphogenesis, and stabilization (**Figure 1 - 2**). Angiogenesis is initiated in nutrient-deprived or hypoxic tissues that demand the formation of new vessels to support their growth⁵⁴. These tissues release angiogenic factors, including VEGF, angiopoietins, and FGF, to activate endothelial cells and promote their exit from quiescence^{48,55,56}. Vessel-supporting cells such as fibroblasts and pericytes secrete matrix metalloproteases to break down the basement membrane, destabilizing the local area of sprout formation^{4,57,58}. Endothelial cells differentiate into two types: tip and stalk ECs⁵⁹. Tip ECs are sensitive to local concentrations of pro- and anti-angiogenic cues such as VEGF and Notch ligands, which act as “gas” and “brake,” respectively, for movement toward the angiogenic source^{60–63}. As the tip cells migrate, they also secrete basement membrane components for the adhesion of stalk cells^{64,65}. The dynamic balance between VEGF and Notch is one of the mechanisms that distinguish between tip and stalk ECs, as Notch signaling supports stabilization of newly formed vessels⁵⁹. As the sprout extends, the stable stalk cells reorganize to form cell-cell contacts in a tube-like structure. These cells undergo polarization to establish a luminal face and outer surface, which contact the lumen and surrounding tissues, respectively⁶⁶. The outer surface contains the proteins required for adhesion to the extracellular matrix. The final steps involve anchoring and stabilization of migrated endothelial cells to the extracellular matrix and one another, promoting barrier stability^{67–69}. A new basement membrane is deposited, replacing the matrix that was degraded earlier in the process. Pericytes and smooth muscle cells are recruited to reinforce the endothelial monolayer⁴⁴.

Vasculogenesis



Angiogenesis

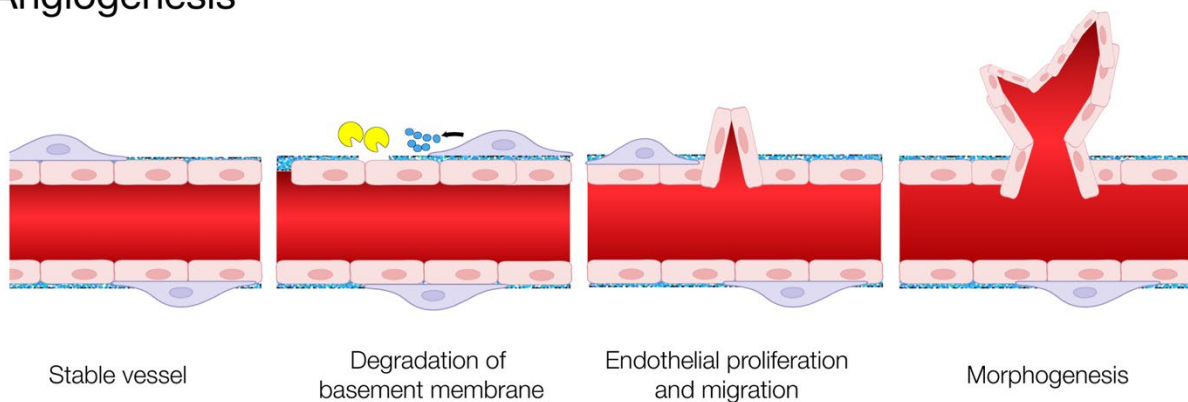


Figure 1 - 2 Vasculogenesis and angiogenesis form and remodels the vascular network. Vasculogenesis is the formation of the initial vascular plexus during embryogenesis. Mesoderm derived angioblasts will differentiate into endothelial cells. In multiple locations of the embryo, endothelial cells will coalesce together and begin to form luminal chambers. Pericytes and smooth muscle cells are recruited to support the growing vessel and maintain luminal pressure. The newly formed vessels join forming the young vascular system. Angiogenesis takes over as the primary process of forming new blood vessels throughout life. This is stimulated by the secretion of various pro-angiogenic signaling factors. The basement membrane is degraded to allow for endothelial cell proliferation and migration. Endothelial cells will migrate toward the source of the growth factor.

Intussusceptive angiogenesis is a form of angiogenesis in which a parent vessel is split to form new vessels (**Figure 1 - 3**)^{53,70-72}. This type of network expansion is predominantly observed within capillary beds to further increase the tissue exchange surface area but also during embryonic development^{52,73,74}. Unlike sprouting angiogenesis, intussusceptive remodeling requires minimal changes to the basement membrane that supports the vessels as endothelial cells do not proliferate into the extracellular matrix or surrounding tissues but across the vessel lumen⁷⁵⁻⁷⁷. The most well-documented form involves the formation of an

intraluminal/intussusceptive pillar via endothelial cells that migrate across the vessel lumen⁷⁸. Scanning electron microscopy facilitated the first documentation of intussusceptive pillar formation using corrosion casting of rat lungs⁷⁹. Completion of pillar formation results in the parent vessel branching into two daughter vessels or pruning of an unnecessary vessel⁸⁰. As there is no migration or invasion of endothelial cells into surrounding tissues, intussusceptive angiogenesis is considered more energetically efficient than sprouting angiogenesis^{71,81}. The formation of intussusceptive pillars is nonrandom and are orthogonal to the vessel, which suggests that the formation location is dependent on blood flow in addition to shear stress⁷⁵. However, it is not completely understood how endothelial cells can migrate in the correct direction within an active vessel to create the desired structure^{75,82}.

Angiogenesis in health and disease

The growth and development of multicellular organisms are highly dependent on the expansion of the vascular network. As described earlier, vasculogenesis predominates in the formation the new vascular plexus in the embryo starting E6.5 (embryonic day) within mice models⁴⁷. From there on, angiogenesis is the primary vascular process responsible for remodeling and maintaining the network throughout adulthood in response to the metabolic demands of the body. In adult tissues, endothelial cells are primarily quiescent but can be activated in response to hypoxic conditions or inflammatory signaling.

The ability to heal is an essential process for animal health. One characteristic of wound healing is the creation of new vessels via angiogenesis to support the large amount of proliferation and metabolic rates^{83,84}. Upon injury, local tissues release pro-angiogenic factors that promote vessel formation toward the site of injury. This blood supply will support the rapid cellular proliferation, migration, and metabolic activities of new cells as well as providing access for immune cells. The rate of capillary formation is aggressive at first but slows over time as hypoxic conditions improve. These vessels undergo pruning and regress until the density of vessels returns to baseline^{85,86}. Tissue repair is coordinated with acute levels of inflammation to clear bacteria, dead tissue cells, and debris. In turn, resident macrophages also secrete pro-angiogenic factors as part of the wound healing response to more easily recruit other immune cells. Impaired vascularization can disrupt the healing process⁸⁷. These nonhealing wounds are associated with chronic inflammation and dysregulated angiogenic signaling.

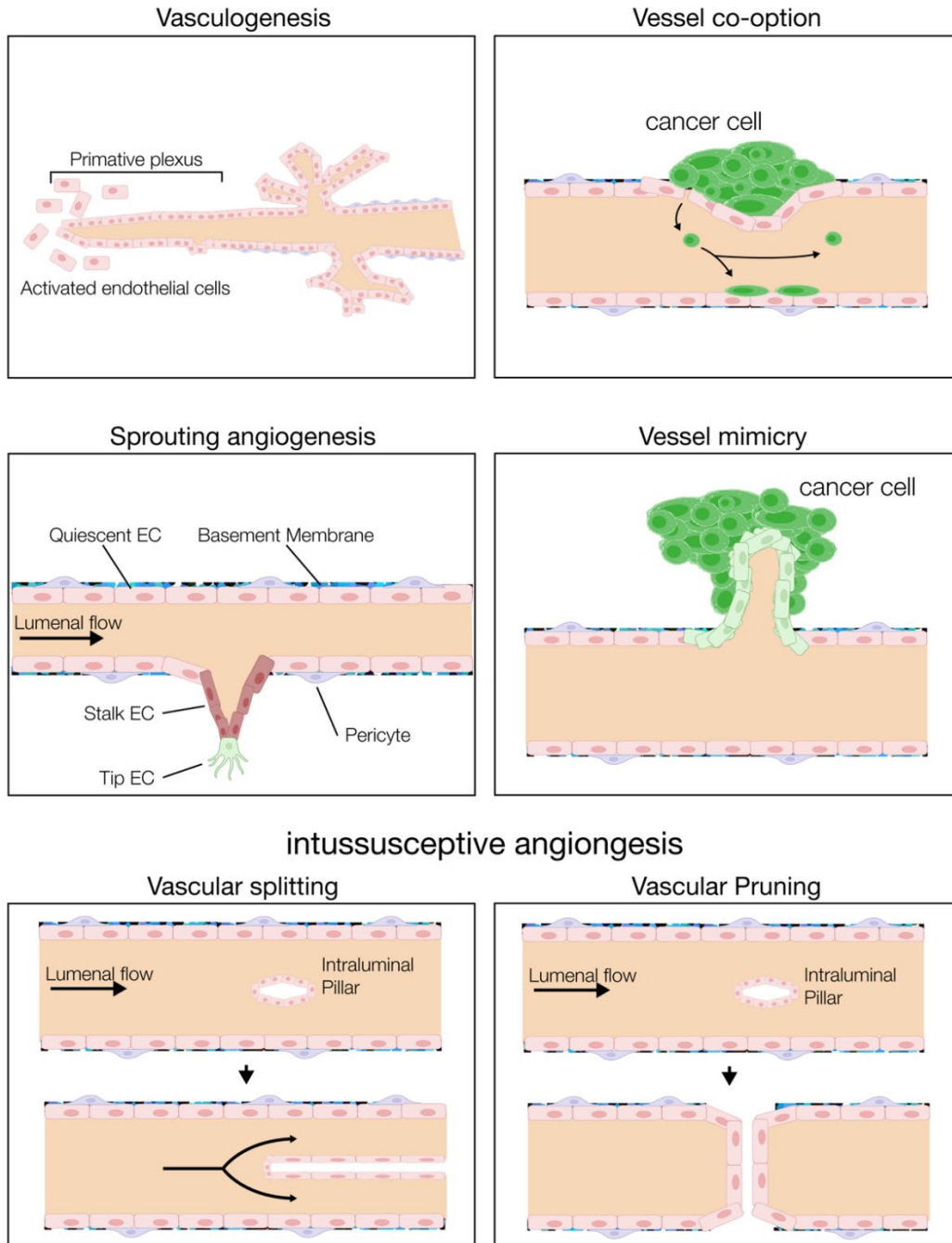


Figure 1 - 3 Sprouting and intussusceptive angiogenesis modes. Vessels grow and remodel in a variety of different morphological methods. Vasculogenesis is the formation of a new and immature vessel system known as the primitive plexus from coalesced endothelial cells. Angiogenesis serves as the primary vessel remodeling process and is by two modes: sprouting and intussusceptive angiogenesis. Sprouting angiogenesis consists of newly formed vessels that bud towards the pro-angiogenic cue from an existing parental vessel. The direction of the vessel is mediated by tip endothelial cells and extension by stalk endothelial cells. Intussusceptive angiogenesis is when the parental vessel splits into two daughter vessels via the growth of an intraluminal pillar. Pillar formation can also be a mechanism to prune unnecessary vessels. Tumors require a substantial amount of blood supply to fuel its growth. Therefore, cancer cells devised mechanisms such as vessel co-option where the tumor cells hijack existing vasculature and vascular mimicry, making their own vessel like structure to secure nutrients. This provides not only their blood supply but also may provide a mechanism of escape to metastasis to other parts of the body.

As the tumor grows, so do its demands for oxygen and nutrients, creating a hypoxic region. This stimulates the secretion of pro-angiogenic factors by tumor and neighboring cells. Angiogenesis is one of the most well studied examples of aberrant angiogenesis^{15,41}. Vessel homeostasis is maintained through the balance between pro- and anti-angiogenic factors that promote vessel formation toward the tumor, shifting the local concentration to pro-angiogenic factors. When pro-angiogenic signaling outweighs the anti-angiogenic signaling, the tumor undergoes an “angiogenic switch”, leaving the dormant state and entering an aggressive proliferative state^{88,89}. The recruited vasculature around the tumor is immature, poorly organized, leaky and inefficiently perfused⁹⁰. These characteristics can perpetuate hypoxia, further stimulating secretion of pro-angiogenic factors. Aggressive tumors have also devised other methods to secure blood supply through co-option and vascular mimicry to support its growth^{91,92}. Vessel co-option is a process where the tumor hijacks nearby vessels to invade surrounding tissue. There have been many instances where vessel co-option has been described with anti-angiogenic therapeutic resistance⁹³. Vascular mimicry takes the tumor cells themselves to form vessel like structure to obtain blood supply, playing a role in stimulating invasion and metastasis⁹². Together, these strategies support the idea of limiting tumor growth via vascularization, providing the rationale for therapeutic development targeting tumor angiogenesis.

As vascular endothelial cells have lower turnover than other cell types, the angiogenic balance of activators and deactivators is tightly regulated^{86,94}. As the angiogenic field has advanced and progressed, angiogenesis is now understood to be implicated in numerous diseases, in addition to cancers⁹⁴. A balance of pro- and anti-angiogenic responses is required for proper function of female reproduction processes, bone health, and retinal development (**Figure 1 - 4**)^{95,97,99,101}. Dysregulation resulting in excessive angiogenesis leads to development of various organ cancers. These tumor vessels are immature as endothelial cells are constantly proliferating and migrating without stabilization. Excessive angiogenesis can also result in psoriasis and high levels of inflammation^{96,98}. On the other hand, insufficient angiogenesis has been identified as a contribution to various disease conditions including chronic wounds, neurodegeneration, and osteoporosis^{83,94,100,102,103}.

This does not necessarily mean that any imbalance is detrimental. Transient shifts between pro- and anti-angiogenic signaling are essential for normal physiology. Upon injury, a rapid increase in pro-angiogenic cues is needed to establish a vascular supply to deliver the

necessary resources and immune cells to support tissue regeneration and clear the site of any potential pathogens. As the wound heals, the balance shifts back in the other direction towards anti-angiogenic signaling as these promoted vessels are no longer required and should be pruned back towards baseline levels. Collectively, these physiological processes highlight that angiogenesis is tightly regulated and that transient imbalance is normal whereas prolonged imbalance promotes abnormal vasculature associated with various diseases.

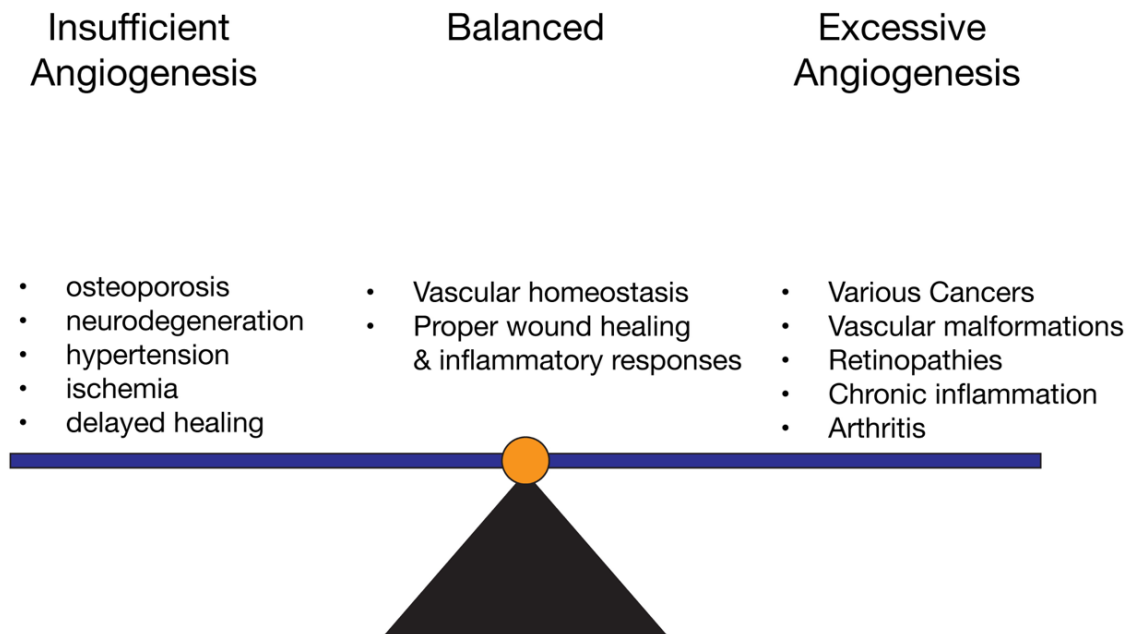


Figure 1 - 4 Balanced angiogenesis results in proper homeostasis and responses. Angiogenic and nonangiogenic factors are in constant flux and the equilibrium of the two dictates the level of angiogenesis that occurs. Prolonged disequilibrium of this balance can result in a variety of venous diseases and conditions. When anti-angiogenic signaling dominates, the vascular structure is reduced due to insufficient angiogenesis. This has been noted in a variety of health conditions ^{83,102–106}. On the other hand, a prolonged shift towards pro-angiogenic results in excessive angiogenesis. These vessels are often characterized as malformed because they are unable to mature properly ^{96,102,107,108}. Short lived imbalances of pro- and anti-angiogenic signaling are hallmarks of vessel homeostasis that allow for wound healing and proper levels of inflammation.

Therapeutic application of angiogenesis

The role of angiogenesis in tumor growth and vascular diseases has established it as an attractive therapeutic target. Several angiogenesis inhibitors have demonstrated efficacy in endothelial cells in vitro and in vivo ^{109–118}. Due to the central role that VEGF and its receptor VEGFR play in angiogenesis, this signaling axis became the leading target ^{48,119}. As our understanding of angiogenesis and the proteins involved increased, the targeting repertoire for

drug development expanded to protein targets outside the VEGF signaling pathway such as FGF, Tie receptors and integrins ^{42,120,121}.

The first approved anti-angiogenesis drug, Bevacizumab (Avastin, Genentech), which targets VEGF, earned approval by the Food and Drug Administration (FDA) in 2004 and by the European Medicines Agency (EMA) in 2006 ^{40,122,123}. Bevacizumab binds specifically to human VEGF-A isoforms, neutralizing the growth factor and disrupting blood vessel formation ¹²⁴. Phase I clinical trials demonstrated low toxicity and its addition to standard chemotherapy did not increase chemotherapy-associated side effects ^{125,126}. Phase II trials assessed Bevacizumab against multiple tumor types, with the strongest efficacy results seen in metastatic colorectal cancer, late-stage non-small cell lung cancer and renal cell carcinoma ^{127–132}. Bevacizumab was later approved for these cancers.

However, in other cancers such as breast, melanoma, pancreatic and prostate cancers, treatment with Bevacizumab did not increase overall survival ^{133–136}. Bevacizumab was highly effective in animal models and preclinical trials, but this did not translate into clinical benefits in humans. The administration of Bevacizumab also shed light on emerging issues with antiangiogenic therapy: tumor drug resistance ⁴⁸. Treatment was effective early on and efficacy declined because angiogenesis signaling is regulated by multiple compensatory signaling that contribute to angiogenic gene activation ¹³⁷. Similar decreases in efficacy are described for other anti-angiogenic molecules ¹³⁸.

The original hypothesis of treating diseases by targeting and inhibiting angiogenesis is valid and many patients have benefited from treatment ^{37,42,139}. With Bevacizumab leading the way, there are now multiple antiangiogenic therapeutics on the market that have been approved by the FDA, including Sorafenib, Sunitinib, Pazopanib, Vandetanib, Axitinib, Regorafenib, Cabozantinib, and Lenvatinib ^{140,141}. However, what Judah Folkman has proposed about targeting angiogenesis as a means of cancer treatment has not been as successful as it should be. There is a large concern about when (not if) tumor drug resistance as other primary or secondary angiogenesis signaling can compensate. At its core, the promotion of angiogenesis stems from the need to relieve localized hypoxia. Following the administration of antiangiogenic therapeutics, hypoxia may continue and eventually worsen, damaging the site at and around the tumor. Growing data suggest that these disappointing results are attributable to tumor microenvironment heterogeneity and complexity ¹⁴¹.

1.3 Cryogenic electron microscopy as a tool for studying angiogenic receptor biology

Many proteins involved in angiogenic signaling start at the surface of the cell ¹⁴². Scientific depictions of these receptors are often simplified as cartoons that project out of the cellular membrane or as static protein models. However, receptors can be highly dynamic molecules, exhibiting breathing or fluctuating motions to find their ligand, analogous to a flip book. Biochemically, we can understand the molecular and protein players that are involved during angiogenic signal transduction. Unfortunately, biochemistry alone cannot fully discern the mechanism of how these receptors can mediate communication. For a more complete understanding, it is vital to understand what the receptor structure is or if there are any conformational changes during ligand binding or receptor function. To complement these studies, structural biology and biophysical methods provide molecular mechanisms of these receptor functions.

For decades, x-ray crystallography has provided high-resolution structural information for all types of proteins and will continue to do so ¹⁴³. For proteins to be prepared for x-ray crystallography, they must be arranged in a crystal lattice, which requires extensive optimization and is difficult to achieve ¹⁴⁴. This can stabilize certain conformations available to the protein and limit the accessible protein states. As a result, crystal packing, which is required for high-resolution information, can also limit our ability to capture multiple snapshots along the conformational equilibrium that these receptors can exhibit ¹⁴⁵. Nuclear magnetic resonance (NMR) can also provide structural information on protein dynamics ¹⁴⁶. However, membrane protein stabilization requires either a detergent or nanodisc, a membrane mimetic, which weakens the signal or increases the size of the protein/complex beyond the effective limit of NMR.

Cryogenic electron microscopy (cryoEM) is an alternative protein structure determination method that has gained considerable traction within the last decade and has been shown to dissect structures of dynamic and heterogeneous proteins (**Figure 1 - 5**). This was largely due to the improvements to both hardware and software during the 2010s “resolution revolution” ¹⁴⁷. Membrane proteins have been traditionally difficult to crystallize due to their instability and dependence on a membrane. Biochemists have developed techniques and technology to

overcome membrane protein instability using detergents as a membrane mimic^{148,149}. However, the dynamic nature of membrane protein receptors can often still limit the crystallization process. In addition, if a crystal is formed, the structure obtained could be the most thermodynamically stable conformation observed rather than a conformational state during function. This severely limits the structural information about other possible conformations that the receptor can adopt. This makes cryoEM a suitable structural determination method as proteins are imaged in near-native conditions and allowed to adopt all possible conformations prior to sample vitrification. Since then, the Protein Data Bank has received an increasing number of structures obtained using cryoEM as it is becoming more mainstream and accessible¹⁵⁰.

The typical cryoEM workflow begins with sample preparation using a purified protein solution. An EM grid is typically 3 mm in diameter that contains a mesh-like arrangement of metal (copper, gold, or nickel-titanium), resulting in 200-400 squares per EM grid (**Figure 1 - 6**). A thin layer of a carbon or gold-based support film is placed on top of the mesh. The support film that covers these squares contains perforations that are equally spaced apart from one another. Prior to sample application, the EM grid is made hydrophilic via glow discharging or plasma cleaning. 3 μ L of protein sample is applied to the glow-discharged EM grid and the solution covers the surface of the grid. The sample is given time to enter the holes. Because the protein is in solution, each protein molecule can adopt any available conformation. Excess solution is blotted away by filter paper such that the only solution remaining is suspended in the holes. The grid is then rapidly plunge-frozen into liquid ethane. Directly prior to the plunge freezing, all protein particles within solution can adopt any conformation and orient themselves randomly in solution. This can be their position, which can be represented in a Cartesian coordinate system (x, y, z; with z being the depth in the layer of ice) within the solution and any rotation about its own center. Upon freezing, the aqueous layer, with the protein particles randomly orientated, turns into non-crystalline or vitreous ice, which has a glass-like appearance.

Using a transmission electron microscope, the EM grid is imaged to acquire a micrograph, which is two-dimensional image that contains the protein particles (**Figure 1 - 6**). The work in this thesis utilizes single particle analysis (SPA), which takes the two-dimensional projections from the micrograph and aligns and averages them into a three-dimensional density map. Briefly, using computer software, each two-dimensional projection can be automatically identified or picked and then extracted from the micrograph, resulting in hundreds of thousands

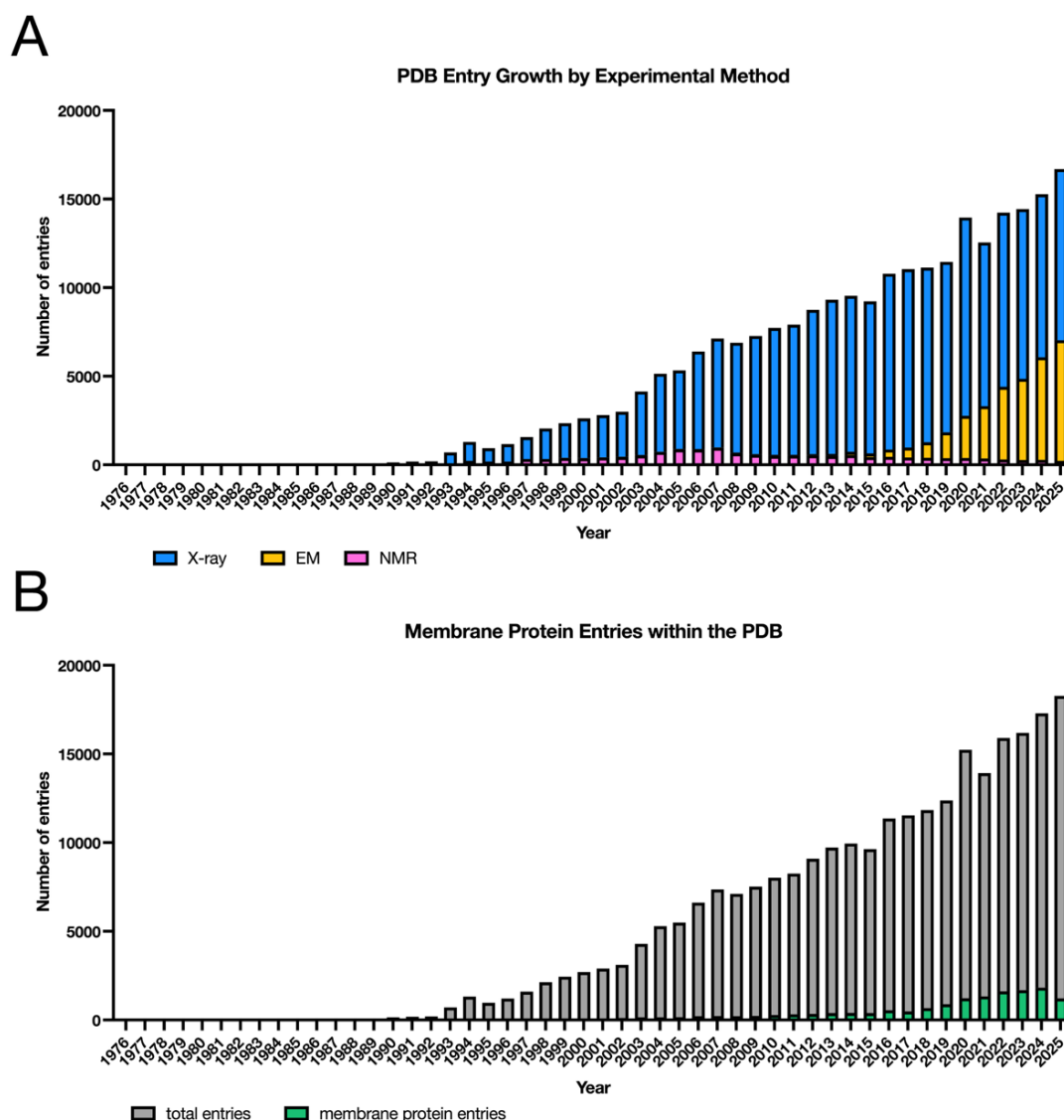


Figure 1 - 5 Protein deposition statistics by method and protein type. The growth of cryoEM determined protein structures have vastly benefitted from the development of direct electron detector coining the era of “resolution revolution”. Displayed is the number of protein structures determined by method that are deposited throughout the history of the Protein Data Bank (upper half). Many of the angiogenic signaling molecules signal through cell surface receptors for cellular communication with one another and the environment. These proteins can be grouped in the larger membrane protein family. This group of proteins has been difficult to structural study due to their dependence on the membrane. Modern developments in protein biochemistry and computational tools have now provided us with tools to stabilize and assess these protein structures (lower half). Full data can be found at <https://www.rcsb.org/stats> using the search API tool. Membrane proteins annotated within the Protein Data Bank based on their membrane-associated regions ^{151–154}.

of individual protein images that retain their orientation and conformation (if any). Using 2D classification algorithms, we can sort particles into classes or groups based on their similarity to each other. Some metrics used for assessment of particle classes are whether they are junk or not, any conformational states (which are unique to each protein) or specific orientations (which are *mainly* random within any micrograph). * The user can select which groups of particles to be used for back projection to create a three-dimensional cryoEM density map.

Particularly with membrane receptors, these molecules are dynamic as they survey a large extracellular space for their ligand. A protein structure determined using either crystallography or cryoEM is only a snapshot of the conformational equilibrium. Unlike crystallography, this equilibrium can still be captured within cryoEM as individual protein particles are utilized for electron density map generation. As cryoEM hardware is improving, so is the software that allows scientists to not only overcome the issues of protein receptor heterogeneity but also analyze and understand it. During cryoEM density map generation, all protein particles are taken into consideration for alignment in three-dimensional space, i.e. protein particles are averaged together. Other cryoEM algorithms are able to further classify and group of protein particles in 3D space. These can be intermediate or the end points of a protein's accessible conformational space. Therefore, not only can cryoEM provide high-resolution structural information about a protein molecule but it can also provide structural information of the conformational changes that the protein undergoes. This analysis is possible due to the development of modern cryoEM tools, such as 3D flex, 3D classification, cryoDRGN and others focused on heterogeneity ^{156–162}.

* When particles are unevenly or unbiasedly distributed across the EM grid this results in the *preferred orientation problem*. This generally arises from the interaction of the particles at the air-water interface during sample preparation. Various techniques and alternative sample preparation methods have been described to reduce this phenomenon ¹⁵⁵.

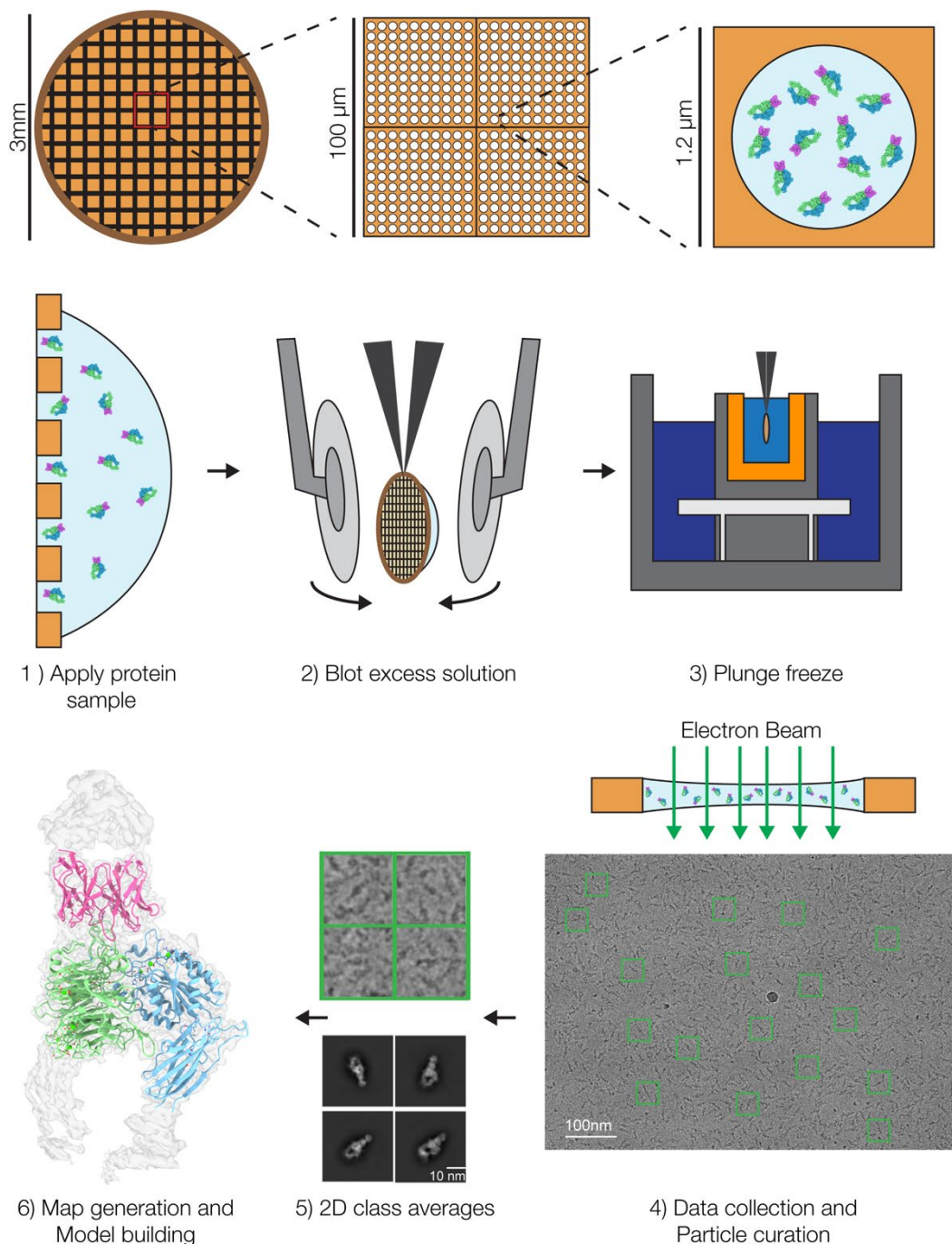


Figure 1 - 6 Typical cryoEM workflow. A cryoEM grid is what holds the protein sample during preparation. The EM grid comprises of a grid of squares which ranges from 200-400 total squares. Inside each of these squares are equally spaced perforations that called holes. The protein sample is adsorbed into these holes and are imaged during data collection. Typical sample preparation begins with applying the protein sample to the grid for protein to enter the holes. Excess protein solution is blotted away prior to being rapidly plunge frozen in liquid ethane to generate the cryoEM sample. This process creates a thin film of suspended protein sample inside the holes. Images of these holes are collected using a transmission electron microscope generating projections of the suspended protein sample. Using computer software and algorithms, protein particles are automatically picked (subset examples are shown) and curated prior to being sorted first in two-dimensions and then three-dimensions. From here, these particles can be used for generation of the 3D cryoEM map and model building can proceed.

1.4 Thesis objective, rationale, and outline

Clinical and biochemical research have significantly enhanced our understanding of the angiogenic signaling pathways and the proteins involved. Notably, this thesis focuses on two angiogenic receptors, integrin $\alpha 5\beta 1$ and Tie2. Integrin $\alpha 5\beta 1$ is a cell surface receptor which mediates cellular adhesion and motility through binding of fibronectin, an extracellular matrix protein. Tie2 is a receptor that regulates endothelial cell stability, permeability and quiescence. These two receptors' biological role in health and disease have been demonstrated. However, they have been difficult to target therapeutically or have limited efficacy as a monotherapy. This may stem from our incomplete understanding of their regulatory and activation mechanisms at the atomic level in the context of angiogenesis. To bridge this gap, high-resolution protein structures can facilitate and complement our biological understanding of this relationship. However, due to the inherent heterogeneous nature of membrane receptors, obtaining high-resolution protein structures has been difficult. With the advances in cryoEM technology, we can now more readily obtain these structures to elucidate the fundamental of this therapeutic to target's conformational dynamic to help guide the development of better therapeutics targeting these receptors.

There are two broad objectives within this thesis. The first objective aims to understand the mechanisms of integrin $\alpha 5\beta 1$ regulation with respect to therapeutic proteins. Integrins are a promising therapeutic target but the consequence of activation or inhibition by a therapeutic on their conformation equilibrium is not completely characterized. Protein function is inherently linked to its conformation. Therapeutic binding can shift the conformational equilibrium by stabilizing certain structural states. These therapeutic-induced conformational changes influence receptor activity, which can have an effect on therapeutic efficacy, specificity or potential allostery. Chapter 2 focuses on published work in therapeutic characterization of integrin $\alpha 5\beta 1$ using inhibitory antibodies and *de novo* design proteins. Leveraging cryoEM, biophysics and cell-based assays, two inhibitory antibodies demonstrate different modes of integrin disruption while having dramatic difference in global conformations via allostery. *De novo* protein design targeting the open conformation of integrin $\alpha 5\beta 1$ demonstrates effectively promoted of cellular attachment and spreading, critical for wound healing. The second objective aims to elucidate the mechanisms of Tie2 activation by its canonical agonist angiopoietin-1 and modulation through

receptor crosstalk with integrin $\alpha 5 \beta 1$. Chapter 3 discusses unpublished work in determining molecular mechanisms underlying Tie2 signal transduction at the atomic level. This thesis concludes with chapter 4, which discusses the current state of the angiogenesis field from a structural biology perspective and the impact of these results in both integrin and Tie2 biology.

Chapter 2 Integrin $\alpha 5\beta 1$ conformation modulated by potentially therapeutic binding proteins

For angiogenesis to proceed normally, endothelial cells must migrate and adhere to the extracellular matrix to provide stability and directionality to the budding vessel. Integrins are a family of receptors that are expressed at the endothelial cell surface. They mediate cellular motility and adhesion and are known to be heavily involved in angiogenesis¹⁶³. Angiogenic dysfunction can lead to pathologies such as cancer and venous malformations. Inhibition of tumor angiogenesis is thought to be an effective strategy to limit the growth of cancer^{164–166}. On the other hand, angiogenesis is critical for the wound healing process by providing increased blood flow for the delivery of nutrients and oxygen to damaged tissues. This duality positions integrins to be an attractive therapeutic target. However, unexpected off-target effects have led to few FDA-approved integrin-targeted therapeutics¹⁶⁷. Integrins are dynamic proteins and their activation and function are directly linked to their conformations. While protein structures of integrins are available, our understanding of the structural mechanisms underlying their regulation and the conformations impacted by potential therapeutics is not complete. This chapter will introduce integrins and present two publications that extensively discuss the modulation of integrin $\alpha 5\beta 1$'s conformational states using therapeutic antibodies and *de novo* designed proteins^{168,169}. Together, these structural and biophysical results shed light on the relationship between target conformational equilibria and therapeutic design, can be used to guide the development of future integrin-targeting therapies.

2.1 Integrin receptors mediate cell adhesion and motility

Cell adhesion and motility underlie many essential processes including development, maintenance and function of a multitude of cell types. Multicellular organisms are organized hierarchically with increasing complexity. Cells are the most basic building blocks, and a group of cells forms tissues. Tissues are organized to form functional organs, which work together to form complex organ systems. For these arrays of structures to exist, there must be some mechanisms to bind cells and tissues together. Integrins are cell surface receptors that mediate the motility and adhesion of cells to the extracellular matrix (ECM) and other cells.

Integrin discovery and subfamilies

The ECM provides a scaffold for cells to adhere and organize into multicellular structures. This matrix is a gel-like, non-cellular network of proteins and carbohydrates¹⁷⁰. One of the primary components of the ECM is a glycoprotein called fibronectin¹⁷¹. It was discovered that fibronectin, an extracellular protein, could induce actin filament rearrangement, suggesting a connection between the ECM and the cytoskeleton^{172,173}. This receptor was one of the first identified integrins, nucleating the field in the 1980s.

Fragmentation of the fibronectin sequence showed that a stretch of amino acids was able to promote cell adhesion, which was later narrowed down to the minimal sequence for binding: arginine-glycine-aspartate (RGD)^{174–176}. The RGD tripeptide motif is found in multiple extracellular matrix proteins¹⁷⁷. This marked an important discovery that defined a subset of integrin as RGD-binding integrins, such as $\alpha 5\beta 1$ ^{178,179}. In parallel studies using functional antibodies, LFA-1 (integrin $\alpha L\beta 2$) was identified as a mediator of adhesion for T cell-mediated cytotoxicity¹⁸⁰. Additional sequencing of these new receptors revealed high similarity to the previously described ECM receptors¹⁸¹. Together, biochemical, immunological, and sequencing studies expanded the integrin family and its ligand repertoire^{182–188}.

In humans, there are 18 α and 8 β subunits that combine to form 24 heterodimeric integrin receptors^{189,190}. The 24 heterodimers can be divided into four subclasses based on their function or ligand class: collagen, laminin or RGD binding and leukocyte integrins (**Figure 2 - 1**)^{191–195}. Due to their roles of cell adhesion and motility, integrins are ubiquitously expressed on all cell types with some heterodimers exhibiting certain cell types (leukocyte integrins)^{194,196,197}. A subset of these integrins contains an additional domain on the α subunit called the I-domain (inserted) with a von Willebrand factor type-A protein fold^{189,198–200}. These integrins cannot bind ligands at the canonical α/β subunit cleft of the non-I-domain integrins. Instead, these integrins use a different mechanism in which the I-domain binds the ligand directly and allosterically conveys binding to the β subunit through an internal ligand for receptor activation and conformational change²⁰¹. Despite the diversity of the integrin receptors, this receptor family exhibits overlapping but non-redundant functions as not all integrins bind the same ligand. For example, some integrins bind a single ligand, whereas others are more promiscuous and recognize a wide range of ligands¹⁶⁴. However, all integrin activation ultimately signals for

restructuring of the actin cytoskeleton to mediate cell motility and adhesion. The work within this chapter will be focused on integrin $\alpha 5 \beta 1$, a member of the RGD integrin subfamily^{178,195}. $\alpha 5 \beta 1$ is also known as the fibronectin receptor.

Overall Integrin structure

Integrins are cell surface non-covalent obligate heterodimers, meaning that a functional receptor requires both α and β subunits. Each integrin subunit is composed of a large extracellular portion connected to a small cytoplasmic domain via a single-pass transmembrane helix (**Figure 2 - 2**). The α subunit contains a seven-blade β -propeller that is followed by a thigh

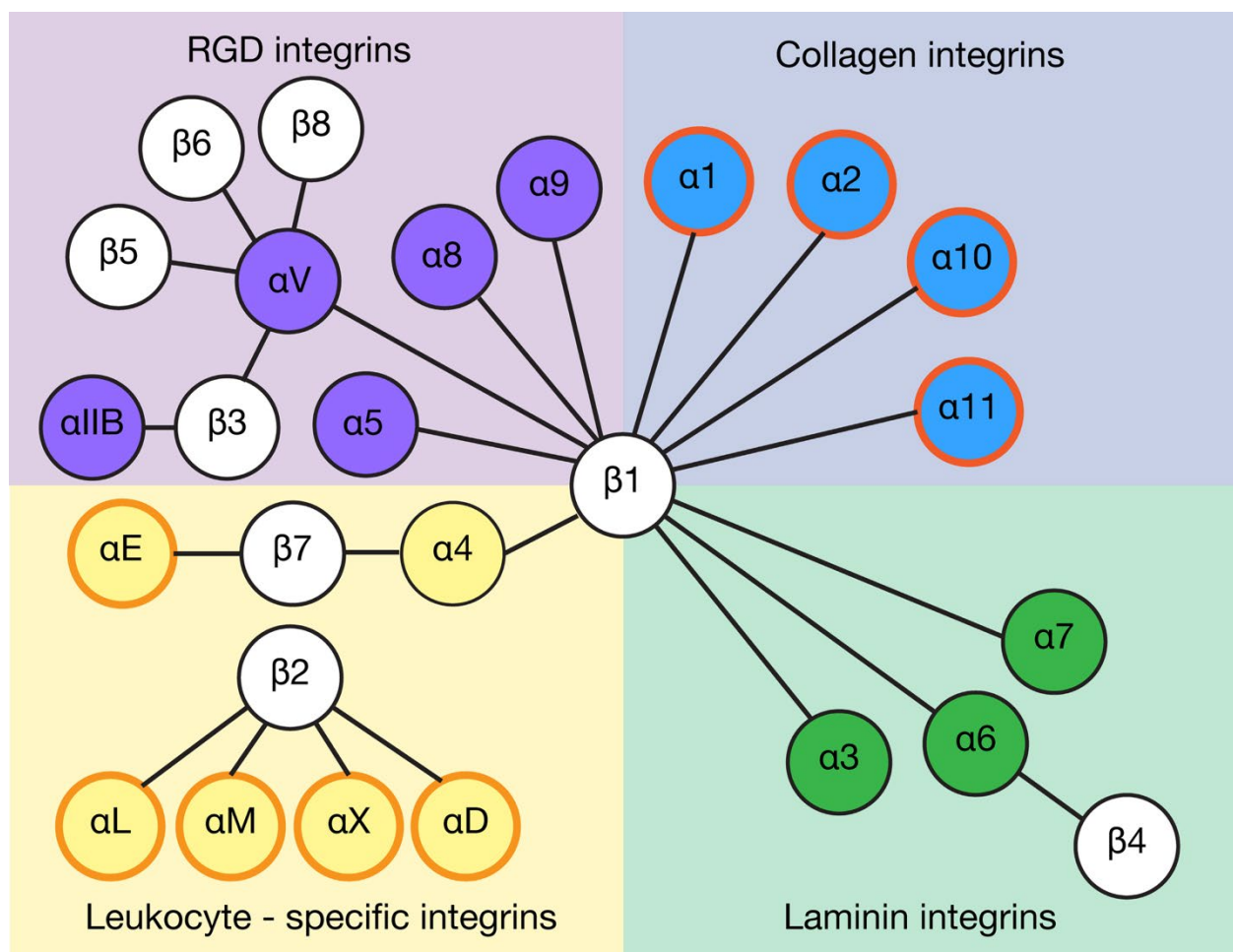


Figure 2 - 1 Mammalian integrin subfamilies and heterodimers. Every integrin receptor is a noncovalently bounded heterodimer consisting of an α and a β subunit. There are 18 α subunits and 8 β subunits, making 24 unique heterodimers (connecting line). The integrins can be classified by their ligand type or tissue expression: RGD (arginine-glycine-aspartate), collagen or Laminin binding integrins and Leukocyte specific expressing integrins. A subset of the α subunits contains the I-domain which are outlined in orange.

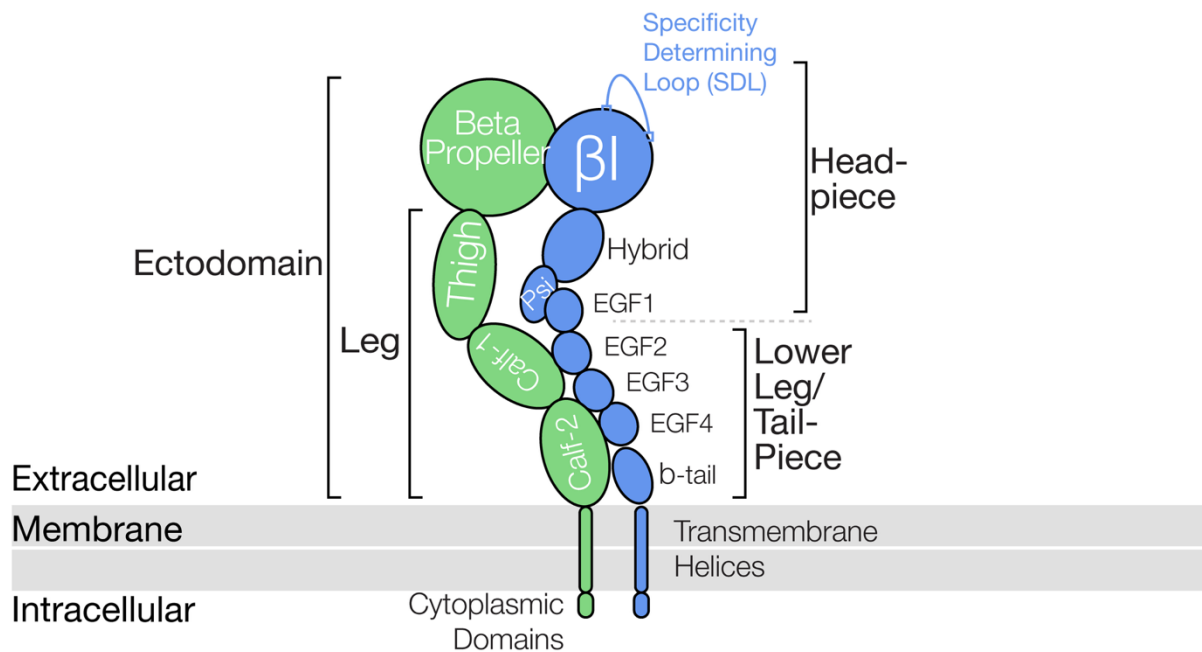


Figure 2 - 2 Integrin structure. The integrin heterodimer is comprised of an α and β subunit that are non-covalently linked shown in green and blue respectively. Both subunits have a large extracellular region that is connected to a small cytoplasmic tail by a single-pass transmembrane helix. The extracellular region or ectodomain can be further divided into two halves, the headpiece and the lower legs. In non-I-domain integrins, ligand binding occurs within the headpiece.

and two calf-domains, which adopt beta-sandwich like folds^{202,203}. For the I-domain integrins, this domain is inserted between the second and third blade of the β -propeller²⁰¹. The β subunit consists of the β -I, hybrid, PSI (plexin, semaphoring, and integrins) and four cysteine-rich EGF (epidermal growth factor) repeat domains^{202,203}. Together, these domains within the α and β subunits make up the extracellular “leg” of the integrin molecule (**Figure 2 - 2**). Lastly, the α and β cytoplasmic tails are small and mainly unstructured. However, any mutation or deletion within these tails can disrupt intracellular signaling²⁰⁴.

Integrin activation and signaling

Integrin receptors are dynamic proteins, and their conformation is linked to their activation states. Broadly, integrins can adopt three primary conformations: bent, extended closed, and extended open. The bent conformation is generally associated with the inactive state, as the headpiece is positioned toward the membrane (**Figure 2 - 3**)^{202,205}. In this conformation, the headpiece is restricted from sampling the extracellular space for fibronectin, resulting in a reduced affinity. The degree of compactness and bending varies across integrins. Crystal

structures show integrin $\alpha\beta 3$ exhibiting an acutely bent conformation in which the legs fold into the headpiece^{202,203,206}. Other integrins, including $\alpha 5\beta 1$, exhibit a half-bent conformation where the degree of flexion at the knee is approximately 90° ^{207,208}. In the absence of ligand, the integrin molecules exist in an equilibrium between the bent and extended closed conformations^{168,208}. Non-I-domain containing integrin receptors bind ligand at the α/β cleft^{202,207,209,210}. For integrin $\alpha 5\beta 1$, the extended open conformation is stabilized upon ligand binding^{207,211}. The β -I domain contains three metal ion binding sites: metal ion dependent adhesion site (MIDAS), adjacent metal ion dependent adhesion site (ADMIDAS), and synergistic metal ion binding site (SyMBS) (**Figure 2 - 3**)^{190,210}. For RGD-binding integrins, this motif is positioned at the α/β cleft and the aspartate residue coordinates with the ion at the MIDAS site^{207,212}. Ligand coordination with the MIDAS is coupled with a rearrangement of the β -I domain $\alpha 1$ and $\alpha 7$, helices leading to the hybrid domain swing out separating the integrin legs^{191,209,213}.

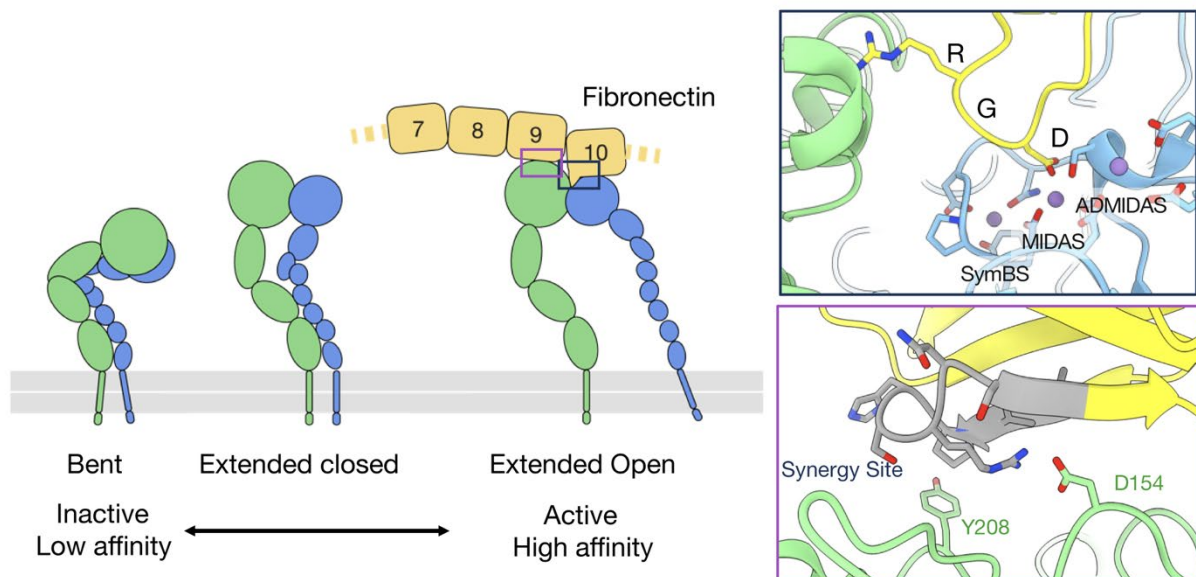


Figure 2 - 3 Integrin $\alpha 5\beta 1$ conformations. Integrins are dynamic transmembrane protein receptors, and their conformational states are a direct reflection of their activity or activation. The bent conformation is identified as the conical resting state of the receptor where the headpiece is tucked toward the membrane. The extended closed conformation exhibits this intermediate where the legs are extended positioning the headpiece out into the extracellular space, but the headpiece remains closed. The extended open conformation is where the headpiece opens through a conformational change with ligand binding. This conformational change is mediated and stabilized by the aspartate of the ligand (Fibronectin for integrin $\alpha 5\beta 1$) coordinating the ion at the MIDAS site within the β -I domain of the β subunit²⁰⁷. Because of the headpiece location, the bent conformation is seen as a low affinity state where the extended open has a high affinity for its ligand. Protein structure within the inset is from PDB: 7NWL.

Integrins are unique receptors that are capable of signaling across the membrane bidirectionally through a conformational change that involves the separation of the legs (**Figure 2 - 3**). These two modes are classified as “outside-in” and “inside-out” signaling (**Figure 2 - 4**)²¹⁴. The direction of signaling will depend on the origin of the ligand binding partner. Outside-in signaling is initiated when extracellular ligand binding promotes integrin conformational changes towards the extended open state. The integrin legs are separated and the receptors cluster together at the cell surface. These events support the formation of focal adhesions through recruitment of focal adhesion kinases (FAK), Src kinases, and talin^{164,215}. This phosphorylation cascade is propagated toward Rho-family GTPase activation for cytoskeletal rearrangement²¹⁶. In contrast, inside-out signaling is mediated by interactions between the cytoplasmic tails and adapter proteins, which in turn alter the conformational change of the extracellular domains^{217–219}. Because the cytosolic tails are linked to the global conformation of integrins, changes on one side of the membrane impact the other. Filamin is an integrin inhibitor that has been shown to compete with talin for β -tail binding²²⁰. When talin interacts with the cytoplasmic tails, this promotes the separation of the integrin legs and brings the extracellular region into the extended open conformation where affinity for ligands is increased. The structure of filamin contains multiple immunoglobulin repeats that have the ability to sequester the cytoplasmic tails away from talin, keeping the integrin clasped^{220,221}. Mechanical force is another regulator of integrin activation and function that contributes to both outside-in and inside-out signaling. Integrins are the physical linkage between the actin cytoskeleton and the extracellular matrix. They experience tensile force during filopodia extension and retraction. The role of force on integrin conformation has been shown to be vital, although the direct impact and regulating function is yet to be described using high-resolution structural information^{213,222–224}.

Integrins in disease and health

The primary function of integrins is to integrate the cellular cytoskeleton with the cellular environment through attachment to the extracellular matrix. In healthy tissues, integrins act as transmembrane adhesion receptors anchoring cells to the extracellular matrix by binding of external and internal ligands and mechanical tension. This interaction causes the formation of focal adhesions, a critical component for the reorganization of the actin cytoskeleton for cellular motility²¹⁶. In addition to cellular motility and adhesion, integrin function extends to white blood

cell trafficking, inflammatory responses and angiogenesis^{121,163,196}. $\beta 2$ integrins found on leukocytes mediate crawling and spreading during transendothelial migration during the inflammatory response¹⁹⁶. Because these processes are essential for development and repair, dysregulated integrin function contributes to multiple pathologies including cancer, inflammation, fibrosis and vascular malformations^{163,167,225}.

Wound healing is a coordinated process involving inflammation, proliferation and tissue remodeling. Integrins contribute to each phase by regulating platelet aggregation, leukocyte recruitment, cellular migration and tissue remodeling^{226–228}. Shortly after injury, platelets seal the site of damage and initiate clot formation. Platelets express primarily $\beta 1$ and $\beta 3$ integrins, particularly, $\alpha I I b \beta 3$ ²²⁹. $\beta 1$ -expressing fibroblasts deposit collagen and fibronectin to replace the damaged extracellular matrix²³⁰. Endothelial cells utilize integrins to facilitate movement toward the source of pro-angiogenic signaling molecules to expand the vasculature in hypoxic tissues¹⁶³. When any part of this process is impaired, the failure of wound healing may lead to chronic wounds²²⁷.

Aside from their role in maintaining tissue structure, integrins are heavily involved in angiogenesis. Within sprouting angiogenesis, the formation of the budding vessel depends on the coordination of migration, proliferation and survival of endothelial cells. These cells are also anchorage dependent, meaning their attachment to the ECM is necessary not only for their movement toward the angiogenic signal but also for their survival²³¹. Endothelial integrins participate in cross-talk with a variety of growth factor receptors and together coordinate the direction of movement during sprouting angiogenesis^{232–235}. Several integrins, typically RGD- and laminin-binding, are upregulated during endothelial cell movement during the angiogenic process¹⁶³. Dysfunction of these integrins can result in malformed or immature vascular networks^{236,237}. In addition, despite being an essential process, dysregulation of integrin-mediated angiogenesis is a hallmark of tumor growth and development^{238–240}.

Cellular adhesion is a fundamental process that supports tissue integrity and vessel stability. There is growing evidence of altered integrin expression in a variety of cancer types including melanoma, lung carcinoma, and glioblastoma^{218,238,239,241–243}. Integrins can contribute to the tumor microenvironment and cancer metastasis through crosstalk with growth factor receptors promoting cellular survival pathways^{242,244}. As the developing tumor increases its

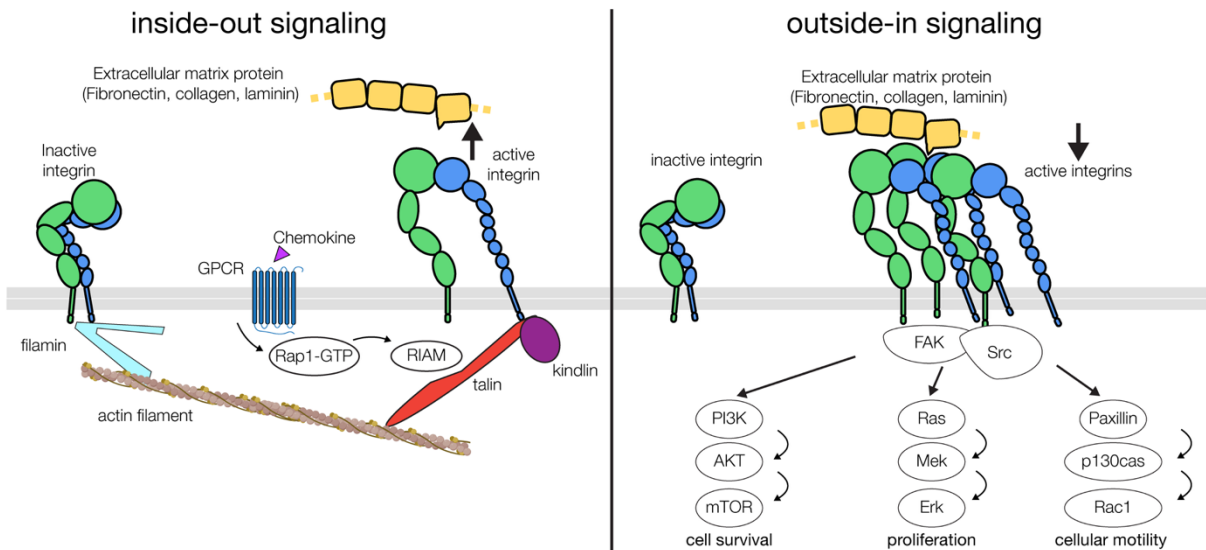


Figure 2 - 4 Integrins are bidirectional signaling receptors. Integrins can transmit signals in both directions across the plasma membrane. Integrin inside-out signaling is initiated through cytoplasmic binding partners that are stimulated by other intracellular cues such as signaling from G protein-coupled receptors (GPCRs)²⁴⁵. This pathway leads to the recruitment of talin and kindlin which induces integrin leg separation switching integrin into the more active conformation for higher ligand affinity. On the other hand, filamin competes with talin binding to the β -tail to keep the heterodimer together. During outside-in signaling, extracellular ligand binding confers a conformational change that promotes the formation of focal adhesion through focal adhesion and Src kinases leading to various downstream signaling pathways for cellular survival, proliferation and motility. In both directions, the activity of integrins is conferred by the conformational change that occurs with ligand binding.

demand for oxygen and nutrients, integrins support both cancer cell expansion and tumor angiogenesis within the microenvironment. The increase in expression of integrins promotes tumor cell survival and invasion of surrounding tissues. As tumor growth exceeds the existing blood supply, reduced oxygen supply results in hypoxic regions promoting angiogenesis^{54,237,246}. However, the tumor-associated vasculature is often malformed and disorganized, further contributing to persistent hypoxia and tumor progression²⁴⁷.

Therapeutic approaches to targeting integrins

Given their multifaceted roles in a variety of critical processes, therapeutic targeting of integrins has attracted significant interest^{167,218,240,248}. As integrins are transmembrane receptors, their extracellular ligand binding domains are positioned such that they are an accessible therapeutic target without requiring drug entry into the cell. Integrin therapeutics have taken multiple design strategies, including small molecules and peptides, antibodies and *de novo* design proteins^{168,169,249–253}.

Small molecules offer advantages in manufacturing, modification and pharmacokinetics. Several small molecules targeting integrins have been developed and tested^{167,249,254,255}. However, some of these molecules have not had consistent effects. Cilengitide is a cyclic RGD mimetic that was designed to compete with the native RGD peptide sequence of ECM ligands targeting $\alpha v\beta 3$ and $\alpha v\beta 5$ integrins²⁴⁹. Preclinical models and studies demonstrated anti-angiogenic and anti-invasive activity but ultimately failed to demonstrate an advantage compared to standard therapy^{256,257}. On the other hand, Tirofiban and Lofitegrast are approved inhibitors for thrombotic events and for dry eye disease respectively^{167,258–260}. Eptifibatide is an RGD mimetic targeting $\alpha IIb\beta 3$ approved for acute coronary syndrome. However a small group of patients have reported severe thrombocytopenia^{248,254,261,262}.

While small molecules are attractive in their tunability and kinetics, antibody-based therapeutics provide high specificity and affinity. In addition they can also complement the immune system targeting through their Fragment crystallizable (Fc) region, to increase effectiveness²⁶³. Successes within this strategy has yielded Natalizumab, which is used for treatment of multiple sclerosis and Crohn's disease^{164,264,265}. Natalizumab is a humanized IgG4 that recognizes the $\alpha 4$ subunit and allosterically inhibits ligand binding at the α/β cleft. Structural studies show that Natalizumab binds at the $\alpha 4$ β -propeller and induces no conformational changes in $\alpha 4$ ²⁶⁵. Vedolizumab, which targets integrin $\alpha 4\beta 7$'s specificity determining loop, has been approved for treatment of inflammatory bowel disease^{266–268}. As with small molecules, not all antibody-based therapeutics have successfully translated to clinical approval. Volociximab is a chimeric monoclonal antibody targeting integrin $\alpha 5\beta 1$ ²⁶⁹. Despite promising preclinical outcomes and safety profiles, there was no improvement in phase II trials^{270–273}.

More recently, *de novo* design proteins are becoming more accessible and have shown promising application in a variety of diseases and conditions^{169,250,274,275}. These engineered proteins have improved binding specificity, stability and offer control of integrin conformation states^{169,250}. Because these are designed in silico, these molecules can be tuned in specificity, chemical properties, size and function. An additional feature of being computationally designed is that screening candidate molecules can be rapidly performed using structural prediction models such as Alphafold²⁷⁶.

2.2 Motivation and rationale

Integrins remain a promising therapeutic target for a variety of diseases and conditions. However, the development of integrin-targeted therapeutics has not been overwhelmingly successful. This may be due in part to the fact that our understanding of integrin conformation in relation to therapeutic binding and effects is not complete. The conformational shifts that $\alpha v\beta 3$ and $\alpha IIb\beta 3$ induced by their inhibitors are known¹⁶⁴. It remains unclear whether this applies to other members of the RGD-binding integrins and their inhibitors or activators. Crystallography provided substantial structural information regarding integrin structure. However, our structural interpretation of these integrin conformations may be confounded due to potential crystal artifacts²⁷⁷.

To this end, integrin $\alpha 5\beta 1$ was selected for study as this heterodimer is associated with angiogenesis and wound healing^{84,121,163,226}. It is also upregulated in a wide variety of cancers^{238,278}. To explore the consequences of therapeutic binding, two integrin $\alpha 5\beta 1$ -targeting antibodies, BIIG2 and MINT1526A, were characterized using cell-based assays and biophysics. MINT1526A is an early integrin $\alpha 5$ therapeutic developed by Genentech that was later dropped due to lack of efficacy²⁷⁹. BIIG2 was developed as a functional inhibitor blocking fibronectin attachment to integrin $\alpha 5\beta 1$ ^{280,281}. To better understand how integrin conformation and how it influences therapeutic binding and vice versa, a comparative study between BIIG2 and MINT1526A was performed using biophysical and cell-based assays.

Current materials for tissue regeneration do not support sufficient cell attachment for optimal tissue integration. Various RGD peptides and mimetics can support attachment, but they are limited in their specificity. De novo protein design allows for the creation of highly specific proteins for a target to overcome traditional limitations of drug designs. To leverage these computational tools, an integrin $\alpha 5\beta 1$ -specific minibinder was developed for tissue regeneration and wound healing by activating the receptor. This study demonstrated the potential of computationally designed proteins not only for regenerative medicine and wound healing but for integrin-targeted therapeutics.

2.3 Structural characterization of integrin $\alpha 5$ targeting antibodies

At the time of writing, this manuscript has been accepted to *Structure* as: “Shared ligand-blocking mechanism but distinct conformational modulation by $\alpha 5$ -targeting antibodies BIIG2 and MINT1526A”.

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Author Contributions:

AN, JBH, MCC, AM and MGC designed the research. CC and ML expressed and purified BIIG2 antibody and prepared BIIG2 and MINT1526A Fab fragments. CC and BM prepared, performed and analyzed mass spectrometry data. AN analyzed the spheroid and tube formation assays. AN, JBH and AM analyzed the tumor xenograft data. JBH performed and analyzed the quantitative PCR experiments. JBH and AM performed and analyzed the immunohistochemistry data. JBH, GC, HJ and UK prepared, collected and analyzed x-ray crystallography data. AN designed, cloned, expressed and purified recombinant $\alpha 5\beta 1$. AN performed and analyzed BLI assays. AN and MGC prepared samples, collected and processed negative stain EM data. AN, CMA and MGC prepared samples and collected cryoEM data. AN and MGC processed the cryoEM data. AN built the cryoEM models. MCC designed, performed and analyzed the molecular dynamics simulations. AN, JBH, AM and MGC wrote the original draft of the manuscript. All authors contributed to reviewing and editing the manuscript.

Summary

Integrins are heterodimeric receptors important for cell adhesion and signaling. Integrin $\alpha 5 \beta 1$ is a key mediator of angiogenesis and its dysregulation is associated with tumor progression and metastasis. Despite numerous efforts, $\alpha 5 \beta 1$ -targeting therapeutics have been unsuccessful due to poor efficacy and off-target effects. A contributing factor is our limited understanding of how integrin conformation influences interactions with therapeutics. Using cell-based functional assays, patient-derived xenografts, biophysics, and electron microscopy, we shed light on these relationships by characterizing two anti- $\alpha 5 \beta 1$ antibodies, BIIG2 and MINT1526A. We show that both antibodies bind $\alpha 5 \beta 1$ with nanomolar affinity, reduce angiogenesis in vitro, and bind overlapping epitopes that block fibronectin binding. However, using electron microscopy, we reveal that while BIIG2 binding does not substantially alter the conformational states, MINT1526A preferentially recognizes the bent conformation and restricts the conformational ensemble. These insights can guide which aspects to prioritize to improve the design of future integrin-targeted therapeutics.

Introduction

Integrin heterodimers, which consist of an α subunit and a β subunit, are type I transmembrane receptors that play vital roles in immune response, cell motility, cell-matrix interactions, signaling, proliferation, and apoptosis^{189,190,214} (**Figure 2 - 2**). Pathologies linked to integrins include autoimmune, cardiac, pulmonary, neurodegenerative, hematologic, and oncologic conditions^{121,240}. Integrins have long been identified as promising therapeutic targets. Although numerous integrin-targeting therapeutics have entered clinical trials, fewer than a dozen have been approved and marketed, while others have been withdrawn due to limited efficacy and adverse effects^{167,218}.

Integrin $\alpha 5 \beta 1$ plays a key role in facilitating cell migration during angiogenesis and is one of several integrins that recognize the conserved arginine-glycine-aspartate (RGD) sequence motif found in several extracellular matrix (ECM) proteins (**Figure 2 - 1**). The primary $\alpha 5 \beta 1$ ligand, fibronectin (FN), is a glycoprotein that scaffolds other matrix proteins and cells. $\alpha 5 \beta 1$ participates in tumor angiogenesis, among other pathologically significant processes related to human diseases^{163,238,282}. Like many other well-studied integrins, $\alpha 5 \beta 1$ adopts three conformations which can be directly linked to $\alpha 5 \beta 1$ activation: bent, extended closed, and

extended open (**Figure 2 - 3**). The extended open conformation is stabilized by FN via two interactions: the canonical integrin binding RGD motif and an additional synergy site. Specifically, a fibronectin domain (type III domain 10, FN10) contains an RGD motif that inserts into the α and β subunit interface^{207,283} and a second domain, FN9 contains the synergy site that facilitates a catch-bond interaction with the β -propeller of the $\alpha 5$ subunit^{172,207,223,284,285}.

The increased expression of integrins $\alpha 5\beta 1$, $\alpha v\beta 3$, and $\alpha v\beta 5$ in new blood vessels during the angiogenic switch in tumors underscores their significance as potential targets for therapeutic intervention^{240,241,286,287}. Early-phase clinical trials involving patients with advanced solid tumors investigated multiple monoclonal antibodies targeting $\alpha 5\beta 1$ function, however, these trials did not demonstrate clear efficacy^{271,279,288}. Among the tested antibodies was MINT1526A (RG-7594), a fully humanized IgG1 antibody specifically formulated to block $\alpha 5\beta 1$ function²⁷⁹. A different $\alpha 5\beta 1$ -inhibiting antibody, BIIG2, was developed to inhibit human JAR choriocarcinoma cells binding to ECM proteins by immunization of Lewis rats²⁸¹. Follow-up studies showed that BIIG2 was effective in both inhibiting fibronectin attachment and identifying invasive cytotrophoblasts within the human placenta^{281,289}. To better understand how integrin conformation influences therapeutic interactions, we sought to characterize BIIG2's functional and mechanistic properties. We compare it to MINT1526A to highlight differences that could contribute to antibody effectiveness, since MINT1526A was well tolerated in patients during phase I clinical trials but was shown to have limited advantages over existing therapies²⁷⁹. Therefore, we evaluated BIIG2 and MINT1526A using structural, biophysical, and cell-based methods to define the mechanisms underlying antibody-mediated $\alpha 5\beta 1$ inhibition. We found that BIIG2 shows more favorable inhibitory outcomes in vitro when compared to MINT1526A, and the two antibodies elicit different effects on conformations accessible to $\alpha 5\beta 1$. Since integrin conformation has been shown to influence the severity of off-target effects^{167,218}, our results provide the molecular basis for the design of future integrin-targeted therapeutics.

BIIG2 and MINT1526A antibodies reduce angiogenesis in vitro

Angiogenesis is the formation of new blood vessels and has been described extensively as critical for tumor growth^{89,163}. To evaluate whether BIIG2 or MINT1526A can inhibit angiogenesis in an integrin $\alpha 5\beta 1$ -dependent manner, we employed a two-dimensional tube formation assay by cultivating human umbilical vein endothelial cells (HUVECs) on Matrigel

and inducing tube formation with vascular endothelial growth factor (VEGF). HUVECs in the basal state where no VEGF is added exhibit limited angiogenesis, forming short connections and minimal junctions (**Figure 2 - 5**). The addition of VEGF (15 ng/mL) dramatically increases the sprouting length and number of junctions creating an extensive capillary-like network after 24 hours at 37°C. Treatment with 1 nM of BIIG2 or MINT1526A was unable to disrupt the VEGF-stimulated tube formation as the segment lengths and the number of tube junctions are minimally

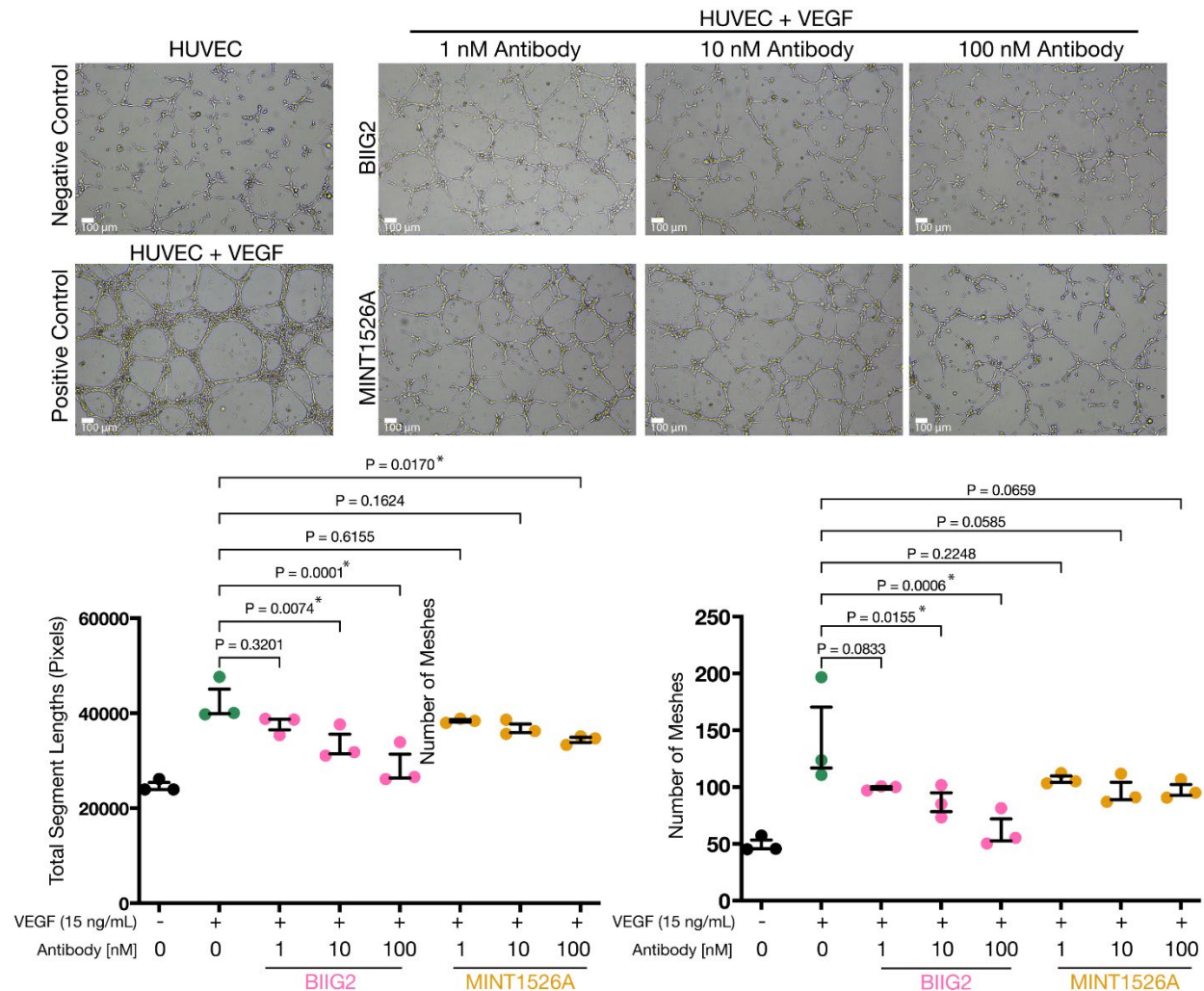


Figure 2 - 5 Effects of BIIG2 and MINT1526A $\alpha 5$ integrin antibodies on angiogenesis in vitro. Representative images of HUVEC tube formation by VEGF on Matrigel after 24 hours of incubation in the presence or absence of antibodies. Scale bar is 100 μ m. HUVEC cultured in medium alone served as the basal control, whereas HUVEC treated with VEGF (15 ng/mL) were used as the positive control. The quantification of tube formation involved measuring the total length of master segments (the cumulative length of tubes formed, measured in pixels) and the number of meshes using angiogenesis analyzer for ImageJ²⁹¹. Master segments and mesh counts were averaged from three fields of view per replicate, n=3 (9 total images, 3 images per replicate). Data are shown as the mean $\pm$ SEM; n=3. One-way ANOVA with Bonferroni correction; adjusted P values are shown.

decreased (**Figure 2 - 5**). BIIG2 at 10 nM and 100 nM reduced both the total segment lengths and the number of junctions whereas MINT1526A only reduced segment lengths at 100 nM. MINT1526A showed a trend towards reducing the number of tube junctions but did not reach statistical significance ($P < 0.05$). Of the concentrations tested, BIIG2 reduces both branching and extension capabilities of HUVECs in a dose-dependent manner. In contrast, MINT1526A only disrupts extension and branching is maintained. Based on these results, we sought to further investigate the inhibitory effects of angiogenesis in three dimensions using HUVEC spheroids (**Figure 2 - S 1**). HUVECs were embedded into a collagen gel, and sprouting was assessed 24 hours after being treated with 25 ng/mL VEGF. Sunitinib, a tyrosine kinase inhibitor known to inhibit angiogenesis²⁹⁰, was used as a positive control and reduced VEGF-induced HUVEC sprouting in a dose-dependent manner. Unlike the two-dimensional tube formation assay, BIIG2 and MINT1526A did not noticeably reduce spheroid sprouting.

BIIG2 localizes to connective tissue-resident fibroblasts and melanoma cells

Integrin $\alpha 5 \beta 1$ is expressed in various tissues at low levels²⁸⁷. We used immunohistochemistry to determine the cellular localization and tissue specificity of BIIG2 and compared it with the polyclonal $\alpha 5$ integrin antibody PA5-82027. As expected, PA5-82027 stains a broad spectrum of human tissues, including placental syncytiotrophoblasts, striated ducts in salivary glands, and alveolar ducts (**Figure 2 - S 2A** and **Table 2 - S 1**). In contrast, BIIG2 exhibits a restricted staining profile, predominantly marking isolated connective tissue-resident fibroblasts across various tissues. Remarkably, BIIG2 also demonstrated intense staining of activated and transformed melanocytes in the epidermis and upper dermis derived from patients with atypical nevi and invasive cutaneous melanoma (**Figure 2 - S 2B**). Tissue specificity in therapeutics is advantageous since it has the potential to reduce off-target effects and BIIG2's specificity supports the targeted treatment of various pathologies, including fibrosis, sclerosis, and melanoma.

BIIG2 reduces tumor volume in a patient-derived xenograft model

Based on the strong BIIG2 immunohistochemical staining localized to human melanoma cells (**Figure 2 - S 2B**), and considering the known tumor-inhibitory effects of other anti- $\alpha 5$ antibodies in rhabdomyosarcoma and glioblastoma mouse models^{292,293}, we established two melanoma xenograft cultures to evaluate the in vivo inhibitory effects of BIIG2 on melanoma

growth. Using brain metastases from patients who had different responses to treatment, we derived two cultures, M15 and M12 (**Figure 2 - S 3A-B**). M15 cells contained a NRAS^{Q61} mutation and exhibited metastatic characteristics and resistance to radiation and systemic therapy. M12 cells contain a BRAF^{V600} mutation and responded well to systemic therapy with the donor patient achieving complete remission ²⁹⁴. Quantitative PCR of M15 and M12 shows differing levels of melanoma markers compared to normal human melanocytes (NHM) such as melan-A (MLANA) and microphthalmia-associated transcription factor (MITF), CDKN2A tumor suppressor as previously described ²⁹⁵⁻²⁹⁷. M15 cells have a higher mRNA expression of extracellular matrix proteins such as fibronectin (FN1) and osteopontin (SPP1), but lower for tenascin C (TNC) compared to M12 (**Figure 2 - S 3C**). Furthermore, cell surface integrin flow cytometry showed that compared to NHM, αv and $\alpha 5$ are upregulated in M15 and M12 cells, with the highest $\alpha 5$ expression in M15 cells agreeing with previous reports (**Figure 2 - S 3D**) ^{286,298}.

M15 and M12 cells were embedded in Matrigel to grow and subsequently injected into the flanks of athymic mice to determine the effect on tumor volumes in vivo (**Figure 2 - 6A**). When tumors became palpable, antibodies were administered intraperitoneally at a dosage of 10 mg/kg twice weekly. Treatments were stopped when the tumors injected with control IgG surpassed an average volume of 1000 mm³. The panel of antibodies tested targeted both $\alpha 5$ and αv integrins as these were seen to be upregulated using flow cytometry (**Figure 2 - S 3D**). P1D6 is an anti- $\alpha 5$ antibody that also inhibits human cell adhesion to fibronectin ²⁹⁹. P1F6, P3G8 and P5H9 targets integrin $\alpha v \beta 5$ blocking cellular binding to vitronectin ³⁰⁰⁻³⁰². In the less aggressive M12 xenograft, BIIG2 demonstrates a measurable anti-tumor effect (**Figure 2 - 6B**). However, it is not more effective than the P1D6 antibody or antibodies targeting integrin αv (**Figure 2 - S 4A**). Expression analysis revealed that in BIIG2-treated mice, the xenografted M12 tumors exhibited an increase in RNA levels of the melanocyte differentiation marker MLANA and p53 by 50% and 32% respectively, in comparison to those treated with control IgG (n = 9 mice per group; T-test p < 0.01; **Figure 2 - S 4B**). In the more aggressive M15 tumor challenge model, no antibodies tested demonstrated a statistically significant impact on tumor growth. In some replicates, BIIG2 demonstrated clear inhibitory effect, however the overall mean did not reach statistical significance. Pentamidine isethionate (Pentam) has been shown to restore free p53 levels in melanoma cells, inhibiting tumor growth and combination targeted therapies have

shown improvement and promise for melanoma treatment^{303,304,305}. We found this was the case for M15 treatment where neither Pentam nor BIIG2 alone reduced tumor volume, however, when combined, a significant reduction of tumor volume was achieved (**Figure 2 - 6C**). In M12, Pentam and BIIG2 were both effective in reducing tumor volumes as monotherapies and their combination demonstrated efficacy but was not more effective. The clinically tested MINT1526A could not be used for comparison due to a shortage of purified antibody, which was kindly provided by Genentech.

BIIG2 and MINT1526A bind $\alpha 5\beta 1$ with sub-nanomolar affinity independent of cation condition

Having established the potential of BIIG2 in angiogenesis and melanoma tumor growth inhibition, we characterized the affinities of BIIG2 and MINT1526A to $\alpha 5\beta 1$, a critical consideration for effective therapeutics³⁰⁶. Integrin function, signaling, and its ability to bind ligands are linked to its conformation and can be influenced *in vitro* using divalent cations^{189,307}. In $\beta 1$ integrins, calcium (Ca^{2+}) is known to promote a bent conformation which is linked to inactive integrins, while manganese (Mn^{2+}) favors an open conformation, which is linked to integrin activity^{208,308,309}. Using recombinant human $\alpha 5\beta 1$ in the presence of 5 mM Ca^{2+} or 1 mM Mn^{2+} , we employed biolayer interferometry (BLI) to determine if the conformation of $\alpha 5\beta 1$ impacts the ability of BIIG2 or MINT1526A to bind. We find both antibodies bind $\alpha 5\beta 1$ in the low nanomolar range irrespective of cations: BIIG2 binds to $\alpha 5\beta 1$ with an affinity of 0.57 nM in Ca^{2+} and 0.64 nM in Mn^{2+} ; MINT1526A binds $\alpha 5\beta 1$ with an affinity of 1.3 nM in Ca^{2+} and 0.23 nM in Mn^{2+} , thus demonstrating that regardless of cation condition, both antibodies bind (**Figure 2 - 7**). Despite these similarities, the antibodies have distinct profiles: in both conditions, BIIG2 associates (K_{on}) and dissociates (K_{off}) slightly more slowly than MINT1526A, the slower K_{off} suggesting better stability. Although for BIIG2, K_D is similar in both cation conditions, for MINT1526A, there is a marked increase in affinity in the presence of Mn^{2+} vs. Ca^{2+} , suggesting Mn^{2+} stabilizes binding (**Figure 2 - 7**).

We hypothesized that the antibody-mediated reduction in angiogenesis is due to steric inhibition of $\alpha 5\beta 1$ binding to fibronectin. To test this hypothesis, we conducted a series of competition experiments using BLI to determine if BIIG2 and MINT1526A prevented $\alpha 5\beta 1$ from

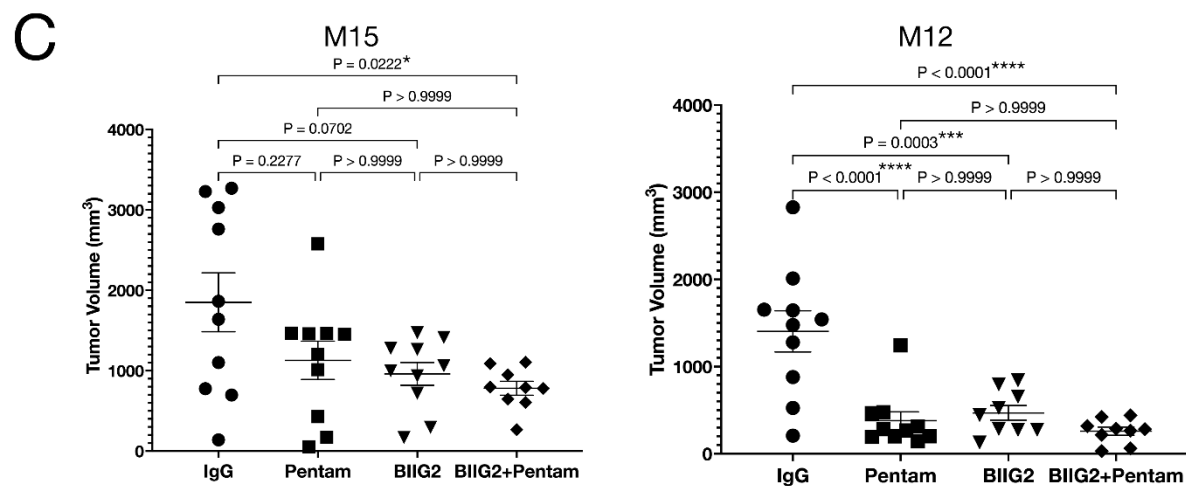
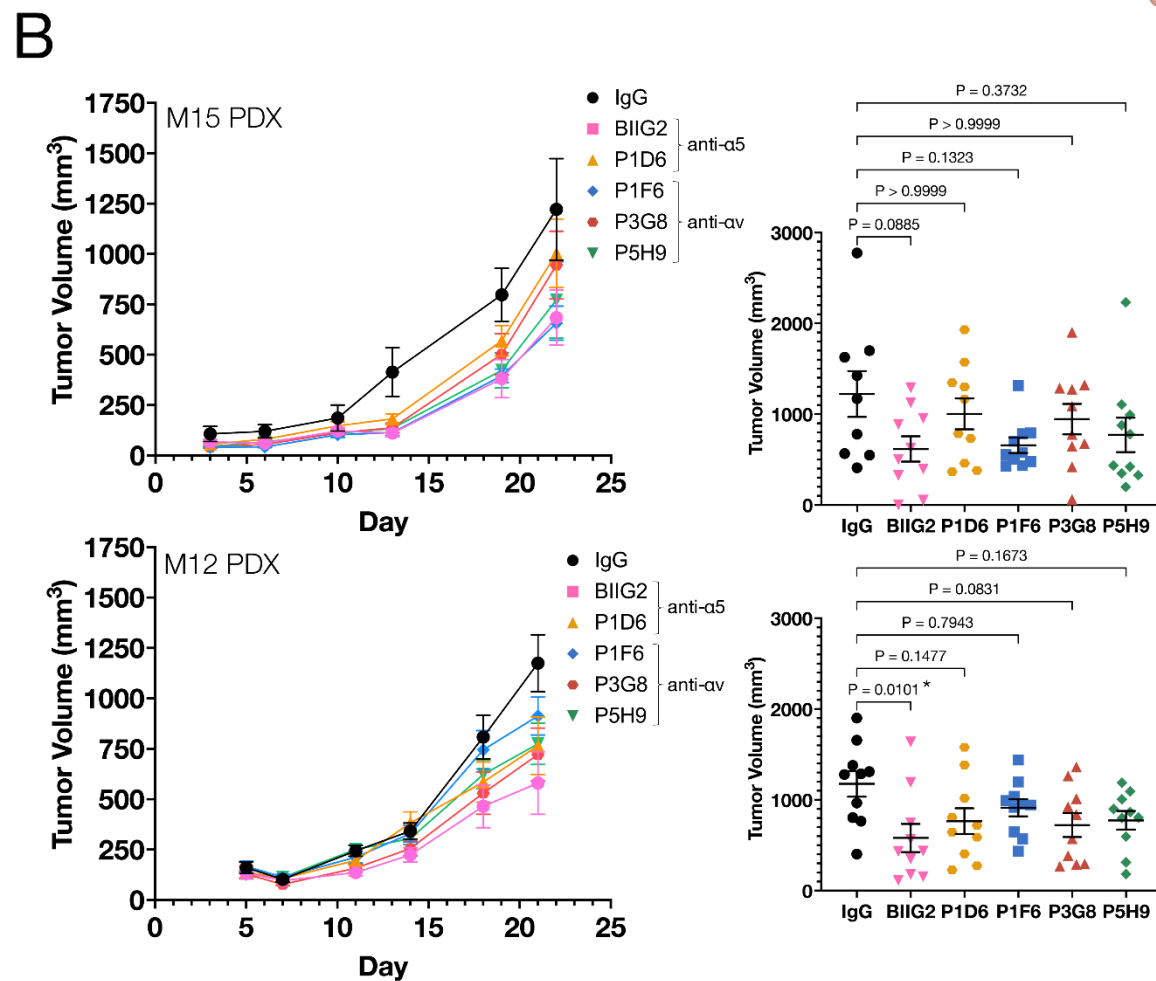
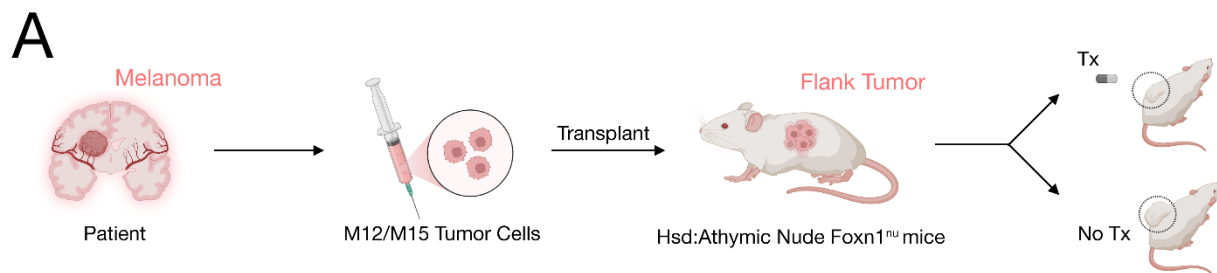


Figure 2 - 6 In vivo anti-tumor activity of BIIG2. (A) Athymic nude mice were xenografted with patient-derived melanoma cells (labeled M15 and M12) from brain metastasis. Upon observation of palpable tumors, we proceeded with the treatment (Tx) of the mice. Illustration created with BioRender.com. (B) Mice were injected intraperitoneally with BIIG2 or other αv and $\alpha 5$ integrin antibodies. Treatment was administered twice weekly at a dose of 10 mg/kg. Data plotted are shown as means $\pm$ SEM; n=10 per day of measurement. Tumor volumes on the final day of observation were plotted as mean $\pm$ SEM; n=10, except for M15 where IgG control n=9. A one-way ANOVA with Bonferroni correction was performed for statistical significance of the complete antibody panel against the control IgG, adjusted P values are shown. Comparisons between BIIG2 and the panel of antibodies were analyzed in the same method in **Figure 2 - S 4**. (C) Pentamidine isethionate, an aromatic diamidine with anti-cancer activity, was administered intraperitoneally at a dose of 10 mg/kg once daily. Data shown are mean $\pm$ SEM; n=10 mice per treatment group; one-way ANOVA with Bonferroni correction; adjusted P values are shown. The clinically tested MINT1526A could not be used for comparison due to a shortage of purified antibody, which was kindly provided by Genentech.

binding to fibronectin and if fibronectin could prevent BIIG2 or MINT1526A binding (**Figure 2 - S 5**). We used a recombinant truncated fibronectin fragment (FN7-10) that contains the necessary domains for $\alpha 5 \beta 1$ binding^{175,207,285} and consistent with prior studies, we show that $\alpha 5 \beta 1$ binds FN7-10 with a high affinity in the presence of Mn^{2+} , and Ca^{2+} significantly reduces the affinity and binding signal (**Figure 2 - S 5A and H**)^{207,208}. In the presence of Mn^{2+} , we find that if $\alpha 5 \beta 1$ is preincubated with either BIIG2 or MINT1526A for 30 minutes at room temperature, there is a marked decrease in binding signal to immobilized FN7-10 (**Figure 2 - S 5B and C**). Likewise, if $\alpha 5 \beta 1$ is preincubated FN7-10, both antibodies do not show binding, suggesting that FN7-10 cannot bind at the same epitope as BIIG2 or MINT1526A (**Figure 2 - S 5E and G**). There may still be association as protein complexes is in equilibrium with the individual protein components. Therefore, free integrin $\alpha 5 \beta 1$ may be available for binding to immobilized BIIG2 (**Figure 2 - S 5E**). When alone, FN7-10 binds $\alpha 5 \beta 1$ with an affinity of 2.2 nM, but if $\alpha 5 \beta 1$ has been preincubated with BIIG2 FN7-10 binds with 9.2 nM affinity and if preincubated with MINT1526A the affinity of FN7-10 is even lower at 13.8 nM (**Figure 2 - S 5A-C**). Likewise, after preincubating integrin with FN7-10, subsequent antibody binding was drastically reduced: immobilized BIIG2 bound $\alpha 5 \beta 1$ with 0.689 nM affinity, but if $\alpha 5 \beta 1$ was preincubated with FN7-10, this dropped to 14.6 nM (**Figure 2 - S 5D and E**). The effect on MINT1526A was most drastic, dropping from 0.18 nM to 25.1 nM (**Figure 2 - S 5F and G**). This suggests that the antibodies, although capable of binding $\alpha 5 \beta 1$ with extremely high affinities, occupy the same epitope on integrin $\alpha 5 \beta 1$ as FN7-10. Taken together, this suggests that the fibronectin, BIIG2 and MINT1526A share a similar or overlapping binding interface. As anticipated, $\alpha 5 \beta 1$ does not bind FN7-10 with high affinity in high Ca^{2+} condition and thus we show that in high Ca^{2+} , BIIG2 and MINT1526A bind integrin with only slightly lower affinity than if FN7-10 is not present (**Figure 2 - S 5H-N**).

MINT1526A preferentially binds compact $\alpha 5\beta 1$; BIIG2 binds multiple conformations

To visualize epitopes and the impact of antibody binding on $\alpha 5\beta 1$'s conformation, we used single-particle negative stain electron microscopy (nsEM) to visualize the protein complexes of purified $\alpha 5\beta 1$ ectodomains bound to BIIG2 or MINT1526A Fabs. We generated 2D class averages that summed thousands of individual particle images (**Figure 2 - S 6A**). As previously hypothesized, our nsEM analysis confirmed that both BIIG2 and MINT1526A bind the $\alpha 5$ subunit and we further localized the epitope to the $\alpha 5$ β -propeller domain^{279,281}. Fibronectin binds the same domain, which further supports the hypothesis that both antibodies inhibit $\alpha 5\beta 1$ function by blocking fibronectin binding through steric hindrance²⁰⁷.

In nonactivating conditions (5 mM Ca^{2+}), most (98%) $\alpha 5\beta 1$ adopts two conformations: 'bent' and 'extended closed' (**Figure 2 - S 6A**), consistent with previous work²⁰⁸. In activating conditions (1 mM Mn^{2+}), we observed an increase in the proportion of $\alpha 5\beta 1$ particles adopting the 'extended open' conformation (24% vs 2%). When $\alpha 5\beta 1$ is bound to the BIIG2 Fab, the ratios of integrins in the three different conformations are similar in both cation conditions (**Figure 2 - S 6A**). In contrast, when MINT1526A binds, we only observe the bent conformation in both activating and nonactivating conditions (**Figure 2 - 7** and **Figure 2 - S 6A**). We considered two possibilities: either MINT1526A binds any integrin conformation and then "locks" it into a bent conformation or it binds only the bent conformation (which is present in both cation conditions due to the dynamic equilibrium) and then locks it. To address this point, we incubated $\alpha 5\beta 1$ with the antibody 9EG7 to induce an extended conformation and then added MINT1526A (**Figure 2 - S 6B**)³¹⁰. The resulting nsEM did not yield any 2D class averages with both 9EG7 and MINT1526A bound, suggesting MINT1526A has a strong preference for the bent conformation. In contrast, when we conducted a similar experiment using BIIG2 we found approximately 33% of particles had both 9EG7 and BIIG2 bound (**Figure 2 - S 6B**).

In summary, although both antibodies inhibit $\alpha 5\beta 1$ function by blocking fibronectin binding through steric hindrance, MINT1526A has an additional conformational effect, restricting the observed conformational equilibrium and preventing integrin extension and headpiece opening.

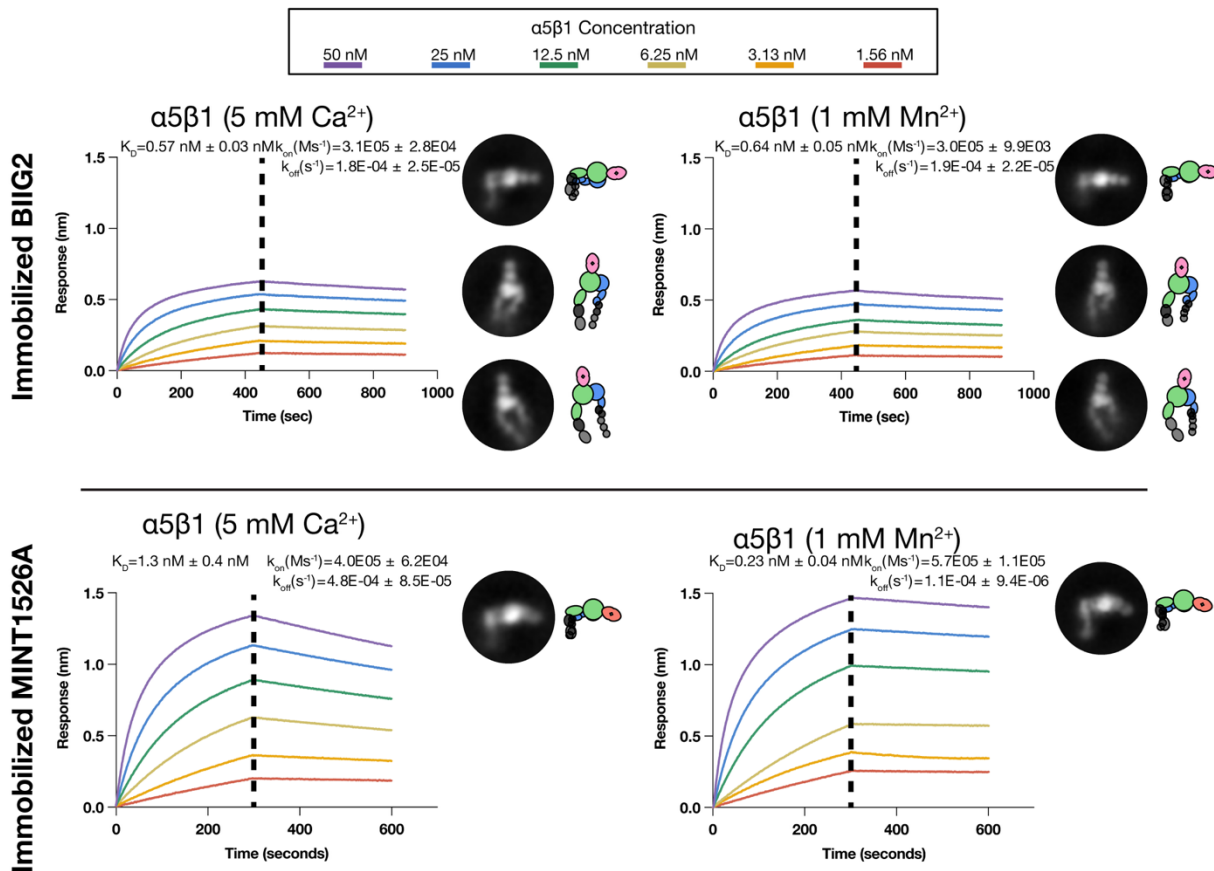


Figure 2 - 7 Biolayer interferometry binding kinetic analysis and negative stain electron microscopy of integrin-Fab complexes. Representative BLI sensorgrams displaying the association and dissociation step of the binding assay between immobilized BIIG2 and MINT1526A and α5β1 integrin ectodomain in the presence of Ca²⁺ (nonactivating) and Mn²⁺ (activating). BIIG2 binds α5β1 ectodomain with a K_D of 0.57 nM and 0.64 nM in nonactivating conditions and activating conditions respectively. MINT1526A binds α5β1 ectodomain with a K_D of 1.3 nM and 0.23 nM in nonactivating conditions and activating conditions, respectively. Each experimental condition was repeated three times with the K_D , k_{on} (1/Ms) and k_{off} (1/s) reported as the mean $\pm$ SEM. Representative negative stain EM 2D class averages of the α5β1 integrin in complex with the antibody Fab fragment in their respective buffer conditions. The complete set of 2D class averages are in **Figure 2 - S 6A**.

High-resolution structural analysis of the BIIG2 Fab reveals occlusion of the integrin binding cleft

We determined the structure of the BIIG2 Fab to 1.4 Å resolution using X-ray crystallography (PDB: 8R38; **Figure 2 - 8F** and **Figure 2 - S 7K-M**). The structure was refined to R/R_{free} values of 19.9/22.4% with good geometry (**Table 2 - S 2**) and is characterized by well-defined electron density from residues 1-210 and 1-214 (Kabat numbering) of the heavy and

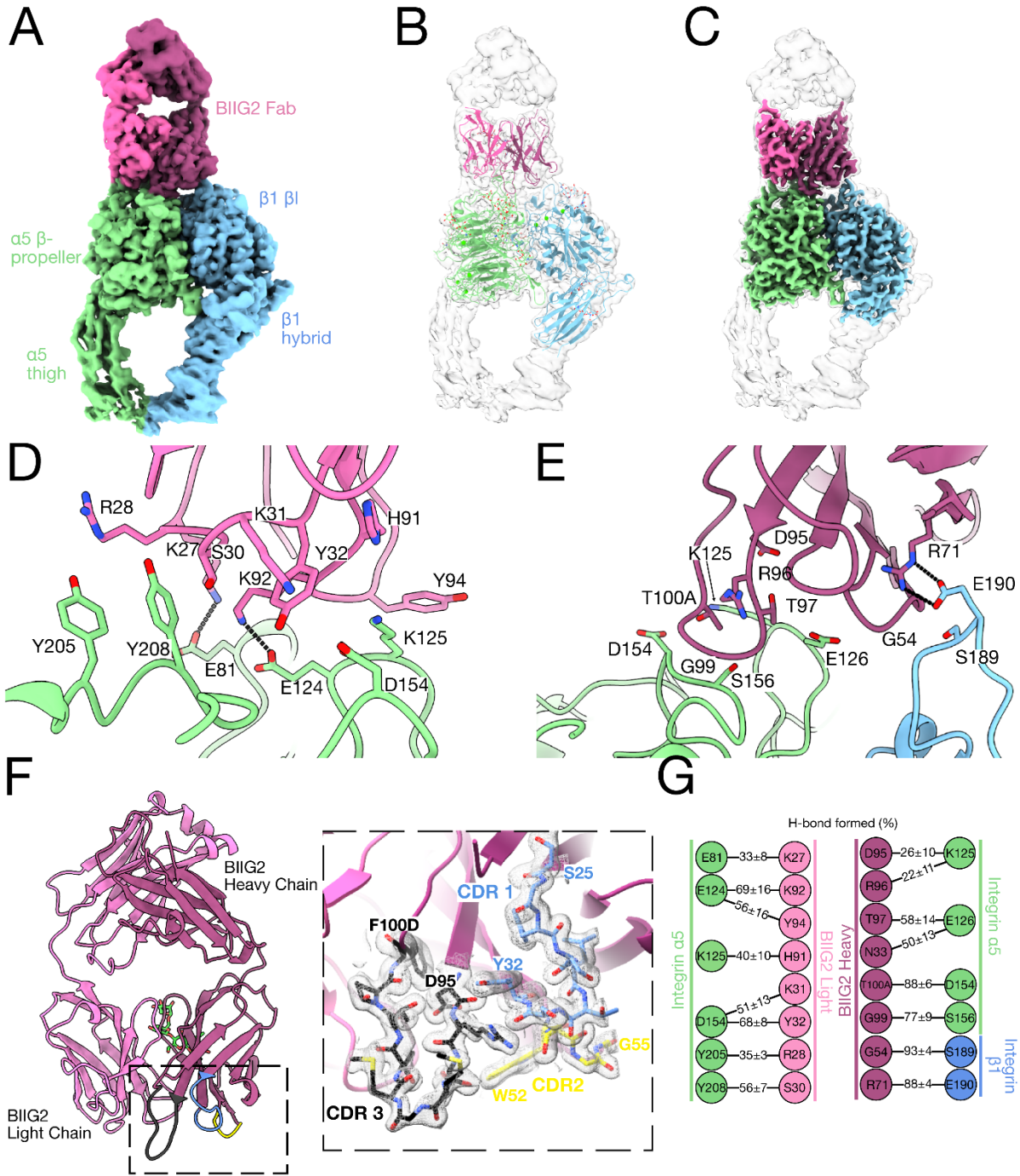


Figure 2 - 8 CryoEM analysis of integrin $\alpha 5 \beta 1$:BIIG2 Fab complex. (A-C) CryoEM maps and model of the $\alpha 5 \beta 1$ ectodomain with BIIG2 Fab bound. The color code is as follows: integrin $\alpha 5$ subunit is green, integrin $\beta 1$ subunit is blue, BIIG2 Fab light chain is light pink and heavy chain is dark pink. (A) The unsharpened map at a low threshold. (B) A ribbon diagram (colors) in the cryoEM map (semi-transparent white). (C) A higher threshold sharpened cryoEM map (colors), superimposed with the unsharpened map (semi-transparent white). (D and E) A close-up view of the interface between $\alpha 5 \beta 1$ and the light chain (D) and heavy chain (E) of the BIIG2. Salt bridges are drawn with dashed lines. (F) The crystal structure of BIIG2 Fab (light chain in light pink, heavy chain in dark pink). The single N-linked

glycan N60 is shown as green stick representation. A close-up view of the Fab crystal structure shown in cartoon representation (dashed box), with σ_A -weighted $2F_o - F_c$ electron density map (grey mesh, contoured at 1.0σ) for selected residues of the CDR loops (CDR1-yellow, CDR2-blue, and CDR3-gray), which are depicted in stick representation; amino acid residues are labeled. **(G)** Percentage of hydrogen bonds and salt bridges formed (mean $\pm$ SEM) between integrin $\alpha 5 \beta 1$ and BIIG2 Fab molecular dynamics simulations using a 3.5 Å cutoff between acceptor and donor-H atoms. Values were averaged across seven simulation repeats, each 2.0 μ s long. Stable interactions are summarized in **Table 2 - S 4**.

light chains, respectively. In particular, the antigen-binding site is well-defined. The binding site of the BIIG2 light chain is dominated by positive charges from three lysine residues (K27, K31, and K92) and R28, whereas the heavy chain CDRs are charge-balanced. The BIIG2 CDR H3 loop is exposed, notably longer than the other CDRs and partially involved in crystal contacts through residues T97, M98, G99, and CDR L1 loop R27 (**Figure 2 - 8F**). The obtained high resolution allowed for accurate modeling of several modified residues. These include a pyroglutamate on the N-terminal position of the heavy chain, a methyllysine on the light chain (L199), and a 5-hydroxyproline modification in the CDR H3 loop (Pro 100C). In addition, we observed well-defined electron density for the only visible glycosylation on BIIG2, located on glycan N60 of the Fv heavy chain.

To map how BIIG2 binds to and inhibits $\alpha 5 \beta 1$ function, we used single-particle cryogenic electron microscopy (cryoEM) to determine the structure of the $\alpha 5 \beta 1$:BIIG2 Fab complex to a global resolution of 2.7 Å (**Figure 2 - 8A** and **Table 2 - S 3**). After focused refinement, the $\alpha 5 \beta 1$ headpiece/BIIG2 Fab variable-region interface was estimated at 2.5 Å resolution, which allows for modeling (**Figure 2 - 8B-C**). The crystal structure of the BIIG2 Fab (PDB: 8R38; this work) and the close $\alpha 5 \beta 1$ closed headpiece structure (PDB: 3VI3; ³¹¹) were docked (rigid body fitting) into our cryoEM density as starting coordinates for model building. From here, we built the $\alpha 5$ β -propeller, the $\beta 1$ β -I domain and a portion of the hybrid domain, and the variable region of the BIIG2 Fab. As with many cryoEM maps of integrins ^{207,250,252,312–315}, our map is anisotropic, with the resolution tapering off away from the well-resolved headpiece (**Figure 2 - 8A and C**). Secondary structural elements for the $\alpha 5$ thigh and $\beta 1$ hybrid domains are visible, but the resolution is insufficient for residue-level modeling. Additional 3D classification reveals populations of BIIG2-bound integrin in the bent (25%) and extended closed conformations (18%). We note that a significant number of particles (57%) contribute to classes that do not have a strong density for the lower leg region (**Figure 2 - S 10B**). We hypothesize that the lower legs could be averaged out due to significant conformational variability or interactions with the air-

water interface that compromise the particle integrity and note that all domains are visible in nsEM averages and the molecular weight estimate from size exclusion chromatography (SEC) is consistent with a full $\alpha 5\beta 1$ ectodomain (**Figure 2 - S 7** and **Figure 2 - S 9**).

The cryoEM map reveals that BIIG2 engages both the $\alpha 5$ and $\beta 1$ integrin subunits through electrostatic interactions, with positively charged residues on BIIG2 and key negatively charged residues on the integrin. Unlike fibronectin, BIIG2 does not contain an RGD motif, nor does it bind to any RGD-interacting residues on $\alpha 5\beta 1$. Two lysine residues on the BIIG2 light chain (K27 and K92) form salt bridges with negatively charged $\alpha 5$ residues E81 and E124 (**Figure 2 - 8D**). This portion of the epitope on the $\alpha 5$ β -propeller is further stabilized by surrounding hydrogen bonds. BIIG2 engages several residues that are necessary for efficient FN binding: D154, a key residue stabilizing the interaction between $\alpha 5$ and the FN9 synergy site, interacts with T100A of the BIIG2 heavy chain, thus occluding FN9 access to this residue (**Figure 2 - 8E**)^{207,311,316}. Additionally, R71 of the BIIG2 heavy chain forms a salt bridge with E190 of the Specificity-Determining Loop 2 (SDL2) of the $\beta 1$ subunit, providing additional stabilizing interactions in the $\alpha 5\beta 1$:BIIG2 complex (**Figure 2 - 8E**). In contrast, the SDL2 does not contact with fibronectin²⁰⁷. BIIG2's CDR H3 methionine residues M98 and M100 exhibit long-range sulfur-aromatic interactions with $\alpha 5$ W157 and F155 respectively, further stabilizing the binding interface (**Figure 2 - S 11D**)³¹⁷. To further support these observations, we employed all-atom molecular dynamics (MD) simulations of the $\alpha 5\beta 1$ headpiece complex with the BIIG2 Fab (**Figure 2 - S 11**). Two microsecond-long trajectories show that the network of hydrogen bonding and electrostatic interactions observed in the cryoEM structure are dynamically involved in maintaining the $\alpha 5\beta 1$:BIIG2 interface (**Figure 2 - 8G**). These interactions, summarized in **Table 2 - S 4**, position BIIG2 to sterically occlude fibronectin from accessing the primary RGD binding site and $\alpha 5$'s synergy binding site for FN9 (**Figure 2 - S 12A**).

MINT1526A exclusively binds the $\alpha 5$ subunit and prevents fibronectin binding by blocking the synergy binding site

To directly compare the binding interfaces with the BIIG2 complex, we next determined the cryoEM structure of the MINT1526A Fab bound to the $\alpha 5\beta 1$ ectodomain to a global resolution of 3.1 Å (**Figure 2 - 9A** and **Table 2 - S 3**). Similar to the $\alpha 5\beta 1$:BIIG2 Fab cryoEM map, the resolution of $\alpha 5\beta 1$:MINT1526A Fab was anisotropic, with the highest-resolution

regions (2.8 Å) in the headpiece and at the epitope/paratope interface (**Figure 2 - 9B-C**, **Figure 2 - S 8** and **Table 2 - S 3**). 3D classification reveals that the integrin angle of rotation about the knee region ranges from 90 to 73 degrees, in agreement with previous data (**Figure 2 - S 10D**)²⁰⁷. MINT1526A exclusively binds the $\alpha 5$ subunit and does not block the canonical RGD binding site cleft. Although MINT1526A also does not directly interact with any residues on $\alpha 5\beta 1$ required to mediate FN binding, its epitope overlaps with one of the known FN binding sites, preventing interactions between $\alpha 5$ and the FN9 synergy site (**Figure 2 - S 12B**; ^{207,316}). $\alpha 5$'s would-be D154 electrostatic and Y208 proline-aromatic interaction with FN are sterically blocked by the CDR H3 of MINT1526A (**Figure 2 - 9E**). The high affinity of $\alpha 5\beta 1$:MINT1526A is achieved primarily by hydrogen bonds, involving residues throughout the epitope (**Figure 2 - 9E-F**). MD simulations of $\alpha 5\beta 1$:MINT1526A Fab indicate that these hydrogen bonding interactions between MINT1526A and $\alpha 5\beta 1$ are stable (**Figure 2 - 9D**). A summary of the interactions can be found in **Table 2 - S 4**.

Discussion

Integrins play an essential role in cell proliferation, adhesion, migration, and survival, and their dysregulation is attributed to the progression of numerous human diseases including cancer²⁴⁰. Due to its important role in tumor angiogenesis, $\alpha 5\beta 1$ has long been identified as a target for cancer therapies^{240,243,287}. More recently, antibodies targeting $\alpha 5$ integrin have been demonstrated to inhibit the progression of the neurodegenerative disorder amyotrophic lateral sclerosis (ALS) and idiopathic pulmonary fibrosis (IPF), underlining and expanding $\alpha 5\beta 1$'s potential as a therapeutic target^{318,319}. Understanding the precise molecular interactions mediating integrin-therapeutic interactions will be key for the development of tailored, specific, and effective therapeutics, however, the molecular details of $\alpha 5\beta 1$ with any function-modulating antibody have so far been lacking. We present a comprehensive functional, structural, and mechanistic characterization of BIIG2, a rat IgG antibody targeting human $\alpha 5$ integrin, demonstrating properties that support further therapeutic evaluation. BIIG2 exhibits high affinity for $\alpha 5\beta 1$ and shows intense, tissue-specific binding to connective fibroblasts and melanocytes in melanoma patient tissues, which are known to express elevated levels of active $\alpha 5\beta 1$ integrin. This staining pattern may result from BIIG2's ability to recognize the full spectrum of $\alpha 5\beta 1$ headpiece conformations. BIIG2 more effectively inhibits angiogenesis in a 2D assay compared

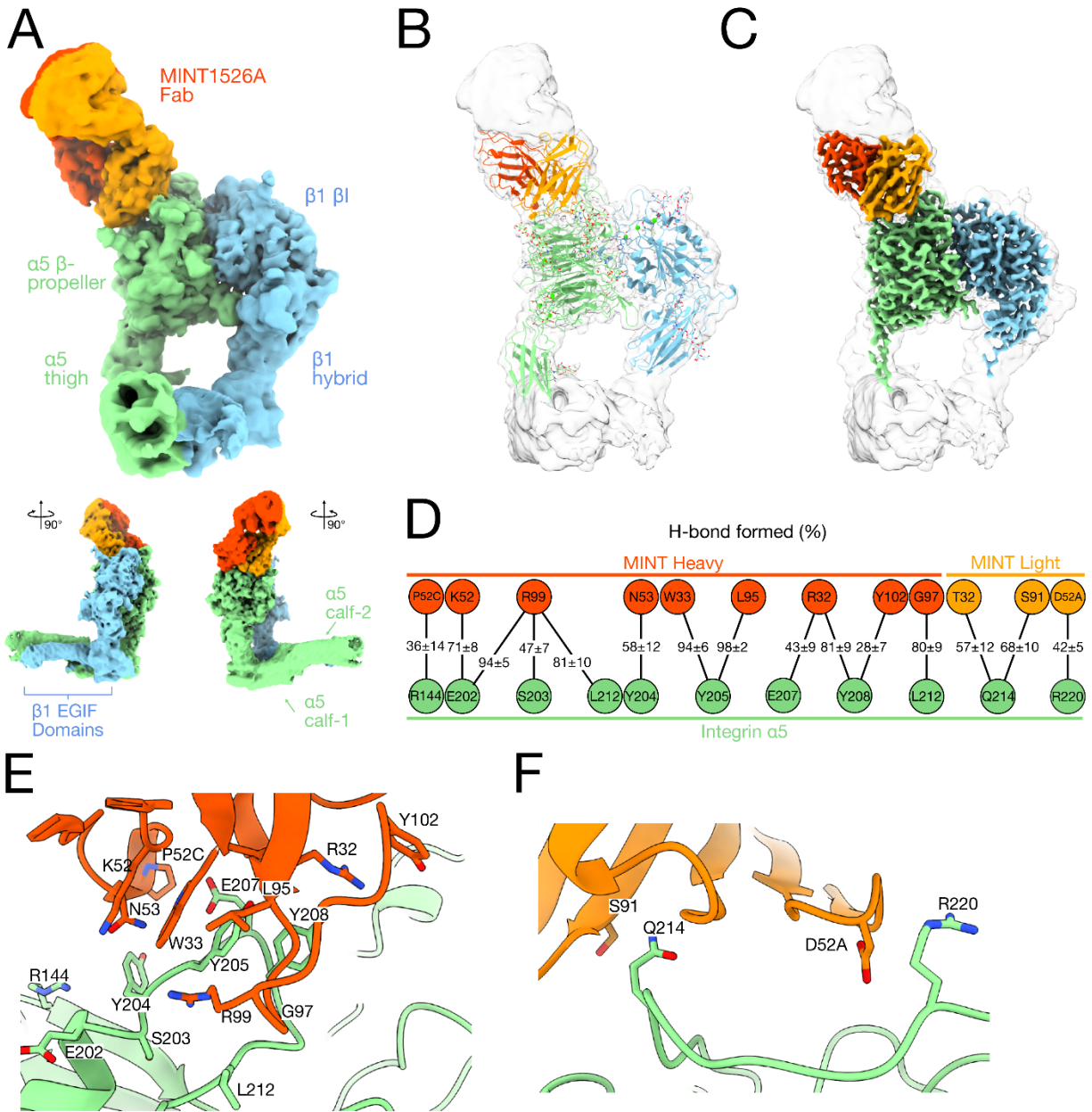


Figure 2 - 9 CryoEM analysis of $\alpha 5\beta 1$:MINT1526A Fab complex. (A-C) CryoEM maps and model of the $\alpha 5\beta 1$ ectodomain with MINT1526A Fab bound. The color code is as follows: integrin $\alpha 5$ subunit is green, integrin $\beta 1$ subunit is blue, MINT1526A Fab light chain is light orange, and heavy chain is dark orange. (A) The unsharpened map at a low threshold. (B) A ribbon diagram (colors) in the cryoEM map (semi-transparent white). (C) A higher threshold sharpened cryoEM map (colors), superimposed with the unsharpened map (semi-transparent white). (D) Hydrogen bonds identified between integrin $\alpha 5\beta 1$ and MINT1526A Fab are supported by molecular dynamics simulations. Percentage of hydrogen bonds formed (mean $\pm$ SEM) between integrin $\alpha 5\beta 1$ and MINT1526A Fab using a 3.5 Å cutoff between acceptor and donor-H atoms. Values were averaged across seven simulation repeats, each 2.0 μ s long. A ribbon diagram shows a close-up view of the interface between $\alpha 5\beta 1$ and the MINT1526A Fab heavy chain (E) and light chain (F). Stable interactions are summarized in **Table 2 - S 4**.

to MINT1526A (RG-7594), an anti- $\alpha 5 \beta 1$ antibody that has already been tested in clinical trials (**Figure 2 - 5**). In the M12 patient-derived xenograft mouse model, BIIG2 demonstrates superior tumor growth inhibition relative to other anti-integrin antibodies, with efficacy comparable to p53 restoration via pentamidine isethionate (**Figure 2 - 6**).

Using single particle cryoEM and BLI, we show that both BIIG2 and MINT1526A have similar affinities but different kinetics for $\alpha 5 \beta 1$, and impede activation by sterically blocking fibronectin binding, albeit through different epitopes (**Figure 2 - S 5**). These kinetic differences suggest that the two antibodies engage $\alpha 5 \beta 1$ through distinct binding pathways. MINT1526A associates more rapidly under both cation conditions, whereas dissociation depends on cation condition (**Figure 2 - 7**). BIIG2 engages both the $\alpha 5 \beta$ -propeller and SDL2, while MINT1526A contacts only the $\alpha 5 \beta$ -propeller, which may contribute to their different kinetic and conformational profiles. Alternatively, this could be because whereas BIIG2 binds most integrin conformations, MINT1526A is more selective. This is consistent with our negative stain analysis, suggesting that MINT1526A has a significant preference for binding bent integrin (**Figure 2 - S 6**). It is intriguing to note, however, that despite preferentially binding the bent conformation, MINT1526A is stabilized in the presence of Mn^{2+} , the cation known to stabilize the active, extended conformation. Although not all three cation binding sites within $\beta 1$ may be occupied by Mn^{2+} ¹⁶⁹. The coordination of the ADMIDAS is critical for the stabilization of the extended open conformation. In retrospect, it would have been ideal to obtain the structure of these antibodies bound to $\alpha 5 \beta 1$ in both the traditional activating buffer with Mn^{2+} and nonactivating buffer (Ca^{2+}) to potentially identify any mechanism for this allostery control.

The dramatic difference in the available conformational states of BIIG2-bound $\alpha 5 \beta 1$ vs MINT1526A-bound $\alpha 5 \beta 1$ was unexpected, since neither Fab interacts with residues that are known to participate in integrin $\alpha 5 \beta 1$ extension or headpiece opening ^{207,283,310,311,320}. The root-mean-square difference (RMSD) of the $\alpha 5 \beta 1$ headpiece region in these two models is 0.7 Å, indicating that there are no significant global structural differences when BIIG2 or MINT1526A is bound. The most notable difference in our two integrin models is a loop (residues 27-33) between two β -sheets in the first blade of the α subunit's β -propeller. It is tempting to speculate that like other flexible loops in the α subunit ³²¹, this positioning could be related to the overall conformation of $\alpha 5 \beta 1$. In the MINT1526A-bound bent $\alpha 5 \beta 1$ structure, the loop points downward, similar to the recent cryoEM structure of $\alpha 5 \beta 1$ in a bent conformation (PDB: 7NXD),

whereas the BIIG2-bound $\alpha 5\beta 1$ has loop points upward, similar to an extended structure from the same study bound to fibronectin (PDB: 7NWL; ²⁰⁷). Most published structures of this loop are more similar to the ‘up’ position of the loop, however, since many of these structures are truncated integrin fragments that do not include the lower leg domains, we cannot be certain if this loop is related to overall conformation ^{169,311,320}. Therefore, the underlying cause of the observed differences in $\alpha 5\beta 1$ conformations is not entirely clear although allosteric changes must either be transmitted or recognized by MINT1526A.

Although integrins have long been identified as promising therapeutic targets, with over 130 clinical trials investigating integrin-targeting therapies, fewer than a dozen therapeutics—either antibodies or small molecules—have reached the market ¹⁶⁴. Several cancer-related integrin therapeutics have progressed to late-stage clinical trials although none have been approved to date ²⁴⁸. Volociximab (M200) was well tolerated in phase 1 trials ²⁶⁹, but demonstrated limited efficacy in phase 2 across multiple cancer types leading to discontinuation of further development ^{271,322}. Many other investigational integrin-targeting therapies in oncology have also been halted due to limited efficacy or safety concerns. Nevertheless, integrins remain validated therapeutic targets, with several approved agents demonstrating significant success in non-oncologic indications ¹⁶⁷. Interest in the field remains strong, with numerous ongoing trials showing promising early results.

One hypothesis posits that the success or failure of some integrin therapeutics depends on selectively targeting specific integrin conformations. This concept is exemplified by Nimotuzumab, an anti-tumor antibody targeting the epidermal growth factor receptor (EGFR). Nimotuzumab permits EGFR to adopt its active conformation and exhibits fewer side effects than other anti-EGFR antibodies, such as Cetuximab, which bind similar epitopes but do not allow the active receptor conformation ³²³. For integrins, the selectivity of therapeutics for specific conformations or their influence on integrin conformation, and how those differences translate into tissue specificity, off-target effects, or toxicity remains incompletely characterized, although recent work has begun to shed light on these mechanisms ^{169,250,253,324}. Structural and biochemical data are available for several late-stage and marketed therapeutics, including high-resolution structures of the epitope or binding site for five of the seven approved integrin therapeutics ^{251–253,258,265,266}. These therapeutics also target the headpiece region near or in the ligand-binding cleft with steric clash with the cognate integrin ligand as the proposed

mechanism, similar to what our structures of BIIG2 and MINT1526A show. Of these complexes, the four solved by crystallography show a single conformation, and of the three determined via cryoEM, Abciximab: α IIB β 3 shows a closed headpiece, Eptifibatide: α IIB β 3 shows an open headpiece, M-tirofiban: α IIB β 3 is in an acutely bent conformation. The only cryoEM structure showing heterogeneity is Tirofiban: α IIB β 3 showing an open headpiece with heterogeneity in the α leg^{251,252,258}. Thus, the conformational landscape available to the therapeutic-bound integrins remains incomplete¹⁶⁷.

Recently, it has been proposed that partial agonism – linked to stabilizing the open conformation – could be responsible for chronic indications of certain failed integrin therapeutics. A comprehensive study of α IIB β 3 small molecule inhibitors showed that of the inhibitors tested, stabilizing the bent conformation has led to better therapeutic outcomes than those that stabilize an open conformation. That study also identified a general chemical principle for the design of bent-stabilizing inhibitors and, excitingly, extended the work to show the principle held true for the distantly related integrin α 4 β 1²⁵³.

On the other hand, studies show that small molecule and antibody inhibitors that stabilize the bent-closed conformation of α v β 6 are not able to lower expression of fibrotic-associated genes in lung epithelial cells as effectively as inhibitors that stabilize the extended-open conformation³²⁵. Clinical advancement of two potential therapeutics that stabilized the closed α v β 6 conformation, antibody BG00011 (the humanized version of 3G9) and the small molecule MORF-720 were halted due to suspected on-target safety signals^{319,326,327}. In contrast, PLN-74809, which binds all conformations of α v β 6 and α v β 1 and GSK3008348, which causes rapid internalization of α v β 6 which is associated with an active conformation, have led to more promising initial results with no serious adverse effects related to the compounds in clinical trials^{328–331}. Similarly, Etaracizumab, an enhanced form of Vitaxin, is well-tolerated in patients and currently under investigation for cancers^{332,333}. Its precursor, LM609 has been shown to bind all conformations of integrin α v β 3³³⁴.

It has become clear that the challenges associated with integrin-therapeutic development remain numerous, however, exciting recent results linking functional and known clinical outcomes to structural interactions have enabled recent leaps in the establishing of principles that govern effective therapeutics²⁵³. Significant efforts have advanced the discovery of novel integrin therapeutics including a new platform for antibody discovery³³⁵ and the development of

de novo structure-based rationally designed integrin miniproteins^{169,250}. In addition to the specific structural and functional details of $\alpha 5\beta 1$ -antibody interactions, the results presented here contribute to the greater understanding of the complex interplay between potential integrin therapeutics, conformation, and downstream effects.

Limitations

While this study advances our understanding of the dynamics of therapeutic antibodies and their impact on integrin conformation equilibrium, it has limitations. For a more complete comparison of efficacy to BIIG2, the xenograft tumor model data using MINT1526A would be needed. While we are able to make a direct comparison within our *in vitro* experiments, we are not able to repeat this for our xenograft experiments due to insufficient quantities of antibody available. Additionally, all structural experiments were conducted using integrin ectodomain constructs and it is possible that full-length integrins (containing the transmembrane and cytoplasmic domains) could show different conformational dynamics. Finally, although we performed a biophysical analysis, an in-depth nsEM study using the native ligand and a conformational antibody, and resolved a high-resolution cryoEM map of the $\alpha 5 \beta 1$ -MINT1526A complex, we were unable to identify the specific allosteric molecular details that lead to the bent-conformation binding preference or the headpiece “locking” we observe.

Resource Availability

All structures and models referenced have been deposited in the EMDB and the PDB. The accession numbers for maps and models are as follows: the $\alpha 5 \beta 1$:BIIG2 complex EMD-44386 and PDB: 9B9J; the BIIG2 Fab crystal structure: PDB: 8R38; the $\alpha 5 \beta 1$:MINT1526A complex EMD-44387 and PDB: 9B9K. Two cryoEM data sets have been deposited in the EMPIAR database, each containing raw movies, aligned micrographs and particle stacks. The accession numbers are as follows: $\alpha 5 \beta 1$:BIIG2 (EMPIAR-12552); $\alpha 5 \beta 1$:MINT1526A (EMPIAR-12553). Analysis scripts for molecular dynamics simulations are available at https://github.com/CampbellLab2020/nguyen_2023. Simulation trajectories and topology files are available on Zenodo at <https://doi.org/10.5281/zenodo.10070033>.

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Methods

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Mouse models:

Harlan Sprague Dawley female athymic nude mice that are recessive for the *nu* mutation in the *Foxn1* gene (Envigo) were used. This mouse strain exhibits a non-functional thymus and are unable to generate cytotoxic effector cells and do not elicit a graft-versus-host response. Mice selected for experimentation were 6 to 7 weeks old, weighing approximately 20 to 30 grams. Their housing conditions involved a 12-hour light/dark cycle with ad libitum access to food and water.

Cell lines:

Hybridoma Cell Lines. All hybridomas were purchased from the Developmental Studies Hybridoma Bank. The BIIG2 hybridoma was deposited by Caroline Damsky²⁸¹. The P1D6, P1F6, P3G8, and P5H9 hybridomas were deposited by Elizabeth Wayner and William Carter^{299,302}. Hybridoma cells were thawed and seeded into 24-well plates with 1 mL of media (IMDM + FBS). Cells were allowed to settle before being placed at 37°C overnight with 5% CO₂. If cells were too dense, they were further split as described. The following day, cells were pooled from confluent wells and transferred into a T75 flask with 10 mL of media. Cells were passed every other day or when confluent. For antibody expression, cells were seeded onto roller bottles.

Patient-Derived Melanoma Cell Lines. Short-term cultures of human primary melanoma cells M12 and M15 were maintained through serial passages in immune-deficient mice via subcutaneous flank implantation, as previously described^{336,337}. Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum (FBS) and antibiotics (penicillin, 100 U/mL, and streptomycin, 100 µg/mL) was used for growing the explant cultures. The cells were kept at 37°C in a humidified incubator with 5% CO₂.

Normal Human Melanocytes. Primary human neonatal epidermal melanocytes were obtained from Lifeline Cell Technology and subsequently cultured using DermaLife M Melanocyte Complete Medium, also provided by Lifeline Cell Technology.

Chinese Hamster Ovary Cells: ExpiCHO-S cells were passaged in ExpiCHO Media, cultured at 37°C with 8% CO₂ according to the manufacturer's protocol. Cells were passaged every 3-4

days and were subcultured down to a density of 3.0×10^5 cells/mL. Before transfection, ExpiCHO-S cells were outgrown to a density of 6.0×10^6 cells/mL.

Human tissue samples:

A 23-core normal human formalin-fixed paraffin-embedded (FFPE) tissue microarray was purchased from Bio SB (BSB 0298). FFPE tissue of patient-derived nevi and melanoma were obtained through an institutional tissue registry. The Mayo Clinic Institutional Review Board approved the human investigations performed in this study following the Department of Health and Human Services requirements, where appropriate.

METHOD DETAILS

2D-tube angiogenesis formation assay:

Endothelial tube formation assays experiments were pursued through Creative Bioarray project CBADY0508. HUVECs (human umbilical vein endothelial cells) were seeded at 5×10^5 cells/well in 2 mL of complete culture medium in 6-well plates and cultured in a 37°C and 5% CO₂ incubator. Prior to experimentation, the culture medium was removed. Cells were subsequently treated with 2 mL of culture medium (negative control), 15 ng/mL VEGF (positive control), and BIIG2 or MINT1526A (1, 10, and 100 nM) supplemented with VEGF (15 ng/mL), then incubated at 37°C for 24 hours. Each condition was performed in triplicate. HUVECs were harvested and suspended in serum-free medium and then seeded in Matrigel-coated wells at a density of 1.5×10^5 /1 mL/well. The cells were incubated at 37°C for 6 hours to allow tube formation.

Spheroid sprouting assay:

Experiments were pursued through Reaction Biology under project PQ20385, with modifications of a previously published protocol³³⁸. HUVECs were seeded at 1×10^4 cells/cm². HUVEC's were subcultured every 3-5 days by splitting 1 volume of cells to 3 volumes of ECGM/ECBM media (endothelial cell growth and basal medium, PromoCell) at 37°C with 5% CO₂. Experiments and spheroid preparation are adapted from previous descriptions^{338,339}. 400 HUVEC were pipetted in a hanging drop to allow overnight spheroid aggregation. 50 HUVEC spheroids were then seeded in 0.9 mL collagen gel and pipetted into a 24 well plate to allow polymerization. The inhibitors combined with 25 ng/mL of VEGF-A were added after 30 minutes by pipetting 100 µL of a 10-fold concentrated working solution on top of the

polymerized gel. Plates were incubated at 37°C for 24 hours before being fixed with 4% paraformaldehyde PFA. Sprouting intensity of HUVEC spheroids were captured using an inverted microscope and the digital imaging software NIS-Elements BR 3.0 (Nikon) by determining the cumulative sprout length per spheroid (CSL). 10 randomly selected spheroids were measured and treated as an individual data point.

Mouse tumor experiment:

The mice were randomized into treatment groups and monitored daily. Tumor cells (M12 or M15) embedded in Matrigel were injected subcutaneously into the flank of the athymic mice. In efficacy studies, groups of mice (10 per group) with flank tumors were administered treatment once the tumor volumes became palpable (approximately 50 to 100 mm³). The flank tumors were measured three times a week. Treatment was administered twice weekly at a dose of 10 mg/kg. The experiments were concluded when the average tumor volume in the control group exceeded 1,000 mm³. All experimental procedures adhered to the Principles of Laboratory Animal Care (NIH) and were approved by the Institutional Animal Care and Use Committee (Mayo Clinic).

Immunohistochemistry:

Proteins of interest were detected using the Leica Biosystems BOND-MAX autostainer platform in combination with primary antibody, post-primary antibody, and a BOND Polymer Refine Red Detection system (DS9390; Leica Biosystems). Heat-induced antigen retrieval was by BOND EDTA-based pH 9.0 epitope retrieval solution 2 (AR9640; Leica Biosystems) applied for 20 minutes. All stains were digitized with a Leica Aperio ScanScope system.

Flow cytometry:

Normal human melanocytes, M12 and M15 melanoma cells were stained using primary antibodies (full antibody list can be found within the online publication) against cell surface proteins as shown and PE-labeled secondary antibodies. The BD Bioscience FACScaliber flow cytometer was utilized for data acquisition, and the acquired data was analyzed using FlowJo software.

Quantitative Microfluidic PCR:

Our approach to PCR was as previously described³⁴⁰. RNA from cells was isolated using the RNeasy Plus Mini Kit (Qiagen). One microgram of total RNA was transcribed into cDNA using the SuperScript III Reverse Transcriptase (Thermo Fisher Scientific) and preamplified

using TaqMan Preamp Master Mix (Thermo Fisher Scientific). Quantitative reverse transcriptase PCR was then performed using the Fluidigm BioMark HD system and dynamic array-integrated fluid circuits (Fluidigm). After thermal cycling, raw Ct (threshold cycles) data were checked for linear amplification. Gene expression was corrected by the mean of housekeeping genes (RLP0, RLP8, and β -actin) using the Δ Ct method. Gene expression input for data analysis was Δ Ct. The complete list of primers (HPLC purified, Integrated DNA Technologies) used for cDNA amplification by quantitative PCR are described within the online publication.

Recombinant α 5 β 1 plasmid design:

The sequence of the α 5 and β 1 subunit wild-type ectodomain was incorporated into a CDNA3.1-Hygro(-)-TET template (Invitrogen). The integrin amino acid numbering starts after the signal peptide. Each integrin subunit was fused with either an ACID or a BASE coiled-coil peptide to assist in heterodimer production³⁴¹. Residues 1 to 954 of the α 5 subunit were cloned followed by an HRV-3C protease cleavage recognition site, an ACID coil, and a Strep-Tag II. The residues 1-728 of the β 1 subunit were put into the same plasmid backbone filled by an HRV-3C protease cleavage recognition site, a BASE coil, and a 6x histidine tag. The list of primers used for construct generation are described within the online publication.

Purification of integrin α 5 β 1 ectodomain:

Soluble integrin ectodomain (α 5 residues 1-954 and β 1 residues 1-728) was expressed using ExpiCHO-S cells (Thermo Fisher) in suspension at a density of 6.0×10^6 cells/mL by transient transfection using the ExpiCHO transfection kit. Cultures were co-transfected with plasmids encoding the α 5 or β 1 subunit in a 2:1 (α : β) ratio with Expifectamine CHO. Expression was according to the manufacturer's 'max titer' protocol. Cells were harvested by centrifugation at $1,000 \times g$ for 15 minutes at 4°C . Phenylmethylsulfonyl fluoride (PMSF) was added to the supernatant at a final concentration of 0.5 mM before undergoing another centrifugation run at $36,000 \times g$ for 45 minutes at 4°C . Clarified supernatant was diluted 1:1 v/v with Histrap binding buffer (20 mM Na_2PO_4 , 500 mM NaCl, and 20 mM imidazole, pH 7.4) and applied to 5 mL Histrap FF Crude column (Cytiva) equilibrated with Histrap binding buffer. The column was washed with 1 column volume (CV) of Histrap binding buffer before eluting protein with 2 CV of Histrap Elution Buffer (20 mM Na_2PO_4 , 500 mM NaCl, and 500 mM imidazole, pH 7.4). Protein fractions were pooled, concentrated and buffer exchanged into integrin storage buffer (20

mM Tris-Base pH 7.4, 150 mM NaCl, 1 mM CaCl₂, and 1 mM MgCl₂). Protease was added 1:20 w/w (protease:α5β1) to cleave the ACID/BASE coils and purification tags at 4°C overnight with rocking. The cleaved α5β1 integrin was further purified via gel filtration chromatography using a Superdex 200 Increase 10/300 SEC column (Cytiva) equilibrated in integrin storage buffer. Peak fractions of α5β1 were pooled, concentrated, flash-frozen with 10% glycerol (v/v), and stored at -80°C until used for experiments.

Purification of BIIG2 and MINT1526A Fab fragments:

BIIG2 antibody was produced at scale through the Mayo Clinic institutional core facility. Briefly, the production process involved culturing the hybridoma cells in roller bottles containing Iscove's Modified Dulbecco's Medium supplemented with gamma globulin-free serum. A single-step Protein G affinity purification was used to isolate antibodies. The eluted antibody fractions were pooled, concentrated, and buffer exchanged into phosphate buffered saline (1x PBS). The concentrated stock solutions of the antibodies were stored at -80°C until used for experiments. Genentech has kindly provided us with purified MINT1526A antibody.

BIIG2 and MINT1526A antibodies were digested with the Pierce Fab Preparation Kit (Thermo Fisher) using papain and following the manufacturer's protocol. BIIG2 Fab fragments were separated from the Fc fragments and intact IgG by loading onto a chromatography column packed with 5 ml of CaptureSelect IgG-Fc (Multi-species) Affinity Resin (Thermo Fisher) in PBS using an NGC FPLC instrument (Bio-Rad). The Fab fragment was present in the flow-through. Separation of the MINT1526A Fab fragment from the Fc fragment was achieved using a NAb Protein A spin column following the manufacturer's protocol. SDS-PAGE gel bands of Fab and Fc samples from both antibodies were digested and analyzed by mass spectrometry (Mayo Clinic Proteomics Core) to verify the identity and separation of the two fragments.

Purification of Fibronectin domains 7-10.

Human fibronectin domains 7-10 (FN7-10) was purified as described previously²⁸⁵. FN7-10 was expressed in *Escherichia coli* BL21 (DE3) in LB media containing ampicillin at 37°C with shaking. Cells were grown until the OD₆₀₀ reached 0.6-0.8. The cells were induced using IPTG at 1 mM final concentration and then grown overnight at 18°C. Cells were pelleted and resuspended in a buffer containing 50 mM Tris-base pH 7.4, 200 mM NaCl, 1% Triton X-100, and a Pierce protease tablet. Cells were then lysed via sonication and clarified by

centrifugation at 76,000 x g for 40 minutes at 4°C. Solid (NH₄)₂SO₄ was added to 40% (final w/v) slowly and the solution was placed on a nutator for 30 minutes at 4°C. This solution was then centrifuged again at 76,000 x G for 40 minutes at 4°C and the pellet was resuspended in Hitrap Q Binding Buffer (20 mM Tris-base pH 7.4 and 50 mM NaCl). The protein sample was applied onto a 5 mL Hitrap Q XL column. The column was washed with 5 CV of Hitrap Q Binding Buffer. The protein was eluted in a gradient profile from 50 mM NaCl to 500 mM NaCl over 20 CV. Protein fractions were pooled, concentrated and buffer exchanged into 50 mM Tris-base pH 7.4 and 150 mM NaCl. FN7-10 was further purified via gel filtration chromatography using a Superdex 200 increase 10/300 SEC column (Cytiva) equilibrated in 50 mM Tris-base pH 7.4 and 150 mM NaCl. Peak fractions of FN7-10 were pooled, concentrated, flash frozen with 10% glycerol (v/v), and stored at -80°C until used for experiments.

Biolayer Interferometry:

All binding experiments were performed on an Octet Red96e (Sartorius). MINT1526A was immobilized on Protein A biosensors (Sartorius). These sensors were hydrated using nonactive running buffer (20 mM Tris-Base pH 7.4, 150 mM NaCl, 0.1% BSA, and 0.02% Tween20 with 5mM CaCl₂) for 15 minutes before subsequently being incubated with fresh nonactive running buffer. MINT1526A was diluted with nonactive running buffer to 5 ug/mL and loaded for 300 seconds before a 120 second baseline equilibration in the nonactive running buffer. Association with integrin $\alpha 5\beta 1$ ectodomain was carried out with a two-fold dilution series from 50nM to 1.56nM in nonactive running buffer for 300 seconds before dissociating for 300 seconds. Experiments were repeated with active running buffer (20 mM Tris-Base pH 7.4, 150 mM NaCl, 0.1% BSA, and 0.02% Tween20 with 1mM MnCl₂). MINT1526A binding experiments were performed in triplicate for both nonactive and active conditions.

Binding assays with BIIG2 began with hydrating AR2G biosensors (Sartorius) in water for 15 minutes, followed by an additional 1-minute incubation. The AR2G biosensors were then activated using 20 mM EDC and 10 mM s-NHS for 300 seconds (following manufacturer's protocol). BIIG2 antibody was diluted with 10 mM sodium acetate pH 5.0 to 5 ug/mL and loaded until a threshold shift of 2nm was achieved. Loading was immediately quenched in 1M ethanolamine for 300 seconds. A baseline equilibration step was performed in nonactive running buffer for 120 seconds. Association with integrin $\alpha 5\beta 1$ ectodomain was performed at a two-fold dilution series from 50nM to 1.56nM in nonactive running buffer for 450 seconds before

dissociating for 450 seconds. Experiments were repeated with active running buffer and each buffer condition was repeated for an additional two times.

Competitive binding assays were carried out where $\alpha 5\beta 1$ was bound to MINT1526A or BIIG2 Fabs or FN domains 7-10 fragment in a 1:2 molar ratio 20 minutes prior to assay running in either active or nonactive buffer. MINT1526A or BIIG2 antibodies or FN 7-10 used as immobilized ligands were loaded onto their respective biosensors (Protein A for MINT1526A and AR2G for BIIG2 and FN 7-10) at 5ug/mL. Experimental conditions and association and dissociation step times follow as described above.

Crystallization of BIIG2:

BIIG2 Fab (7.14 mg/mL in PBS + 0.02% sodium azide) was diluted in CIEX binding buffer (25 mM Bis-Tris-HCl, pH 6.5) and loaded onto a pre-equilibrated 1 mL HiTrap SP HP chromatography column (Cytiva), connected to an ÄKTA Purifier 10 chromatography system (Cytiva). The protein was eluted with a 0-100% gradient over 100 mL of CIEX elution buffer (25 mM Bis-Tris-HCl, 1 M NaCl, pH 6.5). The main protein component eluted as a highly symmetric peak around 12% v/v Buffer B (approximately 120 mM NaCl). Fractions containing BIIG2 Fab protein were pooled and concentrated to 10 mg/mL for crystallization studies.

Preliminary screening of BIIG2 Fab crystallization conditions was performed with pre-formulated commercial screens (JCSG+, Morpheus, PACT Premier, Molecular Dimensions/CalibreScientific Inc.)³⁴²⁻³⁴⁴. Sitting-drop vapor diffusion crystallization experiments were set up on UVXPO Two-Lens Plates (SwissCI) by mixing BIIG2 Fab at 10 mg/mL and the crystallization solutions in a 1:1 volume ratio (300 nL + 300 nL) using an Oryx4 crystallization robot (Douglas Instruments Ltd.) Plates were stored at 20°C in a RI-182 crystal plate hotel (Formulatrix) and monitored at regular time intervals for the formation of crystals. Initial crystals grew from several conditions from the JCSG+, PACT Premier and Morpheus formulations. Two sets of 48 optimized crystallization solutions were designed, combining the chemical components from several PACT Premier hits. A new sitting-drop vapor diffusion experiment was set up by mixing the optimized formulations with CIEX-purified BIIG2 Fab, which was diluted with type I water (Milli-Q) to a protein concentration of 7.6 mg/mL.

Crystallization drops were set up on an MRC Maxi PS (SwissCI) crystallization plate, mixing the diluted protein solution and the crystallization cocktails in a 1:1 v/v ratio (300 nL +

300 nL). Diffraction-quality crystals grew from several conditions. Crystals with the most promising morphologies were harvested in nylon loops or lytholoops, cryoprotected (if appropriate), flash-cooled in a nitrogen cryostream, and sent for synchrotron data collection in a liquid nitrogen-cooled CX 100 dry shipper (Taylor Wharton). The crystal that provided the highest-diffracting data set grew from 20% w/v PEG 3350, 0.2 M sodium chloride, 2.5% v/v DMSO; cryoprotectant solution was prepared by complementing the crystallization solution from the reservoir with glycerol, to a final concentration of 20% v/v.

X-ray data collection and refinement:

Synchrotron data collection on BIIG2 Fab crystals was performed at the BioMAX beamline, MAX IV, Lund, Sweden (at 100 K, 0.9537 Å). Data were initially integrated and scaled with *autoPROC*³⁴⁵, and ranked by their diffraction resolution cut-off, based on the assessment of the Pearson's correlation coefficient ($CC_{1/2} > 10\%$)³⁴⁶.

The highest-resolution data set (1.4 Å), collected on a crystal belonging to space group $P2_12_12_1$, was chosen for structure determination. Many of the programs and tools used belong to the *CCP4* software suite³⁴⁷. Data were integrated and scaled with *DIALS*³⁴⁸; merging and truncation were carried out with *AIMLESS*, a component of the *CCP4* software suite³⁴⁷. A PDB-restricted BLAST³⁴⁹ homology search identified a suitable search model for molecular replacement, the Fab fragment of a rat monoclonal antibody (PDB: 1C5D; VL=80.5 % seq. id.; VH = 79.1% seq. id.). Ligands and water molecules were manually deleted, and alternative conformations removed using PDBSET³⁴⁷. Amino acids were truncated to the last conserved atom using the *CCP4* tool *CHAINSAW*³⁵⁰. The truncated model was segmented into four separate components, the heavy and light chains and their constant and variable regions, which were used as separate search models in *Phaser*³⁵¹. The phased data set contained a single copy of the Fab heterodimer in the asymmetric unit. Search model bias was mitigated by running an extensive initial round (60 cycles) of maximum-likelihood refinement using *REFMAC5*³⁴⁷. Two cycles of real-space model rebuilding were carried out in *Coot*³⁵², each followed by maximum-likelihood isotropic refinement with *REFMAC5*³⁴⁷. Missing atoms from the protein main chain were automatically added with *BUCCANEER*³⁴⁷, followed by several rounds of manual building with *Coot*³⁵², alternating with maximum-likelihood isotropic single-wavelength anomalous diffraction (SAD) refinement with *REFMAC5*³⁴⁷. The final refinement step included TLS parameterization³⁵³.

After correcting inaccuracies in the main protein chain, first and second-coordination sphere water molecules were added to the model, followed by alternative main and side-chain conformations. Water molecules were initially placed using *Coot*:Find waters and then manually inspected for several criteria, including distances from hydrogen-bond donors/acceptors and quality of the electron density. Occupancies for different side chain conformations were assigned during the course of a combined real-space/maximum-likelihood refinement round through *phenix.refine*³⁵⁴. The model was completed by adding ligands, including part of the *N*-linked glycosyl chain visible on N60 and glycerol, which had been used as cryoprotectant. Well-defined saccharide units from the glycan core region were manually positioned in the difference density map and linked by editing the coordinates file. Glutamine H1, the N-terminal amino acid of the Fab heavy chain, was modeled as pyroglutamate. Some of the disulfide bridges were found to be partly reduced (*e.g.*, C23-C88, chain L), due to minor radiation damage; the relevant cysteine residues were modeled as partly reduced, when appropriate. K199 showed residual density on the terminal amino group of its side chain and was modeled as methyllysine. His H100C showed residual density suggesting post-translational modification and was modeled as 5-hydroxyproline. As this residue is not included in the *REFMAC* dictionary, a ligand definition file was created with *AceDRG*³⁵⁵. 5-hydroxyproline (PubChem CID: 14325405) coordinates were generated with *Coot LIDIA*³⁵⁶, starting from an isomeric SMILES string obtained from the PubChem server³⁵⁷. The *AceDRG* output for the new ligand (HY5) was edited, renaming and renumbering atoms to match the standard proline numbering; occupancy refinement suggested an 86% post-translational modification rate for His H100C. Further model improvements were assessed with the PDB_REDO server³⁵⁸, while the diffraction limit was validated by running paired refinement using *PAIREF*³⁵⁹, accessed through the *CCP4i2* GUI³⁶⁰. The overall quality of the model was validated through the PDB Validation server³⁶¹. The percentages of amino acid residues occupying the favored, allowed and outlier regions in the Ramachandran plot are 98/2/0%. The quality of the carbohydrate chain was evaluated by *Privateer*³⁶², a *CCP4* tool accessible through the *CCP4i2* interface³⁶⁰. σ_A -weighted electron density maps were generated using the FFT tool, a component of the *CCP4* software package³⁴⁷. Figures were generated using PyMOL (Schrödinger Inc.), assigning secondary structure elements as defined by the SST web server³⁶³. Position numbering in the final model was adjusted to match the Kabat scheme

³⁶⁴, before depositing the refined coordinates and structure factors in the PDB ¹⁵⁰, with PDB: 8R38. Crystal statistics are summarized in **Table 2 - S 2**.

Negative-stain EM sample preparation, data acquisition and processing:

The $\alpha 5\beta 1$ -Fab complexes were formed using a 1:2 molar ratio of $\alpha 5\beta 1$ to antibody Fab, incubated at room temperature for 20 minutes, and diluted to a final concentration of 7.5 $\mu\text{g/mL}$ in 20 mM Tris-base pH 7.4, 150 mM NaCl supplemented with either 5 mM CaCl_2 or 1 mM MnCl_2 . For experiments determining what integrin conformations MINT1526A binds, we used the commercially available conformation-inducing antibody, 9EG7 IgG (BD Biosciences cat# 553715). Integrin $\alpha 5\beta 1$ ectodomain was buffer-exchanged into nonactivating conditions (5 mM Ca^{2+} , 20 mM Tris-base pH 7.4, 150 mM NaCl) for 10 minutes with rocking at room temperature. 9EG7 IgG was added to integrin $\alpha 5\beta 1$ at a 1:1 molar ratio and incubated for 10 minutes at room temperature with rocking. 9EG7 binding locks $\alpha 5\beta 1$ into the extended state ³¹⁰, then MINT1526A or BIIG2 Fab was added at a 1:1 molar ratio to integrin $\alpha 5\beta 1$ ectodomain and incubated for an additional 10 minutes with rocking at room temperature. Experiments were repeated in activating buffer (1 mM Mn^{2+} , 20 mM Tris-base pH 7.4, 150 mM NaCl).

For all experiments, 3 μL of protein sample was added to a glow-discharged (PELCO easiGlow; 30 seconds; 15 mA) carbon-coated copper grid (homemade carbon layer). The specimens were stained with 3 μL of 2% (w/v) uranyl formate solution as previously described ³⁶⁵. Data were acquired using a Thermo Fisher Scientific Talos L120C transmission electron microscope operating at 120kV and recorded on a 4k x 4k Thermo Fisher Scientific Ceta camera at a nominal magnification of 92,000x with a pixel size of 1.58 $\text{\AA}/\text{px}$. Leginon ³⁶⁶ was used to collect 252 (apo $\alpha 5\beta 1$ nonactivating), 253 (apo $\alpha 5\beta 1$ activating), 385 ($\alpha 5\beta 1$:BIIG2 nonactivating), 399 ($\alpha 5\beta 1$:BIIG2 activating), 422 ($\alpha 5\beta 1$:MINT1526A nonactivating), 482 ($\alpha 5\beta 1$:MINT1526A activating), 368 ($\alpha 5\beta 1$:9EG7:BIIG2 nonactivating), 373 ($\alpha 5\beta 1$:9EG7:BIIG2 activating), 366 ($\alpha 5\beta 1$:9EG7:MINT1526A nonactivating) and 395 ($\alpha 5\beta 1$:9EG7:MINT1526A activating) micrographs with a nominal range of 1.7-2.0 μm under focus and a dose of approximately 50 $\text{e}^-/\text{\AA}^2$. The contrast transfer function of each micrograph was estimated using GCTF ³⁶⁷, and particles were automatically picked unbiasedly using Gautomatch ³⁶⁷ (<http://www.mrc-lmb.cam.ac.uk/kzhang/Gautomatch>). Particles were extracted at 352 pixels and binned to 128 pixels using RELION3 ³⁶⁸ before being imported into cryoSPARC ¹⁵⁸. Particles were subjected to multiple rounds of reference-free 2D alignment and classification to remove

false-positive particle images. The final particle count contributed to 2D class averages were 26,057 (apo $\alpha 5\beta 1$, nonactivating), 31,786 (apo $\alpha 5\beta 1$, activating), 18,200 ($\alpha 5\beta 1$:BIIG2, nonactivating), 16,797 ($\alpha 5\beta 1$:BIIG2, activating), 9,742 ($\alpha 5\beta 1$:MINT1526A, nonactivating), 41,387 ($\alpha 5\beta 1$:MINT1526A, activating), 21,329 ($\alpha 5\beta 1$:9EG7:BIIG2 nonactivating), 25502 ($\alpha 5\beta 1$:9EG7:BIIG2 activating), 12,149 ($\alpha 5\beta 1$:9EG7:MINT1526A nonactivating) and 48,765 ($\alpha 5\beta 1$:9EG7:MINT1526A activating).

CryoEM sample preparation:

$\alpha 5\beta 1$ -BIIG2/MINT1526A Fab complexes were formed using a 1:2 $\alpha 5\beta 1$ to Fab molar ratio in nonactivating buffer (20 mM Tris pH 7.4, 150 mM NaCl, 5 mM CaCl_2) at room temperature with rocking for 20 minutes. nonactivating conditions were used to drive the equilibrium toward a more homogenous sample since we had already established that the epitope binding site was nowhere near the relevant cation coordination sites. These protein complexes were subjected to size exclusion chromatography using a Superdex200 Increase 10/300 GL (Cytiva) equilibrated with nonactivating buffer. Fractions of $\alpha 5\beta 1$:BIIG2/MINT1526A Fab were pooled and concentrated to 0.15 mg/mL before 3 μL of the protein sample was placed on a glow discharged (PELCO easiGlow; 30 seconds; 15 mA) R1.2/1.3 Au 300-mesh grid (QUANTIFOIL), blotted for 8 seconds with a blot-force of 1, and plunge-frozen immediately in liquid ethane using a Thermo Fisher Scientific Vitrobot Mark IV at 4° C and 100% humidity.

CryoEM Data collection and processing:

Two datasets (one each for $\alpha 5\beta 1$:BIIG2 Fab and $\alpha 5\beta 1$:MINT1526A Fab) were acquired on a Thermo Fisher Scientific Glacios cryo-transmission electron microscope operating at 200kV and recorded with a Gatan K3 Direct Detection Camera. Automated data collected was carried out using the SerialEM software³⁶⁹. 99 frame movies were recorded in super-resolution mode with a super-resolution pixel size of 0.561 Å/px at a nominal magnification of 36,000x. Both datasets were collected in a single session with a nominal defocus range of 1.4–1.8 μm under focus and a dose of approximately 50e⁻/Å². 3656 micrographs were collected of the $\alpha 5\beta 1$:BIIG2 Fab complex and 3079 micrographs of $\alpha 5\beta 1$:MINT1526A Fab.

The dose fractionated super-resolution image stacks were motion corrected and binned 2x2 by Fourier cropping using MotionCor2³⁷⁰. Motion corrected images were then processed using CTFFIND4 within the cryoSPARC wrapper^{158,371}. 2,425,530 ($\alpha 5\beta 1$:BIIG2 Fab) and

1,101,393 ($\alpha 5\beta 1$:MINT1526A Fab) particles were initially picked using the unbiased blob picker in cryoSPARC. These particles were subjected to multiple rounds of reference-free 2D alignment and classification in cryoSPARC. Next, particles were polished in Relion¹⁵⁷ and final 3D classification and refinement steps were done in cryoSPARC. The final particle count for $\alpha 5\beta 1$:BIIG2 Fab is 371,066. The final particle count for $\alpha 5\beta 1$:MINT1526A Fab is 342,608. Images showing EM maps were generated using UCSF ChimeraX³⁷². A detailed processing schematic is in **Figure 2 - S 9**.

CryoEM model building:

The closed $\alpha 5\beta 1$ headpiece (PDB: 3VI3) and our solved BIIG2 Fab structure were used as initial models for the $\alpha 5\beta 1$:BIIG2 Fab complex. The half bent $\alpha 5\beta 1$ ectodomain (PDB: 7NXD) was used as an initial model for $\alpha 5\beta 1$:MINT1526A Fab complex. MINT1526A was modeled *de novo* using peptide sequences derived from mass spectrometry. Initial models were fitted into their respective cryoEM density using UCSF ChimeraX³⁷² and manually adjusted in COOT³⁵². Glycosylations were manually added using COOT. Models (including glycans) were refined using Phenix and ISOLDE^{373,374}. Modeling was aided by using sharpened and unsharpened cryoEM maps. BIIG2 and MINT1526A were numbered according to the Kabat numbering scheme³⁷⁵. CryoEM maps and model statistics are found in **Table 2 - S 3**.

Molecular dynamics simulations:

All-atom molecular dynamics simulations were performed using the OpenMM 7.7.0 package³⁷⁶ using AMBER ff19SB parameters and topologies. The initial structure for simulations was constructed from the $\alpha 5\beta 1$:BIIG2 or $\alpha 5\beta 1$:MINT1526A cryoEM models. To reduce the system size, only the headpiece domains of $\alpha 5\beta 1$ and variable domains of BIIG2 and MINT Fabs were simulated. N- and C- terminal residues were capped with acetyl and methyl amide groups, respectively. Protonation states for titratable residues were modeled based on a pH of 7.4. The systems were solvated in a truncated octahedron with the minimal distance between the solute and the box boundary set to 8 Å and 150 mM NaCl. Calcium ions resolved in the cryoEM model were retained. The system was parameterized using the AMBER19ffSB³⁷⁷ force field combined with the OPC water model and 12-6-4 Li Merz ion parameters³⁷⁸. As a control, an $\alpha 5\beta 1$ without Fab bound, referred to as apo $\alpha 5\beta 1$, was modeled and parameterized similarly.

The systems were first energy minimized for 20,000 steps followed by a gradual heating to 298K. During minimization and heating, protein backbone atoms were harmonically restrained by a 5 kcal/mol*Å² force constant. Production simulations were performed using periodic boundary conditions, hydrogen mass repartitioning³⁷⁹, and a Langevin integrator with an integration timestep of 4 fs and collision rate of $\sqrt{2}$ ps⁻¹. Pressure was maintained at 1 atm using a MonteCarlo barostat with an update frequency of 100 steps. Nonbonded interactions were calculated with a distance cutoff of 10Å. Trajectory snapshots were saved every 100 ps during production simulations. A total of 7 replicates, 2μs each in length, were simulated for α5β1 systems.

Trajectories were processed using in-house scripts utilizing the CPPTRAJ³⁸⁰, MDTraj 1.9.7³⁸¹, and GetContacts (<https://getcontacts.github.io/>) packages. Hydrogen bond frequencies were calculated using a 3.5 Å distance cutoff between the donor-hydrogen and acceptor atoms. The first 50 ns of production simulations were not included in analysis.

QUANTIFICATION AND STATISTICAL ANALYSIS

Spheroid assays are reported as means ± SEM. 8-10 spheroids were measured at random per condition and concentration. Cumulative sprout length per spheroid (CSL) was quantified using Wimsprout image analysis software (<https://www.wimasis.com/sprouting-assay>). Significance was determined by a measurable reduction of total sprouting length compared to the stimulated sprouts (+VEGF), with adjusted P values less than 0.05. Statistical analysis was performed using a one-way ANOVA with Bonferroni correction post-hoc. Statistical analysis can be found in the legend of **Figure 2 - S 1**.

2D angiogenesis tube formation assays are reported as means ± SEM. The total master segments length (pixels) and the number of meshes/junctions were counted using Angiogenesis Analyzer ImageJ software²⁹¹. Three representative images were taken for each well at 40x magnification under an inverted microscope and the mean measurement was used as the individual data point. This was repeated for a total of three wells. Statistical analysis was done using a one-way ANOVA with a Bonferroni correction against the control group (HUVEC + VEGF). Significance was determined by a measurable reduction of total tube length and mesh count compared to the VEGF-stimulated control, with adjusted P values of less than 0.05. Statistical analysis can be found in the legend of **Figure 2 - 5**.

Mouse tumor models are reported as means $\pm$ SEM. Total number of mice per antibody per time point equals $n = 8-10$. Statistical analysis performed was a one-way ANOVA with Bonferroni correction against the control group (IgG) and significance was determined if any antibodies tested are capable of reducing tumor volume compared to the control IgG with an adjusted P value less than 0.05. Additional one-way ANOVA testing of the other $\alpha 5$ and αv targeting antibodies against BIIG2 was performed with a Bonferroni correction. Significance was determined if BIIG2 performed better than the panel of antibodies at reducing tumor volumes with an adjusted P value less than 0.05. In experiments with Pentamidine, the means of all conditions were compared to one another via one-way ANOVA with a Bonferroni correction. Total number of mice per experimental group is $n = 8-10$. Significance was determined if any of the treatment conditions were able to reduce tumor volume significantly with an adjusted P value less than 0.05. Statistical analysis can be found in the legend of **Figure 2 - 6** and **Figure 2 - S 4**.

All statistical analyses were performed using the software package Prism 10 (GraphPad Software, San Diego, CA).

Additional figures and tables

This section contains the following supporting data for Chapter 2.3:

- Figure 2 - S 1** Spheroid sprouting assay
- Figure 2 - S 2** Staining pattern of BIIG2 and polyclonal $\alpha 5$ integrin antibody PA5-82027 in diverse human tissue samples
- Figure 2 - S 3** Characterization of patient-derived xenografts of metastatic melanoma for evaluating the efficacy of integrin antibodies on tumor growth
- Figure 2 - S 4** Additional statistical analysis of BIIG2 efficacy and impacts of gene expression in M12 xenograft.
- Figure 2 - S 5** Integrin and antibody BLI competition assay using a fragment of fibronectin (FN 7-10)
- Figure 2 - S 6** Final nsEM 2D classes of apo $\alpha 5\beta 1$, $\alpha 5\beta 1$:BIIG2 Fab and $\alpha 5\beta 1$:MINT1526A Fab, sorted by conformation and 2D classes of $\alpha 5\beta 1$:9EG7:BIIG2 Fab and $\alpha 5\beta 1$:9EG7:MINT1526A Fab complexes
- Figure 2 - S 7** BIIG2- $\alpha 5\beta 1$ complex and structural supporting data.
- Figure 2 - S 8** MINT1526A- $\alpha 5\beta 1$ complex and supporting cryoEM data.
- Figure 2 - S 9** CryoEM Processing Schematic of $\alpha 5\beta 1$:BIIG2 Fab and $\alpha 5\beta 1$:MINT1526A Fab
- Figure 2 - S 10** 3D classification defines the conformational states of integrin bound to BIIG2 or MINT1526A
- Figure 2 - S 11** Molecular dynamics simulations of apo $\alpha 5\beta 1$ or in complex with BIIG2 and MINT1526A Fabs
- Figure 2 - S 12** BIIG2 and MINT1526A occlude Fibronectin binding
- Table 2 - S 1** Staining patterns of anti-human $\alpha 5$ integrin antibodies on normal FFPE tissue microarray (#BSB-0298)
- Table 2 - S 2** X-ray crystallography analysis of BIIG2 Fab fragment
- Table 2 - S 3** CryoEM data collection, refinement, and validation statistics
- Table 2 - S 4** Interactions of $\alpha 5\beta 1$ with BIIG2 and MINT1526A Fabs

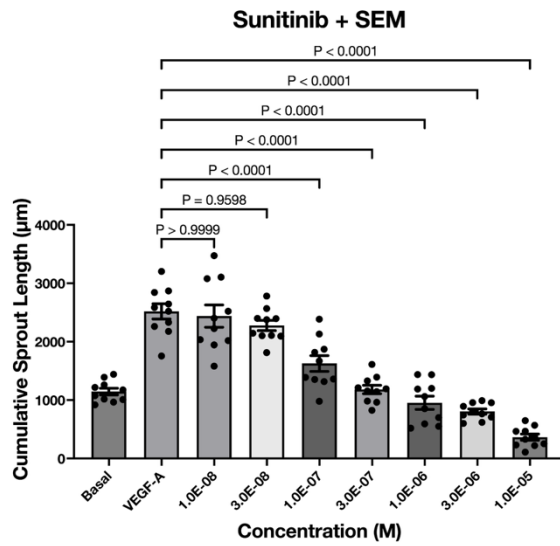
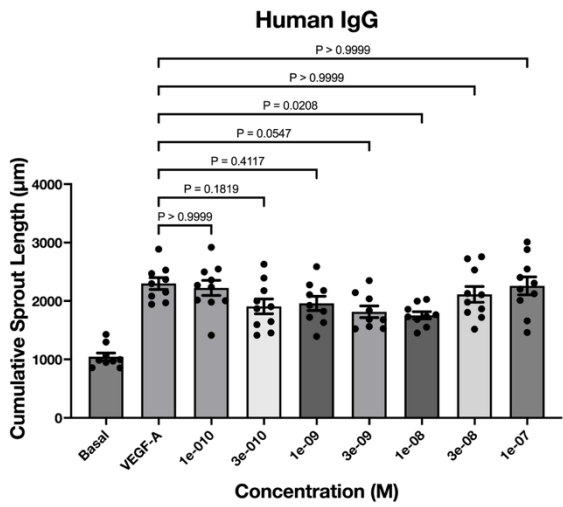
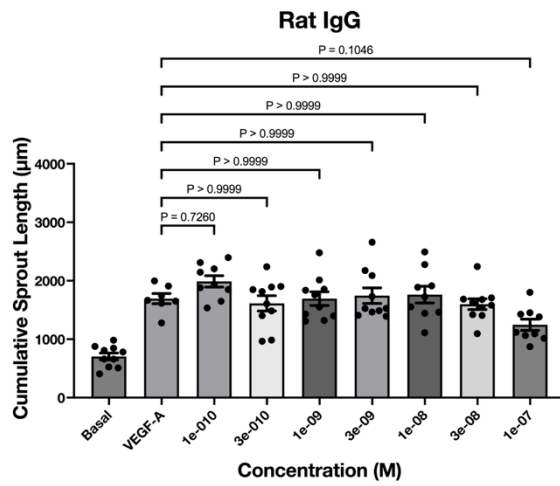
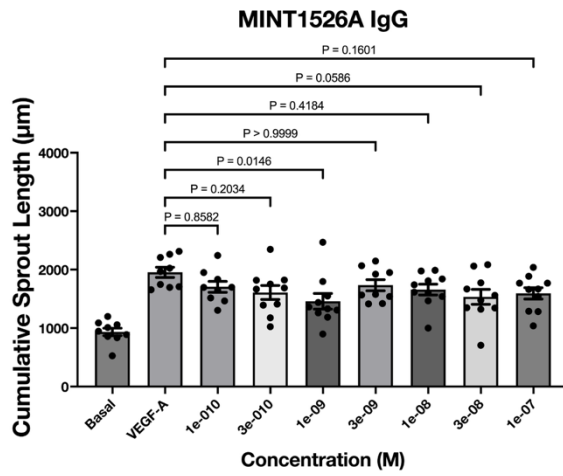
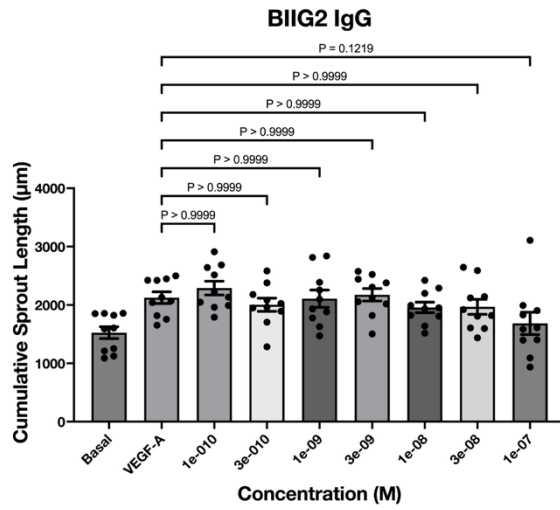
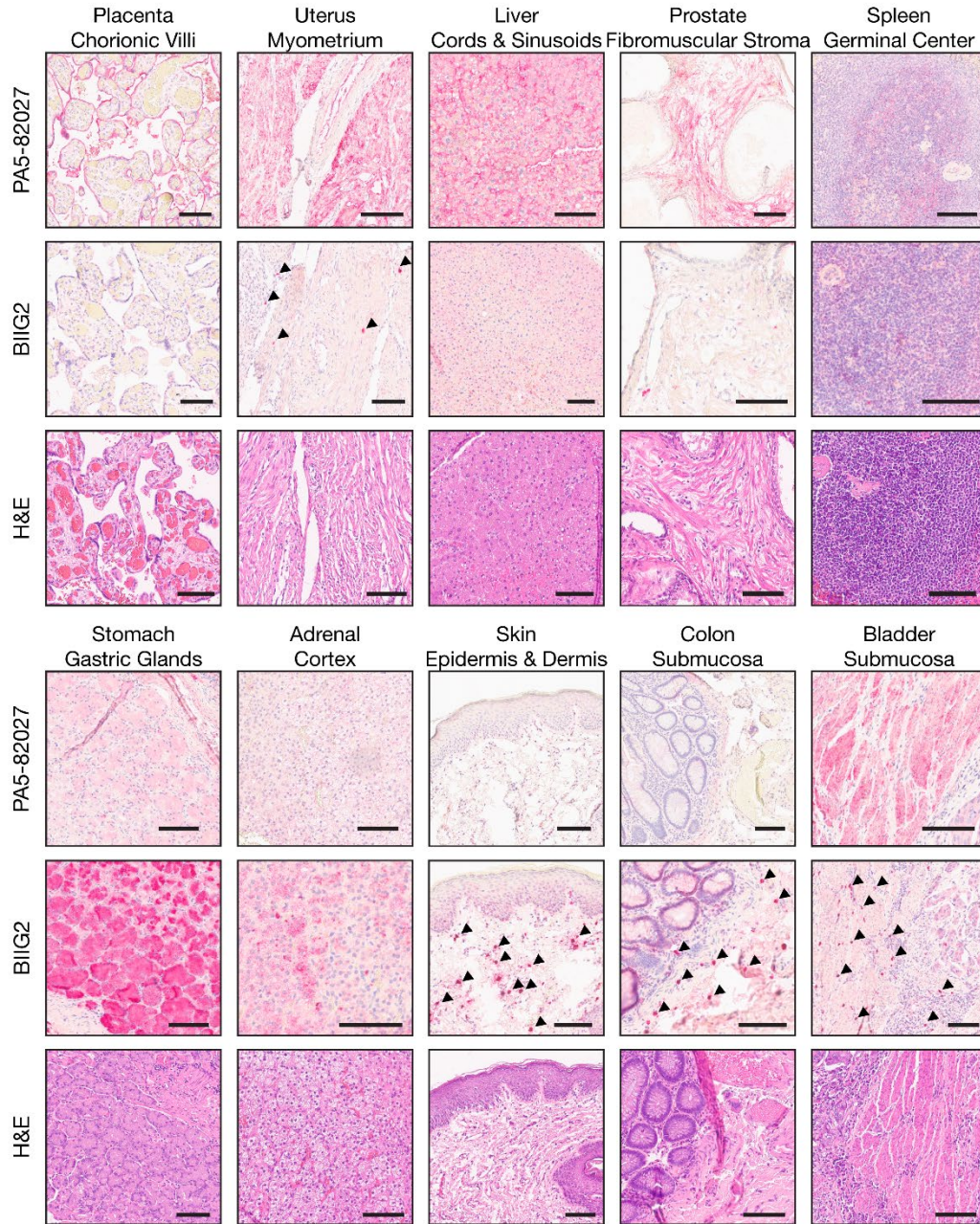


Figure 2 - S 1 Spheroid sprouting assay. HUVEC spheroids were imaged and measured for cumulative sprout length (CSL) in μm using WimSprout analysis software. Concentration of inhibitors are in the presence of 25 ng/mL VEGF-A. Basal conditions do not have VEGF-A. Data shown are mean $\pm$ SEM, n= 9-10 spheroids. Spheroids in which sprouting could not be assessed are excluded. One-way ANOVA was used to determine whether any concentration of antibody or Sunitinib could reduce VEGF-stimulated spheroid sprouting against the control group (25 ng/mL VEGF-A). Multiple comparison testing was used with Bonferroni's correction. Adjusted P values are shown and significance is determined by values less than 0.05.

A



B

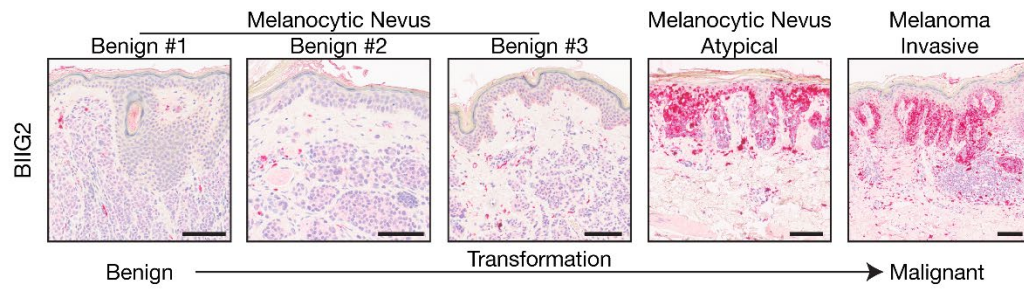


Figure 2 - S 2 Staining pattern of BIIG2 and polyclonal $\alpha 5$ integrin antibody PA5-82027 in diverse human tissue samples. (A) Immunostaining was performed on consecutive sections from a tissue microarray comprising 23 paraffin-embedded, formalin-fixed (FFPE) core samples of normal human tissues using BIIG2 or PA5-82027 as primary antibodies. Hematoxylin and Eosin (H&E) staining was also performed to enhance the visualization of tissue architecture. Arrows indicate specific clusters of fibroblasts within connective tissues that exhibit intense staining with the BIIG2 antibody, highlighting these clusters as regions of interest. In the zona fasciculata of the adrenal gland, spongiocytes—cells rich in lipid droplets—displayed a speckled staining pattern. Gastric glands, containing proteins and mucus, also showed staining with the BIIG2 antibody, but it appeared non-localized. The speckled and non-localized staining patterns observed in spongiocytes and gastric glands suggest potential non-specific staining. (B) Patient-derived biopsies of normal nevi, atypical nevi, and invasive cutaneous melanoma, representing the spectrum of melanocyte malignant transformation, reveal intense BIIG2 staining in atypical melanocytes and melanoma cells within the epidermis and upper dermis. In contrast, benign nevus samples exhibit sparse staining, with only faint staining observed in platelets and vasculature. Scale bars, 100 μ m.

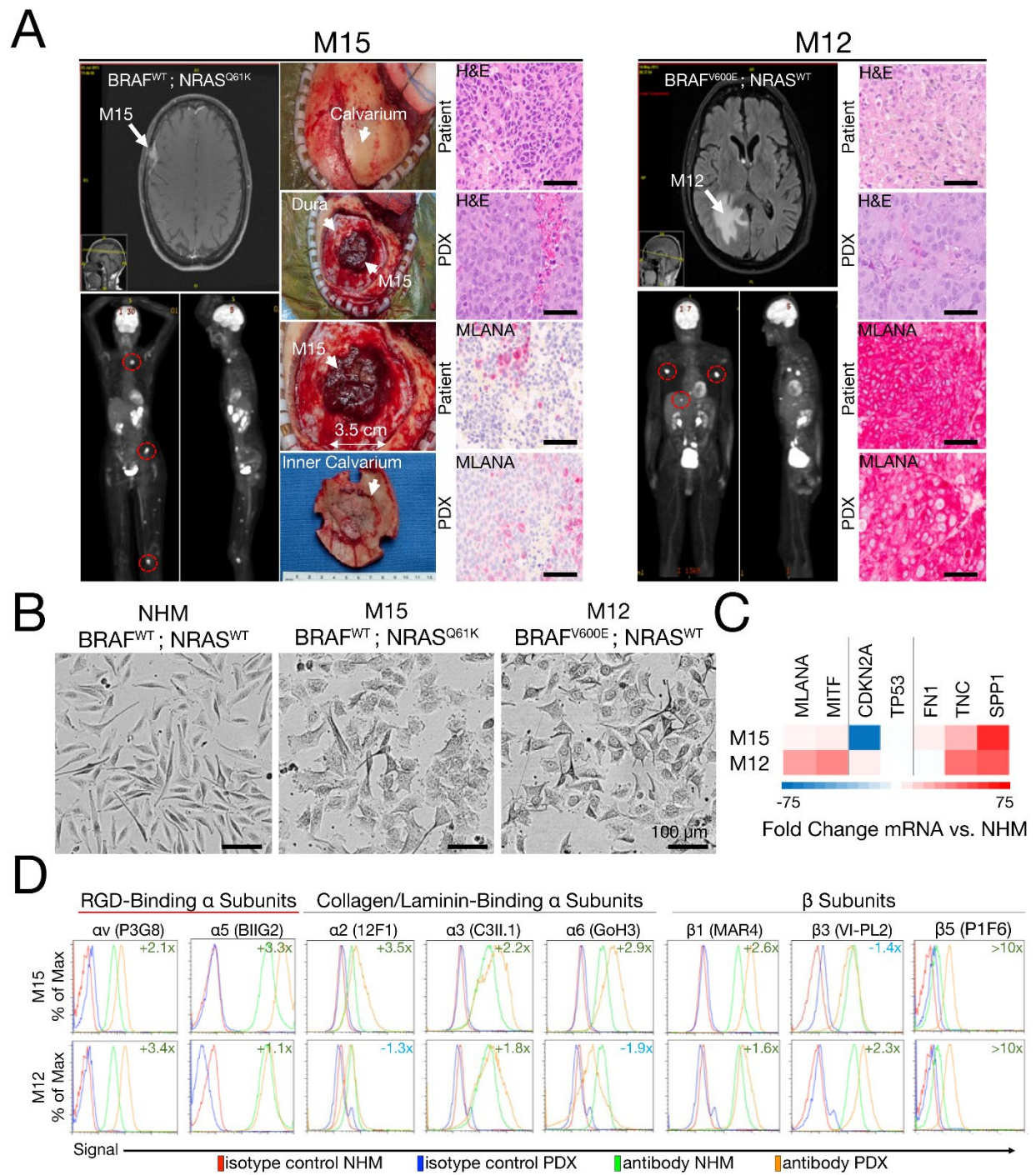
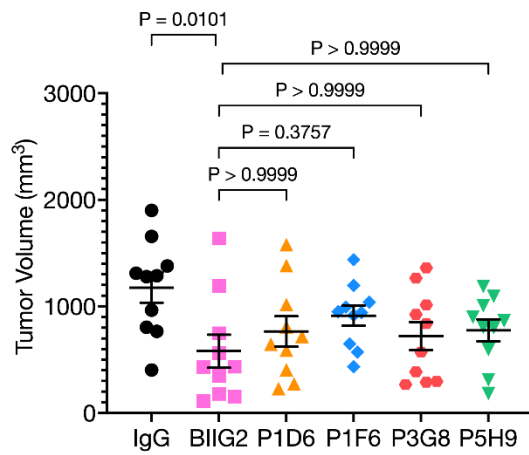


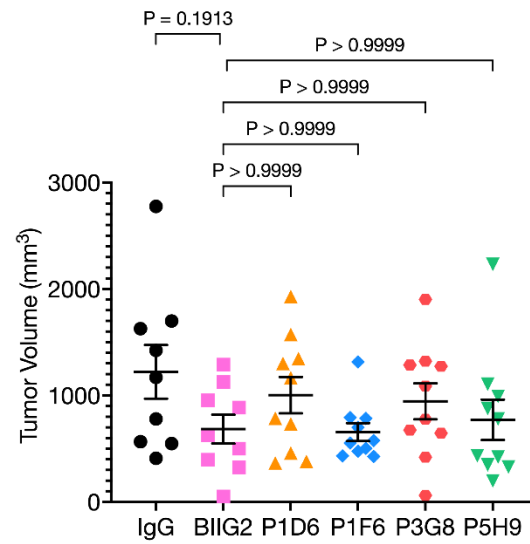
Figure 2 - S 3 Characterization of patient-derived xenografts of metastatic melanoma for evaluating the efficacy of integrin antibodies on tumor growth. (A) M15 cultures were established from a 66-year-old female patient with metastatic melanoma, including brain metastases. The M15 xenograft specifically originated from a right dural-based brain mass with involvement of the overlying calvarium (the dome-like upper part of the skull), as identified through magnetic resonance imaging (MRI; indicated by the arrow labeled M15). Positron emission tomography (PET) imaging further revealed diffuse metastases (some areas of metastasis are marked by red circles). Surgical images depict the exposed calvarium, and upon its removal, the melanoma brain metastasis becomes visible, surrounded by the dura mater (the outermost layer of the meninges). Gross examination revealed that the metastasis had a diameter of approximately 3.5 cm. Images from the surgery also show the inner surface of the calvarium. Histological analysis of the patient's brain tumor and the M15 xenograft grown in mice showed similar characteristics in H&E-stained sections, including densely packed nuclei and occasional mitotic figures. Furthermore, protein expression of MLANA (Melan-A), a melanocyte differentiation marker, was similarly sparse in both the patient's brain tumor and the M15 xenograft. This similarity highlights the fidelity of the PDX model in preserving essential tumor characteristics. The patient's disease began with a primary melanoma on the left ankle, accompanied by microscopic involvement of regional lymph nodes. Over time, the patient developed widespread metastases that did not respond to radiation therapy or immunotherapy with ipilimumab, a monoclonal antibody that targets CTLA-4 (cytotoxic T-lymphocyte-associated protein 4). The patient passed away two years after the initial diagnosis. M12 cultures were established from a 75-year-old male patient who initially presented with a brain metastasis from a metastatic melanoma of unknown primary. MRI identified a paramedian mass in the right parietal region, while PET imaging revealed multiple extracranial metastases (highlighted by red circles). Similar to the findings with the M15 xenograft, both H&E-stained sections and Melan-A-stained sections of the M12 xenograft grown in mice displayed similar characteristics to the patient's brain tumor. Notably, there was strong Melan-A/MLANA staining observed in both the patient's tumor sections and the corresponding M12 xenograft sections, demonstrating the consistency of the xenograft in maintaining one of the tumor's key features. The patient's tumor responded well to treatment with vemurafenib, a BRAF inhibitor, followed by immunotherapy with ipilimumab and pembrolizumab. The patient has remained free of disease for more than ten years since the diagnosis. Scale bars, 100 μ m. (B) Representative phase contrast microscopy images of normal human melanocytes (NHM) and M15 and M12 cultures are shown. NHM cells exhibit a spindled, elongated morphology characteristic of healthy melanocytes. In contrast, M12 cultures display a more varied morphology, with some elongated and some rounded cells, less distinct boundaries, and reduced dendritic processes. M15 melanoma cells are highly variable in shape and size, lacking the typical dendritic morphology and showing increased cell density and clustering, indicative of aggressive melanoma characteristics and a loss of normal melanocyte structure. Scale bars, 100 μ m. (C) Quantitative PCR was used to assess gene expression from extracted mRNA of NHM, as well as M15 and M12 PDX cultures. The fold change in mRNA expression in M15 and M12 cultures relative to NHM is depicted. MLANA, Melan-A; MITF, Melanocyte Inducing Transcription Factor; CDKN2A, Cyclin Dependent Kinase Inhibitor 2A; TP53, Tumor Protein P53; FN1, Fibronectin; TNC, Tenascin C; SPP1, Secreted Phosphoprotein 1. (D) Cell surface integrin expression on NHM, as well as M15 and M12 PDX cultures, using flow cytometry. Fold change in integrin surface expression, normalized for isotype control background, compared to NHM is shown.

A

M12 Xenograft



M15 Xenograft



B

M12 Xenograft

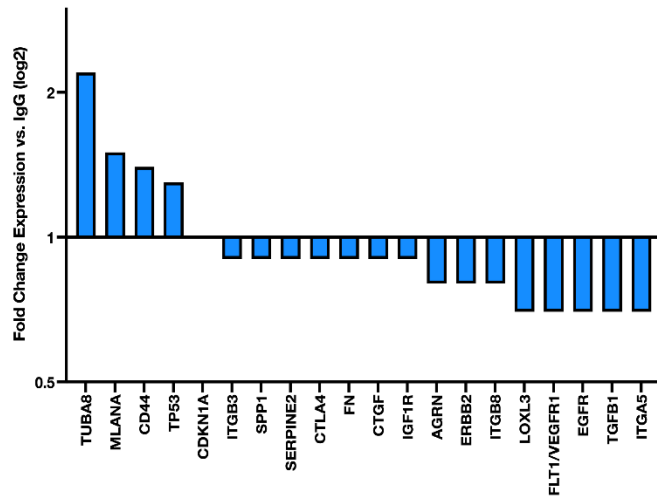


Figure 2 - S 4 Additional statistical analysis of BIIG2 efficacy and impacts of gene expression in M12 xenograft.

(A) Statistical analysis of BIIG2 effectiveness in reducing tumor volume against P1D6 and αv targeting antibodies. Data related to Figure 2B. Data are shown as individual tumor volume values with mean $\pm$ SEM; n=10 per antibody treatment, except for M15 where IgG control is n=9. A one-way ANOVA with a Bonferroni correction was performed to assess statistical significance of BIIG2 against the other antibodies. Adjusted P-values are shown and are considered significant if less than 0.05. **(B)** Analysis of tumors from M12 PDX showed that BIIG2 treatment of mice (n=9; 10 mg/kg twice weekly) induced the expression of the melanocyte differentiation marker Melan-A (MLANA) and cellular tumor antigen p53 (TP53) versus IgG (N=10) while inhibiting the expression of growth factors, growth factor receptors and the pro-angiogenic integrins ($\beta 3$, $\beta 8$ and $\alpha 5$).

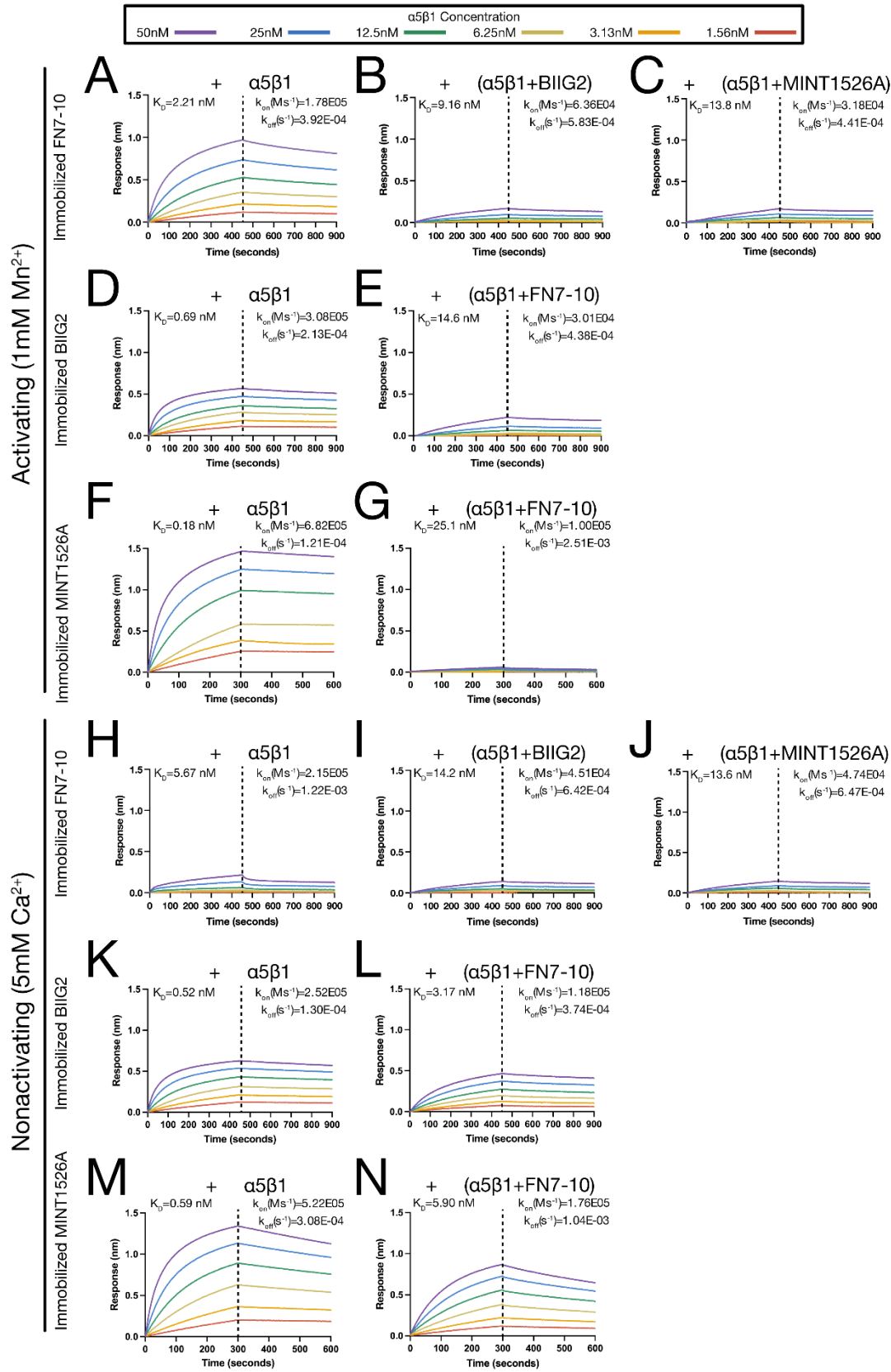
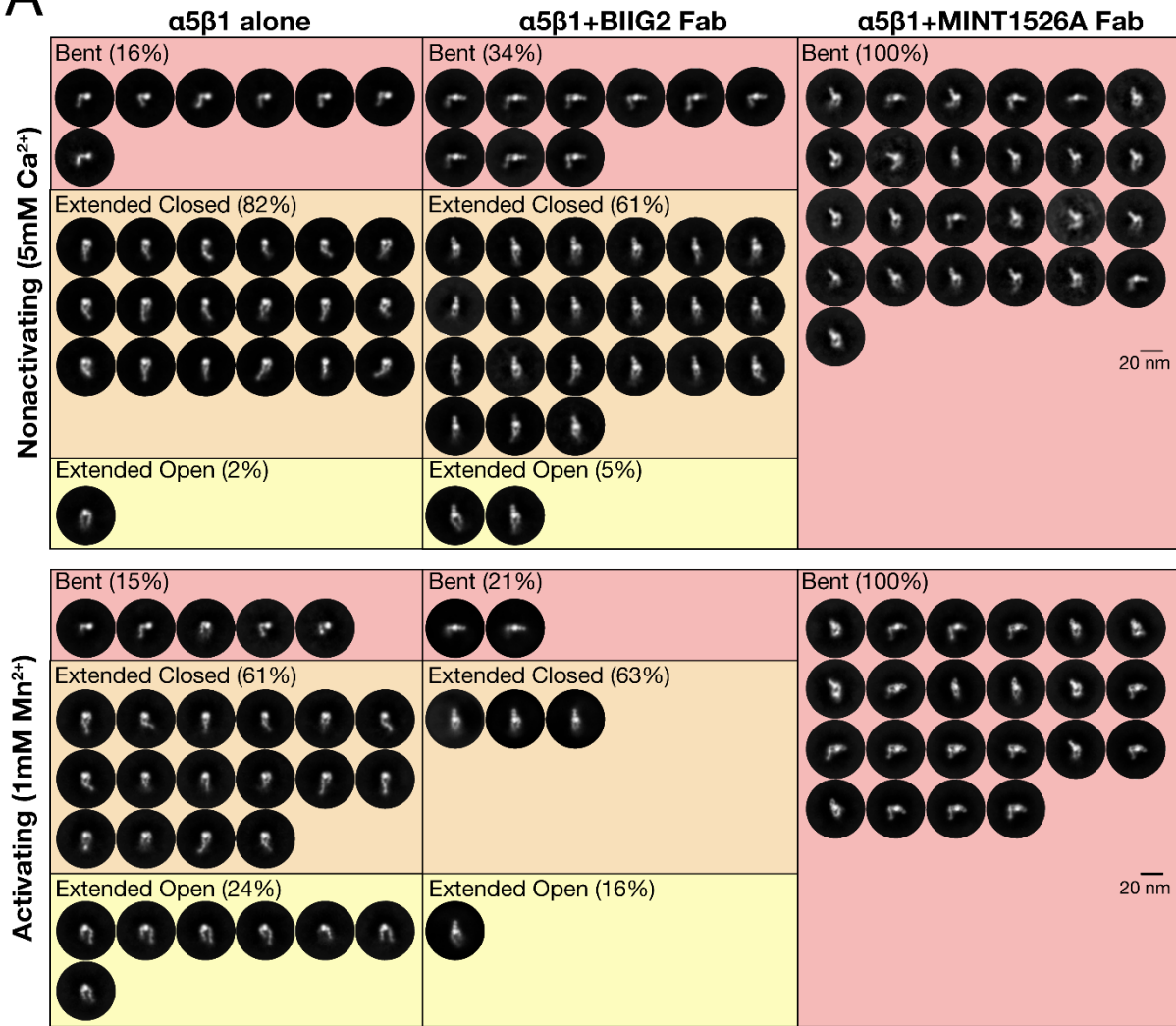


Figure 2 - S 5 Integrin and antibody BLI competition assay using a fragment of fibronectin (FN 7-10). Biolayer interferometry sensorgrams of competitive binding experiments. All experiments were loaded with 5 $\mu\text{g/mL}$ of immobilized protein (BIIG2, MINT1526A, or FN 7-10). The color of the sensorgrams denotes the concentration of $\alpha 5\beta 1$ ectodomain. The immobilized protein (BIIG2, MINT1526A, or FN 7-10) is denoted on the left and the titles of each sensorgram indicates the analyte (integrin $\alpha 5\beta 1$ alone or pre-incubated integrin complex).

A



B

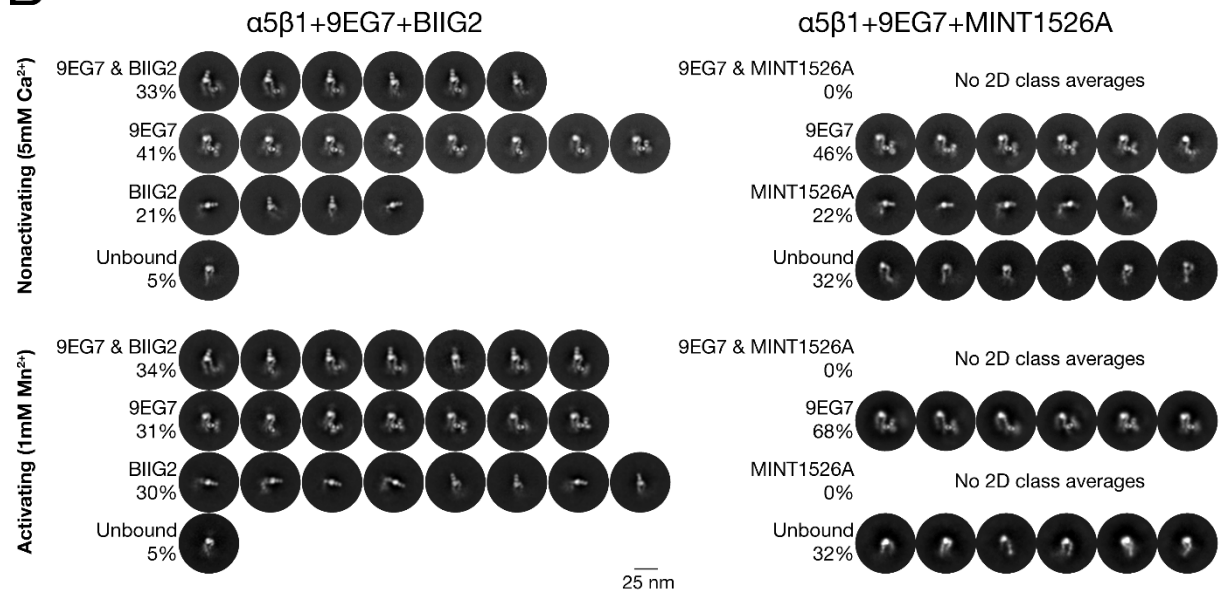


Figure 2 - S 6 Final nsEM 2D classes of apo $\alpha 5\beta 1$, $\alpha 5\beta 1$:BIIG2 Fab and $\alpha 5\beta 1$:MINT1526A Fab, sorted by conformation and 2D classes of $\alpha 5\beta 1$:9EG7:BIIG2 Fab and $\alpha 5\beta 1$:9EG7:MINT1526A Fab complexes. (A) All negative stain EM 2D classes are shown for each condition evaluated to determine whether the binding of the Fabs influences $\alpha 5\beta 1$ conformation. Scale bar is 20 nm. **(B)** Negative stain EM 2D classes of integrin $\alpha 5\beta 1$ pre-incubated with 9EG7 antibody prior to addition of BIIG2 or MINT1526A Fabs. Scale bar is 25nm. Due to the varying particle numbers in each class and condition, the number of classes is not representative of the total number of particles contributing to that conformation; total particle percentages are listed.

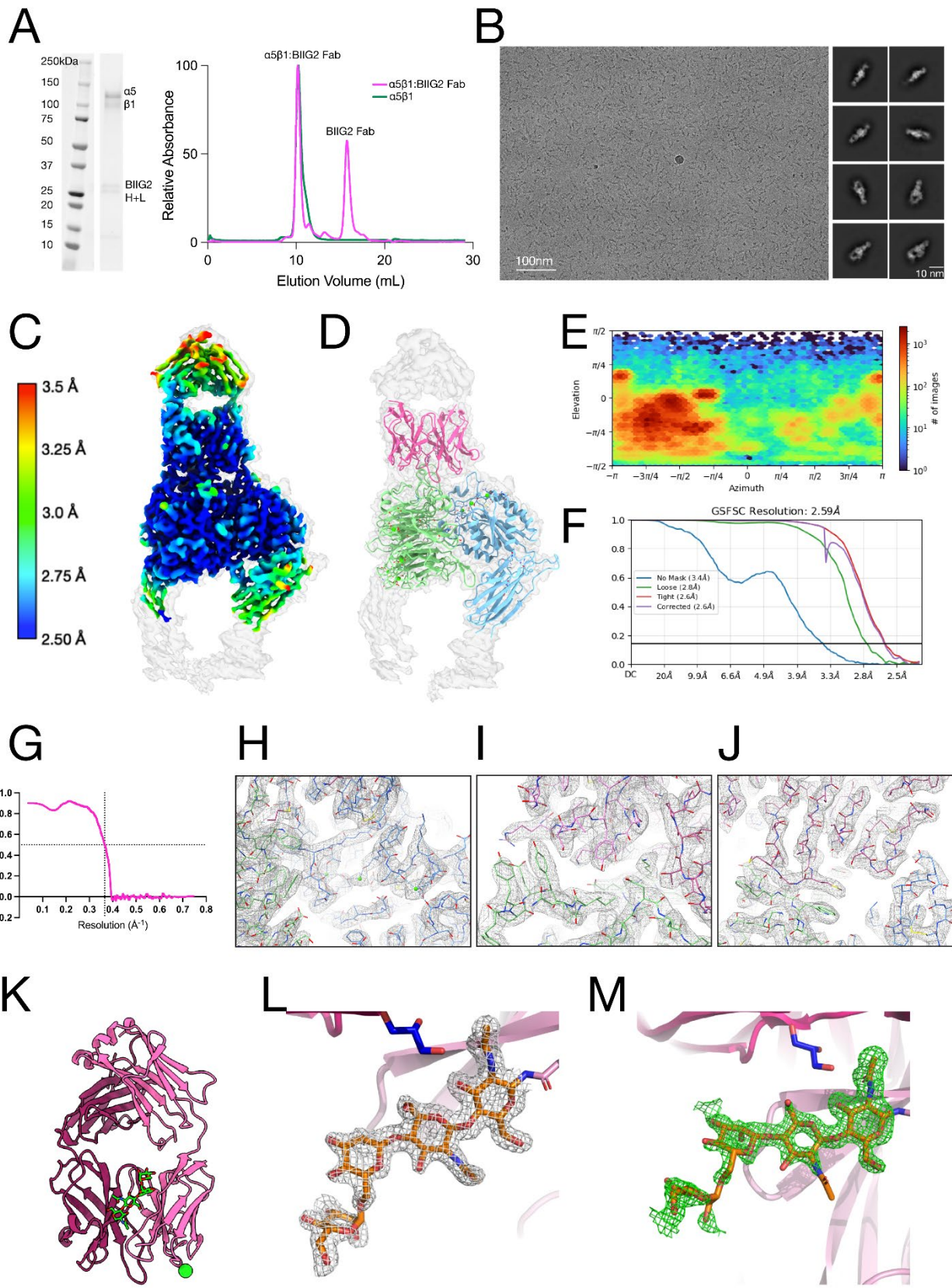


Figure 2 - S 7 BIIG2- $\alpha 5\beta 1$ complex and structural supporting data. (A) SDS-PAGE of the first peak from Size Exclusion Chromatography (SEC) of the BIIG2: $\alpha 5\beta 1$ complex, which contains both $\alpha 5\beta 1$ and BIIG2 Fab (H, heavy chain; L, light chain fragments). SEC chromatogram of $\alpha 5\beta 1$ alone (green) and $\alpha 5\beta 1$:BIIG2 Fab complex (pink), with an excess of BIIG2 Fab. (B) Representative cryoEM micrograph and 2D class averages of the $\alpha 5\beta 1$:BIIG2 Fab complex. Scale bars are 100 nm and 10 nm, respectively. (C,D) The locally refined final cryoEM map colored by resolution (C) or with the atomic model superimposed on the unsharpened map at a low threshold in semi-transparent white (D). $\alpha 5$ is green; $\beta 1$ is blue; BIIG2 is pink. (E) Angular distribution plot of particles used in the final cryoEM reconstruction. (F) Gold standard Fourier Shell Correlation (FSC). (G): Map to model FSC. (H-J) Map and model showcasing clear density for key areas. The coordinated cations SyMBAS, MIDAS, and ADMIDAS (H); the BIIG2 light-chain binding epitope, related to **Figure 2 - 8 (I)**, the BIIG2 heavy-chain binding epitope, related to **Figure 2 - 8E (J)** $\alpha 5$ is green; $\beta 1$ is blue; BIIG2 is light-chain is lighter pink, BIIG2 is heavy-chain is darker pink, cations are neon green. (K) Structure of antigen binding fragment of BIIG2 shown in cartoon representation (paratope on the bottom, light chain in light pink, heavy chain in dark pink) with ligands and solvent molecules depicted in stick representation. Four saccharide units of N-linked glycan N60 are shown in green stick representation and single chloride ion is shown as a green sphere. (L) Close-up view of N-linked glycan, with σ_A -weighted $2F_o - F_c$ electron density map (grey mesh, contoured at 1.0 σ) and N60 shown in stick representation. (M) Close-up view of N-linked glycan with polder (OMIT) map (green mesh, contoured at 3.0 σ) and N60 shown in stick representation. Protein structures and maps were generated using PyMOL (Schrödinger, Inc.) and UCSF ChimeraX.

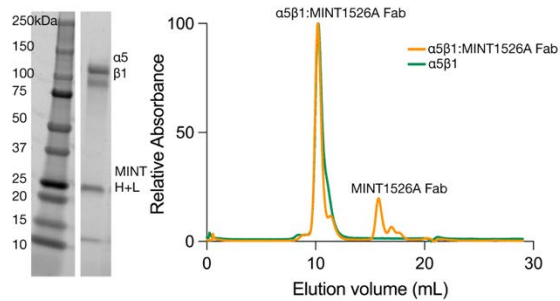
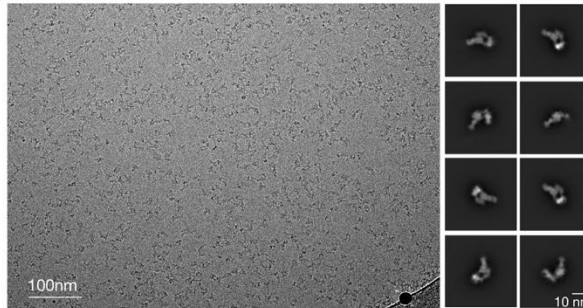
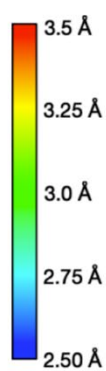
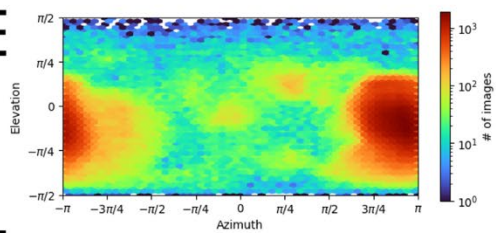
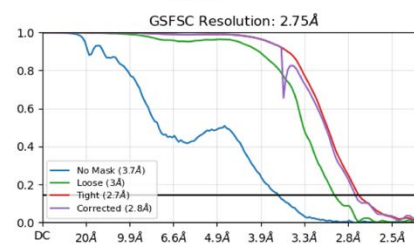
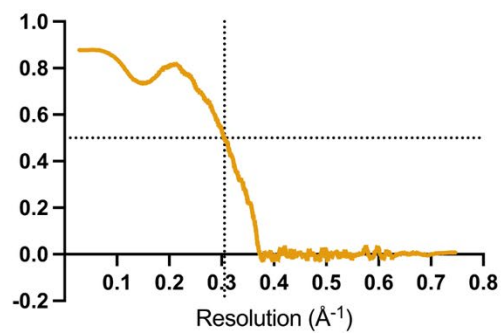
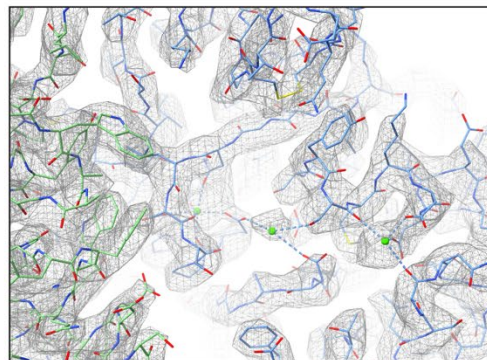
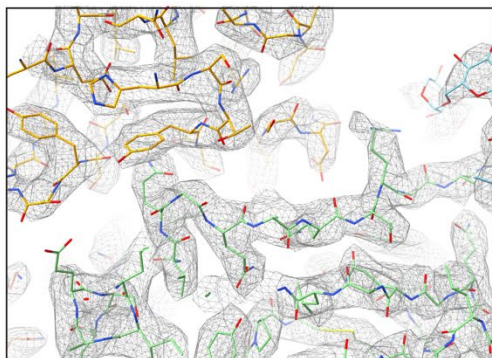
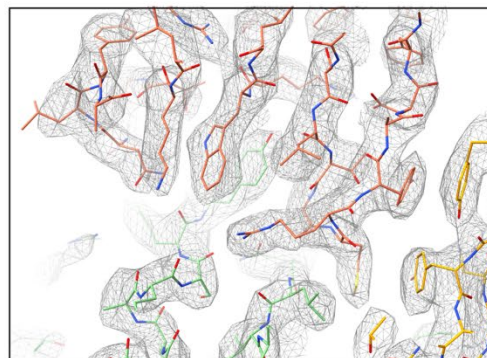
A**B****C****D****E****F****G****H****I****J**

Figure 2 - S 8 MINT1526A- $\alpha 5\beta 1$ complex and supporting cryoEM data. (A) SDS-PAGE of the first peak from Size Exclusion Chromatography (SEC) of the MINT1526A: $\alpha 5\beta 1$ complex, which contains both $\alpha 5\beta 1$ and MINT1526A Fab (H, heavy chain; L, light chain fragments). SEC chromatogram of $\alpha 5\beta 1$ alone (green) and $\alpha 5\beta 1$:MINT1526A Fab complex (orange), with an excess of MINT1526A Fab. (B) Representative cryoEM micrograph and 2D class averages of the $\alpha 5\beta 1$:MINT1526A Fab complex. Scale bars are 100 nm and 10 nm, respectively. (C,D) The locally refined final cryoEM map colored by resolution (C) or with the atomic model superimposed on the unsharpened map at a low threshold in semi-transparent white (D). $\alpha 5$ is green; $\beta 1$ is blue; MINT1526A is orange. (E) Angular distribution plot of particles used in the final cryoEM reconstruction. (F) Gold standard Fourier Shell Correlation (FSC). (G): Map to model FSC. (H-J) Map and model showcasing clear density for key areas. The coordinated cations SyMBAS, MIDAS, and ADMIDAS (H); the MINT1526A heavy-chain binding epitope, related to **Figure 2 - 9E**. (I), the MINT1526A light-chain binding epitope, related to **Figure 2 - 9F** (J) $\alpha 5$ is green; $\beta 1$ is blue; MINT1526A is light-chain is lighter orange, BIIG2 is heavy-chain is darker orange, cations are neon green. Protein structure images and maps were generated using UCSF ChimeraX.

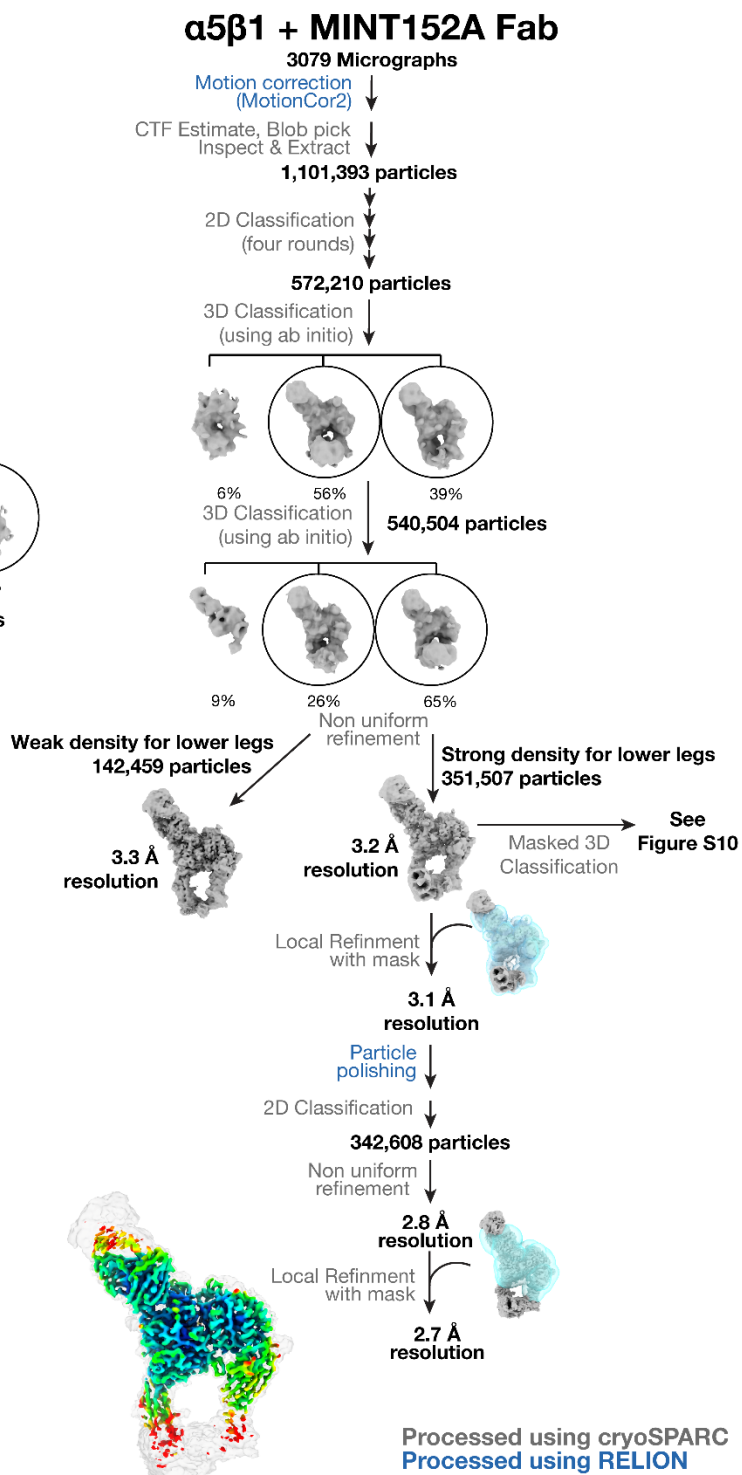
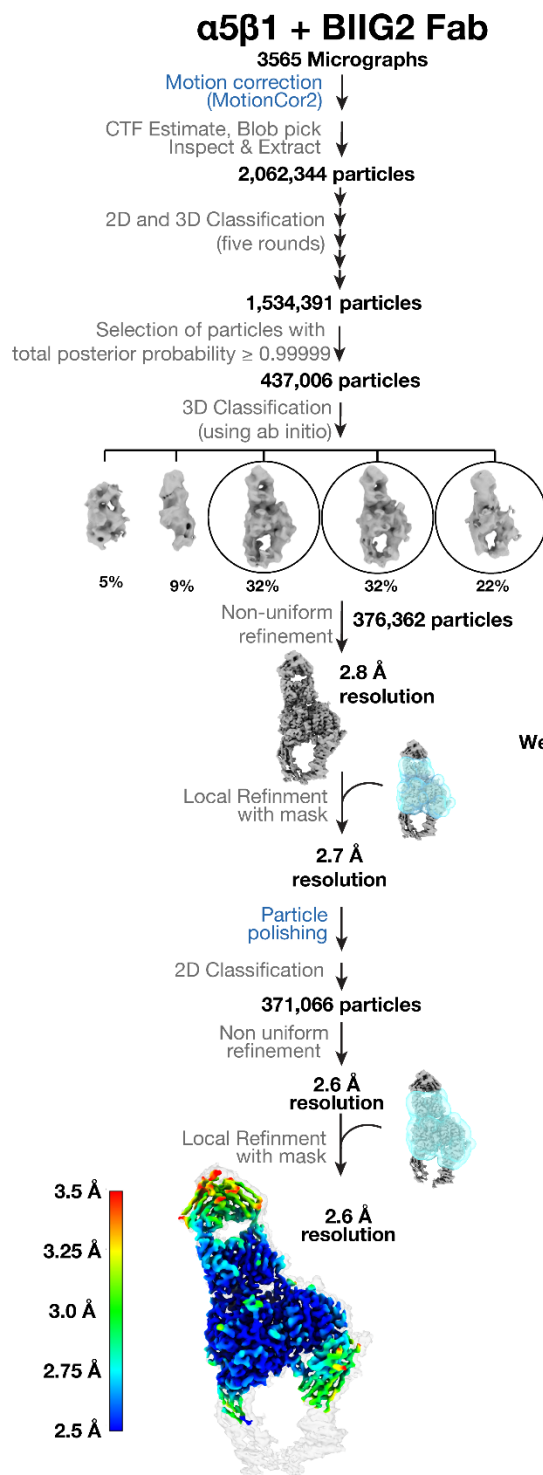
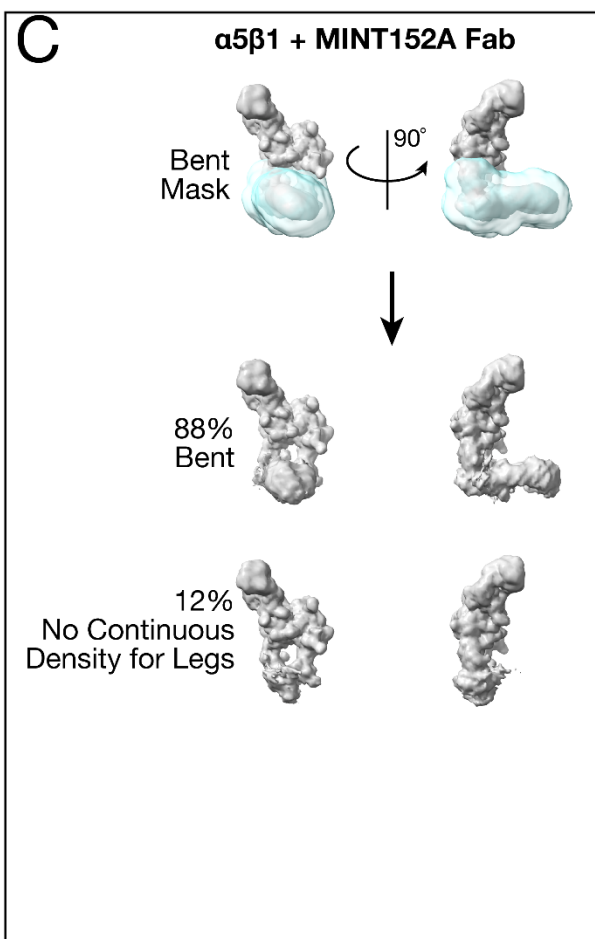
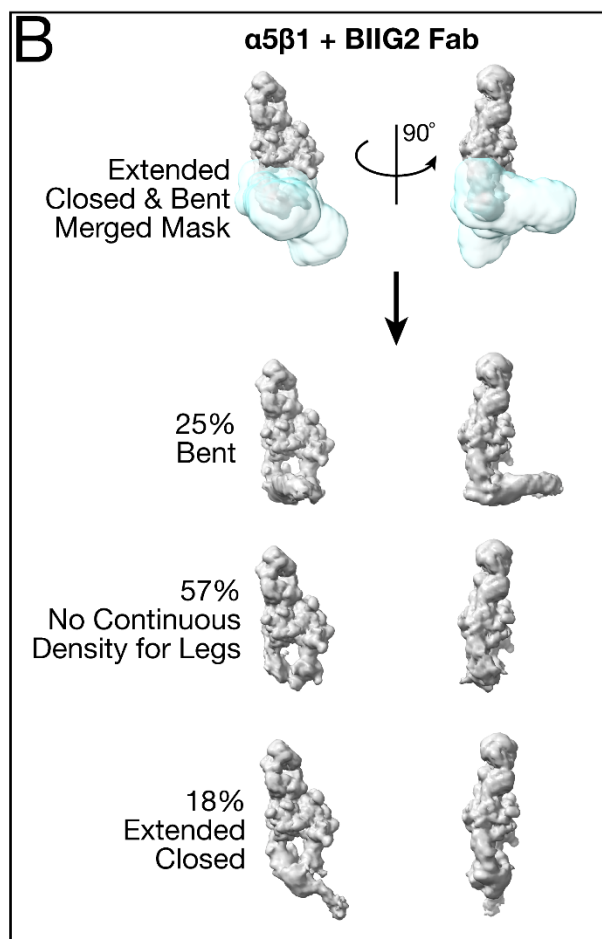
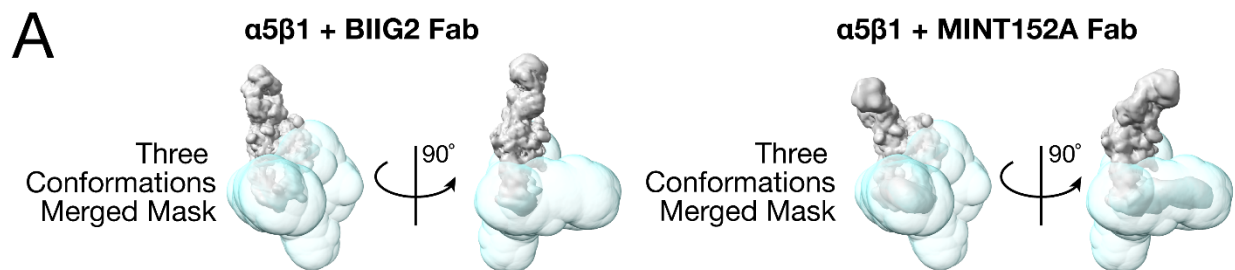


Figure 2 - S 9 CryoEM Processing Schematic of $\alpha 5\beta 1$:BIIG2 Fab and $\alpha 5\beta 1$:MINT1526A Fab. A schematic flowchart highlighting the major classification and refinement steps for the $\alpha 5\beta 1$:BIIG2 and $\alpha 5\beta 1$:MINT1526A complexes. For each complex, a single dataset was collected. Particle numbers at significant steps are shown, and percentages of particles contributing to maps in 3D classification are shown. Intermediate maps are shown in gray and masks used for local refinement in semi-transparent blue. Final maps colored by local resolution at high threshold superimposed on a lower threshold map in semi-transparent white.



D $\alpha 5\beta 1$ + MINT152A Fab, Bent Conformation

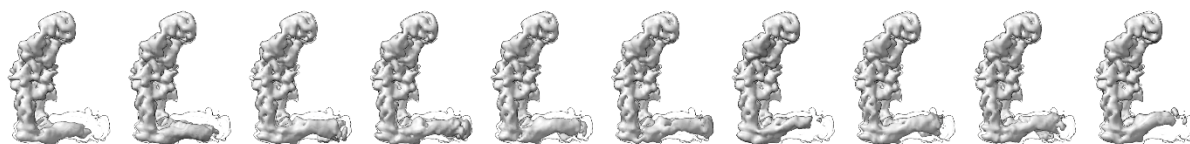


Figure 2 - S 10 3D classification defines the conformational states of integrin bound to BIIG2 or MINT1526A. (A) First, the same soft, dilated mask encompassing the lower leg region was used for all three integrin conformations to determine which conformations were available when integrin was bound to either BIIG2 (left) or MINT1526A (right). The mask was made by merging the following maps: bent integrin ($\alpha 5\beta 1$ + MINT1526A map, this work), extended open integrin ($\alpha v\beta 8$, EMD-7939)³²¹, and extended open ($\alpha 5\beta 1$, EMD-45655)³⁸². The BIIG2-bound dataset revealed lower legs in the bent and extended closed conformation, whereas the MINT1526A-bound dataset only showed lower legs in a bent conformation. (B, C) For a subsequent round of 3D classification, a mask encompassing the lower legs in a bent and extended closed conformation was used for BIIG2-bound integrin (B); for MINT1526A-bound integrin, a mask encompassing the lower leg region in only the bent conformation was used (C). Particles were sorted into twelve classes, and representative classes are shown. Masks are shown in semi-transparent cyan, maps in gray. (D) 3D classification illustrates the flexibility of the integrin lower legs of the $\alpha 5\beta 1$:MINT1526A complex. The ten (of twelve) classes that showed strong density for the entire leg regions are shown. The integrin angle of rotation about the knee region ranges from 90 to 73 degrees, in agreement with previous data²⁰⁷. Each panel depicts a single solid gray map overlaying the nine other maps shown in semi-transparent white.

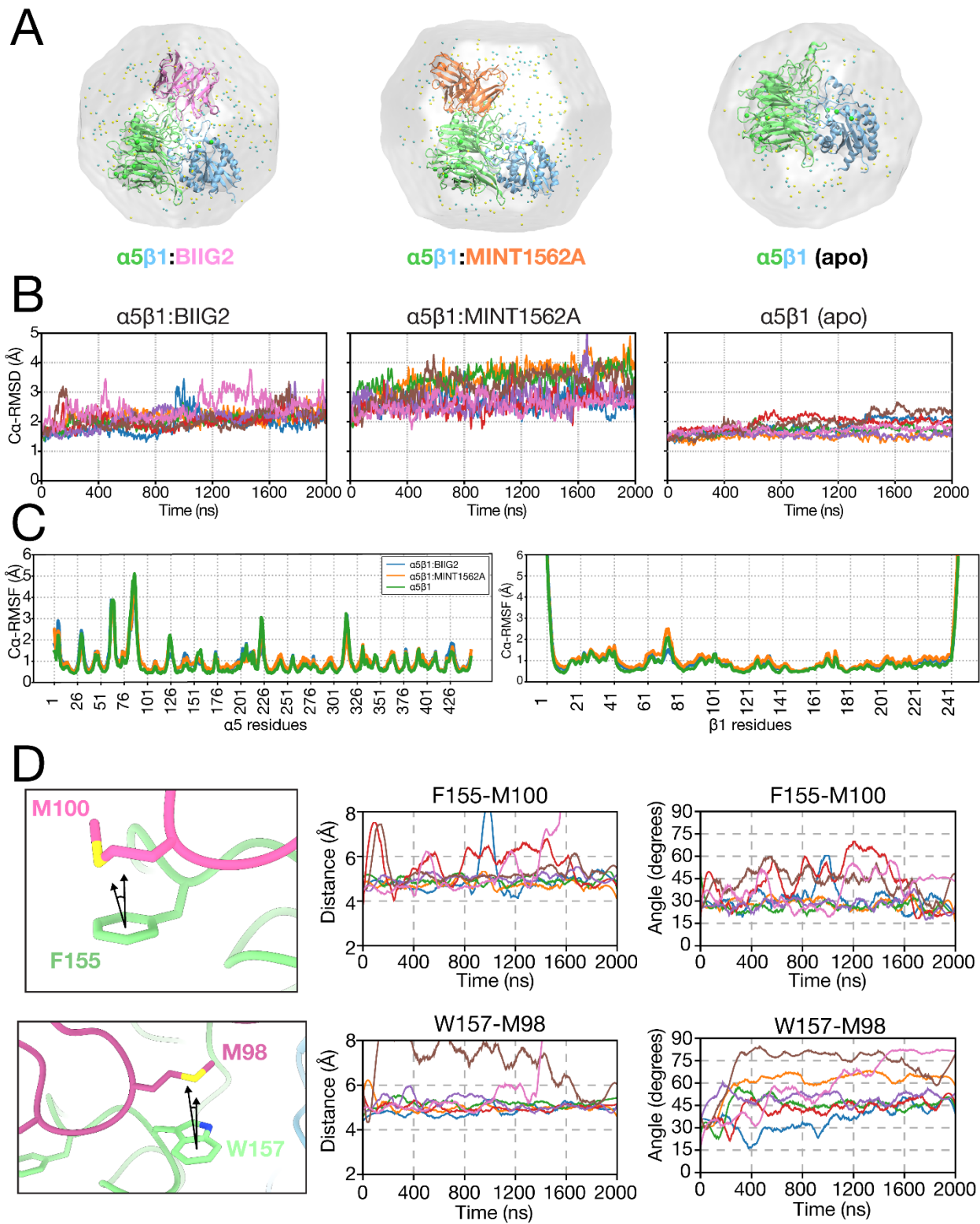
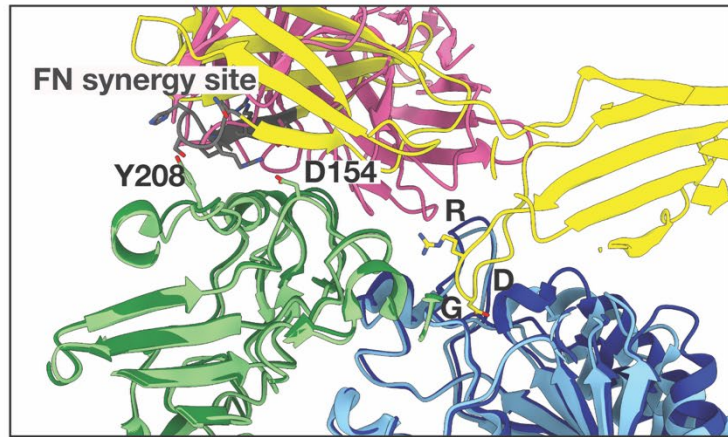
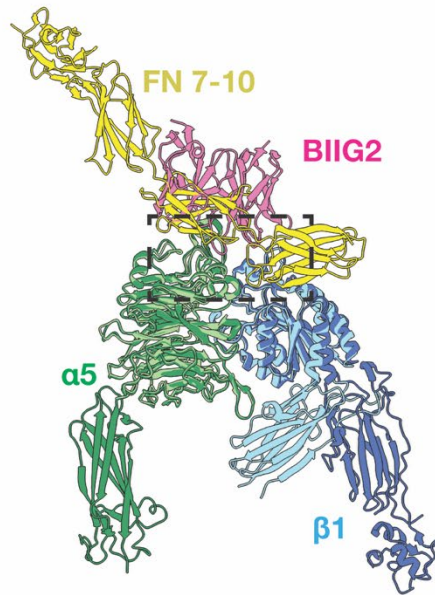


Figure 2 - S 11 Molecular dynamics simulations of apo $\alpha 5\beta 1$ or in complex with BIIG2 and MINT1526A Fabs.

(A) Solvated $\alpha 5\beta 1$:antibody MD system. The protein complexes were solvated in a truncated octahedron and NaCl ions. **(B)** C α -RMSD of $\alpha 5\beta 1$:antibody simulations. The seven MD replicates of 2000 ns are individually plotted. The initial structure was used as the reference structure for RMSD calculations. **(C)** Average per-residue C α -RMSF of the $\alpha 5$ and $\beta 1$ headpiece. Values represent average C α -RMSF across seven 2000 ns replicates. **(D)** The location of the sulfur of the methionine-aromatic interactions between BIIG2 and $\alpha 5\beta 1$. Distances are measured between the center of the aromatic ring of F155 (upper row) and W157 (lower row), and the center of the sulfur atoms. Arrows represent the normal vector of the aromatic plane and the angle measured. Distance and angle measurements in correlation with time, recorded with molecular dynamics (MD) simulations.

A



B

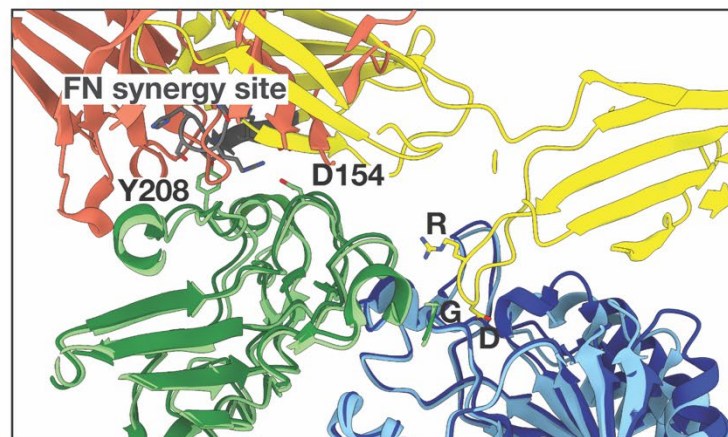
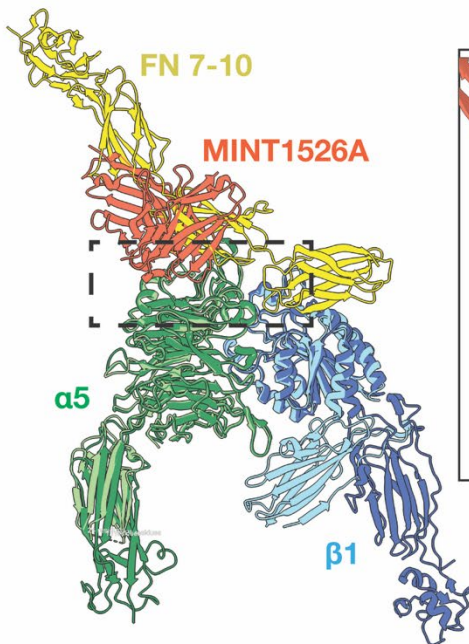


Figure 2 - S 12 BIIG2 and MINT1526A occlude Fibronectin binding. The previously determined structure (PDB: 7NWL) of integrin $\alpha 5 \beta 1$ (dark green, dark blue) bound to fibronectin domains 7-10 (yellow) is superimposed with either our structure of $\alpha 5 \beta 1$ (light green, light blue) bound to **(A)** BIIG2 fab (pink) or **(B)** MINT1526A fab (orange). The steric clash between fibronectin and BIIG2 occurs at the fibronectin type III domain 9; the clash between MINT1526A and fibronectin occurs at the fibronectin type III domain 9 and blocks the synergy binding site.

Table 2 - S 1 Staining patterns of anti-human $\alpha 5$ integrin antibodies on normal FFPE tissue microarray (#BSB-0298). PA5-82027, polyclonal rabbit $\alpha 5$ integrin antibody, antigen-verified; P1D6, monoclonal mouse $\alpha 5$ integrin antibody; BIIG2, monoclonal rat $\alpha 5$ integrin antibody. Related to **Figure 2 - S 2**.

Tissue Type	PA5-82027	P1D6	BIIG2
Chorionic Villi	+++ (Syncytioblast)	-	-
Breast	++ (Ducts)	-	+++ (Fibroblasts)
Myometrium	+++ (Smooth Muscle)	-	+++ (Fibroblasts)
Cervix	-	-	+++ (Fibroblasts)
Fallopian Tube	-	+ (Smooth Muscle)	+++ (Fibroblasts)
Brain	-	-	-
Stomach	+ (Gastric Glands)	++ (Gastric Glands)	+++ (Gastric Glands)
Adrenal Cortex	+ (Cell Surface)	+ (Cytoplasm)	++ (Cytoplasm)
Pancreas	++ (Acini)	-	++ (Acini)
Salivary	++ (Striated Ducts)	+ (Striated Ducts)	+ (Striated Ducts)
Colon	+ (Smooth Muscle)	-	+++ (Fibroblasts)
Liver	+++ (Sinusoids)	+++ (Cords/Cytoplasm)	-
Kidney	+ (Tubules)	+ (Tubules)	+ (Tubules)
Thyroid	-	-	-
Lung	++ (Alveolar Ducts)	-	-
Skin	-	-	+++ (Fibroblasts)
Bladder	+++ (Smooth Muscle)	-	+++ (Fibroblasts)
Testis	-	-	-
Prostate	+++ (Fibromuscular Stroma)	-	-
Spleen	++ (Germinal Center)	-	-
Tonsil	+ (Germinal Center)	-	-
Bone Marrow	++ (Megakaryocytes, Others)	-	+++ (Megakaryocytes, Others)
Thymus	-	-	-

Table 2 - S 2 X-ray crystallography analysis of BIIG2 Fab fragment.

Data Set	BIIG2 Fab ^a (PDB 8R38)
Data collection	
X-ray source	MAX-IV Laboratory - BioMAX
Wavelength, Å	0.9537
Space group	$P2_12_12_1$
Unit cell parameters – a, b, c (Å)	62.9, 80.9, 87.5
Resolution (Å) ^b	43.7 - 1.4 (1.43 - 1.40)
No. of unique reflections ^b	169,111 (8,404)
Multiplicity ^b	6.6 (4.5)
Completeness (%) ^b	99.6 (96.4)
Mean $I/\sigma(I)$ ^b	7.59 (0.23)
$CC_{1/2}$ (%) ^b	99.8 (10.4)
Wilson B -factor (Å ²) ^b	21.3
Refinement	
$R_{\text{work}}/R_{\text{free}}$ (%) ^c	19.9 / 22.4
Average B factors (Å ²)	
Protein	26.1
Ligand	38.0
Solvent	32.9
Number protein chains in the a.u.	2
Number of non-hydrogen atoms	4,183
Protein	3,871
Ligand	89
Solvent	223
r.m.s.d. from ideal geometry	
Bond lengths (Å)	0.01
Bond angles (deg.)	1.7
Ramachandran plot	
Favored (%)	98.1
Allowed (%)	1.7
Outliers (%)	0.2

^a Friedel pairs were treated as different reflections^b Values in parentheses refer to highest-resolution shell^c R_{free} was calculated from 5% of randomly selected reflections

Table 2 - S 3 CryoEM data collection, refinement, and validation statistics.

Data Set	$\alpha 5\beta 1$:BIIG2 Fab (EMDB-44386) (PDB 9B9J)	$\alpha 5\beta 1$:MINT1526A Fab (EMDB-44387) (PDB 9B9K)
Data Collection and Processing		
Magnification	36,000	36,000
Voltage (kV)	200	200
Electron exposure ($e^-/\text{\AA}^2$)	50	50
Defocus range (μm)	1.4 – 1.8 (nominal)	1.4 – 1.8 (nominal)
Pixel size ($\text{\AA}$)	1.122	1.122
Symmetry imposed	C1	C1
Initial particle images (no.)	2,425,530	1,101,393
Final particle images (no.)	371,066	342,608
Map resolution ($\text{\AA}$)	2.7	2.8
FSC threshold	0.143	0.143
Map resolution range ($\text{\AA}$)	2.5 – 6.5	2.6 - 8.0
Refinement		
Initial model used (PDB code)	3VI3 (chains A, B) BIIG2 Fab (this study, 8R38)	7NXD (chains A,B) MINT1526A Fab (this study)
Model resolution ($\text{\AA}$)	2.7	3.3
FSC threshold	0.5	0.5
Model resolution range ($\text{\AA}$)	2.5 – 6.5	2.5 – 8.0
Map sharpening B factor ($\text{\AA}^2$)	-50	-75
Model composition		
Non-hydrogen atoms	8,518	9,150
Protein residues	1,055	1,142
Carbohydrates	32	32
Metal Ions	7	7
B factors ($\text{\AA}^2$)		
Protein	93.3	106.4
Ligand	N/A	N/A
R.m.s deviations		
Bond lengths ($\text{\AA}$)	0.01	0.01
Bond angles ($^\circ$)	2.0	2.0
Validation		
MolProbity score	1.09	1.27
Clashscore	1.02	1.95
Poor rotamers (%)	0.34	0.42
Ramachandran plot		
Favored (%)	95.4	95.7
Allowed (%)	4.2	4.3
Disallowed (%)	0.4	0.0

Table 2 - S 4 Interactions of $\alpha 5\beta 1$ with BIIG2 and MINT1526A Fabs.

α5β1:BIIG2 interactions						
α5β1 Integrin			BIIG2 Fab			Interaction Type
Residue		Subunit	Residue		CDR	
E	81	α	K	27 (L)	CDRL1	Salt bridge
E	124	α	K	92 (L)	CRDL3	Salt bridge
E	124	α	Y	94 (L)	CDRL3	Hydrogen bond
K	125	α	H	91 (L)	CDRL3	Hydrogen bond
D	154	α	K	31 (L)	CDRL1	Hydrogen bond
D	154	α	Y	32 (L)	CDRL1	Hydrogen bond
Y	205	α	R	28 (L)	CDRL1	Hydrogen bond
Y	208	α	S	30 (L)	CDRL1	Hydrogen bond
E	125	α	D	95 (H)	CDRH3	Hydrogen bond
E	125	α	R	96 (H)	CDRH3	Hydrogen bond
E	126	α	T	97 (H)	CDRH3	Hydrogen bond
E	126	α	N	33 (H)	CDRH1	Hydrogen bond
D	154	α	T	100A (H)	CDRH3	Hydrogen bond
S	156	α	G	99 (H)	CDRH3	Hydrogen bond
S	189	β	G	54 (H)	CDRH2	Hydrogen bond
E	190	β	R	71 (H)	Framework	Salt bridge
α5β1:MINT1526A interactions						
α5β1 Integrin			MINT1526A Fab			Interaction Type
Residue		Subunit	Residue		CDR	
R	144	α	P	52C (H)	CDRH2	Hydrogen bond
E	202	α	K	52 (H)	CDRH2	Hydrogen bond
E	202	α	R	99 (H)	CDRH3	Hydrogen bond
S	203	α	R	99 (H)	CDRH3	Hydrogen bond
L	212	α	R	99 (H)	CDRH3	Hydrogen bond
Y	204	α	N	53 (H)	CDRH2	Hydrogen bond
Y	205	α	W	33 (H)	CDRH1	Hydrogen bond
Y	205	α	L	95 (H)	CDRH3	Hydrogen bond
E	207	α	R	32 (H)	CDRH1	Hydrogen bond
Y	208	α	R	32 (H)	CDRH1	Hydrogen bond
Y	208	α	Y	102 (H)	CDRH3	Hydrogen bond
L	212	α	G	97 (H)	CDRH3	Hydrogen bond
Q	214	α	T	32 (L)	CDRL1/ Framework	Hydrogen bond
Q	214	α	S	91 (L)	CDRL3	Hydrogen bond
R	220	α	D	52A (L)	CDRL2	Hydrogen bond

2.4 *De novo* protein design of integrin $\alpha 5\beta 1$ modulating proteins

This section is published in full as: “*De Novo* Design of Integrin $\alpha 5\beta 1$ Modulating Proteins to Enhance Biomaterial Properties”.

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Author contribution: My contributions were preparing integrin $\alpha 5\beta 1$ for electron microscopy studies and analysis the electron microscopy maps and models. Therefore, this section focuses on the results that was a direct output of my contributions within the biophysical assays and cryoEM studies along with the appropriate introduction and discussions. For the complete data and conclusions, refer to the published manuscript.

Introduction

This section aims to leverage the development in computation and machine learning algorithms to de novo design proteins that can be conformationally modulating. Small molecules offer many benefits within the therapeutic spaces such as passing biological membranes for tissue penetration, oral administration and manufacturing. But can fall short with specificity and molecule half-life³⁸³. Antibodies provide high specificity, integration with the human immune system and a long half-life. However, these have limited tissue penetration, high manufacturing costs and cannot be orally administered. With designed proteins, these are small, stable and can be controlled in silico prior. This massively decreases the amount of time spent during screening as this can be performed in silico. The time saved can be focused on the top candidates for in vitro screening.

Integrin $\alpha 5\beta 1$ is one of the principal receptors for fibronectin (FN), a major component of extracellular matrix (ECM) component that is extensively expressed in various cells and tissues. The interactions between $\alpha 5\beta 1$ and FN are vital for cell attachment and migration, making them integral to various stages of development and tissue regeneration, notably in wound healing, bone regeneration, and stem cell therapy^{226,384–386}. However, the clinical use of full-length FN or its main interacting RGD (Arg-Gly-Asp) motif on biomaterials for regenerative purposes has been challenging. Full-length FN, typically derived from human plasma, poses challenges for large-scale production and is vulnerable to protease cleavage^{387,388}. Conversely, while the RGD peptide can be easily manufactured and is widely used in biomaterial coatings, it does not elicit the desired cellular responses and does not consistently enhance bone formation in vivo,^{389–391} perhaps because of the low affinity of RGD peptides to $\alpha 5\beta 1$ compared to FN³⁹² or because of the broad reactivity with the eight RGD/binding integrins^{320,393}. The $\alpha 5\beta 1$, $\alpha 8\beta 1$, $\alpha \nu\beta 1$, $\alpha \nu\beta 3$, $\alpha \nu\beta 5$, $\alpha \nu\beta 6$, $\alpha \nu\beta 8$, and $\alpha \text{IIb}\beta 3$ ³⁹⁴ integrins all have the conserved RGD binding pocket with nearby glycan molecules making the design of integrin specific peptides challenging^{320,393,395}.

Integrin $\alpha 5 \beta 1$ undergoes a large conformational change from the inactive closed state to the active open state when bound to FN^{207,283}.

We reasoned that de novo protein design could enable the creation of small, stable, and easy to manufacture $\alpha 5 \beta 1$ binders that specifically activate $\alpha 5 \beta 1$ on biomaterials. We set out to design $\alpha 5 \beta 1$ protein binders that can bind the interface between $\alpha 5$ and $\beta 1$ subunits, including the RGD-binding pocket and adjacent regions, to stabilize the active conformation of $\alpha 5 \beta 1$. Due to their in-silico origin and outstanding capacity to enhance cell adhesion, we call them NeoNectins (NN).

Computational design and characterization of NeoNectin candidates

During integrin activation, fibronectin RGD tripeptide coordinates the metal ion binding site within the $\beta 1$ β -I domain^{207,311}. The overall position of this loop and surrounding residues fits into the cleft between the α and β subunit. Therefore, the goal of design was to bind this cleft between the $\alpha 5$ and $\beta 1$ subunit to coordinate and stabilize the extended open conformation, mimicking integrin binding with fibronectin. This is done by the RGD -tripeptide motif coordinating the MIDAS with acid residues of the $\beta 1$ subunit²⁰⁷. The extended open state is the active conformation and with leg separation promotes the formation of focal adhesion kinases and outside-in signaling (**Figure 2 - 4**)^{206,219}. Ferredoxin was chosen as the scaffold with two alpha helices and 4 beta sheets, offering high stability (**Figure 2 - 10**). This scaffold provides ideal loop positions for presenting the RGD tripeptide motif and surrounding secondary supporting interactions (**Figure 2 - 11**).

The ferredoxin scaffolds were generated in a piece-wise assembly manner. The backbone information was first extracted for all existing ferredoxin-like proteins reported by the SCOP database to generate guided blueprints for de novo ferredoxin³⁹⁶. For the native ferredoxin-like fold with secondary structure “E-H-E-E-H-E,” there are four beta-sheets (E1, E2, E3, E4) and two helices (H1, H2) as basic elements. These are connected with five loops (L1, L2, L3, L4, L5) (**Figure 2 - 10**). In all instances of designs, there are optimal lengths, torsion angles, and orientations between these secondary structural elements. Combining all possible variables, the top 100 blueprints encoding ferredoxin topology information for the next step was finalized. These blueprints were then applied to Rosetta Blueprinter for backbone constraints.

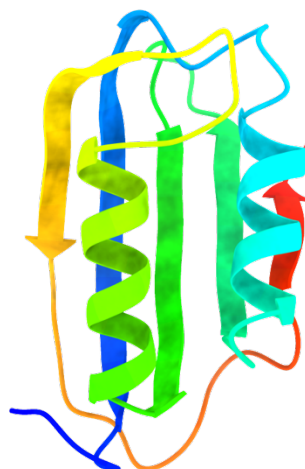


Figure 2 - 10 Ferredoxin Fold. The ferredoxin fold is a common alpha-beta protein fold. This fold has a secondary structure organization of β - α - β - α - β that is connected through five loops in order. The N-terminus is colored in blue where the C-terminus is in red. Visually, the β sheets are orientated in order of 4-1-3-2. PDB: 2ACY.

To improve the success rate, each topology was built with three steps instead of constructing the whole protein in one step. Ferredoxin scaffold proteins were designed either starting with the RGD peptide (Fibronectin residues 1492-1497; PDB: 4WK2) or ferredoxin scaffold library used for RGD-motif grafting in a later step. In the first case step 1, a helix and a beta strand were first built around the RGD peptide (E1+RGD+H1) with blueprint builder. After filtering with the Rosetta metrics, the top fragments were selected for step 2. In step 2, a beta-hairpin at the C-terminus of the H1 and parallel to the E1 (E1+RGD+H1+L2+E2+L3+E3) was further elongated and filtered the outputs with Rosetta monomer metrics. In step 3, the last L4+H2+L5+E4 fragments were built to make the whole ferredoxin. In the end, another round of filtering was imposed to remove designs with cavities and bad compactness. The trajectories that created the best-designed scaffolds were further resampled to increase the number of designs. The filtered backbones generated from the last step were sequence-optimized using Rosetta FastDesign and their structures were further evaluated via DeepAccNet or AFold2^{397,398}. Designs with predicted local distance difference test scores (plddt) <0.85 were dropped. In the second case, the ferredoxin scaffold libraries with altered steps were built. Stepwise building and grafting were two strategies that were employed to generate protein designs^{250,399}. Stepwise building generated 7820 designs and grafting generated 12,674 designs. These designs were

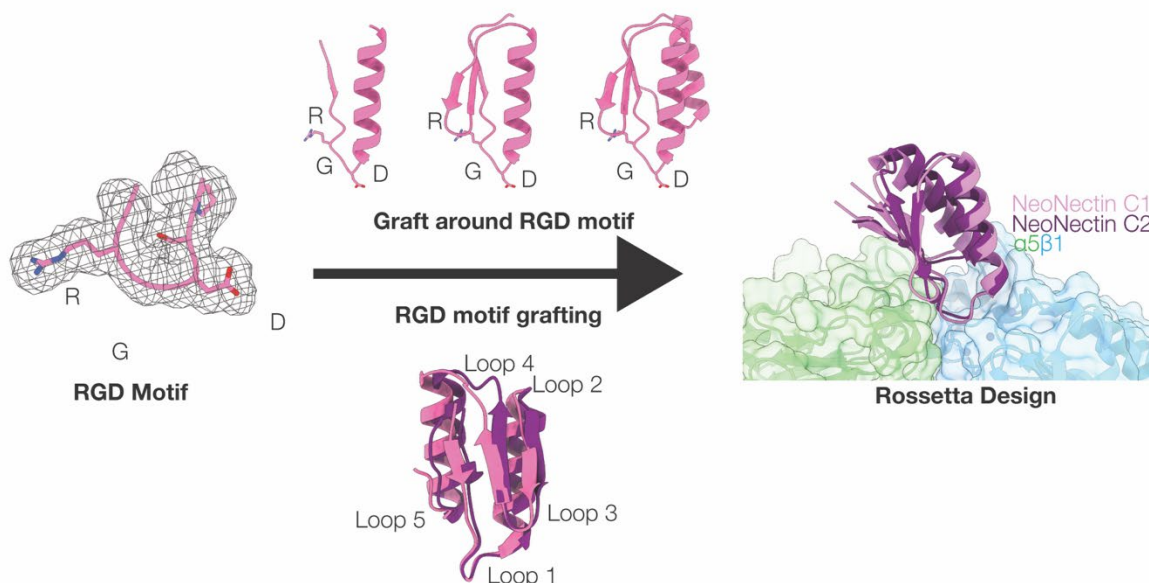


Figure 2 - 11 Computational Design of NeoNectin. Ferredoxin was used previously for integrin $\alpha\beta6$ - and $\alpha\beta8$ -designed binders²⁵⁰. Design campaigns started with the RGD motif from an RGD peptide bound to integrin $\alpha5\beta1$ (PDB: 4WK2). In a stepwise design strategy, the ferredoxin scaffold can be built around the RGD motif (top). Alternatively, the RGD motif can be grafted onto existing ferredoxin scaffold libraries. These design backbones were then optimized using Rosetta flexible backbone design. After filtering designs, the top two candidate are denoted NeoNectin C1 and C2.

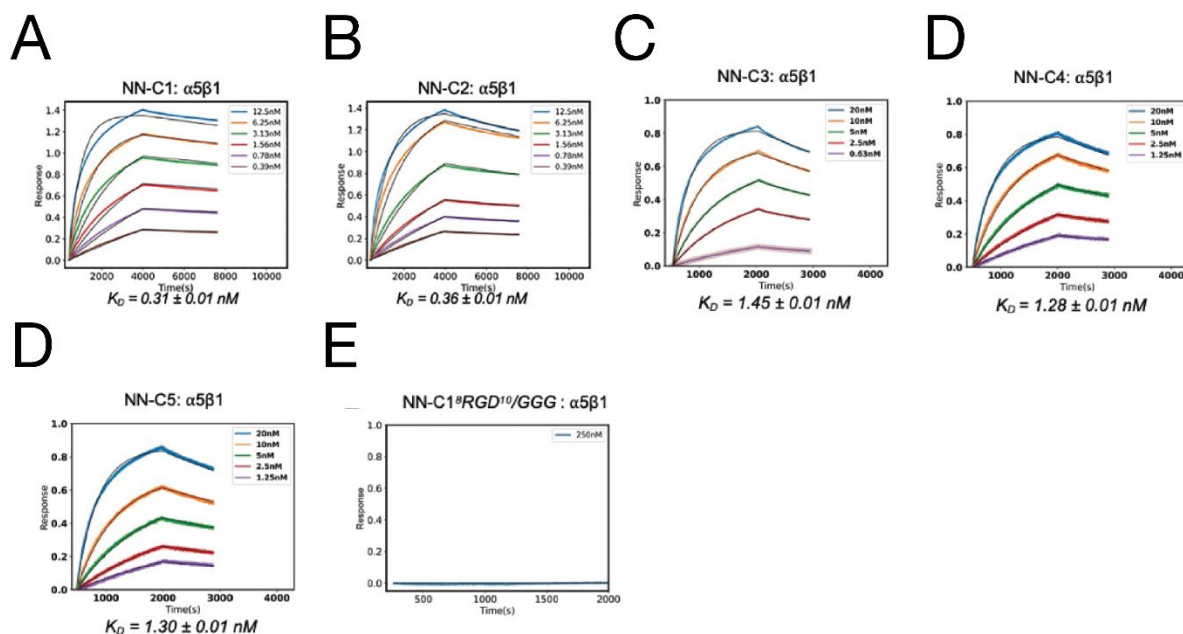


Figure 2 - 12 NeoNectin candidates have high affinity toward integrin $\alpha5\beta1$. A-D) BLI sensorgrams of the immobilized NeoNectin candidates 1 through 5 association and dissociation to $\alpha5\beta1$ ectodomains. Buffer condition is 20 mM Tris, pH 7.4, 1 mM Ca^{2+} , 1 mM Mg^{2+} . Global kinetics fits assuming a 1:1 binding model shown as black lines. E) Site saturation mutagenesis substitution at the RGD motif is not tolerated where binding is abolished.

further optimized using Rosetta flexible backbone protein design and ranked on binding energy, solvent accessibility and molecular contact surface ³⁹⁷.

Yeast surface display (YSD) was used to assess binding of biotinylated integrin $\alpha 5\beta 1$ ectodomain to the designed constructs. YSD offers an advantageous platform as each yeast cell displays one designed protein at its surface ⁴⁰⁰. We can perform multiple rounds of fluorescence-activated cell sorting (FACS) to enrich designs that bound to integrin $\alpha 5\beta 1$ and to ensure that the identified designs are properly folded. The starting and enriched populations at each round were deep sequenced, and the frequency of each design in the starting population and after each sort was determined; this was used to estimate binding dissociation constants (K_D) for each design ³⁹⁹. For the 16 most enriched designs, site saturation mutagenesis libraries (SSMs) were constructed, where every residue was substituted with each of the 20 amino acids. Together with YSD and SSMs, we can optimize the binding affinity of these designs to integrin $\alpha 5\beta 1$. Using biolayer interferometry, five designs showed subnanomolar to nanomolar K_D (**Figure 2 - 12A-D**). Any substitutions at the RGD site are not tolerated as expected, given the central role this motif plays in coordinating integrin binding (**Figure 2 - 12E**). The two highest affinity binders were chosen for downstream analysis. These binders are denoted as NeoNectin candidates 1 and 2 (alternatively NN-C1 and NN-C2 respectively).

NeoNectin candidate 1 bound integrin $\alpha 5\beta 1$ favors extended conformations

To investigate the effects of NeoNectin candidates 1 and 2 on integrin $\alpha 5\beta 1$ conformation and the molecular basis of the interactions, negative stain and cryogenic electron microscopy were used. Many integrins, including $\alpha 5\beta 1$ are known to undergo drastic conformational changes that are linked to activation state. In vitro, it has been established that high Ca^{2+} (5 mM) stabilizes the low-affinity, closed headpiece conformation, while 1 mM Mn^{2+} stabilizes the high-affinity conformation with an open headpiece and extended legs ⁴⁰¹ (**Figure 2 - 3** and **Figure 2 - S 6**). Using nsEM and under both cation conditions, NN-C1 binds and favors the extended open conformation of integrin $\alpha 5\beta 1$ while NN-C2 bound $\alpha 5\beta 1$ showed a mixture of extended open and extended closed conformation (**Figure 2 - 13**).

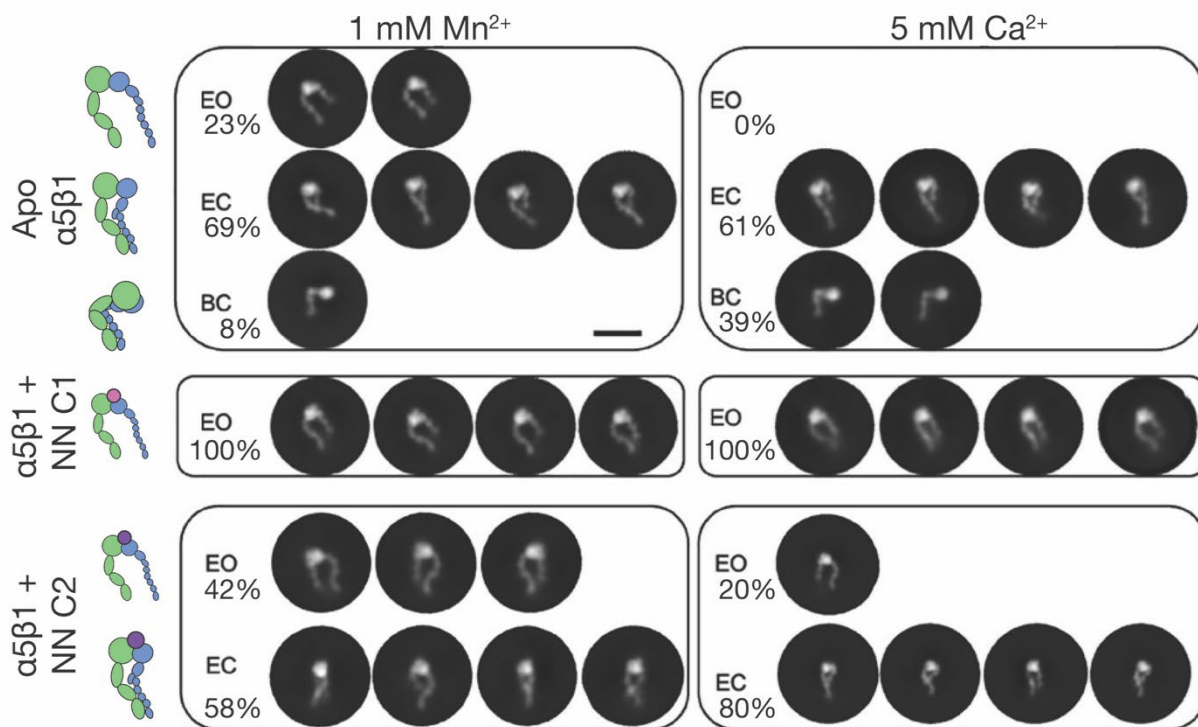
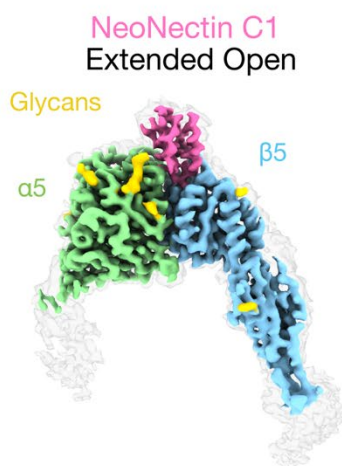


Figure 2 - 13 Negative stain characterization of NeoNectin Candidates 1 and 2 in complex with integrin $\alpha 5\beta 1$. Representative 2D negative stain class averages of $\alpha 5\beta 1$ alone, in presence of NN-C1 or NN-C2 in activating (1 mM Mn^{2+}) and non-activating (5 mM Ca^{2+}) buffer conditions. Integrins are categorized into three canonical conformations: extended open (EO), extended closed (EC), and bent closed (BC). The percentages of each class within each data collection are listed.

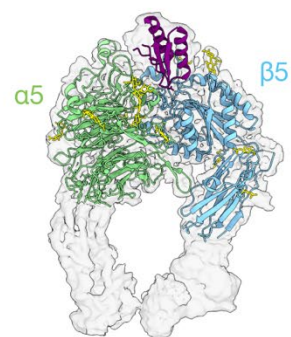
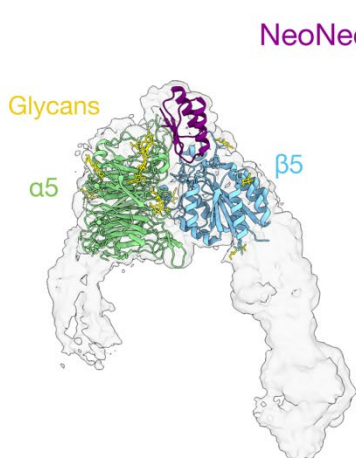
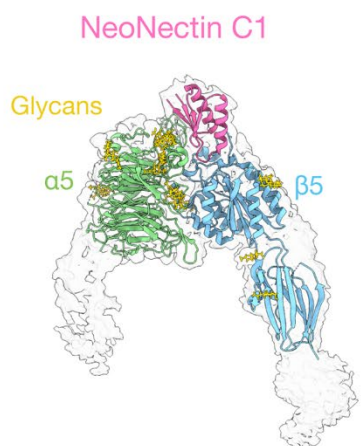
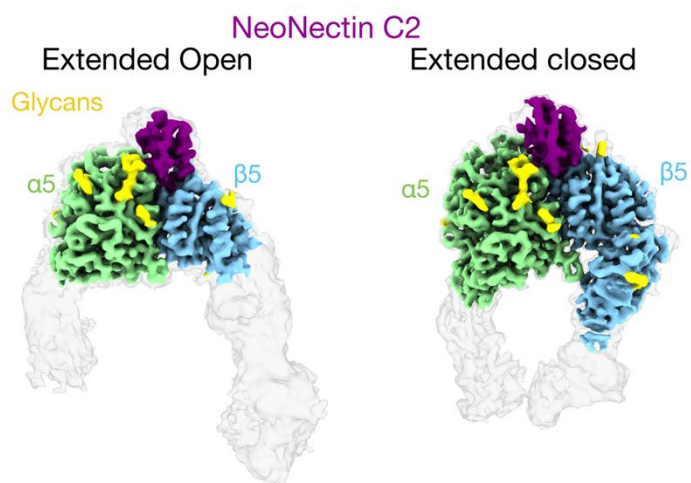
NeoNectin candidate 1 bound to integrin $\alpha 5\beta 1$ in cryoEM

Next, we determined the structure of integrin $\alpha 5\beta 1$ in complex with NN-C1 to a local resolution of 3.2 Å and an overall resolution of 3.3 Å by cryoEM (**Figure 2 - 14A** and **Figure 2 - S 13**). As expected, the dominant class ($\approx 88\%$ of integrin particles) shows the $\alpha 5\beta 1$ headpiece in an open conformation (**Figure 2 - 13**) with NeoNectin making interactions and inducing downstream large-scale structural rearrangements of $\alpha 5\beta 1$ similar to the native fibronectin ligand²⁰⁷. The interaction between NeoNectin-C1 and $\alpha 5\beta 1$ in the experimentally determined cryoEM model is consistent with the designed model in that it centers around three short loops: Loop 1 (G7, R8, G9, D10, F11, P12), Loop 3 (D33, H34, K35), and Loop 5 (G58, I59, W60) (**Figure 2 - 15**). The RGD motif encoded by Loop 1 binds $\alpha 5\beta 1$ via the same entities as the RGD motif in FN or RGD peptide^{207,311}. Specifically, integrin $\alpha 5$ Q221 and D227 bind NeoNectin C1 R8 and $\beta 1$ -coordinated MIDAS cation coordinates D10 (**Figure 2 - 14C** and **Figure 2 - 15**). Loop 3 further stabilizes the interaction through interactions with both $\alpha 5$ and $\beta 1$ subunits:

A



B



C

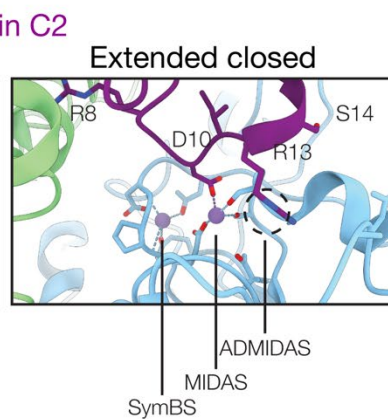
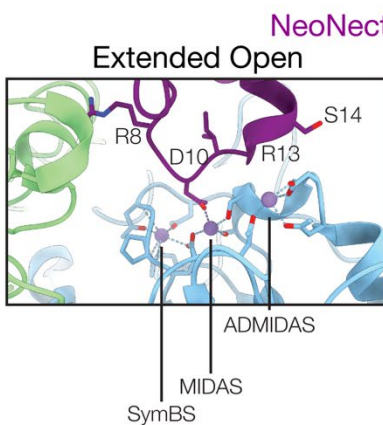
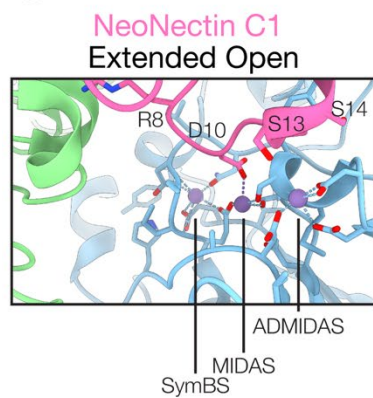


Figure 2 - 14 CryoEM map of $\alpha 5\beta 1$ bound to NeoNectin-C1 and C2. (A) CryoEM map of integrin $\alpha 5\beta 1$ bound to NeoNectin C1 in the extended open conformation. The sharpened, locally refined map is shown in color, superimposed with the unsharpened map in semi-transparent white (left). Below this is the model that was built into the sharpened map. The color code is as follows: $\alpha 5$ (green), $\beta 1$ (blue), Neonectin-C1 (pink), and glycans (yellow). (B) CryoEM map of $\alpha 5\beta 1$ bound to NeoNectin candidate 2 (NN-C2) in extended open and closed states. The sharpened, locally refined maps are shown in color, superimposed with the unsharpened maps in semi-transparent white. Below these are the models that were built into the sharpened maps. (C) Closed-up of the extended open integrin $\alpha 5\beta 1$ -NN C1 binding interface. All three known metal ion binding sites are occupied. When integrin $\alpha 5\beta 1$ is bound to NN C2, the ADMIDAS is unoccupied in the extended closed conformation.

NN-C1 H34 and K35 form a salt bridge triad with integrin $\beta 1$ E320 and NN-C1 H34 forms an additional interaction with integrin $\alpha 5$ L225 backbone (**Figure 2 - 15**). While FN also forms a salt bridge with integrin $\beta 1$ E320 that was suggested to be important for FN-induced headpiece opening through FN R1445 and Y1446²⁰⁷ (**Figure 2 - 15**) mutating NN-C1 H34 and K35 to glycine did not prevent headpiece opening. NeoNectin candidate 1's Loop 5 W60 interacts via shape complementarity with residues in both integrin subunits, including a π - π stacking interaction with $\alpha 5$ W157. It has been shown previously that $\alpha 5$ W157 introduces $\alpha 5\beta 1$ specificity into RGD cyclic peptides; however, W157 is not required for binding to FN^{207,320,402}. Interactions made are summarized in **Table 2 - S 6**.

NeoNectin candidate 2 bound integrin $\alpha 5\beta 1$ exhibits both extended open and closed conformation

Next, we aimed to characterize the interactions of $\alpha 5\beta 1$ with NN-C2, which shares 50% sequence identity with NN-C1. As expected from the nsEM, in cryoEM, we observed both the open (67%) and closed headpiece (33%) conformations when bound to NN-C2 (**Figure 2 - 13** and **Figure 2 - S 14**). We resolved the open headpiece complex to a resolution of 4.0 Å overall and 3.6 Å locally and the closed headpiece complex to a resolution of 3.0 Å overall and 3.0 Å locally (**Figure 2 - 14B**). The contacts made between NN-C2 and $\alpha 5\beta 1$ were strikingly similar to the contacts made by NN-C1. However, while Mn^{2+} is observed at the ADMIDAS site in the open $\alpha 5\beta 1$:NN-C2 complex, no ADMIDAS ion is observed in the closed $\alpha 5\beta 1$:NN-C2 complex (**Figure 2 - 14C**). In the closed $\alpha 5\beta 1$:NN-C2 complex, the additional salt bridges are made by R13_{NN-C2} and D259 _{$\beta 1$} and D138 _{$\beta 1$} . In contrast, the side chain of R13_{NN-C2} is not resolved in the extended open complex, suggesting conformational flexibility. This is consistent with the importance of ADMIDAS engagement in stabilizing the extended open conformation²⁰⁷.

Discussion

Most current biomaterials used for tissue regeneration, such as titanium or 3D hydrogels, do not promote sufficient cell attachment for successful tissue integration in transplanted tissues. Hence, there is a growing need for finding biomolecules that can be used for coating and embedding biomaterials, making them more functional. Various levels of success have been achieved using RGD peptides or FN fragments, but their lack of specificity for various integrins and challenges in manufacturing constrains their application. The 65-amino acid designed NeoNectin, which exhibits high affinity and specificity for integrin $\alpha 5 \beta 1$ and can be produced with high yield, shows considerable promise in overcoming these limitations. NeoNectin stabilizes the EO conformation as does FN (**Figure 2 - 13** and **Figure 2 - 14A-B**)^{207,283}. While FN binds to both the $\alpha 5$ - $\beta 1$ interface and a synergy site on $\alpha 5$, NeoNectin only binds to the interface between $\alpha 5$ and $\beta 1$, suggesting that the synergy site binding is not essential for the conformational switching of integrin $\alpha 5 \beta 1$. Integrin $\alpha 5 \beta 1$ is crucial in early stages for recruiting MSCs in tissue regeneration. A cyclic peptide specific for integrin $\alpha 5 \beta 1$ has also been shown to promote osteoblast adhesion, differentiation, and in vivo bone formation^{403–406}.

Collectively, these results showcase the possibility of using computationally designed proteins for regenerative medicine. Given the growing needs in this field, for example encapsulating therapeutic cells and/or recruiting/differentiating distinct cells during tissue healing, our approach of grafting materials with small, stable, and target-specific designed proteins could be broadly applicable in regenerative medicine.

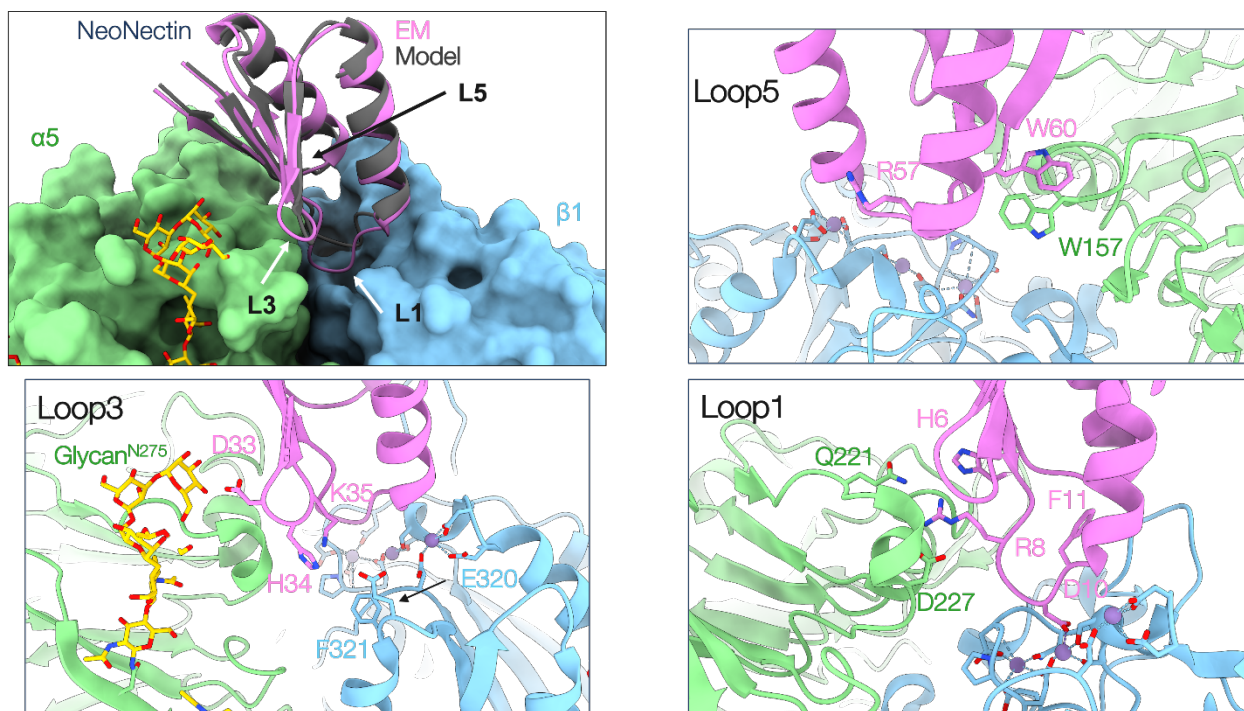


Figure 2 - 15 NeoNectin C1 coordinates the MIDAS Ion site and complementary surfaces as designed. An overlay of the NN-C1 designed model (gray) and the experimentally determined model (pink). **Upper-left:** The three primary loops that create the surface contacts points, L1, 3, and 5 are closely positioned as design. The only discrepancy is Loop 1 position which contains the RGD motif which is slightly different from designed. However, the contains that were designed are maintained (Lower-right). **Upper-right:** L5 Trp60 interacts via shape complementarity with residues in both integrin subunits, including a π - π stacking interaction with Trp157 $_{\alpha 5}$. **Lower-left:** L3 further stabilizes the interaction through interactions with both $\alpha 5$ and $\beta 1$ subunits: His34 $_{NN-C1}$ and Lys35 $_{NN-C1}$ form a salt bridge triad with Glu320 $_{\beta 1}$ and His34 $_{NN-C1}$ forms an additional interaction with Leu225 $_{\alpha 5}$ backbone. **Lower-right:** The RGD motif encoded by L1 binds $\alpha 5\beta 1$ via the same entities as the RGD motif in FN or RGD peptide. Interactions are summarized in **Table 2 - S 6**.

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Methods

The following contains parts of the complete method section and will contain methods relating to the experiments and conclusions described here. For the complete methods details, refer to the published manuscript.

Biolayer Interferometry (BLI)

The BLI experiments were performed on an OctetRED96 BLI system (ForteBio) at room temperature in integrin resting buffer (20 mM Tris pH 7.4, 150 mM NaCl, 1 mM MgCl₂, 1 mM CaCl₂, 0.02% Tween-20) or active buffer (20 mM Tris pH 7.4, 150 mM NaCl, 1 mM MnCl₂, 0.02% Tween-20) or inactive buffer (20 mM Tris pH 7.4, 150 mM NaCl, 5 mM CaCl₂, 0.02% Tween-20) or low-pH buffer (20 mM Tris pH 5, 150 mM NaCl, 1 mM MgCl₂, 1 mM CaCl₂, 0.02% Tween-20). Each BLI buffer was supplemented with 0.2 mg/mL bovine serum albumin (Sigma-Aldrich). Prior to measurements, streptavidin-coated biosensors were first equilibrated for at least 10 minutes in the assay buffer. Protein binders with N-terminal biotin were immobilized onto the biosensors by dipping them into a solution with 100 nM protein until the response reached between 10% and 50% of the maximum value followed by dipping sensors into fresh buffer to establish a baseline for 120 seconds. Titration experiments were performed at 25°C while shaking at 1000 rpm. Association of integrins was allowed by dipping biosensors in solutions containing designed protein diluted in octet buffer until equilibrium was approached followed by dissociation by dipping the biosensors into fresh buffer solution to monitor the dissociation kinetics. In the binding cross specificity assays each biotinylated binder was loaded onto streptavidin biosensors in equal amounts followed by 120 seconds of baseline equilibration. The association and dissociation with all the different binders were allowed for 900–3600 seconds for each step. Global kinetic or steady-state fits were performed on buffer-subtracted data using the manufacturer's software (Data Analysis 12.1) assuming a 1:1 binding model.

Negative-Stain EM Sample Preparation

The integrin-binder complexes were formed using a 1:2 integrin to NeoNectin molar ratio, incubated at room temperature for at least 10 minutes, and diluted to a final concentration of 9 µg/mL (α5β1) in 20 mM Tris-HCl pH 7.4, 150 mM NaCl, supplemented with either 1 mM MnCl₂ or 5 mM CaCl₂. 3 µL of the sample was applied to a glow-discharged 400 mesh copper

glider grid that had been covered with a thin layer of continuous amorphous carbon. The grids were stained with a solution containing 2% (w/v) uranyl formate.

Negative-Stain EM Data Acquisition and Processing

Data were acquired using a Thermo Fisher Scientific Talos L120C transmission electron microscope operating at 120 kV and recorded on a 4k × 4k Thermo Fisher Scientific Ceta camera at a nominal magnification of 92000× with a pixel size of 1.58 Å/px. Leginon⁴⁰⁷ was used to collect micrographs at a nominal range of 1.8–2.2 μm under focus and a dose of approximately 50 e⁻/Å². Data sets collected in activating buffer conditions containing 1 mM MnCl₂ had the following number of micrographs: 264 micrographs α5β1 alone, 331 micrographs α5β1 + NeoNectin, 335 micrographs α5β1 + NeoNectin candidate 2, 319 micrographs α5β1 + NeoNectin H34G/K35G, 474 micrographs α5β1 + NeoNectin W60A. Data sets collected in non-activating buffer conditions containing 5 mM CaCl₂ had the following number of micrographs: 329 micrographs α5β1 alone, 226 micrographs α5β1 + NeoNectin, 301 micrographs α5β1 + NeoNectin candidate 2, 484 micrographs α5β1 + NeoNectin H34G/K35G. Data were processed using Gautomatch (<https://github.com/JackZhang-Lab>), RELION, and cryoSPARC^{158,368}.

CryoEM Sample Preparation

The integrin binder complexes were incubated at room temperature for 30 minutes using a 1:2 integrin to binder molar ratio. From there, complexes were diluted to a final concentration of 90 μg/mL (α5β1) in 20 mM Tris pH 7.4, 150 mM NaCl, 1 mM MnCl₂. For cryoEM grid preparation, UltrAufoil grids (300 mesh, 1.2/1.3) were glow-discharged for 30 seconds at 15 mA, and then 3 μL of each complex were applied to each grid. Complexes were frozen with a Thermo Fisher Scientific Vitrobot Mark IV in 100% humidity at 4 °C and vitrified in liquid ethane cooled by liquid nitrogen.

CryoEM Data Acquisition and Processing

Datasets for 2 complexes were acquired on a Thermo Fisher Scientific Glacios cryo-transmission electron microscope operating at 200 kV and recorded with a Gatan K3 Direct Detection Camera. For the NN-C1 complex, a total of three datasets were collected from three separate grids. For the NN-C2 complex, a single dataset was collected from a single grid. For data collection, the stage was tilted to 30° and all images were recorded using SerialEM software⁴⁰⁸. One hundred frame movies were recorded in super-resolution mode with a super-resolution

pixel size of 0.561 Å/px, a nominal magnification of 36,000 $\times$, a nominal defocus range of 1.2-2.0 μm under focus, and an approximate dose of 50 e⁻/Å². For the NN-C1 complex, 861 micrographs were used for subsequent data analysis (334, 183, and 204 micrographs from the respective data collections). For the NN-C2 complex, 1231 micrographs were used. Dose fractionated super-resolution image stacks were motion-corrected and binned 2 $\times$ 2 using Fourier cropping with MotionCor2 within the RELION wrapper⁴⁰⁹. Motion-corrected stacks were processed using Patch CTF in cryoSPARC. For the NN-C1 complex, 728,379 particles were picked using the unbiased blob picker in cryoSPARC and subjected to iterative 2D and 3D alignment and classification yielding a final map at 3.28 Å resolution (72,604 particles). Additional local refinement of the binding interface gave a final map at an improved resolution of 3.19 Å. For the NN-C2 complex, 115,0338 particles were picked using the unbiased blob picker in cryoSPARC and subjected to iterative 2D and 3D alignment and classification which resulted in two distinct headpiece conformations: closed and open. For the closed conformation, a map using 153,759 particles was determined to a resolution of 2.98 Å. Additional local refinement improved details of the binding interface and had an overall resolution of 2.97 Å. For the open conformation, a map using 69,848 particles was determined to a resolution of 3.95 Å. Additional local refinement resulted in a map with a resolution of 3.56 Å. CryoEM processing details and results are summarized in **Figure 2 - S 13** ($\alpha 5\beta 1$ + NeoNectin C1) and **Figure 2 - S 14** ($\alpha 5\beta 1$ + NN-C2).

Model Building

The initial models for integrin model-building were as follows: For the $\alpha 5\beta 1$ + NN-C1 complex, the open $\alpha 5\beta 1$ headpiece structure (PDB: 7NWL) was used. For $\alpha 5\beta 1$ + NN-C2 complex, the closed $\alpha 5\beta 1$ headpiece structure (PDB: 9B9J) or the open $\alpha 5\beta 1$ headpiece structure (PDB: 9CKV, this work) was used. For all model-building, the predicted structure of the designed binder was used as an initial model. First, these models were fit into their respective cryoEM density using UCSF ChimeraX³⁷² and glycans were manually added. Refinements were performed using COOT³⁵² and ISOLDE³⁷⁴. All maps (sharpened and unsharpened) used for modeling have been deposited. CryoEM and model-building statistics can be found in **Table 2 - S 5**.

Additional figures and tables

This section contains the following supporting data for Chapter 2.4:

Figure 2 - S 13 CryoEM data processing schematic of NeoNectin Candidate 1 + integrin $\alpha 5 \beta 1$

Figure 2 - S 14 CryoEM data processing schematic of NeoNectin Candidate 2 + integrin $\alpha 5 \beta 1$

Table 2 - S 5 Integrin $\alpha 5 \beta 1$ CryoEM Data Collection and Processing Statistics

Table 2 - S 6 Summary of NeoNectin interactions with integrin $\alpha 5 \beta 1$.

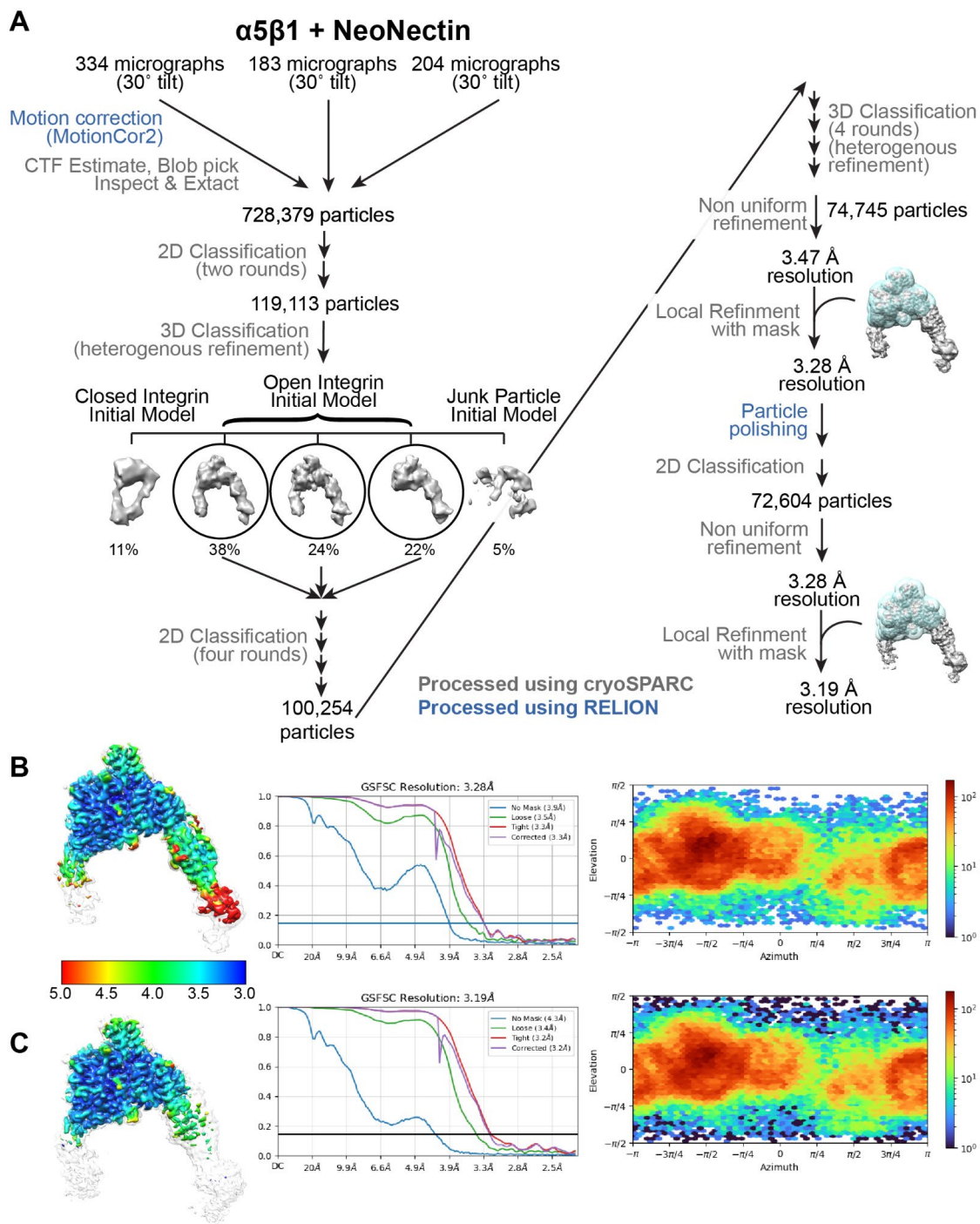


Figure 2 - S 13 CryoEM data processing schematic of NeoNectin Candidate 1 + integrin $\alpha 5\beta 1$. **A)** An overview summarizing cryoEM data processing for the $\alpha 5\beta 1$ +NeoNectin complex. Particle numbers at key steps and percentages for each class are indicated. **B)** Left: Sharpened map of $\alpha 5\beta 1$ +NeoNectin complex colored based on a local resolution, unsharpened map shown in semi-transparent white at a lower threshold. Middle: Gold-standard Fourier shell correlation plot. Right: Orientational distribution plot of the $\alpha 5\beta 1$ in complex with NeoNectin. **C)** Left: Final local refined and sharpened map of $\alpha 5\beta 1$ +NeoNectin complex colored based on a local resolution, unsharpened map shown in semi-transparent white at a lower threshold. Middle: Gold-standard Fourier shell correlation plot. Right: Orientational distribution plot of the locally refined $\alpha 5\beta 1$ in complex with NeoNectin.

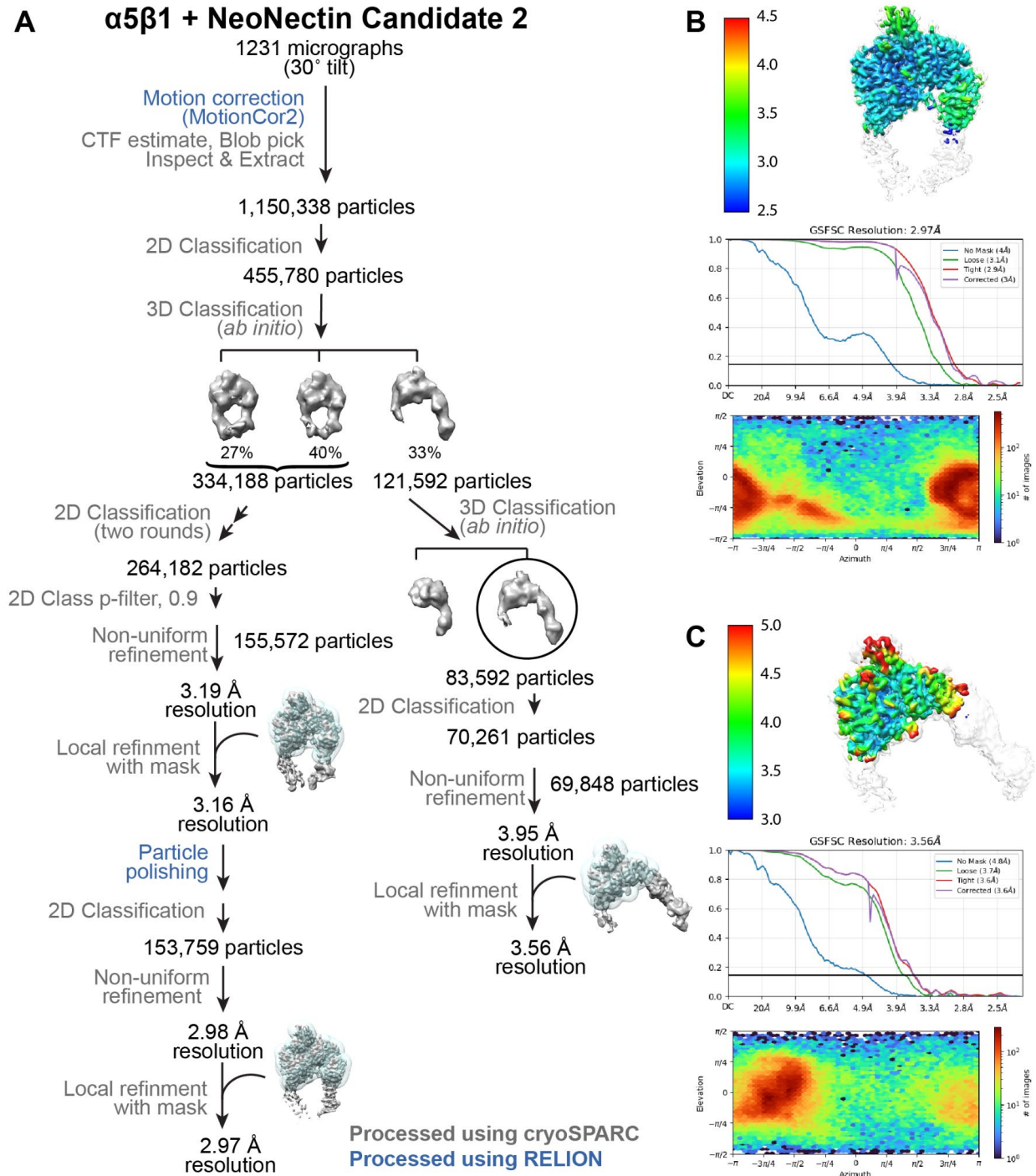


Figure 2 - S 14 CryoEM data processing schematic of NeoNectin Candidate 2 + integrin $\alpha 5\beta 1$. **A)** An overview summarizing cryoEM data processing for the $\alpha 5\beta 1$ +NeoNectin Candidate 2 complex. Particle numbers at key steps and percentages for each class are indicated. **B)** Top: Sharpened map of Closed Headpiece $\alpha 5\beta 1$ +NeoNectinCandidate 2 complex colored based on a local resolution, unsharpened map shown in semi-transparent white at a lower threshold. Middle: Gold-standard Fourier shell correlation plot. Bottom: Orientational distribution plot. **C)** Top: Sharpened map of Open Headpiece $\alpha 5\beta 1$ +NeoNectinCandidate 2 complex colored based on a local resolution, unsharpened map shown in semi-transparent white at a lower threshold. Middle: Gold-standard Fourier shell correlation plot. Bottom: Orientational distribution plot.

Table 2 - S 5 Integrin $\alpha 5\beta 1$ -NeoNectin CryoEM Data Collection and Processing Statistics

	Extended Open $\alpha 5\beta 1$:NeoNectin	Extended Closed $\alpha 5\beta 1$: NN-C2	Extended Open $\alpha 5\beta 1$: NN-C2
EMDB	45655	46902	47968
PDB	9CKV	9DIA	9EF2
Magnification	36,000x	36,000x	36,000x
Voltage (kV)	200	200	200
Electron exposure (e-/Å²)	50	50	50
Defocus range (µm)	1.2 - 2.0	1.0 - 2.0	1.0 - 2.0
Pixel size (Å)	1.122	1.122	1.122
Symmetry imposed	C1	C1	C1
Initial particle images (no.)	728,379	1,150,388	1,150,388
Final particle images (no.)	72,604	153,759	69,848
Map resolution (Å)			
Overall	3.28	2.98	3.95
Local Refinement	3.19	2.97	3.56
FSC threshold	0.143	0.143	0.143
Map resolution range (Å)			
Overall	2.92 - 17.44	2.58 - 39.58	3.25- 56.53
Local Refinement	2.92-9.65	2.59 - 7.86	3.17- 10.21

Table 2 - S 6 Summary of NeoNectin interactions with integrin $\alpha 5\beta 1$.

$\alpha 5\beta 1$:NeoNectin C1 interactions					
Integrin $\alpha 5\beta 1$		NeoNectin C1		Interaction Type	
Residue/Glycan		Subunit	Residue		
F	187	α	R	8 (Loop 1)	Shape Complementarity
Q	189	α	R	8 (Loop 1)	Shape Complementarity
Q	221	α	R	8 (Loop 1)	Shape Complementarity
S	224	α	R	8 (Loop 1)	Hydrogen Bond/ Shape Complementarity
D	227	α	R	8 (Loop 1)	Salt Bridge / Hydrogen Bond
F	187	α	G	9 (Loop 1)	Shape Complementarity
Glycan	275	α	D	33 (Loop 3)	Shape Complementarity
S	224	α	H	34 (Loop 3)	Shape Complementarity
I	225	α	H	34 (Loop 3)	Shape Complementarity
S	156	α	W	60 (Loop 5)	Shape Complementarity
W	157	α	W	60 (Loop 5)	Shape Complementarity
A	159	α	W	60 (Loop 5)	Shape Complementarity
L	225	β	G	9 (Loop 1)	Shape Complementarity
S	132	β	D	10 (Loop 1)	MIDAS Ion Coordination
Y	133	β	D	10 (Loop 1)	Shape Complementarity
S	134	β	D	10 (Loop 1)	MIDAS Ion Coordination
N	224	β	D	10 (Loop 1)	Shape Complementarity
D	226	β	D	10 (Loop 1)	Shape Complementarity
S	227	β	D	10 (Loop 1)	Shape Complementarity
G	229	β	D	10 (Loop 1)	MIDAS Ion Coordination
Y	133	β	F	11 (Loop 1)	Shape Complementarity
Y	133	β	P	12 (Loop 1)	Shape Complementarity
D	137	β	S	None	Shape Complementarity
K	136	β	E	15 (Loop 3)	Hydrogen Bond
E	320	β	H	34 (Loop 3)	Salt Bridge
E	320	β	K	35 (Loop 3)	Salt Bridge
T	188	β	G	58 (Loop 5)	Shape Complementarity

Chapter 3 Structural investigation of Tie2 activation and modulation

The preceding chapter demonstrated the impact of therapeutic targeting of integrin $\alpha 5\beta 1$ and how it changes receptor conformation to promote certain biological outcomes in cellular adhesion. However, integrin receptors are only one of several angiogenic receptors that work in concert for the successful formation of a new vessel. While integrin receptors play a pivotal role in coordinating endothelial cell motility during vessel expansion, these cells also need to prevent the leakage of the growing lumen. Migrating endothelial cells form cell-cell contacts with neighboring endothelial cells (tip ECs or stalk ECs) to establish a semi-permeable monolayer. This cell-cell contact is mediated through the Tie2-angiopoietin pathway. Tie2 is a single-pass transmembrane receptor tyrosine kinase that, upon binding to angiopoietin ligands, triggers a phosphorylation cascade that ultimately signals the activation of genes linked to cellular survival via FOXO1 (forkhead box O1). Our understanding of Tie2 activation is extrapolated from mechanistic studies of other receptor tyrosine kinases (RTKs), such as the epidermal growth factor receptor (EGFR). These receptors have defined general models of ligand-induced dimerization as the primary mechanism of RTK activation. However, the current body of structural information on Tie2 activation and how it is modulated is limited. The work within this chapter describes efforts to determine the molecular mechanism and regulation of Tie2 activation using structural biology and biophysical methods.

3.1 Introduction

Signal transduction is one of the primary mechanisms by which cells can interact with and respond to their environment and other cells. These signaling events are initiated when cell-surface receptors bind their cognate ligands, triggering downstream intracellular signaling pathways through conformational changes. Receptor tyrosine kinases are a group of cell-surface transmembrane receptors that contain a cytosolic kinase domain. These receptors are further classified into 20 subfamilies and are widely expressed across many cell types, where they regulate differentiation, proliferation, survival, and metabolism⁴¹⁰. Among these RTKs, Tie receptors are endothelial-expressed receptors that play a role in angiogenesis.

Angiopoietin-Tie signaling Axis

The Tie receptors are single-pass transmembrane receptor tyrosine kinases that are primarily expressed on vascular and lymphatic endothelial cells and on subsets of pericytes and macrophages^{411–414}. During angiogenesis, endothelial cell migration is initiated by vascular endothelial growth factor (VEGF) signaling through VEGF receptors and supported by integrin-mediated motility. This is followed by remodeling of the newly formed vessel via the angiopoietin-Tie system. This pathway facilitates the formation of tight cell-cell junctions between endothelial cells⁴¹⁵. These cell-cell contacts are critical for maintaining the separation of the vessel lumen and surrounding tissues. The Tie receptor family consists of two members, Tie1 and Tie2, which were isolated in the 1990s (**Figure 3 - 1**)³⁰.

Of the two Tie receptors, Tie2 is better characterized. Tie2 recognizes and directly binds to the angiopoietin growth factor family^{416–420}. There are four identified angiopoietins, angiopoietin-1, -2, -3, and -4 (Ang1-4) with Ang3 being the murine ortholog of Ang4^{417,421}. Because no known ligands bind directly to Tie1, it is classified as an orphan receptor. As a result, Tie1 function and activity remain less well understood compared to Tie2. Despite lacking a cognate ligand, Tie1 is observed to form heterodimeric receptor complexes with Tie2 that are mediated by Ang1 and Ang4^{422–424}. Nevertheless, deletion of the Tie1 gene results in embryonic lethality⁴²⁵.

Angiopoietins share a conserved domain organization. There is a super clustering domain at the N-terminus that is followed by a coiled-coil oligomerization domain⁴²⁶. The C-terminal fibrinogen-like domain (F-domain) serves as the Tie2 receptor binding domain^{427–429}. The human angiopoietins share little sequence similarity outside the F-domain. Despite having the same overall structure, the differences in the oligomerization state of angiopoietins are thought to dictate the activity of Tie2⁴³⁰. Ang1 is the canonical agonist of Tie2 and promotes tight cell-cell junction formation, stabilizing the blood vessel. With the clustering domain and coiled-coils, Ang1 exists in multiple high oligomeric states. Due to the oligomerization, Ang1 can recruit multiple Tie2 in cis on the same cellular membrane and in trans between endothelial cells, enhancing phosphatidylinositol 3-kinase (PI3K) recruitment and activation^{415,430,431}. PI3K phosphorylates Akt, which in turn leads to the nuclear exclusion of the transcription factor FOXO1, reducing expression of vascular destabilizing genes and promoting cell survival

(**Figure 3 - 1**)^{432,433}. However, recombinant angiopoietin expression is difficult due to large amounts of aggregation and solubility issues^{431,434}. Due to these factors, defined and stable variants of angiopoietins were engineered^{430,431,435}.

Ang2, unlike Ang1, does not exhibit higher-order oligomers similar to Ang1, limiting its ability to cluster Tie2. Without clustering, Tie2 activation by autophosphorylation is dramatically reduced. While Ang2 does not oligomerize to the level of Ang1, it can still induce dimerization of Tie2, which can be weakly activated⁴³⁶. Reduced Tie2 phosphorylation limits PI3K recruitment and weakens downstream Akt signaling. As a result, FOXO1 remains in the nucleus and continues to activate vascular remodeling genes resulting in the endothelial cell monolayer being more plastic (**Figure 3 - 1**)^{432,433}. In more recent studies, it is hypothesized that Ang2 is not solely an antagonist but rather a context-dependent agonist^{424,432,437–439}. The dynamic balance of Ang1 and Ang2 regulates the semi-permeable nature of the endothelial cell monolayer as both angiopoietin ligands compete for the same binding site on Tie2^{427,428}. Recent work from the Ruohola-Baker lab demonstrated that the oligomerization states of the angiopoietin ligand control Tie2 activation through scaffolded F-domain⁴³⁰. Trimeric and tetrameric F-domain exhibit Ang2-like signaling and activation, whereas hexamers and above demonstrated Ang1-like activity.

Unlike Ang1 and Ang2, Ang3 and Ang4 are not as well characterized. Although Ang3 and Ang4 are species orthologs, their biological roles have diverged, suggesting that they are not interchangeable across species^{417,419}. Since all angiopoietins bind at the same epitope, all members compete for Tie2 binding. The angiopoietin family differs from many other RTK ligands due to its multiple oligomeric states. These other ligands often induce dimerization, leading to autophosphorylation of the receptor for cytosolic kinase domains⁴⁴⁰. Tie2 uniquely clusters instead of dimerizing, although Tie2 dimers could be the most basic component of these clusters^{410,428,441–443}. The role of these clusters is critical for downstream signaling^{430,443}.

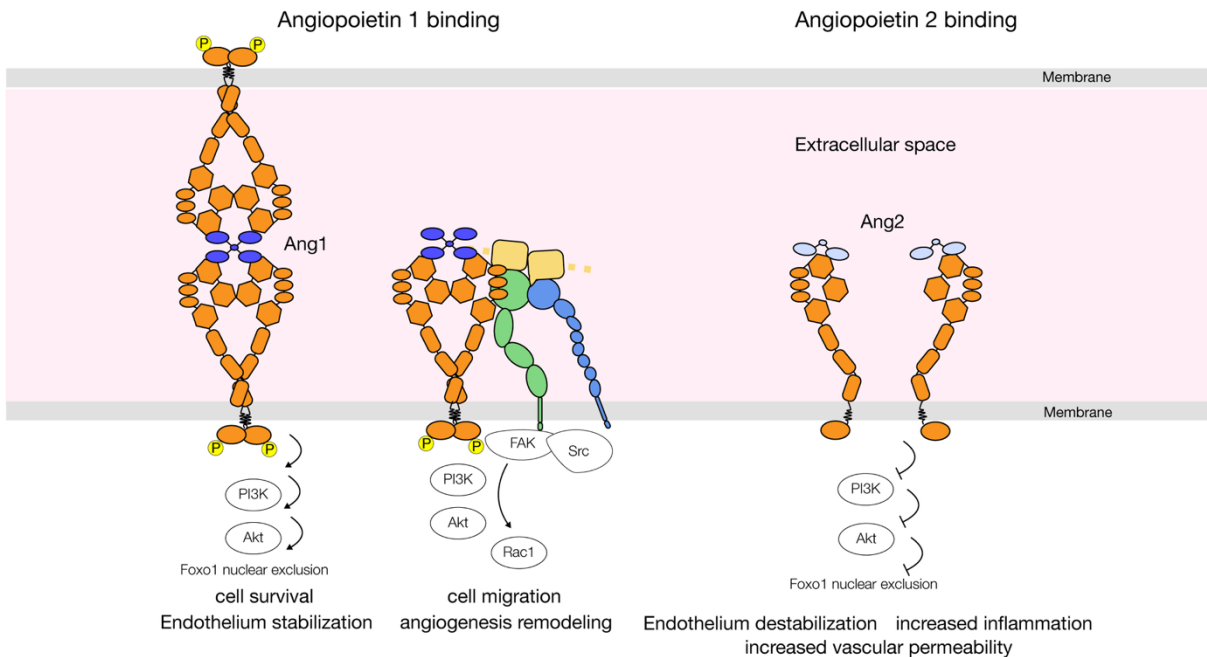


Figure 3 - 1 Angiopoietin-Tie2 signaling. Tie2 is a single pass transmembrane protein that is primarily expressed on the endothelial cells. The activity and function of Tie2 is mediated by growth factors called angiopoietins. The most well described are angiopoietins 1 and 2 (Ang1 and Ang2). Ang1 is the canonical agonist and Tie2 clusters due to the oligomeric states. The clusters of Tie2 can auto-phosphorylation each other. Ang2 is the anti-agonist where it competes with Ang1 for binding with Tie2. The signaling process of Tie2 bound to agonistic angiopoietin-1 (Ang1) or antagonist angiopoietin-2 (Ang2).

Tie Receptor Structural Organization

The extracellular domain architecture of Tie receptors distinguishes them from other receptor tyrosine kinase families. The overall structure of the extracellular domain is “lollipop-shaped,” with the ligand binding head positioned at the N-terminus (**Figure 3 - 2**). The extracellular domain is large and elongated, consisting of the ligand binding head and the stalk or leg regions. The ligand binding head is composed of three immunoglobulin-like (Ig) domains and three epidermal growth factor-like repeat domains. The Ig2 domain forms the primary receptor-ligand binding interface with the F-domain of Ang1 and Ang2^{427–429}. Three fibronectin type III domains anchor the ligand binding head to the membrane^{441,442}. The Tie1 and Tie2 extracellular domains share approximately 30% sequence identity³⁰. To date, there is no experimentally determined protein structure of Tie1’s ligand binding head. However, homology and AFold3 predictions suggest a similar structure and fold⁴²². Their cytosolic kinase domains share a higher sequence similarity of approximately 75%³⁰. The kinase domain is divided into two smaller domains, in which the N-terminal domain binds ATP and the C-terminal mediates

catalysis and substrate binding ⁴⁴⁴. Tie2 phosphorylation recruits PI3K to the membrane to initiate the downstream signaling cascade ⁴⁴⁵.

Angiopoietin-Tie in health and disease

During adulthood, the balance between Ang1 and Ang2 regulates the angiogenic nature of endothelial cells and the formation of a selectively permeable endothelial monolayer. Vascular growth is initiated by VEGF and its receptor, followed by stabilization of newly formed vessels through angiopoietin-Tie signaling ⁴²¹. During angiogenesis, the surrounding tissue secretes both Ang1 and Ang2 to help direct vessel growth. Tip endothelial cells of the budding vessel experience high levels of Ang2, which promote vessel destabilization and reduced cell-cell contacts, promoting motility. Stalk endothelial cells, which lag behind tip cells, provide structure and stability to the vessel. These cells experience a higher concentration of Ang1, which activates Tie2 and promotes cellular stability.

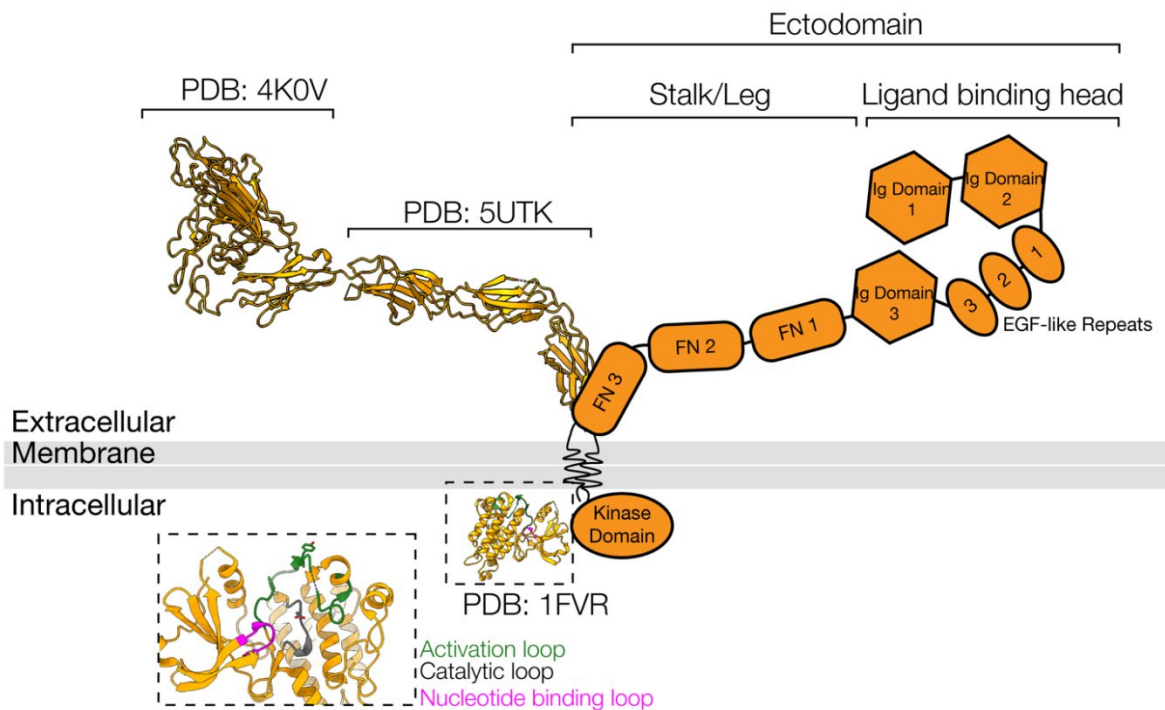


Figure 3 - 2 Structure of the Tie2 receptor. Tie2 is a single pass transmembrane receptor with a large extracellular region and a cytosolic kinase domain connected by a single pass transmembrane helix. The extracellular domain is broken down into two smaller domains of the ligand binding head which contains the binding surface for angiopoietins. Connecting the binding head is a series of three fibronectin repeats making up the stalk. This current structural model of full length Tie2 is a combination of crystal structures of smaller domains and through homology domains are organized.

The growth (or lack of growth) of new blood and lymphatic vessels is a central process in many diseases. Tie2 signaling is essential for the proper development and maintenance of endothelial barrier function. After the vascular plexus is formed through VEGF, the angiopoietin-Tie signaling pathway takes over to remodel the immature vessel network. Mouse models that do not have Tie1 or Tie2 receptors demonstrated a poorly developed vessel network lacking branches and sprouts during embryogenesis, resulting in lethality by E10.5 (embryonic day 10.5)⁴²⁵. Insufficient expression of Ang1 also resulted in embryonic lethality at E12.5⁴⁴⁶. Endothelial cells in Ang1^{-/-} embryos displayed a rounded shape and demonstrated poor attachment to the extracellular matrix. Interestingly, if Ang1 is lost after E13.5, the embryo will proceed to adulthood. However, this comes at a cost of impaired vascular repair and organ damage⁴⁴⁷. Similarly, even though Tie1 does not have an identified ligand, the loss of this receptor results in hemorrhages leading to lethality at E13.5³⁴. The deletion of both Tie1 and Tie2 results in more detrimental impairments of vascular integrity during embryonic development³⁵.

Conversely, disruption of the angiopoietin ligand or Tie receptor function and activity through altered expression or mutation can lead to various forms of vascular malformations. Hypoxia is a common inducer of vascular growth⁵⁴. Unaddressed hypoxic conditions may result in ischemic injury of tissues from insufficient blood and oxygen supply⁴³³. Mutations that disrupt Tie2 function have been reported to cause venous malformations^{421,448,449}. Somatic Tie2 mutations cause sporadic venous malformations that are characterized by unifocal lesions and enlarged vessels⁴⁴⁹. The germline gain-of-function mutant R849W within Tie2 sensitizes the receptor to Ang1 and in some cases induces hyperphosphorylation in a ligand-independent manner resulting in disordered blood vessels^{448,450-452}. Other mutations affect the ability of Tie2 to localize and form clusters at the cell surface and at cell-cell junctions⁴⁴⁹.

As hypothesized by Juddah Folkman, angiogenesis is the rate-limiting step that dictates the growth and size of the tumor¹⁰⁰. Therefore, tumor angiogenesis is pivotal to the development of the tumor. These vessels normally appear malformed and unorganized within the tumor microenvironment. Blocking angiopoietin-Tie signaling appears to be effective at inhibiting tumor angiogenesis by disrupting Ang1 and Ang2 function⁴⁵³. The expression profiles of Ang1 and Ang2 change dramatically to favor Ang2. This increased level of expression, in combination with other angiogenic factors such as VEGF, induces robust tumor angiogenesis during the angiogenic switch⁴⁵⁴.

Due to the critical processes of angiogenesis, the angiopoietin-Tie signaling axis has been implicated in a wide range of vascular conditions, including sepsis, glaucoma, and diabetic retinopathy^{433,455,456}. The angiopoietin-Tie signaling pathway presents as a promising therapeutic target.

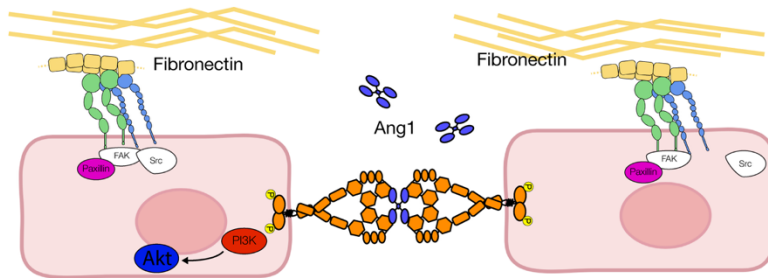
Integrin involvement with Tie2 signaling and activity

Beyond its interaction with its cognate angiopoietin ligands, Tie2 (and, to a lesser extent, Tie1) can engage in receptor crosstalk with other receptors at the endothelial cell surface (**Figure 3 - 3**)^{233,234,457–461}. A cell's response to extracellular stimuli is mediated by ligand binding to cell-surface receptors that induce an intracellular signaling cascade to alter gene transcription. However, as our understanding of receptor regulation and function has expanded, it has become increasingly apparent that cellular signaling in a phosphorylation-mediated manner is complex because multiple pathways use this mechanism⁴⁶². Rather than seeing signaling as a unidirectional flow of information, we now recognize that other cascades can influence one another through receptor crosstalk⁴⁶³. Angiogenesis is a complex process that requires the coordination of multiple protein complexes and pathways to ensure the proper migration of endothelial cells. VEGF signaling initiates angiogenesis, Tie2 signaling stabilizes the vessel, and movement can be mediated in an integrin-dependent manner. As our understanding of endothelial cell signaling has evolved, it has become more apparent that not all signaling axes are linear, and one example of this is the crosstalk between Tie2 and integrin receptors.

Vessel stabilization depends on endothelial cells adhering to the extracellular matrix. Integrins, as alluded to in the previous chapter, are critical receptors for proper angiogenesis. There have been multiple reports of integrins engaging in receptor crosstalk with receptor tyrosine kinases, such as integrin $\alpha\text{v}\beta 3$ and $\alpha 5\beta 1$ with VEGFR^{55,461,464–466}. With both integrins and receptor tyrosine kinases mediating aspects of angiogenesis, it was hypothesized that Tie2 can be modulated by integrins or vice versa (**Figure 3 - 3**)²³³. In 2005, it was demonstrated that Tie2 can constitutively associate with integrin $\alpha 5\beta 1$ on endothelial cells, and when cultured on fibronectin, Tie2 signaling can be initiated with a lower concentration of Ang1 compared to other extracellular matrix proteins. Through immunoprecipitation and fluorescence imaging, Tie2 and integrin $\alpha 5\beta 1$ coimmunoprecipitated and colocalized on the cellular membrane outside of fibrillary contacts. The presence of integrin $\alpha 5\beta 1$ is essential for Ang1-mediated angiogenesis

A

Integrin $\alpha 5\beta 1$ and Tie2 mediates their own specific pathway.

**B**

Integrin $\alpha 5\beta 1$ and Tie2 receptor crosstalk enhances PI3K/Akt and MAPK signaling.

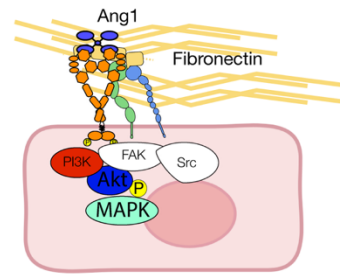


Figure 3 - 3 Integrin $\alpha 5\beta 1$ and Tie2 receptor crosstalk. **A)** Integrin $\alpha 5\beta 1$ and Tie2 mediates their own independently signaling cascades. Integrin is a motility and adhesion receptor and upon activation recruits focal adhesion and Src kinases to form focal adhesion in cellular filopodia for rearrangement of the actin cytoskeleton. Tie2 upon binding to its agonist, angiopoietin-1 (Ang1) activates the receptor to initiate downstream signaling of Akt for cellular survival genes. Together these receptors work independently to maintain the endothelial monolayer as a nonleaky structure. **B)** However, previous literature ^{233,234,457} have identified that integrin $\alpha 5\beta 1$ and Tie2 can directly associate with one another to potentiate their signaling pathways. Clustering of Tie2 and integrin receptors have been identified but their functional role has yet to be elucidated ^{163,234,430,457}.

in vivo using the chick chorioallantoic membrane (CAM) assay ²³³. Follow-up experiments were carried out approximately a decade later, validating the receptor interaction between Tie2 (and Tie1) and integrin $\alpha 5\beta 1$ ²³⁴. The cytosolic kinase domains of both Tie receptors were replaced with green fluorescent protein analogues YFP2 and mTQ (yellow and turquoise). Using Tie1-YFP and Tie2-mTQ constructs, both receptors remained associated with integrin $\alpha 5\beta 1$, suggesting that the extracellular region drives the interaction from endothelial cell lysates. This was further validated using recombinant purified ectodomains of Tie2 and integrin $\alpha 5\beta 1$, showing that the association is weak without full-length fibronectin. This receptor interaction has implications for in vivo vascular development, supporting the idea that Tie2 activity is regulated by integrin $\alpha 5\beta 1$ ^{457,458}. These studies demonstrate that there is a clear connection between integrins and Tie receptors. However, the specifics of this remain uncertain, such as the molecular contacts that govern this receptor-receptor interaction.

3.2 Motivation and rationale

Angiogenesis is a fundamental process involved in regulating blood vessel homeostasis. Since its inception as a prominent field of study in medicine and basic sciences, aberrant angiogenesis has been detrimental to human health by contributing to vessel malformations, cancer, and embryonic lethality^{15,102}. Clinical and traditional biochemical studies of endothelial cell signaling in disease and healthy states revealed that these cascades are critical for the homeostasis of blood vessels. In combination, structural determination of proteins has allowed us to relate molecular mechanisms of these receptors to cellular-level function and phenotype. High-resolution structural information has played a critical role in the development of therapeutics that target cell surface receptors^{467,468}.

Integrin $\alpha 5\beta 1$ has been observed to modulate Tie2 signaling at the cellular level^{233,234,430,457,458}. Despite the established relationship between Tie2 and integrin $\alpha 5\beta 1$, the precise regulatory role of this crosstalk remains elusive. Taking a structural approach will determine the mechanism by which Tie2 and $\alpha 5\beta 1$ regulate each other and dissect the functional roles of receptor crosstalk in cellular signaling.

Tie2 has been difficult to obtain high-resolution structural information due to its dynamic nature. The Tie2 structures that are available stem from a “divide and conquer” approach by solving independent structures of Tie2 domains^{427,428,441–444}. While this approach provided insight into the relevant domains of Tie2, including the binding interface with Ang1, the lack of adjacent and distant domain contexts limits our understanding of inter-domain information transfer (**Figure 3 - 2**)⁴⁶⁹. The structural mechanism underlying how Ang1 binding propagates across the cellular membrane and how Tie2 changes its conformation accordingly has not yet been fully elucidated^{430,441,470–472}. To answer these questions, a structural comparison of Ang1-bound and unbound full-length Tie2 receptor will provide a more complete picture of Tie2 activation without loss of structural context.

3.3 Direct integrin $\alpha 5\beta 1$ and Tie2 association was not detected in the presence of fibronectin or angiopoietin

Previous literature has shown that integrin $\alpha 5\beta 1$ is a noncanonical co-receptor for Tie2 and that integrin $\alpha 5\beta 1$ association/interaction can modulate Tie2 activity^{233,234,457,458}. Early studies concluded that integrin $\alpha 5\beta 1$ and Tie2 engage in crosstalk and fine-tune the effect of Ang1 on vascular development²³³. Dalton et al. replaced the cytosolic kinase domains of Tie1 and Tie2 and demonstrated through pull-down and Western blot assays that the ectodomain of either Tie receptor interacted with integrin $\alpha 5\beta 1$. This was further supported using recombinantly expressed ectodomain constructs. The association of $\alpha 5\beta 1$ -Tie2 complex was strengthened by the presence of fibronectin. The biochemical characterization of this integrin-Tie2 receptor pairing suggests that this complex has a functional role. However, there is currently a lack of structural understanding of how this receptor interaction occurs and which residues mediate this interaction. The addition of fibronectin, particularly the full-length protein, has dramatically influenced the association between integrin $\alpha 5\beta 1$ and Tie2. However, how fibronectin is orientated and engages with this receptor complex remains unknown.

Integrin $\alpha 5\beta 1$ and Tie receptors do not interact with one another directly or with fibronectin

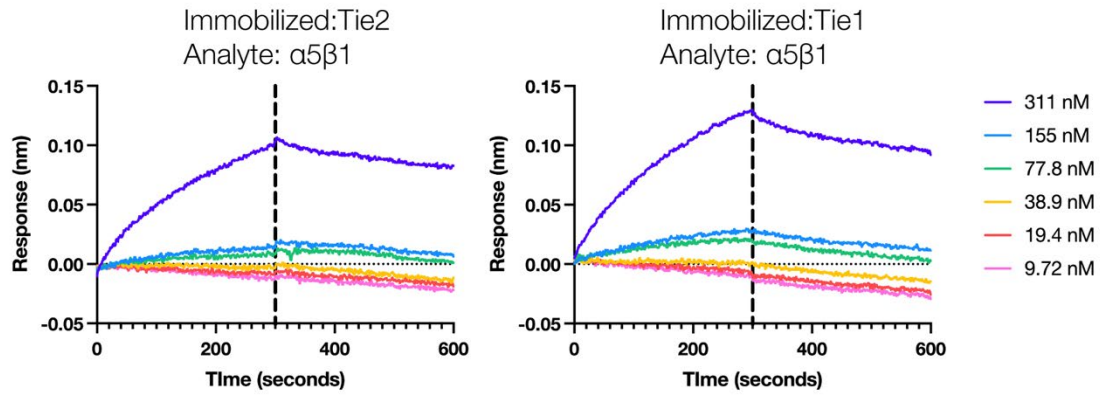
Previous experiments have demonstrated receptor association through immunoblotting and immunoprecipitation^{233,234,457}. These results, while they are specific to the target protein, may not fully recapitulate the necessary components for receptor crosstalk, particularly when using cell lysates. Therefore, to determine whether there is direct binding between integrin $\alpha 5\beta 1$ and Tie2, biolayer interferometry (BLI) was employed. Dalton et al. utilized constructs of integrin $\alpha 5\beta 1$ and Tie receptors in which they had exchanged the cytosolic kinase domains of the Tie1/2 receptors for HA or Myc tags and fluorescent protein variants (YFP and mTQ). They determined, using immunoprecipitation and without native cytosolic domains, that Tie2 can be pulled down with integrin $\alpha 5\beta 1$ ²³⁴. Therefore, this receptor crosstalk is mediated through the extracellular regions of each protein. Using biolayer interferometry and ectodomain constructs of integrin $\alpha 5\beta 1$ and Tie receptors (Tie1 residues 1-759 and Tie2 residues 1-743 with their native signal sequences). We first sought to characterize the binding affinity between $\alpha 5\beta 1$ and Tie2 in

the absence of any ligand or additional protein factors from cell lysates (**Figure 3 - 4**). Within that same study, it was suggested that receptor association in the absence of fibronectin was very weak during immunoprecipitation experiments using recombinant proteins, which could present challenges for cryoEM sample preparation ²³⁴.

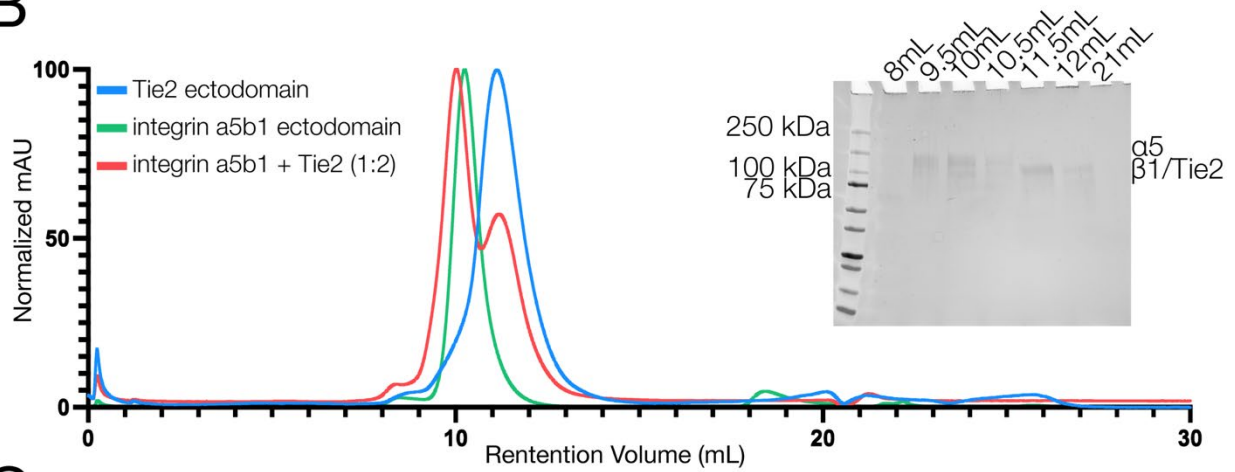
Initial ectodomain constructs of Tie2 and Tie1 were transiently transfected within Expi293 mammalian cells. Transfection followed manufacturer's protocol for 5 days before harvesting via centrifugation at 1,000 x g for 15 minutes at 4°C. The supernatant was further clarified using an additional centrifugation step performed at 18,000 x g for 45 minutes at 4°C. Purification of Tie2 and Tie1 ectodomain is a two-step process with IMAC (immobilized metal affinity chromatography using Ni Sepharose Resin) and size exclusion chromatography (SEC). Clarified supernatant was diluted 1:1 with Histrap binding buffer (20 mM NaPO₃, 500 mM NaCl and 20 mM imidazole) and applied onto a 5 mL Histrap column (Cytiva). The column was washed with Histrap binding buffer for 5 column volumes. Bound Tie2 and Tie1 ectodomains were eluted off the column with Histrap elution buffer (20 mM NaPO₃, 500 mM NaCl and 500 mM imidazole). Protein fractions were assessed by SDS-PAGE and then pooled, concentrated and buffered exchanged into 20 mM HEPES pH 7.4 and 150 mM NaCl. Purified Tie2 and Tie1 were then applied to a Superdex200 10/300 GL size exclusion column for polishing. Peak fractions were assessed by SDS-PAGE and appropriate fractions were pooled and concentrated. Concentrated Tie2 and Tie1 ectodomains were flash frozen in liquid nitrogen and stored at -80°C until use. Integrin $\alpha 5\beta 1$ expression and purification are described previously in Chapter 2.

BLI experiments started with immobilization of Tie receptors on AR2G biosensors (Sartorius), which utilizes amine groups on proteins to form a covalent bond with the biosensor surface via EDC-mediated chemistry. After a baseline measurement, immobilized Tie receptors were dipped into wells of varying concentrations of integrin $\alpha 5\beta 1$ for association and any binding detected resulted in an increase in response. With Tie2 ectodomain immobilized, none of the concentrations of integrin $\alpha 5\beta 1$ aside from the highest tested, 311 nM, displayed any notable increase in binding response (**Figure 3 - 4A**, left sensorgram). However, the magnitude of this shift was low, suggesting that very few binding events were occurring. More stable binding events are recapitulated with higher response as the thickness of the protein layer at the biosensor surface is increasing. While small binding events were measured, this was inconsistent with the

A



B



C

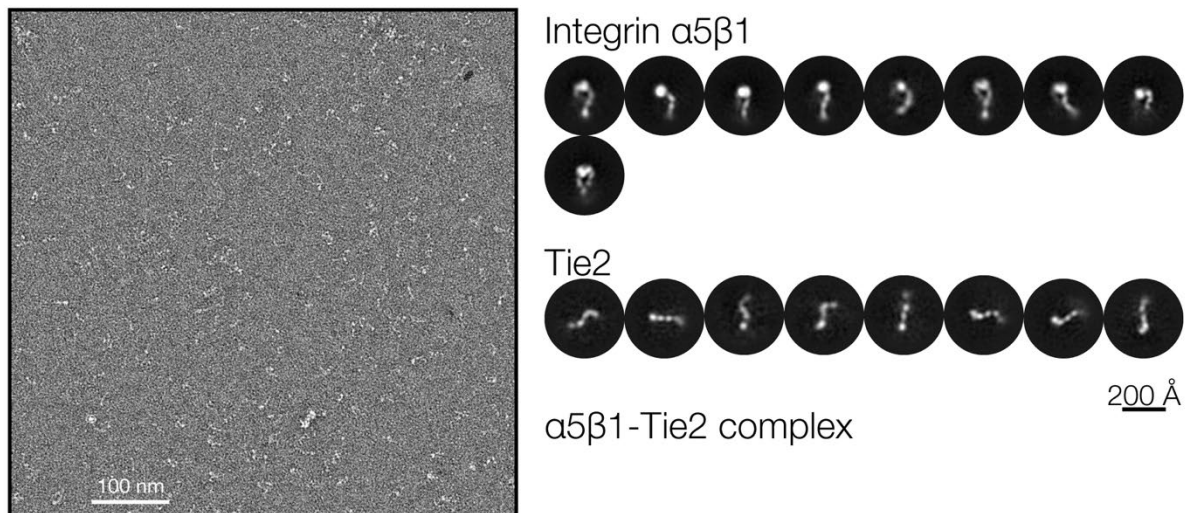


Figure 3 - 4 Tie2 and integrin $\alpha 5\beta 1$ ectodomain do not directly associate. **A)** BLI sensorgrams of immobilized Tie2 via reactive arginine chemistry with AR2G biosensors. Loaded BLI tips were then moved into wells of integrin $\alpha 5\beta 1$ ectodomain at the varying concentrations. In all concentrations tested aside from 311 nM of $\alpha 5\beta 1$, there was minimal to no association between the receptors. **B)** Size exclusion chromatogram of an integrin $\alpha 5\beta 1$ and Tie2 incubated complex for 20 minutes at room temperature prior to injection onto a Superdex200 10/300 column. Two peaks were observed that runs very similar in retention time of the individual ectodomains. SDS-PAGE analysis was partially informative as the integrin $\beta 1$ subunit and Tie2 ectodomains have similar molecular weight, making discerning of whether Tie2's molecular weight band is present within the slightly shifted peak. **C)** Negative stain EM grids were made from the leading peak from size exclusion in panel B (red) to verify the results from SDS-PAGE. A representative micrograph of the data collection is shown. 2D classification of picked particles indicated that no $\alpha 5\beta 1$ -Tie2 complex was formed. And that if there was any Tie2 present within the SDS-PAGE, it could be from the overlapping retention time of Tie2.

remainder of the concentration series, where expected results would show a laddering effect with decreasing response as integrin $\alpha 5\beta 1$ concentrations decrease (see **Figure 2 - 7** and **Figure 2 - S 5** for expected sensorgram characteristics). These results were repeated with Tie1 ectodomains and similar results were obtained (**Figure 3 - 4**, right sensorgram). Because only a single concentration provided a well-defined sensorgram curve, an affinity could not be calculated for either Tie receptor-integrin $\alpha 5\beta 1$ interaction. While these results were unexpected, they do concur with previous findings that in the absence of fibronectin, the direct association with the recombinant ectodomains of Tie receptors and integrin $\alpha 5\beta 1$ is weak²³⁴.

Orthogonally, negative stain electron microscopy (nsEM) can be utilized as a method to assess receptor complex formation directly. Integrin $\alpha 5\beta 1$ and Tie2 ectodomains were incubated together for 20 minutes in integrin active buffer (20 mM Tris-base, pH 7.4, 150 mM NaCl and 1 mM MnCl_2) prior to being subjected to gel filtration (size exclusion chromatography, SEC) using a 10/300 GL Superdex200 size exclusion column (**Figure 3 - 4B**). Analysis of the integrin $\alpha 5\beta 1$ -Tie2 SEC chromatogram shows that there was a very minimal peak shift where integrin $\alpha 5\beta 1$ alone eluted off the column, suggesting a new protein-protein species has formed (**Figure 3 - 4B**, red). When visualizing these fractions using SDS-PAGE, the molecular weight bands correspond with the individual proteins, either integrin $\alpha 5\beta 1$ or Tie2 (**Figure 3 - 4B**). No fractions displayed a clear indication of all three proteins: the $\alpha 5$ subunit, $\beta 1$ subunit and Tie2 ectodomain. This suggests that there is no receptor complex formed using the ectodomain. Nonetheless, for thoroughness, the leading peak fraction was selected for negative stain electron microscopy analysis. The resulting 2D class averages of the selected particles agreed with the SDS-PAGE, where only individual receptors were classified and no receptor complexes were identified (**Figure 3 - 4C**). In a direct 1:1 system of recombinant Tie receptors and integrin $\alpha 5\beta 1$, there was no observable receptor binding or association.

The AR2G biosensors offer a robust method of protein immobilization due to the formation of a covalent bond. This decreases any potential loss in protein during subsequent BLI steps and reduces noisy signaling during data collection. However, the EDC chemistry is nonspecific in its selection of which primary amine will be selected for immobilization. Therefore, any solvent-exposed lysine residues or the amino terminus can be used to form the bond to the biosensors. This nonspecific immobilization of Tie receptors may present suboptimal orientations for integrin $\alpha 5\beta 1$ association or even block the binding epitope. To overcome this, HIS1K biosensors were utilized in place of AR2G, which recognizes His-tagged proteins.

To obtain reliable association data with a sufficiently strong response signal (greater than 0.1 nm), the concentration of integrin $\alpha 5\beta 1$ was increased. Tie2 ectodomains were expressed and purified with a C-terminal 6x His-tag. This immobilization strategy positions the membrane-proximal FN repeat of Tie2 at the surface of the biosensor, allowing the entire ectodomain to be available for integrin binding (**Figure 3 - 2**). With Tie2 immobilized on the HIS1K biosensors, there was a notable increase in association compared to AR2G biosensors immobilized Tie2 (**Figure 3 - 5A**, left sensorgram). Visual inspection of these sensorgrams shows the expected laddering effect with decreasing concentration of integrin $\alpha 5\beta 1$. Using a global fitting analysis, these experiments suggest that the Tie2-His-tag construct and integrin $\alpha 5\beta 1$ ectodomain bind with an affinity of 104 nM. This result is surprising when compared to Tie2 ectodomain that was immobilized via AR2G, where no apparent affinity could be calculated (**Figure 3 - 4A**, left sensorgram). One possible explanation for this discrepancy could be the orientation of the Tie2 receptor with respect to the integrin $\alpha 5\beta 1$ solution. AR2G provides nonspecific immobilization compared to the C-terminal His-tag immobilization using HIS1K biosensors. To cross-validate this result, the reciprocal experiment was performed with the immobilization of integrin $\alpha 5\beta 1$ and varying concentrations of Tie2 ectodomain. The integrin $\alpha 5\beta 1$ ectodomain heterodimer was immobilized via its C-terminal His-tag on the $\beta 1$ subunit. Confusedly, there was no association between integrin $\alpha 5\beta 1$ and Tie2 when integrin is immobilized (**Figure 3 - 5A**, right sensorgram).

The presence of fibronectin (FN) substantially enhances the association between integrin $\alpha 5\beta 1$ and Tie2 when assessed by immunoblotting^{233,234}. It was demonstrated that a truncated fragment of fibronectin (FN9-10), which contains the necessary domains for integrin $\alpha 5\beta 1$ binding, is significantly weaker than the full-length fibronectin²³⁴. These authors concluded that Tie2 association with integrin $\alpha 5\beta 1$ was sensitive to full-length fibronectin²³⁴. Recombinant full-

length fibronectin is difficult to work with as it aggregation-prone^{176,473}. To avoid another layer of complexity, a longer fragment of fibronectin (FN7-10) was used to assess whether there was a minimal amount of fibronectin that enhances receptor association. This fragment has been shown to promote and stabilize the extended open conformation of integrin $\alpha 5\beta 1$ and can be recombinantly expressed in high quantities^{207,283,285}. Integrin $\alpha 5\beta 1$ was incubated with FN7-10 in a 1:1.5 molar ratio in 20 mM Tris-base, pH 7.4, 150 mM NaCl and 1 mM MnCl_2 and subjected to gel filtration to remove excess FN7-10 on a 10/300 GL Superdex200 SEC column (**Figure 3 - 5B**).

A peak shift was observed, indicating that the integrin $\alpha 5\beta 1$ was bound to the FN7-10 fragment. Fractions of integrin $\alpha 5\beta 1$ bound to FN7-10 were pooled and concentrated for BLI experiments. Tie2 6x His-tag ectodomain was once again immobilized using HIS1K and a concentration series of integrin $\alpha 5\beta 1$ -FN7-10 complex was used as the analyte (**Figure 3 - 5B, right**). Unexpectedly, there were reduced levels of association between immobilized Tie2 and the integrin-FN complex. The peak amplitude of the sensorgrams was less than half of the maximal amplitude seen in the absence of FN7-10 (**Figure 3 - 5A, left sensorgram**). This result was consistent with immunoprecipitation experiments using FN9-10 where the amount of integrin $\alpha 5\beta 1$ detected was lower than without the fibronectin fragment²³⁴.

During recombinant protein purification, both Tie2 and integrin $\alpha 5\beta 1$ ectodomains were purified using their C-terminal 6x His-tag followed by size exclusion chromatography (SEC). For the BLI experiments where either protein was utilized as the analyte (non-immobilized component), their 6x His-tag was cleaved prior to gel filtration using 3C protease. This was critical for experiments done with integrin $\alpha 5\beta 1$ as the His-tag was downstream of the acid/base coils used for purification, which keeps the subunits together. If these coils and tags were present, then integrin could not adopt the extended open conformation. This is not as critical for Tie2, as Tie2 is a single peptide chain. Independent data from the lab indicated that treatment with 3C protease was incomplete. This finding raised the possibility that the binding detected with immobilized Tie2-His and integrin $\alpha 5\beta 1$ -His was due to nonspecific binding of His-tagged integrin $\alpha 5\beta 1$. Therefore, any binding seen in **Figure 3 - 5A** may not be attributed to protein-protein binding but rather to additional protein being immobilized. To determine whether this was the case, empty HIS1K biosensors were dipped into wells containing the expected cleaved integrin $\alpha 5\beta 1$ ectodomain during a repeat experiment of the left sensorgram of **Figure 3 - 5A**.

The loading step is expected to be low in signal because no protein (No Tie2-6xHis or integrin $\alpha 5\beta 1$ -6xHis) was being captured by the HIS1K. However, during association (500 to 800 seconds), there was an increase in binding signal that could be attributed to unintentional binding of integrin $\alpha 5\beta 1$ -6xHis (**Figure 3 - 5C**, 62.5 nM NSB-nonspecific binding). This suggests that any association detected in **Figure 3 - 5A** was likely attributed to the immobilization of uncleaved integrin $\alpha 5\beta 1$ rather than actual association.

The discrepancy across the BLI experiments highlights the importance of immobilization methodology. HIS1K provides a convenient immobilization strategy and a lower barrier to entry for His-tagged proteins for BLI. However, nonspecific binding or unintentional immobilization may limit the interpretation of the results and must be controlled for. Our rationale for using HIS1K was that the C-terminal 6xHis on the Tie2 ectodomain positions the protein in such a manner that no potential binding epitope is occluded. In contrast, AR2G biosensors immobilize protein using EDC/NHS chemistry through primary amines (lysine residues and N-terminus). This targeting is random and immobilization at any amine group could occlude possible surfaces. In retrospect, this represents a limitation of the experimental design, and future work should be repeated using protein constructs of high specificity, reducing the ambiguity in interpretation of the results. The use of an Avi tag has been shown to be an effective immobilization strategy for BLI ligands ⁴⁷⁴. The manufacturer of the BLI instrumentation and reagents (Sartorius) now offers anti-FLAG biosensors which immobilizing proteins via the FLAG tag has been effective for other Tie2 experiments. These new sensors combined with unique protein tags may ensure that any nonspecific binding events will be accounted for or removed.

These BLI results also raise the question of whether an alternative binding site on fibronectin is present for Tie2 binding. Recombinant full-length fibronectin is highly heterogeneous but enhances the receptor association markedly compared with truncated fragments or no ligand present compared to a truncated construct. Both the full-length and truncated (7-10) fibronectin are expected to induce the extended open conformation of integrin $\alpha 5\beta 1$. I hypothesized that there must be another site within the full-length fibronectin that enhances this interaction outside of the integrin $\alpha 5\beta 1$ recognizing domains.

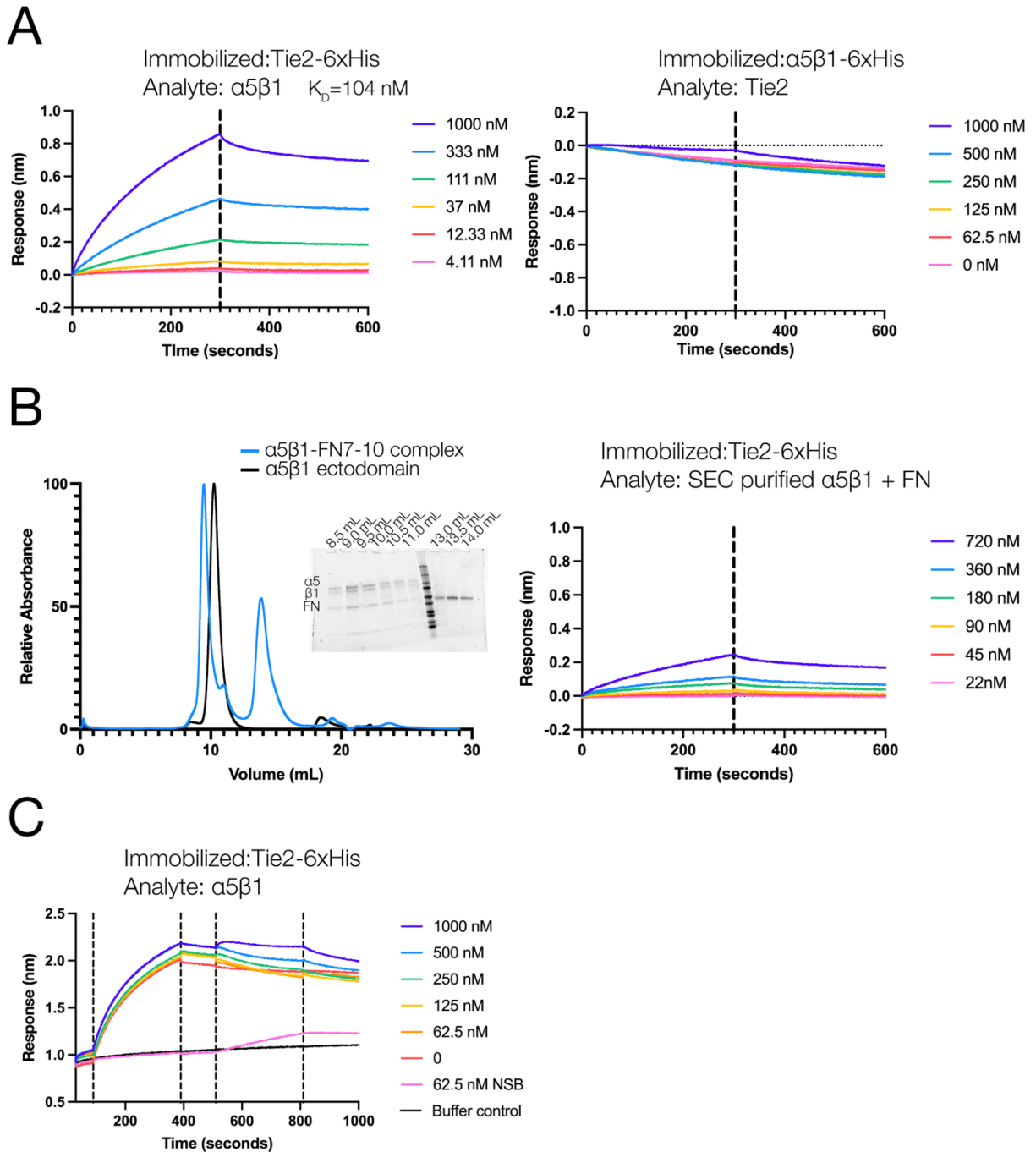


Figure 3 - 5 Fibronectin bound integrin does not associate with Tie2. **A)** Using HIS1K biosensors, association experiments were repeated. Immobilization of Tie2 ectodomain via the C-terminal His-tag proved to be effective at concentrations above 111 nM. Fitting of these sensorgrams yielded an affinity of 104 nM. Performing the opposite experiment where integrin $\alpha 5\beta 1$ was immobilized by the His-tag on the $\beta 1$ C-terminal does not recapitulate any binding. **B)** Integrin $\alpha 5\beta 1$ was mixed with FN7-10 at a 1:6 molar ratio and subjected to SEC purification to capture the integrin $\alpha 5\beta 1$ -fibronectin complex. Peak fractions of the complex were concentrated and directly used for biolayer interferometry experiments with immobilized Tie2. Compared to apo integrin $\alpha 5\beta 1$, FN7-10 bound yielded reduced binding responses which is similar to previous literature using truncated fibronectin²³⁴. **C)** Raw data of immobilized Tie2 on HIS1K and unloaded biosensors. Unloaded biosensors when dipped into a well of low concentration of integrin $\alpha 5\beta 1$, nonspecific binding of incompletely cleaved integrin $\alpha 5\beta 1$ onto these biosensors.

Recombinant Integrin $\alpha 5\beta 1$ does not associate with Tie2 by pulldown assays

To minimize the ambiguity of nonspecific binding prompted by the previous BLI experiments, Tie2 ectodomain was re-cloned to include a 3xFLAG tag in addition to its 6x His-tag used for purification. Purification of Tie2 ectodomain remained the same with a single IMAC step using Ni Sepharose resin (Cytiva) followed by size exclusion chromatography. Instead of biolayer interferometry, pull-down assays using anti-FLAG magnetic resin were used to determine whether integrin $\alpha 5\beta 1$ could be isolated with Tie2. This experimental setup was similar to the published immunoprecipitation results^{233,234}.

Controls were established to ensure that no nonspecific binding was occurring for subsequent pull-down assays. The same “cleaved” integrin $\alpha 5\beta 1$ was applied to anti-FLAG resin in 20 mM Tris-base, pH 7.4, 150 mM NaCl and 1 mM MnCl_2 . The resin was washed with the same buffer before elution with low pH glycine (10 mM glycine, pH 2.5). As expected, integrin $\alpha 5\beta 1$ was found in the flowthrough and wash fractions and not within the elution profile as this construct does not contain a 3xFLAG tag (**Figure 3 - 6A**, left SDS-PAGE). Tie2 ectodomain, which now has the 3xFLAG tag bound to the resin and eluted as expected (**Figure 3 - 6A**, right SDS-PAGE). The effect of full-length fibronectin on receptor association is substantial from immunoprecipitation based on visual assessment²³⁴. Therefore, the following experiments utilized commercially available full-length fibronectin (R&D Systems). Tie2, integrin $\alpha 5\beta 1$ and full-length fibronectin were added to anti-FLAG resin and allowed to incubate for 30 minutes at 4°C with rocking. Unbound material was washed with buffer (20 mM Tris-base, pH 7.4, 150 mM NaCl and 1 mM MnCl_2) before elution with low pH (10 mM glycine pH 2.0). Analysis of the flowthrough and wash fractions showed molecular weight bands that correspond with integrin $\alpha 5\beta 1$ and full-length fibronectin. The only protein species within the elution was Tie2 (**Figure 3 - 6B**). Using this assay, the addition of full-length fibronectin did not enhance or affect the association between integrin $\alpha 5\beta 1$ and Tie2, which contradicts published literature²³⁴. If the integrin open conformation induced by fibronectin is suboptimal for Tie2 association, then exchanging manganese for calcium may resolve this issue. In vitro, calcium shifts the conformational equilibrium of integrin $\alpha 5\beta 1$ toward the bent and extended closed state, reducing affinity for fibronectin^{206,208,283,308}. The same pull-down experiment was repeated under 5 mM

CaCl₂ (nonactivating buffer) in place of MnCl₂ (**Figure 3 - 6B**). In solution, we expected that integrin $\alpha 5\beta 1$ and fibronectin would not be bound. This would also elucidate whether fibronectin contains an alternative binding site for Tie2 as full-length fibronectin enhanced receptor association more effectively than a truncated construct (**Figure 3 - 5B**)²³⁴. Analysis of these fractions demonstrated a similar result as the integrin activating buffer (manganese), where the flowthrough and wash fractions contained molecular weight bands corresponding to both integrin $\alpha 5\beta 1$ and fibronectin. The elution fraction only contained Tie2 (**Figure 3 - 6B**).

Recent work from the Ruohola-Baker laboratory showed that integrin $\alpha 5\beta 1$ was identified within Tie2 clusters mediated by high-order oligomers of angiopoietin-1 (Ang1), the Tie2 agonist^{430,475}. Using a protein-designed scaffold, presentations of six or more copies of the fibrinogen-like domain (F-domain) have agonistic behavior. Alternatively, presentations of three or four F-domains have Ang2 or antagonist behavior. The Ang1 ligand has been reported to influence the receptor association between integrin and Tie2²³³. To test whether the presence of the Ang1 F-domain could facilitate receptor association, the activating *de novo* design oligomer, H-8 Ang1, was added to the pull-down assay (**Figure 3 - 6C**). The construct monomer contains a copy of the F-domain that is connected to an α helix. Together eight copies make up H-8 Ang1 through an α helix bundle. Tie2 and integrin $\alpha 5\beta 1$ ectodomains, H-8 Ang1, and/or full-length fibronectin were added to anti-FLAG resin and allowed to incubate for 30 minutes at 4°C with rocking prior to washing to remove unbound material. The elution profiles in either case only contained molecular weight bands that corresponded with Tie2 ectodomain and the individual components of the H-8 Ang1 (**Figure 3 - 6C**). The presence of H-8 Ang1 supports that the expressed Tie2 ectodomain was properly folded. However, there was no identifiable receptor association.

These results combined with the biolayer interferometry suggest that the ectodomains of integrin $\alpha 5\beta 1$ and Tie2 do not directly bind. The addition of either receptor's ligand did not influence the receptor interaction under the conditions tested. In retrospect, western blotting targeting the specific receptors should have been utilized to complement the SDS-PAGE analysis as the molecular weights between the integrin $\beta 1$ subunit and Tie2 ectodomain are very similar. Additionally, the Tie2 ectodomain construct that was generated here in the lab was not a 1:1 direct comparison with Dalton et al.'s in their recombinant immunoprecipitation assay. Their construct utilized an FC-tag that forces their Tie2 into a synthetic dimer, but their expression

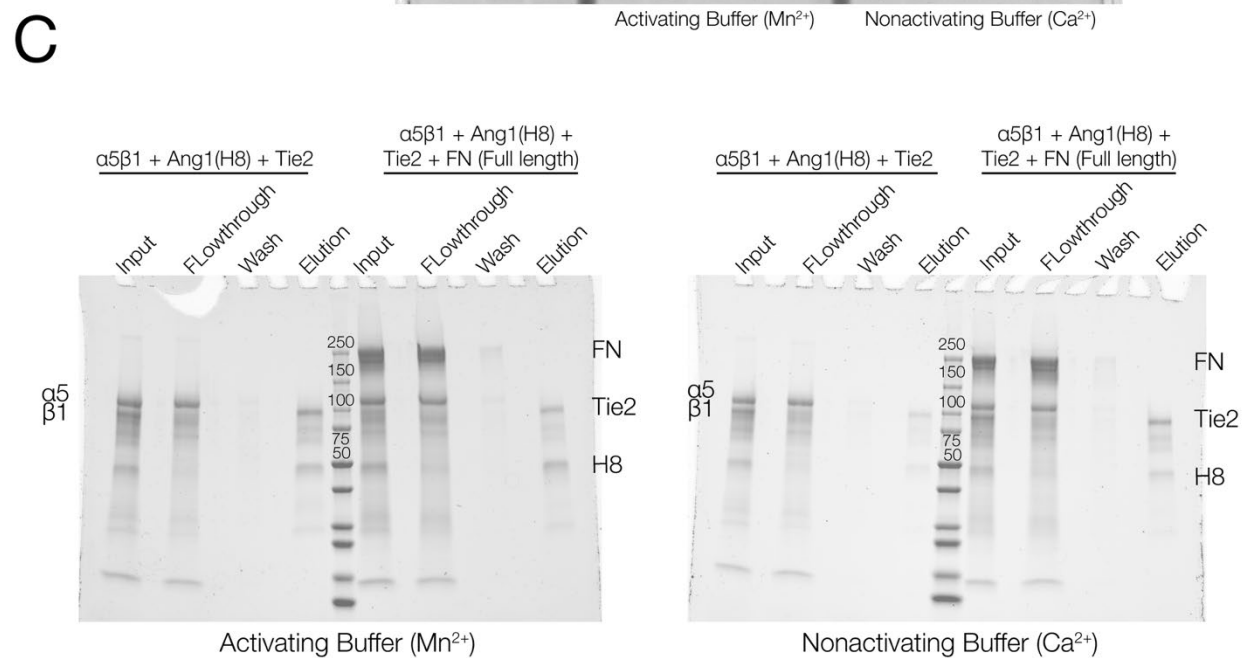
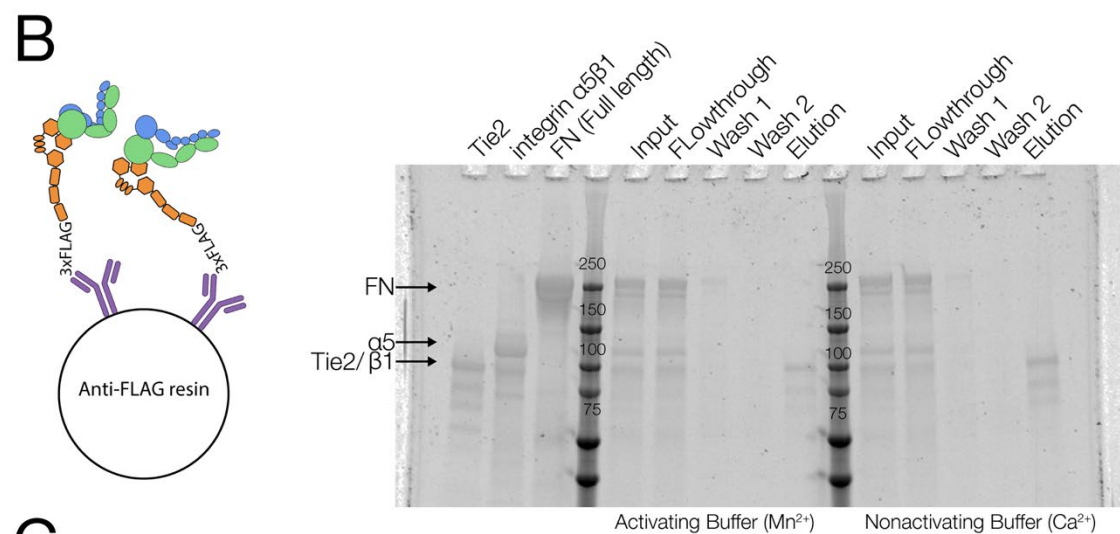
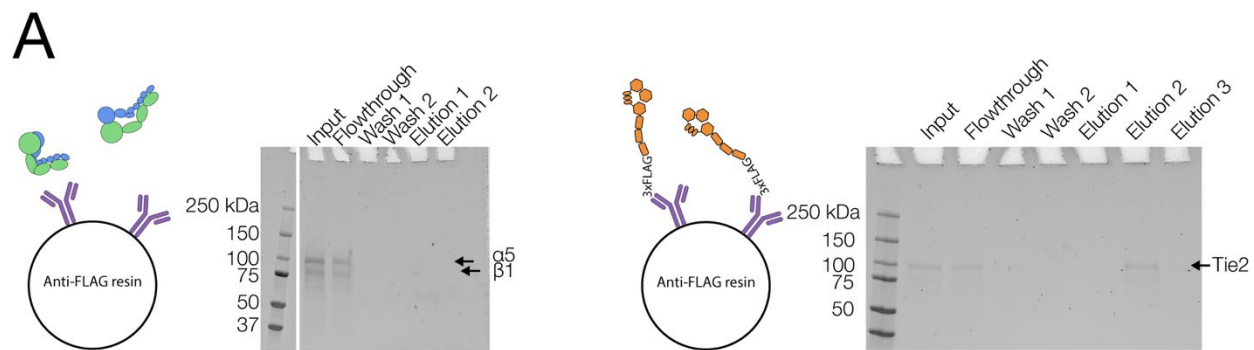


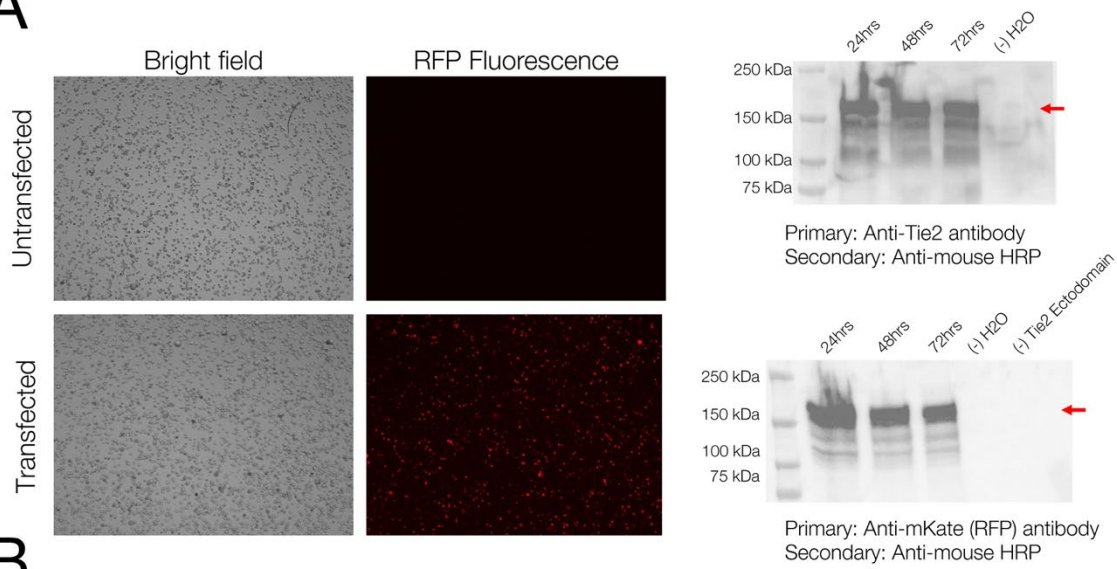
Figure 3 - 6 Pull down assays using anti-FLAG resin. A) Control pull-downs were performed to validate that only Tie2 can be immobilized by the anti-FLAG resin as it has a 3xFLAG tag whereas integrin $\alpha 5\beta 1$ does not. B) All protein components were added to anti-FLAG resin and allowed to equilibrate. The resin was spun down, and the solution was removed and replaced with fresh buffer. The bead mixture was allowed to additionally rock and buffer exchanged was repeated once more. Captured protein was eluted with pH 2.0 10 mM glycine. SDS-PAGE demonstrates that there is integrin $\alpha 5\beta 1$ and the full-length fibronectin is not captured by the Tie2 which is immobilized onto the anti-FLAG resin. C) H-8 Ang1 in both integrin activating and nonactivating buffer, does not facilitate any receptor binding between integrin $\alpha 5\beta 1$ and Tie2. Integrin $\alpha 5\beta 1$ and fibronectin are found in the flowthrough and wash fractions with only the Tie2 ectodomain and H-8 Ang1 in the elution profile. It could be that possible cytosolic binding partners may be involved in this association more than initially realized.

construct of Tie2 did not contain the entirety of the ectodomain²³⁴. It may be that Tie2 oligomerization or the activated dimer conformation of Tie2 is more critical for receptor crosstalk than the presence of the receptor itself.

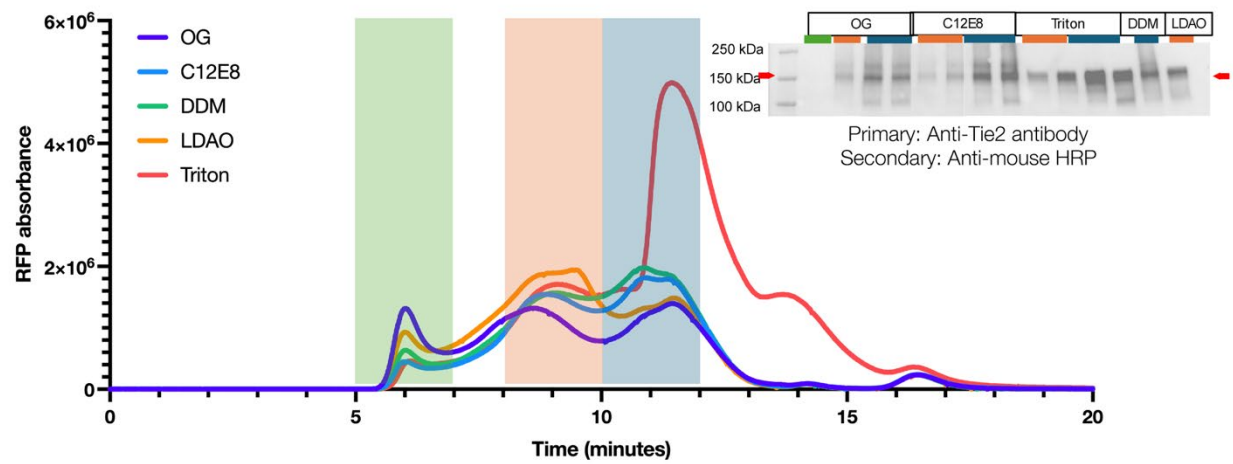
3.4 Molecular mechanisms of Tie2 activation and dimerization

While ligand-induced dimerization is one of the primary activation mechanisms of receptor tyrosine kinases, it is not the only one. Across RTK families, it is becoming more apparent that the multimerization of the receptors into clusters has a functional role in activation^{430,440,443,476}. Regarding Tie2, limited structural information exists about any perturbations that the receptor undergoes during ligand binding. Structural biology of membrane receptors has been historically difficult to study due to low solubility and the dependence on the membrane⁴⁶⁹. The “divide and conquer” strategy is often employed for single-pass transmembrane proteins by determining structures of truncated segments of the receptor of interest. Structures of Tie2 bound to the F-domain of Ang1 and Ang2 demonstrate that they make largely the same contacts with the Tie2 ligand binding domain, yet they have opposing downstream effects^{427,428}. Therefore, if the interface between the receptor and two ligands is largely the same, how is a lower oligomerizing ligand (Ang2) distinguished from a higher order ligand (Ang1)? Furthermore, a single angiopoietin molecule must be capable of bridging the gap between two distinct receptors, which are estimated to be more than 300 Å apart^{442,477}. In addition to ligand-induced dimerization, clustering seems to have a profound impact on Tie2 signaling^{430,431,443}. However, the ligand-bound structures are truncated Tie2 receptor ectodomain constructs of the ligand-binding head or the stalk regions^{427,428,442,477}. It is unclear from these current protein structures whether Tie2’s stalk region of the fibronectin type III repeat domains and the transmembrane

A



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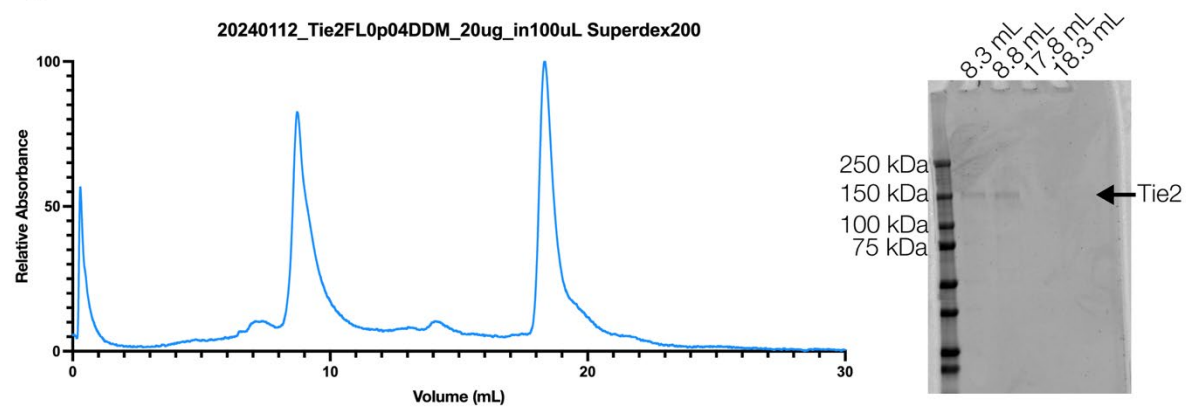


Figure 3 - 7 Tie2 Full-length expression and purification. **A)** Full-length Tie2 (residues 1-1124) with a C-terminal mKate (RFP) tag were transfected into Expi293 mammalian cells. Because the protein is not secreted into the supernatant, prolonged overexpression of membrane proteins can be toxic to the cell on top of the transfection process. Expression was carried out according to manufacturer's protocol for 3 days. Western blot analysis verified expression of full-length Tie2 with the mKate tag. **B)** There are many types of detergents that can be utilized to extract membrane from the plasma membrane. FSEC chromatograms are shown to display the effectiveness of a panel of common detergents used to solubilized membrane proteins. Interestingly, Tie2 was found to be present in all peaks owing to potentially different populations of aggregated or properly folded full-length Tie2. **C)** Size exclusion chromatogram of FLAG affinity purified full-length Tie2 injected onto a 10/300 GL Superdex200 SEC column. Due to the resolving power of the SEC column and molecular weight and hydrodynamic radius of Tie2, the purified protein runs near the void (~ 9 mL). Downstream purifications utilized a 10/300 GL Superose6 SEC column instead. SDS-PAGE analysis of peak fractions retained the molecular weight band from FSEC.

helix change (or not) due to angiopoietin binding and whether they have any impact on clustering.

To overcome this obstacle, a full-length protein structure of Tie2 bound to Ang1 would provide a clear mechanism of any conformational changes. Protein structure determination of membrane proteins has been historically difficult. To do so, multiple strategies have been developed to circumvent the membrane requirement. These range from mimicking the membrane environment with detergents to replacing the native membrane with a synthetic phospholipid bilayer^{478,479}. This section aims to take advantage of modern developments to stabilize single-pass transmembrane receptors to study them structurally. Applying these strategies to Tie2 may elucidate more clearly Tie2's conformational changes during activation induced by angiopoietin-1 and how this binding signal is transmitted across the membrane.

Detergent Solubilized Tie2 yields low resolution structural information

Detergent solubilization is the most common way to stabilize membrane proteins and in comparison to alternative methods, it is the simplest^{148,478,479}. The objective is to exchange the membrane protein from its native lipid environment and composition in favor of nonnative detergent micelles. These are amphipathic molecules that have polar and nonpolar properties. There are several different detergents available for extraction, which can be classified into three major classes: ionic, nonionic and zwitterionic^{478,480}. Each of these has its own properties that make them advantageous and a suitable alternative to native lipids from the membrane⁴⁸¹. Transmembrane domains of the receptors are the most susceptible to aggregation in aqueous solutions due to a high abundance of nonpolar residues. The nonpolar domains of the detergent molecules will pack against the transmembrane domain, sheltering them from water or polar

solutions. The choice of detergent for the stabilization of membrane proteins is in part guided by its critical micelle concentration (CMC). The CMC is defined as the concentration at which the detergent molecules spontaneously self-assemble into micelles. Higher CMC values will require a higher detergent concentration to maintain micelle formation, which can affect protein stability⁴⁸². Despite these guiding principles, there is no systematic method to determine the optimal detergent for stabilization of a given membrane protein. Thus, each membrane protein's optimal solubilizing and stabilization detergent must be empirically determined. Full-length Tie2 (residues 1-1124) fused with a C-terminal 6x His-tag and mKate RFP were expressed using Expi293 mammalian cells by transient transfection (**Figure 3 - 7A**). Using RFP, the expression of full-length Tie2 could be tracked over time. Cells were harvested three days post transfection to avoid possible cytotoxic effects of membrane overcrowding. Western blot analysis was performed orthogonally to validate expression (**Figure 3 - 7A**). Blotting for Tie2 or mKate showed that protein expression did not appear to increase 48 hours post transfection. Overexpression of membrane proteins is often on a shorter time scale compared to soluble proteins as their presence on the membrane can contribute to membrane overcrowding, which can be toxic to the cells on top of their transfection conditions. To limit the amount of aggregation or unfolded protein, transfections are often limited to a maximum of 72 hours.

A screen of n-dodecyl β -D-maltoside (DDM), octaethylene glycol monododecyl ether (C12E8), octylglucopyranoside (OG), lauryldimethylamine-N-oxide (LDAO) and Triton-X 100 was performed as these are common detergents used for solubilization of membrane proteins⁴⁸². Transfected Expi293 were harvested at 1000 x G at 4°C for 15 minutes before being resuspended in 1x HBS buffer (20 mM HEPES, pH 7.4, 150 mM NaCl). 1x HBS supplemented with each detergent was then added to the cellular solution to obtain a final detergent concentration of 2%. The cellular-detergent mixture was rocked gently for four hours at 4°C to solubilize and extract Tie2. Insoluble material was clarified using centrifugation at 36,000x G at 4°C for 30 minutes. To rapidly determine whether a detergent was effective at extracting Tie2 from the cellular membrane, fluorescent size exclusion chromatography (FSEC) was employed (**Figure 3 - 7B**). FSEC is similar to traditional size exclusion chromatography where protein particles are separated based on molecular weight and hydrodynamic radius. In addition to a UV detector, which tracks the elution profile of proteins off the column, a fluorescence detector is also utilized. This allows tracking of fluorescent protein with respect to retention time and can be

combined with UV detection to track specific target proteins. The solubilized and clarified cell lysate was injected onto a 3.2/300 Superdex200 3 mL SEC column. Analysis of the FSEC chromatograms shows that all detergents were able to extract RFP-tagged proteins. When these fractions were taken for western blot analysis to see if Tie2 is present within these fractions, all fractions contained Tie2 (**Figure 3 - 7B**). This is puzzling as Tie2 was present across a range of molecular weights, which could be an indication of heterogeneity. While all detergents were successful, not all detergents protected membrane proteins equally and are compatible with cryoEM. Triton-X 100 appears to solubilize the greatest amount of Tie2. However, TritonX-100 is an aggressive detergent and oxidation sensitive and forms peroxides, which are damaging to protein stability⁴⁸³. This is likely due to multiple states that Tie2 may be experiencing under solubilization conditions. Therefore, n-dodecyl β -D-maltoside (DDM) was selected as it is a milder detergent and is compatible with cryoEM due to its low CMC.

Protein expression was scaled accordingly, and extraction was repeated as described above but SEC was performed using a 10/300 GL Superdex200 column instead. Initial purification using Ni-NTA Sepharose resin provided a workable amount of solubilized full-length Tie2. However, upon assessment of SEC peak fractions and via negative stain EM, protein particles were highly heterogenous, and a large amount of aggregation was observed. This was likely caused by the high concentration of imidazole that was used for protein elution from the nickel column. While this buffer reagent is widely used within the lab and other proteins, concentrations this high may be unstable for Tie2. With expression of full-length Tie2 determined, the C-terminal RFP tag, mKate was exchanged for 3xFLAG.

Protein extraction proceeded as described above, and the cell lysate was clarified through ultracentrifugation at 100,000x g for 1 hour at 4°C. Soluble material was then applied to equilibrated anti-FLAG resin and allowed to incubate for 2 hours at 4°C with rocking. Unbound material was washed away with buffer (20 mM HEPES, pH 7.4, 150 mM NaCl) before elution with 0.5 mg/mL of 3xFLAG peptide. Peptide elution was opted for in place of low pH elution as it is gentler on the already semi-stable full-length Tie2. Elution fractions were pooled and concentrated. This was applied onto a 10/300 GL Superdex200 size exclusion column where peak fractions were assessed by SDS-PAGE for purity and validation (**Figure 3 - 7C**). The SEC chromatogram shows two peaks, the first being full-length Tie2 when assessed by SDS-PAGE

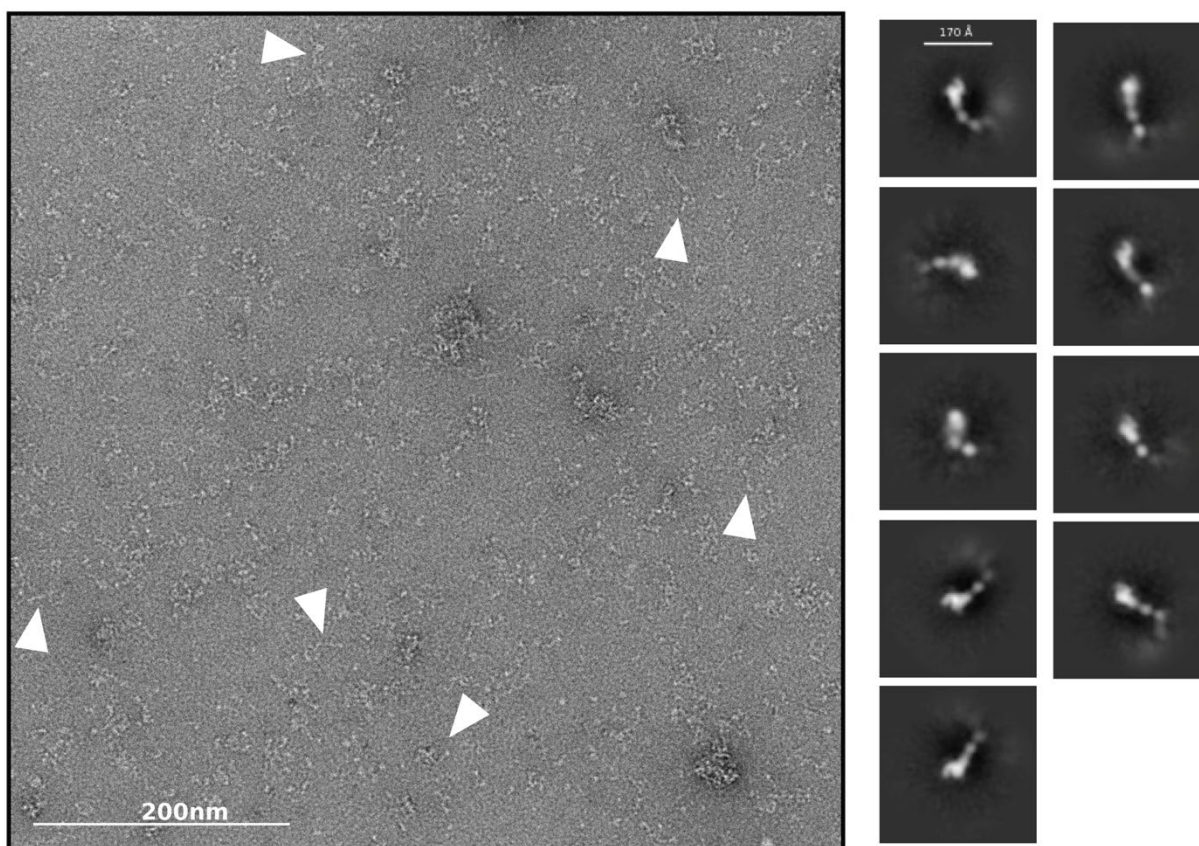


Figure 3 - 8 Negative stain micrograph and 2D class averages of full-length Tie2. Shown is a curated micrograph where protein aggregation was limited and individual full-length Tie2 particles can be identified. Particles that were used for 2D classification were automatically picked using cryoSPARC's blob picker. 2D class averages show the ligand binding head (has a triangular shape) of the extracellular region along with the first two fibronectin type III repeats of the Tie2 stalk.

and the latter peak corresponding to the 3xFLAG peptide that was used for elution from affinity resin.

Initial assessment using nsEM showed that detergent micelles were present and Tie2 was found in isolation or within clusters (**Figure 3 - 8**). Through particle curation and 2D classification, the ligand binding head was identified. In a few instances, the lower domains of the FN repeat stalks are present but at low-resolution, suggesting heterogeneity as predicted. Nonetheless, this result demonstrates that full-length Tie2 can be isolated from mammalian cells and purified with the proper protein folds for downstream nanodisc incorporation in a more native-like system. The full-length Tie2 eluted near the void volume through the 10/300 GL Superdex200 SEC column. This makes interpretation of the separation between aggregated or larger species and full-length Tie2 difficult. In retrospect, a 10/300 Superose6 SEC column

should have been switched to earlier to provide a clear distinction between large potential protein aggregates and full-length Tie2. This became apparent within the initial nanodisc incorporation conditions as the peaks were maintaining retention volumes approximately at the void volume. However, upon thawing full-length Tie2 aliquots from -80°C storage, protein quality under negative stain EM became significantly worse and aggregation was consistent throughout the grid and confirmed by SEC. Therefore, for each nanodisc preparation and trial, Tie2 would have to be purified fresh.

Tie2-nanodiscs exhibit high heterogeneity

Nanodiscs are synthetic phospholipid bilayer mimetics⁴⁸⁴⁻⁴⁸⁶. A nanodisc is a two-component system that is composed of lipids and an amphipathic helical protein that acts as a belt to encapsulate lipids within a defined area and volume. One of the most common types of scaffolding protein is the membrane scaffold protein (MSP), which is derived from apolipoprotein-A1⁴⁸⁷. The MSP of choice will determine the size of the nanodisc, which can range from 6 nm to 20 nm in diameter. Unlike the detergent micelles, nanodiscs preserve the lipid bilayer, maintaining any protein-lipid interactions. In addition, the detergent molecules within micelles are arranged radially, creating a suboptimal environment compared to biological membrane bilayers. The general process of nanodisc incorporation involves the extraction of the target membrane protein via detergents (**Figure 3 - 9**). This can be done directly by adding detergents to the cell lysate or to purified membranes obtained by sonication and ultracentrifugation. The membrane protein can be purified away from impurities before the addition of MSP and a lipid mixture. This lipid mixture will exchange with the detergent molecules that are protecting the transmembrane domain, and the excess detergent will be removed. The removal of free detergent initiates the spontaneous formation of the nanodisc by the MSP, encapsulating lipid molecules. Occupied nanodiscs can then be purified away from unoccupied nanodiscs in aqueous buffer solution without the need for detergents.

MSP-1E3D1 (and other MSP variants tested *) was cloned through PCR amplification into a bacterial expression vector. *Escherichia coli* BL21 was grown until an OD600 of 0.6-0.8 was reached before being placed on ice and induced with isopropyl (β)-D-1-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. The bacterial cultures were

* Other membrane scaffold protein (MSP) variants that were tested are 1D1, 1D1ΔH5 and 2N2.

brought back into the shaker for overnight expression at 18°C. *E. coli* were harvested via centrifugation at 4800x G for 15 minutes at 4°C before being resuspended with 50 mM Tris-base, pH 7.5, 150 mM NaCl, 1% TritonX-100, 1 mM EDTA and one protease inhibitor tablet. MSP was extracted from cells using sonication followed by centrifugation at 36,000 x G for 45 minutes at 4°C to remove cellular debris. The protein extract was diluted 1:1 with Histrap binding buffer (20 mM NaPO₃, 500 mM NaCl and 20 mM imidazole) and applied onto a Cytiva 5mL Histrap column. The column was washed with 2 column volumes of binding buffer before being eluted with Histrap elution buffer (20 mM NaPO₃, 500 mM NaCl and 500 mM imidazole). Peak fractions were assessed by SDS-PAGE, pooled and concentrated and buffer exchanged into MSP buffer (50 mM Tris-base pH 7.5, 150 mM NaCl). Due to the purity of MSP protein from Histrap purification, protein was flash frozen at -80°C and stored until use.

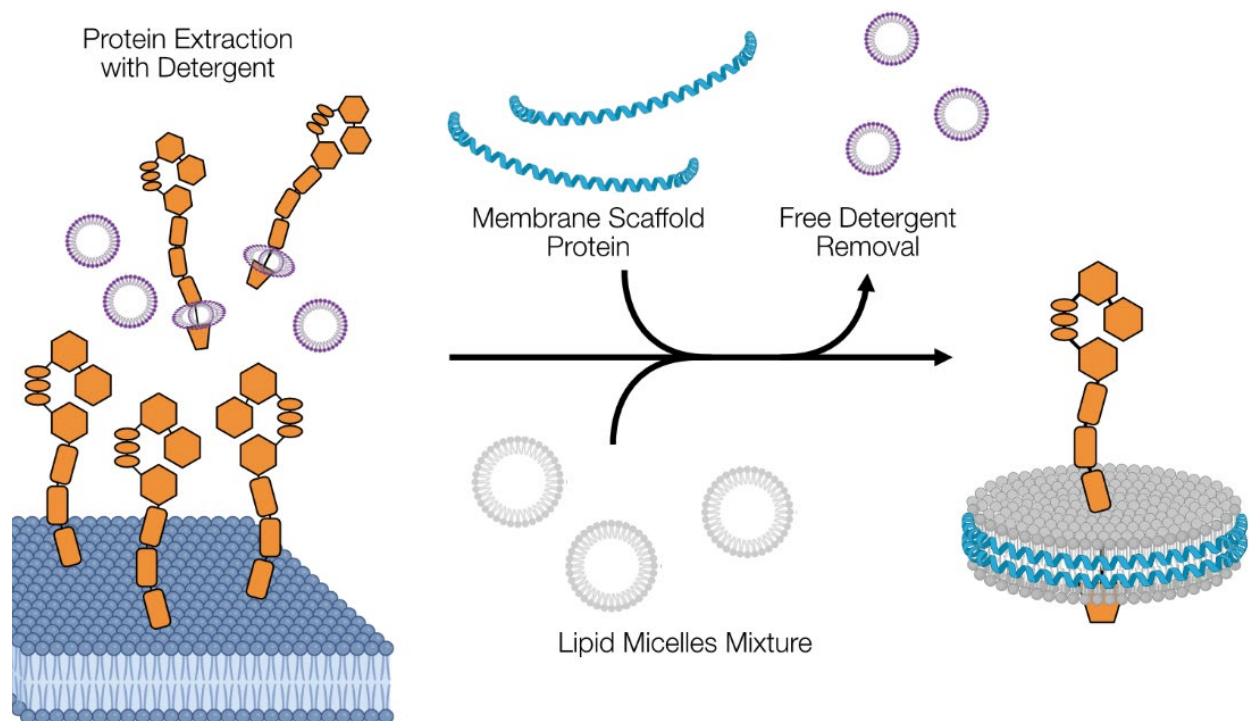


Figure 3 - 9 Process of membrane protein extraction using detergent for nanodisc incorporation. Tie2 are expressed on Expi293 mammalian cells via transfection. Tie2 and other membrane proteins can be solubilized or extracted from the plasma membrane using detergent solutions, or more recently copolymers. Non Tie2 proteins can be purified away using affinity purification and size exclusion chromatography. Using purified Tie2, membrane scaffold proteins (MSP) can be added to Tie2 alongside a lipid micelle mixture that is commercially available for custom made. The detergent molecules that were used in solubilization and purification will exchange with the lipid micelle mixture and get encased by the MSP molecules. Excess detergent can be removed from the system using adsorbent beads. Tie2-nanodiscs can be polished using a final SEC purification.

Due to the wide range of molecular weights of phospholipids, a nanodisc stoichiometry calculator was developed to assist in experimental setup and lipid selection. This takes into consideration the size of the MSP, the membrane protein and the chosen phospholipids. As with detergent solubilization and stabilization of membrane proteins, nanodisc incorporation and assembly are guided by various principles, but the optimal conditions must be determined empirically⁴⁸⁸. Lipid mixtures that were used for nanodisc incorporation included soybean polar lipids, porcine brain polar lipids, and synthetic lipid mixtures. Soybean polar lipids is a popular choice as the composition of the lipid mixture is mostly defined with a small percentage of the lipids denoted as unknown. However, with Tie2 as a mammalian protein, the plant-based membrane from soybeans may not be a perfect match for transmembrane helix lipid interactions. On the other hand, porcine brain polar lipids, is also mammalian, but the composition of this lipid mixture is approximately 70% unknown. With synthetic lipid mixtures, the researcher can define the composition by mixing in defined lipids to achieve their ratio of interest, allowing more control during experimentation. **Table 3 - 1** summarizes the nanodisc assembly conditions that have been tested and major takeaways.

Across all nanodisc preparations, lipids, either single phospholipid components or phospholipid mixtures, must be prepared. Lipid stocks dissolved in chloroform were dried the day before under a stream of nitrogen gas, followed by overnight desiccation under vacuum in the presence of Drierite (CaSO₄). The lipid film is then rehydrated with buffer containing small amounts of detergent (DDM) or sodium cholate⁴⁸⁸. This mixture is resuspended via a water bath sonicator first at 30 kHz for 5 minutes before a second 5-minute at 80 kHz. This process is repeated until the lipid solution is transparent or nearly transparent. The lipid solution is added to full-length Tie2 protein at the desired molar ratio before adding the MSP protein. The Tie2-phospholipid-MSP solution was incubated with rocking at 4°C for 1 hour before detergent removal. To initiate assembly, the detergent or sodium cholate is slowly removed using hydrophobic adsorbent resin such as Bio-beads or Amberlite. To assess for successful incorporation of Tie2 into MSP nanodiscs, size exclusion chromatography is used for the separation of empty nanodiscs and any free MSP protein.

A cryoEM structure of integrin $\alpha 5\beta 1$ within a 1E3D1 nanodisc served as a starting point for lipid ratios because integrin subunits and Tie2 are both type-1 transmembrane proteins²⁰⁷. Initial optimization of lipid ratio started with soybean polar lipids at a protein:MSP:lipid ratios of

1:30:2000, 1:30:3500, and 1:30:5000 (**Figure 3 - 10A**). After detergent removal, the protein solution was subjected to SEC purification from empty nanodiscs and other unincorporated materials. Across the protein:MSP:lipid ratios tested, the quantity of the empty nanodiscs dwarfs the occupied nanodiscs. The fractions of the occupied nanodiscs exhibit a high degree of heterogeneity. Superimposition of the retention times of full-length Tie2 solubilized in DDM and MSP 1E3D1 conditions suggests very few nanodiscs are formed and a large amount of aggregation occurred, which is seen by negative stain EM (**Figure 3 - 10B**). Despite the aggregation, there are a few nanodiscs that do have Tie2 incorporated successfully. However, these were infrequent and no 2D class averages could be generated from this low number of particles. The utilization of high molar ratios of lipids is suboptimal, and nsEM micrographs show large amounts of aggregation. This reinforces that determining optimal conditions for nanodisc incorporation is protein-dependent and performed empirically.

The nanodisc formed by 1E3D1 was initially chosen because it provides a sufficient bilayer area for Tie2 incorporation. With large bilayer area, Tie2 can experience increased movement within the bilayer, which can have complications during particle alignment in cryoEM⁴⁸⁹. With consultation from the Atkins Laboratory at the University of Washington, MSP 1D1 was identified as the optimal size for Tie2 nanodisc assembly. The diameter of 1D1 nanodiscs is approximately 9-10 nm compared to that of 1E3D1, which are 12-14 nm. The lipid ratio was drastically reduced as well. This finding highlighted the empirical nature of optimization of protein occupancy as conditions are not directly transferrable despite similar protein structure where integrin subunits and Tie2 are single-pass transmembrane receptors.

With these results and adjusted parameters in mind, we further refined the nanodisc assembly process. We took advantage of the unique 3xFLAG tag on the C terminus of Tie2 by performing an additional affinity purification. After nanodisc assembly and removal of detergent by poly-adsorbent beads (Biobeads or Amberlite), all remaining Tie2 should be encased within a nanodisc, and all empty discs will be in the flowthrough. This sample can then be injected onto SEC for final polishing to assess homogeneity. SDS-PAGE analysis of the SEC fractions

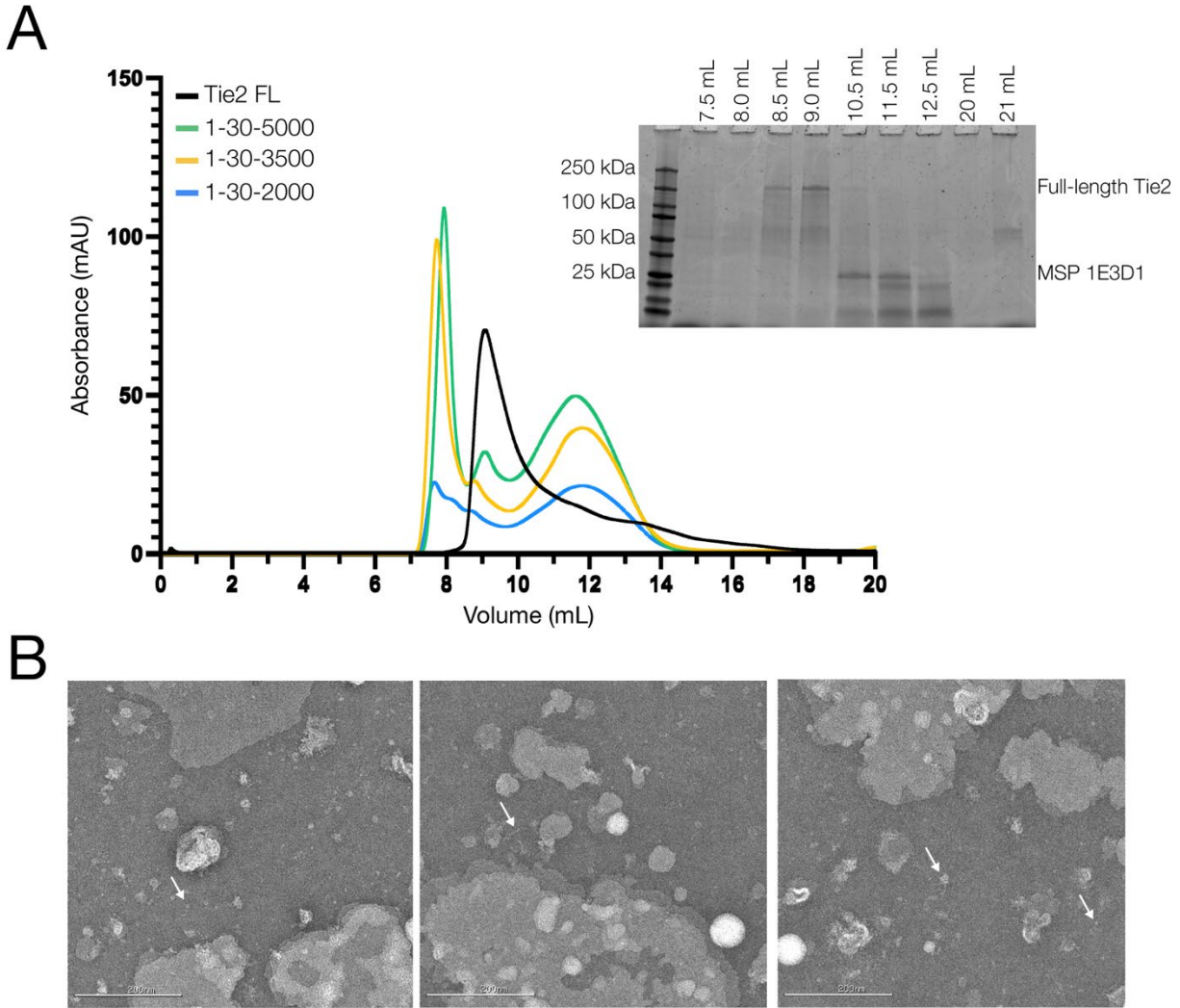


Figure 3 - 10 Initial Tie2 nanodisc incorporation SEC and nsEM. Initial nanodisc incorporation experiments were inspired by full length integrin $\alpha 5 \beta 1$ as it is also a single pass transmembrane receptor that undergoes dynamic conformational changes. **(A)** Size exclusion chromatograms of different molar ratios of full length Tie2, MSP, and lipids used during reconstitution of nanodiscs. The SEC fractions of 1-30-3500 were taken for representative SDS-PAGE analysis. The first eluted peak is within the void volume of the Superdex200 10.300 GL and contains aggregates. The following peak around 8.5-9 mL retention time was suggested to be the Tie2 encased nanodisc when compared to full-length Tie2 in detergent (black line). The final peak was broad and from the SDS-PAGE, suggests empty nanodiscs. **(B)** Representative negative stain electron microscopy micrographs of the second peak fractions (8.5 mL retention time). While most of the sample contained high amounts of aggregation, there are some rare instances of Tie2 encased within a nanodisc suggested by a large circular body with a protein projecting from it.

Table 3 - 1 Conditions experimented to incorporate Tie2 into nanodiscs. All conditions that were tested during optimization of incorporation of Tie2 into membrane nanodiscs. These are conditions where full-length Tie2 purification was successful and had enough protein for nanodisc incorporation. Conditions such as detergent solubilization optimization and purification are not presented here.

Tie2	MSP	Lipids	Detergent removal	MSP type	lipid rehydration	Lipid type	Purification	Things learned and outcomes
1	30	3500	biobeads	1E3D1	DDM	soybean polar lipids	Flag-SEC	-lipid ratio too high, ratio of potentially occupied nanodisc is significantly less than empty disc -negative stain
1	30	5000	biobeads	1E3D1	DDM	soybean polar lipids	Flag-SEC	-huge amount of aggregation and low nanodisc occupancy
1	30	2000	biobeads	1E3D1	DDM	soybean polar lipids	Flag-SEC	-large excess amount of empty ND
1	20	5000	biobeads	1E3D1	DDM	soybean polar lipids	Flag-SEC	-lowering the amount of MSP by 33% did not have an effect
1	20	2000	Biobeads	1E3D1	DDM	Porcine Brain polar lipids	Flag-SEC	- SEC chromatogram largest peak is in the void volume (aggregation?) - secondary peak needs to be reevaluated due to low mAU
1	30	3000	biobeads	1D1	DDM	soybean polar lipids	Flag-SEC	-new method of membrane isolation rather than purifying directly from cells. This provided a better sample for FLAG purification -large excess amount of empty ND
1	20	2500	biobeads	1D1	DDM	soybean polar lipids	Flag-flag-SEC	-ratio of the occupied vs empty nanodisc improved -seeing some instances of Tie2 on its own within 2D class averages, suggesting that there is a large amount of protein not getting incorporated into nanodiscs
1	20	1000	biobeads	1D1	DDM	soybean polar lipids	Flag-flag-SEC	- significant amount of nanodisc on negative stain EM and stain is thick
1	20	500	biobeads	1D1	DDM	soybean polar lipids	Flag-flag-SEC	-2D class averages improve with less aggregation. -cryoEM grids were highly heterogenous... upon assessing all potential ice thickness, the medium thickness provides the best "particle" contrast -2D class averages of cryoEM grids depict some classes of extracellular domain with no visual of nanodisc. Or the complete opposite where there are some visualizations of nanodisc but no Tie2
1	20	1800	Biobeads	1E3D1	DDM	soybean polar lipids	Flag-flag-SEC	Able to isolate circular objects presumed to be nanodisc, but there are no protein projecting from them
1	20	1000	biobeads	1D1	DDM	soybean polar lipids	Flag-flag-SEC	-2D class averages depict classes of a large circular object (likely nanodisc) with small portion of Tie2 -high amounts of aggregation
1	5	100	biobeads	1D1	Cholate	soybean polar lipids	Flag-flag-SEC	-sodium cholate is an alternative to using detergents during lipid rehydration ⁴⁸⁸ . -little to no nanodisc formed via SEC Lipid solution remains cloudy after sonication
1	5	250	biobeads	1D1	Cholate	soybean polar lipids	Flag-flag-SEC	-cholate buffer worked this time around and the solution is clear, highly variable? -2D class average shows IgG and Fab classes, suggesting that the anti-FLAG resin is beginning to degrade

1	5	250	biobeads	1D1	Cholate /DDM	soybean polar lipids	Flag-flag-SEC	-EGFR paper successfully used a combination of sodium cholate and DDM as lipid rehydration buffer ⁴⁹⁰ - there does not appear to large difference in the amount of aggregation seen on negative stain compared to just having cholate or DDM. Go back to just sticking with one or the other
1	20	500	biobeads	1D1	Cholate	soybean polar lipids	Flag-flag-SEC	-obtained N2 gas for drying down lipids. This made lipid rehydration much more uniform and the solution is clear afterwards.
1	20	500	biobeads	1D1	DDM	soybean polar lipids	Flag-flag-SEC	- two conformation of Tie2 in ND? -low resolution data
1	4	250	biobeads	1D1	DDM	POPC/POPS 80/20	Flag-SEC	- Tried PES concentrator units instead of regenerated cellulose to reduce aggregation -very few MSP on SDS page
1	4	250	biobeads	1D1	DDM	POPC/POPS 80/20	Flag-Flag-SEC	-concentrating protein after SEC and blotting for anti-his (as both Tie2 and MSP are His-tag), only Tie2 is present... no incorporation
1	10	500	amberlite	1D1	DDM	POPC / POPS 80/20	Flag-SEC	-Tie2 is not at the center of ND which is something seen prior with the biobeads. -2D class average in negative stain depicts the nanodisc with a small density of potentially Tie2 -cryoEM particles are only the membrane proximal FN repeats at low resolution with the presumably nanodisc density or un-incorporated Tie2 which is shown as the ectodomain -some 2D cryoEM class averages show that Tie2 above (in z height) a fuzzy density which is presumed to be the nanodisc (top-down view) -mass photometer shows that a larger and heavier species is present in solution, but this is in low abundance
1	20	500	amberlite	1D1	DDM	POPC / POPS 80/20	Flag-SEC	-very few MSP on SDS page
1	10	500	amberlite	1D1	cholate	POPC/POPS 80/20	his-Flag	- His-tag does not provide clean tie2
1	NA	NA	SMA25 Copolymer	NA	NA	native Expi293	Histrap	No extracted Tie2
1	NA	NA	SMA30 Copolymer	NA	NA	native Expi293	Histrap	Poor extraction of Tie2
1	NA	NA	DIBMA Copolymer	NA	NA	native Expi293	Histrap	-High purification quality after FLAG batch bind -2D class averages in negative stain EM, has few Tie2 classes with a large proportion being aggregation -many classes are uninterpretable
1	NA	NA	AASTY Copolymer	NA	NA	native Expi293	Histrap	Acceptable amount of purified protein. Falls short during SEC

demonstrates that all the necessary components are present (**Figure 3 - 11A**). To minimize aggregation, the protein sample is applied directly onto negative stain grids and imaged for Tie2 nanodiscs without concentrating. The amount of aggregation is drastically reduced, although some aggregation remains and is manageable. Individual particles are now clearer in this sample than in previous samples. 2D classification and ab initio reconstruction of picked particles demonstrated promising structural features (**Figure 3 - 11C**). Peak 3 was also assessed by negative stain electron microscopy to validate the formation of empty nanodisc (**Figure 3 - 11B**). Based on the encouraging classification results from negative stain electron microscopy, Tie2-1D1 was taken for cryoEM analysis. With the improved particle quantity seen in negative stain EM, cryoEM micrographs displayed continued heterogeneity and sample fragility with aggregation. 2D classification of picked particles exhibits low-resolution of possible Tie2 projecting from the 1D1 nanodisc (**Figure 3 - 11D**). A small portion of the particles that were curated through 2D classification contain density for the nanodisc and the membrane proximal domains of the Tie2 stalk. However, the limited resolution and the lack of features make this cryoEM density map difficult to interpret further. Interestingly, another population of particles align to form the extracellular region of the Tie2 molecule with no density for the nanodisc. This has been seen with other single-pass transmembrane protein receptors in nanodiscs, although others display density^{207,469,490}.

Although incorporation of full-length Tie2 into MSP nanodiscs was achieved, cryoEM screening revealed that sample heterogeneity remains to be completely solved, and optimization of the sample is required. Immediate steps would involve the optimization of cryoEM sample preparation. From our experiments, aggregation was present in various amounts. Nevertheless, optimization of the nanodisc system identified several favorable conditions, including the use of MSP1D1 and a defined POPC:POPS (80:20) lipid mixture, which improved particle occupancy and sample behavior relative to earlier preparations.

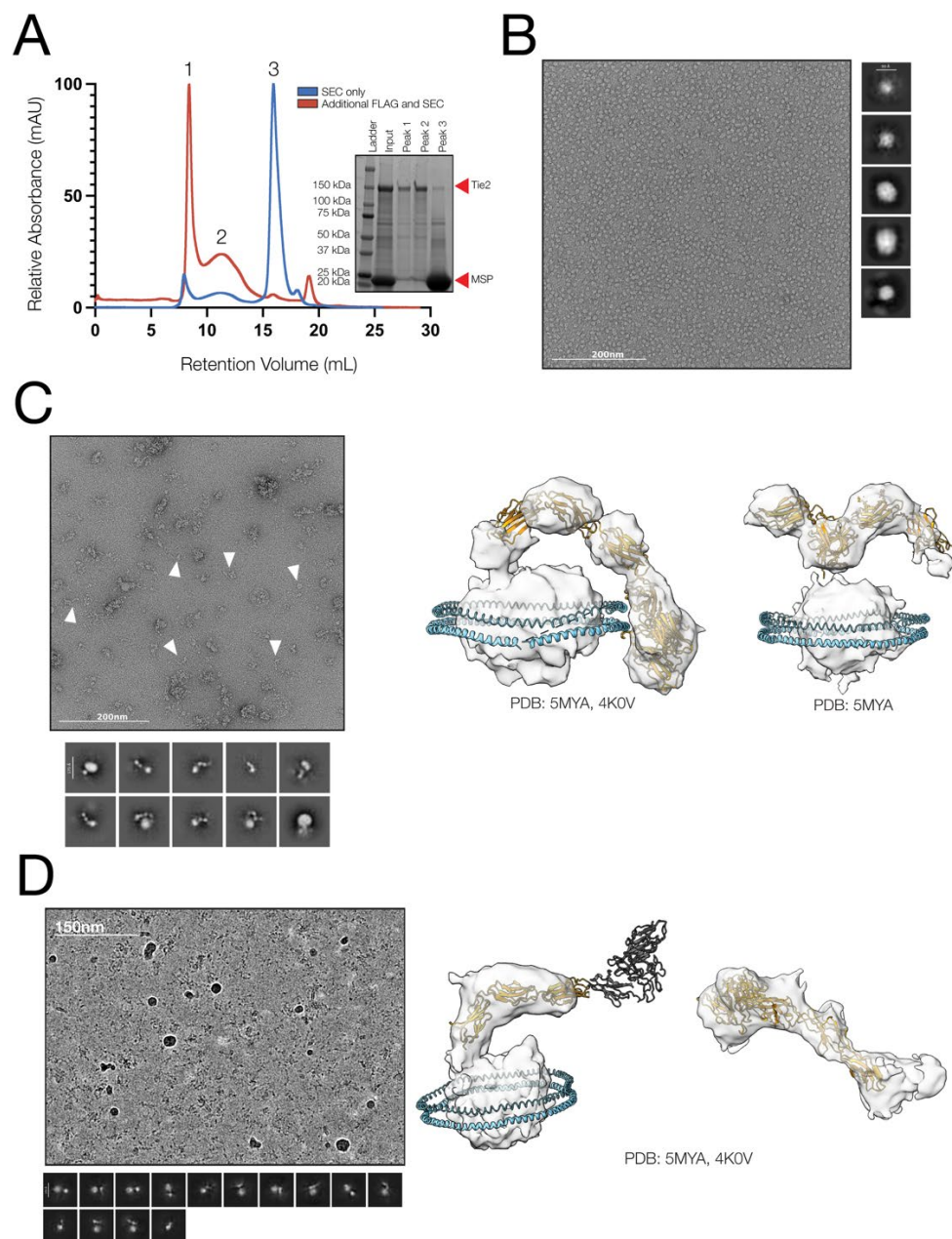


Figure 3 - 11 Secondary purification of Tie2-nanodisc massively improved yield and protein quality in negative stain. **A)** Purification of Tie2-nanodisc again using FLAG affinity drastically reduced the amount of empty nanodisc relatively to occupied nanodiscs. **B)** As validation, negative stain was performed on peak three of panel A. Shown is a representative micrograph and 2d class averages of empty nanodiscs. **C)** Peak two was taken for negative stain analysis and yielded promising micrographs and 2D class averages with protein density projecting from a nanodisc. Ab initio modeling of these classes of particles resulted in the two electron density maps shown here. **D)** However, within cryoEM imaging, the micrographs were distinctly noisier resulting in suboptimal 2D classifications. Multiple rounds of particle picking and curation have led to an initial model that has partial density of the 1D1 nanodisc and the membrane proximal fibronectin repeats. In parallel, particles obtained from data collection also resulted in a cryoEM density map of the extracellular region of the Tie2 protein. Indicating that despite performing secondary purification, there are still Tie2 that are not encased within nanodiscs, which could be contributing to the aggregation during detergent removal.

Stabilizing the Tie2 ectodomain using nonfunctional IgG

While full-length Tie2 encased within a nanodisc would have provided a near-native condition for structural determination to elucidate the domain organization of Tie2, the ectodomain can provide complementary information. Tie2, like other receptor tyrosine kinases, possesses a large extracellular region⁴¹⁰. Although the ectodomain construct of Tie2 cannot fully contextualize the ectodomain in relation to the cytoplasmic kinase domain, it can provide structural information on how the extracellular region is oriented with respect to the membrane during ligand engagement. Our current understanding of Tie2's extracellular domain organization stems from the docking of two crystal structures of the ligand-binding head to the fibronectin repeat stalk to formulate a composite structural model (PDB: 4K0V and 5UTK/5MYA)^{442,477}. A structural analysis of the complete ectodomain would represent a step forward in understanding the mechanisms of Tie2 activation and dimerization and reduce any ambiguity in domain organization.

As seen in the full-length experiments, Tie2 is inherently flexible, leading to conformational heterogeneity during structural analysis. While cryoEM can tolerate protein heterogeneity and flexibility, it comes at a cost of resolution and consistent density. To complicate this further, Tie2 is irregularly shaped, resulting in a low signal-to-noise particle images for cryoEM imaging compared to globular proteins. This, compounded with the noise from the detergent micelles within the DDM-solubilized Tie2 resulted in poor-resolution reconstruction and 2D class averages (**Figure 3 - 12**). Until recently, the polarity of the Tie2 molecule was difficult to determine consistently due to the irregular nature of the protein. Resolution of the unbound ectodomain is limited, potentially due to a lack of defining features. Tie2 protein domains are predominantly beta sheets which generally scatter weakly compared to alpha helices, whose geometry provides a stronger signal during cryoEM imaging. To provide additional contrast and stability, the use of ligands and IgG or Fab fragments is often employed for cryoEM⁴⁹¹. Here, we utilized a Tie2 monoclonal antibody: clone 33.1, which has been used to detect Tie2 on endothelial cells in immunohistochemistry experiments, demonstrating that this antibody recognizes a conformational epitope⁴⁹². Although the binding epitope has not yet been reported.

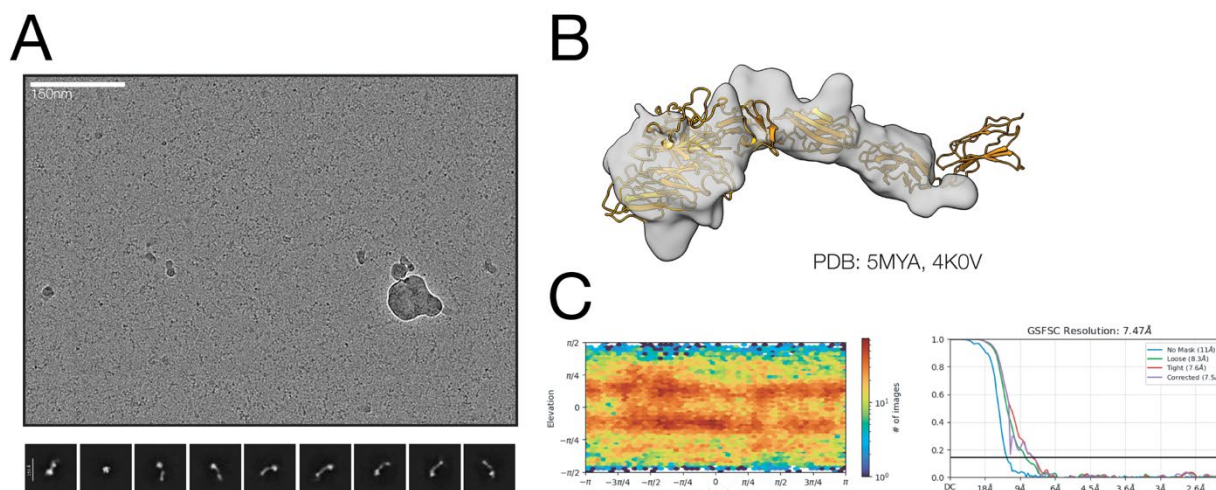


Figure 3 - 12 Apo Tie2 achieves medium to low resolution cryoEM map. (A) A representative cryoEM micrograph shows particle density using quantifoil 300 mesh R1.2/1.3 cryoEM grid. 2D class averages lack discernable features that assist in defining particle polarity. The ligand binding head is larger than the stalk region, but from these averages, it may be misleading resulting in inclusion of potential junk particles during refinement. (B) Further curation of particles in two- and three-dimensions resulted in a final refinement that allowed rigid body docking of homology built Tie2 ectodomain using PDB: 4K0V and 5UTK. (C) Angular distribution and refinement statistics.

SEC and non-reduced SDS-PAGE analysis confirmed the formation of the Tie2 ectodomain-33.1 IgG complex through a clear peak shift (**Figure 3 - 13A**). Using SDS-PAGE in non-reducing conditions where the IgG remains intact at 150 kDa coelutes with the Tie2 ectodomain at approximately 87 kDa (**Figure 3 - 13A**). nsEM showed that the Tie2-33.1 is of sufficient quality for cryoEM analysis. An initial cryoEM dataset of 2,535 micrographs was collected and processed. This led to a final refinement of a cryoEM density map with an estimated resolution of 5 Å (FSC cutoff of 0.143). However, this exhibited preferred orientation as many of the particles contribute to the side view of the Tie2-33.1 complex with few particles viewing “down the Tie2 axis”. To improve the angular distribution, an additional two datasets were collected to increase particle counts and angular distribution. 2,579 of these added micrographs contained 0.05% CHAPS, which has been shown to be effective in improving the orientation distribution of irregularly shaped proteins²⁰¹. The benefit of 33.1 IgG binding became clear during cryoEM analysis. The addition of 33.1 dramatically increased the quality of the 2D classification results and the cryoEM density map exhibited identifiable backbone density (**Figure 3 – 13C and E**). cryoEM analysis provided the first visualization of the epitope of 33.1 at the interface between the fibronectin type III repeats 1 and 2, with much of the binding

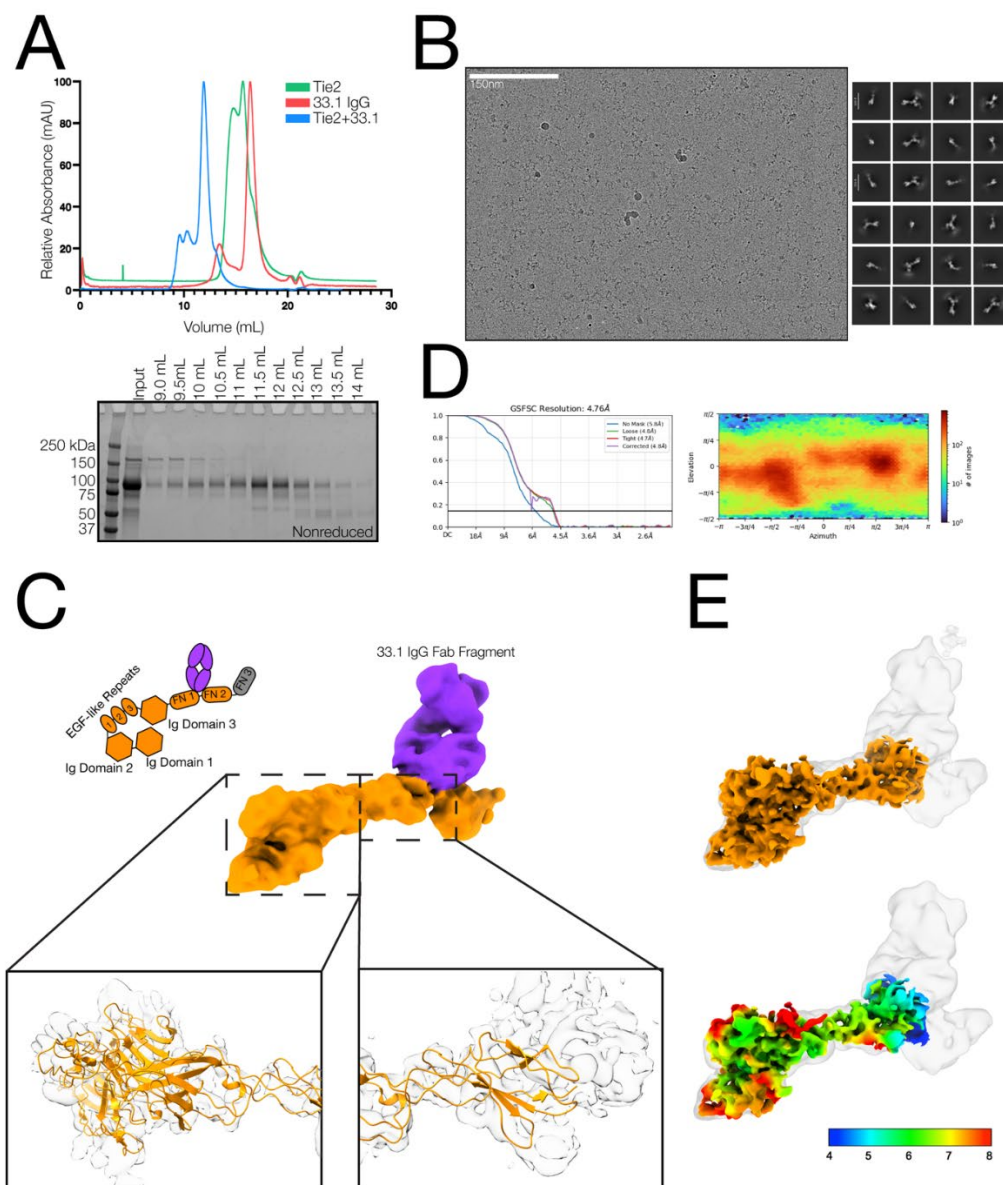


Figure 3 - 13 Tie2 ectodomain in complex with 33.1 IgG. **A)** SEC chromatogram of Tie2 ectodomain incubated with 1.5 times molar excess of 33.1 IgG. SDS-PAGE analysis confirms that the formation of a complex by the co-elution of 33.1 and Tie2 under nonreducing conditions. **B)** A representative micrograph of data collection. The total number of micrographs was a combination from three datasets was 5,866 micrographs where 2579 micrographs came from a dataset where 0.05% CHAPS was added to the sample to aid in particle distribution and orientation. Representative 2D class averages depict the epitope of the 33.1 to be at the interface between the first fibronectin like repeat and second. Due to the flexibility of whole IgG, only the fab is visible within the averages. **C)** Current cryoEM map of Tie2 ectodomain in complex with 33.1 IgG at low threshold. **D)** Angular distribution and gold standard Fourier Shell Correlation (FSC) plots of particles used in the current cryoEM reconstruction. **E)** The local refinement cryoEM density map at a medium threshold superimposed with the refinement map at low threshold. **F)** The current resolution and quality of the cryoEM map is sufficient for rigid body docking of PDB: 4k0V which contains the ligand binding head and the first fibronectin repeat domain.

surface on the former repeat. The overall quality and resolution of the map improved significantly compared to the apo unbound state (**Figure 3 - 13C and E**). Final refinements from combining datasets resulted in an estimated cryoEM density map at 4.6 Å resolution (FSC cutoff of 0.143) where features of the protein backbone are visible and a protein model can be docked confidently (**Figure 3 - 13E**). Additional particles contributed by more data and 0.05% CHAPS improved the angular distribution (**Figure 3 - 13D**). Currently, the cryoEM density map is sufficient for rigid body docking of the crystal structures of the ligand binding head and some of the fibronectin repeats (PDB: 4K0V and 5UTK). Additional work and refinements should be done to obtain residues that are appropriate for model building.

3.5 Next steps, hindsight and futures directions

Tie2 is a critical protein receptor for the proper progression of angiogenesis and vascular stability. Receptor crosstalk has become more critical in our understanding of cellular signaling. Integrins have been observed to associate and interact with receptor tyrosine kinases ^{233,234,244,457,465,493}. There is a clear connection between the extracellular matrix and the angiopoietin-Tie system. Previous work established the biochemical basis for the interaction between integrin $\alpha 5\beta 1$ and Tie receptors. Although an interaction was identified and then potentiated with fibronectin and angiopoietin, the molecular underpinnings of this receptor complex remain unresolved. What role does fibronectin play in this receptor interaction? Does fibronectin act as a scaffold or mediator protein to engage integrin $\alpha 5\beta 1$ and Tie2 together? Aim 1 of this project sought to answer these questions. Despite the efforts described here, the answers to these questions remain unclear.

Due to the success of resolution improvement using the 33.1 IgG for ectodomain of Tie2, the idea of an additional fab or IgG may provide further stabilization benefits. A recent Fab, 3H7, was developed against the fibronectin repeat domains of Tie2 to study the mechanism of clustering ⁴⁴³. The variable region of this Fab was grafted onto a Fab construct within the lab and expressed via transfection of Expi293 cells. By combining this Fab with 33.1 IgG, this increases the total mass of the Tie2 particle. With additional mass, cryoEM imaging of Tie2 becomes easier* (**Figure 3 - 14A**). Combining Tie2 ectodomain, 33.1 IgG and 3H7 Fab coeluted on SEC

* Larger protein particles can diffract electrons more readily compared to smaller or irregular shaped proteins. The increased in electron scattering results in protein particles with higher contrast relative to the background. This can aid in particle alignments in both two- and three-dimensions for classification and refinement respectively. Although, this still comes with caveats such as ice thickness and imaging conditions.

with all associated molecular weight bands present within the same fraction (**Figure 3 - 14B**). Assessment by negative stain EM and 2D classification shows that both the 33.1 IgG and 3H7 fab is bound to Tie2 (**Figure 3 - 14C**). Next steps would be to optimize conditions for cryoEM imaging to obtain a density map in which the entire ectodomain can be visualized and built into a protein model.

There is also protein extraction using copolymer as an alternative to membrane nanodisc, these copolymers offer the capability of native membrane nanodiscs. The process of detergent solubilization of membrane proteins is inherently difficult, and the likelihood of reduced stability is high. During the optimization of nanodisc formation, a brief trial of copolymer extraction was performed. DIBMA Glycerol provides a clean SDS-PAGE gel of extracted Tie2 (**Figure 3 - 15**). Additional studies should follow up by using negative stain EM and cryoEM to assess this method of protein extraction. This is the ideal case of extraction as the immediate surrounding lipids of Tie2 are also extracted. Therefore, any important helix-lipid interactions are maintained.

With the advancements in cryoEM and membrane mimetic system, the field of structural biology is at an optimal time for investigating the molecular mechanisms of multiple receptor proteins. From previous reports, Tie2 activation is suggested to be similar to that of other receptor tyrosine kinases where ligands bring together homodimer formation for transphosphorylation and thus receptor activation^{442,477}. But there have been reports that this may not be the case with ligand-independent dimerization and clustering playing a role in Tie2 signaling^{430,475,494}. Additionally, the heterodimeric receptor pairing between Tie1 and Tie2 is also of functional importance⁴⁷⁷. How does Tie1 influence the clustering of Tie2? Does Tie1 serve more of a scaffolding role than a signaling role? Thus far, the dimerization and clustering of the Tie receptors discussed have been from the point of view within the same cellular membrane. How the angiopoietin ligands facilitate Tie2 homo- or hetero-association with Tie1 across cell junctions at the molecular level remains unclear. Structural characterization of full-length and/or ectodomain of Tie2 (and Tie1) represents an important step towards understanding these mechanisms.

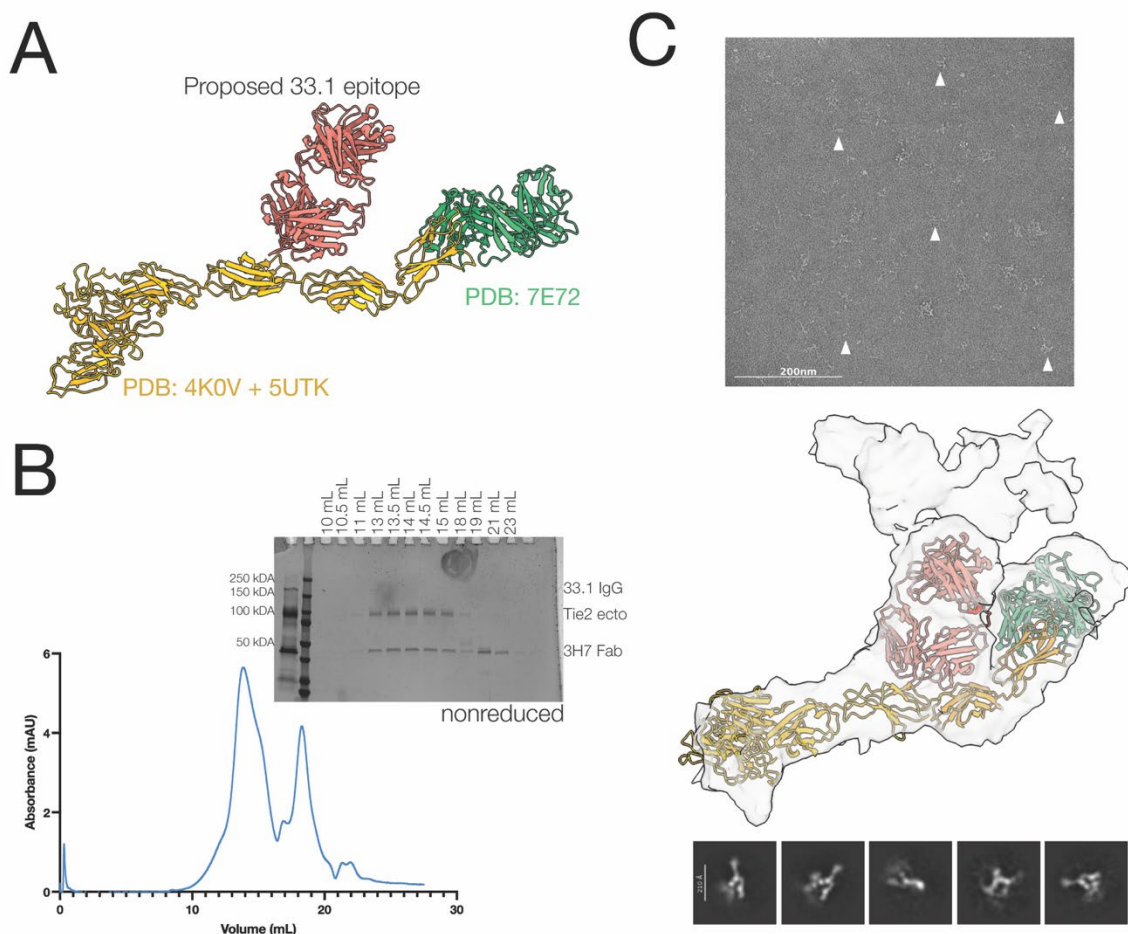


Figure 3 - 14 Tie2 ectodomain bound with 33.1 IgG and 3H7 Fab. **A)** Antibody 33.1 proved effective at stabilizing and improving the resolution of Tie2. However, what this cryoEM map lacks (likely due to flexibility) is the membrane proximal fibronectin repeat (FN type III repeat 3; see **Figure 3 - 2**). The 3H7 fab was developed to study the mechanisms of Tie2 clustering and a crystal structure shows that it binds to the third repeat⁴⁴³. By adding 3H7 to the 33.1 bound Tie2 may provide enough mass and stability to resolve these membrane proximal domains. **B)** Tie2 ectodomain, 33.1 IgG and purified 3H7 was mixed in a ratio of 1:1:1.5 and allowed to incubate together for 30 minutes at room temperature before being injected on a 10/300 GL Superose6 SEC column. SDS-PAGE analysis was performed on peak fractions. **C)** A representative micrograph of fraction obtained at 13 mL retention volume from size exclusion chromatography in panel B. Micrograph and particle quality is moderate which allowed for automated particle picking. Particles were curated by 2D classification and selected particles were used for ab initio modeling to generate an initial model. EM density was sufficient to rigid body dock the homology model of Tie2 bound to 33.1 and 3H7 from (A). Representative 2D class averages are shown.

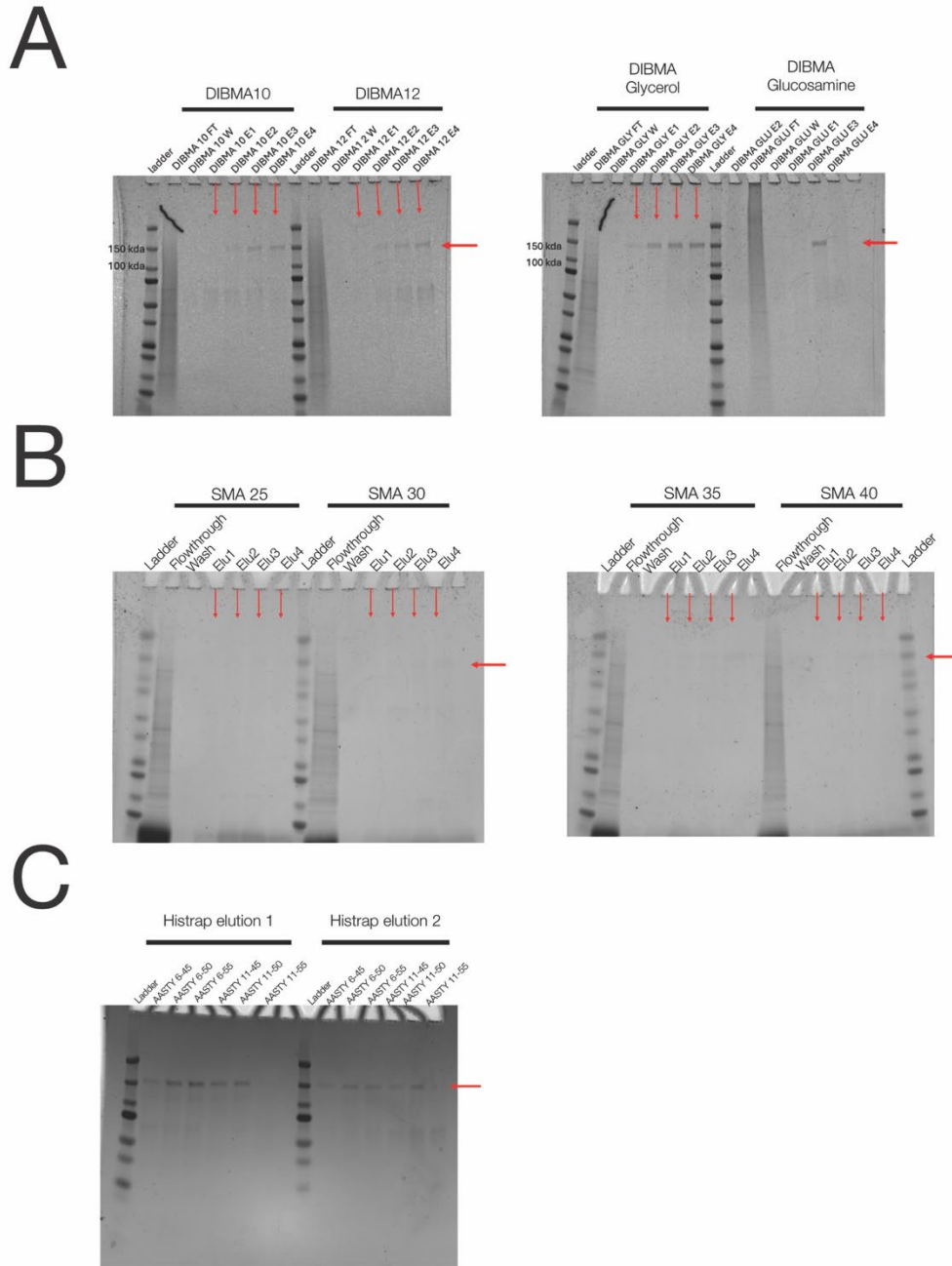


Figure 3 - 15 Full-length Tie2 extraction using copolymers. SDS-Page gel of post anti-FLAG batch binding of FL Tie2 expressing Expi293 cells solubilized by DIBMA (A), SMALPS (B) or AASTY (C). Batch binding was performed at 4°C with rocking. Elution was performed with 3xFLAG peptide. DIBMA and AASTY were successfully able to solubilize and extract full-length Tie2. NOTE: only AASTY purification was performed using NI-NTA resin and eluted with 500 mM imidazole containing buffer.

Chapter 4 Concluding remarks

Membrane proteins are an essential component of human biology. They mediate communication between the intracellular and extracellular environments. Membrane proteins account for approximately 20 to 30% of the human proteome, yet they represent up to 60% of identifiable druggable targets⁴⁹⁸. Cell surface receptors such as integrins and Tie receptors regulate multiple biological processes. Their involvement in angiogenesis and vascular remodeling has made them attractive therapeutic targets. However, targeting these receptors has been mechanistically challenging. This thesis focused on elucidating the structural basis of integrin conformation as a consequence of therapeutic binding and described efforts to delineate the mechanisms of Tie2 activation and modulation. From a structural perspective, this work highlights how binding of potential therapeutics can influence integrin conformational states to achieve desired outcomes. At the same time, the in-progress work of resolving full-length single-pass transmembrane receptors is an ongoing challenge and an active area of structural investigation. Integrin and Tie2 engagement at the cell surface bring insights of receptor crosstalk for the flow of information through the cell. Taken together, integrin $\alpha 5 \beta 1$ and Tie2 provide a unique case where endothelial cell integrates multiple extracellular stimuli to regulate vascular biology

Therapeutic Design with computational tools and protein design

Advances in structural biology have transformed our ability to investigate membrane protein dynamics at high resolution⁴⁶⁹. With these advances, the number of high-resolution membrane protein structures has begun to rise, further shedding light on the structure-function relationship within the membrane environment. However, there are still many challenges and hurdles to obtaining these structures, particularly for single-pass transmembrane receptors^{276,398,499}. The growth of experimentally determined protein structures help gives rise to structural databases for development and training of computational tools and approaches to complement and assist in understanding their biology.

In recent years, the introduction of artificial intelligence and computational models propelled advances in the field and our understanding of protein structure. The computational

design space presented an initial hurdle. Proteins can take on an almost limitless number of conformations. However, in 2024, the Nobel Prize in Chemistry was awarded to pioneers of computational protein design and protein structure prediction. At this time, AlphaFold and RoseTTAFold have become mainstays within the field, demonstrating their capabilities¹⁵⁰. However, these predictive and design models are trained on published protein structures within the Protein Data Bank^{500,501}. The development of therapeutics is beginning to utilize virtual screening of candidate compounds and biologics. At the time of writing, the fields of structural biology and protein prediction are merging. The tools that were developed can substantially overcome the inherent traditional limitations of therapeutic design of high-throughput screening or directed evolution in favor of accurate in silico validation, saving valuable resources and time.

Receptor crosstalk

Traditional models of signaling have largely been described as a linear relationship between ligand binding and the transduction outcome. Within this model, ligand binding to a receptors result in a cascade of phosphorylation events (or other signaling modes such as ubiquitination, methylation or acetylation) that lead to a change in gene expression or protein activity. A growing body of literature suggests that not all signaling is linear^{502–504}. Independent signaling pathways can converge on a common group of secondary messengers such as the mitogen-activated protein kinase family (MAPK). This group of kinases is involved in various cellular processes^{233,466,505,506}. Other times, crosstalk can be initiated at the membrane (including the nuclear membrane), where receptors form hetero-oligomers to modulate the signaling cascades^{466,507–512}. Integrins are an essential class of receptors that mediate cellular adhesion and motility. This fundamental process directly influences interactions with other receptors for cellular processes necessary for survival^{244,462,513–515}. Tyrosine kinase receptors also participate extensively in receptor crosstalk⁵¹⁶.

However, the dynamic nature of receptor crosstalk makes this process difficult to study. Crosstalk is a highly variable process that can occur in specific cell types and tissues or at certain stages of cellular development^{517,518}. One of the mechanisms of crosstalk occurs at the membrane with receptors hetero-oligomerizing with one another, forming clusters. While some mechanisms of hetero-receptor clustering are defined in immune systems, other mechanisms that govern specific sites of clustering remain elusive^{463,500,501,516,519}. Other nuances include the

context-dependent behavior of crosstalk, the complexity of the signaling network, and the effects of crosstalk on the signaling trajectories over time ^{463,520}. Recent developments in computational biology have begun to dissect the signaling maps across the human proteome ⁵²¹. These studies may provide a springboard for identifying receptor pairs that engage in crosstalk to investigate to decipher the functional importance and regulation of this phenomenon.

Challenges in full-length membrane protein structure characterization

Single-pass transmembrane proteins are the most abundant and diverse group of membrane proteins ^{484,487}. These proteins participate in a wide range of cellular and biological processes. Membrane protein structures remain underrepresented compared to soluble proteins. While detergent solubilization provides a relatively accessible route to structural determination, membrane mimetics, such as nanodiscs, offer a more native-like environment ^{522–524}. Nanodiscs are effective tools, and a wide range of membrane proteins such as multi-pass receptors, channels, and transporters have benefited from their use ^{488,525–527}. However, structures of single-pass transmembrane receptors within nanodiscs are less common than multi-pass or larger proteins. One possible hypothesis is that these larger proteins have a stronger dependence on the lipid environment compared with single-pass transmembrane proteins as their function is critically linked to membrane composition. The cross-sectional area of a helix is significantly less than that of a multi-pass protein, resulting in a potential mismatch between single-pass and nanodisc dimensions. In addition, membrane protein stabilization in vitro either through detergents or membrane mimetic systems is unique to every protein. There is currently no systematic method to optimize the conditions for stabilization, making the process laborious. Although there are reports of method and protocols to assist researchers in finding their optimal conditions ^{528–530}. Polymer-based solubilization strategies are actively being developed to extract membrane proteins directly from native membranes, preserving any protein lipid interactions or protein binding partners ^{156,159,162,531}. These polymers exhibit both hydrophilic and hydrophobic properties, making them potentially ideal for directly partitioning into the membrane and extracting the membrane protein. The hydrophobic portions stabilize the transmembrane region of the protein, while the hydrophilic component protects the protein molecule from solvent.

Technological developments in both hardware and software have transformed cryoEM into a powerful and versatile tool for determining high-resolution protein structures. However, single-pass transmembrane protein receptors remain a challenge due to their inherent flexibility and dynamic nature. CryoEM can capture not only a single structure but also an ensemble of conformations. This dynamic nature and heterogeneity may have implications for protein regulation and function. In recent years, multiple software tools have been developed to access this information^{156,159,162,531}. The transmembrane helices are often resolved at low resolution as they are buried behind stabilizing detergent and lipid molecules. Therefore, understanding the conformations of these helices will shed light on their roles in regulation beyond anchoring the protein to the membrane. While these tools have become indispensable for resolving conformational heterogeneity of flexible proteins, including membrane-associated receptors, their refinement is dependent on continued efforts to experimentally determine these structures across a wide range of proteins.

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