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Capillary Morphogenesis Gene Protein 2 (CMG2) Mediates Matrix Protein Uptake and is Required for Endothelial Cell Chemotaxis in Response to Multiple Vascular Growth Factors

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A dissertation submitted to the faculty of Brigham Young University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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ABSTRACT

Capillary Morphogenesis Gene Protein 2 (CMG2) Mediates Matrix Protein Uptake and is Required for Endothelial Cell Chemotaxis in Response to Multiple Vascular Growth Factors

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Doctor of Philosophy

Pathological angiogenesis, or new blood vessel formation, is involved in many pathologies, including cancer and serious eye diseases. While traditional anti-angiogenic therapies target vascular endothelial growth factor receptors to reduce or inhibit new vessel formation, this approach has several downsides, including unpleasant side effects and low efficacy over time. Therefore, identifying new targets to treat pathological angiogenesis is still needed. CMG2, one of the two identified anthrax toxin receptors, has been proposed as an alternative target to treat pathological angiogenesis. CMG2’s role as a cell surface receptor that mediates anthrax toxin internalization is very well documented. One physiological function for CMG2, not related to anthrax intoxication, is suggested by the observation that loss-of-function mutations in CMG2 cause hyaline fibromatosis syndrome (HFS), a genetic disease that results in accumulations of extra-cellular matrix (ECM) protein in different parts of the body.

While the complete molecular mechanism for CMG2’s role in regulating angiogenesis has not been determined, this dissertation addresses multiple ways CMG2 regulates pathological angiogenesis. We have discovered that CMG2 plays a role in mediating ECM homeostasis via endocytosis of ECM proteins and protein fragments as a way to generate angiogenic signals from the cell. We have also demonstrated that a fragment from Col IV, S16, is endocytosed into the cells by interacting with CMG2, and S16 treatment to endothelial cells leads to a significant reduction in cell migration. Also, an endothelial cell migration assay with CMG2 knockout cells results in abolished directional migration, indicating that CMG2 is required for endothelial cell chemotaxis. Notably, we have identified that bFGF, VEGF, and PDGF are involved in CMG2 mediated chemotaxis but not insulin and sphingosine-1-phosphate (SIP). While recent literature reports show that CMG2 works closely with RhoA GTPase, which is commonly known to regulate cell migration, we have also observed that inhibition of RhoA also reduced cell chemotaxis towards VEGF but not SIP. These results could be leveraged to develop new classes of therapeutic molecules to treat pathological angiogenesis induced by multiple various growth factors via targeting CMG2.

Keywords: capillary morphogenesis gene 2 (CMG2), pathological angiogenesis, extracellular matrix, growth factors, chemotaxis, RhoA GTPase
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In addition, various colleagues and collaborators also contribute to different parts of this dissertation, as listed below:

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1. Introduction:

Angiogenesis is a highly complex process that is crucial to body development. However, in adults, angiogenesis occurs most commonly in numerous pathologies. Pathological angiogenesis causes serious retinal and corneal eye diseases that lead to blindness. Also, tumor growth and metastasis depend closely on oxygen and nutrients supplied by new vessels; therefore, new blood vessel formation is a crucial process for cancer development. Effective treatments to inhibit angiogenesis thus play an important role in improving the health outcomes for multiple diseases. The current anti-angiogenic treatments use inhibitors that target vascular endothelial growth factor (VEGF) to reduce angiogenesis. However, anti-VEGF treatments may produce harsh side effects, including hypertension, proteinuria, slow wound healing, and cardiological events. Besides, the efficacy of anti-VEGF drugs over time is relatively low, reflecting drug resistance from the emergence of alternate angiogenic pathways. Therefore, there is still a need for new therapeutic targets that can provide higher efficacy and lower toxicity.

Capillary Morphogenesis Gene 2 (CMG2) is upregulated during vessel formation, suggesting that it has a role in mediating angiogenesis. Our lab has observed that targeting CMG2 with a known binding partner, anthrax protective antigen (PA), or other small molecules, reduces angiogenesis both in vitro and in vivo. These observations suggest CMG2 is a possible target to treat pathological angiogenesis. However, CMG2 has only been thoroughly studied as one of the two anthrax toxin receptors (ANTXR), leaving the molecular mechanism for CMG2 regulated angiogenesis unresolved. Fortunately, research efforts throughout the past two decades have revealed multiple CMG2 related pathologies and intracellular interactors, which are critical clues to elucidate the physiological roles of CMG2 related to angiogenesis. Here, the
current state of the scientific literature will be used to discuss different roles, functions, and signaling that CMG2 may involve/utilize in mediating angiogenesis.

1.1. CMG2 related pathologies illuminate its potential physiological functions and their possible link to angiogenesis

CMG2 was identified as a protein that potentially mediates angiogenesis over a decade ago;\textsuperscript{3, 4} however, the biological function and biochemical signaling that links CMG2 to angiogenesis remain unknown. Published reports have suggested both direct and indirect relationships between CMG2 and angiogenesis including 1) anthrax intoxication via receptor-mediated endocytosis;\textsuperscript{16, 17} 2) CMG2 inhibition reduces pathology angiogenesis; 3) loss of function mutations cause a buildup of matrix proteins;\textsuperscript{10, 18} and 4) overexpression of CMG2 was linked to cancer cell invasion, and metastasis.\textsuperscript{13, 19-21} These observations illuminate the potential physiological functions of CMG2 and their links to angiogenesis.

1.1.1. CMG2 internalizes anthrax toxin via receptor-mediated endocytosis

CMG2 and its homolog, Tumor Endothelial Marker 8 (TEM8), are type I transmembrane proteins that share 60% sequence homology within the extracellular domain and 40% throughout the entire protein.\textsuperscript{22} They were identified as anthrax toxin receptors soon after the terrorist attacks in 2001,\textsuperscript{22, 23} resulting in the additional titles ANTXR1 (TEM8) and ANTXR2 (CMG2). Since then, these two receptors and their roles in mediating anthrax intoxication have been extensively studied.
These two primary ANTXRs interact with the tripartite anthrax toxin, composed of the enzymatic moieties lethal factor (LF) and edema factor (EF), together with the receptor-binding subunit PA. The toxin is a product of the gram-positive, endospore-forming, rod-shaped bacterium Bacillus anthracis. The anthrax spores usually enter the host through skin contact, inhalation into the respiratory tract, or via the gastrointestinal system. Macrophages will take up spores and transport them to the lymph nodes, where they germinate, proliferate, and produce the toxin throughout their vegetative lifetime. The two enzymatic subunits LF and EF are harmless unless they are assembled into the toxin complex with PA and transported into the cell, which is a process that depends on the interaction between PA the ANTXRs.

During anthrax intoxication, the 83 Kd PA binds to the ANTXRs (TEM8 and CMG2) on the cell surface, a 20 kDa fragment from the PA N-terminus is then cleaved by a furin-like protease. The remaining 63 kDa PA oligomerizes to form a PA-receptor heptamer, which acts as a binding platform for LF and EF, and transports the toxin complex into cells via endocytosis. The PA-heptamer complex initially is in a pre-pore structure that rearranges to form a pore structure when the pH drops in the endosome. The PA pore structure becomes the channel that LF and EF use to enter the cytosol. Once LF and EF enter the cytosol, they become active and affect their substrates. LF is a zinc-dependent endoprotease that inactivates MEK (a mitogen-activated protein kinase kinase; MAPKK) by proteolytically cleaving the N-terminus of MEK, which eventually lead to apoptosis. EF is an adenylate cyclase that increases cellular cAMP concentration, which disrupts water homeostasis and severely affects intracellular signaling. Both enzymatic toxin moieties severely impair intracellular signaling pathways and upset cell functions and are fatal for the host.
Interestingly, the process of endocytosis is shown to be dependent on the post-translational modifications (PTMs), ubiquitination and phosphorylation, of the cytosolic tail.\textsuperscript{16, 33, 34} Clathrin-mediated endocytosis is triggered upon PA binding to the ANTXRs. Once PA binds and oligomerizes, a src-like kinase phosphorylates the cytosolic tail of the ANTXRs and recruits β-arrestin.\textsuperscript{34} Next, the cytosolic tail gets ubiquitinated by an E3 ubiquitin ligase, which is important for recruiting the adaptor protein, AP1, to the cytosolic tail.\textsuperscript{16, 33} Mutations to the four identified Tyr phosphorylation sites on CMG2 diminished ubiquitination of the cytosolic tail and significantly reduced the amount of oligomerized CMG2, a marker of endocytosed CMG2—PA complex.\textsuperscript{34, 35} Furthermore, it was identified that src and fyn, but not yes, are the two responsible kinases that phosphorylate both ANTXRs. Knocking out src or fyn results in similar phenotypes as the phospho-mutants.\textsuperscript{34} Similarly, ubiquitination mutants also diminish oligomerized PA. Cbi was identified as the E3 ubiquitin ligase of TEM8, while the CMG2 ubiquitin ligase remains unknown.\textsuperscript{33} Together, these data indicate that anthrax toxin internalization is strictly regulated by PTMs. These CMG2 PTMs were later observed to be important for intracellular signaling,\textsuperscript{14, 15} which will be discussed later.

While both CMG2 and TME8 are recognized as anthrax toxin receptors, CMG2 is likely the primary receptor for anthrax toxin \textit{in vivo}.\textsuperscript{36} Studies with CMG2- or TEM8-depleted mice showed that anthrax toxin lethality occurs mainly through CMG2. This likely reflects the relative binding affinity of CMG2 and TEM8 for PA. While the affinity between PA and CMG2 is relatively high, 200 pM,\textsuperscript{37} TEM8 affinities for PA is around 100 fold weaker (Kd = 20 nM).\textsuperscript{38} Thus, CMG2 is thought to be the major receptor for mediating anthrax intoxication.\textsuperscript{36}

To this point, all of the information summarized above helps to understand the fundamental role of CMG2 as an ANTXR. The critical PTMs on the CMG2 cytosolic tail that mediates
endocytosis of anthrax toxin further suggests that CMG2 may be involved in intracellular signaling. Interestingly, PA triggers CMG2-mediated endocytosis and, at the same time, inhibits angiogenesis. This suggests that CMG2 mediated endocytosis may play a role in mediating angiogenesis. The exact mechanistic link behind the two observations is not yet answered; more research will be required to elucidate this mechanism.

1.1.2. Angiogenesis and CMG2

Angiogenesis, or vasculature development, is a fundamental process for development and wound healing. This complex process is usually initiated in poorly perfused areas, which induces either vessel splitting or endothelial sprouting towards an angiogenic growth factor such as vascular endothelial growth factor A (VEGF-A). During sprouting angiogenesis, extracellular matrix (ECM) proteins surrounding the vessels will be degraded by various proteases, including matrix metalloproteinases (MMPs). Afterward, endothelial tip cells play a critical role in guiding cells towards the stimuli, also called chemoattractants. Endothelial stalk cells then proliferate as they follow behind the tip cells. Then, when tip cells reach the destination, they fuse and form a continuous lumen to allow blood flow. These newly formed capillaries will recruit pericyte cells and lay down matrix protein to mature and stabilize the new vasculature. While this process is regulated by various growth factor signaling pathways, many anti-angiogenic therapies mainly target the VEGF pathway, limiting the choices for long-term and effective therapy. Thus, the field is still looking for new targets to treat pathological angiogenesis.

CMG2 has been suggested to have a role in sprouting angiogenesis, an observation supported by the 2001 identification of CMG2 as a gene upregulated during tube formation in a
3D collagen matrix.\textsuperscript{3} In CMG2’s role as an ANTXR, CMG2 binds PA, a non-toxic subunit of anthrax toxin. Hence, the PA interaction with CMG2 provides a tool to probe whether ligand binding to CMG2 impacts angiogenesis. Results indicate that treatment with either PA-WT or PA\textsuperscript{SSSR} (a mutant that is resistant to furin-like protease cleavage) leads to a reduction of corneal neovascularization in mice.\textsuperscript{4} Notably, PA\textsuperscript{SSSR} demonstrated a more potent anti-angiogenic effect than PA-WT (PA-WT 40%, PA\textsuperscript{SSSR} 60%) in the mouse corneal micro-pocket assay\textsuperscript{4}. Additionally, small molecules have been identified that bind CMG2 and also show anti-angiogenic effects in both the corneal mico-pocket and endothelial migration assays.\textsuperscript{6, 7, 41} It is worth noting that these inhibitors, including PA\textsuperscript{SSSR}, inhibited serum-induced endothelial cell migration but not proliferation. Furthermore, the concentration of PA\textsuperscript{SSSR} treatment in the cell migration assay was 200 pM, a concentration that will target 50% of available CMG2 while providing minimal to no effect through other anthrax toxin receptors, including the lower affinity CMG2 homolog, TEM8. Together, these data suggest that targeting CMG2 can produce anti-angiogenic effects.

1.1.3. CMG2 defective mutants cause HFS, implicating CMG2 may regulate ECM homeostasis

The CMG2 extracellular domain (ECD) contains a vWA domain, a common protein domain in various integrin cell-surface receptors. However, unlike integrins, the pool of physiological CMG2 ligands has not yet been fully identified. Previously published reports show that CMG2 binds collagen IV, collagen VI, fibronectin, and laminin.\textsuperscript{3, 8} Also, a recent study suggests that CMG2 acts as a receptor for collagen VI, and regulates its uptake by cells.\textsuperscript{8} This report is consistent with observations included in Chapter 3, which show that CMG2 interacts with a collagen IV derived peptide and internalizes the ECM-derived peptide by endocytosis.
The strongest evidence that CMG2 interacts with ECM proteins is suggested by data from patients with hyaline fibrosis syndrome (HFS). This disease is characterized by the accumulation of hyaline materials in the skin and different organs, and was first reported in the 18th century. Currently, HFS describes two separate diseases: infantile systemic hyalinosis (ISH) and juvenile hyaline fibromatosis (JHF). ISH is a type of HFS that comes with more severe pathological phenotypes accompanied by diarrhea and respiratory infections. It usually causes patients to die during infancy, thus, earning the name infantile systemic hyalinosis. JHF patients have a relatively mild phenotype and usually live longer lives. In 2003, with the help of genomic sequencing, the cause of HFS was finally revealed. Genomic sequencing of both ISH and JHF patients revealed that the disease is closely related to loss of function mutation(s) in CMG2. CMG2 mutations that result in HFS are mostly found in the CMG2 exons, are widely spread in different domains of CMG2 throughout the whole protein, and are classified into four separate classes. Class I are missense mutations located in the vWA domain of CMG2 that disrupt the ligand binding of CMG2. These vWA domain mutations (e.g., D50N and T118K) impair the divalent cation coordination in the CMG2 MIDAS and are predicted to disrupt the ligand binding of CMG2. For example, unlike CMG2 WT, the CMG2 D50N mutant cannot bind series of extracellular binding partners, including PA, laminin, and collagen VI. In addition, six out of seven of the known class I HFS mutations are amino acid substitutions in the vWA domain and are related to the more severe type, ISH. Together, the observed CMG2 mutation data suggest that the interactions between CMG2 and its ligands (ECM proteins) play important roles in CMG2’s physiological functions.
Class II HFS mutations are other missense mutations found on the vWA and Ig-like domain of CMG2. The Ig-like domain contains disulfide bond bridge and palmitoylation sites, which are important for the proper folding and trafficking of CMG2. These mutations prevent the formation of disulfide bonds, disrupt proper protein folding, and reduce the structural stability of CMG2. Thus, these CMG2 mutants are often retained in the ER and then targeted to the ER degradation (ERAD) pathway rather than being delivered to the cell surface where they can bind extracellular ligand. Interestingly, when patients’ primary fibroblast cells are treated with proteasome inhibitors, cell surface CMG2 expression level was restored, and the HFS phenotype was reduced. Also, class II mutations include the only reported missense mutation in the transmembrane domain (TMD) (L329R). This mutation potentially prevents CMG2 plasma membrane targeting and therefore avoids CMG2 clustering, which is believed to be important for CMG2-mediated endocytosis. All these data suggest that functional CMG2 must be present at the cell surface to avoid the accumulation of hyalinosis materials or ECM proteins. Therefore, in addition to binding ECM proteins, CMG2 must also play a role in ECM homeostasis.

Class III mutations are all frameshift mutations that cause premature stop codons and splicing mutations. These mutants often lead to unstable mRNA, which is targeted for degradation soon after synthesis. Thus, the mutated CMG2 protein expression level is very low or undetectable.

Lastly, the few Class IV mutations are missense mutations identified on the CMG2 cytosolic tail. Unlike Class II and III mutations, Class IV mutations do not affect protein and mRNA stability, nor plasma membrane targeting. However, these cytosolic tail mutations do affect CMG2 function, presumably by disrupting the interactions of CMG2 intracellular binding partners and the downstream signaling cascade. It is notable that one of the class IV mutations that
leads to JHF, Y381C, is one of the four putative phosphorylation sites of CMG2.\textsuperscript{34} This PTM was previously reported to be critical for anthrax toxin internalization\textsuperscript{34, 35} and supports CMG2-related intracellular signaling, which will be discussed later in this chapter.

Overall, CMG2 mutants that cause a buildup of hyaline materials and lead to HFS suggest a role for CMG2 in regulating ECM homeostasis. ECM protein homeostasis is regulated by a group of calcium-dependent endopeptidases called matrix metalloproteinases (MMPs),\textsuperscript{50} and CMG2 associates with some MMPs. For example, MT1-MMP (also called MMP14) co-immunoprecipitates with CMG2, an observation that suggests the possibility of direct interaction between this MMP and CMG2. The interaction with CMG2 modestly increased MMP14 activity, which correspondingly increased other MMP expression and activity.\textsuperscript{50} Also, other reports suggest that lower levels of CMG2 expression decreased MMPs activities\textsuperscript{20, 50, 51} These results have not been reproduced,\textsuperscript{8} which suggests that these observations with MMP could potentially be an artifact of overexpression of the two proteins. However, this link between CMG2 interactions with MMPs and MMP activity provides one possible mechanism by which CMG2 could impact ECM homeostasis. While the biochemical mechanism for the role of CMG2 in ECM homeostasis remains unresolved, the link between CMG2 and ECM homeostasis is clear, given observations that a CMG2 knockdown leads to accumulations of hyaline materials in multiple studies.\textsuperscript{8, 50, 52}

One role that CMG2 may take in ECM homeostasis is to take up hyaline materials and target them to lysosomes\textsuperscript{8} for degradation. Consistent with that observation, our lab also identified a fragment of Col-IV that specifically interacts with CMG2 and gets taken into the cell via receptor-mediated endocytosis (Chapter 3). Together, these data presented in this section strongly suggest that CMG2 interacts with different ECM proteins and has a role in regulating ECM homeostasis, potentially by internalization of ECM proteins or ECM protein fragments and
targeting them for degradation through the endocytic pathway. However, more work is needed to elucidate the actual mechanism for CMG2-dependent clearance of hyaline materials, and its relationship to angiogenesis.

1.2. CMG2 is involved in cancer metastasis, a process that involves cell adhesion and migration.

Multiple literature reports suggest that CMG2 plays a role in cancer development and invasion. In addition to its role in developing vasculature around the tumor microenvironment, increased CMG2 expression enhanced cancer cell proliferation and metastasis for some cancers. Relatedly, reduced CMG2 expression suppressed gastric cancer and nasopharyngeal carcinoma (NPC) invasion, while enhanced CMG2 expression leads to increased cell invasion in glioma cells. One potential mechanism for these phenotypes is through ERK activation. These data above suggest that CMG2 has a role in mediating cancer invasion or, in other words, cell migration. In addition to cancer cell migration, we have also observed that CMG2 mediates cell migration in different cell lines. For example, when treating cells with a CMG2 antagonist, either with PA or other small molecules, results in a significant reduction in endothelial cell migration. Similarly, a study with human uterine smooth muscle cells showed that a CMG2 knockout significantly reduced cell migration. At the same time, CMG2 overexpression enhanced migration. Together, these data suggest that CMG2 plays a critical role in mediating cell migration.

There are three categories of cell migration: directional migration (chemotaxis), random migration (chemokinesis), and adhesive substrate-induced directional migration (haptotaxis).
Previously, it was not known how CMG2 impacted these types of cell migration or what chemoattractant(s) stimulated CMG2 mediated angiogenesis. These critical gaps in knowledge will be discussed in later chapters to characterize how CMG2 mediates angiogenesis.

During cell migration, the bottom of the cell adheres to matrix proteins and changes cytoskeleton dynamics to spread out, pulling itself to move. Thus, in addition to cell migration, CMG2 also plays a role in regulating cell adhesion and spreading. For example, CMG2 silencing leads to significantly reduced prostate cancer cell adhesion.\textsuperscript{19} Although there are limited reports on how CMG2 mediates these cell adhesion phenotypes, there are published studies describing the roles of the CMG2 homolog, TEM8, in mediating adhesion and spreading by acting as a link between ECM proteins and actin.\textsuperscript{57, 58} Werner et al. has reported that mammalian cells expressing TEM8, enhance cell adhesion and spreading on collagen I.\textsuperscript{58} It was also observed that actin and TEM8 colocalize at the base of lamellipodia during cell spreading, suggesting a TEM8—actin interaction. Intriguingly, the TEM8 cytosolic tail has an actin interaction domain (AID) that binds actin, removing the TEM8 cytosolic tail impaired cell spreading. However, the remaining receptors (e.g., integrins) were still sufficient to maintain cell adhesion.\textsuperscript{58} Also, actin-binding to the cytosolic tail of TEM8 can influence the conformation of the TEM8 extracellular domain and subsequently affect TEM8 binding to its ligands, including PA.\textsuperscript{59-61} These data suggest that TEM8 is a cell surface receptor that regulates cell adhesion and spreading by coupling the actin and cytoskeleton.

Combining the role of CMG2 in HFS (i.e., uptake of ECM proteins) and the structural homology between CMG2 and TEM8, CMG2 may mediate cell adhesion by binding to matrix proteins. Also, CMG2 has an AID, suggesting CMG2 may also interact with the cytoskeleton similar to TEM8.\textsuperscript{10} In fact, a recent study has observed interactions between CMG2 and actin via a complex with Talin and Vinculin.\textsuperscript{14} Moreover, CMG2 mediates spindle orientation during
mitosis in zebrafish and mammalian cells, supporting the idea that CMG2 has a role in cytoskeleton dynamics. However, more work is required to elucidate the role of CMG2 in regulating cell adhesion, spreading, and cytoskeleton dynamics.

1.3. CMG2 structure

The canonical CMG2 has four domains. Four CMG2 isoforms are also predicted by mRNA splicing, with variations throughout the four domains\textsuperscript{10, 22} (Table 1). Our understanding is limited in both the distribution and function of the different CMG2 domains and isoforms in angiogenesis. Below we will discuss the current knowledge about the different isoforms and domains of the CMG2.

Isoform 1 is considered to be the canonical CMG2, but it shares high homology with isoform 4; the only difference found in isoform is the last twelve amino acids, and isoform 1 is one amino acid longer than isoform 4 (Table 1-1). These two isoforms of CMG2 (1 and 4) are the most commonly studied in CMG2 related research. Isoform 2 is similar to isoform 1, except it is missing amino acid residues between 213 and 315 in the extracellular domain (Table 1-1). However, no functional differences between isoform 1 and 2 have been reported. Isoform 3 is the most different from the others. The amino acid sequence from 290 to 322 is entirely different from isoform 1 and 4. It is also missing the amino acids from 323 to 489, which is the whole transmembrane domain and cytosolic tail; therefore, it is considered to be a secreted form of CMG2. This secreted form of CMG2 was mainly used as an anthrax toxin inhibitor previously,\textsuperscript{22, 62} with very little knowledge on its physiological function. A recent study of prostate cancer progression indicated that the soluble CMG2 concentration is highly related to the progression of prostate cancer.\textsuperscript{12}
CMG2 is composed of four domains, plus a signal peptide at the N terminus. The four domains are the von Willebrand Factor A (vWA) domain, Immunoglobulin (Ig)-like domain, Transmembrane domain (TMD), and an unstructured cytosolic tail. However, the functions of each of these domains are not fully understood. The signal peptide on the N terminus plays an important role in CMG2 targeting and transport within the endoplasmic reticulum during synthesis. Next, the vWA factor domain, which is involved in CMG2 binding to ligand(s) and is, therefore, the most studied domain of CMG2. The vWA factor of CMG2 has a published crystal structure along with its binding partner PA. Both ANTXRs bind PA at their metal ion-dependent adhesion site (MIDAS), which is embedded within the vWA domain. The CMG2 MIDAS domain usually coordinates an Mg²⁺ or Ca²⁺ ion with the help of various negatively charged amino acids present in the MIDAS, such as aspartate and glutamate. Literature reports have suggested that CMG2—PA interaction is significantly lower when cations are absent, implying that cation coordination in the MIDAS plays a critical role in CMG2 ligand binding. Also, as mentioned previously, mutations in the CMG2 MIDAS that block the cation coordination (e.g. D50N) not only disrupt PA binding but also lead to a build-up of ECM proteins as in HFS. Together, these data indicate that the MIDAS in the vWA factor domain is a ligand-binding site for CMG2.

<table>
<thead>
<tr>
<th>CMG2 Isoforms</th>
<th>Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMG2⁴⁰⁸ Isoform 1</td>
<td>Canonical Sequence</td>
</tr>
<tr>
<td>CMG2⁵⁸⁶ Isoform 2</td>
<td>213-315 missing.</td>
</tr>
<tr>
<td>CMG2²²² Isoform 3</td>
<td>290-322 TLTVSVSFGKGSVIGSLVATECSNGIAAI → WGLTVQAGYKWHDLTHCTFGLSGDPPTSAS 323-489 missing. Considered to be secreted protein since missing TMD</td>
</tr>
<tr>
<td>CMG2⁴₈₈ Isoform 4</td>
<td>477-489 VCWECEKEELTA → GRCINFOVPSQ</td>
</tr>
</tbody>
</table>

**Table 1-1.** The differences predicted for the difference isoforms result from alternative splicing. Isoform 1 is considered to be the canonical sequence, while isoform 2 and 4 are mostly similar to isoform 1. Isoform 3 does not have a transmembrane domain, which is considered as a secreted form of CMG2.
Integrins also have this vWA factor domain structure and use it to mediate the interaction with ECM proteins.\textsuperscript{67}

The other CMG2 extra-cellular domain is the Ig-like domain, which follows the vWA domain; its function remains unknown. Literature reports suggest that the Ig domain has potential glycosylation sites,\textsuperscript{47} which could be important for post-translational trafficking to the cell surface,\textsuperscript{68} but not much more is known about the Ig-like domain.

The CMG2 transmembrane domain (TMD), is a 23 residue single helix that inserts through the cell membrane and connects the extracellular domains to the cytoplasmic tail. The CMG2 TMD may play a role in oligomerization and CMG2 clustering,\textsuperscript{9, 69} a process that may be important for the anthrax intoxication. Also, the palmitoylation sites found at the end of TMD were shown to be essential for anchoring CMG2 on the cell membrane and helps regulate the endocytosis of anthrax toxin.\textsuperscript{33}

Finally, the CMG2 cytosolic tail is enriched with charged amino acid residues (40%), leading to a prediction of it being intrinsically disordered.\textsuperscript{10} As discussed previously, this unstructured region of CMG2 contains multiple post-translational modification (PTM) sites, including phosphorylation, ubiquitination, and palmitoylation.\textsuperscript{10} Literature reports indicate that these PTM sites play a critical role in mediating the proper functions of ANTXR2.\textsuperscript{33, 34} In fact, a recent report indicates that a PTM on the cytosolic tail affects the binding of different intracellular interactors and is regulated by ligand binding at the extracellular domain of CMG2.\textsuperscript{14} Together, these reports suggest that PTMs are vital for CMG2 function and are probably involved in CMG2 intracellular signaling.
1.4. Signaling downstream of CMG2

To understand how CMG2 may regulate angiogenesis and cell migration, we first need to identify the angiogenic mediator(s) downstream of CMG2. Fortunately, literature reports describe several CMG2 intracellular interactors that potentially provide clues to the downstream signaling. These clues include: 1) CMG2 post-translational modifications (PTMs), 2) a role for CMG2 mediating YAP activity in the Hippo pathway, 3) a functional interaction of CMG2 with a Wnt signaling receptor LRP6, and lastly, 4) a cytosolic interaction with the RhoA GTPase that mediates cytoskeleton dynamics. Since the PTMs on CMG2 cytosolic tail were already discussed earlier, we will focus on the other signaling molecules that directly or indirectly work with CMG2.

1.4.1. CMG2 mediates YAP activity

Recently, researchers have identified a role for CMG2 in regulating the activity of YAP, a well-known transcription factor downstream of the Hippo pathway. Many genes involved in regulating cell proliferation, apoptosis, migration, and adhesion were identified as targets of YAP. When the Hippo pathway is inactive, YAP will localize in the nucleus and interacts with DNA through an additional protein called TEAD to mediate gene expression. Upon the activation of Hippo, a cascade of signaling will turn on the kinases upstream of YAP and phosphorylate YAP. Phosphorylated YAP (p-YAP) is inhibited by 14-3-3 protein binding and stays localized in the cytosol. When p-YAP remains in the cytosol, it can promote the ubiquitination of YAP that leads to proteasomal degradation. Both mechanisms keep YAP localized away from the nucleus and blocks its role as a transcription factor.
Notably, researchers have observed that CMG2 protein expression is upregulated in tissue samples extracted from glioblastoma patients\(^{53}\) and endometriosis patients.\(^{70}\) In glioblastoma, glioma cells with higher levels of CMG2 expression have significantly increased migration and invasion abilities when compared to cells without CMG2.\(^{21, 53}\) RNA sequencing data and bioinformatic analysis of gene profiling suggest that there is a link between CMG2 and YAP, indicating CMG2 may have a role in the Hippo signaling pathway.\(^{53, 70}\) Multiple reports suggest that CMG2 mediates the activity of YAP; the amount of p-YAP was significantly reduced upon CMG2 over-expression, while nuclear YAP abundance increased.\(^{21, 53, 70}\) Either knockdown or inhibition of CMG2 reversed the effect.\(^{53, 70}\) In addition, upon activation of YAP via CMG2 overexpression, other genes that contribute to cell migration, adhesion, and angiogenesis were upregulated. The effect was reversed with CMG2 inhibition or knockdown. These data suggest a potential role for CMG2 in the Hippo signaling pathway, where CMG2 mediates the activity of YAP.

### 1.4.2. CMG2 and Wnt signaling

A little over a decade ago, it was reported that low-density lipoprotein receptor-related protein 6 (LRP6) was involved in the process of anthrax intoxication through interactions with CMG2 and TEM8.\(^ {73}\) LRP6 is a well-known receptor involved in Wnt signaling. LRP6 interacts with another receptor call Frizzled and mediates the activity of β-catenin, a transcription factor impacting genes that mediate cell growth.\(^ {74}\). Interestingly, reduced LRP6 expression results in cells with lower sensitivity to anthrax toxin, and that showed impaired binding and internalization of PA.\(^ {73}\) Specifically, LRP6 interacts with both anthrax toxin receptors (TEM8 as well as CMG2) and can form complexes.\(^ {73, 75}\) These interactions promote the internalization of anthrax toxin.\(^ {73}\)
Interactions between CMG2/TEM8 and LRP6 appears to play a role in regulating Wnt signaling separate from anthrax intoxication. When both CMG2 and TEM8 are knocked down, LRP6 is down-regulated, which also significantly lowers the stability of β-catenin, and thus decreases Wnt signaling. Consistent with a link between CMG2, LRP6, and Wnt signaling, a recent report indicates that CMG2 is highly expressed in gastric cancer (GC) cells, where the receptor promotes stem cell-like properties by activating Wnt signaling. Also, CMG2 colocalizes and interacts with LRP6 in GC cells; silencing CMG2 reduces the level of β-catenin in the nucleus, while overexpression of CMG2 significantly increases nuclear β-catenin. Together, these data suggest that CMG2 works closely with LRP6 to mediate anthrax intoxication and to alter the stability of β-catenin in the Wnt signaling pathway.

1.4.3. **CMG2 and Rho signaling**

Rho GTPases play a significant role in regulating cytoskeletal dynamics, cell shape, phagocytosis and the immune response. Other data suggest that Rho GTPase also plays a role in anthrax intoxication and a possible link between CMG2 and Rho GTPase activity. For example, inhibition of Rho GTPases by statins or by expression of dominant-negative mutants of Rho GTPases increases cell viability in the presence of anthrax toxin by reducing LT activity. Also, depletion of ARAP3, a downstream effector of the Rho GTPase, RhoA, reduced anthrax toxin uptake. This information links RhoA and anthrax toxin entry and suggests a potential relationship between anthrax toxin receptors (primarily CMG2) and RhoA. A study of the role of CMG2 in mitosis concluded that CMG2 interacts with RhoA to regulate spindle position during mitosis in zebra fish and mammalian cells. Specifically, during cell division, CMG2 colocalizes with the F-actin cap, where it interacts with RhoA and activates mDia and ROCK to align
microtubule spindles with the actin cap, a process required for appropriately-oriented mitosis.\textsuperscript{14,15} Notably, CMG2 interacts with RhoA, via its cytosolic tail, specifically to the GTP bound (active) form. This CMG2-RhoA interaction is dependent on ligand binding at the extracellular domain: cells under serum-free condition or which express a ligand-binding defective mutant do not demonstrate this interaction.\textsuperscript{14} Under ligand-free conditions, CMG2 forms an alternate protein complex containing CMG2 and talin, vinculin, and actin (TVA). Upon CMG2 binding, this TVA complex may mask the cryptic binding domain of RhoA. Also, upon ligand binding to CMG2, tyrosine kinases (Src/Fyn) induce phosphorylation of the CMG2 cytosolic tail and trigger the release of the TVA complex, thus allowing RhoA binding for downstream signaling.\textsuperscript{14} These data indicate a link between CMG2 and RhoA, which provides a possible mechanism for the impact of CMG2 on cell migration.

Since the Rho GTPase is known to mediate cytoskeleton and cell migration, our lab has proposed that a CMG2—RhoA interaction may be involved in CMG2-mediated chemotaxis. RhoA and its downstream effectors Rho-associated coiled-coil kinase (ROCK) and a mammalian homolog of Drosophila diaphanous) mDia are known to be the major regulators of cytoskeleton reorganization during cell migration.\textsuperscript{79-81} During cell migration, chemotactic stimuli direct cells to extend protrusions from the leading edge and make focal adhesions to ECM (lamellipodia). Cells use these adhesion sites on ECM to pull themselves forward, then disassemble and retract the tail as they move on.\textsuperscript{79} mDia is responsible for nucleating and polymerizing actin filaments in lamellipodia, while ROCK reverses what mDia does and induces actomyosin contraction at the rear of the cell.\textsuperscript{79-81} The balance between ROCK and mDia depends on the concentration of GTP bound RhoA. At high GTP-RhoA concentrations, both mDia and ROCK are activated to promote retraction. In contrast, low concentrations preferentially enhance mDia activity to induce cell
stretching, which is also supported by the published Kd of ROCK and mDia for Rho (130 and 6nM, respectively). These previous reports show CMG2 interacts with RhoA, and forms complexes with different downstream RhoA effectors upon ligand binding. We have shown that endothelial cells lose their ability to migrate in serum-free (e.g., no ligand) medium, suggesting that the CMG2—RhoA interaction plays a critical role in CMG2 mediated migration. Additional studies of RhoA and CMG2 mediated cell migration will be further discussed in chapter 4.

1.4.4. CMG2 impacts endothelial cell migration induced by multiple growth factors

Our lab has observed that CMG2 inhibition reduces serum-induced migration. While there are many different chemoattractants in the serum, including different protein growth factors and lipids, the identities of the chemoattractants that stimulate CMG2 mediated migration is not fully understood. Literature reports have shown that silencing CMG2 in uterine smooth muscle cells results in reduced migration towards PDGF. Also, reports have shown that both ANTXRs work closely with EGFR. Depleting CMG2 in NPC cells significantly decreases EGF induced migration.

Furthermore, inhibiting CMG2 with PA also leads to significant anti-angiogenic effects on both bFGF and VEGF induced cornea neovascularization. All these data indicate that CMG2 potentially mediates angiogenesis by regulating cell migration induced by a series of different growth factors. Interestingly, activation of RhoA is also induced by different cell surface receptors, including tyrosine kinase receptors (such as EGFR, PDGFR, and VEGFR) and GPCRs (such as LPA and S1P receptors). These reports lead us to hypothesize that various stimuli, including different growth factors and/or lipids, may involve in CMG2—RhoA mediated chemotaxis. Recent
research has observed that RhoA knockout or inhibition by exoenzyme C3 toxin reduces VEGF induced endothelial cell migration, but not S1P. Together, the information above suggests that CMG2 may work with different protein growth factors but not lipids.

1.5. Final comments

CMG2 has been identified as an angiogenic mediator for almost two decades; however, the biology of how CMG2 mediates angiogenesis is not fully understood. Different CMG2 studies published over the years provides critical clues to understand the physiological role of CMG2 in mediating angiogenesis. CMG2 mutants lead to HFS (a genetic disease causing a build-up of ECM proteins), consistent with a series of reports indicating that CMG2 interacts and takes up ECM proteins into the cell. These observations provide a link between CMG2 and ECM homeostasis, a critical step for angiogenesis. Furthermore, various literature reports indicate that CMG2 is heavily involved in cell migration, another critical process in angiogenesis. Since RhoA GTPase, which is well known as a regulator in cell migration, binds to CMG2 cytosolic tail, CMG2 may mediate angiogenesis via RhoA. CMG2 was also observed to be involved in signaling pathways that include Wnt and Hippo. RhoA was also found to involve in these intracellular signaling pathways, and the CMG2—RhoA axis may act as a central mediator in multiple angiogenic signaling pathways. These data provide clues to build a model for how CMG2 mediates angiogenesis. Experimental work presented in this dissertation provides further evidence to establish a link between CMG2, RhoA, endothelial cell chemotaxis, angiogenesis, and ECM homeostasis, hence illuminating the physiological role of CMG2.
2. Monitoring Endothelial Cell Migration using a Gradient of Chemoattractants on a Microfluidic Platform

2.1. Abstract:

Cell migration assays have been used to monitor angiogenic phenotypes under different conditions. Traditional migration assays, including the wound scratch assay and the transwell migration assay, are commonly employed to monitor endothelial migration under a variety of conditions. However, these methods have significant limitations, including significant hands-on time, poor repeatability and reproducibility, and minimal physiological relevance to detecting angiogenic phenotypes. The method described herein provides a straightforward, repeatable, and more physiologically relevant way to perform cell migration assays. Our optimized migration assay utilizes a commercially available microfluidics platform coupled with time-lapse microscopy to monitor and measure endothelial migration of individual cells towards a chemoattractant. Importantly, the readily available gradient-generating microfluidic devices provide a stable gradient of chemoattractants for cells and allow measurement of both total cell motility (chemokinesis) and migration towards the chemotactic gradient (chemotaxis) under controlled conditions. Results from this assay can provide information about how different antiangiogenic molecules affect endothelial cells, a critical phenotype that can ultimately affect vessel formation. The protocol below describes the detailed procedures to perform an endothelial cell migration assay using a microfluidic platform. Data analysis is performed using readily available and free software to provide both measurements and statistics of various aspects of cell migration.
2.2. Introduction:

Angiogenesis involves a series of complex physiological processes. Angiogenesis is initiated by a group of growth factors that diffuse towards the nearby blood vessels and bind the growth factor receptors on the endothelial cells.\textsuperscript{87, 88} Once the angiogenic signaling is turned on; then matrix metalloproteinases will be expressed, resulting in remodeling of the basement membrane surrounding the endothelial cells.\textsuperscript{87, 89, 90} An endothelial tip cells will then start migrating along the gradient of growth factors, while the endothelial stalk cells propagate behind the tip cells to form new blood vessels.\textsuperscript{91} Therefore, endothelial cell migration is one of the key determinants of angiogenesis. Multiple varieties of cell migration assays have been developed and used in screening for inhibitors that have anti-angiogenic effects; however, the traditional approaches have limitations,\textsuperscript{92, 93} thus, there remains a need for more robust, reproducible, and sensitive approaches.

The most common migration assays in current use for angiogenesis-related research are wound scratch migration assays and trans-well migration assays.\textsuperscript{92} The wound scratch migration assay takes a simple approach, which involves using a pipette tip to produce a cell-free region (wound) in a confluent layer of cells, then measures cell migration towards the wound over time.\textsuperscript{92} Advantages of this approach include its low cost and minimal time to perform. Matrix protein-coated assay plates help with cell adhesion and seeding; however, that coating can be easily damaged during the wound creation, which reduces its physiological relevance. Also, wound formation can vary between wells, causing inconsistencies measuring cell migration. Most importantly, this approach does involve a growth factor gradient critical to the in vivo model of angiogenesis. In the absence of a gradient, there is also no way to monitor chemotaxis. Thus, when research requires the measurement of chemotactic migration, this assay is insufficient.
The transwell migration or the Boyden assay is performed in a two-compartment well plate; a porous filter membrane separates the two compartments. Cells are seeded on one side and a solution with a chemoattractant is placed on the other side of the experimental chamber. Cells that migrate onto across the filter membrane are fixed and stained, and the number of migrated cells counted is the output of assay. While the transwell migration assay is more physiologically relevant than the wound scratch migration assay due to its ability to measure migration towards a chemoattract, such a gradient is not stable during the assay timeframe, as it comes to equilibrium through diffusion over time. Also, this assay only provides an endpoint readout, which does not provide a visualization of cells during the migration. This approach can require more hands-on time to complete a single assay due to the time to fix, stain, and count individual filter membranes.

The combination of traditional migration assay limitations makes them incapable of direct visualization of cell responses to a particular chemoattractant. To overcome these limitations, researchers have used custom microfluidic devices for measuring cell migration. These devices better mimic the local microenvironment during angiogenesis compared with traditional methods and allow better cell migration to be observed at the single-cell and population level. However, in our experience, these custom-fabricated microfluidic devices require cleanroom access and significant competencies to produce and operate.

Our lab has utilized a commercially available microfluidics platform (CellASIC ONIX2) accompanied by a pre-fabricated microfluidic gradient device. We have successfully used this integrated control system and devices to monitor endothelial cell migration. The objective of this optimized method is to provide an easy-to-use, repeatable and reproducible, and more physiologically relevant way to measure endothelial cell migration. This system allows cell culture
to occur in a microfluidic device, which also can rapidly generate a diffusion gradient across the cell chamber and remains stable well beyond the typical assay time, even for the slowly moving endothelial cells. Combining this system with time-lapse microscopy allows observation of individual cell movement in response to a gradient of chemoattractant. This system provides a user-friendly platform, which requires less hands-on time along with higher repeatability and reproducibility and tunable sensitivity. Data collected from this cell migration system provides information for both chemotaxis and chemokinesis and allows both single cell and population data that increases the statistical power of the measurements. The optimized protocol, below, is a more robust and efficient way to measure more physiologically relevant endothelial migration phenotypes.

2.3. Methods:

2.3.1. Materials and Reagents:

Cell culture medium, buffers, and syringe filters were purchased from Genesee Scientific. Fluorescein-NHS powder was purchased from Fisher Scientific (Catalog no. 46410). The CellASIC ONIX2 platform (Catalog no. CAX2-S0000), along with its temperature-controlled manifold (Catalog no. CAX2-MXT20), was purchased from Millipore Sigma. The CellASIC ONIX microfluidic plates for mammalian cell culture were also purchased from Millipore Sigma (Catalog no. M4G-02-5PK).

2.3.2. Protein Preparation

A protective antigen mutant ($\text{PA}^{\text{SSSR}}$) was used to treat the endothelial cells in cell migration assays since it is a high-affinity antagonist of capillary morphogenesis gene 2 (CMG2)
and is known to reduce angiogenesis in vivo.\textsuperscript{4} PA\textsuperscript{SSSR} was cloned into pET-22b (Novagen) and was expressed in NEB T7 Express (New England Biolabs) \textit{E.coli} in a bioreactor in LB + 10 mM glucose. PA\textsuperscript{SSSR} was purified from periplasmic lysates and purified by an anion exchange chromatography (Q-sepharose) as previously reported.\textsuperscript{97} Briefly, the ion exchange column was equilibrated with 20 mM Tris-HCl pH 8 and 20 mM NaCl, and the lysate was loaded at 5 mL/min, then eluted using gradient elution with 20 mM Tris-HCL pH 8 and 1 M NaCl. The eluted PA was then concentrated with an Amicon 30 kDa centrifugal filter from Millipore Sigma (Catalog no. UFC903024) and stored at -80°C in storage buffer (2 mM Tris pH8, 20 mM NaCl, 50% glycerol).

A fluorescein-bovine serum albumin (BSA) conjugate was prepared per the manufacturer protocol. The labeling reaction was performed at a ratio of protein: label (1:10) in PBS. Excess free dye was removed with a PD-10 desalting column from GE Healthcare (Catalog no. 17085101) per the manufacturer protocol and followed by concentration with an Amicon 30 kDa centrifugal filter from Millipore Sigma (Catalog no. UFC903024) and stored in PBS with 50% glycerol.

2.3.3. Cell Cultures

\textit{EA.hy926} (ATCC CRL-2922) cells are a somatic hybrid of human umbilical vein cells with A549 lung carcinoma cells and were purchased from the American Type Culture Collection.\textsuperscript{98} The cell line was cultured in 10% FBS + DMEM (Genesee Scientific, catalog no. 25-500) and incubated at 37°C in a humidified environment with 5% CO\textsubscript{2} until ready for passaging.

2.3.4. Microscopy
Fluorescence images were acquired using an IX73 epifluorescence microscope (Olympus Corp., Tokyo, Japan), with an Orca Flash V4.0 CMOS camera (Hamamatsu, Shizuoka, Japan). The light source and camera were controlled using Slidebook 6.0 (Intelligent Imaging Innovation, Denver, Co). Bright field time-lapse images were captured on an IQcrew Stem Science Discovery inverted microscope and an Amscope MU130 camera that was inserted into the ocular port. Migration images exported from the Amscope 3.7 software were further analyzed using Image J and Chemotaxis and Migration Tool (ibidi).

2.3.5. FITC Gradient formation

The fluorescein-BSA conjugate was diluted to 1mg/mL in PBS then filtered through a 0.22\(\mu\)m syringe filter. The fluorescein-BSA working solution (200\(\mu\)L) was added to either well 2 (or 3) on the CellASIC migration plate, which goes to the top perfusion channel. PBS (200\(\mu\)L) was placed into either well 4 (or 5), which goes to the bottom perfusion channel. Once the manifold was sealed on top of the plate and secured on the microscope platform, the ONIX program was customized to run both well 2 and 4 at 0.5psi for 12 hours (Table 1A). The fluorescence was measured using fluorescence microscopy (480nm excitation, 530nm/30nm), and images were acquired with a 200 ms exposure 6 times every hour for 12 hours. The fluorescence gradient across the cell chamber was measured in Slidbook 6.0 and plotted in SigmaPlot 11.

2.3.6. Microfluidic Plate Preparation and Setup

The CellASIC gradient microfluidic plate (EMD Millipore Cat# M04G-02-5PK) was transferred and opened in the biosafety cabinet. The storage solution (PBS supplemented with
0.05% azide) in wells 1, 6, 7, and 8 were removed by aspiration making sure the fluid was completely removed from wells 6 and 8. The channels were primed by adding 10 µL of DMEM supplemented with 10% FBS into the ring of well 6, and then the plate priming program (0.25psi for 2 minutes) was started. After the plate was primed with medium, plates were incubated at 37°C, 5% CO₂, and >90% humidity for at least an hour before cell seeding (Table 1B). This incubation allows the plate to warm up and enhances the cell seeding. When indicated by the CellASIC controller, the cell channel were coated with extracellular matrix protein before cell loading. The extracellular matrix protein coating procedure uses 10 µL of 20 µg/mL solution of the desired protein diluted in PBS. In these experiments, human fibronectin (HFN) was loaded into the ring structure of well 6 and followed by running the ONIX priming program (0.25psi for 2 min). After the protein was introduced into the cell chamber, the CellASIC plate was incubated at 37°C for 1 hour or overnight at 4°C (Table 1C). Following the channel coating procedure, culture medium priming can proceed. Annotated well numbers are shown in the plate layout (Figure 1A).

2.3.7. Cell loading and migration

EA.hy926 cells resuspended at a concentration of 3,000,000 cells/mL in warm medium (10% FBS in DMEM + 10 mM HEPES; migration assay medium) for the migration assay. Migration assay medium was loaded into wells 1, 2, 5, 7, and 8 (10 µL was loaded into the ring in well 1; 200µL was loaded into wells 2 and 5; 100µL was loaded into wells 7 and 8). Then 10 µL of the EA.hy926 cells suspension was loaded into the ring in well 6. Liquid loading into wells 7 and 8 should occur before adding cells in well 6 because it provides backpressure to prevent premature cell loading into the cell chamber (Figure 1C). After loading all solutions, the cell
loading program on the ONIX platform was run (0.25 psi from well 1 and 6 for 30 seconds) while monitoring the cell loading via the microscope (Figure 1D). Once cells were loaded into the chamber, wells 2 and 5 (both were loaded with migration assay medium) were activated at 0.5 psi, and the temperature set to 37°C overnight to allow cells to adhere and spread onto the channel surface (Figure 1E).

The next day, the plate was detached from the manifold to replace the migration assay medium into well 2 and to add serum-free migration assay medium into well 4. The migration assay medium and serum-free migration assay medium containing 200 pM PASSSR were also filtered and loaded into wells 5 and 3, respectively. The migration plate was then returned to the microscope, and the ONIX manifold was replaced. Next, a region of the cell channel that had approximately 50% - 80% confluent cells in the field of view was selected for the first assay (control) by pressurizing wells 2 and 4 at 0.5 psi for 12 hours (or 8 hours when indicated). At the conclusion of the control experiment, wells 3 and 5 were pressurized (0.5 psi) for another 12 hours that delivered the same gradient, but with 200 pM PA supplemented into both wells (Figure 1F-G; Table 2).

2.3.8. Migration data analysis

Cell migration assay images were exported as TIFF series (72 pictures per condition, or 48 when indicated), and then imported into Image J for analysis using the Manual Tracking plugin. The x/y calibration was set to 2 μm and the interval 10 minutes before beginning to track individual cells. Forty cells were tracked per condition, and these data were exported as a CSV file. Data files from different conditions were then imported into the Chemotaxis and Migration Tool v2.0.
(ibidi.com). The following settings were applied to initialize the data sets: Number of slides 72 or 48 (12 hours and 8 hours respectively); x/y calibration 2µm (Hamamatsu camera) or 0.98µm (Amscope MU130 camera); Time interval 10 minutes. The output from the Chemotaxis and Migration Tool was plotted as cell track plots, and the measured chemotaxis (directness) and measured chemokinesis (distance) were recorded under the “Statistics” tab. Quantified measurements were exported and plotted in SigmaPlot 11. Statistical p-values were calculated using the Student t-test.

2.4. Results

2.4.1. Plate Layout and Gradient Formation

The CellASIC ONIX platform and accompanying gradient migration plate is a commercially available microfluidic device that has been used to measure cell responses to a gradient. An initial report using a mammalian epithelial cell line (MDA-MB-231) was published as a non-peer reviewed application note in Nature Methods in 2015.99 The microfluidic device allows monitoring of cell motility in a controlled temperature environment under a stable chemoattractant gradient over many hours. The gradient migration plate has four rows (one row per experiment) with six inlets on the left and two outlets on the right (Figure 1A). Fluids in the inlets are perfused into the device by pressurizing addressable wells. The cell chamber portion of each individual assay channel is located in the center, with perfusion channels on the top and bottom sides of the cell chamber separated by a pillar array that keeps cells in the main channel. Still, it allows free diffusion from the top and bottom channels containing cell culture media or buffer (Figure 1B). Fluids flow in the top and bottom channels but diffuse through the perfusion barrier to reach the middle cell chamber. As a result, a gradient can be formed when perfusing different fluids from both perfusion channels (Figure 1F-G). However, in the initial report, the
stability of such a gradient was not tested. Hence, to investigate the stability and magnitude of the generated gradient, we used a fluorescein-conjugated BSA (BSA-FITC) to visualize the gradient over time. The resulting data demonstrate that the fluorescence gradient started to form quickly, 15 minutes after starting the flow, then remained relatively stable for up to 16 hours (Figure 2A). The fluorescence intensity was quantified in Slidebook. The quantified gradient showed that a steep gradient was formed within an hour after the initiation of flow stabilized after 8 hours (Figure 2B).

2.4.2. Monitoring Endothelial Cell Migration in the Microfluidic Device

In this study, we used a microfluidic system to measure endothelial cell (EA.hy926) chemotaxis and chemokinesis with and without a cell migration inhibitor. Previously, we observed that a mutant of *Bacillus anthracis* protective antigen (PA\(^{\text{SSSR}}\)) has a potent anti-angiogenic effect on both corneal neovascularization in vivo. PA\(^{\text{SSSR}}\) treated primary endothelial cells (HMVEC) also show significantly reduced migration in the transwell cell migration assay.\(^4\) Since the primary target of PA\(^{\text{SSSR}}\) is CMG2, similar reductions in cell migration have been with several small molecules.\(^6,\,7\) Thus, a CMG2-expressing human endothelial cell was chosen as a model to evaluate cell migration in the CellASIC microfluidic migration assay. Initially, the optimization of the cell loading protocol was needed. After several trials, loading 30,000 cells in 10 µL of cell medium at 0.4 psi for 25 seconds provided the best results. After successfully loading cells into the central chamber, we evaluated how cells migrated in response to a serum gradient with and without PA. In this case, DMEM supplemented with 10% fetal bovine serum was in the top perfusion channel while serum-free DMEM was on the bottom (Figure 1F). Within the first four hours, cell tracking plots showed the cells migrated in a random pattern regardless of the treatment (Figure 3). After 6 hours, untreated cells began to migrate uniformly towards the serum gradient. However, cells
treated with PA\textsuperscript{SSSR} showed a random migration pattern. The difference between the untreated cells and PA\textsuperscript{SSSR} treated cells became more significant over time (Figure 3), indicating that assay sensitivity appears to increase proportionally with time. At the end of 12 hours, all of the untreated cells migrated toward the serum gradient, while cells treated with PA\textsuperscript{SSSR} still showed a random migration pattern (Figure 3). Measured values of endpoint \( y \)-displacement (chemotaxis) and accumulated distance (chemokinesis) were plotted for comparison. As was observed in the tracking plots, no significant difference was observed for chemotaxis at the early time point (4 h). However, as expected, when assay time increased, the difference in chemotaxis between the untreated and treated cells also increased (Figure 4A). While chemokinesis of the PA\textsuperscript{SSSR}-treated cells decreased compared to the untreated control cells, the magnitude of the difference was small compared to the effects PA\textsuperscript{SSSR} treatment had on chemotaxis (Figure 4B).

2.4.3. Monitoring Endothelial Cell Migration Against Individual Chemoattractants.

Since endothelial cells migrated towards a serum gradient, we next tested whether we could monitor migration towards individual growth factors. We tested endothelial cell migration towards several growth factors, including bFGF, VEGF, PDGF, insulin, and boiled FBS supplemented serum-free medium, respectively. In all cases, cell migration with PA\textsuperscript{SSSR} treatment was normalized to the untreated control cells to indicate whether the growth factor-induced migration was affected by a CMG2 blockade (Figure 5A-B). In the case of bFGF, VEGF, and PDGF, PA\textsuperscript{SSSR} treatment strongly inhibits chemotaxis towards the growth factor gradient, while there is no effect on chemokinesis. Migration towards insulin and DMEM supplemented with 10% boiled FBS were unaffected by PA\textsuperscript{SSSR} treatment.
Since heating FBS to 100°C caused significant precipitation of the protein fraction of serum, and the precipitate was removed by centrifugation before supplementing the medium, this suggests that CMG2-mediated chemotaxis is affected by molecules in the serum subject to heat-denaturation (e.g., bFGF, VEGF, PDGF). Molecules that are not heat denatured or precipitate upon heating can still drive endothelial cell chemotaxis. Similarly, insulin-induced chemotaxis was also not affected by PA^{SSSR} treatment, suggesting that CMG2 is not involved in the regulation of all chemotaxis, but instead responds to specific growth factor signals as previously reported. We note that 200 pM PA^{SSSR}, as used here, would only result in 50% occupancy of CMG2 and essentially no occupancy of TEM8, the CMG2 homolog. Thus, the inhibition of CMG2-mediated chemotaxis observed here is likely a specific CMG2 effect on endothelial cell migration and chemotaxis specifically.

2.5. Discussion

Here, we have shown that the CellASIC microfluidic gradient migration assay is a sensitive and reproducible method for performing cell migration assay. Unlike the traditional migration assays, the microfluidic gradient approach allows us to monitor individual cell migration under a stable gradient formed across the chamber. While changes in migration were the same in both the wound scratch and microfluidic gradient migration assay, the microfluidic gradient migration assay provided substantially more details about different aspects of cell migration (e.g., separating chemotaxis and chemokinesis). Also, while traditional migration assays can be labor-intensive and hard to perform, the microfluidic gradient migration assay allows the user to perform migration assays with minimal hands-on time. Data reproducibility is a significant concern for any migration assay, particularly for the transwell assay, where cell counts on the membrane vary significantly.
between replicates. In contrast, this microfluidic approach also allows for monitoring of the same group of cells migrating under both control (untreated) and treatment conditions, therefore, reducing variation between the two conditions. Importantly, this microfluidic gradient migration assay provides a stable gradient (about 5-fold) across the chamber, which allows for a more physiological environment that is not the case for the traditional migration assays. Notably, we have demonstrated that this method is capable of testing a variety of chemoattractants with relevance to a specific receptor, CMG2. One disadvantage of the microfluidic gradient migration assay compared to well plate-based assays, is that the assay throughput is much lower, and can only be multiplexed to four simultaneous assays using an automated microscope stage and appropriate software. However, the readily available microfluidic gradient migration assay using the CellASIC ONIX platform remains an incredibly potent tool that allows researchers to study the effects chemoattractants on cell migration, chemotaxis, and chemokinesis.
Technical Notes:

During the protocol optimization, we found that filtering the medium through a 0.2 µm syringe filter is critical to the success of the assay. We observed no gradient formation occasionally when the medium was not filtered (data not shown), which was probably caused by aggregated proteins from serum blocking the perfusion barrier. The filter also helps to sterilize any liquid that will go into the system and minimize contamination.

We also observed that cell density in the culture chamber is critical for cell migration. A CellASIC application note suggested that cells tend to migrate better when they are in a cluster, and cells also migrate farther when they are at a lower density. We observed that when loading cells at a concentration higher than 3,000,000 cells/mL, cell density became too high for observing EA.hy926 cell migration. However, since cell size and morphology varies from one cell line to the other, cell loading optimization experiments are recommended when working on a new cell line. In our hands, it was always better to load cells at a lower density and repeat the loading to add more cells if needed. Also, monitoring liquid priming and cell loading under a microscope is also recommended.

We note that cells’ response to chemoattractants and inhibitors can be different from cell line to cell line. When working on a new cell line, it is important to run an optimization assay to find the total assay time that provides robust responses to chemoattractants. An experiment such as this can be performed, as described in the methods section, by running a twelve-hour or longer assay, and then comparing cell migration versus a known migration inhibitor at various time points (e.g., 4, 6, 8, 10, and 12 h).
2.6. Figures and Tables

Table 2-1: Gradient test and plate preparation

<p>| Table 1A: BSA-FITC Gradient Perfusion Protocol |  |</p>
<table>
<thead>
<tr>
<th>Channel Number</th>
<th>Content</th>
</tr>
</thead>
<tbody>
<tr>
<td>1, 3-6</td>
<td>PBS with 0.05% azide</td>
</tr>
<tr>
<td>2</td>
<td>1 mg/mL BSA fluorescein</td>
</tr>
</tbody>
</table>

Perfuse channel 2 and 4 at 0.5 psi for 16 hours.

Table 1B: Matrix Protein Coating Protocol

<table>
<thead>
<tr>
<th>Channel Number</th>
<th>Contents</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10% FBS DMEM with 25 mM HEPES</td>
<td>10 µL</td>
</tr>
<tr>
<td>6</td>
<td>20 µg/mL Collagen IV in PBS</td>
<td>10 µL</td>
</tr>
<tr>
<td>3-5</td>
<td>PBS with 0.05% azide</td>
<td>250 µL</td>
</tr>
<tr>
<td>7,8</td>
<td>Empty</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Perfuse channel 6 at 0.25 psi for 2 minutes, then incubate at 37°C for 1 hour or 4°C overnight.

Table 1C: Cell Medium Priming Protocol

<table>
<thead>
<tr>
<th>Channel Number</th>
<th>Contents</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10% FBS DMEM with 25 mM HEPES</td>
<td>10 µL in ring</td>
</tr>
<tr>
<td>6</td>
<td>10% FBS DMEM with 25 mM HEPES</td>
<td>10 µL in ring</td>
</tr>
<tr>
<td>3-5</td>
<td>PBS with 0.05% azide</td>
<td>250 µL</td>
</tr>
<tr>
<td>7,8</td>
<td>Empty</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Perfuse channel 6 at 0.25 psi for 2 minutes, then incubate at 37°C until use.
### Table 2A: Cell Seeding Protocol

<table>
<thead>
<tr>
<th>Channel Number</th>
<th>Content</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10% FBS DMEM with 25 mM HEPES</td>
<td>10μL in the ring</td>
</tr>
<tr>
<td>6</td>
<td>EA.hy926 cells at 3,000,000 cells/mL</td>
<td>10 μL in the ring</td>
</tr>
<tr>
<td>2, 4</td>
<td>10% FBS DMEM with 25 mM HEPES</td>
<td>200 μL</td>
</tr>
<tr>
<td>3, 5</td>
<td>PBS with 0.05 azide</td>
<td>200 μL</td>
</tr>
<tr>
<td>7, 8</td>
<td>10% FBS DMEM with 25 mM HEPES</td>
<td>50 μL</td>
</tr>
</tbody>
</table>

Perfuse channels 1 and 6 at 0.25psi for 30 seconds then immediately switch to channels 2 and 4 at 0.5psi for overnight at 37°C.

### Table 2B: Cell Migration Protocol

<table>
<thead>
<tr>
<th>Channel Number</th>
<th>Content</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>10% FBS DMEM with 25 mM HEPES</td>
<td>200 μL</td>
</tr>
<tr>
<td>3</td>
<td>0% FBS DMEM with 25 mM HEPES, 200 pM PA</td>
<td>200 μL</td>
</tr>
<tr>
<td>4</td>
<td>0% FBS DMEM with 25 mM HEPES</td>
<td>200 μL</td>
</tr>
<tr>
<td>5</td>
<td>10% FBS DMEM with 25 mM HEPES, 200 pM PA</td>
<td>200 μL</td>
</tr>
<tr>
<td>7, 8</td>
<td>Empty</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Perfuse channels 2 and 4 at 0.5psi for 12 hours at 37°C, then repeat with the same setting on channels 3 and 5.
Figure 2-1. CellASIC microfluidic gradient plate and experiment layout. (A) Gradient plate layout. White wells are both gravity or pressure-driven wells. Blue and red wells are only pressure driven. Blue wells connect to the top perfusion channel while the red wells connect to the bottom perfusion channel. (B) Zoom-in view of the migration channel where the yellow portion is the cell chamber. Two pillar arrays are separating the cell chamber and the perfusion channel. (C-E) Cell loading procedures. (C) Fluids in wells 7 and 8 generate a higher pressure towards the left side of the plate, which is important to keep cells from premature loading before pressurizing wells 1 and 6. (D) Cells flow into the main chamber from well 6. In contrast, the medium flows from well 1 to keep the cells in the middle of the cell chamber and prevent them from blocking the pillar arrays. (E) 10% FBS DMEM with 25 mM HEPES is then flowed in both perfusion channels to allow cell seeding and spreading. (F-G) Forming a gradient of serum by flowing complete medium on one side and serum-free on the other.
Figure 2-2: FITC-BSA gradient formation in the CellASIC microfluidic gradient plate. (A) An image of FITC-BSA in PBS flowing in the top perfusion channel and PBS was flowing on the bottom channel over 16 hours. A sharp gradient forms within an hour and is maintained at a similar level for at least 15 hours. (B) Quantitation of the FITC-BSA signal across the cell chamber, where the gradient was formed. The signal remains at a similar level, and the gradient magnitude was maintained at about 5-fold across the channel. Together, this suggests that a sharp and stable gradient can be generated by these microfluidics gradient devices.
Figure 2-3: Migration track plots of EA.hy926 cells migrating towards serum at different time points. Representative track plots of endothelial cell migration induced with FBS supplemented medium. Some cells were treated with 200 pM PA^{SSSR}. Cells were monitored in the microfluidic gradient migration plate for 12 hours per condition. At least 40 cells were tracked from each experiment in Image J and further quantified for both directional migration and total migration distance using the chemotaxis and migration tool (ibidi.com).
Figure 2-4: Quantified endothelial cell migration data over 12 hours period. Directional migration towards a serum gradient where chemotaxis (A) and chemokinesis (B) were quantified and compared between untreated and PA\textsuperscript{SSSR} treated cells. Image J was used to track the cells from each replicate, and a total of three replicates were analyzed. The Student T-test was used to compare conditions. * p<0.05; ** p<0.01; *** p<0.001; n.s not significant.
Figure 2-5. Monitoring the effects of CMG2 inhibition on endothelial cells migration towards various growth factors. EA.hy926 cells were cultured in the microfluidic gradient migration plate and a gradient formed using different growth factors with or without 200 pM PA$_{SSSR}$. Quantified migration data for the effects of PA$_{SSSR}$ treatments were normalized to the no PA$_{SSSR}$ control to obtain normalized PA$_{SSSR}$ inhibited migration.
3. A Canstatin-Derived Peptide Provides Insight into the Role of Capillary Morphogenesis Gene 2 in Angiogenic Regulation and Matrix Uptake

3.1. Abstract

Capillary Morphogenesis Gene 2 protein (CMG2) is a transmembrane, integrin-like receptor and the primary receptor for the anthrax toxin. CMG2 also plays a role in angiogenic processes. However, the molecular mechanism that mediates the observed CMG2-related angiogenic effects is not fully elucidated. Previous studies have reported that CMG2 binds type IV collagen (Col-IV), a vital component of the vascular basement membrane, as well as other ECM proteins. Here, we further characterize the interaction between CMG2 and individual peptides from Col-IV and explore the effects of this interaction on angiogenesis. Using a peptide array, we observed that CMG2 preferentially binds peptide fragments of the NC1 (non-collagenous domain 1) domains of Col-IV. These domains are also known as the fragments arresten (from the α1 chain) and canstatin (from the α2 chain) and have documented antiangiogenic properties. A second peptide array was probed to map a putative peptide-binding epitope onto the Col-IV structure. A top hit from the initial array, a canstatin-derived peptide, binds to the CMG2 ligand-binding von Willebrand factor A (vWA) domain with a sub-micromolar affinity (peptide S16, K_d = 400 ± 200 nM). This peptide competes with anthrax protective antigen (PA) for CMG2 binding and does not bind CMG2 in the presence of EDTA. Together these data suggest that, like PA, S16 interacts with CMG2 at the metal-ion dependent adhesion site (MIDAS) of its vWA domain. CMG2 specifically mediates endocytic uptake of S16; both CMG2−/− endothelial cells and WT cells treated with PA show markedly reduced S16 uptake. Furthermore, S16 dramatically reduces directional endothelial cell
migration with no impact on cell proliferation. These data demonstrate that this canstatin- derived peptide acts via CMG2 to elicit a marked effect on a critical process required for angiogenesis.

3.2. Introduction

CMG2, also known as ANTXR2, is an integrin-like receptor initially identified as a gene highly upregulated during endothelial cell tube formation in vitro. Like integrins, CMG2 contains an extracellular vWA domain that coordinates a divalent metal ion in a MIDAS domain and binds various extracellular matrix proteins. The initial report qualitatively showed CMG2 binding to Col-IV, fibronectin, and laminin; subsequent reports indicate that CMG2 also binds Col VI. However, the affinity, specific binding sites on each ECM, and the cellular relevance of these interactions have received a limited examination.

CMG2, like its close homolog TEM8, is most studied its role as an anthrax toxin receptor. These two cellular receptors bind the non-toxic anthrax toxin subunit, protective antigen (PA), and subsequently mediate intracellular delivery of the catalytic toxin subunits. Experiments that challenged mice lacking full-length CMG2 or TEM8 with B. anthracis spores indicate that CMG2 is the primary receptor of anthrax toxin. Biophysical characterization also demonstrates that PA has a substantially higher affinity (100 fold) for CMG2 than for TEM8.

Abbreviations: CMG2, Capillary Morphogenesis Gene 2; MIDAS, metal ion dependent adhesion site; ECM, extracellular matrix; BM, basement membrane; VBM, vascular basement membrane; BLI, bio-layer interferometry; TEM8, Tumor Endothelial Marker 8; Col-IV, Type IV Collagen; Col-VI, Type VI Collagen; ANTXR2, Anthrax toxin receptor 2; vWA, von Willebrand factor type A; PA, anthrax protective antigen; PA-SSSR, a furin protease resistant version of PA; HFS, hyaline fibromatosis syndromes; NC1, non-collagenous domain; CMG2-GST, a glutathione-s-transferase fusion with the vWA domain of CMG2;
While the interaction of CMG2 with PA does not illuminate the role of CMG2, the high-affinity and specific interaction of CMG2 with PA has been used to probe the role of ligand interaction with CMG2 in angiogenesis. Notably, the binding of a mutant PA, (PA-SSSR) to CMG2 substantially inhibits growth-factor induced angiogenesis in the cornea, reduces tumor volume \textit{in vivo}, and blocks endothelial cell migration \textit{ex vivo}.\textsuperscript{4} These data indicate that the interaction of ligands with CMG2 has a profound effect on angiogenic processes. Further, knockdown of CMG2 in endothelial cells inhibits proliferation and tubule formation,\textsuperscript{54} and several additional studies have demonstrated the relevance of CMG2 targeting in the inhibition of angiogenesis.\textsuperscript{6, 37, 41, 102}

Substantial data indicate that CMG2 is also crucial for extracellular matrix (ECM) homeostasis. Mutations in CMG2 that result in loss of CMG2 function are responsible for a severe genetic disorder known as hyaline fibromatosis syndrome, or HFS.\textsuperscript{10, 35, 44, 45} HFS results in an aberrant accumulation of hyaline material under the skin and in other organs. A recent report showed that wild-type CMG2 mediated cellular uptake and clearance of type VI collagen (which accumulates in HFS patient nodules), without affecting mRNA levels.\textsuperscript{8}

Here has previously been no functional connection between the role of CMG2 in ECM homeostasis and the role of CMG2 in angiogenic regulation. However, remodeling of the vascular basement membrane (VBM, composed predominantly of Col-IV, laminins, and other glycans) is an essential step in angiogenesis.\textsuperscript{103} Hence, CMG2 may regulate angiogenesis through VBM/ECM remodeling, which may occur in part via uptake of ECM materials, including protein hydrolysis products. This hypothesis is consistent with the observation that CMG2 facilitates internalization of Col-VI and/or Col-VI fragments and with the buildup of Col-VI in HFS patients that lack functional CMG2.\textsuperscript{8}
Individual ECM fragments themselves can impact angiogenesis. Specifically, liberated Col-IV C-terminal NC1 domains are potently anti-angiogenic. These domains appear to act in a negative feedback loop: as angiogenesis proceeds, the VBM remodeling takes place to allow vessel outgrowth, while at the same time newly-generated Col-IV NC1 fragments engage endothelial receptors to suppress further vessel sprouting. Importantly, Col-IV NC1 domains are essential for both proper triple-helix formation (through NC1 domain trimerization) and ECM network formation (through dimerization of adjacent NC1 trimers). While the 6 distinct Col-IV chains can trimerize in 3 different combinations, the most abundant Col-IV isoform, including within the VBM, is composed of two α1 chains and an α2 chain. The NC1 domains of the α1 and α2 chains are the angiogenesis inhibitors arresten and canstatin, respectively.

Aspects of the mechanism(s) by which arresten and canstatin inhibit angiogenesis have been described, including identification of specific integrin receptors and downstream signaling pathways. However, the ultimate fate of these Col-IV NC1 domains, including potential receptor-mediated endocytosis and degradation pathways, has not been outlined. Given both the established interaction between CMG2 and Col-IV and the involvement of CMG2 in both ECM homeostasis and angiogenic processes, peptide fragments of these domains may interact with CMG2 and are taken into cells via interaction with CMG2. If so, this interaction could be in part responsible for the anti-angiogenic effects of CMG2 targeting. Hence, we undertook investigations designed to determine whether possible proteolytic peptide fragments of the NC1 domain could interact with cell-surface CMG2 and thereby influence angiogenesis.

Here, we report and characterize a novel interaction between CMG2 and a peptide fragment of canstatin. This unanticipated observation occurred via analysis of overlapping peptide arrays of the Col-IV α1 and α2 sequences, which identified peptide sequences in the Col-IV NC1
domain as possible CMG2 binding sites. Peptide array hits with the highest binding intensity were synthesized for further analysis and characterization of their anti-angiogenic effects. Significantly, a canstatin-derived 15-mer peptide (denoted as S16) exhibited both high affinity for CMG2 and potent blocking of endothelial cell migration, suggesting that this small peptide can mimic the anti-angiogenic behavior of full-length NC1 domains. CMG2 mediates endocytosis and perinuclear-lysosomal delivery of this peptide fragment, consistent with a role for CMG2 in ECM/VBM fragment clearance. These findings suggest that CMG2 may play a role in the regulation of angiogenesis by Col-IV NC1 fragments and that uptake and degradation of these antiangiogenic VBM fragments could be a functional explanation for the pro-angiogenic behavior of CMG2.

3.3. Material and Methods

3.3.1. Protein preparation

Human CMG2 vWA-GST (CMG2-GST), CMG2 vWA R40c and C175A, and PA were expressed and purified as previously described. Briefly, CMG2 vWA (40-217) was expressed with R40C and C175A mutations (R40C provides an exposed site for maleimide labeling, and C175A removes a buried cysteine to ensure a 1:1 labeling ratio) from a pGEX-4T1 vector. Human CMG2-GST was expressed in BL21 T7 Express E. coli (New England Biolabs) with 0.5 mM IPTG induction in a 5L bioreactor (Sartorius). Cells were lysed via French press and sonication, and CMG2-GST was affinity purified with Glutathione Superflow Agarose (Thermo Fisher). Glutathione was removed, and CMG2 exchanged into HBS-T with 50% glycerol using Sephadex G50 (GE Life Sciences) on an Äkta Start chromatography system.
Briefly, His tagged mCitrine-TEM8, cloned into pQE30, was expressed in BL21 T7 Express E. coli (New England Biolabs) with 1mM IPTG induction in a shake flask with 1L LB supplemented with 10mM glucose. Cells were lysed in 10mM imidazole PBS via French press, centrifuged to obtain a cleared lysate, then purified with a nickel column. The column was washed with eight column volumes of 20mM imidazole PBS, then eluted with 4 column volumes of 250mM imidazole PBS. Eluted protein was concentrated using a 30kDa centrifugal filter unit (Millipore Sigma, Cat: UFC903024), and stored in 50% glycerol PBS at -20°C.

PA-SSSR and PA-E733C were expressed from pET-22b (RRID: Addgene 11079) into the periplasm and purified from the periplasmic lysate via anion exchange chromatography (Q-sepharose, GE Life Sciences Cat: 25236), using 20 mM Tris-HCl pH 8.0 with 20 mM NaCl (Buffer A) and Buffer A + 1 M NaCl. Endotoxin was removed by passing twice through poly-lysine coated cellulose beads (Thermo Fisher Cat: 88275), followed by an endotoxin test using the gel clot method and appropriate dilutions.

MG2-GST (AlexaFluor 488 or biotin) and PA-E733C (AlexaFluor 546) were conjugated to a single reactive cysteine with maleimide-labels (Thermo Fisher) after the GST was cleaved by using thrombin. Label:protein ratio was 5:1 in the reaction mixture. Conjugated protein was then purified from the free label by size exclusion (Sephadex G50). All protein stocks were stored at -80°C in 50% glycerol.

Peptide S16 (PAIAIAVHSQDVISP) was synthesized (Genscript and Biomatik). Peptide U12 (VSIGYLLVKHSQTDQ) and scrambled S16 peptide (SPIAVDVQSAPIHAI) were synthesized (Genscript). S16 conjugated at the N-terminus to HiLyte-488 was also synthesized (Anaspec).
3.3.2. **Membrane-based peptide array with 10 residue sliding window**

An overlapping peptide array (15 residue peptides, with a 5-residue overlap) containing the Col-IV α1 (Uniprot P02462) and α2 (Uniprot P08572) sequences was created by direct synthesis of peptides onto an amino-PEG cellulose membrane (ABIMED peptide array; Koch Institute Biopolymers and Proteomics Facility). The membrane was blocked in TBST with 1% milk, then probed with 250nM CMG2-biotin. Bound CMG2-biotin was detected by avidin-HRP. Spot intensities were visually scored from 0 (no observed binding) to 5 (max binding). Hits were quantified and compared by calculating both a hit ratio and weighted hit ratio for each domain. Hit ratio was calculated by dividing the total number of hits by the total number of peptides in the array for that domain; the weighted hit ratio divided the sum of the intensity scores of all the peptide hits in each domain by the total number of peptides in the array for that domain.

3.3.3. **PEPperPRINT peptide micro-array with 2-residue sliding window**

A peptide array containing the Col-IV α1 and α2 NC1 domains (15 residue peptides with a 13-residue overlap) was printed by PEPperPRINT and probed according to the manufacturer recommendations with 2μM CMG2-GST then anti-GST-DyLight 800 4X PEG (RRID: AB_2537633). The raw TIFF image was analyzed by PepSlide® Analyzer (Sicasys).

From the quantified data and visual inspection, peptide hits flanked by adjacent hits are considered a potential binding surface. Hits with irregularly shaped noise or background were eliminated from further analysis. Epitope mapping molecular graphics were made in the PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC.110
GibbsCluster-2.0\textsuperscript{111,112} was used to perform clustering analysis on array hits. Parameters were systematically varied in order to evaluate the effect of motif length and insertions and deletions on the quality of the alignment. A visual representation of these analyses was generated by Seq2Logo.\textsuperscript{113}

3.3.4. **CMG2-PA FRET assay**

Interaction between CMG2 and peptides were measured using an in vitro FRET assay to detect competition with PA, as described previously.\textsuperscript{41} Briefly, 10 nM each of CMG2-488 and PA-546 were incubated in the presence of peptide of varying concentration, with DMSO as vehicle control. FRET ratio = \( \frac{I_{485/590}}{I_{548/528}} \). FRET ratio with 10 mM EDTA was set to 0.

3.3.5. **TEM8-PA FRET assay**

Interaction between TEM8 and peptide S16 were measured using in vitro FRET assay to detect competition with PA, as described previously.\textsuperscript{38} Briefly, 250nM mCitrine-TEM8 and 325nM PA-546 were incubated in the presence of peptide of varying concentrations, with DMSO as vehicle control. FRET ratio = \( \frac{I_{485/590}}{I_{548/528}} \).

3.3.6. **Bio-layer interferometry**

Bio-layer interferometry was used to characterize the interaction of peptides with CMG2. Assay buffer was 50 mM HEPES, 150 mM NaCl, pH 7.2, 0.1% Tween-20, 1 mg/mL BSA, 2 mM CaCl\textsubscript{2}, 1 mM MgCl\textsubscript{2}, 0.02% NaN\textsubscript{3}. First, streptavidin biosensors (ForteBio) were coated with 5-
10 µg/mL CMG2-GST-biotin overnight at 4°C. Eight sensors were loaded and run in parallel (6 sensors for S16 binding, 2 for reference controls). Binding assays were performed the following day, using an Octet RED96 biolayer interferometer (ForteBio) and the Octet 8.2 Data Acquisition software. Assays were performed at 30°C and 1000 rpm shaking. The CMG2-GST-biotin loaded sensors were equilibrated in assay buffer (1200 sec) followed by an association step with a serial dilution (1-90 µM) of the peptide (300-1200 sec), and dissociation in assay buffer (600-1800 sec). Binding data for S16 concentrations below 1µM were not obtainable, due to the low signal/noise ratio at sub-micromolar concentrations. Data were processed and analyzed in the Octet Data Analysis 8.2 software. Processed data were fit to a 1:1 binding model to obtain kinetic and thermodynamic parameters. Residuals were examined to assess the quality of fit and no systematic deviation was observed.

3.3.7. Cell lines and culturing technique

EOMA (CRL-2586, RRID:CVCL_3507) is a murine hemangioma endothelial cell line. EA.hy926 (CRL-2922, RRID:CVCL_3901) cells are the result of a fusion of human umbilical vein cells with lung carcinoma cells. Both cell lines were cultured in 10% FBS + DMEM and incubated at 37°C in a humidified environment with 5% CO2 until ready for passaging.

3.3.8. Endothelial cell binding assays by flow cytometry

Cells were cultured in well-plates to 30-70% confluence. Cells were incubated in 10% FBS DMEM and treated with 200nM PA-SSSR when indicated. Cells were co-treated with S16-Alexa Fluor 488 conjugate (2µM), transferrin-Alexa Fluor 633 conjugate (10µg/mL ThermoFisher, Cat:
T23362), or Dextran 10,000 MW Texas Red (100µM ThermoFisher, Cat: D1863), and incubated at 37°C for indicated times. When indicated, a well plate with staining media supplemented with 20mM HEPES was left on ice for the indicated time. After incubation, cells were washed, trypsinized, and spun down, then resuspended in cytometry buffer (PBS, 1% BSA, 5 mM glucose) and analyzed in an Attune Acoustic Focusing Cytometer, equipped with 488nm and 633nm lasers. Flow experiments that involved with Texas Red conjugated dextran were performed with the Beckman Coulter CytoFLEX cytometer that equipped with 405nm, 488nm, 561nm, and 638nm lasers. Cytometry data were analyzed in FlowJo.

3.3.9. Confocal microscopy to track ligand endocytosis

Cell preparation and staining for confocal microscopy were as described above for flow cytometry, except that cells were seeded in glass-bottom culture dishes. Staining with S16-488 (2µM) and transferrin-633 (10µg/mL) was performed at 37°C for 1-3 h, washed three times with serum-free media, then incubated with a low fluorescence media supplemented with ProLong live-cell antifade reagent (ThermoFisher Cat: P36975) at 37°C for at least 1 hour. Live-cell images were acquired on a Leica DMi8 confocal microscope, using 488nm and 633nm lasers. Images were acquired with the Leica Application Suite X (LAS X), deconvoluted in Huygens Essential, and further analyzed in LAS X to examine colocalization.

3.3.10. Wound-scratch migration assay

EOMA cells were seeded in 96 well plate and cultured in 10% FBS DMEM. Cells were incubated at 37°C in a humidified environment with 5% CO₂ until completely confluent. A 200 µL pipette
tip was used to scratch a wound in the monolayer, followed by three washes with PBS. Cells were then treated with 10% FBS DMEM with or without S16, and serum-free DMEM as negative control. Images were acquired every four hours, and the change in wound area was quantified using ImageJ.

3.3.11. Cell proliferation assay

15,000 EOMA cells were seeded into each well in a 96 well plate and incubated for 1 h to attach. After cell attachment, media with treatments were added. Ethanol-fixed cells were used as negative control. After a 24 h incubation, 20µL CellTiter-Blue Reagent (Promega Cat: G8080) was added to each well for 4h. Fluorescence signal (Ex: 560nm / Em: 590nm) was measured using a BioTek Synergy H2 plate reader. All readings were normalized to the non-treated control.

3.3.12. CellASIC migration assay

The assay protocol followed the CellASIC ONIX M04G-02 Microfluidic Gradient Plate User Guide. All media put into the plate (except the cell suspension) was filtered through a 0.2 µm syringe filter. EA.hy926 cells (3 x 10^6 cells/mL) were loaded in and incubated overnight with DMEM + 10% FBS under flow at 37°C. Assays were then performed with a stable gradient of DMEM + 0 – 10% FBS with or without peptide treatment. Brightfield images at 10x magnification were taken every 10 min over 12 h on an Olympus IX73 microscope and the ORCA-Flash 4.0 camera (Hamamatsu). Individual cells were tracked with ImageJ (RRID: SCR003070) manual tracking plugin. Data was transferred to the ibidi Chemotaxis and Migration Tools 2.0 to export
endpoint ‘y’ displacement and accumulated displacement. P-values were calculated using Student’s t-test, and error bars are standard error of the mean (n=40).

3.4. Result and Discussion

3.4.1. CMG2 interacts with peptides within the anti-angiogenic NC1 domains of Col-IV as determined by peptide array

CMG2 has emerged as an important regulator of angiogenesis, but there is still little insight into its mechanism. Given reports that CMG2 binds matrix proteins, including Col-IV, and that the Col-IV fragments have anti-angiogenic properties, we investigated the interaction of the CMG2 vWA domain with Col-IV peptide sequences to shed light on the possible relevance of this interaction for angiogenic regulation. To identify peptide sequences on Col-IV that can interact with CMG2, we constructed a scouting peptide array in which the linear peptide sequences of human Col-IV α1 and α2 chains were directly synthesized onto a nitrocellulose membrane. The array consisted of 15-mer peptide units, with a 10-residue shift between subsequent peptides, scanning the entire length of the α1 and α2 chains of Col-IV. The array was probed for CMG2 interaction with 250 nM CMG2-biotin and read out with avidin-HRP; spots representing binding of CMG2 to individual peptide sequences were scored with intensities assigned from 0 (min) to 5 (max) (Fig 1a-b, SI Figure 1). When these intensity values were plotted against the complete protein sequence in the Col-IV α1 and α2 chains, we observed sparse CMG2 binding throughout the Col-IV 7S and triple-helical regions, but heavily concentrated binding within the Col-IV NC1 domains (Fig 1, Table 1). We further quantified this binding by calculating both a hit ratio and an intensity-weighted hit ratio for each domain. When the peptide array binding
data were evaluated this way, we observed that arresten and canstatin exhibited much higher hit ratios and intensity-weighted hit ratios than their corresponding triple-helical or 7S domains (Table 1). Specifically, hit ratios and intensity-weighted hit ratios for arrestin were 14-fold and 3-fold higher, respectively, than the triple-helical domain and 25-fold and 6-fold, respectively, higher than the 7S domain. These observations indicated that CMG2 preferentially binds the NC1 domain-derived sequences on the peptide array.

Recognition of Col-IV NC1-derived peptides by CMG2 suggests the possibility that CMG2 could interact with one or more of these peptides in the intact NC1 domain. To investigate this possibility, we mapped CMG2-binding peptides onto the NC1 domain. While the initial peptide array strongly indicated that CMG2 binding is enriched in canstatin and arresten versus the rest of the Col-IV chains, the large sliding window for different peptides in this array made the individual CMG2-binding residues within each peptide hit challenging to identify. To identify the individual peptides in the NC1 domain that could be binding CMG2, we synthesized an additional peptide array on a glass chip, with the NC1 domains arrayed as 15-mer peptides in a 2-residue sliding window (SI Figure 2). The goal was to use hits from the overlapping peptides to confirm the identity and sequence of CMG2-binding peptides in the NC1 domain. We defined a putative peptide hit as 3 or more consecutive peptides with binding to CMG2 and mapped identified CMG2-binding sequences onto a previously solved structure of the Col-IV NC1 domain hexamer (Fig 1c, PDB: 1LI1). Notably, both canstatin and arresten fragment peptides identified as putative CMG2 interactors are localized on the accessible surface of the NC1 domain (Figure 1c, left), and therefore could be recognized by CMG2. Peptide fragments from arresten were also identified on the solvent-exposed surface (Fig 1c, right) although the CMG2-binding surface of arresten was sparser than for canstatin, consistent with its lower hit rate. Importantly, identification
of individual CMG2 binding peptide fragments of arresten and canstatin, combined with their localization on the solvent-accessible surface of the NC1 domain, suggests that CMG2 could interact with intact NC1 domains via these peptide sequences.

CMG2 has high structural homology to many integrins,\textsuperscript{65} whose ligands commonly have an established RGD motif.\textsuperscript{116} Hence, we evaluated the 2-residue sliding window peptide array data to identify a possible consistent CMG2-interacting motif. Alignment of all hits from this array produced a consistent binding element distinct from the RGD motif of integrins that contained an aliphatic residue at the n\textsuperscript{th} position, followed by an acidic residue at the (n+2)\textsuperscript{th} position (SI Figure 3). The presence of acidic residues in this motif suggests that, like PA, CMG2 peptide ligands could participate in binding the divalent cation in the CMG2 MIDAS domain.\textsuperscript{117}

\textbf{3.4.2. Canstatin-derived peptide S16 binds with high affinity to CMG2 via the MIDAS}

To further understand the relevance of CMG2 interaction with NC1-derived peptides, we subjected the top NC1 hits from the initial peptide array for further evaluation. Peptides from the NC1 region showing the maximum binding score of 5 were selected and characterized using two \textit{in vitro} assays: 1) a PA-CMG2 competition FRET assay,\textsuperscript{41} and 2) bio-layer interferometry (BLI) kinetic binding assay. Of the peptides tested, two showed activity in both assays at the concentrations tested. These peptides are both from canstatin and are denoted here as S16 and U12. Both U12 and S16 bind CMG2 and compete with PA for binding to CMG2 (Figure 2, SI Figure 4). Peptide U12 showed a weak affinity for CMG2, both by BLI (K\textsubscript{d} = 80 µM) and the FRET competition assay with PA-AF546 conjugate (IC\textsubscript{50} = 35 µM; SI Figure 4). Peptide S16 bound CMG2 with much higher affinity. Analysis by BLI revealed that S16 binds CMG2 with sub-
micromolar affinity: $K_d = 0.4 \pm 0.2 \, \mu\text{M}$ (Fig 2b, mean $\pm$ SD, n = 3 independent experiments). This interaction was characterized by a relatively slow on-rate ($\approx 2 \times 10^2 \, \text{M}^{-1}\text{s}^{-1}$) and off-rate ($\approx 1 \times 10^{-4} \, \text{s}^{-1}$). Further discussion on the observed kinetics is provided in SI Note 1. Binding of CMG2 by S16 was corroborated by FRET competition with PA, in which the S16 IC$_{50}$ was 2.7 $\mu\text{M}$ (Fig 2a, 95% CI: 1.9-3.7), which corresponds to a $K_d$ of 100 $\pm$ 55 nM, in rough agreement with that measured by BLI.

To confirm the specificity of S16 for CMG2, we synthesized a scrambled S16 peptide comprised of the same residues as S16 but in random order, and probed for the interaction of this scrambled peptide with CMG2 via BLI. Unlike S16, the scrambled version of S16 peptide showed no clear binding to CMG2, even at concentrations up to 30 $\mu$M (SI Figure 5). Additionally, the scrambled S16 binding data fit poorly to all available binding models tested (1:1, 1:2, and 2:1 bivalent models). These studies are consistent with a sequence-specific interaction between S16 and CMG2.

The ability of S16 to compete with PA for CMG2 binding, as identified in the FRET competition assay (Fig 2a), suggests that S16 interacts with the ligand-binding surface of the CMG2 vWA domain at the MIDAS, as does PA. BLI experiments were used to verify that S16 binds in competition with PA. Specifically, when CMG2-loaded biosensors were treated with PA before exposure to S16, CMG2 binding to S16 was reduced (Fig 2c). As further support of MIDAS interaction, EDTA prevents S16 binding to CMG2 (Fig 2d), confirming that the interaction is metal ion ($\text{Ca}^{2+}$ or $\text{Mg}^{2+}$) dependent. Indeed, S16 contains an aspartate (preceded by several aliphatic residues) that could participate in metal coordination.

Since CMG2 shares high sequence and structural homology with its competing ANTXR, TEM8$^{22, 65}$ and both bind PA (albeit with dramatically differing affinities), we asked whether S16
could also bind TEM8. FRET showed that S16 is unable to compete with PA for TEM8 binding, indicating that S16 is not recognized or bound by TEM8 (SI Figure 6). The conclusion that this Col-IV peptide fragment interacts with CMG2 rather than TEM8 is perhaps not surprising; while CMG2 has been reported to bind Col-IV, there is no published evidence for interaction of TEM8 with Col-IV.

These multiple independent assays demonstrate a high-affinity specific interaction of S16 with CMG2 (~ 400 nM Kd). The interaction appears to involve the MIDAS divalent cation since binding disappears in the presence of EDTA, consistent with a mode of binding similar to the CMG2-PA interaction. Considering the established role of CMG2 in angiogenesis and the anti-angiogenic activity of anthrax toxin PA and the Col-IV NC1 canstatin, we asked whether this small fragment of canstatin (S16) could mimic PA and inhibit angiogenic processes in endothelial cells.

3.4.3. Peptide S16 inhibits endothelial cell migration, but not proliferation

Proliferation and migration of endothelial cells are essential to angiogenesis. We examined the impact of S16 on both proliferation and migration of EOMA cells. Previous work has shown that binding of individual ligands to CMG2, including PA and small molecules, inhibits migration but not the proliferation of microvascular endothelial cells. Like PA, S16 did not impact endothelial cell proliferation in EOMA cells (Fig 3a). However, in a standard wound scratch assay with EOMA cells, S16 exhibited potent inhibition of endothelial cell migration compared with the untreated, full-serum control (Fig 3b). Indeed, the migration of S16-treated EOMA cells was statistically indistinguishable from the migration of EOMA cells without serum, indicating that
S16 potently reduces cell migration in this cell type. Given that S16 competes with PA for CMG2 binding, and PA is known to inhibit cell migration and other angiogenic processes, the observation that S16 also inhibits migration suggests that S16 exerts its angiogenic impact via interaction with CMG2.

To further characterize the anti-migratory effect of S16 on endothelial cells, we performed additional assays in a microfluidic platform that allows for tracking of individual cell migration within a serum gradient. Real-time tracking of human EA.hy926 cells demonstrated strong migration in the absence of S16 (Fig 3c). In contrast, when S16 was present in the cell medium, migration toward serum was reduced by ~90% (Fig 3c); migration was similarly inhibited in medium containing 200pM PA-SSSR (Figure 3d). In these experiments, we chose PA-SSSR rather than PA-WT because it has been demonstrated to be a better antagonist of angiogenesis than PA-WT and would, therefore, be expected to have a more significant impact on migration. To confirm that the loss of EA.hy926 cell migration results specifically from S16 interaction with CMG2, we repeated the microfluidic gradient migration assay using the same scrambled peptide generated as a CMG2-binding control. We observed no difference in migration between scrambled-peptide treated cells and the vehicle control (SI Figure 7), a result demonstrating that the anti-migratory effect depends on sequence-specific interaction with CMG2. Notably, S16 does not bind TEM8, and the anti-migratory effect observed in these studies depends on the interaction of S16 with CMG2.

3.4.4. CMG2 mediates endocytosis of S16 in endothelial cells
Having determined that S16 binds to CMG2 with high specificity and affinity (Fig. 2) and that S16 inhibits endothelial cell migration (Fig. 3), we sought to confirm that, like other CMG2 ligands, S16 is internalized following receptor binding. We quantified internalized S16 via flow cytometry analysis of EA.hy926 cells treated with a fluorescently conjugated S16 construct (S16-488) and compared the S16 signal to that of Transferrin-633 (Tf-633), a commonly used endocytic marker. We first compared S16-488 and Tf-633 fluorescence in EA.hy926 cells at 37°C to that of cells treated on ice, a condition that inhibits all endocytic pathways, including both receptor-mediated uptake and (nonspecific) fluid-phase pinocytosis. The robust S16-488 and Tf-633 flow cytometry signal exhibited at 37°C was completely eliminated by incubation on ice (Fig 4a). Hence, S16, like transferrin, is endocytosed by these endothelial cells.

To confirm S16 endocytosis requires interactions with CMG2, we compared S16 internalization by EA.hy926 cells in the presence and absence of 200 nM PA-SSSR. As shown in Figure 4b, S16-488 showed a time-dependent interaction with EA.hy926 cells that was drastically reduced (~60%) by co-incubation with PA-SSSR at concentrations high enough to compete with S16 for binding to cell-surface CMG2. In contrast, the binding of Tf-633 was unaffected by the presence of PA-SSSR (Fig 4c). These data show that S16 and PA-SSSR share a cell-surface receptor. Since our *in vitro* FRET analysis indicates that S16 does not compete with PA for TEM8 binding (SI Figure 6), TEM8 cannot be the primary receptor responsible for S16 endocytosis.

To further validate that CMG2 mediates S16 endocytosis, we have developed a CMG2 knockout EA.hy926 cell line (CMG2<sup>−/−</sup>, SI Figure 8) and evaluated its ability to internalize S16. CMG2<sup>−/−</sup> cells display significantly less S16-488 signal than WT EA.hy926 cells (50%; p<0.05; Figure 4d; SI Figure 9). This reduction is statistically indistinguishable from the reduction in S16-488 fluorescence observed in the presence of CMG2-saturating PA-SSSR concentrations that
completely block CMG2-S16 interactions. Hence, we conclude that CMG2 mediates endocytosis of S16.

To gain insight into possible endocytic mechanisms involving CMG2, we used confocal microscopy to visualize the cellular uptake of S16-488 and Tf-633. We observed clear punctate intracellular colocalization of S16-488 and Tf-633 (Fig 4e, SI Movie 1A-D) in vesicles visible throughout the cell and concentrated around the nucleus in a pattern consistent with perinuclear lysosomes. Tf-633 is taken into cells via receptor-mediated clathrin-dependent endocytosis and can either be recycled through recycling endosomes or delivered to lysosomes. Colocalization of S16 and transferrin in the same vesicular structures indicates that S16-488 is likely taken into cells via clathrin-dependent endocytic uptake, like transferrin, and therefore delivered to the same endosomes as transferrin. This mechanism of S16 endocytosis is consistent with the CMG2-mediated PA uptake, which also occurs via clathrin.\textsuperscript{10}

We note that residual S16 uptake is significant in the presence of both a PA-SSSR competitor and in CMG2–/– cells. Because these uptake assays were performed with a high concentration of S16 (2µM), pinocytosis likely contributes this background signal. To evaluate levels of non-CMG2-mediated uptake, we performed additional assays with Texas-red conjugated dextran. Both WT EA.hy926 and CMG2 knockout cells showed significant and identical uptake of fluorescent dextran (SI Figure 10) indicating that pinocytosis is significant in this cell type and could account for the residual uptake of S16 in CMG2 knockout cells.
3.5. Conclusion

Our BLI, flow cytometry, and confocal microscopy data show that CMG2 binds and mediates the internalization of the peptide S16. Endothelial cells participate in ECM remodeling during angiogenesis and are known to induce proteolytic cleavage of ECM materials and the resulting release of ECM peptides. Such peptide cleavage products are then available for interaction with endothelial cell surface receptors, including CMG2. Indeed, many of these interactions and their subsequent signaling effects have been documented previously. While there is no evidence that S16 itself is a physiological ligand of CMG2, peptides containing relevant portions of the S16 primary amino acid sequence could, like other peptide proteolytic products, be available for binding and subsequent endocytosis by CMG2 during the process of ECM remodeling. A role for CMG2 in ECM homeostasis and uptake of ECM molecules and/or fragments has been previously proposed, particularly in the context of Col-VI. Data presented here show that this CMG2-mediated process is not unique to Col-VI or Col-VI fragments, and may be a consistent cellular response to other ECM materials and/or peptides that also bind CMG2. CMG2-mediated endocytosis of a CMG2-binding molecule, as observed here, is consistent with the established role of CMG2 in mediating clathrin-dependent endocytosis of the anthrax toxin. Indeed, co-localization of intracellular S16 with transferrin, as observed here, suggests that endocytosis of ECM-derived peptides like S16 could utilize the same mechanism as endocytosis of anthrax toxin.

Together, the findings included here suggest that CMG2 could bind and internalize Col-IV NC1 peptide fragments, as is observed for S16, resulting in their eventual lysosomal degradation. This process provides one potential functional explanation for the anti-angiogenic effects of CMG2 targeting. CMG2 interaction with, and endocytosis of, anti-angiogenic peptide fragments and/or protein domains such as arresten and canstatin would be expected to reduce their local concentrations, and thus, their anti-angiogenic impact. CMG2-binding molecules such as PA, S16, and small molecules may thus inhibit CMG2-mediated endocytosis of anti-angiogenic fragments and therefore magnify their anti-angiogenic impact. More work is needed to examine the mechanistic function of CMG2 in cell migration and to identify
possible additional molecules involved in this process, which could be additional therapeutic targets for the modulation of angiogenesis.
3.6. Acknowledgement

The plasmid pGEX4T1-CMG2-GST was a generous gift from the John Collier lab. This work has been made possible in part by a grant from the Simmons Research Endowment at Brigham Young University.

3.7. Figures and Tables

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‡ Tukeys post-hoc – 7S vs Triple-helical: p = 0.9394, NC1 vs 7S: p < 0.0001, NC1 vs Triple-helical: p < 0.0001

‡‡ Tukeys post-hoc – 7S vs Triple-helical: p = 0.9827, NC1 vs 7S: p = 0.0009, NC1 vs Triple-helical: p < 0.0001

Figure 3-1. Binding profile quantification for CMG2 interaction with Col-IV-derived peptides. Hit ratio was calculated as # hits (intensity > 0) divided by the total number of peptides in the domain. Intensity-weighted hit ratio was calculated as the total measured intensity of domain divided by the total number of peptides in the domain.
Figure 3-2. Peptide arrays identify CMG2 binding to Col-IV NC1 domains. (a-b) A peptide array of 15-mer peptides with a 10-residue sliding window, covering the sequences of Col-IVα1 (a) and Col-IVα2 (b). This array was probed with 250 nM CMG2-biotin and read out using avidin-HRP. Spot intensities were scored from 0 (no observed binding) to 5 (maximal binding). Domain topology is shown below. There is a greater proportion of CMG2-binding peptides within the c-terminal NC1 domains than the collagenous and 7S domains. (c) PEPperPRINT peptide array with a 2-residue sliding window identifies a putative CMG2-binding surface. NC1 hexamer shown (PDB 1LI1) consists of two trimers, each composed of two arresten (Col-IVα1) and one canstatin (Col-IVα2). Arresten is shown in orange; canstatin in green. Binding sequences are shown in darker shades. Left shows canstatin-exposed face, demonstrating a large, uniform epitope for CMG2 to canstatin. Right displays arresten-exposed face, where CMG2-binding peptides are present, but are more sparse.
Figure 3-3. Peptide S16 binds to the CMG2 MIDAS with sub-micromolar affinity. (a) CMG2-488/PA-546 FRET inhibition assay confirms S16 binding to CMG2. IC50 shown with 95% CI. (b) A representative experiment of S16 (concentration indicated) binding to CMG2-GST-biotin loaded BLI sensors. Kd shown is mean ± std (3 independent experiments). (c-d) Peptide S16 interacts with the MIDAS domain of CMG2. (c) CMG2-GST-biotin loaded BLI sensors equilibrated in buffer with or without 500 nM PA, followed by incubation in 10 µM S16 (n=3 per condition). (d) CMG2-GST-biotin loaded BLI sensors were equilibrated either in buffer with 2 mM CaCl2 and 1 mM MgCl2, or 10 mM EDTA, followed by incubation with 10 µM S16 (n=3 per condition). Error bars are the standard deviation of replicates.
Figure 3-4. Peptide S16 abolishes the migration of endothelial cells, with no effect on proliferation. (a) The proliferation of EOMA cells was monitored. Treatment with S16 resulted in no change in proliferation relative to the untreated control. The negative control cells were fixed in ethanol. Error bars are SD of n=7 replicates from two independent experiments. (b) Wound scratch migration assay. S16 results in 60% reduction in wound closure and is not statistically distinguishable from the serum-free (negative) control. Error bars are SD of n=3 replicates. (c-d) Microfluidic serum gradient migration assay with EA.hy926 cells. Data show that cells were migrating towards serum in the vehicle control, but a significant loss in migration towards serum with S16 treatment (c) or 200 pM PA-SSSR (d). Error bars are SEM of n=40 cells for each condition. **, p<0.01, ***, p < 0.001; ****, p < 0.0001; ns, not significant.
Figure 3-5. CMG2 is a relevant cell surface receptor for S16 and mediates S16 internalization
(a) Cold treatment abolishes the signal of both S16-488 and transferrin-633, indicating that, as transferrin, S16 is being internalized. Conditioned EA.hy926 cells were incubated with 2 µM S16-488 and 10 ug/mL transferrin-633 in DMEM (with 20 mM HEPES and 1% FBS) for 6 hrs, either at 37C or on ice. Cells were harvested and analyzed by flow cytometry. The plot shows the mean fluorescence intensity of four replicates. (b-c) Flow cytometry analysis on time course of conditioned EA.hy926 cells treated with S16-488 (b) and transferrin-633 (c) with or without 200 nM PA-SSSR. Treatment with PA-SSSR specifically reduces the binding of S16-488 to endothelial cells but does not reduce the binding of transferrin-633, demonstrating that at least 60% of S16 cell-binding occurs through the anthrax toxin receptors. MFI, mean fluorescence intensity. n=6 from two independent experiments. (d) Flow cytometry analysis on EA.hy926 WT and CMG2−/− cells treated with S16-488. The depletion of CMG2 significantly reduced S16 binding to S16 in a similar manner as CMG2 inhibition by PA-SSSR. (e) Conditioned EA.hy926 were incubated with 2 µM S16-488 and 10 ug/mL Tf-633 at 37C for 3 hours, then incubated in imaging buffer (SDM79
with 7.5 mM glucose and ProLong™ antifade) for 1 hr at 37°C, then imaged by confocal microscopy. Representative images of two individual cells (top and bottom) are shown. Scale bar is 10 µM. Consistent colocalization between S16 and Tf in endosomes and apparent perinuclear lysosomes was observed. These findings were consistent across n=20 cells from 2 independent experiments; in every cell, endosomal and lysosomal colocalization was also observed. ****, p < 0.0001; ns, not significant.
Supplementary Figure 3-1. Membrane-based peptide array for CMG2 binding. 15-mer peptides in a 10aa sliding window were synthesized directly coupled to a nitrocellulose membrane. This membrane was then probed with 250 nM CMG2-biotin and read out using avidin-HRP. Left, low exposure image. Right, high exposure image. Proteins arrayed include: PA (blue), human Col-IV α1 (orange), human Col-IV α2 (green), and human fibronectin (black). Circled peptides include S16 (green) and U12 (red). Peptide in yellow circle is from triple-helical region of Col-IV α2, and thus was not further characterized here. Binding evident in PA-array is not surprising. Binding evident in fibronectin-array is currently being further validated and investigated.
Supplementary Figure 3-2. PEPperPRINT peptide array for CMG2 binding. 15-mer peptides in a 2aa sliding window were synthesized with N- and C-terminal flanking linkers (GSGSGSG) in duplicate and coupled to a polyethylene-based graft copolymer. This array was then probed with 500 nM CMG2-GST-biotin and read out using an anti-GST-Dylight-800 conjugate. Top left, raw array image. Bottom left, heat map used to determine binding. Sequences including portions of S16 are circled in red. Proteins arrayed include: human Col-IV α1, human Col-IV α2, human laminin α1, α2 and α3, and selected epitopes from PA. Though S16-representing peptides did not exhibit high intensity compared to some other peptides assayed in this array, a clear binding pattern was observed in the S16 epitope (right, exposed amino acids represented in red). Binding evident in other proteins assayed is under current investigation.
Supplementary Figure 3-3. Identification of a potential CMG2-binding motif using PEPperPRINT array. Top hits (65 peptides, false hits identified by visual inspection and removed) from array were pooled and subjected to clustering analysis with GibbsCluster-2.0. Top, 5-residue motif, with no insertions or deletions allowed. Bottom, 7-residue motif with up to 3 insertions and deletions allowed. Consistent motif consists of an enriched acidic position, preceded two positions by a hydrophobic/aliphatic residue.
Supplementary Figure 3-4. FRET and BLI demonstrate comparatively weak interaction of U12 with CMG2. (top) PA-CMG2 competition FRET assay demonstrates an IC50 ≈ 35 µM for U12, over an order of magnitude weaker than S16. (bottom) Representative BLI sensorgram and fit for U12 interacting with CMG2-loaded SA sensors. Legend shows U12 concentration in µM. Baseline drift due to extremely low signal makes concentrations for <30 µM not usable. Parameters for this fit are: $K_d = 83 \pm 6 \mu M$, $k_{on} = 150 \pm 10$ (Ms)$^{-1}$, $k_{off} = 0.0125 \pm 0.0002$ s$^{-1}$. While the fit quality is poor, it is clear from replicates that $K_d > 30 \mu M$, in agreement with the FRET IC50.30 µM, in agreement with the FRET IC50.
Supplementary Figure 3-5. BLI analysis of CMG2–scrambled S16 interaction. A scrambled peptide, made by synthesizing a randomly shuffled S16 sequence, was tested on BLI for binding to CMG2. Assay conditions used were identical to those tested with S16–CMG2. Binding was compared across 3 independent experiments at 30 µM scrambled S16. In all experiments, the magnitude of the scrambled peptide signal was found to be minimal compared to that of S16, and data did not fit any binding models (shown above). Additionally, we note that scrambled S16 association and dissociation was assayed on a longer timescale (1200s) than the S16 assays. Any drift observed between samples is likely due to nonspecific, hydrophobic interactions of scrambled S16 with the sensor. These data provide evidence that the S16–CMG2 interaction is sequence-specific.
Supplementary Figure 3-6. S16 does not interfere with TEM8-PA binding. (Left) Inhibition of TEM8-GFP and PA-546 FRET was negligible at S16 concentrations between 313 nM and 40 μM, differing from that of CMG2–PA as shown in Figure 2. TEM8-GFP and PA-546 mixed with DMSO vehicle only was used as normalization control. (Right) Activity of TEM8-GFP used in FRET inhibition assay was validated by measuring quenching by PA-QSY9. Measured K_D was approximately 100 nM (116 ± 111 nM), and matches the published affinity for TEM8–PA.\textsuperscript{38}
Supplementary Figure 3-7. EA.hy926 cells in the microfluidic serum gradient migration assay with the scrambled peptide. The scrambled peptide is composed of the same amino acids as S16, but in a randomized sequence. Quantified directional migration of EA.hy926 cell migration in 0.3% DMSO (vehicle) and 10µM scrambled peptide had no significant difference. This data indicates that unlike S16, the scrambled S16 has no effect on endothelial cell migration towards serum.
Supplementary Figure 3-8. EA.hy926 CMG2 −/− characterization via PA uptake assay.
CMG2 knockout in EA.hy926 cells was confirmed by PA uptake assays. EA.hy926 WT and CMG2 −/− cells were treated with 200 pM of PA-WT-Cy5 conjugate, and the PA signal was measured via flow cytometry (Top). As observed above, the CMG2−/− peak (green) is shifted to the left when compared to the WT peak (red). WT cells treated with PA on ice (blue) was also used as a control to compare with WT cells with PA at 37°C (Bottom). Endocytosis is expected to be stalled when cells are placed on ice. Data show that the peak position of CMG2−/− and WT cells on ice are very similar, indicating that CMG2−/− does not uptake PA at the concentration that only targets CMG2.
Supplementary Figure 3-9. CMG2 −/− cells have less S16 uptake than WT cells. S16-488 uptake in both EA.hy926 WT and CMG2−/− cells was measured in a flow cytometer. The two cell lines were pulsed with 2 µM S16-488 conjugate on ice for 1 hour, then chased at 37°C with 5% CO2 for 4 hours then analyzed by flow cytometry. Data show that the peaks of WT and CMG2−/− unstained cells (green and red) overlay onto each other, in the AF-488 channel. While S16 treated CMG2−/− (blue) and WT (orange) cells showed a shift when compare to their unstained controls. WT cell with S16 shift was further away, suggesting that the CMG2−/− cell had a reduced S16 uptake.
Supplementary Figure 3-10. Fluid phase uptake contributes to the non-specific uptake of S16. Dextran uptake in both EA.hy926 WT and CMG2−/− cells. The two cell lines were pulsed with 100 µM Dextran Texas Red conjugate on ice for 1 hour, then chased at 37°C with 5% CO2 for 4 hours. Texas Red signal was measured by flow cytometry. The data show that the peak shift in the Texas Red channel was similar between WT and CMG2−/− cells (Top left). Quantified data shows the mean fluorescent intensity difference between the two cell lines was insignificant (Top right). Therefore, CMG2−/− does not significantly affect rates of endocytosis in the cell and decreases in S16 uptake are via some other mechanism, most likely via abolished S16–CMG2 interactions. Additionally, these data show that non-specific uptake of material surrounding the cells is independent from CMG2-mediated endocytosis and can contribute to nonspecific S16 uptake.
4. Capillary Morphogenesis Gene 2 Mediates Multiple Pathways of Growth Factor-Induced Angiogenesis by Regulating Endothelial Cell Chemotaxis

4.1. Abstract

Pathological angiogenesis contributes to diseases as varied as cancer and corneal neovascularization. The vascular endothelial growth factor (VEGF) - VEGF receptor 2 (KDR/VEGFR2) axis has been the major target for treating pathological angiogenesis. However, VEGF-targeted therapies exhibit reduced efficacy over time, indicating that new therapeutic strategies are needed. Therefore, identifying new targets that mediate angiogenesis is of great importance. Here, we report that one of the anthrax toxin receptors, capillary morphogenesis gene 2 (ANTXR2/CMG2), plays an important role in mediating angiogenesis induced by bFGF, VEGF, and PDGF. Inhibiting physiological ligand binding to CMG2 results in a significant reduction of corneal neovascularization, endothelial tube formation, and cell migration. We also report the novel finding that CMG2 mediates angiogenesis by regulating endothelial chemotactic migration without a strong effect on overall cell motility.

4.2. Introduction

Pathological angiogenesis plays a role in many diseases, including cancers and various ocular diseases that lead to blindness. Most of the current treatment strategies are targeting the VEGF - VEGFR2 axis. However, such therapies only provide modest long-term efficacy and are accompanied by undesirable side effects. Thus, identification of new anti-angiogenic targets is needed and critical for the development of alternative therapeutic strategies. It was previously demonstrated that one of the anthrax toxin receptors, CMG2, has a role in angiogenesis. Our
previous work demonstrated that molecules binding to CMG2 potently reduce angiogenesis in the cornea and inhibits endothelial cell migration. Thus, we were interested in investigating the role(s) of CMG2 in angiogenesis.

While knowledge of CMG2’s exact role in angiogenesis remains limited, CMG2’s function as one of anthrax toxin receptors is very well established. CMG2 and its homolog, tumor endothelial marker 8 (ANTXR1 / TEM8), are the primary facilitators of anthrax toxin entry into cells. CMG2 and TEM8 share 40% amino acid homology, including high homology within an intracellular domain that is not shared with other proteins in the mammalian genome. Within the cell-surface von Willebrand Factor A (VWA) ligand-binding domains, homology between CMG2 and TEM8 rises to 60%. During anthrax intoxication, the 83 kDa protective antigen (PA), one of three toxin subunits from *Bacillus anthracis*, binds to the VWA ligand-binding domain of the receptor. PA is then cleaved by a furin-like protease that cuts and releases a 20 kDa fragment, leaving a 63 kDa PA remnant at the receptor surface. The proteolyzed PA then oligomerizes to form a PA-CMG2 heptamer, which acts as a binding platform for the other two toxin subunits, lethal factor (LF) and edema factor (EF). Together, these toxin subunits form the complete anthrax toxin, which is then trafficked into the cell via clathrin-mediated endocytosis.

The endogenous functions of the anthrax toxin receptors are still poorly understood; although, it has been suggested that these receptors interact with extra-cellular matrix (ECM) proteins. In particular, a series of studies have observed that mutations on CMG2 or TEM8 lead to a build-up of hyaline materials that result in an alteration of skeletal growth and/or changes in vascular patterns. For example, loss-of-function mutations in TEM8 cause GAPO syndrome, a disease characterized by vascular anomalies, skeletal defects, growth retardation, and progressive fibrosis of various organs. Loss-of-function mutations in human CMG2 causes Hyaline
Fibromatosis Syndrome (HFS), a rare but serious autosomal recessive disorder that is characterized by the accumulation of hyaline material in connective tissue in the skin and other organs and by the presence of non-cancerous nodules\textsuperscript{10, 125, 126} containing excess collagen I, collagen VI, and glycosaminoglycans. It has been hypothesized that HFS patients with a CMG2 mutation have a defect in either the synthesis or degradation of ECM,\textsuperscript{126, 127} presumably related to CMG2 dysfunction.

In addition to ECM binding and trafficking anthrax toxins, it has been suggested that ANTXRs have angiogenic related functions.\textsuperscript{4, 37} Our previous work demonstrated that a furin-cleavage resistant mutant of protective antigen (PA\textsuperscript{SSSR}) inhibits basic fibroblast growth factor (bFGF)-induced corneal neovascularization, VEGF-induced corneal neovascularization, and tumor growth.\textsuperscript{4, 11} However, because PA\textsuperscript{SSSR} is known to bind both anthrax toxin receptors (CMG2 and TEM8) as well as integrin β1,\textsuperscript{23, 66} which of these receptors was responsible for mediating the observed anti-angiogenic effect of PA\textsuperscript{SSSR} \textit{in vivo} was not firmly established. CMG2 has a much higher affinity for PA than the other two receptors,\textsuperscript{66, 101, 128} and has been considered the major receptor for PA.\textsuperscript{36} Thus, we hypothesized that CMG2 was the likely receptor responsible for the anti-angiogenic effects of PA\textsuperscript{SSSR}. Work described here outlines experiments that firmly establish CMG2 as the receptor mediating the observed anti-angiogenic impact of PA\textsuperscript{SSSR} in the cornea and provide insight into the role of CMG2 in mediating angiogenesis in endothelial cells.
4.3. Methods

4.3.1. Protein preparation

PA$^{SSR}$ and PA$^{SSR \ E73C}$ were expressed using the pET-22b (RRID: Addgene 11079) expression system resulting in recombinant protein expression in the periplasm. Both proteins were purified from a periplasmic lysate via anion exchange chromatography (Q-sepharose, GE Life Sciences Cat: 25236), using 20 mM Tris-HCl pH 8.0 with 20 mM NaCl (Buffer A) and Buffer A + 1 M NaCl. Endotoxin was removed by passing twice through poly-lysine coated cellulose beads (Thermo Fisher Cat: 88275) followed by an endotoxin test using a gel clot assay.

4.3.2. Cell culture

EA.hy926 (ATCC, CRL-2922) cells are the result of a fusion of human umbilical vein cells with A549 lung carcinoma cells. These cells were cultured in 10% FBS + DMEM and incubated at 37°C in a humidified environment with 5% CO$_2$ until ready for passaging.

4.3.3. EA.hy926 cell proliferation assay

EA.hy926 cells were seeded at 15,000 cells per well in a 96 well plate and incubated at 37°C for 1 h to attach. After cell attachment, media with various treatments were added to each well. Ethanol fixed cells were used as negative control. After a 24 h incubation, 20 µL CellTiter-Blue Reagent (Promega) was added to each well and incubated again for 4 h. The fluorescence signal (Ex: 560nm / Em: 590nm) was measured using a BioTek Synergy H2 plate reader. All readings were normalized to the non-treated control.
4.3.4. *Microfluidic gradient migration assay*

The assay protocol followed the CellASIC ONIX M04G-02 Microfluidic Gradient Plate User Guide. All media put into the plate (excepting the cell suspension) was filtered through a 0.2 µm syringe filter. EA.hy926 cells (3 x 10⁶ cells/mL) were loaded in and incubated overnight with DMEM + 10% FBS underflow at 37°C. Assays were then performed with a stable gradient of DMEM + 0 – 10% FBS with or without treatment. Brightfield images at 10x magnification were taken every 10 min over 12 h on an Olympus IX73 microscope and the ORCA-Flash4.0 camera (Hamamatsu). Individual cells were tracked with the Image J manual tracking plugin. Data were transferred into the ibidi Chemotaxis and Migration Tools 2.0 to measure endpoint y-displacement and accumulated displacement. P-values were calculated using Student’s *t*-test, and error bars represent the standard error of the mean (n=50).

4.3.5. *CMG2 ECM ELISA*

All steps were performed at room temperature unless otherwise indicated. For binding assays, matrix protein (Rockland Immunochemicals, Corning, EMD Millipore) was adsorbed onto 96-well polyethylene plates (Greiner) by incubating 2 µg/mL matrix protein in HBS with 2 mM Mg²⁺ and Ca²⁺ (Buffer A) in individual wells at 4°C overnight. PASSSR was treated similarly but was incubated at 1 µM in HBS. After the incubation, wells were washed 3x with Buffer A and blocked with 5% BSA (GoldBio) in HBS with 2 mM Mg²⁺, 2 mM Ca²⁺, and 0.1% Tween-20 (Buffer B) for 1 hour. Blocking was followed by 3 washes with Buffer B, after which varying concentrations of a biotinylated CMG2-AviTag construct (for matrix, 2nM to 10 µM; for PA, 4 µM to 1 pM) were prepared in Buffer B and incubated in wells for 4 hours. After incubation with
CMG2-AviTag, wells were again washed 3 times, after which streptavidin-HRP (Thermo Scientific) diluted in Buffer B with 5% BSA was incubated in wells for 1 hour. Wells were then washed 6 times with Buffer B, after which 1x TMB solution (Thermo Scientific) was added to wells. Once the color was visible in wells, the reaction was quenched with 0.2 M H₂SO₄. Wells were read out using a BioTek H4 Hybrid plate reader (BioTek) by quantifying absorbance at 450 nm. Data were analyzed in Microsoft Excel, and Kd values were calculated in MATLAB using the sigm_fit function.

4.3.6. Adhesion assay

EA.hy926 cells were grown in DMEM supplemented with 10% FBS. 20 µg/mL collagen I, IV, VI, laminin, fibronectin, PA^{SSR}, and BSA were prepared in PBS and used to coat the wells of a 96 well plate (100 µg/well) overnight at 4°C. After coating, collagen I, IV, VI, laminin, fibronectin, PA^{SSR}, and BSA were removed and 5% BSA was added to wells for 1 hour. Cells were washed out of serum-containing media by first scraping using a cell scraper to harvest the cells, then centrifuging cells at 100x g for 10 min. After discarding the supernatant, cells were resuspended in at 2-3 x 10⁵ cells/mL in DMEM. Cells (100 µL) were then added to each of the coated wells. The plate was incubated at 37°C for 1 hour to allow the cells to adhere to the coated well surface. Each well was then washed gently two times using warm DMEM using a multi-channel pipettor. DEME (100 µL) and 20 µL of cell-titler blue (Promega Cat#G8080) were then added into each well and incubated for 4 hours at 37°C. Following the incubation, the fluorescence signal was measured at 560 Ex/590 Em using the plate reader.
4.3.7. *EA.hy926 CMG2 knockout (CMG<sup>−/−</sup>) development*

HEK293T cells (3.8 x 10<sup>6</sup> cells) were seeded in a 10 cm tissue culture dish and incubated for 18 hours at 37°C in the tissue culture incubator. Next, 12 µg of pCMV (packaging), 5 µg of pVSV-G (envelope), 12.5 µg of Lenti-CRISPRv2 (transfer plasmid targeting CMG2 exon 1, sgRNA sequence: GCACCAACAGCCACAGCCCCG), 90 µL of 1 mg/mL PEI and 600 µL of serum-free DMEM were mixed into a tube and incubated for 15 minutes before adding to the 10 cm culture dish. The media was replaced with 10 mL of 10% FBS DMEM after 4 hours, which was followed by a 48-hour incubation in the cell culture incubator. The virus-containing media was removed from the transfected HEK293T cells, placed into a centrifuge tube and spun at 2500xg for 3 minutes to remove HEK293T cells and debris. The virus-containing supernatant was supplemented with 10 µg/mL Polybrene and added to 40% confluent EA.hy926 cells and incubated for 24 hours. The next day, the media was removed and replaced with fresh 10% FBS DMEM and 1 µg/mL puromycin and incubated for 3-5 days. After 3-5 days, the surviving cells (after puromycin selection) were plated in tissue culture well plates using a limiting dilution approach to approximately 1 cell/well in a 96 well plate and grown for several weeks. Wells with single clones of selected cells were then expanded, and further tested the CMG2 KO efficiency by flow cytometric PA endocytosis assay.

4.3.8. *Flow cytometric PA endocytosis assay*

CMG2 KO EA.hy926 cells and WT EA.hy926 cells were split and plated into a 12 well plate and grown until 50% confluent. Next, PA-Alexa Fluor 546 was added to 200 pM in each well and incubated overnight. After the cells were trypsinized and resuspended into a
microcentrifuge tube, cells were washed once with complete media. Then 50,000 cells were measured for each condition using the Cytoflex flow cytometer (RIC facility at BYU). These data were analyzed in our lab by using FlowJo.

4.3.9. Corneal micropocket assay

The corneal micropocket assay was performed as described using pellets containing 80 ng of basic fibroblast growth factor (bFGF) or 180 ng of human carrier-free recombinant vascular endothelial growth factor (VEGF; R&D Systems) in C57BL/6J mice. The treated groups received daily or twice daily i.p injections for 5 (bFGF treated) or 6 (VEGF treated) consecutive days of PASSR in PBS. Treatment was started on the day of pellet implantation; control mice received only vehicle i.p. The area of vascular response was assessed on the 5th (bFGF treated) or 6th (VEGF treated) postoperative day using a slit lamp. Typically, at least 10 eyes per group were measured.

4.3.10. HMVEC proliferation assay

Human microvascular endothelial cells (Cambrex) were maintained in EGM-2 (Cambrex) according to the vendor's instructions and used before passage 7. On day 0, proliferating cultures of BCE or HMVEC-d cells were seeded at ~10% confluence into 96-well plates. After attachment, the medium was exchanged for medium containing 1 pmol/L to 1 μmol/L of the indicated protein. Cells were allowed to grow for 7 days and then quantified using CyQUANT (Invitrogen) according to the manufacturer's directions. The degree of proliferation in culture was measured by comparing wells in each plate fixed in absolute ethanol on day 0 with experimental wells, with fold
proliferation calculated by dividing CyQUANT fluorescence in experimental wells by that in day 0 wells. Groups were compared using Student's t-test, with Bonferroni correction where appropriate.

4.3.11. *HMVEC* transwell migration assay

Human microvascular endothelial cells were maintained as above. Polycarbonate Transwell inserts, 6.5 mm diameter with 8.0 μm pores, were coated with fibronectin (BD Biosciences). Cells were harvested and resuspended in EBM (Cambrex) containing 0.1% bovine serum albumin (Fisher Chemical). Cells (10,000 per well) were plated onto wells containing medium alone or medium containing the molecule to be tested. These wells were suspended above wells containing 5 to 10 ng/mL recombinant human VEGF (R&D Systems) or full serum medium. Cells were allowed to migrate for 4 h. Membranes were rinsed once in PBS and then fixed and processed using Diff-Quick (Dade Diagnostics). Cells on the top of the membrane were removed using cotton-tipped applicators, and the membrane was removed from the insert using a scalpel. Membranes were then mounted on slides, and the number of cells in a microscopic field was counted manually.

4.3.12. *HMVEC* CMG2 / TEM8 knockdown

CMG2 and/or TEM8 knockdown in HMVEC was achieved using Dharmacon smartpools (Dharmacon, Lafayette, CO) and RNAimax (ThermoFisher, Waltham, MA) in OPTI-MEM with Glutamax (ThermoFisher, Waltham, MA). Approximately 6 hours after the addition of the
transfection complex, an equal volume of EBM-2 was added to the cells, and media was changed to EBM-2 ~18 hours later.

4.3.13. HMVEC tube formation assay

Human microvascular endothelial cells were maintained as above. Before the assay, a 1- to 2-mm layer of Matrigel was plated into the wells of a 12-well cluster. Approximately $10^5$ cells were plated on this layer in EGM-2. Plates were examined at 12, 14, 16, 18, and 24 h for differences in network formation. In each experiment, good network formation was observed in untreated control wells.

4.3.14. CMG2 and TEM8 knockout mice

CMG2 and TEM8 knockout mice were a kind gift from Stephen Leppla. They were housed in the ARCH facility at Boston Children’s Hospital on standard food and bedding (CMG2) or standard food and bedding, with supplemental soft food available (Nutra-gel mouse diet, Bio-Serv, Flemington, NJ). Genotyping was performed by Transnetyx (Cordova, TN).

4.3.15. Western blotting

Near-confluent HMVEC WT and CMG2 / TEM8 KD cells were washed three times in cold PBS, and lysed for 30 min at 4°C in PBS supplemented with 1% Triton X-100 and a protease inhibitor cocktail (cOmplete mini protease inhibitor cocktail, Roche). Lysates were cleared by centrifugation at 16,000x g and the supernatant protein concentration was measured using the BCA
total protein assay. Protein (40 µg) from each sample was used for SDS-PAGE and blotted onto PVDF. Following blocking, the membranes were probed with antibodies against CMG2 (1:1000 16723-1-AP, Proteintech, Rosemont, IL) or TEM8 (1:1000 ab21269, Abcam, Cambridge, UK), then stripped and reprobed for beta-actin (1:10,000 A5441, Sigma) as a loading control. Blots were imaged using a ChemiDoc imager (Bio-Rad, Hercules, CA).

4.4. Results and Discussion

4.4.1. Comparison of roles for CMG2 and TEM8 in corneal angiogenesis.

We have previously demonstrated that PA<sup>SSSR</sup> administration inhibits angiogenesis, as measured by endothelial cell migration, corneal neovascularization, and tumor growth<sup>4</sup>. However, PA<sup>SSSR</sup> can interact with both anthrax toxin receptors: CMG2 and TEM8 (ANTXR1).<sup>23, 66, 129</sup> Either of these interactions could, in theory, be responsible for the observed anti-angiogenic effects of PA<sup>SSSR</sup> <i>in vivo</i>. Therefore, we performed a series of experiments to compare the relative contributions of CMG2 and TEM8 to corneal neovascularization in mice, and to determine whether blockade of either of these receptors is sufficient to explain the observed anti-angiogenic effects of PA<sup>SSSR</sup>. First, we used either a CMG2 or TEM8 extracellular domain fused to an antibody Fc-domain to disrupt the ligand-receptor interaction, by competition for binding to endogenous ligand(s). In the corneal micropocket assay, the administration of CMG2-Fc significantly inhibited bFGF-induced vessel growth when compared to the untreated control, but the TEM8-Fc fusion did not (Fig. 1A). Hence, targeting CMG2 binding sites impacted corneal angiogenesis, while targeting TEM8 binding sites did not. We confirmed this observation by administering antibodies specific to either the CMG2 or TEM8 extracellular domains. An anti-CMG2 antibody significantly
reduced bFGF-induced corneal neovascularization in a concentration-dependent manner (Fig. 1B). In contrast, treatment with the anti-TEM8 antibody (L2), at a dose and schedule shown to inhibit tumor growth in mice (20 mg/kg/week), resulted in no significant decrease in corneal neovascularization compared to the vehicle control (Fig. 1C). Together, these results show that inhibiting the interaction of CMG2 with its endogenous ligand(s) significantly inhibits corneal angiogenesis, while inhibition of the TEM8-ligand interaction does not.

To confirm the importance of CMG2 rather than TEM8 as a mediator of angiogenesis in the cornea, we next sought to identify phenotypic changes that result from a CMG2 or TEM8 knockout. We performed the corneal micropocket assay in both WT and CMG2 knockout (CMG2$,^−/−$,) mice (Fig. 1D), using either bFGF or VEGF to induce vessel growth. CMG2$,^−/−$, mice exhibit a striking reduction in both bFGF-induced (Fig. 1E) and VEGF-induced (Fig. 1F) corneal vascularization versus WT mice. This result indicates a role for CMG2 in corneal angiogenesis. Interestingly, female mice were particularly susceptible to this effect (Fig. S1A-B) and exhibited a dramatic (>85%) reduction in their response to VEGF (Fig. S1B). In contrast, the TEM8 knockout (TEM8$,^−/−$,) mice exhibited no significant reduction when stimulated with VEGF (Fig. 1H) and only modest reductions in bFGF-induced neovascularization (15%; p<0.05;Fig. 1G, S1C-D). These data confirm that CMG2 plays a substantial role in corneal neovascularization, while TEM8 does not.

Finally, we evaluated the activity of PA$^{SSSR}$ in CMG2$,^−/−$, or TEM8$,^−/−$, mice. Importantly, in CMG2$,^−/−$, mice, PA$^{SSSR}$ did not further reduce bFGF-induced corneal angiogenesis (Fig. 2A). In contrast, PA$^{SSSR}$ treatment significantly reduced corneal neovascularization in TEM8$,^−/−$, mice (Fig. 2B). We conclude that CMG2 is the only target mediating the anti-angiogenic effects of PA$^{SSSR}$. 
To establish whether the effect of a CMG2 knockout or blockade is intrinsic to endothelial cells, we performed tube formation assays in Matrigel with HMVEC cells, which express both CMG2 and TEM8. We compared the anti-angiogenic effects of PA<sup>SSSR</sup> on cells selectively expressing only one of the two receptors (Fig. 2C). In the TEM8 knockdown (KD) cells (which then primarily express CMG2), PA<sup>SSSR</sup> administration resulted in a concentration-dependent reduction in the extent of tube network formation (Fig. 2D-E). In contrast, tube formation in the CMG2 KD cells was not altered by PA treatment (Fig. 2D-E). Together with the knockout mouse data described above, these results demonstrate that PA<sup>SSSR</sup> retains its anti-angiogenic effect in both TEM8<sup>−/−</sup> mice and TEM8 KD HMVECs but not in CMG2<sup>−/−</sup> mice or CMG2 KD cells. Hence, we conclude that PA<sup>SSSR</sup> exerts its anti-angiogenic effects by inhibiting CMG2 on endothelial cells.

4.4.2. CMG2 binds multiple ECM ligands.

In addition to the anti-CMG2 antibodies and PA, both small molecules and peptides that compete with PA for CMG2 binding inhibit angiogenesis. These data support the idea that PA<sup>SSSR</sup> exerts its anti-angiogenic effects <i>in vivo</i> by competing with interaction(s) between the CMG2 von Willebrand Factor A (vWA)<sup>65,67</sup> domain (including the metal ion-dependent adhesion site (MIDAS)<sup>22,131</sup>) and endogenous ligand(s). However, the specific ligands that regulate angiogenesis by binding CMG2 remain unidentified. ECM proteins are likely candidates for CMG2 binding <i>in vivo</i> because of CMG2’s homology with integrins<sup>67</sup> and modest available data showing CMG2 interactions with ECM proteins<sup>3,8</sup> and the observation that in individuals with hyaline fibromatosis syndrome (HFS) resulting from CMG2 mutations in the vWA domain result in widespread accumulation of extracellular matrix (ECM) proteins, including Col VI<sup>8</sup>. It has been alternately proposed that CMG2 binds Col IV, fibronectin<sup>3</sup> and Col VI<sup>8</sup> However, previous
single-concentration assessments of CMG2 binding to different ECM materials cannot distinguish differences in affinity from differences in the number of binding sites attached to the assay substrate. Here, we used an ELISA to measure the CMG2 $K_d$ for a series of different ECM proteins, including Col I, Col VI, laminin, and fibronectin (Fig. S2). Consistent with the previous reports, we found the highest intensity binding on the collagen VI-coated wells. In contrast, measurements of equilibrium binding affinities shows that each of the ECM proteins tested (Collagens I, VI, laminin and fibronectin) interact with CMG2 with indistinguishable near-micromolar $K_d$ values (Table 1). Both positive and negative control assays (CMG2 + PA$_{SSSR}$ and CMG2 + PA$_{SSSR}$ in the presence of EDTA, respectively) mirrored previously published $K_d$ values. The similar CMG2 affinity for the different matrix proteins tested indicates that CMG2 is capable of binding to multiple ECM proteins. Such promiscuous binding of ECM proteins also indicates that CMG2 could, like integrins, play a significant role in cell adhesion and motility through direct interactions with multiple components of the ECM.

4.4.3. **CMG2 mediates cell adhesion and migration ex vivo.**

Next, we worked to illuminate the CMG2-mediated endothelial process that PA$_{SSSR}$ disrupts to decrease angiogenesis. We have previously demonstrated that PA$_{SSSR}$ affects cell migration, but not proliferation in HMVEC, a primary endothelial cell type. We also observed no reduction in cell proliferation in EA.hy926 endothelial cells treated with PA$_{SSSR}$ under conditions that selectively target CMG2 (Fig. S4A). Together, these data suggest that CMG2 inhibition must impact angiogenesis by modulating endothelial cell migration, which directly impacts tube formation, rather than affecting cell proliferation.
Tube formation is closely related to endothelial cell proliferation, adhesion, and cell migration. Having eliminated proliferation as the mediator of CMG2 targeting, we evaluated another important angiogenic phenotype: endothelial cell adhesion. CMG2 targeting with 200 pM PA<sup>SSSR</sup> significantly reduced EA.hy926 cell adhesion on plates coated with different ECMs (Collagens I, IV, and VI, Human Fibronectin, and Laminin-111), but not on the BSA-coated control plates (Fig. S4B). Together, these data suggest that CMG2 plays a role in mediating cell adhesion to ECM proteins<sup>67</sup> and that PA<sup>SSSR</sup> binding to CMG2 inhibits that phenotype.

A major function of cell adhesion is to enable cell migration, and we find that PA<sup>SSSR</sup> significantly reduces cell migration on multiple ECM substrates (Fig. S4C-D). Using a microfluidic device that allows stable gradient generation, we monitored the migration of individual cells towards concentration gradients of chemoattractants over time. As expected, PA<sup>SSSR</sup> treatment resulted in decreased migration toward FBS over nearly all ECM-coated surfaces (Fig. S4C-D), even at the 200 pM concentration expected to result in only 50% occupancy of CMG2. Combining these adhesion and migration data, we conclude that interactions between CMG2 and ECM proteins mediate endothelial cell adhesion and migration and that PA<sup>SSSR</sup> disrupts this interaction. Importantly, CMG2 involvement in adhesion and migration should then also impact tube formation. Hence, CMG2 involvement in cell adhesion and migration observed here provides a functional explanation of the decrease in tube formation upon targeting of endothelial cells with PA<sup>SSSR</sup>, discussed above (Fig. 2D-E).

Since the microfluidic gradient migration assay tracks the motion of individual cells over time, we were able to compare two different aspects of motility: total cell movement (chemokinesis) and directional migration towards serum (chemotaxis). Targeting CMG2 in EA.hy926 cells with PA<sup>SSSR</sup> at the CMG2 K<sub>d</sub> (200 pM) resulted in a significant decrease in
observed chemotaxis over serum-coated plates (70%, P<0.01; Fig. S4C). In contrast, the total distance traveled by PA
SSSR treated cells was only modestly reduced (~20%) compared to untreated cells (Fig. S4D). Thus, CMG2 targeting with PA
SSSR alters chemotaxis with little effect on chemokinesis.

The effect of CMG2 knockout on chemotactic migration in EA.hy926 cells was next examined. We used CRISPR to target the exon 1 of CMG2 and isolated cell clones that exhibit loss of PA uptake as measured by flow cytometry (Fig. 3A). We then compared the migration of WT and CMG2
−/− EA.hy926 cells in our gradient migration assay. While CMG2 WT cells migrated towards serum (Fig. 3B), CMG2
−/− cells showed complete abolition of chemotaxis (P<0.001, Fig. 3C, F) with only slight alteration in chemokinesis (Fig. S5). To confirm that loss of chemotaxis in CMG2
−/− cells was not a consequence of off-target CRISPR effects, we complemented the CMG2
−/− cells with a vector expressing a CMG2-mClover fusion protein and found that the CMG2 add-back restored both PA uptake (Fig. 3A) and EA.hy926 cell chemotaxis (Fig. 3D-F). Together, these data demonstrate that a CMG2 knockout abolishes chemotaxis without substantially altering chemokinesis, a phenotype that echoes the effect of targeting CMG2 with PA
SSSR in vivo. Thus, CMG2 is a critical component of chemotaxis ex vivo and may play a role in regulating endothelial cell chemotaxis in vivo. It was previously reported that CMG2 orients the microtubule-based spindle in developing zebrafish embryos, and endothelial microtubules orientation may play a role in directing cell migration.

4.4.4. CMG2 knockout results in altered vascular development in vivo.
Alteration of cell chemotaxis mediated via a CMG2 knockout could be expected to result in altered vessel construction during embryonic development. As a test for apparently altered vessel development, we compared retinal vessel structures between the WT and CMG2\(^{-/-}\) mice. The total vascular area was not measurably different between the two genotypes (Fig. 4). However, the vascular growth pattern was subtly different between the knockout and WT mice. Careful quantitation of these mouse retinas showed that while the total number of veins in the retina of WT and CMG2\(^{-/-}\) mice is similar (Fig. 4A-B), CMG2\(^{-/-}\) mice have slightly fewer arteries (Fig. 4A, C), but those arterioles exhibit 2.5-fold more arteriolar branches than those of WT mice (Fig. 4A, D). Compared to the excurrent-like growth habit in WT mice, the retinal vessels in knockout mice have a more decurrent-like pattern characterized by increased branching in primary vessels. The observed difference in vessel patterning could be consistent with decreased precision in chemotactic migration.

4.4.5. **CMG2 mediates chemotaxis in response to several key angiogenic growth factors**

Chemotaxis is driven by chemoattractants, however, the stimuli that involve CMG2 mediated chemotaxis are not fully understood. While previous gradient migration assays used serum as the chemoattractant, which contains multiple chemoattractants, including protein growth factors and various lipids. We decided to measure endothelial cell migration in a gradient of media supplemented with boiled FBS, which has many proteins removed while keeping lipids and other thermally non-denaturable substances. Interestingly, we have observed no differences in either chemotaxis and chemokinesis between PA\(^{SSSR}\) and non-treated cells (Fig. S7). This indicates that CMG2 mediated chemotaxis is driven by chemoattractants that were removed from the serum, but not lipids. We have previously demonstrated that CMG2 targeting inhibits VEGF- and bFGF-
induced corneal neovascularization⁴ (Fig. 1), and a CMG2 knockdown has been reported to reduce migration of uterine cells towards PDGF-B.⁵⁶ We evaluated the impact of CMG2 targeting CMG2-mediated chemotaxis towards each of VEGF, bFGF, and PDGF, as well as a lipid chemoattractant, S1P. As shown in Figure 5 A-D and Figure S7, EA.hy926 cell chemotaxis towards VEGF, bFGF and PDGF is dramatically inhibited by PA⁶⁹, while chemokinesis remains almost unchanged (Fig. S8A-D). Similar results were obtained in CMG2⁻/⁻ cells, confirming that loss of directionality results from CMG2 loss of function, rather than novel PA-induced signaling (Fig. 5 A-D and Figs. S8–9A-D).

4.4.6. CMG2 mediation of chemotaxis and angiogenesis involves RhoA.

In developing zebrafish embryos, CMG2 is required for orientation of the microtubule-based epiblast mitotic spindle and, thus, for appropriately polarized cell division. Microtubules also control the direction of migration. RhoA influences microtubule organization via interaction with microtubule capping proteins,¹⁵ and is an established regulator of chemotaxis.⁷⁹, ⁸¹ The complete lack of chemotaxis we observe in CMG2 knockout cells or by PA and anti-CMG2 treatments suggests that CMG2 could be influencing RhoA signaling. Indeed, evidence for the direct interaction between CMG2 and RhoA is provided by coimmunoprecipitation data that suggests the formation of a complex between CMG2 and RhoA and downstream RhoA effectors¹⁴, ¹⁵. To investigate the possibility that CMG2 acts upstream of RhoA to influence chemotaxis, we sought to determine whether the disruption of RhoA phenocopied the effect that CMG2 targeting had on chemotaxis. As shown in Figure 5E and Figure S8A-B, treatment with the RhoA inhibitor C3 exoenzyme abolished EA.hy926 cell chemotaxis towards VEGF (Fig. 9E, Fig. S10A-B), with only a modest effect on chemokinesis (Fig. S9E). Notably, the effect of RhoA targeting observed
here was indistinguishable from that observed with either CMG2<sup>−/−</sup> cells or treatment with PA<sub>SSSR</sub> (Fig. 5C).

To further confirm a link between CMG2 and RhoA signaling, we compared the impact(s) of a CMG2 knockout or targeting to that observed for a RhoA knockout. Zahra et al. have recently observed that RhoA knockout abolishes the migration of endothelial cells towards VEGF, but not towards S1P<sup>86</sup>. As described above (Fig. 5A-D), either CMG2<sup>−/−</sup> or treatment with PA<sub>SSSR</sub> replicates this effect (Fig. 5A-D). In contrast, S1P-induced chemotaxis, which is not sensitive to either a CMG2 knockout or PA treatment, was also not altered by the presence of C3 exoenzyme (Fig. 5F, Fig. S10C-D). Zahra et al. have also analyzed the impact of a RhoA knockout on retinal development, similar to studies we carried out in CMG2<sup>−/−</sup> mice (Fig. 4A-B, Fig. S6). The retinal images presented by Zahra et al. (Fig. 6C in Zahra et al<sup>86</sup>) evince more branching in the primary arteriole of RhoA<sup>−/−</sup> mice vs. WT controls, a phenotype very reminiscent of our observations in CMG2<sup>−/−</sup> mice (Figs. 4 and S6). Together, these data demonstrate a striking concordance between the effects of CMG2 targeting and RhoA targeting and suggest that CMG2 probably lies upstream of RhoA in the same chemotaxis signaling pathway.

Here we show that the anti-angiogenic effects of PA<sub>SSSR</sub> treatment result from targeting the function of CMG2, which both disrupts cellular adhesion to ECM materials and causes cells to lose their ability to orient toward chemotactic cues from angiogenic growth factors. Indeed, CMG2 targeting can completely abolish RhoA mediated directional migration towards at least three growth factors. Together, these data suggest that CMG2 is an attractive target for the development of anti-angiogenic therapies. Notably, the bulk of existing anti-angiogenic therapies target a single growth factor, VEGF, and its receptor, and are subject to the development of drug
resistance caused by the breakthrough of alternate angiogenic pathways. Since the development of resistance may be completely inhibited if at least three angiogenic pathways are inhibited,\textsuperscript{132} CMG2 targeting holds promise as a broad spectrum anti-angiogenic therapeutic strategy that is less susceptible to the development of drug resistance than existing anti-VEGF therapy.
4.5. Figures

Figure 4-1. Blocking the interactions of CMG2 with its natural ligand inhibits both FGF and VEGF induced corneal neovascularization in vivo. (A) Corneal micropocket assay on mice treated with the soluble extracellular domain of either CMG2 or TEM8, via intraperitoneal injection. (B-C) Corneal micropocket assay on mice treated with either anti-CMG2 antibody (B) or anti-TEM8 (L2) anti-body (C). Corneal neovascularization in these assays was induced by bFGF before treatment; vessel area on both the left and right corneas were measured from each mouse. (D) Representative image of corneal neovascularization in both wild type (WT) and CMG2$^{-/-}$ male mice. (E-F) Comparison of corneal neovascularization between WT and CMG2$^{-/-}$ mice induced by either bFGF (E) or VEGF (F). CMG2$^{-/-}$ mice showed significant reductions in neovascularization for both bFGF- and VEGF-induced angiogenesis. (G-H) A similar experiment was performed with WT and TEM8$^{-/-}$ mice with either bFGF (G) or VEGF (H). Data presented are pooled from both genders. Error bars are standard error of mean, * p<0.05; ** p<0.01; *** p<0.001.
Figure 4-2. CMG2 is the main mediating receptor of PA^{SSSR}-induced angiogenic inhibition. (A) Comparative levels of bFGF-induced corneal neovascularization between CMG2^{+/−} and WT mice treated with or without PA^{SSSR}, and (B) TEM8^{+/−} and WT mice treated with or without PA^{SSSR}. Results showed that PA^{SSSR} reduced vessel formation on TEM8^{+/−} mice but not CMG2^{+/−} mice. Error bars are the standard error of the mean. (C) Western blot of HMVEC lysates with either CMG2 knockdown (CMG2si) or TEM8 knockdown (TEM8si). Band intensity was normalized to control (untreated cells). (D) EGF- and VEGF-induced tubule formation assays with both CMG2si- and TEM8si-HMVEC, treated with or without different concentrations of PA^{SSSR}. (E) Quantification of tube formation assays by counting the number of networks on each field. Error bars are the standard deviation.
Table 1

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Figure 4-3. CMG2 binds several broadly expressed matrix proteins with high affinity. Results of an ELISA-based quantification of CMG2 binding to several broadly-expressed matrix proteins, including collagens, laminin-111, and fibronectin. For the positive control, PA was assayed for binding to CMG2. Such similar affinities between matrix proteins indicate that CMG2 shows minimal preference for binding to any one of the matrix proteins assayed.
Figure 4-4. PA treated and CMG2 KO EA.hy926 endothelial cells lose the ability to migrate toward serum. (A) Differential PA-AF586 conjugate uptake by wild type (WT), CMG2 add back (AB) CMG2 knockout (CMG2−/−) EA.hy926 cells via flow cytometry (10,000 cells per condition). The median fluorescence intensity of each condition was normalized against the WT signal after subtracting from the unstained control. Error bars are normalized standard deviation from three individual replicates. (B-E) Aggregated track plots of individual EA.hy926 cell migration in the CellASIC migration chamber for 8 hours (Wild type cells: WT; CMG2 knockout cells: CMG2−/− cells; CMG2 add back cells: AB; and Wild type cells treated with 200 pM PA^{SSSR}. (F) Quantification of EA.hy926 chemotaxis (displacement toward gradient) in the CellASIC migration assay. * p<0.05; ** p<0.01; *** p<0.001; n.s not significant
Figure 4-5. CMG2 knockout increases vessel branching in the mouse retina. (A) Representative images of vessel formation in the retina of both WT (left) and CMG2<sup>−/−</sup> (right) mice. (B-D) Quantified vessel formation from retinal assays. (B) Comparison of vascular radius, normalized to the retinal radius, between WT and CMG2<sup>−/−</sup> mice. (C) Quantification of arterial branching in both WT and CMG2<sup>−/−</sup> mouse retinas. (D) Artery and vein counts per retina as in WT and CMG2<sup>−/−</sup> mice. CMG2<sup>−/−</sup> mouse retinas exhibit fewer veins and arteries than WT, but only artery count is significantly lower than WT mice.
Figure 4-6. CMG2 and RhoA targeting leads to endothelial cells losing the ability to migrate towards vascular growth factors, but not S1P. (A-D) Quantified chemotaxis towards various chemoattractants between WT, WT cells treated with 200 pM PA<sup>SSSR</sup>, and CMG2 knockout (CMG2<sup>−/−</sup>) cells. No difference was observed between the treatments under S1P stimulation (A). Significant reductions in chemotaxis were observed with PA<sup>SSSR</sup> treatment and CMG2<sup>−/−</sup> cells with bFGF, VEGF, and PDGF as chemoattractants (B-D). EA.hy926 WT cells treated with C3 exoenzyme, a RhoA inhibitor, reduced chemotaxis towards VEGF (E) but not S1P (F). * p<0.05; ** p<0.01; *** p<0.001; n.s. not significant.
Supplementary Figure 4-1. Gender-specific quantification of bFGF- and VEGF-induced corneal vascularization in WT, CMG2⁻/⁻, and TEM8⁻/⁻ mice. (A-B) Quantification of (A) bFGF-induced and (B) VEGF-induced neovascularization in male and female CMG2⁻/⁻ mice as compared to WT. (C-D) Quantification of (C) bFGF-induced and (D) VEGF-induced neovascularization in male and female TEM8⁻/⁻ mice as compared to WT. For both genders, greater decreases in corneal vascularization were observed in CMG2⁻/⁻ than for TEM8⁻/⁻.
Supplementary Figure 4-2. Binding of CMG2 to extracellular matrix proteins, as determined by ELISA. Proteins were coated on wells, after which CMG2-GST-biotin was added and the signal readout was with streptavidin-HRP/TMB. $K_d$ values were calculated by fitting each dataset to a 4-parameter logistic curve. (A) positive control (PA); (B) fibronectin; (C), collagen I; (D), collagen VI. PA x-axis scale is logarithmic with pM concentration units; all others are logarithmic with nM concentration values. Y-axis is normalized absorbance at 450 nm. Collagen IV and laminin-111 were also assayed for binding, but curves are not displayed due to poor fit. In each case, similar binding affinities (500 nM-1 uM) were observed (see Supplementary Table 4-1).
Supplementary Figure 4-3. Effects of CMG2 and TEM8 siRNA-mediated knockdown on HMVECs cell proliferation. (A-B) Western blot analysis of HMVECs lysates for (A) CMG2 and (B) TEM8 before and after the introduction of siRNA. (C-D) The proliferation of both WT and siRNA-treated EA.hy926 cells with both (C) CMG2-specific and (D) TEM8-specific siRNA. Proliferation was not significantly altered from WT in either CMG2-targeted or TEM8-targeted cells.
Supplementary Figure 4-4. Matrix binding to CMG2 is an important component of cell adhesion and migration. (A) EA.hy926 endothelial cell proliferation in different concentrations of PA^SSSR. Ethanol (EtOH) treated cells was used as negative control. (B) EA.hy926 endothelial cell adhesion to plates coated with various matrix proteins, or no ECM proteins (“NT”), both with and without 200 pM PA. The addition of PA significantly inhibits adhesion to matrix-coated plates. Adhesion to uncoated plates was not affected by PA^SSSR treatment. (C-D) EA.hy926 migration on different ECM coated surfaces and compared to the PA^SSSR treatment for 12 hours per condition. Chemotaxis is the vertical displacement measured towards a serum gradient across the migration chamber (C). While chemokinesis is measured by total distance, cells migrated in a random pattern (D). Statistical significance between untreated and PA treated cells was calculated by Student’s t-test. Error bars are the standard deviation, * p<0.05; ** p<0.01; n.s. not significant.
Supplementary Figure 4-5. CMG2 knockout and PASSR treatment do not affect endothelial chemokinesis induced by serum. FBS induced migration of wild type EA.hy926 (WT), CMG2 knockout (CMG2<sup>−/−</sup>), CMG2-mClover fusion protein add back (AB), and PASSR treated WT cells were monitored in the microfluidic gradient migration assay for 8 hours per condition. Image J was used to track 40 cells per condition, and three biological replicates per condition. Total distance migrated, or chemokinesis, was quantified from the chemotaxis and migration tools 2.0 (ibidi.com). Student’s t-test was used to test significance. * p<0.05; ** p<0.01; *** p<0.001; n.s. not significant.
Supplementary Figure 4-6. Comparison of vessel pattern on the retina from WT and CMG2−/− mouse. Representative pictures of the full retina from WT and the CMG2−/− mouse. Artery branches from these pictures were quantified and compared between WT and the CMG2−/− mice.
Supplementary Figure 4-7. Targeting CMG2 did not affect endothelial migration towards the boiled serum. EA.hy926 cells were put under a gradient of boiled fetal bovine serum (FBS), in which proteins were precipitated and removed by centrifugation. (A-B) Representative track plots of cell migration under boiled FBS only (A) and boiled FBS with the presence of 200pM PA (B). Quantified chemotaxis (C) and chemokinesis (D) showed no significant difference between control and PA treated cells. 40 cells were tracked under each replicate and three replicates were performed in total.
Supplementary Figure 4-8. Migration track plots of CMG2 directs cell chemotaxis towards bFGF, VEGF, PDGF, and S1P. Representative track plots of endothelial cell migration stimulated with bFGF, VEGF, PDGF, and S1P. Cells were monitored in the microfluidic gradient migration plate under various chemoattractants gradient for 8 hours per condition. 40 cells were tracked from each experiment in Image J and further quantify directional migration and total migration distance from chemotaxis and migration tool (ibidi.com).
Supplementary Figure 4-9. CMG2 and RhoA targeting do not reduce to endothelial cells chemokinesis stimulated by various chemoattractants. (A-D) Quantified chemokinesis towards various chemoattractants between wild type cells (WT), wild type cells treated with 200pM PA\textsuperscript{ASSR}, and CMG2 knockout (CMG2\textsuperscript{−/−}) cells. A slight reduction was observed in CMG2\textsuperscript{−/−} cells under S1P stimulation (A). No significant reduced chemokinesis was observed in different cell types when stimulated with bFGF, VEGF, and PDGF (B-D). EAhy926 WT cells treated with C3 toxin, a RhoA inhibitor, slightly reduced chemokinesis towards VEGF (E) but not S1P(F). * p<0.05; ** p<0.01; *** p<0.001; n.s not significant.
Supplementary Figure 4-10. Migration track plots of RhoA directs cell chemotaxis towards VEGF and S1P. Representative track plots of endothelial cell migration stimulated with VEGF and S1P, treated with or without a Rho inhibitor (C3 toxin, 1 μg/mL). Cells were monitored in the microfluidic gradient migration plate under VEGF or S1P gradient for 8 hours per condition. 40 cells were tracked from each experiment in Image J and further quantify directional migration and total migration distance from chemotaxis and migration tool (ibidi.com).
5. Conclusion:

Capillary Morphogenesis Gene 2 is commonly known as the major anthrax toxin receptor that regulates toxin entry into the cell via receptor-mediated endocytosis\textsuperscript{10, 36}. Also, CMG2 is involved in angiogenesis, however, the details on how it influences angiogenesis have remained unclear. Clues from the literature indicate that CMG2 is important for ECM homeostasis\textsuperscript{8, 10} and regulating cell migration\textsuperscript{4, 21, 56}; however, efforts to link these diverse situational roles of CMG2 mediated angiogenesis are needed.

To evaluate one potential role of CMG2 in angiogenesis, an effective method to assess endothelial migration, a key indicator of angiogenesis, was vital for our studies. Data presented in Chapter 2 report an optimized method to monitor endothelial cell migration in a microfluidic gradient cell migration assay. We have shown that this platform can generate a stable gradient of serum or vascular growth factors for long periods (12+ hours), which allows us to monitor cell migration towards a stable gradient of chemoattractants, including serum, growth factors, and lipids. Unlike other migration assays, this cell migration assay provides critical information on cell migration, including the ability to separate chemotaxis and chemokinesis. Therefore, it was a critical technique in our studies to evaluate the role of CMG2 in endothelial migration.

CMG2 and its homolog, TEM8, both bind to PA\textsuperscript{SSSR}. Although CMG2 has a lower $K_d$ than TEM8, it remained unclear which receptor was the most relevant to the PA\textsuperscript{SSSR} elicited anti-angiogenic effects. Our data show that in both CMG2 knockout mice and CMG2 knockout endothelial cells that were insensitive to PA\textsuperscript{SSSR} treatment, while TEM8 knockouts still responded to PA treatment. This strongly suggests that CMG2 is the receptor that is responsible for the observed anti-angiogenic effect of PA\textsuperscript{SSSR}. 
Existing data show that defective mutants of CMG2 lead to HFS, a major pathology of which is the accumulation of ECM proteins. While ECM remodeling is a critical step during angiogenesis, CMG2 may play a role in ECM homeostasis, which may also be related to its role in angiogenesis. Our data presented herein support such a hypothesis. We have identified a peptide, S16, from the Col-IV NC1 domain that interacts specifically with CMG2, but not its homolog TEM8. Kinetic studies showed an S16 \( K_d \) for the CMG2 vWA domain of about 400 nM. Also, this interaction strongly elicits an anti-migratory effect in endothelial cells while not affecting proliferation. These data suggest that, like PA^{SSSR}, S16 binds to CMG2 and inhibits serum-induced endothelial migration. Furthermore, our flow cytometry and confocal microscopy data indicate that CMG2 binds and facilitates endocytosis of S16. Together, these data suggest that CMG2 mediated ECM fragment clearance may also take part in the CMG2-dependent angiogenic regulation.

Finally, we were also able to provide new evidence for how CMG2 participates in angiogenic signaling. Here, we have identified that CMG2 is critical for endothelial cell chemotaxis. A knockout of CMG2 abolished endothelial cell chemotaxis towards serum or three different vascular growth factors, while chemokinesis was only minimally affected. Moreover, other previously reported data suggest that CMG2 is putatively involved in multiple signaling pathways, including Wnt, Hippo, and RhoA signaling. RhoA was especially interesting because it is a well-known regulator of cell chemotaxis. We have identified nearly identical phenotypes from our CMG2 knockout experiments and recently published RhoA knockout data. Both knockouts (CMG2 and RhoA) significantly decrease endothelial cell migration and increases developmental arterial branching in the retina. It appears that both CMG2 and RhoA are involved in the responses of the cells to growth factors, like VEGF, but
not the lipid, S1P, further suggesting that CMG2 works closely with RhoA signaling to affect endothelial chemotaxis, which may be the signaling that CMG2 uses to regulate angiogenesis.

Taken together, the data and analysis in this dissertation provide critical information that may act as a catalyst to the field to accelerate and enlarge the understanding of the physiological roles of CMG2. The experimental work presented herein is fundamental to the development of a molecular mechanistic model to explain how CMG2 impacts angiogenesis and can be leveraged to future therapeutic developments to treat pathological angiogenesis.
6. Appendix

*Overview:*

Other research efforts are presented below as individual figure panels with a short overview of the work, methodology, and data analysis.
6.1. EOMA assays

6.1.1. Overview:

Before using EA.hy926 cells, our lab used a mouse endothelioma cell line called EOMA. We used these EOMA cells in proliferation and gradient migration assays in the early stages of the research. These results remain important to validate that the anti-angiogenic effects of \( \text{PASSR} \) observed in EA.hy926 cells are similar at least one other endothelial cell line.

6.1.2. Methods:

For the gradient cell migration assay, 30,000 cells were seeded into the microfluidic gradient plate with DMEM supplemented with 10% FBS and 25 mM HEPES (complete medium) in both perfusion channels. Positive controls were performed by flowing complete medium in one perfusion channel and serum-free DMEM with 25 mM HEPES (serum-free medium) on the other for 12 hours. Similarly, \( \text{PASSR} \) treated complete medium and serum-free medium (200 nM) flowed in the perfusion channels for 12 hours. Images were acquired every 10 minutes and analyzed with ImageJ using the manual tracking plugin. A total of 40 cells were tracked per condition were then imported into Chemotaxis and Migration Tools 2.0 (ibidi.com) software, to obtain quantified data.

For the proliferation assay, 10,000 cells were plated in each of the wells of a 96 well plate. After incubating for 4 hours, the medium in each well was replaced by a medium with or without \( \text{PASSR} \) ranging from 1 nM to 10 \( \mu \text{M} \). The cell-free medium was used as a negative control, while no \( \text{PASSR} \) treatment was used as a positive control or vehicle. After 48 hours of incubation, 10 \( \mu \text{L} \) of Cell Titer Blue was added into each well followed by further incubation for 4 hours. Fluorescence (ex: 560 nm, em: 590 nm) was read out in a BioTek H4 Hybrid plate reader (BioTek). The signal was normalized against the vehicle control.

6.1.3. Results:

EOMA cell migration was inhibited by \( \text{PASSR} \) treatment, but not proliferation. Before running gradient cell migration assays in the microfluidic platform, we optimized the assay time that maximizes assay sensitivity and minimizes total assay time relative to measuring chemotaxis (Appendix Figure 6.1A). These data showed that assay sensitivity to chemotaxis began to drop after 12 hours (i.e., increased ~10 \( \mu \text{m} \) between 8 to 12 hours; increased ~6 \( \mu \text{m} \) between 12 to 16 hours). Thus, the migration assays were run for 12 hours. After treatment with \( \text{PASSR} \), chemotaxis decreased significantly in a serum gradient, but not as large an effect as was observed in the no gradient control (Appendix Figure 6.1B).
This suggested that PA SSSR treatment was inhibiting EOMA cell migration towards the serum gradient. Next, we investigated whether PA SSSR also affected EOMA cell proliferation. The results indicated that PASSSR treatment does not significantly affect EOMA cell proliferation over 48 hours, even at 10 µM (Appendix Figure 6.1C)

6.1.4. Appendix Figure 6.1

Appendix Figure 6-1. EOMA cell chemotaxis can be inhibited by PA SSSR treatment but not proliferation. (A-B) EOMA gradient migration assay optimization and effects of PA SSSR treatment on chemotaxis. (C) EOMA cell proliferation was not affected by PA SSSR treatment. Proliferation was normalized against the vehicle control (no treatment). Negative control was medium only. P-values were calculated using the Student’s t-test; *** p<0.0001.
6.2. 1,2,3,4,6-penta-O-galloyl-β-d-mannopyranose (PGM) inhibits endothelial cell migration, but not proliferation, in a dose-dependent manner.7

6.2.1. Overview:

Our lab has previously identified that CMG2 is a target for an antiangiogenic polyphenol, 1, 2, 3, 4, 6-Penta-O-galloyl-β-D-glucopyranose (PGG), and it inhibits endothelial migration.6 We tested a recently synthesized analog, PGM, which substitutes mannose for glucose. We measured the effects of PGM on endothelial cell migration and proliferation, critical aspects of angiogenesis. These experiments have been reported in a recent publication in the Journal of Medicinal Chemistry.7

6.2.2. Method:

Using the microfluidic cell migration assay described in detail in Chapter 2 and throughout the main parts of the dissertation. Briefly, EA.hy926 cells were seeded in the microfluidic gradient plate with DMEM supplemented with 10% FBS and 25 mM HEPES (complete medium) perfusing from both perfusion channels. Vehicle controls were performed by flowing in complete medium and in serum-free DMEM with 25 mM HEPES (serum-free medium) in their respective perfusion channels for 12 hours. Similarly, PGM was supplemented in both complete medium and serum-free medium (concentration range from 2.4 µM to 1 nM) and flowed in the respective perfusion channels for 12 hours. Time-lapse images were acquired every 10 minutes and cell tracks were analyzed with ImageJ using the manual tracking plugin. A total of 40 cell tracks per condition were analyzed using the Chemotaxis and Migration Tool 2.0 (ibidi.com), to obtain chemotaxis and chemokinesis data.

A cell proliferation assay was performed by seeding 10,000 cells in each well of a 96 well plate. After incubating at 37°C for 4 hours in the cell culture incubator, the medium in all wells was replaced by a medium with various concentrations of PGM or a vehicle control. A 5 min ethanol treatment in some wells was used as a negative control. Then, after a 48 hour incubation, 10 µL of Cell Titer Blue (Promega) was added to each well followed by 4 additional hours of incubation. The fluorescence intensity (ex: 560 nm, em: 590 nm) was readout in a BioTek H4 Hybrid plate reader (BioTek). The measured intensity was normalized versus the DMSO (vehicle) control.

6.2.3. Results:

Endothelial cell migration towards serum was inhibited upon PGM treatment in a dose-dependent manner (Appendix figure 6.2A). The IC50 was determined to be 365±129nM, which is over 2-fold better than the other analog PGG (IC50: 830nM).6 Similar
to the PGG results reported previously,\textsuperscript{6} cell proliferation was not affected by PGM (Appendix figure 6.2B).

\textbf{6.2.4. Appendix Figure 6.2}

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure6.2.png}
\caption{PGM activity in a serum gradient endothelial cell migration assay and PGM effects on cell proliferation. (A) PGM inhibits endothelial cell migration against a serum gradient (10% FBS to serum-free medium). Quantified migration displacement towards the serum gradient were normalized against the vehicle control (n=40). The curve was fitted in SigmaPlot to determine the IC\textsubscript{50}: 365±129 nM. (B) EA.hy926 cell proliferation in various concentrations of PGM using the Cell Titer Blue cell viability assay. The proliferation signal was then normalized towards DMSO control (n=3). Error bars in both A and B represent the standard deviation normalized to the untreated control.}
\end{figure}
6.3. CMG2–BioID Shotgun Proteomics and Analysis

6.3.1. Overview:

Preliminary experiments were performed to determine the putative CMG2 interactome using a CMG2–BioID fusion protein expressed in Human Embryonic Kidney 293T cells (HEK293T).

6.3.2. Methods:

The human CMG2 gene in the pcDNA3.1-CMG2-V5 plasmid (a gift from the Michael Rogers’ lab) was transferred to the MCS-BioD2-HA plasmid (a gift from the Daniel Simmons’ lab) using restriction digest cloning utilizing KpnI and EcoRI. This C-terminal fusion protein (CMG2-BirA*) was then transfected into HEK293T cells using PEI (DNA:PEI ratio was 1:3). The MCS-BioD2-HA (BirA-WT) was also transfected into HEK293T cells as a negative control. Transfected cells were grown to approximately XX% confluency, then 12 hours prior to lysis, biotin was supplemented to 50 µM. Cells were harvested and lysed in RIPA lysis buffer plus a protease inhibitor cocktail and the lysate cleared by centrifugation. Total protein concentration in the cleared lysate was quantified using the BCA total protein assay. The same amount of total protein (10mg) from both the CMG2-BirA and BirA-WT samples were added to 50 µL of streptavidin magnetic beads (New England Biolabs) and incubated with mixing at 4°C for 1 hour. The beads were captured using a strong magnet and washed 3X in PBS. Protein were then eluted from the beads by boiling in 2% SDS for 5 minutes. The eluted proteins were analyzed via SDS-PAGE and western blotting, then sent to the Duke Proteomic Center for sample preparation and LC-MS/MS analysis.

6.3.3. Results:

Western blot of the pulldown from CMG2–BirA* and BirA WT indicated that CMG2–BirA was expressed (~80 kDa). Interestingly, there were more biotinylated proteins isolated from the lysate in CMG2-BirA* expressing cells between 70 to 40kDa region than the BirA-WT expressing cells, suggesting that CMG2 interacts with different proteins in the HEK293T cells (Appendix Figure 6.3A-B). The analyzed proteomics data provided more than 2000 protein IDs. Among them, we have identified proteins that are at least 3-fold enriched in the CMG2-BirA sample, when compared to the BirA-WT control. We observed that among those enriched proteins, half of them are known to play a role in endocytosis. We also observed some proteins that are involved in Wnt signaling and cell migration (Appendix Figure 6.3C). Other proteins that have been shown or suggested to directly interact with CMG2, including RhoA and Tln, were also observed. These
preliminary data suggest that CMG2 has intracellular interaction partners, including RhoA signaling proteins.
Appendix Figure 6-3. Identifying putative intracellular binding partners of CMG2 using the BioID approach. Western blot analysis of lysate and pulldown of CMG2-BirA* and BirA WT with streptavidin (A) and anti-CMG2 (B). A list of protein IDs that are at least 3-fold enriched in the CMG2-BirA sample (C).
6.4. Using BLI to measure TNK1-UBA interaction with K48 and K63 tetra-ubiquitin

6.4.1. Overview:

In collaboration with the Josh Andersen lab, a protein they study in their laboratory, TNK1, was shown to have a ubiquitin associated (UBA) domain. Preliminary data show that the TNK1 UBA binds to both K48 and K63 tetra-ubiquitin in a pull down assay. Therefore, we are interested to measure and compare the binding kinetics between TNK1 UBA and the two tetra-ubiquitins using BLI.

6.4.2. Methods:

BLI was used to characterize the binding of GST-TNK1-UBA and GST-TAB2-UBA to the K63 and K48 tetra-ubiquitins. These fusion proteins were expressed and purified by the Andersen lab. Lysates were aliquoted and stored at -20°C. For the BLI experiments, assay buffer was optimized. The final assay buffer was 50 mM Tris-HCl, 150 mM NaCl, pH 7.2, 0.1% NP-40, 0.25 mg/mL BSA, and 5 mM DTT that was prepared each day. Assays were performed using the Octet RED96 biolayer interferometer (ForteBio) at 30°C and 1000 rpm shaking. First, Anti-GST biosensors (ForteBio) were loaded from the GST-TNK1-UBA lysate (8 sensors, 6 for tetra-ubiquitin binding, 2 for reference controls) or the control lysate (GST-TAB2-UBA; 4 sensors, 3 for tetra-ubiquitin binding, 1 for reference control) for 60 seconds. The loaded sensors were then equilibrated in assay buffer (360 sec) followed by an association step with various concentrations of the tetra ubiquitins. For the GST-TNK1-UBA, K48 tetra ubiquitin concentrations ranged from 25 nM to 0.8 nM; the K63 tetra ubiquitin concentrations ranged from 20 nM to 0.6 nM. The association step for both tetra ubiquitins was 60 seconds, followed by a dissociation step in assay buffer for 300 seconds. The GST-TAB2-UBA, a K63 specific UBA, was used as a positive control. For the control assays, the K63 and K48 tetra ubiquitins ranged in concentration from 200 nM to 50 nM. These assays had an association time of 5 or 60 seconds respectively, followed by a 60 second dissociation step. Data was processed and analyzed in the Octet Data Analysis 8.2 software. Processed data was fit to a 1:1 binding model to obtain the kinetic and thermodynamic parameters. Residuals were examined to assess the quality of fit and no systematic deviation was observed.

6.4.3. Results:

The experimental data indicates that the TNK1-UBA binding to both the K63 and K48 tetra ubiquitins have high affinities compared to the TAB2-UBA control. Comparing the curve fits, the dissociation rates of both the K63 and K48 tetra ubiquitins are much slower with TNK1 than TAB2 (Appendix Figure 6.4), suggesting a lower K<sub>d</sub> than TAB2. The calculated K<sub>d</sub> of TNK1-UBA to K63 and K48 are 0.6 nM and 2 nM respectively, while TAB2-UBA binding to K63 resulted in only a modest affinity (200 nM). The control, TAB2, is considered a K63 specific UBA, and showed no apparent binding to the K48 tetra ubiquitin as expected.
Surprisingly, TNK1 binds the K63 tetra-ubiquitin 300-fold tighter than TAB2 and the observed subnanomolar affinity is among the tightest measured interactions between a UBA domain and the K63 tetra-ubiquitin. Unlike TAB2, TNK1-UBA also binds the K48 tetra-ubiquitin tightly, indicating it is neither a K63 nor K48 specific binding UBA.
Appendix Figure 6.4. Measurements of the TNK1-UBA binding to the K48 and K63 tetra-ubiquitins via BLI. BLI curve fits of GST-TNK1-UBA binding to the K63 (A) and K48 (B) tetra-ubiquitins. BLI curve fits of the GST-TAB2-UBA binding to the K63 (C) and K48 (D) tetra-ubiquitins. Measured $K_d$ values (E) from the curve fits for the different UBA domains and tetra-ubiquitins. All measured $K_d$ values were averaged from three individual runs and errors are the standard deviation of the measured $K_d$. 

<table>
<thead>
<tr>
<th>UBA</th>
<th>Tetra-Ubiquitin</th>
<th>$K_d$</th>
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</thead>
<tbody>
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</tr>
<tr>
<td>TNK1-UBA</td>
<td>K48</td>
<td>2 ± 0.7nM</td>
</tr>
<tr>
<td>TAB2-UBA</td>
<td>K63</td>
<td>200 ± 100 nM</td>
</tr>
<tr>
<td>TAB2-UBA</td>
<td>K48</td>
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</tr>
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6.5. TNK1 knockout in A549 cells affect migration

6.5.1. Overview:

In collaboration with the Josh Andersen lab, we explored how TNK1 affects cell migration. Using the cell migration assay developed and deployed as part of my dissertation work, cell migration assays were performed to understand whether the depletion of TNK1 in A549 cells affects cell migration.

6.5.2. Method:

Briefly, 30,000 A549 cells were seeded in the microfluidic gradient assay device, followed by flowing DMEM supplemented with 10% FBS and 25 mM HEPES (complete medium) in both perfusion channels for 4 hours to allow for cell adhesion and spreading. Migration assays were performed using wild-type cells, TNK1 knockout cells, and a TNK1 addback as a CRISPR knockout off-target control. The migration assays were performed by perfusing complete medium in one perfusion channel and serum-free DMEM plus 25 mM HEPES (serum-free medium) on the other for 8 hours. Time-lapse images were acquired every 10 minutes and analyzed with ImageJ using the manual tracking plugin. A total of 40 cell tracks per condition were imported into the Chemotaxis and Migration Toolss 2.0 (ibidi.com), to quantify these data.

6.5.3. Result:

To understand the role of TNK1 in mediating cancer cell migration, we performed cell migration assays in the microfluidic gradient migration assay. We observed that the TNK1 knockout completely abolished directional cell migration or chemotaxis towards the serum gradient (Appendix figure 6.5A), while also providing a 50% reduction in total cell motility or chemokinesis (Appendix figure 6.5B) These phenotypes were both rescued upon re-expressing TNK1 in A549 cells (TNK1 Addback), indicating that TNK1 plays a crucial role in both directional migration and cell motility. These data suggest a link to TNK1 as an oncogene that could make cancer cells more likely to increase metastasis.
Appendix Figure 6-5. TNK1 knock out significantly reduced chemotaxis and chemokinesis in A549 cells. The microfluidic gradient cell migration assay using a serum gradient was performed on WT, TNK1 knockout, and a TNK1 addback cells. Here, the A549 WT cells were used as a positive control to compare the effects of a TNK1 knockout (KO) and a TNK1 addback (AB) control. These data indicate that the TNK1 KO significantly reduced chemotaxis (A) and cell motility or chemokinesis (B) when migrating towards a serum gradient. The re-expression of TNK1 in the addback cells rescued these phenotypes. Students’ t-test was used to determine statistical significance, * p<0.05; *** p<0.0001; n=40. Error bars are standard deviation of the replicates in each condition.
6.6. Using BLI to measure Ferritin affinity to anti-Ferritin polyclonal antibodies

6.6.1. Overview:

In collaboration with the Adam Woolley lab, I have assisted them in developing a BLI assay to determine the binding affinity (K_d) of antibodies for the biomarkers, Ferritin and lactoferrin, using BLI.

6.6.2. Methods:

A BLI assay was developed and optimized to characterize the binding affinity of biotinylated-ferritin or lactoferrin with associated polyclonal antibodies. All assays were performed using an Octet RED96 biolayer interferometer (ForteBio) in an optimized assay buffer (PBS pH 7.2) at 30°C with 1000 rpm shaking. First, streptavidin biosensors (ForteBio) were loaded with 5-10 µg/mL biotinylated ferritin or lactoferrin (5 sensors, 3 for antibody binding, 2 for reference control) for 300 seconds (ferritin) and 60 seconds (lactoferrin). The loaded sensors were then equilibrated in assay buffer (360 sec) followed by an association for 300 seconds for anti-ferritin or 120 seconds for anti-lactoferrin. Antibody concentrations ranged from 500 nM to 125 nM. Next, the dissociation step of the assay was implemented in assay buffer for 600 to 1200 seconds. All association and dissociation kinetics were processed and analyzed using the Octet Data Analysis 8.2 software. The processed data was fit to a 1:1 binding model to obtain kinetic and thermodynamic parameters. Residuals were examined to assess quality of fit and no systematic deviations were observed.

6.6.3. Results:

The preliminary measured K_d for ferritin to the polyclonal anti-ferritin was 20 nM. While this indicates that this is a relatively low affinity antibody, the affinity was not outside the expected range for a polyclonal antibody. Overall, if this antibody is to be used to detect ferritin as a biomarker, the relative concentration of ferritin in the sample influence biomarker detection limits and assay sensitivity.
6.6.4. Appendix figure 6.6

Appendix Figure 6-6. BLI association and dissociation data and fitting for ferritin and lactoferrin binding to their respective polyclonal antibodies. Streptavidin sensors loaded with either biotinylated ferritin or biotinylated lactoferrin were dipped into wells with diluted anti-ferritin antibody (A) or anti-lactoferrin (B) at 500 nM, 250 nM, and 125 nM. The preliminary calculated K_d for the anti-ferritin antibody was 20 nM while the anti-lactoferrin antibody was 700 pM. Uncertainty for the K_d is not available yet without sufficient replicates.
6.7. Treponema pallidum shotgun proteomics analysis to identify lipidated proteins

6.7.1. Overview:

In collaboration with the Parveen lab at the Rutgers New Jersey Medical School, we prepared and ran lysates from *T. pallidum*, the causative agent of syphilis to potentially identify lipated proteins from the organism. This organism is difficult to culture and these samples came from infected rabbit testes.

6.7.2. Methods:

The lysate was prepared by adding 6 M guanidine-HCl to the frozen cell pellets and then using a bead beater to homogenize the sample for 30 seconds. A BCA protein assay was used to quantify the total protein concentration of the lysates. The soluble proteins were reduced by addition of DTT to 5 mM, and then alkylated with iodoacetamide. The reduced and alkylated lysates were then transferred onto a 30 kDa ultrafiltration spin filter and washed 5x with 25 mM ammonium bicarbonate (ABC). The sample was then resuspended in 100 µL of ABC and 2 µL of 1 mg/mL trypsin was added and incubated at 37ºC overnight. Peptides were then separated by centrifugation and dried in a Speedvac for 2 hours. Peptides were then resuspended at a final concentration of 1 mg/mL in 3% acetonitrile and 1% formic acid in mass spectrometry grade water. Samples were run on the LC-MS/MS at the Fritz B. Burns Mass Spectrometry facility at BYU. Proteomics data were processed using PEAKs and Search GUI combined with a UCSF database to search for lipid-modified proteins.

6.7.3. Results:

The results show some proteins from *T. pallidum* with lipid modifications from both the PEAKS and the UCSF database. These experiments highlight the difficulty in doing proteomics on *T. pallidum* as the samples were heavily contaminated by rabbit proteins, which are residual from growing the bacteria in an animal host. The prevalence of rabbit proteins likely masked the less abundant *T. pallidum* proteins. Although we only managed to collect a few *T. pallidum* protein IDs, preliminary analysis indicates that the IDs recovered have the correct lipid modifications (Appendix Table 6.7).
<table>
<thead>
<tr>
<th>IDs</th>
<th>Lipid Modifications</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNAK, FLAA, RPOB, TMPA</td>
<td>Palmitoylation</td>
</tr>
<tr>
<td>ENO, FTSZ, FLAB1, FLAB3, HTPG, PCKG, TA47, TMPA, TRXB,</td>
<td>Myristoylation</td>
</tr>
<tr>
<td>CFPA_TREPA</td>
<td>Glycosyl-phosphatidylinositol</td>
</tr>
</tbody>
</table>

**Appendix Figure 6-7.** Protein IDs of *T. pallidum* proteins that were identified with lipid modifications from the preliminary shotgun proteomics analysis.
6.8. EAhy.926 cell migration towards an insulin gradient

6.8.1. Overview:

The Christensen lab remains interested in understanding how different growth factors affect CMG2-regulated cell migration. Therefore, we have analyzed the effects of different protein growth factors on endothelial cells. Tested growth factors include, bFGF, VEGF, PDGF, and insulin. Preliminary results with insulin was compared using non-treated and PA-treated EA.hy926 cells, but not with CMG2\(^{-/-}\) cells. These results appear in the appendix as there were insufficient, yet interesting, results to include in the draft publication in Chapter 4.

6.8.2. Method:

For the migration assays, 30,000 EA.hy926 WT cells were seeded in the microfluidic gradient migration assay device, followed by flowing DMEM supplemented with 10% FBS and 25 mM HEPES (complete medium) from both perfusion channels for 4 hours to allow for cell adhesion and spreading. The migration assay was performed by perfusing with 1.9 \(\mu\)M insulin supplemented DMEM in one perfusion channel and DMEM alone in the other channel for 8 hours. Cells were then treated with 200 pM PA\(^{SSSR}\), followed by running the assay a second time. Images were acquired every 10 minutes and analyzed with ImageJ using the manual tracking plugin. A total of 40 cell tracks per condition were analyzed in the Chemotaxis and Migration Tool 2.0 (ibidi.com), to obtain quantified data.

6.8.3. Result:

These data indicate that neither insulin-induced chemotaxis or chemokinesis was affected by targeting CMG2 (Appendix Figure 6.8A-D). This suggests that CMG2 mediated chemotaxis does not involve the insulin signaling pathway. Also, CMG2 exclusively regulates chemotaxis towards a specific set of chemoattractants (notably vascular growth factors).
Appendix Figure 6-8. Targeting CMG2 did not affect endothelial migration towards insulin. EA.hy926 cells treated with a gradient of 1.9 μM insulin in the microfluidic cell migration assay device. (A-B) Representative track plots of cell migration towards an insulin gradient (A) and migration towards an insulin gradient after treatment with 200 pM PA (B). Quantified chemotaxis (C) and chemokinesis (D) showed no significant difference between the control and the PA treated cells. In each case, 40 cells were tracked in each assay and three replicates were performed in total for each condition. Error bars represent the standard deviation from the three replicates of each condition.
7. References:


[42] Murray, J. (1873) On three peculiar cases of Molluscum Fibrosum in Children in which one or more of the following conditions were observed: hypertrophy of the gums, enlargement of the ends of the fingers and toes, numerous connective-tissue tumours on the scalp, &c, *Med Chir Trans* 56, 235-254 231.


[74] Nusse, R., and Clevers, H. (2017) Wnt/beta-Catenin Signaling, Disease, and Emerging Therapeutic Modalities, Cell 169, 985-999.


