LGN-DEPENDENT MICROTBULE REGULATION INFLUENCES ENDOTHELIAL CELL MIGRATION, ADHESION, AND SPROUT INTEGRITY

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ABSTRACT

Catherine Elizabeth Wright: LGN-Dependent Microtubule Regulation Influences Endothelial Cell Migration, Adhesion, and Sprout Integrity
(Under the direction of Dr. Victoria Bautch)

Blood vessels form during organismal development and maintain integrity to provide oxygen and nutrients to the tissues. Vessels are comprised of endothelial cells that coordinate their individual behaviors to generate functional sprouts. Endothelial cells undergo directional migration, oriented divisions, and lumen formation through organization of the microtubule network. Microtubules are actively growing and shrinking polymers that direct the shape and movement of cells. Disruption of the microtubule network is detrimental for the cell. Here I investigated the role of the mitotic polarity protein LGN in endothelial cells and sprouting angiogenesis. To study LGN in the vasculature, I utilized a three-dimensional model for sprouting angiogenesis. Surprisingly, loss of LGN did not affect oriented division of endothelial cells within a sprout, but perturbed overall sprouting and branching. I utilized two-dimensional assays to investigate the cause behind three-dimensional sprout defects in LGN KD endothelial cells. At the cellular level, LGN KD resulted in reduced endothelial cell migration and dysregulated cell-cell adhesions. Endothelial cells with LGN knockdown displayed stabilized microtubules at the growing plus-end. The data fits a model in which LGN promotes turnover of microtubules in endothelial cells, which in turn regulates migration, cell-cell adhesion, and angiogenic sprouting.
I dedicate this work to Eric and Cody
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>AJs</td>
<td>Adherins junctions</td>
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<tr>
<td>aPKC</td>
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<tr>
<td>MDCK</td>
<td>Madin Darby Canine Kidney cells</td>
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MT – Microtubule

MTOC – Microtubule Organizing Center

NHLF – Normal Human Lung Fibroblasts

NuMA – Nuclear mitotic apparatus protein

Par3 – partitioning defective 3

Par6 – partitioning defective 6

PBS – phosphate buffered saline

PECAM – platelet endothelial cell adhesion molecule

PFA – paraformaldehyde

PH3 – phospho-histone H3

s.e.m – standard error about the mean

tdTomato – tandem dimer tomato

TPR – tetratricopeptide repeats

VE-Cadherin – Vascular Endothelial Cadherin

VEGF – Vascular Endothelial Growth Factor

ZO-1 – Zonula Occludens protein 1
CHAPTER I – GENERAL INTRODUCTION

A. Mechanisms of Vascular Development

The vascular system is essential for providing oxygen and nutrients to the body\textsuperscript{1–3}. The vascular network is organized into a hierarchy of veins, arteries, and capillary vessels. These vessels are formed when endothelial cells undergo cell migration, changes in adhesion, and proliferation\textsuperscript{2,4,5}. Endothelial cells migrate and coalesce during initial vessel formation to generate a primitive network\textsuperscript{4,6,7}. To expand the primitive network, endothelial cells generate branches by sprouting from the parent vessel\textsuperscript{8}. Endothelial adhesions are formed and maintained through a cycle of externalization and recycling of adhesive proteins to promote vessel integrity\textsuperscript{2,9}. Vessel elongation occurs through migration and oriented cell divisions\textsuperscript{10}. The coordination of migration, adhesion, and cell division promotes healthy vasculature that supports the organism. Understanding these processes and how their disruption contributes to disease states is crucial\textsuperscript{11}.

Growth factor signaling, particularly vascular endothelial growth factor (VEGF), promotes overall vessel network formation and integrity\textsuperscript{2,6,12}. Mice lacking VEGF ligand or receptors die during embryonic development, due to failure of vessel patterning and hemorrhaging\textsuperscript{13}. Excessive VEGF activity disrupts downstream signaling pathways, inhibits vascular sprouting, and leads to randomized division orientation\textsuperscript{10,14}. While many endothelial-specific pathways have been studied in relation to VEGF, it is unknown what is downstream of VEGF in regulation of division orientation. Common pathways in division orientation have been elucidated in \textit{C. elegans, Drosophila melanogaster}, and epithelial tissues, but none have been
reported in endothelial cells\textsuperscript{15–17}. Unpublished data suggests that endothelial cells can orient spindles when some cell polarity proteins are genetically reduced, but further work needs to be done to fully understand the exact mechanism (C. Lee and V. Bautch, unpublished).

B. Cell Polarity and Division in Development

Polarity influences cell behavior throughout development, including cell and tissue migration, spatial identity, and oriented division\textsuperscript{18,19}. Cell polarity is generated and maintained by the asymmetric localization and activation of protein complexes\textsuperscript{20–22}. One polarity complex is Par3/Par6/aPKC, which facilitates the selective positioning of fate-determining and spindle orientating factors, among other functions\textsuperscript{23,24}. Par3/Par6/aPKC polarity is maintained through mitosis to ensure proper spindle placement and establish daughter cell polarity\textsuperscript{25–27}.

When a cell undergoes division, the cytoplasm is segregated just as the genetic material\textsuperscript{16}. The cytoplasm can be divided symmetrically or asymmetrically, which can dictate the identity, function, and position of the daughter cells (Figure 1.1A,B)\textsuperscript{20,28}. Asymmetric division is used to structure tissues, differentiate cell types, and maintain stem cell progenitor populations. During division, the microtubules organize into a spindle, which can be aligned specifically to ensure daughter cells receive the proper material\textsuperscript{15}. Spindle orientation can be established in response to asymmetrically polarized factors at the cell membrane. Spindles in endothelial cells orient along the long axis of the vessel, but the mechanism dictating this orientation is not known (Figure 1.1C)\textsuperscript{10}. Endothelial cell shape or vessel polarity via flow could be contributing factors in spindle alignment.

Endothelial cells are polarized on two axes: 1) proximal-distal as established by flow and growth away from the point of origin and 2) lumenal/apical-ablumenal/basal wherein the apical
side faces the lumen and the basal side faces the external environment\textsuperscript{29}. Proximal-distal polarity is important in the migration of ECs and the generation of new vessels while apical-basal polarity establishes lumens in new sprouts\textsuperscript{29–31}. The Par3/Par6/aPKC polarity complex establishes and supports lumen formation in the vasculature\textsuperscript{19,31}. Loss of aPKC in the vasculature leads to a delay in lumenization of the vessel, but no observed change in oriented division\textsuperscript{31}. Downstream of aPKC and cell polarity is LGN, a protein necessary in many cell types for oriented cell division, which has not been characterized in endothelial cells\textsuperscript{32–34}.

C. Regulation of Cell Division by LGN

LGN was first identified as a binding partner of Inscuteable (Insc) in \textit{Drosophila melanogaster}\textsuperscript{32}. LGN complexes with Insc and Par3/Par6/aPKC to establish asymmetry in \textit{Drosophila melanogaster} neuroblast cell division\textsuperscript{33,35,36}. LGN acts in complex with G-alpha-i and NUMA to direct and anchor the astral MTs (MTs extending from the spindle to the cell membrane) (Figure 1.2). In mammalian epithelial cyst culture, Par3/Par6/aPKC excludes LGN from acting on astral MTs\textsuperscript{26}. LGN is phosphorylated by aPKC, preventing it from binding G-alpha-i and NUMA. By limiting where the LGN complex forms, the astral MTs only anchor at specified regions of the membrane\textsuperscript{26,36}. In mammalian epithelium, the LGN complex is distributed on both sides of the spindle poles, while in \textit{Drosophila melanogaster} neuroblasts, the complex is restricted to one daughter cell\textsuperscript{26,37,38}. When LGN activity is disrupted (either through depletion, truncation, or mislocalization), the spindles fail to properly orient\textsuperscript{26,37–39}. Roles for LGN are established in \textit{Drosophila melanogaster} and mammalian epithelium, but its role in angiogenic sprouting is not understood.
I. LGN acts with MTs during Cell Division

At the start of mitosis, the nuclear envelope breaks down, releasing NUMA into the cytoplasm\(^{40,41}\). NUMA traffics to the spindle poles in complex with dynein and dynactin\(^{42}\). From there, NUMA is brought out to the membrane where it binds LGN and creates a bridge between the membrane and the astral microtubules\(^{43}\). The bridge consists of NUMA bound to LGN, which binds to G-alpha-i (in the GDP-bound state) at the membrane. Once the bridge has formed, the minus-end directed motor dynein moves toward the spindle pole while in complex with NUMA/LGN/G-alpha-i, generating the forces to pull the spindle poles apart\(^{37,42,44}\).

At the spindle poles, LGN is phosphorylated by Aurora A kinase, which recruits Discs Large (Dlg)\(^{45}\). Discs large is normally present at the membrane during interphase and acts to promote cellular junctions\(^{36,46}\). Dlg function changes during mitosis to help direct spindle orientation by complexing with LGN and plus-end MT motor protein, kinesin-heavy-chain 73 (khc73)\(^{17,46,47}\). Kinesins align and shorten MTs during mitosis, generating additional force for separating the spindle poles\(^{48}\).

These examples establish that LGN acts with the MT network to influence cell behavior during mitosis. The current body of work on LGN has focused primarily on cell division, with limited evidence showing that LGN was non-functional during interphase. However, some recent studies challenge that model, and early studies that excluded LGN from interphase function no longer fits with the current model for LGN. LGN is required for neutrophil polarization and chemotaxis in mammalian immune response. Neutrophils rely on the actin cytoskeleton to generate pseudopods in the direction of migration, facilitated by LGNI/G-alpha-i signaling\(^{77}\). Neutrophil movement is regulated through G-beta/gamma downstream signaling and does not
likely require LGN. The next section will discuss the early and current studies in context with LGN protein structure.

D. LGN Structure and Function

LGN is a 74kDa protein consisting of 3 distinct structural domains: the TPR motif, the Linker, and the GoLoco repeats. LGN conformation can be closed or open based on the proximity of the TPR and the GoLoco repeats and dependent on the presence of binding partners\textsuperscript{43,49,50}. The Linker region does not contain any secondary protein folding, acting as a hinge for LGN’s conformational changes. Each domain has documented interactions to facilitate LGN function.

The tetratricopeptide repeats (TPR) are a series of alpha-helices that arrange in parallel to form an amphipathic channel\textsuperscript{50,51}. The channel creates a binding pocket that allows for protein-protein interaction based on amino-acid charges. Charge-based interactions are flexible, and allow for LGN to interact with multiple proteins via the TPR\textsuperscript{51}. Due to the flexibility of the TPR binding channel, it is unlikely that all possible binding partners of LGN are known. Several of the known binding partners of the TPR, including NuMA and Insc, are involved in orienting the mitotic spindle\textsuperscript{34,52}. Expression of LGN-truncation mutants lacking the TPRs mimic loss-of-function phenotypes in MDCK cysts\textsuperscript{26,37}.

The GoLoco repeats are a series of highly specific binding pockets for G-alpha, which is tethered to the membrane\textsuperscript{53}. G-alpha is a member of the heterotrimeric G-protein signaling cascade, but its LGN interactions are G-protein signaling-independent\textsuperscript{54}. There are multiple G-alpha isoforms, but G-alpha-i has the highest affinity for binding to LGN\textsuperscript{54} and can only bind to LGN in the GDP-bound state. Mammalian LGN contains multiple GoLoco repeats, each capable
of binding a single G-alpha\textsuperscript{55}. LGN-mediated division orientation is abrogated when the GoLoco domains cannot bind G-alpha-i\textsuperscript{37,56}.

The TPR domain and GoLoco repeats are extensively characterized. Less attention has been given to the flexible Linker region between the TPR and GoLoco domains. Previous studies truncated LGN within the Linker region to study the function of the TPR or the GoLocos. However, recent findings support the Linker having target sites for phosphorylation and downstream function\textsuperscript{26,45}. It is not known if LGN conformation influences kinase activity on the Linker region. Linker-dependent function suggests that LGN truncations must be carefully designed to eliminate any bias. There are limited studies of the Linker’s contribution to LGN function, and it will be interesting to monitor the research published in the field in the future.

E. Microtubules Regulate Cell Behavior

I. Microtubule Morphology and Formation

Microtubules (MTs) are essential in cell migration, vesicle and protein trafficking, and cell division. MTs are hollow, tube-shaped polymers of alpha and beta tubulin heterodimers. MTs alternate between states of growth and catastrophe, a behavior termed dynamic instability\textsuperscript{57}. MT growth is characterized by the addition of heterodimers to the plus-end, located distally from the centrosome. The centrosome is also referred to as the microtubule-organizing center (MTOC). MT catastrophe occurs at the plus end, and involves sudden MT depolymerization, which can be spontaneous or facilitated based on cellular needs.
II. Microtubule Organization in Cell Division

Microtubules are essential during cell division, as they establish the bipolar spindle and anchor the spindles to the cell membrane to generate pulling forces that separate the chromosomes. One of the two centrosomes migrates away from the other during prophase (early mitosis), which positions it on the opposite side of the nucleus. This is done using overlapping microtubules linked by motor proteins that push against each centriole until the pushing forces equalize. The microtubules connecting the spindle poles to the chromosomes are the spindle microtubules while the MTs that grow from the spindle toward the cortex are the astral microtubules. Anchoring of the MTs to the cortex leads generates the forces necessary to separate the spindle poles. Once the astral MTs are anchored, they begin to shorten, pulling the spindle poles toward the cortex. Failure to shorten astral MTs leads to spindle instability, however it was not determined what effect this had on completing division\textsuperscript{58}. Once the chromosomes separate fully, MTs concentrate between the separated chromosomes and generate a contractile ring to initiate cytokinesis.

III. Microtubules Direct Cell Migration

The MTOC position during migration follows the direction of movement. This allows the majority of nucleating MTs to grow towards the direction of migration\textsuperscript{59}. Additionally, polarization of the MTOC provides cues to other organelles, leading to their re-positioning\textsuperscript{57,60,61}. The Golgi orients in response to MTOC polarity to traffic vesicles to the leading edge\textsuperscript{62}. Cell migration is promoted through directed trafficking of proteins (including Cdc42, WASP, and Arp2/3) in Golgi-derived vesicles along microtubules\textsuperscript{62,63}. These factors act to promote MT polymerization towards the direction of migration\textsuperscript{64}. 
F. Vascular Adhesions

Focal adhesions (FAs) are cytoskeletal complexes containing adhesion proteins (vinculin, paxillin), integrins, and actin. FAs are present at the leading edge of cells and at the ends of actin stress fibers. The formation, maturation, and degradation of FAs are necessary for cell signaling, migration, and cell cycle progression. FAs are capable of integrin cross-talk which coordinates the actin cytoskeleton with the MT network. During cell migration, cues from the extracellular matrix trigger the integrin signaling network. This leads to the production and turnover of FAs at the leading edge. These FAs provide a physical anchor for the cell to pull the cell body during movement. Stabilization of FAs, through reduced MT dynamics, lead to reduced cell migration and defects in cell division, as proper anchoring of the cell to its environment is critical for establishing the pulling forces for cytokinesis. FA size correlates with stability, with larger FAs being more mature and stable.

Adherens junctions (AJs) are cell-cell junction complexes that protect against barrier disruption and leakiness in vessels. In blood vessels, flow generates tension on the surface of the ECs, which activates the formation and remodeling of AJs. AJs are formed when adhesion molecules, such as Vascular Endothelial Cadherin (VE-Cadherin, EC specific) are deposited at the membrane. VE-Cadherin acts by dimerizing its extracellular domain with the extracellular domain of VE-Cadherin on adjacent cells. The extracellular domains mediate homophilic interactions between cells while the cytoplasmic domains generate scaffolding with catenins and the actin cytoskeleton. Constant recycling of VE-cadherin at the membrane promotes strong adhesions. Endothelial cell adhesions have stereotypical VE-cadherin localization patterns, and quantification of those patterns provides insight to adhesion dynamics.
G. Summary

Endothelial cell behavior is dependent upon coordinated molecular and cytoskeletal activity. Molecular components of cell polarity work in conjunction with the cytoskeleton to regulate division, migration, and adhesion. LGN, a known regulator of cell division, interacts with the microtubule network to orient cell division. There are multiple known binding partners that are involved in cell division orientation, but these binding partners and others have additional functions. This introduces the potential that LGN has a more diverse function that is facilitated by the variety of interactions. This thesis proposes that LGN influences MT behavior outside of mitosis and acts upstream of cell migration and adhesion in endothelial cells.
A. Cell divisions that are symmetrical have equal distribution of their contents. Symmetrical divisions are utilized to maintain progenitor cell populations.

B. Asymmetric divisions have unequal distribution of cellular contents. Daughter cells can assume different identities, sizes, or positions.

C. Endothelial cell divisions polarize along the proximal/distal sprout axis. Daughter cells contribute to lengthening of the sprout.
During mitosis, LGN localizes to the membrane, where it binds to G-alpha-i and NUMA. This complex promotes astral MT anchoring and shortening of the astral MTs to separate the spindle poles. Par3/Par6/aPKC localizes to the membrane asymmetrically, and excludes LGN from binding to G-alpha-I at the membrane. aPKC-phosphorylated LGN is sequestered to the cytoplasm through 14-3-3 binding.
REFERENCES


CHAPTER II – MATERIALS AND METHODS

Cell Culture

Human Umbilical Vein Endothelial Cells (HUVEC) were obtained from Lonza, cultured in EBM2 (Lonza) supplemented with EGM2 bullet kit (Lonza) and 1X Antibiotic-Antimycotic (Gibco), and used between passages 2-6. For starvation conditions, OptiMEM (Gibco) was supplemented with 0.5% fetal bovine serum (FBS, Gibco) and 1X Anti-Anti. HEK293T (Clontech) and Normal Human Lung Fibroblasts (NHLF, Lonza) were cultured in DMEM with 10% FBS and 1% Anti-Anti and used between passages 4-12.

Lentiviral Constructs and Production

A tdTomato reporter was introduced into LGN KD and EV constructs at the GFP reporter site. Lentivirus was produced by the UNC Lentiviral Core. Additional LGN targeting constructs (TRCN0000011025, TRCN0000006469) were obtained from Thermo Scientific. Targeting constructs were co-transfected with viral packaging plasmids pRSV Rev, pMDL RRE, and pVSV-g (Addgene) into HEK293T cells, and viral supernatants were collected 48 hr post transfection.

In Vitro Angiogenesis Assay

The sprouting angiogenesis assay was performed as described. HUVEC were infected with virus 72hr prior to or infected at the start of the assay, with no significant difference in data
analysis. 10⁶ HUVEC were coated onto Cytodex microcarrier beads and allowed to settle overnight, then suspended in 2mg/mL fibrinogen (Sigma, Fisher) plus 0.15 units/mL aprotinin (Sigma) in PBS. Upon addition of 0.625 units/mL thrombin (Sigma), the fibrinogen clotted to form a fibrin matrix. NHLF were plated on top of the fibrin, and media (EBM2 supplemented with EGM2 bullet kit) was added and changed every 2 days.

Random Migration Assay

HUVEC were sparsely plated on coverslips treated with 1μg/mL fibronectin 4 hours prior to imaging. Single cells expressing the viral reporter were selected, and images were acquired at 10 minute intervals over 12 hours. Cells that migrated out of frame or underwent mitosis were excluded. The center of the nucleus followed, and migration coordinates were obtained using Manual Tracking plug-in in FIJI and quantified in Excel.

Immunofluorescence

Cultured HUVEC were fixed in 4% PFA for 10 min followed by 10 min permeabilization in PBS/0.5% Triton X-100. Sprouting HUVEC were fixed in 2% PFA for 20 min followed by 2 hours permeabilization in PBS/0.5% Triton X-100. Samples were blocked in staining solution (PBS/0.5% Triton X-100/1% BSA/1% Goat Serum/0.2% sodium azide) for 2 hours at RT or overnight at 4°C. Primary antibodies (Table 1) in stain solution were incubated at 4°C overnight. Samples were washed 3X 10 min and incubated in Alexa-fluor 305, 568, and 647 (Life Technologies) (1:250 dilution for 1 hour at 37°C in cultured HUVEC and 1:50 dilution overnight at 4°C for sprouting HUVEC). Phalloidin (1:50 in stain solution, Life Technologies) was
incubated overnight, and Dapi and Draq5 (1:5000) were incubated 1 hour at RT. Conjugated phosphohistone H3 488/555 (Cell Signaling, 1:100) was incubated overnight at 4°C.

**Nocodazole Washout and MT Nucleation Assays**

HUVEC expressing control or LGN KD were incubated in OptiMEM plus nocodazole (5μg/ml in DMSO; Sigma) for 3 hr at 37°C. Cells were rinsed 2X in cold OptiMEM then incubated in EBM2 at 37°C and fixed in 100% cold methanol at the following timepoints: 2 min for microtubule nucleation and stained with alpha-tubulin-555 and 10 min for acetylated tubulin and Alexa-567.

**Focal Adhesion Analysis**

HUVEC were treated with nocodazole and fixed in 2% PFA after incubating in EMB2 at 37°C for 20 min. Cells were stained with vinculin and Alexa 567 Images were captured at the same zoom factor, 15 images per condition. Static properties of focal adhesions were analyzed using FAAS (http://faas.bme.unc.edu/) with the following parameters: detection threshold 2, minimum adhesion size 2 pixels, and minimum FAAI ratio 3. Output was processed in Excel.

**PlusTip Tracking and Analysis**

Cultured HUVEC were co-infected with control or LGN KD-tdTomato and EB1-GFP and imaged as described³. Imaging utilized a PerkinElmer UltraView spinning disk confocal ORCA-ER camera, Nikon 60× Plan Apo NA 1.4, and MetaMorph software. Briefly, images were captured at 2 sec intervals and the first 30 sec were analyzed. Analysis was done using
plusTipTracker in MatLab. The entire cell was analyzed with the region of interest outlining the cell perimeter.

**Adherens Junctions and EDTA Recovery Assay**

Control or LGN KD HUVEC were plated at confluency and treated with 2.5 nM EDTA for 2 hours prior to release (Goh et al., 2010). Cells were fixed and analyzed pre-, during, and 1 hour post-EDTA release. Cells were stained with VE-Cadherin, PECAM, or ICAM2 with or without permeabilization.

**Imaging and Quantification**

Cultured HUVEC were imaged on Leica DMI 6000B and Olympus LSM5 confocal microscope. Sprouting HUVEC were imaged on Olympus LSM5. Live imaging of HUVEC was performed on Olympus FV10 and Olympus VivaView. Images were processed in LSM Image Browser and FIJI with Manual Tracking, Metamorph, and Chemotaxis plug-ins. Quantification of cell detachment, sprout length, branchpoint frequency, transwell migration, and line scans were done in FIJI. Graphing and statistical analyses were done in Excel and Prism.
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REFERENCES


CHAPTER III – RESULTS

A. INTRODUCTION

Understanding how endothelial cells (ECs) cooperate to form and maintain the vasculature is crucial for disease prevention. Disease states, such as cancerous tumors, utilize the normal processes that promote the vessel network to grow and metastasize. Formation of vessel networks requires intricate coordination of endothelial cell migration, adhesion, and polarization. In response to angiogenic cues, EC assume a pro-migratory phenotype; altering cell polarity and de-stabilizing cell-cell and cell-matrix junctions to facilitate vessel growth. However, to maintain the integrity of growing blood vessels, individual ECs must strike a delicate balance between growth and vascular stability.

The generation of a new sprout is controlled by molecular and morphological mechanisms. New sprouts form by re-orienting their polarity and activating pro-migratory pathways. Proximal/distal polarity promotes the formation and elongation of sprouts; it is also critical in cell migration. When ECs undergo mitosis, they divide along the proximal/distal axis, which contributes to the lengthening of the sprout. The formation of the lumen is promoted by apical/basal polarization within the sprout. Apical/basal polarization allows the EC to distinguish and respond to luminal and extracellular matrix signals.

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This chapter is adapted from a manuscript that was submitted in October 2014. I designed, performed, and analyzed experiments, made figures, and wrote this draft. Dr. Kevin Mouillesseaux provided valuable help with draft edits. Dr. Erich Kushner provided help with experimental analysis, Dr. Quansheng Du provided essential materials. Dr. Victoria Bautch designed and analyzed experiments.
Various signaling pathways, including VEGF, provide environmental cues to ECs to regulate the formation of branched networks\textsuperscript{1,14}. The VEGF signaling pathway has been extensively studied for its requirement in EC survival and morphogenesis\textsuperscript{4,7,10,15,16}. EC sprouts exposed to VEGF break down their adhesions and migrate toward the signal, proliferating and lumenizing to generate a new sprout\textsuperscript{13,14,17}. The VEGF signaling cascade is complex and influences many aspects of EC behavior, making it difficult to dissect the exact mechanisms behind each phenotype. Studying mechanisms that directly affect sprouting, migration, oriented division, and adhesion in the context of endothelial cells will provide a more thorough understanding of EC behavior. Because ECs alter their division orientation in response to VEGF, we were interested in studying factors known to influence division in the context of developing structures.

LGN, an adapter protein previously shown to function in mitotic orientation, has not been previously studied in endothelial cells. LGN acts to anchor astral microtubules, which emanate from the spindle pole towards the cortex\textsuperscript{18–22}. LGN is necessary for asymmetric division in two separate mouse epithelial tissues, the epidermis and the neuroepithelium, to maintain progenitor cells and generate differentiated epithelial cells\textsuperscript{23,24}. Without proper distinction between the two cell populations, both epithelial tissues are unable to correctly form. LGN binds to G-alpha-\textit{i} and NuMA (or Discs Large) to create a bridge between the microtubules and the cortex\textsuperscript{21,25,26}. Microtubules are similarly regulated at the cortex during cell migration and adhesion dynamics\textsuperscript{27}. As in the epithelium, we predict that LGN promotes endothelial sprout formation and maintenance through the microtubule network.

Here we present the first studies of LGN in angiogenic sprouting and EC behavior. We found that endothelial sprouts do not require LGN for spindle orientation. Endothelial sprouts
mosaic for LGN knockdown displayed reduced sprouting and branching behavior and increased occurrence of isolated ECs present in the matrix. We determined that two EC behaviors important in sprout formation, migration and cell adhesion, were disrupted in a manner consistent with reduced sprout maintenance. We challenged microtubules in LGN KD HUVEC and concluded that LGN is required for proper MT dynamics, a molecular mechanism upstream of migration and adhesion in ECs. We propose that LGN control EC behavior through the microtubule network during interphase, upstream of migration and adhesion.

B. LGN SUPPORTS ANGIOGENIC SPROUT FORMATION

To explore the role of LGN during sprouting angiogenesis, we took a genetic knockdown approach. We obtained a previously characterized LGN shRNA lentivirus, and validated its efficacy in Human Umbilical Vein EC (HUVEC). HUVEC infected with LGN KD virus showed over a 10-fold decrease in LGN expression levels 72 hours post infection compared to control empty vector virus (Fig 2.1A).

In order to examine LGN in endothelial sprouts, we utilized a sprouting angiogenesis model that has been previously characterized. Mosaic sprouts where quantified if at least half of participating cells were LGN KD. By looking at mosaic sprouts, we were able to determine whether LGN was globally required within a sprout for morphogenesis. We quantified the number of sprouts that emerged from individual beads in each condition (Fig 2.1B-D). LGN KD beads formed significantly fewer sprouts than control beads. Additionally, the sprouts that did form in LGN KD beads displayed reduced branching (Fig 2.1E). To determine if the phenotype we observed in the sprouting angiogenesis assay was due to loss of LGN, we obtained two alternate shRNAs against LGN. These shRNA both reduced LGN expression in HUVEC (Fig
2.2A) and led to a reduction in sprouting and branching in the sprouting angiogenesis assay (Fig 2.2B-D). Taken together, these data suggests that LGN promotes the generation of new sprouts and branches.

During the analysis of LGN KD and control beads, we observed that LGN KD cells were more likely to be dissociated from a sprout (Fig 2.1F, 2.2E). We initially hypothesized that the dissociated cells might represent branching EC that failed to remain connected to the parent vessel. We compared the frequency of isolated cells against branching frequency within the same bead and saw that there was no correlation between the parameters (Fig 2.2F). We concluded that the isolated cells were not a result of failed branching attempts, but an independent effect of LGN KD.

LGN directs spindle orientation during mitosis in epithelial tissue development\textsuperscript{23,24}. Therefore, we determined if the sprouting defects might be due to disrupted mitotic orientation. However, in contrast to previous studies, we found that LGN was dispensable for orienting the spindle (Fig 2.1G, H) in endothelial sprouts in our model. This suggests that LGN promotes endothelial sprout formation through mechanisms other than spindle orientation.

C. LGN IS REQUIRED FOR CELL MIGRATION AND DIRECTIONAL CHANGE

The process of endothelial sprouting involves multiple cellular events, including cell migration to generate branches and networks\textsuperscript{1}. We hypothesized that reduced sprouting in LGN KD sprouts was a consequence of defective cell migration. To determine the effect of LGN KD on HUVEC migration, we used live-imaging to measure the distance cells traveled in 2D (Fig
LGN KD HUVEC traveled a significantly shorter distance than control cells over the same time period (Fig 2.3B and Fig 2.4A,B), suggesting LGN facilitates cellular motility.

Reduced cell migration has many potential root causes, including defects in altering directional migration, a cell behavior critical in the tip cell competition that guides growing sprouts. Therefore, we sought to determine if LGN had any influence on the ability of EC to change direction during migration. To quantify directional changes, we generated vectors for individual cells’ movement from one time-point to the next in live-imaging movies, then calculated the magnitudes of angles between vectors (Fig 2.3C). Loss of LGN significantly impaired the ability of HUVEC to make large (>30°) directional changes. Instead, almost 25% of directional changes were of 30° or less (Fig 2.3D and Fig 2.4C,D). These data support a role for LGN in re-orienting the cytoskeleton to initiate and alter migration.

D. LGN KD HUVEC HAVE ENHANCED MT NUCLEATION

Cell migration requires dynamic remodeling of the cytoskeleton by the microtubule organizing center (MTOC) and microtubules (MTs). Since our observations of LGN KD HUVEC were consistent with cytoskeletal defects, we hypothesized that the MT network was impaired in LGN KD HUVEC, contributing to the migration defects. Our lab has previously shown that excess centrosomes can alter MT dynamics and EC migration. Therefore, we quantified centrosome numbers in interphase LGN KD and control HUVEC, and found them to be indistinguishable. (Fig 2.5A). We then tested the nucleation capacity of the centrosomes. We quantified the length and number of nucleations in control and LGN KD HUVEC 1 minute after nocodazole washout, a common method to destabilize MTs and then monitor their re-growth. LGN KD cells had the same number of nucleations but significantly longer microtubules.
compared to control HUVEC (Fig 2.5A-C). We hypothesize that LGN KD leads to more stable MTs.

We interrogated steady-state MT dynamics through live-imaging of HUVEC infected with an EB1-GFP lentiviral vector, which labels the plus-end of growing MTs\textsuperscript{32,33}. Consistent with our data from the nocodazole washout assay, we observed significantly longer comets and no difference in nucleation rate in LGN KD HUVEC (Fig 2.5E-F). Combined, these data reinforces our hypothesis that LGN regulates MT length.

Microtubules in LGN KD cells are longer, which could be due to increased rate of MT polymerization or elevated MT stabilization\textsuperscript{30,32}. We quantified the velocity of EB1 comets in both control and LGN KD HUVEC and we observed no global difference in comet velocity (Fig 2.6B). This suggests that the longer MTs are due to stabilization. When MTs are categorized by their growth rate and lifetime, we observed a significantly larger population of fast, longer-lived MTs in LGN KD (Fig 2.6C). This further supports a model in which LGN regulates MT stability.

Our data suggest that LGN influences MT stabilization (Fig 2.5A, E, 2.6C). Nucleations polarize toward the cell membrane and in the direction of migration\textsuperscript{27,34,35}. Because of this, we hypothesized that MT polarity would be skewed in LGN KD HUVEC. However, we failed to see a difference in the ability of MT comets to polarize, suggesting that LGN does not influence the
directional growth of MTs (Fig 2.6D). Overall, we conclude that LGN promotes the dynamic instability necessary for MT growth and turnover.

E. FOCAL ADHESIONS ARE ENHANCED IN LGN KD HUVEC

In addition in influencing migration, the microtubule network regulates the formation, maturation, and recycling of cellular adhesions. Adhesions require signaling cross-talk between MTs and the actin cytoskeleton. Focal adhesions (FAs) provide anchors to the extracellular environment to promote cell movement, and require active MT polymerization and catastrophe. Based on our observation that LGN KD HUVEC have longer and more stable MTs, we hypothesized that FA morphology was disrupted in LGN KD HUVEC. To investigate FAs in LGN KD HUVEC, we pretreated the cells with nocodazole to halt FA turnover. Once the cells were released from nocodazole, FA turnover resumes. We quantified FA length in control and LGN KD HUVEC, and observed that LGN KD cells had a higher frequency of long focal adhesions (Fig 2.7A). LGN KD HUVEC not only have more long focal adhesions, but the longest FAs in LGN KD cells are significantly longer than in control cells (Fig 2.7B). Longer focal adhesions after washout suggests that turnover is reduced, which impairs cell migration. In contrast, Ras activation produces larger FAs and also increase cell migration. Because LGN
KD cells have longer FAs and reduced cell migration, it is likely a mechanism independent from Ras activation, which relies on the actin cytoskeleton, not MT.

**F. VE-CADHERIN LOCALIZATION IN LGN KD HUVEC SUGGESTS JUNCTIONAL INSTABILITY**

In addition to FAs, endothelial cells form adherens junctions (AJs) when in contact with another cell\(^\text{40}\). The AJs are crucial in preventing leakiness and promoting structural integrity during branching and sprouting\(^\text{5,41}\). VE-Cadherin promotes the formation of AJs and its localization pattern can be used to extrapolate junctional stability\(^\text{42}\). VE-Cadherin contains an extracellular domain that dimerizes with other VE-Cadherins on cells at the junction\(^\text{15}\). VE-Cadherin gets recycled back into the cell, leading to separate populations of VE-Cadherin\(^\text{43}\). We immunostained confluent monolayers with VE-Cadherin to determine the integrity of junctions in control and LGN KD HUVEC. We observed that LGN KD junctions had a higher VE-Cadherin intensity and wider signal peak than control junctions, consistent with having a less stable junction (Fig 2.8A-B)\(^\text{42}\).

To distinguish between internal and junctional VE-Cadherin, we repeated the immunostaining without permeabilizing the cells, which will only label cell-surface VE-Cadherin (Fig 2.8A-B). Under these conditions, LGN KD junctions had more VE-Cadherin at the junction (Fig 2.8C-D). Combined, this suggests that LGN KD HUVEC have more VE-Cadherin adjacent to the junction, suggesting rapid turnover and instability. Excess VE-Cadherin
could be caused by stabilized MTs, because there is evidence that VE-cadherin trafficking to and from the cell membrane requires dynamic MTs\textsuperscript{44}.

Our initial analyses of VE-Cadherin in LGN KD HUVEC focused on borders between LGN KD positive and negative cells. We questioned how LGN KD cells contributed excess VE-Cadherin at the AJ. To address this, we performed additional analyses of junctions between two LGN KD cells. We observed an LGN-dependent increase in total VE-Cadherin present at the junction, while only one LGN KD cell was necessary to observe higher intensity signals (Fig 2.8E-F).

**G. VE-CADHERIN TRAFFICKING IS ENHANCED IN LGN KD HUVEC**

We showed that VE-Cadherin has disrupted localization in steady-state LGN KD monolayers (Fig 2.8). We sought to determine if the rate of junction formation was increased in LGN KD monolayers. We investigated VE-Cadherin trafficking to the junctions through modification of Ca\textsuperscript{2+} signaling\textsuperscript{45}. By blocking Ca\textsuperscript{2+} signaling, VE-Cadherin is internalized and junctions begin to break down. Once Ca\textsuperscript{2+} signaling resumes, VE-Cadherin re-localizes to the junction. When HUVEC monolayers were treated with EDTA (a Ca\textsuperscript{2+} chelator) and released, we quantified the rate of VE-Cadherin localization to the junctions. The rate of VE-Cadherin re-localization in LGN KD junctions was increased two-fold compared to control junctions (Fig 2.7C-E), suggesting more rapid turnover as described above. VE-Cadherin recycling is
dependent on proper MT turnover, suggesting that the VE-Cadherin localization in LGN KD cells is downstream of the MT phenotype\(^{46}\).

**H. OVERALL MEMBRANE ADHESION IMMUNOSTAINING IS DISRUPTED**

We observed that VE-Cadherin was not properly localized in LGN KD AJs. However, VE-Cadherin is not the only adhesion molecule involved in EC junctions. We sought to determine if LGN KD effect was VE-Cadherin-specific. We immunostained for PECAM and ICAM-2 and observed that LGN KD junctions had significantly different patterns than control junctions (Fig 2.7G-L). Additionally, PECAM localization following EDTA treatment recovers to pre-treatment levels in both control and LGN KD HUVEC (Fig 2.7C-D, F). These data suggests that overall regulation of adhesion molecule trafficking is disrupted in LGN KD HUVEC, but that VE-Cadherin and PECAM are affected differently. Because multiple adhesion molecules are not properly localizing at LGN KD AJs, the effect that LGN has on junction formation is likely more general and not directed at specific adhesion pathways.

**I. DISCUSSION**

In this study, we show that LGN influences endothelial cell functions that support blood vessel formation. EC sprouts rely on effective cell adhesion and cell migration\(^{3,47}\), which are both impaired by MT stabilization\(^{46,48}\), a feature observed in LGN KD HUVEC. This study is the first to characterize LGN in endothelial cells, and the first to identify a requirement for LGN in non-canonical functions. Additionally, this study shows that LGN has the potential to act outside of mitosis to regulate cell behavior.
The role that LGN plays in anchoring astral microtubules during mitosis is extensively detailed. Previous studies would predict that LGN is indispensable for establishing spindle polarity in a tissue. Here, we show that EC sprouts do not require LGN to establish and undergo oriented divisions. We predict that HUVEC rely on alternate mechanisms to promote oriented divisions. Cell shape during interphase can dictate the formation, orientation, and maintenance of a bipolar spindle. Endothelial cells have highly elongated cell shapes. We predict that EC shape promotes oriented division in the sprouting angiogenesis model. The sprouting angiogenesis model as a tool for observing division orientation led us to conclude that LGN was not required for EC division orientation, but instead had novel influences on cell behavior.

Although LGN was dispensable for division orientation in a sprout, we observed that loss of LGN altered focal adhesion patterns and adherens junction protein localization, which are important for maintaining sprout shape and integrity. We predict that the elongated focal adhesions and enhanced VE-cadherin localization are result from increased MT stability observed in LGN KD HUVEC. Microtubules actively target focal adhesions to promote their growth and disassembly, but stabilization of MTs produced excessive focal adhesion growth. Microtubules are also necessary in adhesion receptor recycling, which promotes stable junctions in endothelial cells.

LGN associates with the astral MTs during mitosis through binding Discs Large. Discs Large is recruited to the spindle poles and binds to phosphorylated LGN. This binding promotes astral MT positioning and orientation in Drosophila S2 cells. The mammalian homolog of Discs Large, ZO1, localizes to focal adhesions and promotes their life cycle, and loss of ZO1 drastically reduces cell migration, consistent with our observations in LGN KD.
HUVEC. Another documented interaction that we might consider is pLGN/14-3-3 binding, however this complex has no documented MT association. 14-3-3 has other functions, which includes the stabilization of focal adhesions. If LGN interacts with 14-3-3 in mammalian cells during interphase, the binding of LGN and 14-3-3 would remove the complex from FAs and promote FA disassembly. In LGN KD HUVEC, 14-3-3 would be maintained at the membrane and continue stabilizing FAs, leading to reduced cell migration. While we predict that the primary effect of LGN is on the MT network, we do not exclude the possibility that LGN may participate directly in adhesion turnover.

We predict that LGN influences EC behavior through regulation of the MT network, thus influencing downstream cell migration and adhesion. Previous studies have shown that LGN was required for primary cilia migration during interphase. Another study implicated LGN in pseudopod formation in neutrophils. Our studies utilized established tools and assays to directly characterize LGN function in endothelial cells, and further, we uncoupled LGN function from mitosis in EC. With the evidence that LGN can act during interphase in angiogenic sprouting, we anticipate an expansion of future LGN studies beyond cell division orientation.
Figure 2.1 – Loss of LGN leads to loss of sprout integrity in sprouting angiogenesis

A. qRT PCR showing relative expression of LGN in control and LGN KD treated HUVEC. Samples were normalized to TBP1. Error bars show SEM. B,C. Confocal images showing representative whole beads containing control HUVEC. B shows compressed z-stacks showing GFP reporter (green) and phalloidin (purple) in control (B) or LGN KD (C) beads. The second panel shows colorized z-projection of the phalloidin to distinguish sprout/branch identity. D. Quantification of the number of sprouts per bead in control and LGN KD samples. Bars show SEM. **, p<0.01. E. Quantification of the branching frequency per bead in control and LGN KD samples. Bars show SEM. **, p<0.01. F. Quantification of the frequency of unattached cells per bead in control and LGN KD samples. Bars show SEM. ****, p<0.0001. G. Confocal image showing LGN KD cells undergoing mitosis in a sprout (arrow) and on the bead (arrowhead). H. Quantification of the division angles for control and LGN KD HUVEC undergoing division in sprouts.
Figure 2.2 – Additional shRNA targeted to LGN show defects in angiogenic sprouts.
A. qRT PCR showing relative expression of LGN with two separate targeting sequences against LGN. B. Confocal projections of representative whole beads stained with phalloidin. They are depth projected to distinguish individual sprouts and branches. C. Bar graph showing frequency of branchpoints per bead in control and shRNA treated beads. Bars show SEM. *, p<0.05 D. Bar graph showing frequency of sprouts per bead in control and shRNA treated beads. Bars show SEM. *, p<0.05; **, p<0.01 E. Bar graph showing frequency of unattached cells per bead in control and shRNA treated beads. Bars show SEM. **, p<0.01
Figure 2.3 – LGN KD HUVEC display reduced migratory capacity and directional change.

A. Plots showing traces of individual cell migration tracks for control and LGN KD, axes in μm.
B. Scatterplot showing total distance traveled by control and LGN KD cells over a 12-hour period. Bars show average and 95% CI. *, p<0.05. C. Schematic showing how directional change was measured. Angles were measured between the new direction and the previous direction. D. Distribution plot showing the frequency of events that control and LGN KD cells changed direction.
Figure 2.4 – Additional shRNA against LGN lead to reduced migration in HUVEC

A. Plots showing individual cell movements during the 12-hour imaging period. Axes in μm. B. Scatterplot showing total distance traveled by control and shRNA-treated HUVEC. Bars show mean and 95% CI. **, p<0.01, ****, p<0.0001 C. Distribution plot of directional change with shRNA 6469 compared to control cells. D. Distribution plot of directional change with shRNA 11025 compared to control cells.
Figure 2.5 – Microtubule Dynamics are Disrupted in LGN KD HUVEC

A. Quantification of microtubule length 1 minute post-Nocodazole washout; EV n=210, LGN KD n=188; p<0.0001

B. Scatter plot showing number of microtubule nucleations per cell 1 minute post-Nocodazole washout; bars show 95% CI; n=38 per condition; ns

C. Representative images of EV and LGN KD post-Nocodazole washout; stained with alpha-Tubulin to mark microtubules; scale bar=5 microns

D. Time projections of 60 second movies looking at EB1-
GFP labeled MT plus-ends in EV and LGN KD HUVEC E. Scatter plot showing length of MT growths at the plus end; EV n=10 cells, LGN KD n=11 cells; p=0.0109; bars show SEM F. Scatter plot showing number of nucleations per unit area, bars show SEM; ns.
Figure 2.6 – LGN KD HUVEC maintain some regulation of MT dynamics

A. Quantification of excess centrosomes in control and LGN KD HUVEC. Bars show SEM. B. Scatter plot showing average velocity of microtubule comet growth in control and LGN KD HUVEC. Bars show mean and 95% CI. C. Distribution of MT plus ends based on lifetime length and growth speed. **, p<0.01; ***, p<0.001 D. Rose plot showing the distribution of MT plus end growth angles in control and LGN KD HUVEC. NS.
Figure 2.7 – Focal Adhesions, Adherens Junctions and Membrane Markers are disrupted in LGN KD HUVEC

A. Distribution graph of focal adhesion length in control and LGN KD HUVEC. Bars show SEM. ***, p<0.001; *, p<0.05; **, p<0.01

B. Scatter plot showing the longest 2% FAs in both control and LGN KD HUVEC. Bars show mean and 95% CI. ***, p<0.001

C. Confocal images

D. Confocal images

E. Graph showing VE Cadherin Total Signal

F. Graph showing PECAM Total Signal

G. Graph showing PECAM Total Signal

H. Graph showing PECAM Max Intensity

I. Confocal images

J. Graph showing ICAM2 Total Signal

K. Graph showing ICAM2 Max Signal

A. Distribution graph of focal adhesion length in control and LGN KD HUVEC. Bars show SEM. ***, p<0.001; *, p<0.05; **, p<0.01 B. Scatter plot showing the longest 2% FAs in both control and LGN KD HUVEC. Bars show mean and 95% CI. ***, p<0.001 C. Confocal images
showing VE Cadherin, Dapi, and PECAM staining in confluent EV HUVEC before, during, and after EDTA treatment. Scale bar is 50nm. D. Confocal images showing VE Cadherin, Dapi, and PECAM staining in confluent LGN KD HUVEC before, during, and after EDTA treatment. Scale bar is 50nm. E. Time course showing total VE Cadherin levels with EDTA treatment and recovery. ****, p<0.0001 F. Time course showing total PECAM levels with EDTA treatment and recovery. **, p<0.01; ***, p<0.001 G. Bar graph showing total PECAM signal in control and LGN KD line scans. ****, p<0.0001 H. Bar graph showing maximum signal intensity of PECAM in control and LGN KD line scans. I. Confocal images showing PECAM immunostaining in EV and LGN KD HUVEC monolayers. Scale bar is 50nm. J. Bar graph showing total ICAM2 signal in control and LGN KD line scans. K. Bar graph showing maximum signal intensity of ICAM2 in control and LGN KD line scans. ****, p<0.0001 L. Confocal images showing ICAM2 immunostaining in EV and LGN KD HUVEC monolayers. Scale bar is 50nm.
Figure 2.8 – VE-Cadherin localization is misregulated at Cell-Cell Borders in LGN KD HUVEC

A) Maximum VE-Cadherin signal intensity taken across cell-cell borders; **, p<0.01; bars show SEM. B) Total amount of VE-Cadherin signal above baseline levels; ****, p<0.0001; *, p<0.05. C) Width of VE-Cadherin peak in microns. Widths of highest VE-Cadherin peak determined by start and end of peak at threshold level; p<0.0001; bars show SEM. D) Immunofluorescence of Empty Vector or LGN KD monolayers with and without Triton X-100 treatment. E. Comparison of total VE-Cadherin signal between cell borders of two control cells, one control and one LGN KD cell, and two LGN KD cells. Bars show SEM. **, p<0.01 compared to WT:WT; ***, p<0.001 compared to WT:WT F. Comparison of maximum VE-Cadherin signal between cell borders as described in E. Bars show SEM. **, p<0.01 compared to WT:WT; ****, p<0.0001 compared to WT:WT
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CHAPTER IV – DISCUSSION

A. LGN IS REQUIRED FOR ENDOTHELIAL SPROUT MORPHOLOGY

I have presented data supporting an interphase role for LGN in endothelial cells, via microtubules and downstream cellular behaviors. LGN is required for vascular sprouting, cell migration, and adhesion stability. Endothelial cells require proper coordination of migration and adhesion to generate and maintain sprouts, and these functions are impaired by MT stabilization\(^1,2\). Previous studies established that LGN is in a complex that interacts with microtubules at the plus end, but in the context of cell division\(^3-5\). Here I show that LGN is not required for orientation of cell division in 3D sprouts, but it does contribute to sprouting via interphase effects.

Early studies of LGN determined that the TPR and GoLoco domains were in loose association during interphase and when no other binding partners were present\(^3\). This earlier study concluded that the closed conformation coincided with LGN inactivity because only NUMA, G-alpha-i, and Insc were identified binding partners\(^6-8\). Additionally, LGN/NUMA binding only occurred during mitosis, further establishing the precedent of closed/inactive versus open/active LGN. At the time of that study, there was little known about the Linker region, and it was thought that the Linker simply provided structural flexibility\(^3,9,10\). Over the last several years, two groups have identified sites of phosphorylation in the Linker region that are necessary for LGN function\(^10,11\). These studies did not determine the conformational state of LGN when phosphorylated. Two separate groups have since investigated LGN structure in greater detail, yet failed to address phosphorylation states\(^12,13\). I predict that the Linker region can be
phosphorylated regardless of LGN’s conformational state. This prediction fits with previous studies that suggest that is closed during interphase without restricting LGN activity to mitosis, as previous models have done.

I showed that LGN is involved with MT dynamics in endothelial cells, which has not been previously shown. Previous studies of LGN in the developing mouse epithelium focused on LGN during fate specification, showing a spatial and temporal requirement for LGN\textsuperscript{14,15}. Temporally, angiogenesis occurs after endothelial differentiation\textsuperscript{16}. It is reasonable to assume that post-differentiated endothelial cells adapt mitotic polarity proteins for functions separate from division orientation. Polarity proteins, including aPKC and the Par polarity complex, direct cell fate specification through asymmetric divisions, yet remain active in terminally differentiated cells\textsuperscript{17,18}. Par polarity proteins, which are also necessary for oriented division in the epithelium, are required for endothelial cell sprout, branching, and migration, but seemingly not for endothelial division orientation (Pelton, observations)\textsuperscript{11,19,20}. I predict that endothelial cells utilize LGN to moderate MT turnover in order to regulate EC migration and morphogenesis.

**B. LGN REGULATES MICROTUBULE TURNOVER**

LGN facilitates astral microtubule anchoring during mitosis\textsuperscript{4,11,21,22}. Previous studies predicted a requirement for LGN in establishing spindle polarity in a tissue\textsuperscript{23–25}. However, I showed that angiogenic sprouts successfully oriented their divisions despite severely reduced LGN levels. Additional unpublished evidence from the Bautch lab shows that G-alpha-i is not required for division orientation (DalPra et al, in preparation). Endothelial divisions are able to orient despite loss of established mechanisms of mitotic polarity. I predict that endothelial cells rely on their shape and morphology to establish division orientation. Cell shape dictates bipolar
spindle formation, and aberrant changes in shape results in spindle defects\textsuperscript{26–28}. Endothelial cells are elongated along the proximal/distal sprout axis\textsuperscript{29} and LGN KD did not alter EC elongation in the sprouting angiogenesis assay (Wright, observations). The results from the sprouting angiogenesis model led us to conclude that LGN is not required for orientation of cell division, but instead has novel influences on EC behavior.

Endothelial cells require careful coordination of the microtubule network to effectively participate in sprouting and other cellular behaviors\textsuperscript{30–32}. The microtubules participate in the localization and assembly of complexes at the membrane to influence cell migration, adhesion, and overall cell morphology\textsuperscript{33–35}. However, the inverse is also true, any changes to cell adhesion and morphology will influence the microtubules. It is difficult to predict if LGN affects the MT directly, or if the MT phenotype is downstream of an LGN effect. Because there are no known direct LGN/MT interactions, I would not predict that LGN influences MT stability directly. It is unlikely that all LGN interactions are known, so I cannot exclude the possibility that LGN could directly act with MT growth regulators (Fig 3.1).

In LGN KD HUVEC, I observed stabilized MTs, suggesting that LGN acts on the growing MT to regulate turnover. MT nucleation occurs at the centrosome and the switch from catastrophe to MT growth occurs near the membrane\textsuperscript{36}. Both MT nucleation and MT plus-end frequency were not affected by LGN KD, which suggests that LGN acts on MTs to promote turnover and catastrophe. It is not currently feasible to directly measure catastrophe in cells, but imaging purified tubulin in the presence of cell extract with and without LGN would provide more conclusive evidence\textsuperscript{37}.

LGN undergoes directional movement along microtubules between the spindle pole and the membrane\textsuperscript{38}. LGN transport is only documented during mitosis; however, I predict that LGN
transport along MTs also occurs during interphase. LGN plus-end transport is mediated through Dlg and khc73, which are not cell-cycle limited, and LGN-Dlg binding is observed in unsynchronized, whole-tissue *Drosophila melanogaster* lysates\(^4,10,39\). Dlg is present at cell junctions and khc73 moves Dlg toward the membrane where Dlg binds to LGN. Recent evidence shows khc73 localizes to the MT plus-ends, generating a link between MT and the membrane; however, Dlg does not localize to the plus-ends\(^40\). The LGN/Dlg/khc73 complex has not been characterized in mammalian cells, but I predict that this complex may mediate LGN effects in endothelial cells.

C. MODELING A MECHANISM FOR LGN ACTIVITY THROUGH A PREDICTED BINDING PARTNER, ZO-1

I propose that ZO-1, the mammalian homolog of Dlg, is a candidate binding partner for LGN and I present a model wherein LGN cooperates with ZO-1 at the migratory front to promote cell migration through MT turnover\(^41\). ZO-1 contains the LGN-binding sites that were identified in Dlg\(^13\). ZO-1 acts at the migratory front to promote cell migration and focal adhesion turnover\(^42,43\). Depletion of ZO-1 inhibits cell migration and leads to accumulation of focal adhesions, just as I observed with LGN KD in ECs. LGN is phosphorylated by Aurora A kinase specifically at the spindle poles during mitosis to generate the Dlg/ZO-1 binding site\(^10\). This timing potentially excludes LGN/ZO-1 binding from occurring at the migratory front and during interphase. However, the kinase target site on LGN is the same for Aurora A and aPKC, creating potential for aPKC-mediated phosphorylation enabling LGN/Dlg(ZO-1) binding. Spatially and temporally, aPKC and ZO-1 are co-localized and active in migrating cells\(^43,44\). I propose that aPKC phosphorylates LGN, leading to the stabilization of ZO-1 at the membrane to promote
migration (Fig 3.1). A caveat to this proposal is that it is dependent upon hypothetical interactions that have not been directly observed. As attractive as this proposal is, it would be prudent to consider other possible LGN mechanisms that are based on existing data.

D. AN ALTERNATE MODEL FOR AN LGN MECHANISM THROUGH A KNOWN BINDING PARTNER, 14-3-3

LGN binds to 14-3-3, a known promoter of focal adhesion growth and stability. This binding occurs in mammalian epithelial cells and leads to the removal of LGN and 14-3-3 from the membrane. Removing 14-3-3 from the membrane prevents FA stabilization, a feature seen in cells with hyper-migratory behavior. I predict that LGN/14-3-3 binding is necessary to re-direct 14-3-3 localization to the cytoplasm and promote FA turnover. FA turnover occurs at the migratory front, downstream of cdc42 and aPKC signaling. Coincidentally, phosphorylation of LGN by aPKC generates the 14-3-3 binding site. I propose that aPKC phosphorylates LGN at the migratory front to generate the LGN/14-3-3 complex and promote migration (Fig 3.1). This model is a strong alternative to LGN/ZO-1 acting to regulate the microtubules and relies on more conclusive evidence.

E. EVIDENCE THAT LGN FUNCTIONS THROUGHOUT THE CELL CYCLE

This thesis presents evidence that LGN influences EC behavior by regulating the MT network during interphase, directly influencing downstream behaviors. The early idea that LGN is active only in mitosis is outdated, and surpassed by a growing body of evidence that NUMA is not the only possible interacting partner with LGN via the TPR domain. While LGN[NUMA interactions are restricted to mitosis, other binding partners (Dlg, 14-3-3, G-alpha-i) are present
and available throughout the cell cycle\textsuperscript{45–47}. There is no direct evidence that LGN cannot bind with the aforementioned partners during interphase. Additionally, LGN mechanistic studies in terminally differentiated mammalian cells are limited to the MDCK epithelial cyst model\textsuperscript{3,11,22}. MDCK cysts are highly polarized and depend on apical/basal/lateral polarity to maintain ideal morphology. It would be prudent to directly investigate LGN mechanisms in endothelial cells.

LGN-protein binding occurs in vitro and in vivo. Experiments that identified and characterized LGN interactions drew conclusions from unsynchronized samples, and therefore, non-mitotic interactions cannot be fully excluded\textsuperscript{3,10,11}. LGN does exhibit cell-cycle-dependent expression changes in HeLa, with an increase in protein levels during mitosis\textsuperscript{3}. To further understand LGN behavior in ECs, LGN expression patterns throughout the cell cycle would be useful information. I predict that LGN expression will remain significant in EC throughout the cell cycle because I observed interphase effects of LGN KD in endothelial sprouts.

Recent studies of LGN in primary cilia indicate that LGN is necessary for cilia migration\textsuperscript{48}. However, the phenotype presented in daughter cells following LGN-null mitoses. The dividing cells failed to properly segregate Notch signaling components and the resultant daughter cells were not sufficiently polarized for migration. Similar to primary cilia, the developing mouse epithelium requires LGN to properly segregate members of the Notch pathway\textsuperscript{14}. The primary cilia and epithelium require a series of divisions to stratify the developing tissue whereas endothelial cells use mitoses to elongate vessels. I have shown that endothelial cells do not require LGN for mitosis in the 3D sprouting model, yet LGN is expressed, suggesting that ECs utilize LGN differently.

LGN may have a role in neutrophil chemotaxis in the mammalian immune system\textsuperscript{49}. Neutrophils respond to chemical changes in the environment and migrate toward the signal,
through the generation of pseudopods. Pseudopod formation requires directional migration and cytoskeletal organization through the actin network. G-alpha-i signaling promotes neutrophil polarization through AGS3 and possibly LGN. However, the studies failed to make clear distinctions between AGS3 and LGN, which are related, but non-homologous proteins. AGS3 is involved in G-protein signaling and downstream activation of G-protein-dependent pathways, whereas LGN does not influence the signaling cascade. Still, it is interesting that AGS3 knockout in neutrophils showed reduced cell migration and failure to polarize, similar to LGN KD in EC. It is not clear if AGS3 and LGN are interchangeable in any pathway, but it is an important distinction to make, especially given the growing evidence that LGN has a broader function.

F. ROLE OF LGN IN MAMMALIAN EPITHELIUM AND ENDOTHELium

The in vivo study of LGN has generated complex conclusions about the importance of LGN during development. In Drosophila melanogaster, LGN-null flies reach adulthood with no global phenotype, despite significant defects in oriented division at different stages of development. A genetic mouse model globally truncated LGN, leaving only the TPR and the Linker domains intact, which was not lethal, despite measurable changes in neuroepithelial development. Selective depletion of LGN in the mouse epidermis during embryonic development halts epidermal development and leads to postnatal lethality. It is difficult to speculate about the consequences of LGN loss in the vasculature because the current in vivo data have conflicting conclusions for LGN dependency. Studies of polarity in vivo and the vasculature are a stronger predictor for how endothelial cells would respond in an LGN null experiment. I predict that LGN depletion in the mouse vasculature will phenocopy aPKC and
planar cell polarity knockout models. I predict that LGN acts downstream of aPKC and PCP polarity, both of which have been studied in the mouse endothelium. Perturbation of these pathways showed overall delay in vessel plexus development and defects in MTOC polarization, coincident with LGN KD in vitro data (C. Lee and Bautch, observations)\textsuperscript{55}.

I predict that endothelial-specific loss of LGN in the mouse will result in reduced vascularization of the organism. Proper vascularization depends on EC responses to hypoxic signals to generate vessel networks via sprouting and branching (Fig 3.2A). The formation of sprouts require that ECs remain adhered to each other while migrating toward a stimulus. LGN KD HUVEC display both a reduced ability to maintain stable adhesions and directional migration, suggesting that LGN KD vessel networks will have reduced coverage (Fig 3.2B).

G. SUMMARY

Endothelial cells require LGN in order to properly maintain migration and adhesions, potentially downstream of the microtubule network. Despite extensive work indicating that LGN is essential for oriented cell division, LGN is dispensable for endothelial cell division in three-dimensional sprouts. Based on previous studies of LGN, I predict that ECs utilize LGN during interphase via polarized aPKC phosphorylation. I predict that LGN is necessary for formation and maintenance of the vasculature because endothelial cells require polarization at the cellular level to generate vessel networks at a tissue level. Future studies of LGN will determine if it is required at an in vivo level and establish the exact mechanism for LGN function in endothelial cells.
LGN is phosphorylated by aPKC near the membrane at the migratory front. This facilitates LGN binding to 14-3-3 or Discs Large/ZO-1 leading to downstream effects on cell adhesions and migration. There is also potential for LGN having direct interactions with a microtubule-growth regulator, which would account for LGN KD phenotypes.
Figure 3.2 – Endothelial cells require LGN for proper migration and adhesion to generate an effective vessel network.

A) Wild type ECs have tightly regulated adhesions which promote cell-cell contacts. Wild type ECs migrate away from the parent vessel in a dedicated direction without losing contact to the vessel. B) LGN KD ECs fail to maintain stable junctions and are unable to effectively migrate, leading to reduced branching and vessel integrity. I predict this will generate a sparse, inefficient vascular network that is incapable of supporting tissue health.
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