THE FUNCTIONAL AND COMPARATIVE STUDIES OF SONIC HEDGEHOG SIGNALING PATHWAY IN ANOLIS SAGREI AND FORMING AND MANAGING GROUPS IN UNDERGRADUATE LABORATORY CLASSES

by

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(Under the Direction of DOUGLAS B. MENKE AND

JONATHAN EGGENSCHWILER)

ABSTRACT

The primary goal of this research is to increase the repertoire of molecular tools and resources for functional and genetic experiments in *Anolis sagrei*, a reptilian model system for developmental and evolutionary studies. I applied these new tools to investigate mechanisms of Hedgehog (Hh) pathway induction in reptiles and the role of a limb specific *shh* enhancer in reptilian limb development. To understand the mechanism of Hh induction in reptiles, I evaluated the role of primary cilia in Hh signaling using a novel cell culture system that I generated for this purpose: an immortalized, clonal lizard embryonic fibroblast cell line (ASEC-1). Using CRISPR/Cas9, I successfully created targeted mutations in the *ift88* gene, which is known to be required for ciliogenesis in other species. The *ift88* mutant cell lines that I generated lack cilia, have lost their sensitivity towards Hh signaling, and show increased basal expression of *gli*1. My data suggests that in reptiles *gli*1 might be the major activator of the Hh pathway and, potentially, the predominant role of primary cilia reptiles may be to silence the pathway.

Beyond my cell culture studies, I tested the functional importance of *ZRS*, a deeply conserved limb enhancer for the *shh* gene. Recent studies in snakes suggest that the *ZRS* underwent gradual sequence degradation that contributed to the loss of *shh* expression in the developing limbs and the evolution of limb reduction in this group of squamate reptiles. However, functional tests of snake *ZRS* elements have only been performed in mice. Ideally, the phenotypic consequences of sequence changes in the snake *ZRS* should be assessed in limbed reptiles rather than mammals. Using the CRISPR/Cas9 and CRISPR/Cas12a system, I have successfully deleted portions of the brown anole *ZRS* and ~33% of these mutants show a range of limb-related phenotypes.

Along with these projects, I worked on an education research project related to group work from the instructor's point of view in an Undergraduate Biology laboratory class. My work explores differences in students' behavior in self-selected vs randomized groups and suggests a strategy to identify poor group members for instructor intervention.

INDEX WORDS: Shh, ZRS, Primary cilia, Reptiles, Lizards, Anolis sagrei, Limb loss, gene editing, CRISPR, enhancers, group work, group conflicts, CRISPR/Cas9, CRISPR/Cas12a

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DEDICATION

I dedicate this work to all the lizards and mice who contributed to this quest for knowledge.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Anolis sagrei as a reptilian model system

Squamate reptiles consisting of lizards and snakes show tremendous diversity in a variety of morphological traits including but not limited to limb length, digit morphology and numbers, body plan, and pigmentation. According to the reptile database, there are around 11,000 recognized species of squamates (Uetz P et al.,2022). Squamates have been subjects of studies of evolution, ecology, behavior, physiology, development, and neuroendocrinology for many decades (Kabelik & Hofmann, 2018; Losos et al., 2001; Sanger & Kircher, 2017; Wadet, 2005). Among squamates, *Anolis* lizards have become a leading model system to study reptile evolution, behavior, and biology (Sanger & Kircher, 2017). The convergent evolution studies of "ecomorphs" on different Caribbean islands have generated rich literature about the biology of this species (Losos et al., 1994, 2001). However, functional genomic experimental studies have not been possible in this group of organisms for the lack of a model system where gene editing and transgenic approaches are possible.

Among the known 400 species of the *Anolis* genus, we opted to work with *Anolis sagrei. Anolis sagrei* or brown anole is a small lizard native to Cuba and the Bahamas. It is invasive and found in abundance in Florida and southeastern states in the USA. This species breeds well in captivity (Sanger et al., 2008). A well-annotated genome assembly

for *Anolis sagrei* is available. The small size, ease of husbandry, abundant availability, high fertility, and continuous egg production makes brown anole almost the perfect candidate for experimental genetics and development studies (Sanger et al., 2008). Recently, a novel approach for CRISPR/Cas9 mediated gene editing in *Anolis sagrei* lizard was established (Rasys et al., 2019). This makes brown anole an attractive model for developing more tools for testing functional genetics. The current study aims to develop new reagents and increase the repertoire of molecular tools for gene editing and genomic studies in *Anolis sagrei* to establish brown anole as a reptilian model system. This model will hence become a valuable resource to study the genetic basis of convergent evolution and gene function to explore reptilian biology.

Decades of research in developmental biology and the growing availability of complete genome sequences from a wide range of organisms provided insight into the evolution of key signaling pathways regulating the complexity of form and function both across the phyla and within individual species (Ingham et al., 2011). Hedgehog signaling (Hh) is among the limited number of signaling pathways that have a central role in the development of metazoans (Ingham et al., 2011). Mutations in Hh genes were first identified in the genetic screen of *Drosophila* which affected larval body plan (Nüsslein-Volhard & Wieschaus, 1980). Since its discovery, the Hh signaling pathway components have been studied extensively in both invertebrates and vertebrates. Genetic studies in *Drosophila*, mice, and zebrafish revealed that the core components and framework of the Hh signaling pathway are highly conserved (Huangfu & Anderson, 2005; Ingham et al., 2011). Squamate reptiles are evolutionarily positioned between fish and mammals. Studying Hh signaling in squamate reptiles will provide new information about the complex

mechanism, regulation, and components of the Hh signaling pathways. Hence, in this study, we focused on functional and comparative studies of Hh signaling in *Anolis sagrei*.

Hh signaling in vertebrates

The name Hedgehog is derived from the short and "spiked" phenotype of the cuticle of Hh mutant *Drosophila* larvae (Nüsslein-Volhard & Wieschaus, 1980). There are three homologs of Hh ligands in mice and chicken namely *Desert hedgehog (Dhh)*, *Indian hedgehog (Ihh), and sonic hedgehog (Shh)*. *Ihh* and *Shh* are more closely related to each other than *Dhh*. *Dhh* is closer to *Drosophila Hh*. All these three genes are involved in regulating embryonic tissue patterning (Varjosalo & Taipale, 2008). *Shh* has a regulatory role in tooth and hair development, cell proliferation, neural tube, and limb patterning (Chiang et al., 2001; Dassule et al., 2000; Dessaud et al., 2008; St-Jacques et al., 1998). *Ihh* has an important role in skeletal development (Long et al., 2001). The role of *Dhh* is restricted to gonads and is involved in the regulation of spermatogenesis (Bitgood et al.,1996, Chiang et al.,1996, Zhang et al.,2001, Nygaard et al.,2015). Zebrafish have 5 Hh homologs: *shh*, *Ihh, dhh, echidna hedgehog(ehh)* and *twiggywinkle hedgehog (twhh)* (http://zfin.org). Shh signaling is required for tooth morphogenesis in squamates and *Ihh* has a significant role in lizard tail regeneration (Handrigan & Richman, 2010; Lozito & Tuan, 2015, 2016).

All Hh family proteins act as morphogens and regulate the cell fates in an autocrine and paracrine manner. Immature Hh proteins undergo autoproteolytic cleavage followed by lipid modification to link cholesterol moiety at its carboxyl terminus. Cholesterol modification is then followed by palmitoylation. Lipid modifications restrict the free mobility of Hh proteins by their retention in the plasma membrane. The post translational

modifications of Hh proteins are necessary for their secretion from the cells synthesizing them by the activity of a transmembrane 'Dispatched'(Disp) transport proteins (reviewed in Briscoe & Thérond, 2013). The mechanism of movement of Hh proteins over the long range is unclear. It appears to involve passive diffusion, active transport and /or transcytosis (Varjosalo & Taipale, 2008). The secreted Hh proteins are received by cells with transmembrane protein receptors. Patched1 (Ptch1) and the vertebrate specific HH-interacting protein 1 (Hip1) are the known receptors for Hh ligands. These proteins anchor the Hh ligand, restricting its spread and hence the availability of the ligand. Both these receptors are induced by Hh signaling and create a negative feedback loop further restricting Hh mobility (Briscoe & Thérond, 2013; Chuang & McMahon, 1999).

The basic regulatory logic of the Hh signaling is conserved between invertebrates and vertebrates. Hh ligand binds to its receptor transmembrane protein Patched1 (ptch1). Ptch1 is a negative regulator of the Hh signaling pathway. In absence of the Hh ligand, Ptch1 inhibits activation of another seven transmembrane protein Smoothened (Smo). Smo acts as a positive regulator of the Hh signaling pathway. Active Smo in turn regulates the modification of a bifunctional transcription factor Cubitus interruptus (Ci) in *Drosophila* and Gli proteins in vertebrates (Huangfu & Anderson, 2005; Taipale et al., 2002). However, in vertebrates Hh signaling requires primary cilia, a microtubule-based single organelle protruding out of the vertebrate cell surface, for transduction of the pathway (Huangfu et al., 2003). The trafficking of core components in and out of the cilia indicates that Smo and downstream proteins may require localization in the primary cilia for their activity (Corbit et al., 2005; Haycraft et al., 2005; Rohatgi et al., 2007).

Ptch1 is located on the ciliary membrane. In the absence of the ligand, Ptch1 inhibits the activation of Smo by excluding it from the cilium. Upon binding of the Hh ligand, Ptch1 exits the cilium allowing Smo to accumulate in the cilium. The enrichment of primary cilium is a necessary step for the activation of Smo (Corbit et al., 2005; Rohatgi et al., 2007, 2009). Activation of Smo, in turn, regulates the processing of three Gli transcription factors (Gli1, Gli2 and Gli3). In absence of the Hh ligand, the suppressor of fused (SUFU) forms a protein complex with full length Gli proteins which then are moved out of the cilium, and then their phosphorylation is mediated by Protein kinase A (PKA). Phosphorylation of Gli proteins triggers its proteolytic cleavage to the repressor form (GliR) which in turn can translocate into the nucleus and repress transcription of the target genes (Bangs & Anderson, 2017; Briscoe & Thérond, 2013; Humke et al., 2010; Svärd et al., 2006). Upon accumulation of Smo in the primary cilium, a G protein coupled receptor protein GPR161 exits the cilium. GPR161 is a negative regulator of the Hh pathway as it increases cAMP levels by activating adenylyl cyclases and hence helps in the activation of PKA (Corbit et al., 2005; Mukhopadhyay et al., 2013). Upon activation, Smo dissociates GLI-SUFU complex full-length activated GLI proteins (GliA) exit cilia to translocate to the nucleus where they promote transcription activation of Hh target genes (Bangs & Anderson, 2017; Briscoe & Thérond, 2013; Wheway et al., 2018). There are additional components of the Hh pathway and ciliary proteins which are responsible for the transport of Gli proteins in and out of cilium and processing of the Gli proteins, which are not described here. Accumulation of Gli proteins at the tip of the cilium in response to Smo activation is a key step for the activation of the Hh pathway (Haycraft et al., 2005; Liu et al., 2005).

In vertebrates, primary cilia act as a specialized center for receiving the Hh signal and processing Gli proteins. The ratio of Gli activators and Gli repressors decides the cell's graded response to the Hh signal. Hence, in vertebrates, primary cilia mediate Hh signaling by modulating Gli protein activities. This response is tissue-specific and developmental time specific. *Gli1* lacks the repressor domain and hence always acts as an activator. *Gli*2 and *Gli*3 can act as both activators and repressors (Falkenstein & Vokes, 2014).

Within the vertebrate lineage, the role of Gli proteins has diverged. In mice, *Gli*2 is a major activator of the pathway and *Gli*3 majorly acts as a repressor (Bai et al., 2002; Ding et al., 1998; Mo et al., 1997). Activation of *Gli*2 in mice depends on cilia (Eggenschwiler & Anderson, 2007; Santos & Reiter, 2014). In zebrafish, *gli*1 is the major activator of the pathway and *gli*2 can act as a minor activator or repressor (Huang & Schier, 2009; Tyurina et al., 2005). In chicken *gli*2 and *gli*3 can act as both activators and repressors (Yt et al., 2016). As the use of distinct Gli proteins for aspects of Hh signaling appears to be evolving in vertebrate lineages, we are interested in studying Hh signaling and the role of Gli proteins in reptiles which diverged from mammals more recently than did ray-fin fishes (~320 vs. ~450 Mya).

Primary cilia structure, the role of intraflagellar transport, and Hh signaling

Primary cilia are microtubule-based single organelle protruding out of the vertebrate cell surface (Sorokin,1968). Cilia and flagella are highly conserved ancient structures that are found in a diverse range of organisms from single-celled eukaryotes to humans (Pazour et al., 2000, 2002). The ciliary structure can be divided into subdomains- basal body, transition fibers, and axoneme. At the exit of the cell cycle, an older centriole of the centrosome transforms into a basal body and is responsible for the position and orientation

of the primary cilium on the cell surface (Sorokin 1968; Lechtreck et al.,1999). The basal body consists of 9 microtubule triplets, two of which are extended to form the axoneme, which is the backbone of the ciliary structure. Axoneme consists of 9 doublets of α and β tubulin polymers (Fawcett & Porter,1954; Manton and Clark, 1952; Taschner et al., 2012). Typically, motile cilia have a 9+2 axoneme composition and primary, non-motile cilia have a 9+0 composition. The basal body is attached to the plasma membrane by proteinous fibers called 'Transition fibers'. (Nachury et al.,2010, Rosenbaum et al.,2002). Distal to the basal body transition zone is present which comprises microtubule fibers of axonemes connecting it to the ciliary membrane. Transition zone functions as a diffusion barrier for inappropriately targeted proteins creating an exclusive ciliary environment (Lechtreck et al.,1999; Gigante & Caspary, 2020; Garcia-Gonzalo and Reiter, 2017; Reiter et al., 2012). The ciliary membrane is biochemically unique and has a lipid and protein composition distinct from the plasma membrane. This feature is crucial for the sensory function of the primary cilium (Taschner et al., 2012).

There is no molecular machinery present for protein synthesis in the primary cilium. Hence to synthesize and maintain ciliary structure, cilia depend on the intraflagellar transport (IFT) system. IFT is an evolutionarily conserved process required for the assembly and maintenance of cilia in all the ciliated species from *Chlamydomonas* to mammals (Pazour et al., 2000, 2002). It is a bidirectional molecular cargo to transport proteins to and from cilia (Rosenbaum & Child,1967). Anterograde IFT particles move proteins toward the distal tip of the cilium via the kinesin-2 motor and retrograde IFT particles move proteins from the tip of the cilium via dynein-2-motor (Kozminski et al.,1995, Pazour et al.,1995, Gigante & Caspary, 2020). There are 6 IFT-A proteins IFT140, IFT144,

IFT143, IFT121, IFT122, and IFT139 involved in anterograde transport. Retrograde transport IFT- B core complexes contain 9 proteins namely IFT88, IFT70, IFT74, IFT81, IFT52, IFT46, IFT27, and IFT25. There are five peripheral IFT-B proteins IFT172, IFT180, IFT57, IFT54, and IFT20 (Taschner et al., 2012). I recommend referring to Taschner et al. (2012) for a detailed review of the IFT proteins.

Any disturbances in IFT proteins along with other ciliary proteins affect ciliogenesis and ciliary length. In humans, several genetic syndromes associated with abnormalities in the ciliary assembly with phenotypic consequences, including but not limited to the kidney and skeletal development, are commonly called ciliopathies (Bangs & Anderson, 2017). Targeted null alleles of *Ift88* and *Kif3a* completely ablated cilia in mice with loss of *Shh*-dependent ventral neural cell types (Huangfu et al., 2003). *Ift172* null mice display *Shh*-dependent loss of ventral neural cell type (Huangfu et al., 2003) *Dync2h1* functions in retrograde IFT dynein motor. Mice lacking *Dync2h1* have shortened bulbous cilia and loss of *shh* dependent ventral neural cell type (Huangfu & Anderson, 2005). Similarly, mutations in *Ift52,54* and *57* inhibited cilia formation, and mice showed loss of *Shh* dependent ventral neural cell types (Berbari et al., 2011; Houde et al., 2006; Liu et al., 2005). *Ift88* null zebrafish lack cilia but have milder phenotypic defects associated with the Hh-dependent patterning. This phenotype was different in severity than mice and humans as the role of Gli proteins is diverged in fish and mammal lineage (Huang & Schier, 2009). The double mutant studies in mice involving *Ift172*, *Ift88,* and *Kif3a* indicate the role of the IFT proteins is downstream of Ptch1 and Smo axis but upstream of Gli proteins (Huangfu et al., 2003).

Cilia dynamics is closely associated with the cell cycle. The primary cilia are generally characteristics of cells in G0/G1 phase and often during the S/G2 phase (Plotnikova et al.,2009). Ciliary assembly occurs in the G1 phase of the cell cycle, and they disassemble before mitosis (Ho & Stearns, 2021), In a given cell cycle the temporal dynamics between ciliary assembly and disassembly generates variability in cilium formation and hence in Hh signaling. The developmental consequences of the cell cycle, cilia formation, and Hh signaling are worth investigating (Ho & Stearns, 2021).

Evolution of cilia and vertebrate-specific link to Hh signaling

In invertebrates, Hh signaling is independent of the cilium. *Drosophila melanogaster* does not require primary cilia for Hh signaling (Goetz & Anderson, 2010). RNAi experiments to reduce Cos2/Kif27/Kif7, Fused, or Iguana proteins in planarians affected the ciliogenesis however, Hh signaling remained unaffected. This indicates that cilia are not required for Hh signaling but there is a conserved link mechanism between Hh signaling (Rink et al., 2009). Hh signaling in echinoderms like sea urchins is mediated via motile cilia during embryonic musculature patterning (Walton et al., 2009; Warner et al., 2014). Fuzzy mutants in *Xenopus* provide indirect evidence of the requirement of cilia for Hh signaling. Ciliogenesis is affected in fuzzy mutants, and they show Hh signaling defects as a secondary phenotype indicating the requirement of cilia for Hh signaling (Park et al., 2006). Milder phenotypic defects associated with the Hh-dependent patterning in *ift88* null zebrafish lacking cilia are observed (Huang & Schier, 2009). *Talpid3* encodes a centrosomal protein essential for cilia formation. *Talpid3* mutant chickens have abnormalities in *shh* associated limb formation associated with an inability to receive signals (Yin et al., 2009). The association of cilia and Hh signaling in mice and humans is discussed in the previous

section. The components of the Hh signaling also evolved and diverged in the vertebrate lineage. Fused is a positive regulator of the pathway in *Drosophila* and zebrafish and does not have any role in mammal Hh signaling. Fused however is required for ciliary motility in both zebrafish and mice (Wilson et al., 2009; Wolff et al., 2003). There is a possibility that Hh signaling coevolved with the motile cilia (Warner et al., 2014).

To explain the coupling of non-motile primary cilia and the Hh signaling pathway, Goetz & Anderson (2010) propose that *Kif7*, the vertebrate homolog of *Drosophila Costal2 (Cos2) kinesin*, as a connecting link. *Cos2* and *Kif7* carry out similar functions as positive and negative regulators of the pathway. Cos2 can bind to microtubules, but it does not have a motor function. Kif7 on other hand has a motor domain which is typical of the kinesin motors (Endoh-Yamagami et al., 2009; Farzan et al., 2008). Goetz & Anderson (2010) propose a model that in absence of the ligand, Kif7 functions as a ciliary motor transporting Gli2 out of the cilium to avoid its activation. In presence of the ligand when the pathway is activated, Kif7 functions as an Shh pathway component and promotes Gli activation at the ciliary tip by inhibiting SUFU activity. This dual role of Kif7 might be the reason for the connection between primary cilia and Hh signaling in vertebrates.

In vertebrates, Hh signaling requires primary cilium for receiving ligand signal, pathway activation and, processing Gli transcription factors (Bangs & Anderson, 2017; Corbit et al., 2005; Haycraft et al., 2005; Ho & Stearns, 2021; Huangfu et al., 2003; Liu et al., 2005; Rohatgi et al., 2007; Santos & Reiter, 2014). This role in Hh signaling has also diverged in the vertebrate lineage in the context of Gli protein function. In mammals, *Gli1* and *Gli2* both are the activators of the pathway and Gli3 has the repressor role. However, *Gli2* is the predominant activator of the pathway. (Bai et al., 2002; Ding et al., 1998; Hui &

Joyner, 1993; Matise et al., 1998; Mo et al., 1997; Wang et al., 2019). Genetic evidence in mouse embryos lacking cilia shows a pronounced loss of ligand-induced Hh pathway activation and mild ligand-independent (derepressed) activity (Chen et al., 2009; Jia et al., 2009). In these mutants, cilia dependent *Gli2* activation is lost which in turn reduces the activation of *Gli1* drastically. Hence the derepression observed in mice is through the loss of the Gli activator function. Hence, in mammals, the major role of primary cilia appears to be the activation of the pathway. In zebrafish, *gli1* is the essential activator of the Hh signaling pathway while *gli2* has a minor role as an activator or repressor depending on the tissue context (Karlstrom et al., 2003). Depending upon tissue specificity and developmental time, *gli3* can function as both an activator and repressor (Tyurina et al., 2005). In contrast, zebrafish lacking cilia display strong ligand independent Hh derepression that cannot be further increased by Hh ligands (Huang & Schier, 2009). The derepression of the pathway observed in zebrafish is through a reduction in gli repressor activity as a result of loss of cilia and ligand independent activation of *gli1*. Hence, in fish, the role of primary cilia appears to be repression of the pathway. Our experimental model system *Anolis sagrei* provides a unique evolutionary position between fish and mammals to study the divergence of Gli protein functions and the role of primary cilia in the vertebrate lineage.

Limb development and shh

Tetrapod limbs are diverse structures whose development is regulated by complex gene regulatory networks (Tickle, 2015)**.** The basic structure of the tetrapod limbs comprises a single stylopod (Humerus/ Femur), paired zeugopods (radius and ulna/ tibia and fibula), and multiple autopod elements (hand plate/foot plate) (Zhu & Mackem, 2017).

The early limb bud is derived from the lateral plate mesoderm cells and arises in the flank region of the embryo with the core mesenchymal cells surrounded by epidermis along the anterior-posterior axis (Tickle, 2015). Before the formation of the limb buds, the cells in the early limb fields have already established the differential developmental potential across an antero-posterior and dorso-ventral axis. As a result, the early limb bud has two organized /signaling centers that regulate early limb bud patterning and growth (McQueen & Towers, 2020).

John Saunder characterized these two singling centers as the apical ectodermal ridge (AER), (the thickened epithelium that arises at the boundary between dorsal and ventral ectoderm and then surrounds the limb bud tip) and the zone of polarizing activity (ZPA) (Saunders, 1948; Saunders and Gasseling, 1968; Tickle, 2015). The importance of ZPA in defining the anterior-posterior axis of the limb bud was identified by embryological experiments in chicken wing buds. Saunders grafted the tissue from the posterior margin of the wing bud into the anterior margin of the other wing bud resulting in a mirror-image digit pattern (Tickle & Towers, 2017). Later it was discovered that mesenchymal cells in ZPA secrete a morphogen Shh that regulates the anterior-posterior axis of developing vertebrate limb bud. Shh is required to establish the anterior-posterior axis and digit identity (Riddle et al., 1993). The dorsal-ventral axis of the limb bud is established by *Wnt7a* in the dorsal ectoderm and BMP in the ventral ectoderm (Parr & McMahon, 1995; Pizette et al., 2001). AER secretes fibroblast growth factors (FGFs) which regulate the proximodistal axis of the limb bud and outgrowth. The *Fgf10 -F*gf8 feedback loop controls the early limb bud growth (Ohuchi et al., 1997). AER and ZPA signaling centers interact and maintain each other's activity via interlinked feedback loops hence maintaining limb

patterning and growth (Benazet & Zeller, 2013; Ohuchi et al., 1997; Pizette et al., 2001; Tickle, 2015).

Homeobox family genes (Hox) genes are involved in determining the position of the limbs (Tickle, 2015). Functional inactivation of *Hoxb5* in mice and disruption of the *Hox4- 9* gene expression pattern in chicken shifts the position of forelimbs more anteriorly (Moreau et al., 2019; Rancourt et al., 1995). The hindlimbs were repositioned because of the ablation of *Hoxc8* in mice (van Den Akker et al., 2001). Evidence suggests that RA affects the Hox gene expression (Langston & Gudas, 1994; Moreau et al., 2019) and influences anterior -posterior patterning of the limb field. Experiments in quails and chicken embryos indicate retinoic acid deficiency reduces shh expression (Stratford et al., 1996, 1999). In mice removal of *Raldh2* enzyme required for retinoic acid biosynthesis resulted in early death and lack of forelimbs. When the mice were rescued by RA treatment, shh expression was not restricted posteriorly. Taken together the data suggests that RA has a role in determining the anterior-posterior axis before the initiation of shh expression. (Tickle & Towers, 2017).

Identification of limb specific cis-regulatory element of *Shh* provided new insights about how *Shh* is regulated and restricted in ZPA (Lettice et al., 2002). This long-range enhancer element is called ZPA Regulatory Sequence (ZRS, also known as MFCS1). It is located around 1000kb away from the *Shh* promoter in intron 5 of the *lmbr1* gene. Disruption of ZRS by insertion of a transgene caused preaxial polydactyly in mice and complete removal of ZRS lead to truncated limbs in mice (Lettice et al., 2002; Lettice, 2003; Sagai et al., 2005). Identification of multiple transcription factor binding sites in ZRS refined our understanding of spatiotemporal regulation of *shh* expression in limbs. Deletion

of *Hand2* and *Hox* binding elements from ZRS resulted in the loss of *Shh* expression (Galli et al., 2010; Lettice et al., 2017). The genetic and molecular evidence indicates that co-interaction between *Hox5* and Zinc finger transcription factor *Plzf* restricts *Shh* expression in forelimb patterning. Point mutations in the binding site of *Ptzf* are reported in 'Cuban mutation' (Xu et al., 2013). The Phenotype of Cuban mutations recapitulates the anterior limb phenotypes of *Hox5* mutations in mice. In vitro studies also indicate that *Hox5* proteins interact with *Plzf* through ZRS to restrict *shh* expression during forelimb patterning (Xu et al., 2013). *Gata6* is responsible for the suppression of *shh* in anterior limbs by directly binding to ZRS (Kozhemyakina et al., 2014). Previous studies have shown that ZRS is primed for activity in both anterior and posterior margins of the limb bud (Amano et al., 2009). ZRS has ETS translocation variant transcription factors binding sites which play a major role in establishing the boundary of shh expression in ZPA. ETS translocation variant transcription factors *ETV4* and *ETV5* expression is regulated by FGF signaling. These ETV transcription factors are expressed broadly across the AER. These factors when bound to ZRS repress the activity of the primed ZRS enhancer by interacting with histone deacetylase (HDAC2) (Peluso et al., 2017). ETS transcription factor binding sites when bound by *GABPα* and *ETS1* regulate the position of shh expression in the limbs. Hence, *ETS1* and *GABPα* establish ZPA boundary position and *ETV4/5* restricts *Shh* expression outside ZPA. Point mutations in ETS binding sites result in ectopic expression of *Shh* outside ZPA and, hence, preaxial polydactyly (Lettice et al., 2012).

The point mutations in the ZRS region led to a spectrum of limb defects in humans collectively called ZRS-associated syndromes (Anderson et al., 2012). The role of SHH in regeneration has been of interest for decades. Targeted mutation in the ZRS region in

Newts indicated potentially different regulation of *Shh* via ZRS in embryonic development and regeneration (Suzuki et al., 2018). In *Xenopus*, the hypomethylated state of ZRS in tadpoles is associated with complete regenerative ability. In adults, however, the loss of regenerative ability is associated with the hypermethylated state of ZRS. The data is indicative of epigenetic regulation of *Shh* expression or repression during limb patterning and regeneration (Yakushiji et al., 2007).

Evolution of limb lessness -digit loss and ZRS

ZRS is highly conserved in terms of sequence and topological position across a large evolutionary time scale, including sharks, skates, and rays (Dahn et al., 2007; Gehrke & Shubin, 2016). Studies in mice discover that the functional activity of ZRS comprises two domains: one domain predominantly acts over short range and the other promotes long range chromosomal conformation changes associated with the gene activity (Lettice et al., 2014). Evidence suggests that the changes in the sequence of the Cis Regulatory Elements (CREs), specifically transcriptional enhancers, are involved in the morphological evolution of vertebrate traits (Chan et al., 2010; Cooper et al., 2018; Cretekos et al., 2008; Gehrke & Shubin, 2016; Guerreiro et al., 2016; Infante et al., 2018; Lopez-Rios et al., 2014; McLean et al., 2011). The limb specificity of the ZRS region makes it an ideal candidate for evolutionary modification without altering the body plan to create marvelous limb morphology diversity (Tickle & Towers, 2017). In squamate reptiles the diversity of limb length and digit numbers is fascinating. Partial to complete limb loss has evolved independently in multiple lineages of squamates (Infante et al., 2018). The ancient origin of limb loss in snakes can be found through fossil records as well as sequence analysis of limb-specific genes (Infante et al., 2018). Investigation of sequence analysis of two claw

keratin genes *HA1* and *HB1* indicated that these genes are completely lost in snakes and are completely intact in limbed reptiles (Emerling et al., 2017; Infante et al., 2018). The sequence divergence of ZRS in limbless advanced snakes is extremely high while in basal snakes displaying vestigial limbs is extremely low. The sequence analysis narrowed down a 17bp snake-specific deletion in the ZRS region (Kvon et al., 2016; Leal & Cohn, 2016). The functional analysis of enhancers with this 17bps deletion in mice lead to the generation of 'serpentized' mice indicating a complete loss limb specific activity. This 17 bps deletion overlaps with the *ETS1* transcription factor binding site (Kvon et al., 2016). The loss of *HoxD13* binding sites in the ZRS region of python is implicated for the weak ZRS activity in python limbs (Leal & Cohn, 2016). The progressive loss of *HoxD13* binding sites along with the loss of *ETS1* binding sites in ZRS might have been a probable reason for the limb loss in the advanced snakes (Kvon et al., 2016; Lettice et al., 2017).

Infante et al. (2015) identified, by systematic investigation of 65 limb-specific enhancer elements from the VISTA enhancer database, around 40 enhancer elements conserved in the reptiles. The sequence divergence between snakes and limbed reptiles was moderate. The probable reason for this conservation in limbless squamates is that these limb enhancers are active in other non-limb tissues. Functional analysis of the snake orthologs of *HLEB, Prox* and *Isl*I enhancers have lost limb activity but have maintained genital activity (Infante et al., 2015, 2018).

Over the course of evolution, subtle changes in *Shh* expression and cell's responsiveness to *shh* have been suggested as reasons for the digit loss. In cows, two artiodactyl specific insertions were found in *Ptch1* enhancer in lateral plate mesodermal cells. These insertions altered the evolutionarily conserved Gli protein binding sites.

Hence, *Ptch1* was not upregulated as a response to the Shh signal. As a result, Shh is not sequestered and is not restricted to the posterior part of the bud. Thus, instead of graded Shh signaling, these cells experience more or less uniform Shh signaling. Hence, cows have two symmetric digits. Loss of lateral digits is the response to the failure of AER extension. (Lopez-Rios et al., 2014). Similar to the cows, in pigs, *ptch1* is downregulated and restricted to the posterior part. However, unlike cows, there is no apparent increase in cell death resulting in the formation of the 4 digits (Cooper et al., 2014). The evolutionary digit loss in Australian skink *Hermiergis* is the result of changes in the temporal regulation of *Shh*. The comparative analysis suggested that the skink species with the reduced number of digits have experienced a shorter duration of shh exposure developmentally (Shapiro et al., 2003). The diversity of limb reduction in gymnophthalmid lizards morphologically separated them into two groups. One group has lizard-like pentadactyl species, and the other group has a drastic loss of limb elements. *Calyptommatus sinebrachiatus* is a serpentiform lizard in this genus (Roscito et al., 2015). The forelimb bud develops in these lizards and then degenerates while the hindlimb bud develops into a one-digit hindlimb with a femur, tibia, and fibula (Roscito & Rodrigues, 2012). In situ hybridization studies showed that *Shh* is not expressed in the forelimb buds for the stages analyzed while it is expressed in the posterior mesenchymal region of the hind limb bud (Roscito et al., 2015).

To understand the functionality of limb-specific genes and enhancer elements in the context of the evolution of limb reduction, limb loss, and digit loss in squamates, it is imperative to test their activity in phylogenetically closely related squamate species. The feasibility of gene editing in anoles makes it an attractive model to perform functional

testing of ZRS activity. Hence, we created targeted mutations in the 17bps snake-specific deletion region in *Anolis sagrei* ZRS region to test its effect on limb length.

Technology development for studying Shh signaling in squamates

There is a lack of literature available studying shh signaling in squamates. From an evolutionary standpoint, the mechanism of shh signaling has evolved from invertebrates to vertebrates and within the vertebrate lineage. *Anolis* lizards provide insight into reptilian biology and shh signaling. There are three distinct approaches to explore and answer biological questions in anoles. As it is an oviparous species, we can use eggs and manipulate the Shh signaling to understand targets of the signaling pathway, or we can perform functional genetic testing in vivo or we can use a cell culture approach.

The role of primary cilia in fish and mammals is distinct. Squamates being in the middle position between them will provide evolutionary insight into the role of primary cilia. To explore reptilian Shh signaling, we wanted to use pharmaceutical manipulations to induce shh signaling hence we needed a system with more experimental control. The cell culture system had the inherent advantage of being fast and having more control over the experiments. Hence, we decided to use the cell culture system to answer the evolutionarily conserved mechanism of shh signaling. The challenge of this approach was that there is a lack of an established tissue culture system, immortalized cell line, and gene editing approaches. Hence, we decided to develop tools for studying reptilian biology questions in vitro.

I performed preliminary experiments to understand which mammalian promoters can drive gene expression in lizard cells. This was important so that we could use commercially easily available Cas9 plasmids with mammalian promoters. To perform gene

editing I needed a Cas9 plasmid that can robustly drive gene expression in lizards. I also developed an immortalized cell line as a novel reagent. This reagent will be a resource for the scientific community who wants to explore reptilian biology and explore evo-devo questions.

To understand the evolution of limb lessness we wanted to perform functionality testing of 17 bps snake-specific deletion in the ZRS region. We opted to do gene editing in vivo to answer this question. Previously Menke lab developed a method for gene editing by accessing unfertilized oocytes in *Anolis sagrei* (Rasys et al., 2019). CRISPR/Cas9 has been used robustly in the Menke lab to create targeted mutations in multiple gene loci. However, there are sequences of regulatory elements which are AT-rich or do not have any suitable PAM site for Cas9 CRISPR targeting. This has proven to be a limitation for our functional genetic studies. We wanted to increase our repertoire of CRISPR toolbox to increase the flexibility of the use of *Anolis sagrei* as a model system. Hence, I opted to work with the Cas12a enzyme. Along with the *Shh* enhancer element I also created a targeted mutation in another ancient *Tbx4* HLEB enhancer element. This enhancer is involved in the regulation of limb length. Creating targeted mutations in two enhancer elements regulating limb length gave us an insight into how robustly Cas12a can work in our model organism. We observed the efficiency of Cas12a experiments at room temperature was much lower as compared to Cas9 experiments. We can now try other Cas12a varieties which are more active at room temperature. Cas12a creates an overhang at the cut site. We can use this to our advantage and can design experiments with homology directed repair mechanisms.

The rationale for group work-related education research project

During graduate school, I was a graduate laboratory assistant (GLA) for the introductory Biology 1108 lab for about three semesters. As an instructor, I struggled with motivating students for group work. A lot of undergraduates I talked to strongly disliked group work in this lab. Other GLAs informed group conflicts among students. After talking to multiple people, I realized none of us were equipped with intervention strategies. Hence, with the mentorship from Dr. Brickman, I chose to explore BIOL1108 lab sections to understand when conflicts occur in these labs, what type of conflicts occur among the students, and how students and instructors approach the conflicts. We also wanted to explore group formation criteria in this lab. The curriculum of this lab section encourages students to mingle and work with other students before forming formal groups. Students rarely do this by themselves. Instructors also infrequently initiate this. So, we wanted to know if we can provide evidence that there was an inherent advantage of spending time randomizing students for a couple of initial lab weeks. The goal of my study was to understand group conflicts in the labs and help GLAs with intervention strategies.

CHAPTER 2

THE ROLE OF PRIMARY CILIA IN HEDGEHOG PATHWAY INDUCTION IN *ANOLIS SAGREI*

Introduction

The hedgehog family of proteins acts as secreted ligands and regulates a large number of developmental processes in both vertebrates and invertebrates (McMahon et.al, 2003). Mutations in Hh genes were first identified in the genetic screen of *Drosophila* which affected larval body plan (Nüsslein-Volhard & Wieschaus, 1980). Since its discovery, the Hh signaling pathway components have been studied extensively in both invertebrates and vertebrates. Genetic studies in *Drosophila*, mice, and zebrafish revealed that the core components and framework of the Hh signaling pathway are highly conserved. Hh ligand binds to its receptor transmembrane protein Patched1 (ptch1). Ptch1 is a negative regulator of the Hh signaling pathway. In the absence of the Hh ligand, Ptch1 inhibits the activation of another transmembrane protein Smoothened (Smo). Smo acts as a positive regulator of the Hh signaling pathway. Active Smo, in turn, regulates the modification of a bifunctional transcription factor Cubitus interruptus (Ci) in *Drosophila* and Gli proteins in vertebrates (reviewed Huangfu & Anderson, 2005; Ingham et al., 2011).

Even though the core Hh signaling pathway is conserved between vertebrates and invertebrates, there are some key differences. For instance, genes encoding components of the Hh pathway are duplicated in vertebrates. There are three homologs of Hh ligands

in mice and chicken, *Desert hedgehog (Dhh)*, *Indian hedgehog (Ihh)* and *Sonic hedgehog (Shh)* (Varjosalo & Taipale, 2008). *Shh* and *Ihh* have roles in embryonic development while *Dhh* is restricted to the gonads and is involved in the regulation of spermatogenesis (Bitgood et al.,1996, Chiang et al.,1996, Zhang et al.,2001, Nygaard et al.,2015). Zebrafish have 5 Hh homologs *shh*, *Ihh, dhh*, echidna *hedgehog(ehh)* and *twiggywinkle hedgehog (twhh)* (http://zfin.org). Both mice and zebrafish have 2 homologs of *ptch*. *Ptch1* acts as the predominant receptor of ligands during embryonic development (Goodrich et al.,1997; Wolff et al., 2003). Zebrafish *ptch2* mutants have mild phenotypes (Koudijis et al.,2005). Vertebrates have three homologs of Ci protein namely Gli1, Gli2, and Gli3 (Bai et., al,2004; Motoyama et al.,2003). Gli1 acts as an activator and Gli2 and Gli3 can act as both as activators and repressors (Karlstrom et al., 2003; Matise et al., 1998; H. L. Park et al., 2000; Tyurina et al., 2005; Yt et al., 2016). Another distinct and unique feature of vertebrate Hh signaling is the requirement of primary cilia for the transduction of the pathway (Huangfu et al., 2003)

The primary cilium is a microtubule-based organelle that protrudes from the surface of vertebrate cells. Primary cilia are non-motile with a 9+0 microtubule structure. The primary cilia are generally characteristics of cells in G0/G1 phase and often during the S/G2 phase (Plotnikova et al.,2009). The components of ciliary structures are highly conserved throughout evolution (Pazour et al., 2000, 2002). The ciliary structure can be divided into subdomains, including the basal body, transition fibers, and the axoneme. The axoneme consists of 9 doublets of α and β tubulin polymers (Fawcett & Porter,1954; Manton and Clark, 1952). At the base of the cilium, the basal body is located in the "ciliary pocket". Distal to the basal body, there is a region (the transition zone) that contains

transition fibers connecting the basal body microtubules to the cilium membrane. The transition zone gates the entry and exit of cilium proteins and lipids excluding improperly targeted proteins from entering the cilium (Lechtreck et al.,1999; Gigante & Caspary, 2020; Garcia-Gonzalo and Reiter, 2017; Reiter et al., 2012). Thus, an exclusive ciliary environment is generated by separating the cilium from the main cell body (Gherman et al., 2006).

Since molecular machinery for protein synthesis is absent from primary cilia, they rely on a specialized transport mechanism called intraflagellar transport (IFT) for transporting proteins to and from the cilia (Rosenbaum & Child,1967). IFT is a bidirectional transport system. Anterograde IFT particles move proteins toward the distal tip of the cilium via kinesin-2 motor and retrograde IFT particles move proteins from the tip of the cilium via dynein-2-motor (Kozminski et al.,1995, Pazour et al.,1995, Gigante & Caspary, 2020). IFT is a highly conserved process required for the assembly and maintenance of cilia in all the ciliated species from *Chlamydomonas* to mammals (Pazour et al., 2000, 2002).

In vertebrates, *Ptch1*, the receptor of the Hh ligand is located on the ciliary membrane. In the absence of the ligand, Ptch1 inhibits the activation of Smo by excluding it from the cilium. At the tip of the primary cilium, Suppressor of Fused (SuFu) sequesters Gli transcription factors hence suppressing their activation (Haycraft et al., 2005; Zeng et al., 2010). Gli proteins are proteolytically cleaved into their repressor form GliR. GliR is then transported to the nucleus where it represses the expression of the target genes (Gerdes et al.,2009). In the presence of the ligand, Ptch1 binds to it removing the repression of Smo. Smo then enters the cilium while Ptch1 leaves the cilium (Corbit et al., 2005; Rohatgi et

al., 2007). Upon activation, Smo prevents the inhibition of the Gli activator (GliA) state by Sufu, which allows modified Gli proteins to avoid cleavage and stimulate the pathway. Once formed, Gli activators are then in turn transported from the tip of the cilium to the nucleus. Gli activators then regulate the expression of downstream target genes of the pathway (Wheway et al., 2018).

In an evolutionary context, sea urchins provide the first evidence of the requirement of cilia for Hh signal transduction. However, they use motile cilia for signal transduction (Warner et al., 2014). In vertebrate lineage, data from *Xenopus*, zebrafish, chicken, and mouse provide evidence that nonmotile primary cilia are required for Hh transduction (Ben et al., 2011; Huang & Schier, 2009; Huangfu et al., 2003; T. J. Park et al., 2006; Yin et al., 2009). In humans, mutations in cilia genes and disruption of ciliogenesis lead to disease conditions collectively called ciliopathies. Mutations in the dynein-2 complex are associated with mild Hh pathway defects and cause short rib dysplasia with or without polydactyly and Jeune Asphyxiating Thoracic Dystrophy (Dagoneau et al.,2009; Merrill et al.,2009; Bangs & Anderson, 2017).

In vertebrates, cilia act as a specialized center for receiving the Hh signal and processing the gli proteins. The ratio of Gli activators and Gli repressors decides the cell's graded response to the Hh signal. Hence, in vertebrates, primary cilia mediate Hh signaling by modulating Gli protein activities. This response is tissue-specific and developmental time specific. Gli1 lacks a site for proteolytic cleavage and hence always acts as an activator. Gli2 and Gli3 can act as both activators and repressors. (Falkenstein & Vokes, 2014). Within the vertebrate lineage, the role of Gli proteins has diverged. In mice, *Gli*2 is a major activator of the pathway and *Gli*3 majorly acts as a repressor (Bai et al., 2002; Ding

et al., 1998; Mo et al., 1997). Activation of *Gli*2 in mice depends on cilia (Eggenschwiler & Anderson, 2007; Santos & Reiter, 2014). In zebrafish, *gli1* is the major activator of the pathway, *gli2* can act as a minor activator or repressor (Huang & Schier, 2009; Tyurina et al., 2005) and *gli*3 can function as both activator and repressor in context to tissue specificity and developmental time (Tyurina et al., 2005).

In chicken *GLI2* and *GLI3* can act as both activators and repressors (Yt et al., 2016). As the use of distinct Gli proteins for aspects of Hh signaling appears to be evolving in vertebrate lineages, we are interested in studying Hh signaling and the role of Gli proteins in reptiles which diverged from mammals more recently than did ray-fin fishes (~320 vs. ~450 Mya).There is a lack of literature to understand the role of primary cilia and shh signaling in reptiles. Hence, we chose to work with a reptilian model *Anolis sagrei* lizard. *Anolis* lizards have been used as model organisms for studies of evolution, behavior, physiology, development, and neuroendocrinology for many decades (Kabelik & Hofmann, 2018; Losos et al., 2001; Sanger & Kircher, 2017; Wadet, 2005). The brown anole lizard, *Anolis sagrei*, is ideal for developmental studies due to its small size, ease of husbandry, continuous egg production, high fertility, and low maintenance cost. A fully annotated genome assembly for *A. sagrei* has been generated. This makes brown anole an attractive model for developing tools for functional genetics. In 2019, Rasys et al established a novel approach for gene editing in vivo.

To develop more tools for functional and genomic experiments in reptiles, we wanted to develop a tissue culture approach. Hence, we established an immortalized cell line to study Hh signaling in lizards. We wanted to study the role of primary cilia in Hh signaling reptiles using anole as a model from an evolutionary context. We edited the *ift*88

gene which is required for the generation of primary cilia (Ben et al., 2011; Han YG et al.,2003; Huang & Schier, 2009; Huangfu et al., 2003; Pazour et al., 2000, 2002;) Our findings indicate that *ift*88 in lizards is required for ciliogenesis and Hh signaling as in fish and mice.

Results

Establishing an experimental system to study Hh signaling using in ovo approach in A.sagrei.

To manipulate Hh signaling in anoles, we exposed the 24hr laid eggs to a pharmaceutical compound Smoothened Agonist (SAG). We exposed a total of 9 eggs to a bolus dose of 100μM SAG and 9 eggs to a bolus dose of volume equivalent DMSO. Polydactyl embryos were observed in 8 out of 9 eggs treated with 100μM SAG (Figure 2.1B). In four of these embryos, polydactyly was observed in both forelimbs and hindlimbs. In the remaining four embryos, polydactyly was observed in hind limbs only. Two of the 8 embryos showed left-right asymmetry in context to the number of extra digits on either forelimbs or hindlimbs. The one embryo which did not show polydactyly was in a later stage of development (stage 16) as compared to the rest of them (Table S2.1). Out of the 9 eggs treated with an equivalent volume of DMSO, one egg died in 24 hours and the rest 8 showed no polydactyly. We speculate that the death of the egg was not associated with DMSO treatment but with the husbandry and overall initial health of the egg. Both SAG treated and DMSO treated eggs had a comparable mix of stage 13, stage 14, and stage 16 embryos.

Establishing a primary tissue culture system to study Hh signaling in A.sagrei.

To have more control over experimental conditions to manipulate Hh signaling in anoles, we optimized a primary cell culture system collecting lizard embryonic fibroblasts (LEFs) from *A.sagrei* torso and limb region (Figure 2.1C). In a reproducible manner, we have shown that primary LEFs can be grown robustly at 29ºC/5% CO_2 with the culture conditions described in the methods. As a response to SAG exposure, the known direct targets of the shh pathway, *gli*1 and *ptch*1 were induced in primary LEFs from the torso region as well as from limb buds. The relative fold induction was calculated relative to *gli*1 or *ptch*1 expression in the primary LEFs from the same embryo exposed to DMSO. The mean value of *gli*1 relative fold induction in primary torso LEFs was 20.79 with SD ± 8.96 and *ptch*1 fold induction was 5.12 ± SD 1.63 (Figure 2.1D). The mean value of *gli*1 relative fold induction in primary limb LEFs was 135.80 with SD ± 47.72 and *ptch*1 fold induction was 8.33 ± SD 3.07(Figure 2.1E). The variation in the extent of *gli*1 induction may be explained by the heterogeneity of the primary cells, and possibly subtle differences in the developmental stages between the individual embryos. The trend of increase in *gli*1 and *ptch*1 expression observed in the primary LEFs indicates that the lizard cell culture system is responsive to the pharmacological manipulations and thus can be used as an assay to study Shh signaling in *A. sagrei*.

RNAseq analysis for DE genes in A .sagrei and M. musculus after Shh pathway induction

Once we optimized a primary LEF tissue culture system, we wanted to study Shh signaling in lizards and mice through an evolutionary context using the cell culture approach. To understand the extent to which Shh signaling pathway target genes are shared between *A. sagrei* and *M. musculus*, we stimulated the Shh signaling pathway in primary

limb LEFs and primary limb MEFs (mouse embryonic fibroblasts) with 200nM Smoothened Agonist (SAG) treatment. To perform a more controlled comparison between these two evolutionarily distant species, we collected primary LEFs and MEFs from stage-matched embryos. Limb bud size and shape were used for this comparison. *M.musculus* E11.5 embryo is equivalent to *A. sagrei* late stage 5/early stage 6. DE genes between SAG and DMSO treatment were identified based on an FDR-adjusted p-value cutoff of 0.05. (Figure 2.2A,2.2B). We found 275 genes were upregulated and 116 genes were downregulated as a response to 200nM SAG treatment in *A. sagrei* cells. In *M. musculus*, 1056 genes were upregulated, and 844 genes were downregulated in response to 200nM SAG treatment. We then compared upregulated genes in both species to identify shared upregulated and downregulated genes in response to inducing the Shh signaling pathway. We found 133 were upregulated in both species and 20 genes were downregulated in both species (Figure 2.2C,2.2D, Table S2.3, Table S2.4). In mice, *Gli2* is known to be a major activator of the Shh signaling pathway (Bai et al., 2002; Ding et al., 1998; Mo et al., 1997). *Gli*2 was upregulated in mice, but it was not differentially expressed in *Anolis* cells. This finding may have implications for the role of Gli proteins in an evolutionary context. The other known direct targets of the pathway *gli1*, *ptch1,* and *ptch2* were upregulated and *hip*1 was downregulated in both species. *Gas*1 and *Boc* were downregulated in mouse, but not *Anolis*, cells. We then performed gene ontology analysis using the gProfiler program. The shared upregulated gene data set of 133 genes (Table S2.3) is enriched with known genes associated with cell cycle regulation. We found 13 genes which were upregulated in *Anolis* cells yet downregulated in mouse cells. *Snai2* was one of the genes in this data set. There were 15 genes downregulated in *Anolis* but upregulated in mouse cells. *Fbn*2 was one of

the genes in this data set (Table S2.5). The RNAseq data was validated by qRT-PCR in primary LEFs with three biological replicates (Table S2.2). We used RNAseq data to identify and select reference genes for qPCR. Transcript levels of *gli*1 and *ptch*1 were comparable with *tbp* and *atp5f1d*. The RNAseq data indicates that *tbp* and *atp5f1d* were stably expressed in DMSO and SAG treated LEF samples. The stable expression was defined as a coefficient of variance below 0.5 within the biological replicates.

Generation of an A. sagrei immortalized cell line and characterization

To perform functional genetics experiments, we needed an immortalized cell line from *A. sagrei.* The strategy we used to create immortalized cell lines was serial passaging of primary LEFs collected from *A .sagrei* stage 6 embryo torso region. This strategy was adapted from Xu, 2005. The primary cells slowly started undergoing senescence during serial passaging. A few cells which escaped senescence started growing after around 15 days in culture. These cells were then flow sorted to collect single cell clones. The single cell clones were allowed to grow. We generated 40 cell lines from one *A. sagrei* embryo. To understand the responsiveness of these cell lines to pharmaceutical manipulations, we chose three cell lines to work with and subjected them to 200nM SAG treatment for 24 hours. All three clones we worked with showed a significant increase in *gli*1 and *ptch*1 expression as a response to SAG treatment (Figure S2.2 A and B).

In the additional experiments, we worked with one of these cell lines, ASEC-1. RNAseq analysis of the ASEC-1 cell line (data not shown) indicated the presence of transcripts of common fibroblast markers like fibroblast-specific protein-1 (a.k.a. *s100a*4), *col1a*1, *col1a*2, *col5a*1, *loxl*1, *lum*, *fbln*1, and *fbln*2, the cell surface receptors *cd*34 and *pdgfra* (Muhl et al., 2020). Transcripts for common mural cell markers were also found

namely *des*, *mcam, notch*3, *pdgfrb*, and *anpep* (*cd*13) (Muhl et al., 2020). No evidence of expression of pericyte specific marker, *rgs*5 transcript was observed (Muhl et al., 2020). The fibril-like morphology of the cells and evidence of commonly expressed fibroblast molecular markers provide evidence for the fibroblast nature of the cell line.

We performed sex genotyping to confirm the sex of the cell line. Figure S2.2E shows an agarose gel image with a Y chromosome-specific 156bp PCR band. RNAseq analysis also confirmed the presence of the Y chromosome.

To develop an assay to study Shh signaling in LEFs we quantified the responsiveness of the ASEC-1 cell line to different concentrations of SAG. We measured *gli*1 and *ptch*1 expression in SAG-exposed cells relative to the cells exposed to volume equivalent DMSO by qRT-PCR. We used *tbp* and *atp5f1d* as reference genes as they showed stable expression ASEC-1 cell line exposed to SAG and DMSO (Figure S2.1B). Figure 2.3B and C show a trend of increase in *gli*1 and *ptch*1 expression with respect to increasing SAG concentration. We exposed the cells with 200nM SAG at different time intervals and measured relative *gli*1 and *ptch*1 expression (Figure S2.2C and D). The relative *gli*1 expression increased from 6hr to 72 hr. In both SAG dosage and time curve, relative *gli*1 expression was more sensitive to the perturbations of the experimental conditions than *ptch1* expression. The overall relative *gli*1 and *ptch*1 induction values were lower than primary torso LEFs exposed to similar conditions.

Hh signaling is functionally linked to the primary cilium in vertebrates (Huangfu et al., 2003; Huangfu & Anderson, 2005). To determine whether the ASEC-1 cell line generates cilia, we optimized the immunostaining protocol for visualization of the structure. We used two established ciliary markers namely α acetylated tubulin and *ift*88.

Figure 2.3D shows ASEC-1 cells co-immunostained for the detection of primary cilia. Both the antibodies we used showed *Anolis* reactivity and specificity.

CRISPR/Cas9 gene editing in ASEC-1 cell line generating ift88 mutants

The *ift*88 gene is required for ciliary assembly and maintenance (Huangfu & Anderson, 2005; Pazour et al., 2000, 2002). To address the requirement of the primary cilium in the ASEC-1 cell line in Hh signaling, we mutated the *ift*88 gene by CRISPR/Cas9 gene editing to disrupt ciliogenesis. *A .sagrei* has one copy of the *ift*88 gene containing 26 exons. Exon 1 is noncoding. We did not find any evidence from the *A.sagrei* RNAseq data previously generated from different tissues by Menke lab, suggesting the possibility of an alternate splicing event near exon 4. There was RNAseq evidence for an alternative splice site near exon 2. Hence, we chose exon 4 for CRISPR/Cas9 gene editing. Gene editing was performed by transfection. We collected a total of 46 clonal cell lines at the end of the gene editing experiment. These were screened by PAGE for detecting mutants (Figure S2.3 A). The nature of the mutation was then confirmed by Sanger sequencing. We used Genious and DECODR software for deconvoluting the chromatograms. We identified a total of 19 clonal cell lines with indels in *ift*88. 17 of which had biallelic frame-shifting mutations. The details of the mutations can be found in table S2.7. We then analyzed three mutant clones- clone #28, clone #30, and clone #25 which had biallelic mutations (Figure 2.4C, S2.5-A) along with two WT clones, #12 and #35. Clones #12 and 35 were also Sanger sequenced to confirm they did not harbor mutations in *ift*88.

ift88 expression and primary cilium in mutants

Immunofluorescence was used to detect *ift*88 expression and visualize primary cilium. To detect and confirm primary ciliary structure we used three features. We

identified primary cilia structure based on α acetylated tubulin antibody staining, the position of the nucleus, and the microtubule organization center in context to a ciliary structure. We then confirmed the primary cilia structure by ift88 antibody staining. We could identify with confidence and visualize the primary cilium in ≥70% of the cells of the parental ASEC-1 cell line, clones *ift*88$^{+/+}$ #12 and #35. WT Clone #12 and 35 both showed ift88 expression and localization to the tips and base of cilia (Figure 2.4D i-vi). Primary cilia were also visualized by scanning electron microscopy in *ift88* WT clones (Figure S2.3B). Cells from *ift*88 $^{-4/-2}$ clone #28 and *ift*88 $^{+1/-5}$ clone # 30 did not show ift88 staining. Roughly 90% of the cells in both clones #28 and #30 failed to show any ciliary structures (Figure 2.4D vii-xii), although 11 % of cells in clone #28 and 5% of cells in clone # 30 showed ambiguous structures which we could not be confidently called as primary cilia. Interestingly, 40% of cells from *ift*88 $^{-7/-1}$ clone #25 showed primary cilia (Figure S2.4B), 23% of cells lacked primary cilium structure, and ~ 37% of cells showed ambiguous structures. In clone #25 cells with primary cilia, ciliary ift88 staining was detected, indicating that some functional ift88 is made in these clones, despite the biallelic frame-shifting mutations in this line.

The observations in *ift*88 $^{-7/-1}$ clone #25 were puzzling. To clarify the nature of the transcripts in this clone, we sequenced cDNA from all the mutants and WT clones (data not shown). Sanger sequencing results of genomic DNA and cDNA from clones # 12,35,28 were identical. DECODR analysis of clone # 30 cDNA sequence suggested the same indels as that of genomic DNA, along with a 38bp deletion in 6.3% alleles. This deletion overlapped partially with exons 4 and 5 but would still shift the reading frame and result in nonsense codons. DECODR analysis of clone # 25 cDNA sequencing suggested the

same indels as seen in genomic DNA, along with a 57bp deletion in 19.6% alleles. This deletion overlaps with the entire exon 4. Hence, in clone #25, it appears there is novel alternate splicing (not seen in wild type) whereby exons 3 and 5 are joined. Such a transcript preserves the reading frame and thus is likely to generate an ift88 isoform retaining at least some biological activity.

SAG responsiveness in ift88 mutant and control cell lines

To understand how the loss of *ift*88 and primary cilia may affect Shh signaling in mutant cell lines, we studied the SAG responsiveness of these cell lines. We exposed parental cell line ASEC-1, clone #12 *ift*88$^{+/+}$, clone#35 *ift*88$^{+/+}$,clone #28 *ift*88$^{-4/-2}$, clone # 30 *ift*88$^{+1/-5}$ and clone #25*ift*88 $^{-7/-1}$ with 400nM SAG for 24 hr. For each cell line a volume equivalent DMSO was used as a control treatment. The levels of *gli*1 and *ptch*1 transcripts were quantified relative to this DMSO control by qRT-PCR. Both the WT clones, clones #12 and #35 had an increase in *gli*1 expression as a response to the SAG treatment, although the increase in relative *gli*1 expression was lower than that in the parental cell line (Figure 2.5A). The relative levels of *ptch*1 expression increased in WT clones #12 and #35 similar to the ASEC-1 cell line (Figure 2.5B). Clone #30 showed no change in *gli*1 and *ptch*1 expression in response to the SAG treatment, whereas Clone #28 potentially showed a slight increase (37% and 14%, respectively, $p<0.05$) in relative *gli*1 and *ptch*1 expression in response to the SAG treatment (Figure 2.5A and B). Relative *gli*1 and *ptch*1 expression increased in response to SAG treatment in clone #25, consistent with the retention of cilia in this cell line. The magnitude of the increase in Clone#25 was comparable to responses observed in WT clones and 35 (Figure S2.4C).

In mice and zebrafish, the primary cilium also plays a role in repressing the Hh signaling pathway in the absence of ligand stimulation, leading to a higher level of unstimulated, basal Hh pathway activity in mutants lacking cilia/ift88. To determine if there is a difference in basal *gli*1 expression levels in ASEC-1, WT clones, and *ift*88 mutants, we analyzed *gli*1 expression in these cell lines (treated with DMSO) relative to the parental cell line (treated with DMSO). Basal relative *gli*1 expression in WT clone #12 is significantly lower than the parental cell line ASEC-1. There is no difference between basal *gli*1 expression in WT clone #35 and the parental cell line. *ift88* mutant clones #28 and 30 showed significantly elevated basal *gli*1 expression as compared to WT clones #12 and #35 (Figure 2.5C). Interestingly ift88 mutant Clone #25 also showed elevated levels of relative basal *gli*1 expression compared to the WT clones, even though this clone retained appropriate responses to SAG treatment (Figure S2.4D).

Together the data indicate that 90-100% of the cells in *ift88* mutant clones #28 and #30 did not generate primary cilia and they largely failed to respond to SAG treatment with respect to the expression of Hh target genes. Moreover, the basal *gli*1 expression in these cell lines is elevated in these lines compared to WT clones with primary cilia, suggesting that cilia in *A. sagrei* are also required for repression of the Hh pathway in the absence of induction. In the third *ift88* mutant clone, #25, roughly 40% of the cells expressed *ift*88 and developed primary cilia, and responded to the SAG induction normally. However, the basal *gli*1 expression clone #25 cells is elevated, as in *ift88* mutant clones #28 and #30. It is important to note that in a given experiment all 6 cell lines had comparable passage numbers. The difference between the passage numbers, if any, for that experiment was not more than 3-5 passages.

Discussion

Establishment of in-ovo and primary cell culture

To study Hh signaling in lizards using *A.sagrei* as a model system, we wanted to explore *in ovo*, in vivo and tissue culture approaches. We wanted to explore Shh signaling in anoles by manipulating the pathway using pharmaceutical compounds like SAG. To establish *in ovo* experimental system we have optimized a method for the uptake of chemical compounds by capillary action in *A. sagrei* eggs. Our method generated reproducible results in terms of digit morphology. Shh is known to regulate digit patterning and identity in a developing limb bud. Polydactyly in several naturally occurring mutants and KO mice is a result of perturbations in Shh expression in the developing limb buds (Anderson et al., 2012; Hill et al., 2003; Lettice, 2003; Lettice et al., 2012; Tickle et al., 1975; Tickle & Towers, 2017). 8 out of 9 embryos dissected from SAG-treated eggs showed polydactyly in our experiment (Figure 2.1B, table S2.1). The effect of SAG exposure was very specific and reproducible. The variation in the number of digits in the forelimb and hindlimb can be accounted to the fact that the developmental stage of the embryo collected in 24 hr laid eggs varies. It can be stage 2/3/4. Depending upon how early or how late the embryo was in development, its response to the SAG and/or concentration of SAG will vary. This is one of the probable reasons why one embryo did not show any differences in digit numbers. This was the only embryo that was at stage 16 after dissection. We believe that the developmental stage of that embryo before exposure to SAG was different from other embryos. We believe this method can be used effectively to study the effects of environmental pollutants and pharmaceutical chemicals on signaling pathways in reptiles using anoles as a model system. There are limitations with this method

as we have no control over how much SAG is biologically available to the developing embryo.

Establishment of a tissue culture system to study Shh signaling

For more controlled experiments we established a tissue culture system for studying Shh signaling in reptiles using *A.sagrei* as a model. We used a similar approach to that of the mouse embryonic fibroblast cells. The primary cell culture system was used to optimize tissue culture conditions in terms of the growth medium, temperature, and CO_2 % for optimal growth. Primary LEFs from both the torso and limb region responded robustly to the treatment SAG (Figure 2.1D and E). The biological variation between the samples can be the result of subtle differences in the developmental stage of the embryos from which primary LEFs were collected. It is also possible that the heterogeneous nature of the primary LEFs themselves is responsible for the biological variation. The SAG responsiveness of the system opened an avenue for pharmacological manipulations of the Shh pathway. This was used to develop a qRT-PCR assay to study Shh signaling.

The primary cell culture provided an opportunity to study the conservation of the Shh signaling targets in reptiles and mammals which diverged from each other ~320 mya. Primary LEFs from limb buds are more biologically representative of limb buds than our immortalized cell lines. We used SAG to induce the Shh signaling pathway to understand how many target genes of Shh signaling pathways are shared between reptiles and mammals. Among the upregulated DE genes, *gli*2 was not upregulated in *A.sagrei* samples. The other known direct targets of the pathway *gli*1*, ptch*1 and *ptch*2 were upregulated in both species. In mammals, *Gli1* and *Gli2* both are the activators of the pathway and *Gli3* plays a more important role in transcriptional repression. Mammalian *Gli*2 is the

predominant activator of the pathway as *Gli*1 is largely dispensable when *Gli*2 is present (Bai et al., 2002; Ding et al., 1998; Hui & Joyner, 1993; Matise et al., 1998; Mo et al., 1997; Wang et al., 2019). In zebrafish, *gli*1 is the essential activator of the Hh signaling pathway while *gli*2 has a minor role as an activator or the repressor depending on the tissue context (Karlstrom et al., 2003) and *gli*3 can function as both activator and repressor in zebrafish. The role is governed by tissue specificity and developmental time (Tyurina et al., 2005).

Our RNAseq data suggests that in reptiles *gli*1 is upregulated as a response to activation of the shh pathway while *gli*2 is not appreciably induced. The difference in *gli*2 induction between lizards and mammals, coupled with the difference in significance between zebrafish and mammalian *Gli*2 in Hh pathway control raises the possibility that *gli*1, rather than *gli*2, might be a major activator of the pathway in lizards, as it is in zebrafish. However, further genetic analysis needs to be done to understand the function of Gli proteins in lizards.

RNAseq analysis also revealed that *snai*2 and *fbn*2 are differentially expressed in LEFs and MEFs in response to activation of the Shh pathway. *Snai* 2 is an SNAI family protein and is involved in many cellular and developmental processes involving epithelial-to-mesenchymal transition, chondrocyte-to-osteocyte development, inhibition of cell apoptosis (reviewed in Razmara et al., 2021). It is upregulated in LEFs and downregulated in MEFs. *fbn*2 is involved in limb patterning. *Fbn*2 null mice show bilateral syndactyly (Arteaga-Solis et al., 2001). *fbn*2 expression is downregulated in LEFs and upregulated in MEFs. Curiously, the genes involved in limb patterning and long bone development are differentially expressed in opposite directions in mammals and lizards as a response to the induction of the pathway. The transcriptomic data, showing conservation and divergence

in Shh pathway targets when comparing reptiles and mammals, indicates that exploration of the evolution of gene regulatory networks involved in limb development and cell proliferation among tetrapods is warranted. This would provide for hypothesis generation and the development of additional research questions to understand how vertebrate limb development has evolved.

Generation of Immortalized lizard embryonic fibroblast cell line ASEC-1 and ift88 mutant cell lines

To design functional genetics and genomics experiments, the generation of an immortalized cell line was necessary. Hence, we created an immortalized cell line from *A.sagrei* torso LEFs (Figure 2.3A). To understand Shh signaling in reptiles we created targeted mutations in the *ift*88 gene to disrupt ciliogenesis. Primary cilia are required for the transduction of Hh signaling in vertebrates (Ben et al., 2011; Chung et al., 2012; Huangfu et al., 2003; T. J. Park et al., 2006; Yin et al., 2009). The *Ift88* gene is required for the biogenesis and maintenance of the cilium (Huang & Schier, 2009; Huangfu et al., 2003; Huangfu & Anderson, 2005; Pazour et al., 2000, 2002). By creating targeted mutations in the *ift*88 gene by CRISPR/cas9, we created mutant cell lines with out-of-frame biallelic indels. ~41% of the collected clonal cell lines had biallelic *ift*88 mutations (Table S2.7). 90-100% of the cells in two *ift*88 mutant cell lines, clones #28 and #30, lacked primary cilia (Figure 2.4D). Unlike control cells, *ift88* mutant clones #28 and #30 showed little or no increase in g*li*1 or *ptch*1 expression upon SAG exposure (Figure 2.5A and B). These results are similar to the results observed in MEFs collected from *Kif3a* null mice and *Ift*88 null mice where SAG treatment of the cultured cells failed to activate the Shh signaling pathway as a result of loss of cilia (Chen et al., 2009; Ocbina et al., 2009). Hence,

we provide genetic evidence that lizards require primary cilia for proper transduction of Shh signaling as they are in fish and mammals (and suggested for amphibians and birds).

In clone #25, we observed primary cilia formation, apparently as a result of functional /partially functional ift88 protein generated due to alternative splicing/exon skipping. We did not find any RNAseq evidence suggesting alternative splicing near this exon in any lizard samples aside from in clone #25. This includes RNAseq data from a variety of different lizard tissues in the Menke lab as well as primary LEFs, the parental clone, and *ift88* mutant clones 28 and 30. However, the human and mouse *IFT88* gene model data in the UCSD genome browse shows that this homologous exon can alternatively be spliced in those systems. It is possible that in culture conditions, this specific clone (#25) developed novel epigenetic modifications resulting in some degree of alternative splicing which skips the 57 bp exon and generates a version of ift88 lacking only 19 amino acids. Although these cells were able to assemble cilia and showed an appropriate increase in *gli*1 and *ptch*1 expression in response to SAG, they appear to be deficient in repression of the Hh pathway in the absence of stimulation. This raises the possibility that the IFT88^{D19aa} version is sufficient for building cilia that can activate the Hh pathway but cannot properly restrain it when the inducer is not present.

Role of primary cilia in lizards

Genetic evidence in mouse embryos lacking cilia shows a pronounced loss of ligand-induced Hh pathway activation and mild ligand-independent (derepressed) activity in the neural tube (Chen et al., 2009; Jia et al., 2009). In these mutants, cilia dependent *Gli2* activation is lost which in turn dramatically suppresses the activation of *Gli*1. The formation of *Gli3* repressor is greatly attenuated in mouse cells lacking cilia. The data

indicates that failure to induce the pathway is due to loss of Gli activator function whereas mild derepression (seen in the ventral neural tube, Huangfu et al. 2003) is due to defective formation of Gli repressor. In contrast, zebrafish lacking cilia display stronger ligand independent Hh pathway derepression, which cannot be further increased by Hh ligands (Huang & Schier, 2009). The derepression of the pathway observed in zebrafish *ift88* mutants appears to be primarily mediated by Hh-independent activation *gli1* rather than by a reduction in Gli repressor activity. Reptiles diverged from mammals more recently than did ray-fin fishes (~320 vs.~450 Mya). Hence, we were curious about the extent to which cilia are important for Hh pathway repression in reptiles. We observe a roughly 3-to-4-fold increase in basal *gli1* expression in unstimulated *ift88* mutant lizard embryonic fibroblasts (Figure 2.5C and S2.4C) whereas *Ift88* mutant mouse embryonic fibroblasts (without stimulation) do not exhibit detectable derepression of the Hh pathway (Ocbina 2009 pmid:19718259). Thus, it appears that cilia may be more important for Hh pathway repression in zebrafish and lizards than in mice, perhaps due to the constitutive activation of *gli1* in lizards that lack primary cilia as shown for zebrafish (Huang and Schier, 2009).

The RNAseq data from primary limb LEFs and MEFs (Figure 2.2) shows that *gli*2 is not differentially expressed in lizard cells in response to ligand SAG. RNAseq data from the ASEC-1 cell line (data not shown) corroborates that *gli*2 is not differentially expressed in lizard cells exposed to SAG. Taken together we suggest that in lizards *gli*1 is potentially a major activator of the Hh signaling pathway, as in zebrafish but not mice. More genetic experiments to explore the role of *gli*1/*gli*2 and *gli*3 proteins need to be done.

To study the sequence divergence of Gli transcription factors in vertebrates, phylogenetic analysis was performed by Abbasi et al.(2009). They discovered three aspects

of Gli evolutionary patterns. All three Gli proteins suggested a relatively relaxed selection in the fish lineage. They found that fish *gli*1 evolved at a significantly higher rate than mammalian *Gli1*. *Gli*2 and *Gli*3 experienced equivalent functional constraints in mammalian and fish lineages and thus evolved at a considerably slow rate (Abbasi et al., 2009). Functional studies from zebrafish, *Xenopus* indicate *gli*1 is the predominant activator of the Hh pathway in these species. If, as we suggest, *gli*1 is the predominant activator of the pathway in reptiles, it is possible that the role of *gli2* as the predominant activator of the pathway emerged after the divergence of reptiles and mammals. Moreover, the greater importance of cilia in Hh pathway repression in lizard cells compared to mouse cells suggests they use the cilium in a manner more similar to zebrafish where cilia prevent *gli1* activating targets in the absence of ligand stimulation.

Conclusions

We have successfully established an immortalized lizard cell line to study reptilian biology. We also provided evidence of efficient gene editing using this cell line. We have provided evidence indicating that primary cilia are required for Hh signal transduction in *A. sagrei* and, likely other reptiles. The specific roles of Gli proteins in lizards still need to be addressed and we are currently addressing that in the ASEC-1 cell line. For reptilian biologists who wish to perform genetic studies but cannot do so in vivo, this cell line will be an easily accessible tool. To explore the biology of thermoregulation this cell line provides a useful tool as it grows at a lower temperature than cells from endothermic organisms. For biomedical research, studying evolutionarily conserved pathways and gene functions in different species is crucial. Our cell line is a novel tool representing reptilian biology. We believe this tool will be used by evolutionary geneticists, developmental

biologists, and molecular biologists for understanding human health questions from an evolutionary perspective.

Methods

Establishing an experimental system to study Hh signaling using in ovo approach in A.sagrei.

A total of 18 eggs were collected within 24 hr of being laid from the lizard colony. Eggs were carefully cleaned with Kim wipes to remove any dirt on them. In a 24-well plate, each egg was carefully placed in an individual well. A 10nM SAG stock solution in DMSO solvent was prepared (CAS 912545-86-9 - Cayman Chemical). A 100µM SAG solution from 10nM SAG stock and a 'control DMSO' solution with an equivalent volume of DMSO was prepared. In each well 80µl of either 100µM of SAG or control DMSO solution was placed below the eggs carefully making sure that the egg was in contact with the liquid (Figure 2.1A). It is necessary for capillary action. 9 eggs received 100µM of SAG solution and 9 received control DMSO solution. Eggs were incubated at 29 ºC. After 24 hr, SAG and DMSO solutions were replaced by 80µl of water. The eggs were incubated for 14 days at 29 ºC. The eggs were monitored every day and 80µl of water was added as and when required to avoid drying of the eggs. On the 15th day, eggs were dissected in 1X PBS solution, and embryos were analyzed for any morphological differences. The developmental staging was done according to Sanger et al., 2008. Embryos were imaged and fixed in 4% PFA. Embryos were then dehydrated in serial dilution of MeOH starting from 25% to 100%. Embryos were then stored in 100%MeOH at 4ºC.

Establishing a primary tissue culture system to study Hh signaling in A.sagrei.

A. sagrei egg was cleaned with a Kim wipe soaked in 70% EtOH. All the vermiculite was removed, and the egg was given 2-3 quick washed with 1X PBS to clean the eggshell. The egg was dissected in sterile 1X PBS. A stage 6 embryo was then transferred into a clean petri dish with sterile 1X PBS containing 1X Pen/strep/amphoterin B antibiotics. The embryo was gently washed by swirling the dish. The process was repeated at least 3 times. For collecting LEFs from the limb tissue, with the sterile forceps, the head was removed and limb buds were separated from the embryo. All four limb buds were aseptically transferred in a 1.5 ml Eppendorf tube with 0.5 ml 0.05% Trypsin-EDTA. For collecting LEFs from the torso region (Figure 2.1C), the head, tail, and limb buds were removed with sterile forceps. The rest of the tissue was eviscerated. The tissue was then teased with the forceps and aseptically transferred into a 1.5 ml Eppendorf tube with 0.5 ml 0.05% Trypsin-EDTA. The tissue was then incubated at 29 ºC for 45 mins -1hr. For facilitating the tissue disintegration, the tissue was teased by pipetting the trypsin solution up and down gently at every 15 minutes intervals. After the incubation, the trypsin is neutralized by adding an equivalent volume of LEF growth medium [1X DMEM with 4.5g/l glucose, without L-glutamine, 10% heat-inactivated fetal bovine serum (Benchmark), 1X glutamine,1X Pen/Strep/amphoterin B]. The cells were collected by centrifuging at 1200-1300rpm at RT. The cell pellet was resuspended in 100µl of LEF growth medium. The cells were uniformly plated on a 24-well plate and incubated at 29 ºC at 5% CO_2 for 1-1.5hr. This ensured that the cells were attached to the plate. Afterwards, 1 ml of LEF growth medium was added to each well, and cells were allowed to grow. This is passage # 0. When the cells became

confluent, they were split in a 1:2 ratio and plated again. This was passage # 1. Cells from an individual embryo are considered as one biological replicate.

Pharmacological manipulation of shh signaling pathway in primary LEFs

Primary limb and/or torso LEFs were collected from *A.sagrei* stage 6 embryo. Primary LEFs from an individual embryo were grown till passage 3. After passage 3, the cells were seeded with equal density into two wells in a 24 wells plate. After the cells from passage #4 become confluent, they were serum starved for 48 hr by adding serum starvation medium [1X DMEM with 4.5g/l glucose, without L-glutamine, 1% heat-inactivated fetal bovine serum (Benchmark), 1X glutamine,1X Pen/Strep/amphoterin B]. Serum starvation results in cell cycle exit inducing ciliogenesis (Pugacheva al,2007). 200nM SAG solution was prepared in a serum starvation medium. The volume equivalent of DMSO solution was also prepared in the serum starvation medium. 200nM SAG was added to one well and a DMSO solution was added to another well. Both wells had primary LEFs collected from the same embryo. 24 hr after the addition of SAG, RNA was collected from these cells.

RNA isolation, cDNA synthesis, and qRT-PCR

Cells were lysed in 500µl Invitrogen™ TRIzol reagent and total RNA was isolated according to the Invitrogen™ TRIzol reagent user guide. RNA yield was measured using Qubit reagents. cDNA was synthesized using ProtoScript® II First Strand cDNA Synthesis Kit (New England BioLabs). We used both oligo dT and random primers to synthesize cDNA. Depending upon the yield, up to 500ng of the total RNA was used as a template per reaction. *gli*1 and *ptch*1 expression in the cells exposed to SAG were measured by qRT-PCR using Roche Lightcycler(R) 480 instrument. Roche Lightcycler(R) 480 SYBR green I Master reaction mix along with the respective primers (Table 2.1) used and 10ng of cDNA

template was used for every reaction. The PCR conditions were Pre-incubation at 95 ^{0}C- 5 mins followed by 44 amplification cycles of [95 ^{0}C-10 sec,58 ^{0}C-10 sec,72^{0}C-10 sec], for the melting curve analysis 95^{0}C- 5mins -65^{0}C- 1 min-40 ^{0}C- 30sec. For each biological replicate, three technical replicates were used. Delta ct value was calculated by normalizing *gil*1 and/or *ptch*1 expression with the appropriate reference gene. When more than one reference gene was used as a normalizer, the geometric mean of delta ct values with the individual normalizer gene was calculated. This geometric mean of delta ct value was then further used for 2^-delta delta ct calculations. The statistical analysis was performed on delta ct values.

RNAseq analysis for DE genes in A.sagrei and M.musculus after shh pathway induction

We stage-matched *A.sagrei* and *M.musculus* embryos based on their limb morphology at the given developmental stage. 5 embryos from *A.sagrei* stage late 5/early 6 (Sanger et al., 2008) were collected and limb buds were dissected out. Primary limb LEFs were collected as described before. 5 embryos from *M.musculus* were collected at stage E11.5. Primary limb MEFs were collected in a similar fashion to the primary LEFs. MEFs were grown at 37 ^{o}C at 5%CO2. For both LEFs and MEFs cells from individual embryos were divided into two wells. 200nM SAG was added to one well and the volume equivalent was added to another well. The embryos were processed in a pair-wise manner and analyzed the same way. 24 hours after the SAG exposure, we collected total RNA using the mirVana miRNA Isolation Kit (ThermoFisher Scientific). mRNA libraries were prepared using the TruSeq Stranded mRNA Library Prep Kit (Illumina). We generated 5 biological replicates for each treatment namely, primary LEFs treated with SAG, primary LEFs treated with DMSO control, primary MEFs treated with SAG, and primary MEFs treated with DMSO

control. These were then sequenced at the Georgia Genomics and Bioinformatics Core. The quality of the reads was checked with FastQC v0.11.8. *A.sagrei* reads were aligned to the A.sag2.1 genome using HISAT2 v2.1.0 and transcripts were counted using the feature count function from Rsubread v2.10.5. *M.musculus* reads were aligned to the mm10 genome. We then examined sample similarity and batch effect with principal component analysis. Differentially expressed genes between SAG treated and untreated samples in both species were determined using DESeq2 v1.36.0. The batch effect due to sampling from different embryos was removed by putting batch in the design formula when constructing DESeq Dataset from the count matrix.

Selection of reference genes for qRT-PCR

We used RNAseq data to identify genes that were stably expressed in DMSO, and SAG treated LEF samples. The stable expression was defined as CV below 0.5. We initially identified 7 candidates as reference genes. We then compared the number of transcripts for *gli*1 and *ptch*1 to all 7 candidates. We then shortlisted 3 candidate genes namely *tbp*, *atp5f1d*, and *atp5f*. The stability of the expression level of these 3 candidate genes was determined by qRT-PCR. Three independent biological replicates for primary LEFs were used to calculate the average ct value. We performed a similar experiment with the ASEC-1 cell line when exposed to different SAG concentrations (n=3). We calculated primer efficiencies using a 2-fold dilution series of the cDNA samples from the ASEC-1 cell line.

Generation of immortalized lizard embryonic fibroblast cell lines

We collected primary LEFs from the torso region of stage 6 *A.sagrei* embryo as described before. We grew the cells from a 24-well plate to a 10cm plate keeping track of the passage number. After the cells were confluent in a 10cm plate (around passage #6),

we serially passaged the cells with a 1:3 split ratio till passage #24. After the 24th passage, we changed the split ratio to 1:6. The growth rate of the cells was slowed down at this time point as cells started undergoing senescence. At passage #26 very few cells were attached to the plate. There was no obvious visible sign of cell growth for 13-15 days. We changed the LEF growth medium frequently during this time. After this 'no growth period', visible cell growth was observed in the form of patches or colonies of the cells. The few cells which escaped the cellular senescence started growing at this point. We allowed these cells to grow and become confluent. Within 7-8 days the cells in the 10cm plate were confluent. Cells were then trypsinized and single cells were sorted using the MoFlo Astrios EQ (Beckman Coulter) cell sorter at the cytometry shared resource laboratory at UGA. Each cell was sorted into a well pre-filled with LEF growth medium in a 96-well plate. The cells were incubated at 29^{o}C at 5%CO2. These single cell clones started growing in ~7 days. Once cells started growing, we expanded the clones, froze them, and stored them in Liq.N2 till further processing.

Pharmacological manipulation of shh signaling pathway in immortalized LEFs

Out of 40 clonal cell lines, we used the ASEC-1 clonal cell line for pharmacological manipulation of the shh signaling pathway. We seeded the wells with an equal density of the ASEC-1 cells. Each well is considered a biological replicate. Cells were prepared and serum starved as described before. We treated the cells with different SAG concentrations from 50nM to 500nM and the volume equivalent of DMSO. For every SAG concentration, three biological replicates were assayed. RNA was collected after 24 hr. We exposed the ASEC-1 cell line to 200nM SAG for different time intervals from 6 hr to 72 hrs. For each time interval, a volume equivalent of DMSO was used as a control. The experiment was

repeated twice. For SAG responsiveness of the cell lines, three biological replicates were treated with 400nM SAG, and three biological replicates were treated with volume equivalent of DMSO for 24 hr. This experiment was repeated three times.

CRISPR/Cas9 gene editing of ASEC-1 cell line

We designed gBlocks with IDT guidelines with a human U6 promoter followed by the gRNA sequence (5'-GTTCTTGAACTGGATGTACC-3'). We transfected ASEC-1 cells with Cas9 plasmid with puromycin resistance (Addgene pSpCas9(BB)-2A-Puro (PX459) V2.0) and gBlock in a 1:1 ratio. We used lipofection as a transfection method. Lipofectamine LTX Plus kit from Thermo fisher was used for lipofection. 48 hours after the transfection, the cells were put under puromycin selection at the concentration of 25µg/ml for 24 hours. After 24 hr, puromycin-resistant cells were allowed to grow and become confluent. Cells were then trypsinized and single cells were sorted as described before in a 96-well plate. The cells were incubated at 29ºC at 5%CO_2. These single cell clones started growing in ~7 days. Once cells started growing, we expanded the clones, froze them, and stored them in Liq.N2 till further processing.

PAGE screening for detection of mutant cell line

We collected DNA from each clone. The targeted ift88 region was then PCR amplified with primers: ift88-screen-F1-5'-GATAATTCTGAATTAATTATAAACTCTTG-3' and ift88-screen-R1-5'-TAACATTTAGGGCTCACTGG-3'. PCR conditions were

95^0C-2 min; [95^0C- 30 sec-58^0C-30 sec-72^0C-30 sec]X30 cycles; 72^0C-5min.

PAGE protocol was adapted from VanLeuven et al. (2018). We performed electrophoresis at 150mV for 7 hr.

Immunostaining

The cells were plated on a gelatin-coated coverslip in a 24-well plate. Once the cells were confluent, we serum starved them for 48 hrs to promote ciliogenesis, as described previously. After 48 hrs, the cells were washed with 1X PBS twice and fixed in EMS grade 4% PFA for 15 min at RT. The cells were then washed with 1X PBS three times. Cells were incubated at RT for 1 hour in freshly made blocking solution (10% heat-inactivated goat serum with 0.1% Triton X100 in 1X PBS). Cells were then washed with wash buffer (1% heat-inactivated goat serum with 0.1% Triton X-100 in 1X PBS) for 10 mins at RT. The cells were incubated with primary antibody solution 1:8000 dilution of acetylated α tubulin antibody (Sigma Aldrich T7451) and 1:125 dilution of ift88 antibody (Abcam AB 184566) overnight at 4ºC. The next day the primary antibody solution was removed. We washed the cells three times with the wash buffer at RT. The cells were then incubated in the dark for 1 hr at RT with secondary antibody solution with anti-mouse cy3 α mouse (1:125 dilution, Jackson immunology 715.167-003), anti-rabbit (1:125) Alexa 488 fluor and DAPI as a nuclear stain (Sigma Aldrich 1mg/ml). After 1 hr of incubation with the secondary antibodies, we mounted the coverslips on a slide. A drop of Vectashield was used to protect the fluorescence. The slides are stored at 4ºC till imaging. The primary cilia were observed and imaged with Zeiss LSM 880 Confocal Microscope.

Scanning Electron Microscopy

The cells were plated on a gelatin-coated coverslip in a 24-well plate. Once the cells were confluent, we serum starved them for 48 hrs to promote ciliogenesis, as described previously. After 48 hrs, the cells were washed carefully with 1X PBS at least 3 times. This is to ensure all the traces of serum are washed away carefully. The cells were then fixed in

a fixative (2% glutaraldehyde and 2% PFA solution) provided by the Georgia Electron Microscopy core facility at UGA. The cells were processed and imaged by the core facility using the Thermo Fisher Scientific (FEI) Teneo, a field emission scanning electron microscope (FESEM).

Sex Genotyping

Following primers were used in equimolar concentration in a multiplex PCR reaction using a DNA template from the ASEC-1 cell line.

AsagB-F1-5' GAAGACCAGGAGAGCAARGTC 3';

AsagB-R1-5' GATGTCGGCAGCYTTGCGTAC 3' ;

kank1_AcF - 5' CCTTCCTTTGTAGGATCCAGTG 3' ;

kank1_AcR - 5' GGAGCACAGGGATAGTTTTGAC 3'.

The PCR conditions were 95ºC for 30 sec for initial denaturation; 35 cycles of 95ºC for 30 sec; 58 ºC for 30 sec; and 72ºC for 30 sec; 72ºC for 2 min for the final extension.

FIGURES AND TABLES

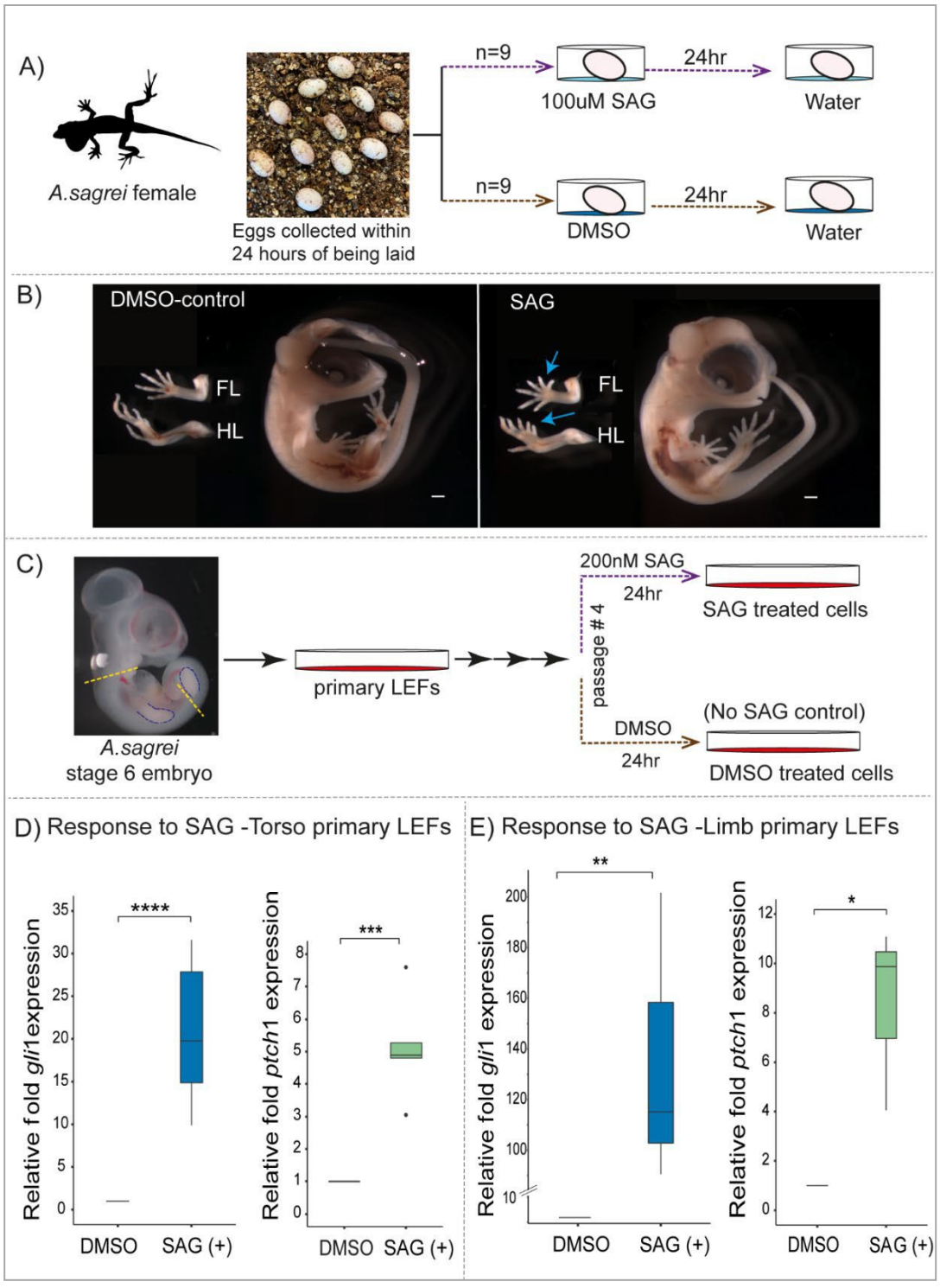

Figure 2.1: Establishing in ovo and a cell culture system for studying the Hh signaling pathway in *Anolis sagrei*

(A) Schematic representation of the experimental design for in ovo manipulation of the shh pathway in *A.sagrei.* Each well represents a single well from a 24-well plate with an egg placed on top of 80µl of either DMSO or 100 µM SAG solution. A total of 18 eggs were processed in a single 24-well plate at 29^0C. DMSO or SAG solution was replaced by water after 24hr. The embryos were dissected out on the 15^{th} day. (B) Representative images of the stage-matched embryos from the eggs exposed to a bolus DMSO and SAG treatment. The SAG treated embryo is polydactyl. Blue arrows emphasize the presence of extra digits. FL= forelimbs, HL-hindlimbs. Scale bar =500µm. (C) Schematic illustration of the experimental design for inducing the shh pathway in *A.sagrei* primary embryonic fibroblast cells (LEFs). Cells from either the torso region (indicated by the region between two dashed yellow lines) or limb buds (outlined by blue dotted lines) from a single embryo were plated on a 24-well plate. After passage 3, the cells were divided into two equal parts and plated. As indicated in the figure, one part received DMSO, and the other received SAG treatment. (D) qRT-PCR data for relative fold *gli*1(blue) and *ptch*1(green) expression in *A.sagrei* torso primary LEFs when exposed to 200nM SAG for 24 hr. *gli*1 and *ptch*1 expression was normalized to *gapdh* expression. The fold gene expression values are relative to DMSO treated primary torso LEFs from the same embryo. n=5, **** p value<0.0001, *** p value <0.001. p values represent the statistical significance between delta ct values by paired t-test. (E) qRT-PCR data for relative fold *gli*1(blue) and *ptch*1 (green) expression in *A.sagrei* limb primary LEFs when exposed to 200nM SAG for 24 hr. *gli*1 and *ptch*1 expression was normalized to *gapdh* expression. The fold gene expression values are relative to DMSO treated primary limb LEFs from the same embryo. n=3, **p value <0.01, *p value <0.05. p values represent the statistical significance between delta ct values by paired t-test.

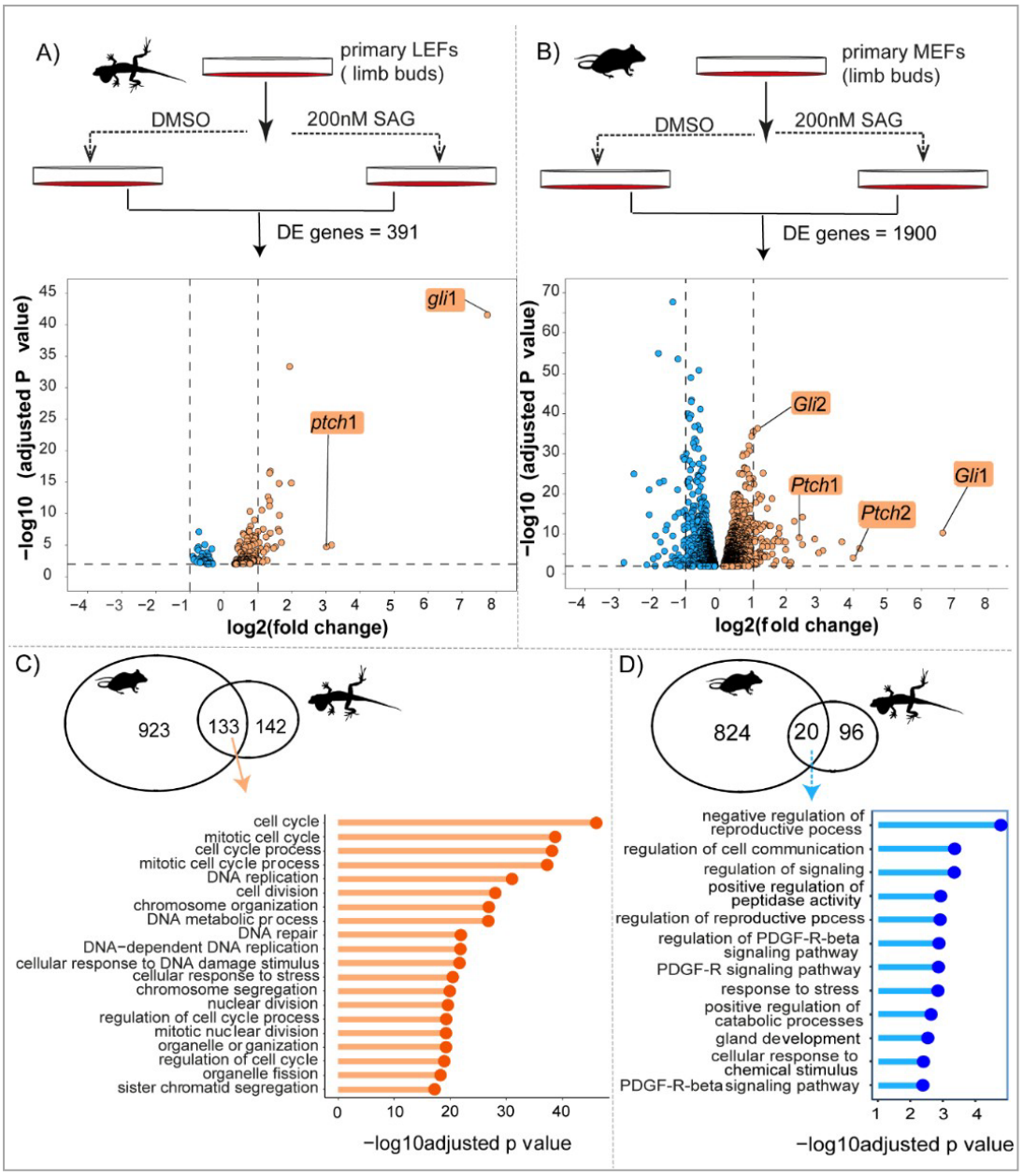

Figure 2.2. Shared differential gene expression in *A.sagrei* and *M.musculus* limb primary embryonic fibroblast cells in response to 200nM SAG treatment for 24 hr. (A)-(B) Schematic presentation of the experimental design and volcano plot of DE genes in *A.sagrei* and *M.musculus*. Primary LEFs were collected from stage 5 embryo limb buds.

Primary MEFs were collected from E11.5 embryo limb buds. Cells from a single embryo were divided into two parts. One part received DMSO and the other received 200nM SAG. n=5. The data was analyzed in a pairwise manner. The volcano plot shows the DE genes which have adjusted p value <0.05. The circles represent DE genes. The orange color shows upregulated genes and the blue color shows downregulated genes. (C) Venn diagram shows shared and distinct upregulated genes in *A.sagrei* and *M.musculus.* The lollipop plot shows the top 20 biological processes GO terms associated with the shared upregulated genes. (D) Venn diagram shows shared and distinct downregulated genes in *A.sagrei* and *M.musculus.* The lollipop plot shows all the biological processes GO terms associated with the shared downregulated genes.

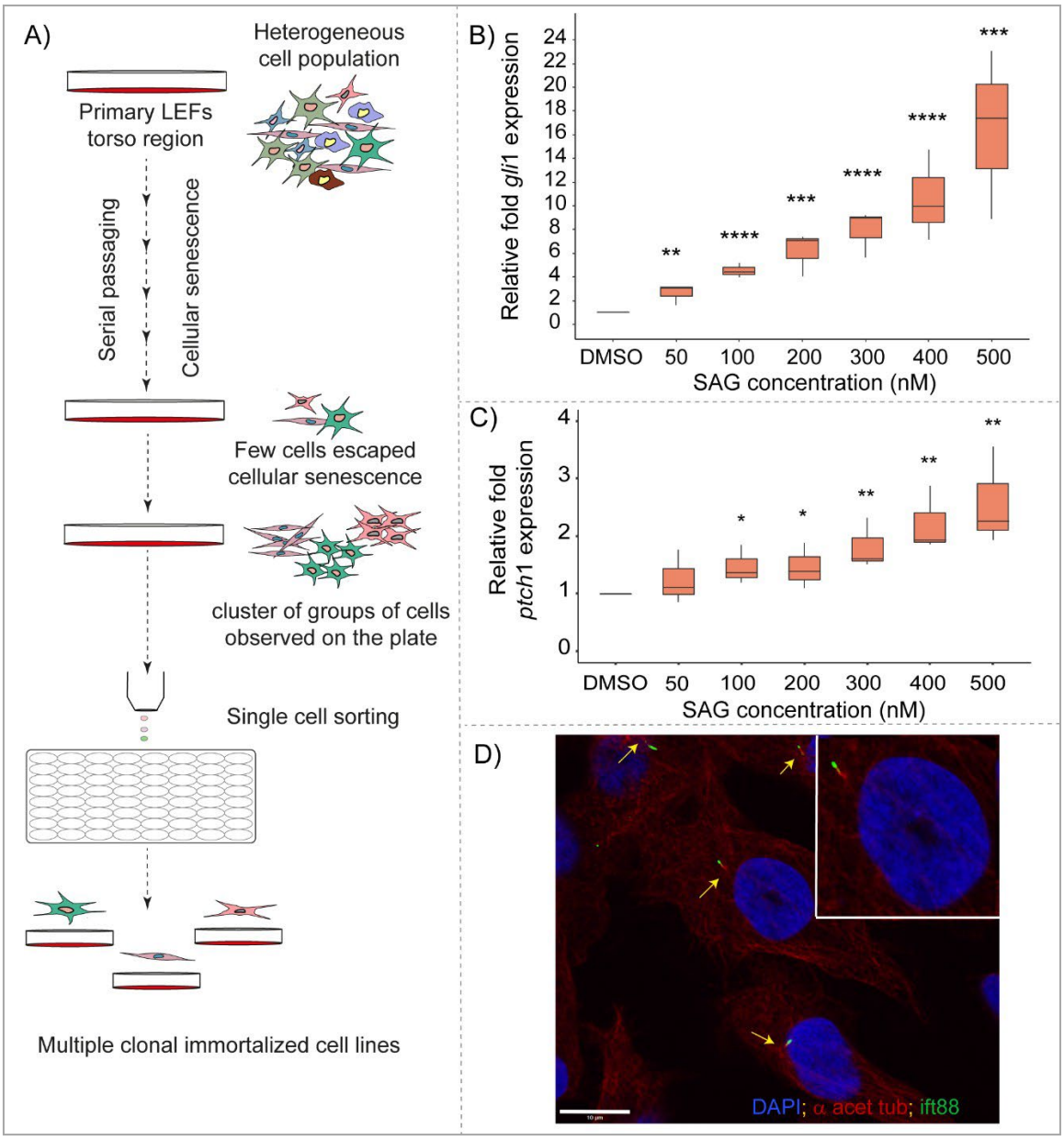

Figure 2.3. Generation of immortalized lizard cell lines and SAG responsiveness of ASEC-1 cell line with IF image of the primary cilium.

(A) Illustration representing the protocol of generation of an immortalized cell line from stage 6 *A.sagrei* torso primary LEFs. (B) qRT-PCR data showing relative fold *gli*1 expression in ASEC-1 cell line in response to various SAG concentrations. The *gli*1 expression is relative to DMSO treated cells. n=3.*gli*1 expression is normalized with two

reference genes namely *tbp* and *atp5f1d*. **** p value <0.0001, *** p value <0.001, **<0.01. p values represent the statistical significance between delta ct values of DMSO and SAG treated samples by paired t-test. (C) qRT-PCR data showing relative fold *ptch*1 expression in ASEC-1 cell line in response to various SAG concentrations. The *ptch*1 expression is relative to DMSO treated cells. n=3. *ptch*1 expression is normalized with *tbp* and *atp5f1d* expression. **** p value <0.0001, *** p value <0.001, **<0.01. p values represent the statistical significance between delta ct values of DMSO and SAG treated samples by paired t-test. (D) Representative image of co-immunostaining for visualization of the primary cilium in ASEC-1 cell line with α acetylated tubulin antibody(red), ift88 antibody (green), and nuclear stain DAPI (blue). The primary cilium indicated by yellow arrows shows ciliary axoneme stained with α acetylated tubulin antibody and the tip of the cilium stained with ift88 antibody. Scale bar = 10µm.

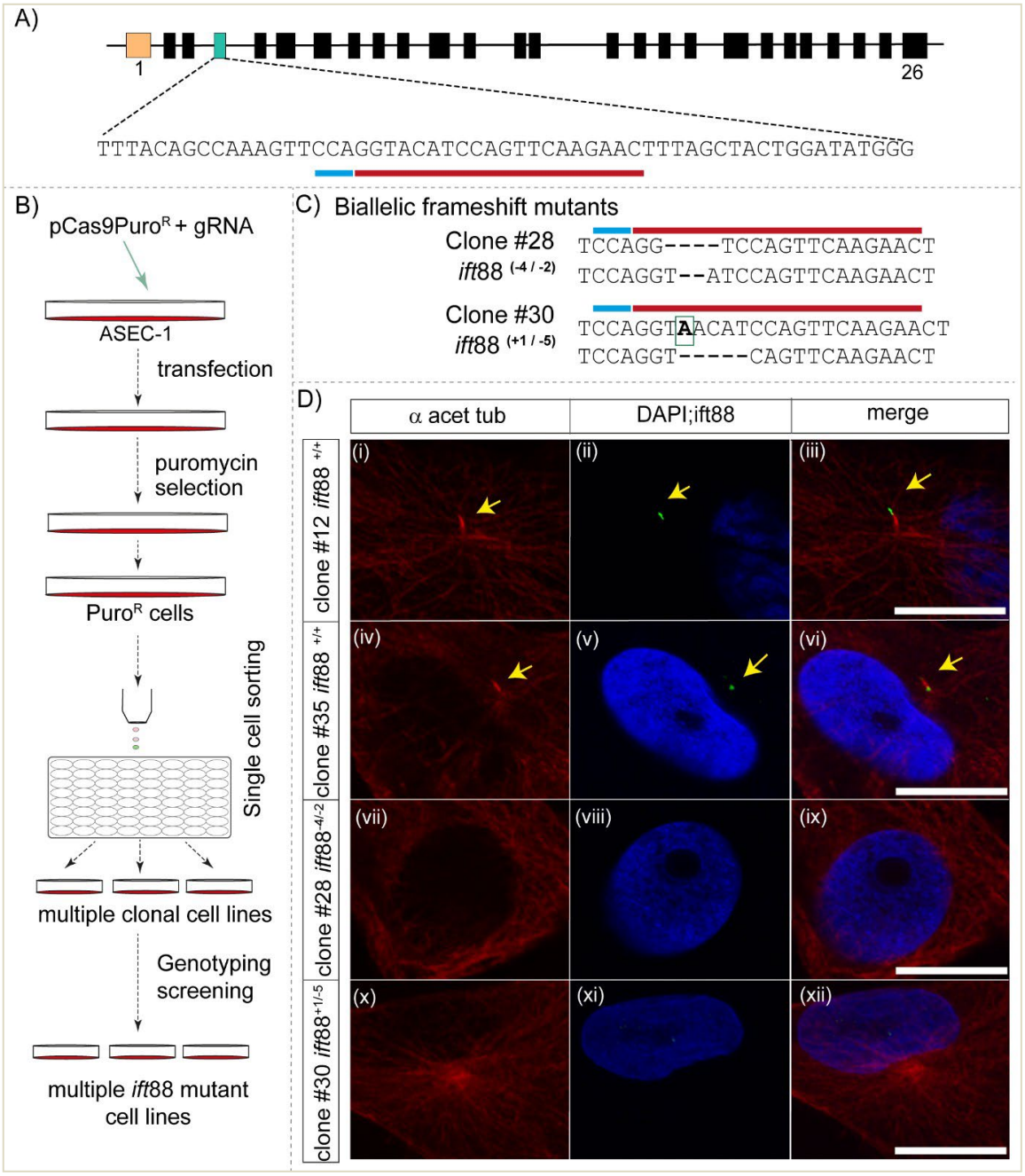

Figure 2.4. CRISPR/cas9 editing of *ift*88 gene in ASEC-1 cell line and *ift*88 mutant cell lines (A) Schematic representation of *A.sagrei ift*88 gene (not to the scale). Black rectangles indicate exons. The first exon is noncoding and is indicated by orange color. The fourth exon was targeted for gene editing and is represented by green color. The gRNA

sequence is specified by a red line segment followed by the PAM site specified by a blue line segment. (B) Illustration of the protocol used to generate clonal *ift*88 mutant cell lines. The ASEC-1 cell line was transfected with puromycin-resistant plasmid and gRNA in a 1:1 ratio. 48 hours after the transfection, cells were exposed to 25µg/ml puromycin for 24 hours. Cells which survived puromycin selection were allowed to grow followed by single cell sorting. The individual clones were screened for detection of indels in *ift*88. (C) Representation of sanger sequence analysis of idels in biallelic mutants clone #28 and clone #30. The insertion of the base is highlighted by a bold letter and green rectangle. Each dash represents the deletion of a base. gRNA and PAM site positions are indicated by red and blue line segments respectively. (D) Representative images of IF staining for detection and visualization of the primary cilium in *ift*88 clones. In WT clones #12 and #35, α acetylated tubulin antibody (red) stained primary cilium axoneme (i) and (iv) respectively. In these clones, the ift88 antibody (green) stained the tip of the cilium (ii) and (v). No obvious primary ciliary structure was observed in clones #28 and #30 with α acetylated tubulin antibody staining(vii,x). ift88 antibody staining was not observed in clone #28 and clone #30 (viii,xi). Images iii, vi, ix, and xii show co-immunostaining with DAPI as nuclear stain, α acetylated tubulin, and ift88 as ciliary markers. Yellow arrows indicate the primary cilium. Scale bar = 10µm.

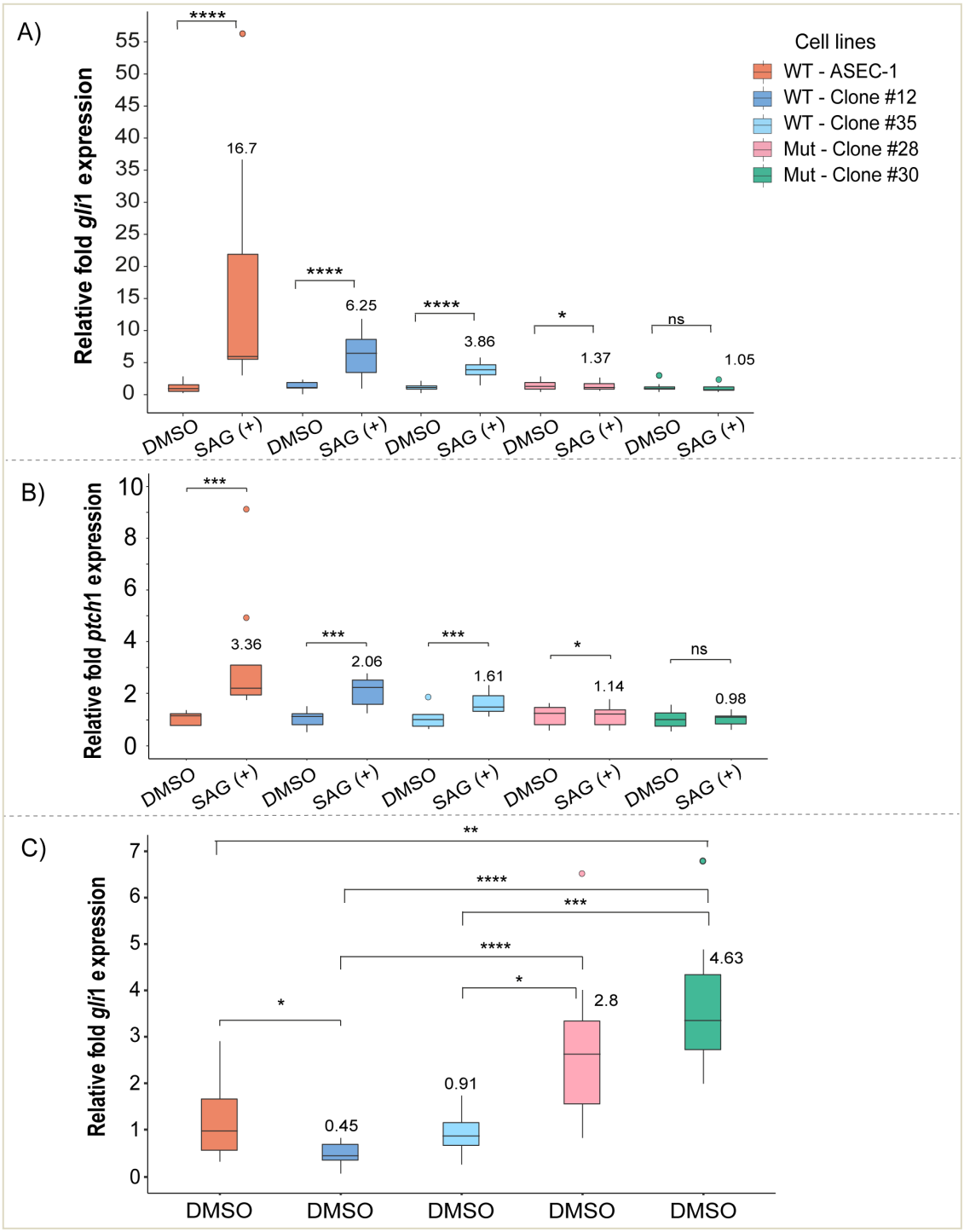

Figure 2.5. Relative *gli*1 and *ptch*1 expression by qRT-PCR analysis of ASEC-1 and *ift*88 clones in response to 400nM SAG exposure for 24 hr.

The cell lines are color coded, and the key is shown in panel A. (A)-(B) Relative fold *gli*1and *ptch*1expression (n=9). The data is normalized to the two reference genes *tbp* and *atp5f1d*. *gli*1or *ptch*1 induction in SAG treated samples is relative to the average delta ct values of *gli*1 or *ptch*1 expression in DMSO control of that cell line. The numbers on the top of the box plots are the mean values of fold induction in SAG treated cells. The x axis indicates SAG treatment. **** $p<0.0001$, ***$p<0.001$, ** $p<0.01$, *$p<0.05$ p values represent the statistical significance between delta ct values of DMSO and SAG treated samples using paired t-tests. Parental ASEC-1 cell line as well as two WT clones show an increase in *gli*1 and *ptch*1expression in response to the SAG treatment. Clone #30 does not show any significant change in *gli*1 and *ptch*1 expression while Clone #28 shows a very small increase in *gli*1 and *ptch*1expression. (D) Relative basal *gli*1 expression in DMSO treated ASEC-1, clone #12, clone #35, clone #28, and clone #30 cells (n=9). The data is normalized to the two reference genes *tbp* and *atp5f1d*. The *gli*1 expression is relative to the average delta ct value of *gli*1 expression in ASEC-1 DMSO treated cells. The numbers on the top of the box plots are the mean values of fold induction. **** $p<0.0001$, **$p<0.01$, *$p<0.05$ p values represent the statistical significance between delta ct values calculated by one-way ANOVA and Tukey's HSD post hoc test.

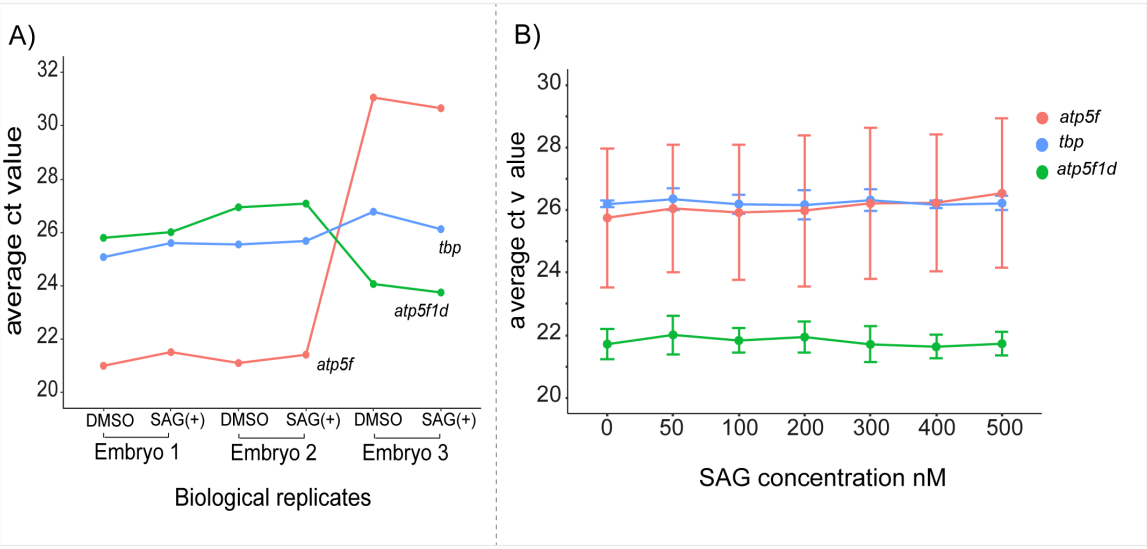

Figure S2.1: Selection of reference genes for qRT-PCR

(A) Expression of *tbp*, *atp5f1d,* and *atp5f* in primary limb cells. X axis indicates SAG treatment in three biological replicates. The Y axis indicates the average ct value corresponding to each reference gene in each biological sample. For each biological sample, there were three technical replicates. (B) Expression of *tbp*, *atp5f1d,* and *atp5f* in ASEC-1 cells. The X axis represents the SAG concentration to which the cells were exposed for 24 hrs. Y axis indicates the average ct value corresponding to each reference gene. Each data point indicates the average ct value of three biological replicates and error bars indicate the SD. For each biological sample, there were three technical replicates. *tbp* and *atp5f1d* had stable expression and hence were selected as reference genes for further data analysis.

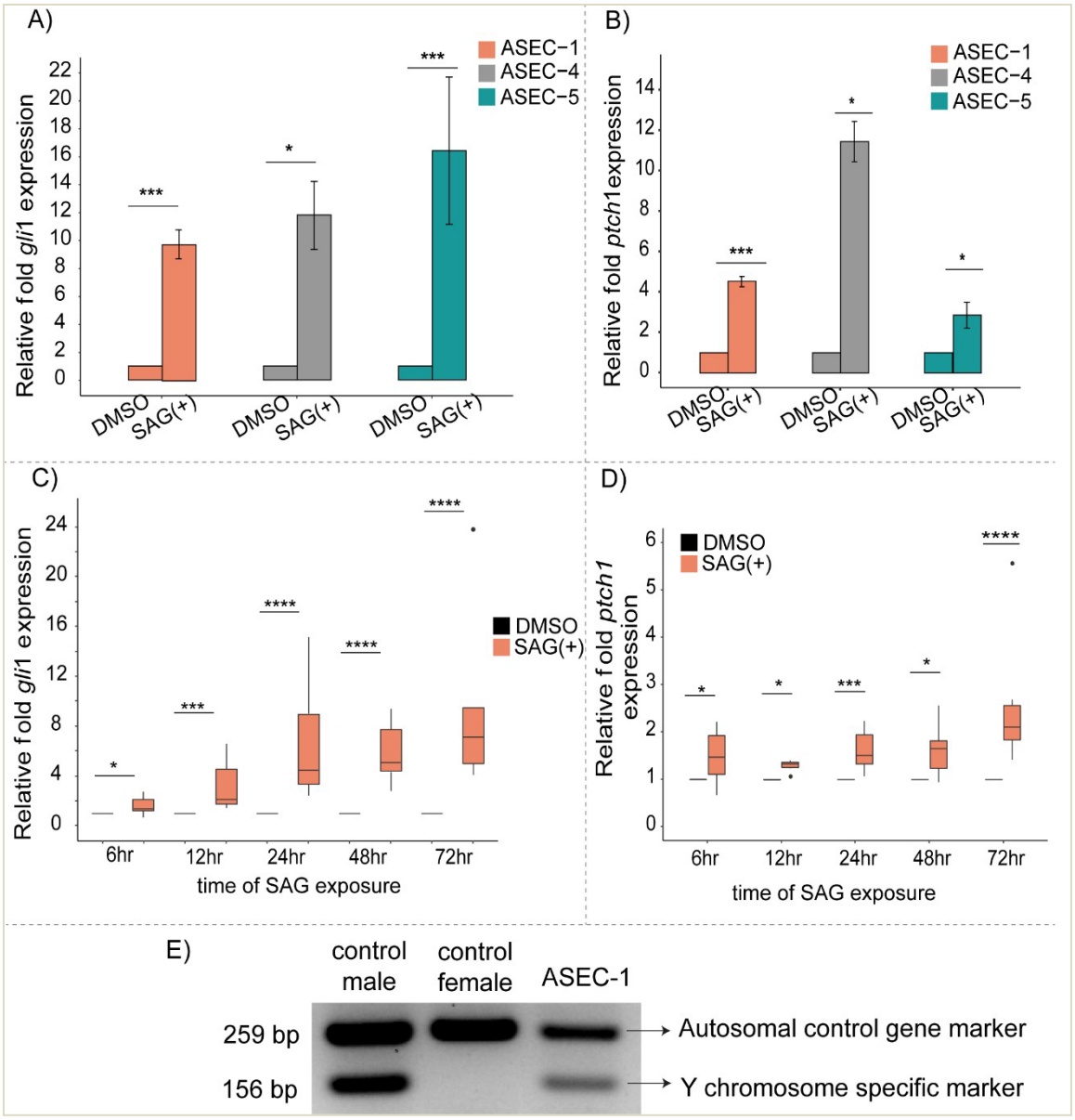

Figure S2.2: SAG responsiveness of immortalized cell lines assayed by qRT-PCR and sex genotyping. (A)-B) Relative fold *gli*1 and *ptch*1expression in three immortalized clonal cell lines generated. The bar graph represents the average value of *gli*1 and *ptch*1induction in SAG treated samples relative to DMSO control. The data was normalized with *gapdh*. Error bars represent SD. Biological replicates n=3 for ASEC-1, n=2 for ASEC-4, and n=3 for ASEC-5. For each biological replicate, there were three technical replicates.

(C)–(D) Relative fold *gli*1 and *ptch*1 expression in ASEC-1 cell line in response to 200nM SAG exposure for different time intervals. The box plots represent *gli1* and *ptch*1 induction in SAG treated samples relative to DMSO control for that time point. The data was normalized using two reference genes *tbp* and *atp5f1d*. The X axis represents the time of SAG exposure in hours. n=6. p values represent the statistical significance between dct values using paired t-tests. * $p < 0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$ (E) Representative gel image confirming the presence of Y chromosome in ASEC-1 cell line.

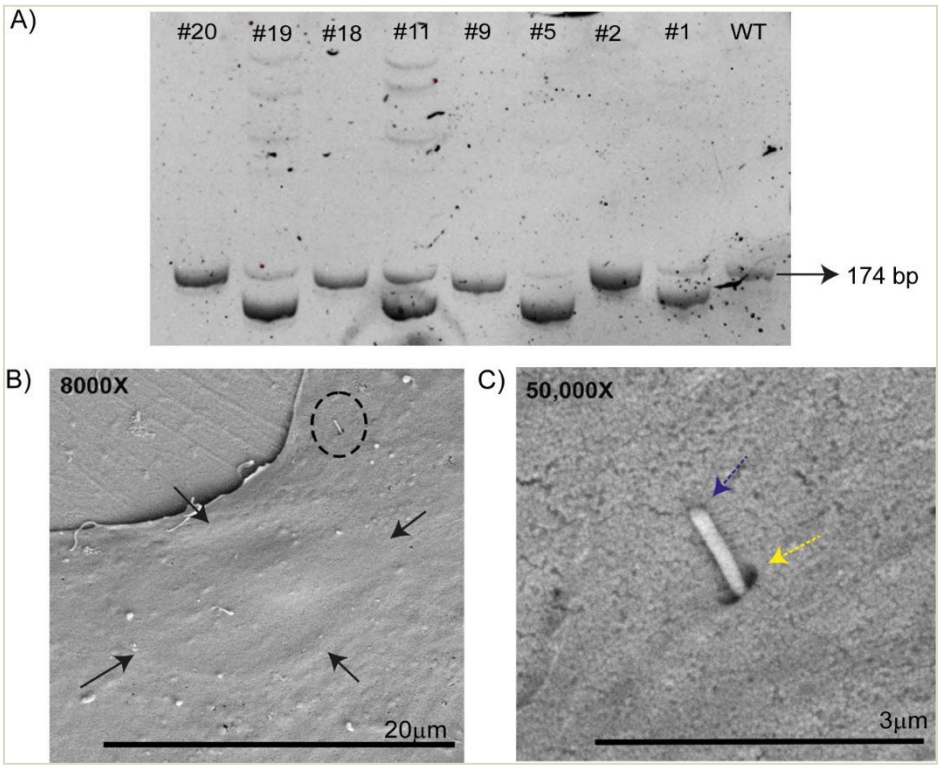

Figure S2.3: (A) Representative gel image of genotype screening by PAGE for *ift*88 mutants. Numbers on the top of the gel represent the unique ID numbers of the clones collected. Clones with deviation from the 174bp band were sequenced to confirm the nature of the mutation. (B) SEM image of a primary cilium in WT clone #12 *ift*88$^{+/+}$ at 8000 X magnification. The black arrows circle the position of the nucleus in a cell. The dotted black circle indicates the position of the primary cilium. (C) SEM image of the primary cilium

shown in figure panel B at 50,000X magnification. The yellow arrow indicates the ciliary pit, and the blue arrow indicates the tip of the cilium.

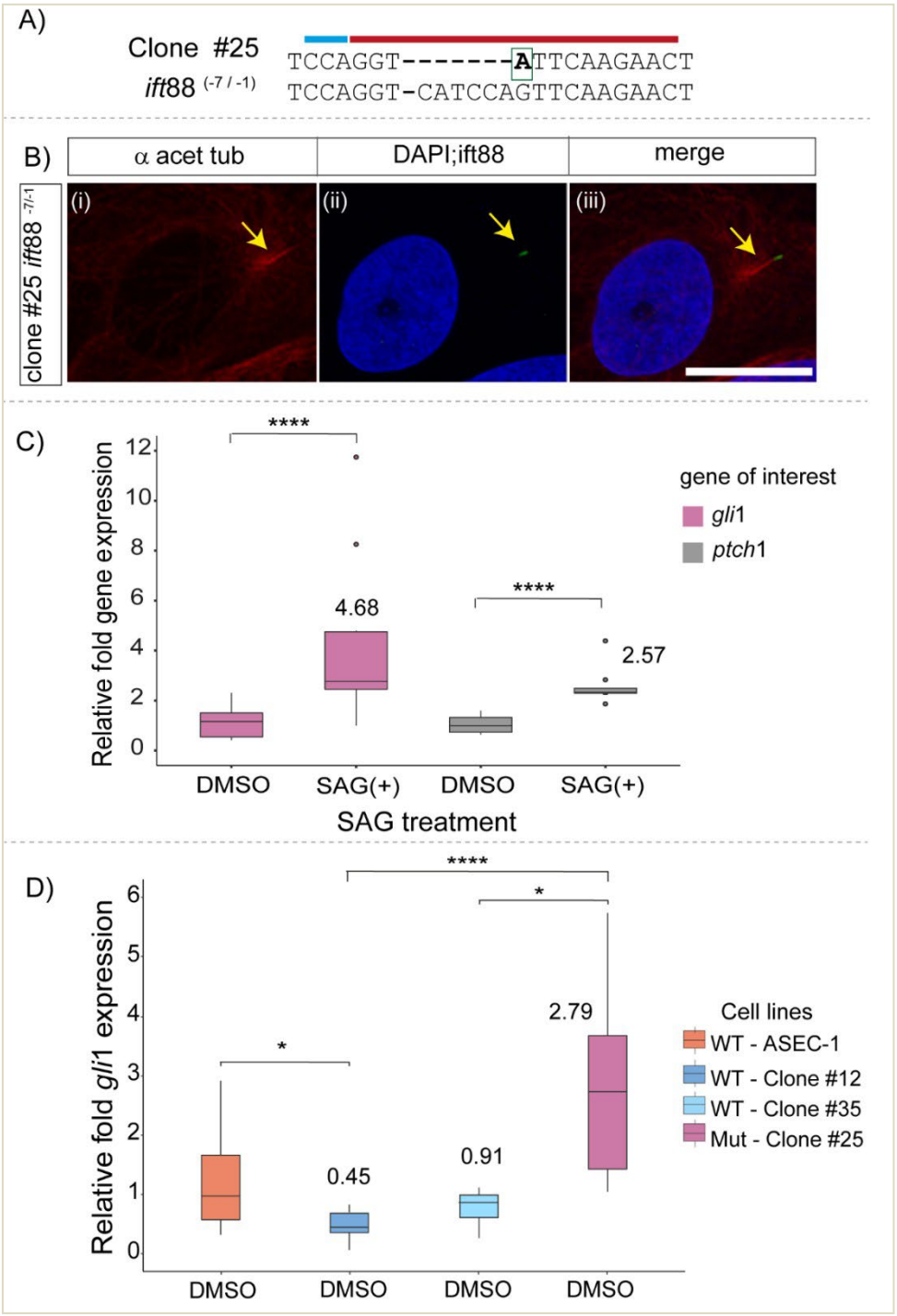

Figure S2.4: Presence of the primary cilium and SAG responsiveness of biallelic mutant cell line - Clone #25 *ift*88 $^{-1/-7}$ (A) Representation of nature of the mutation in *ift*88

gene in clone #25. The red line segment represents the position of the gRNA sequence and the blue line segment indicates the position of the PAM site. Insertion of the base is indicated by the bold letter and green rectangle surrounding it. Each dash represents the deletion of a base. On the one allele there was 8 bp deletion and one bp insertion (indicated by a green rectangle), hence a total of 7 bp deletions. On the second allele, only one bp deletion was observed. (B) Representative image of IF staining for visualization of the primary cilium (i) shows primary cilium stained with α acetylated tubulin antibody (ii) represents the nucleus and tip of the primary cilium stained with DAPI and ift88 antibody respectively and (iii) represents co-immunostaining with α acetylated tubulin antibody, ift88 antibody, and DAPI. Yellow arrows indicate the primary cilium. Scale bar = 10μm. (C) Relative fold *gli*1and *ptch*1expression by qRT-PCR in the cells exposed to 400nM SAG for 24hr (n=9). The data is normalized to the two reference genes *tbp* and *atp5f1d*. *gli*1and *ptch*1 induction in SAG treated samples is relative to the average delta ct values of *gli*1 or *ptch*1 expression in DMSO control. The numbers on the top of the box plots are the mean values of fold induction in SAG treated cells. **** $p<0.0001$ p values represent the statistical significance between delta ct values of DMSO and SAG treated samples using paired t-tests. (D) Relative basal *gli*1 expression by qRT-PCR in DMSO treated ASEC-1, clone #12, clone #35, and clone #25 cells (n=9). The data is normalized to the two reference genes *tbp* and *atp5f1d*. The *gli*1 expression is relative to the average delta ct value of *gli*1 expression in ASEC-1 DMSO treated cells. The numbers on the top of the box plots are the mean values of fold induction. **** $p<0.0001$, *$p<0.05$. p values represent the statistical significance between delta ct values calculated by one-way ANOVA and Tukey's HSD post hoc test.

Table 2.1: qRT-PCR primer sequences

Gene name	Primer name	Sequence
gapdh	A.car-gapdh-fwd	ATCGGAGTCAACGGATTTGG
	A.car-gapdh-rev	CATGTAGACCATGTAGTTCAGG
tbp	A.sag-tbp-fwd	TCTCCAATGACTCCCATGAC
	A.sag-tbp-rev	CAGCCAAGATTTACCGTAGA
atpf1d	A.sag-atpf1d-fwd	AGACTCTTCCGTCCAACTCC
	A.sag-atpf1d-rev	TCAGACAAGGCCTTCTCCAG
gli1	A.sag-gli1-fwd	GCTCAGTACATGCTGGTTGTC
	A.sag-gli1-rev	CCGTGAGTAGGCTTTATTGCAG
ptch1	A.sag-ptch1-fwd	GTGGAGTTTACGGTTCACATTG
	A.sag-ptch1-rev	CACAGGTGCAAACATGTGTTCT

Table S2.1: Digit numbers observed in polydactyl embryos from the eggs treated with 100 µM SAG

No.	Number of digits on hindlimbs		Number of digits of forelimbs		Developmental stage
	Left	Right	Left	Right	
1	5	5	5	5	Late 16
2	6	6	5	5	13
3	6	6	6	6	Late 13
4	6	6	6*	5*	14
5	6	6	6	6	13
6	6	6	5	5	14
7	6	6	5	5	13
8	6*	9*	6	6	Late 11
9	6	6	5	5	Late 13

* asymmetry observed between left and right digit numbers

Table S2.2: Validation of RNAseq data by qRT-PCR

Embryo no.	Fold induction relative to DMSO control			
	***gli*1**	***ptch*1**	***cldn*1**	***ramp*2**
1	12.6992	2.81539	1.37872	1.64338
2	65.7993	9.9406	2.16845	3.81055
3	66.0277	9.56877	1.25266	3.52674
p value =	0.00014	0.00408	0.01709	0.00729

p values are associated with a two-tailed paired t-test. The analysis was performed using delta ct values.

Table S2.3: The list of shared upregulated genes between *A.sagrei* and *M.musculus* in primary limb cells.

gli1	*ptx3*	*chaf1a*	*pclaf*	*ercc6l*	*acat2*	*ncapg*	*mad2l1*	*kif22*
mcm6	*lss*	*aacs*	*rnf157*	*pkmyt1*	*ndc80*	*fgf7*	*fancc*	*mcm8*
mcm5	*hmgcs1*	*esco2*	*nsd2*	*pcna*	*fdft1*	*incenp*	*pole*	*h2az1*
mcm4	*clspn*	*fdps*	*cped1*	*kif20b*	*ccn2*	*spc24*	*rad51ap1*	*bub1*
mcm3	*cldn1*	*shcbp1*	*cdc6*	*mybl2*	*atad2*	*dlgap5*	*jcad*	*plk4*
rrm2	*kif11*	*ckap2*	*dhcr24*	*dck*	*ncaph*	*atad5*	*nsdhl*	*kif14*
insig1	*msmo1*	*spc25*	*orc1*	*tacc3*	*nuf2*	*mcm7*	*cxxc5*	*cit*
cdca7	*mcm10*	*kif15*	*pdgfc*	*atp10a*	*tpx2*	*ncapg2*	*ptch2*	*gen1*
idi1	*top2a*	*hmgcr*	*fasn*	*hells*	*srebf2*	*gtse1*	*cyth3*	*hspd1*
mcm2	*ptch1*	*mki67*	*cip2a*	*nusap1*	*wdhd1*	*kif2c*	*melk*	*kif20a*
tyms	*pmp22*	*fst*	*ldlr*	*pola1*	*bub1b*	*ccna2*	*ect2*	*fanci*
uhrf1	*mms22l*	*itga11*	*rrm1*	*hmga2*	*cdk1*	*kif23*	*lrig1*	*brca1*

smc2	*stard4*	*ets1*	*dlc1*	*rad51*	*bard1*	*lig1*	*rfc4*	*pik3cb*
sqle	*dhcr7*	*wdr76*	*prc1*	*fabp5*	*cyp1b1*	*psat1*	*hspa9*	--------
tk1	*ccne2*	*mvk*	*asf1b*	*prim1*	*dhfr*	*timeless*	*rbl1*	--------

Table S2.4: The list of shared downregulated genes between *A.sagrei* and *M.musculus* in primary limb cells.

cpt1a	*adcy7*	*glis1*	*pxdn*	*cdkn1b*
plat	*bcl2l11*	*lox*	*ada*	*nfat5*
dapk1	*antxr1*	*dhrs3*	*gja1*	*hip1*
plxdc2	*adamts8*	*tgfbi*	*vasn*	*clmn*

Table S2.5: The list of shared DE genes in opposite directions between *A.sagrei* and *M.musculus* in primary limb cells.

Sr. No	**DE genes upregulated in *A.sagrei* and downregulated in *M.musculus***	**DE genes downregulated in *A.sagrei* and upregulated in *M.musculus***
1	*clec11a*	*pdk4*
2	*Fap*	*mgat3*
3	*adgre5*	*zfp36l2*
4	*tmem132a*	*phlda1*
5	*anxa4*	*rasl11b*
6	*snai2*	*Thbd*
7	*mapk6*	*Rflnb*

8	*krt75*	*slc27a3*
9	*rftn2*	*angpt4*
10	*Rerg*	*ncam1*
11	*ism1*	*rnf24*
12	*plekhg1*	*fbn2*
13	*arfgef3*	*gsg1l*
14	--------	*fth1*
15	--------	*sh3glb2*

Table S2.6: Primer efficiency values

Sr.No	Primers	E value
1	gli1primers	1.88
2	ptch1primers	1.90
3	gapdh primers	1.97
4	tbp primers	2.12
5	atp5f1d primers	1.95
6	atp5f primers	1.91

Table S2.7: Number of *ift*88 mutants clones and types of indels

Sr.no.	Type of indel	Number of clones	Biallelic and out-of-frame mutation
1	+ 1 / - 5	5	yes
2	- 1 / - 7	1	yes
3	- 2 / - 4	1	yes
4	+ 1 / - 2	1	yes
5	+ 1 / - 4	1	yes
6	+ 1 / - 1	3	yes
7	+ 1 / - 9	6	No
8	+ 227 / (?)	1	No

CHAPTER 3

TECHNOLOGY DEVELOPMENT TO TEST FUNCTIONALITY OF ENHANCERS ZRS AND HLEB DURING LIMB DEVELOPMENT IN LIZARDS USING CRISPR-CAS GENOME EDITING.

Introduction

Tetrapod limbs are diverse structures whose development is regulated by complex gene regulatory networks (Tickle, 2015). Naturally occurring mutations in humans and genetic studies in a variety of model organisms have identified different genes and cis-regulatory elements involved in the development of limbs. The genetic basis of the developmental mechanisms involved in the diversity of limb morphologies over the course of tetrapod limb evolution is a growing area of research. Spatiotemporal control of precise differential gene expression during embryonic development is governed by cis- regulatory elements (CREs) (Gehrke & Shubin, 2016). Evidence suggests that the changes in the sequence of the CREs, specifically transcriptional enhancers, are involved in the morphological evolution of vertebrate traits (Chan et al., 2010; Cooper et al., 2018; Cretekos et al., 2008; Gehrke & Shubin, 2016; Guerreiro et al., 2016; Infante et al., 2018; Lopez-Rios et al., 2014; McLean et al., 2011).

Among the many tetrapod lineages, squamate reptiles exhibit a spectrum of limb morphologies associated with the reduction in limb elements from minor loss of phalanges

to complete limblessness. This complete or partial limb loss has evolved in multiple lineages of Squamata independently. Hence, they provide a powerful system to study the morphological evolution of limbs and gene regulation (Infante et al., 2018; Roscito et al., 2015). Rasys et al.(2019) developed a method to perform gene editing in the lizard *Anolis sagrei* by microinjecting unfertilized oocytes. To test the functionality of the conserved ancient tetrapod limb enhancers to understand limb reduction, we chose to work with *Anolis sagrei* as a model system. We focused on two ancient limb enhancers: Zone of Polarizing Activity [ZPA] Regulatory Sequence (ZRS, also known as MFCS1) for *Shh* and Hind Limb Enhancer B (HLEB) for *Tbx4*.

ZRS is a long-range limb-specific enhancer of the sonic hedgehog (*shh*) gene. It regulates the spatiotemporal expression of shh in limb development and it is required for digit identity and specification. It is located around 1000kb away from the shh promoter in intron 5 of the *lmbr1* gene (Lettice, 2003; Sagai et al., 2005). This enhancer is around ~ 780bps in length and is highly conserved in terms of sequence and topological position across a large evolutionary time scale, including sharks, skates, and rays(Dahn et al., 2007; Gehrke & Shubin, 2016). Studies in mice discover that the functional activity of ZRS comprises two domains: one domain predominantly acts over short range and the other promotes long-range chromosomal conformation changes associated with the gene activity (Lettice et al., 2014). The point mutations in the ZRS region lead to a spectrum of limb defects in humans collectively called ZRS- associated syndromes (Anderson et al., 2012). The binding sites for the transcription factors HoxD, ETS, and E box have been identified in the ZRS region (Kvon et al., 2016; Leal & Cohn, 2016; Lettice et al., 2017).

In basal snakes, like boa and python which have vestigial limbs, the ZRS sequence is highly conserved. In advanced snakes, like cobra and viper which are limbless, extremely high levels of sequence divergence in ZRS are observed. The result of the sequence analysis identified a 17 bps snake-specific deletion in the ZRS region (Kvon et al., 2016; Leal & Cohn, 2016). The functionality of the snake ZRS region and 17bps snake-specific deletion was tested in mouse transgenic reporter assay and knockin models. Transgenic mouse reporter assay with basal snake ZRS region drove the expression of the reporter gene in mouse limb buds while advanced snake ZRS region did not (Kvon et al., 2016). When the mouse ZRS region was swapped with the cobra ZRS region by CRISPR knock-in experiment, the mice showed a 'serpentized' phenotype. When the mouse ZRS region was swapped with the python ZRS region by CRISPR knock-in experiment, the mice had vestigial limbs just like pythons. The python ZRS was resurrected by adding 17 bps and was knocked into these mice. The resurrected ZRS rescued limb development in mice with python ZRS (Kvon et al., 2016). The 17bps snake-specific deletion consisted of an ETS1 factor binding site (Kvon et al., 2016; Leal & Cohn, 2016). The progressive loss of HoxD13 binding sites along with the loss of the ETS1 binding site might have been a probable reason for the limb loss in the advanced snakes (Kvon et al., 2016; Lettice et al., 2017).

HLEB is an enhancer element of the *Tbx4* gene. The Tbx4 transcription factor is crucial for normal hindlimb and vascular development. HLEB is highly conserved from fish to mammals and was sufficient to drive the expression of a reporter gene in transgenic mouse reporter assay in hindlimbs and genital tubercle (Menke et al., 2008). In amniotes, there is growing evidence of overlap between genes regulating limb and phallus development (Infante et al., 2018). *Anolis* lizard HLEB enhancer mouse reporter assays

also revealed a similar pattern of expression in hindlimbs and genital expression (Infante et al., 2018). In snakes HLEB is conserved and it is active in the genitalia. This indicates the ancestral amniote activity pattern of CRE (Infante et al., 2015). In snakes, the loss of HLEB activity in the limbs can be associated with mutations that deleted limb-specific transcription activator binding sites or gain in binding sites for transcription repressors. Functional studies to understand the mechanism of the loss of function of HLEB are necessary (Infante et al., 2018).

All the functional studies to understand limb loss in snakes and conserved enhancer function in snakes have been performed using transgenic and knock-in mouse models (Guerreiro et al., 2016; Infante et al., 2015; Kvon et al., 2016; Leal & Cohn, 2016). While addressing the nature of cis-regulatory elements in interspecies transgenic experiments the evolutionary distance between the two species is an important variable to consider. The evolution of trans-regulatory machinery is an important aspect to consider. Mammals and reptiles have diverged from a common ancestor around 300mya. Ideally, we need to evaluate the role of these cis-regulatory elements in the context of the organism itself or in the closely related reptile. Hence, we decided to study the functionality of ZRS and HLEB enhancers in lizards to understand limb reduction in squamates.

Rasys et al. (2019) developed a method to perform gene editing in *Anolis sagrei* by microinjecting unfertilized oocytes. To test the role of 17bps snake-specific deletion in limb loss, we aimed to create targeted mutations in this region of ZRS in anoles. Recently Menke lab identified microdeletions in HLEB enhancer elements in short-limbed anoles from different islands. These microdeletions are evolved independently and are responsible for reduced *Tbx4* expression and length of hindlimbs in anoles (Infante et al., unpublished

data). To test if these naturally occurring deletions in HLEB enhancer are responsible for limb length reduction in anoles, we aimed to create targeted mutations in these regions. To increase our repertoire of gene editing tools in *Anolis sagrei* we used CRISPR/Cas12a system along with CRISPR/Cas9 system. We successfully generated mutants using both the CRISPR system. We report the gene editing efficiency of the CRISPR/Cas9 system is higher. The phenotypic analysis of the mutants generated is in progress.

Results

Targeted mutations in the ZRS region via CRISPR/Cas12a enzyme

We annotated the ZRS region in *Anolis sagrei* as described in the methods section. We designed two Cas12a gRNAs to create targeted mutations in a 17bp snake-specific deletion region to test its functionality (Figure 3.1A). We microinjected 5μM of RNP solution containing gRNA1, gRNA2, and cas12a enzyme initially in 33 unfertilized oocytes (7 lizards). The initial genotyping screening by polyacrylamide gel electrophoresis (PAGE) identified two mutant lizards ZRS#13 and ZRS#18 (Figure 3.1B). ZRS#13 had a drastic phenotype. Both the forelimbs showed loss of digits and severe shortening of the stylopod/zeugopod. The hindlimbs developed digits although the length of the digits on both hindlimbs was reduced. Both hindlimbs appeared to be twisted/malformed. ZRS #13 also had craniofacial abnormalities associated with jaw morphology (Figure 3.1C). The developmental stage for ZRS #13 was late 16 and for ZRS #18 was late 17. ZRS #18 appeared like a WT lizard on cursory examination (data not shown). Sanger sequencing of both the mutants identified 13 bp and 12 bp deletions associated with the gRNA 1(Figure 3.1D). Both deletions resulted in the removal of 2 bps from the 3' end of the ETS1 binding motif. The gene editing efficiency for 5μM RNP solution (Cas12a; gRNA1;gRNA2) was

6.06%. We further injected 41 follicles (11 lizards) with 10µM RNP solution with Cas12a enzyme and only gRNA1. The initial genotyping screening by PAGE identified four mutant lizards ZRS# 41,47,52 and 53 (Figure 3.2A). ZRS #41 was a dead egg and hence no phenotypic information was available. The rest of the three mutants appeared wild type with no obvious limb and/or craniofacial abnormalities. The developmental stage of ZRS#47 was 16, and ZRS #52 and 53 were late 17. We then confirmed the nature of the mutation by sanger sequencing (Figure 3.2B). The gene editing efficiency for 10µM RNP solution (Cas12a; gRNA1) was 9.8%. It appears that doubling the concentration of the RNP solution to be injected increased the gene editing efficiency. All the indels deleted some part of the ETS1 binding site motif. However, because of the nature of the indels, the binding motif sequence was restored in most of the cases apart from ZRS #13 and 52. We successfully generated 6 mutants using the CRISPR /cas12a system. This was the first time Cas12a was used to perform gene editing in *Anolis sagrei*. Increasing the concentration of the reagents appears to have increased gene editing efficiency. Hence going forward, we propose to microinject 10µM RNP solution in unfertilized oocytes.

Targeted mutations in the ZRS region via CRISPR/Cas9 enzyme

We designed Cas9 sgRNAs to create targeted mutations in a 17bps snake-specific deletion region to test its functionality (Figure 3.3A). We microinjected 10µM of RNP solution containing gRNA1 and cas 9 enzyme in 46 unfertilized oocytes (14 lizards). All the 24 hr laid eggs were collected and incubated for 30 days at 280C. I dissected out all the eggs on day 30 to synchronize the developmental stages of any potential mutants. I screened all the embryos for mutations by Sanger sequencing. We generated 7 mutants with targeted mutations in the ZRS region (Figure 3.3C). On the initial screening, three mutants

showed subtle limb abnormalities. ZRS #97 had two digits fused on both forelimbs. The jaw morphology was abnormal, the tip of the tail was twisted and curled, and the embryo had hunched back. ZRS # 98 had one abnormal hindlimb. ZRS # 109 had one extra partially formed digit on one forelimb (Figure 3.3B). The rest of the mutants did not show any obvious morphological abnormalities and they all appeared like wild-type embryos. The indels in the Cas9 mutants did not affect the sequence of the ETS1 binding motif which may explain the lack of strong phenotypes. The CRISPR/Cas9 system appears to be more efficient than the Cas12a system in generating targeted mutations in the ZRS region (data shown in Table 3.1).

Targeted mutations in the HLEB region via CRISPR/Cas12a enzyme

We wanted to generate targeted mutations mimicking naturally occurring deletions in short-limbed lizards. We designed two Cas12a gRNA (Figure 3.4A). We microinjected 5µM of RNP solution containing gRNA1,gRNA2, and cas12a enzyme initially in 33 unfertilized oocytes (6 lizards). Eggs were collected weekly and incubated at 290C till hatching. The genomic DNA from the tail was PCR amplified and sequenced for screening for the mutants. We identified two mutants HLEB #60 and 94 by Sanger sequencing (Figure 3.4B). HLEB #60 had 22 bp deletions which removed sequences partially overlapping the location of Del V and Del Ch. HLEB #94 had 46 bp deletion which completely removed the region overlapping Del V and Del Ch sites. The gene editing efficiency for 5µM RNP solution (Cas12a; gRNA1;gRNA2) was 6.06%. Thus, we successfully generated mutants using the CRISPR/Cas12a system in anoles.

Targeted mutations in the HLEB region via CRISPR/Cas9 enzyme

To generate targeted mutations mimicking naturally occurring deletions in short-limbed lizards (Del V and Del Ch) we designed three Cas9 gRNA (Figure 3.4A). We micro-injected 5µM of RNP solution containing gRNA-D,gRNA-E,gRNA-F, and cas9 enzyme in 71 unfertilized oocytes (12 lizards). Eggs were collected weekly and incubated at 29^0C till hatching. The genomic DNA from the tail was PCR amplified and sequenced for screening for the mutants. We identified 11 mutants. Figure 3.4B summarizes the nature and position of indels observed in these mutants. The data from HLEB #138 and HLEB# 142 is not presented in the figure. These two mutants had chromatograms which indicated the mosaic nature of the deletion. To understand the nature and extent of mosaicism of the mutations present, we performed amplicon based NGS sequencing. Table 3.2 summarized the data from all the mutants. HLEB #94,110 and 115 were dead eggs and hence were not sequenced further. The data indicate that HLEB 138,142,143 were heterozygous for the mutation while the rest of the HLEB mutants were homozygous (Table 3.2, Figure 3.4B). The mutations created, partially or completely removed the sequences overlapping the Del Ch location (Figure 3.4B). The gene editing frequency for the Cas9 system was 15.5% (Table 3.3). The data indicate that the gene editing efficiency of Cas9 is more than Cas12a system.

Discussion

Rasys et al. (2019) developed a method to perform gene editing in *Anolis sagrei* by microinjecting unfertilized oocytes. They used the CRISPR/Cas9 system for gene editing. To increase our repertoire of gene editing tools for genetic and functionality experiments in Anolis sagrei we wanted to further optimize the method they developed as well as try

other CRISPR systems. The use of the Cas12a enzyme offers an opportunity to specifically target AT-rich regions increasing the flexibility of gene editing. Hence, we used and optimized the CRISPR Cas12a system to create targeted mutations in two enhancers ZRS and HLEB known to be crucial for limb development. We successfully generated mutants in both cases using the Cas12a system. We also used the Cas9 system to create targeted mutations in ZRS and HLEB to understand the working gene editing efficiency of both Cas9 and Cas12a systems in lizards. The 17bps snake-specific region we targeted in ZRS is a highly AT-rich region. To use the Cas9 enzyme for gene editing purposes, there were only two possible NGG PAM sites. Designing specific sgRNA for this AT-rich region raised some challenges. Compared together, our data indicate that Cas12a gene editing efficiency is lower than Cas9. However, the use of Cas12a allowed us more flexibility to create targeted mutations. This was the first time Cas12a was used to perform gene editing in *Anolis sagrei*. The phenotypic analysis of HLEB mutants in context to limb length and size is currently in progress at Kingsley lab at Stanford University. Preliminary data suggests that several of the mutants generated have reduced limb length. If the mutations are created to mimic the naturally occurring deletions in short-limbed lizards, we can establish a mutant lizard line. This mutant lizard line can then be used to understand the biomechanics of the locomotory behavior in anoles.

None of the mutants we generated in the ZRS region had deletion which knocked out the whole 17 bp snake-specific region. The mutants generated by the CRISPR/Cas12a system had indels that removed some part of the ETS1 binding site motif. However, because of the nature of the indels, the binding motif sequence was restored in most of the cases apart from ZRS #13 and 52. Our analysis indicated that ZRS#13 and ZRS#52 have the

same mutation however, only ZRS#13 showed a drastic phenotype. It is possible that there was a big deletion associated with ZRS-Cas12a-gRNA2 which potentially removed the primer binding site and hence only one allele was amplified during a PCR reaction. To understand the nature of the mutation, we propose to perform next-generation sequencing of a larger amplicon size so that the biallelic or mosaic nature of the indels in these mutants can be confirmed. Apart from ZRS# 13, all the other mutants appeared wild type on cursory observation. The phenotype of ZRS #13 is peculiar, because of the perturbations of the ZRS region and specific limb abnormalities in this crispant, we do not think this is an off-target effect. The development of both forelimbs and hindlimbs in this crispant is affected, without severe limb truncation which was observed in ZRS-deleted mice (Sagai et al., 2005) and mice with its original ZRS replaced by cobra ZRS (Kvon et al., 2016).

Unfortunately, none of the mutants generated using the CRISPR/Cas9 system had any mutations in the 17 bp snake-specific deletion region or the ETS1 binding motif. Most of these mutants appeared WT. Two out of seven mutants had abnormalities in digit formation and one out of seven mutants had malformed hindlimbs near the stylopod-zeugopod junction. We cannot comment on the length of the skeletal elements in limbs in these mutants. We propose to perform micro-CT analysis on these mutants to understand changes in ZRS and its potential effect on limb morphologies in anoles.

ZRS is a highly conserved limb specific shh enhancer element. The mutations in ZRS are responsible for polydactyly in humans, mice, chicken, dogs, and cats (Anderson et al., 2012; Johnson et al., 2014; Lettice, 2003; VanderMeer & Ahituv, 2011; Xu et al., 2020). In humans, syndactyly and triphalangeal thumb is also observed as a result of mutations in ZRS (VanderMeer & Ahituv, 2011). Suzuki et al. (2018) reported a reduction in the

number of digits during limb development and regeneration in *Pleurodeles waltl* in response to the deletion mutations near the 17bp snake-specific sequence. In *Anolis sagrei,* we generated targeted mutations in the ZRS region, and we have mutants exhibiting a variety of digit malformations like polydactyly, syndactyly, and loss of digits. Evolutionary digit loss in the Australian Lizard *Hemiergis* was associated with the decreased expression of Shh (Shapiro et al., 2003). The variety of these phenotypes is associated with the region and type of mutations created in the ZRS region.

In our study, we wanted to test the functionality of 17bp snake-specific deletion in the context of the evolution of limb loss in squamates. The gRNA design for all our experiments was focused on the potential deletion of this region which includes the ETS1 binding motif. However, none of our mutants have created perturbation in this region. In the future, we would like to perform ChIPseq analysis to test the direct binding of ETS1 factors in this region in developing forelimbs and hindlimbs. We propose to develop more precise gene editing of this 17bp region using the Cas12a system and an HDR donor.

Overall, we have successfully generated mutants in the ZRS and HLEB enhancer region using CRISPR/Cas12a and Cas9 systems. This allows us more flexibility in creating targeted mutations in regions of our interest. This provides more genetic tools to perform genetic experiments and functionality testing to answer these questions.

Methods

Designing sgRNAs

For targeted mutations in the ZRS region, we searched *A.sagrei* genome assembly 2.0 for the ZRS sequence and coordinates. For this purpose, we used the published *A.carolinensis* ZRS sequence (Kvon et al., 2016). We then aligned the *A.sagrei* ZRS

sequence to the *A.carolinensis* ZRS sequence. Using published data by Kvon et al (2016) and the above mentioned sequence alignment we annotated 17 bp snake-specific deletion. We also annotated deletion B in the same region published by (Leal & Cohn, 2016). Both these deletion regions overlap. To create targeted mutations in the region surrounding 17bp snake-specific deletion, we carefully scanned the region and identified potential guide sequences by identifying PAM sites. We manually designed gRNAs for both the Cas12a and Cas9 systems making sure to avoid known polymorphic sites. Once the gRNAs were designed, we used CRISPOR software to detect potential off-target effects. We used the chicken genome and mouse genome to understand the possibilities of the off-target effects.

For creating targeted mutations in the HLEB region, Menke lab had already annotated naturally occurring deletions in short limbed lizards. The sgRNAs were designed to create mutations in regions associated with Del V and Del Ch deletion (Figure 3.4A). The off-target effects and the quality of the sgRNA were assessed using the CRISPOR program and known polymorphic sites were avoided while designing. For CRISPR/ Cas12a experiments, we used sgRNAs from Integrated DNA Technologies (IDT). As Anolis genome assembly is not yet part of the CRISPOR platform, I used chicken and mouse genomes to predict the quality of sgRNA and the potential off-target effects. This strategy was used because the ZRS region is highly conserved in these species. For the CRISPR/Cas9 system, we used sgRNAs from Synthego. The sequences of the sgRNAs are in table 3.1.

Preparation of RNPs and in vitro testing of RNPs

To prepare RNPs, we adapted the protocol from IDT and Synthego. The details of the protocol can be found in Lizard Gene-Editing Handbook (2021) prepared by Menke

Lab. For preparing Cas9 RNPs, the equimolar concentration of the Cas9 enzyme and sgRNAs was used. For preparing Cas12a RNPs, we used a 1:1.26 molar ratio of enzyme to sgRNAs. For Cas12a experiments, I have used both 5μM and 10μM RNP solutions. We added 0.2% phenol red in an injection mixture of RNPs for visibility purposes.

For in vitro testing of RNPs we used the protocol from IDT. The details are available on the IDT website as well as in the Lizard Gene-Editing Handbook (2021). We assembled a 10μL of reaction with 1μL of IDT's 10X nuclease reaction buffer, 0.5μL of 10μM RNPs (or 1μL of 5μM RNPs), 1μL of 250nM DNA substrate (PCR amplified and purified targeted region) and the volume was made with the nuclease-free water. After incubating this mixture for 1 hour at 37^0C, 1μL of 20 mg/mL Proteinase K was added. The reaction was then incubated for 10 minutes at 56^0 C. We visualized the digested product by agarose (1.5%) gel electrophoresis. At least 80% of the DNA template was expected to be digested by RNPs in vitro. This was used as a quality control check before using RNPs for gene editing experiments. If the RNPs failed to digest the DNA substrate, we re-tested them by stringently controlling the temperature at which we assemble the reaction and the concentration of EDTA used in 1XTE buffers. We also made sure the working bench,1X PBS, and water used were nuclease-free (Lizard Gene-Editing Handbook,2021). I freshly prepared RNPs for the experiments and used them within a week of preparation. If it has been more than two weeks since I last tested the RNP solutions, I repeated in vitro testing before gene editing experiments. For every new batch of RNPs prepared, I tested it *in vitro* before gene editing experiments.

Lizard anesthesia,surgery, and microinjections

Rasys et al (2019) developed a method for lizard anesthesia, surgery, and microinjections. Menke lab has optimized and adapted the method for further gene editing experiments. The details of the protocol with information about anesthesia drugs, pain management, and surgery can be found in Lizard Gene-Editing Handbook (2021) prepared by Menke Lab. We sterilized the surgical tools and surgery area. We weighed an individual reproductively healthy lizard and calculated the anesthesia drug dose for that lizard (3.2µL/g of lizard). We subcutaneously administered anesthesia drugs [Alfaxalone 30µg/g+ Dexmedetomidine 0.1µg/g 15:1 ratio]. We scrubbed the anesthetized lizard 5 times on each side of the body with surgical scrub iodine and 70% EtOH, alternating between the two. We then subcutaneously administered the calculated dose (1µL/g of lizard body weight) of pain management drug [Rimadyl 4ug/uL]. We monitored the respiration rate of the lizard every 15 minutes since the anesthesia was administered. When the respiration rate was 20 breaths /minute, we positioned the lizard for the surgical incision and applied 15µL 0.2% xylocaine/lidocaine topically to the intended surgical site. We made a surgical incision using iris scissors, cutting through the skin, rib musculature layer, and pigmented layer. The incision on the skin was skin along the side from ventral to dorsal not more than ~8 mm. Once the internal organs were visible, we maneuvered the body cavity to find the follicles. We carefully grasped the small cluster of follicles using forceps and, using the microinjection needle, injected the 3 largest follicles (including the yolky follicle) with RNPs. Our microinjection parameters were 20µm needle, psi 21.7, and 20msec injection time. We measured the follicle size with respect to the needle diameter and injected 1 to 1.5 times the follicle size pulses of the RNP solution. We then placed the

follicles back in the body cavity and aligned all three layers carefully so that both sides of the cut touch each other but do not overlap. We then closed the surgical incision by using a small droplet of Vet Bond glue. Once the glue dried, we dyed the lizard with a unique identifier pattern using food dye and allowed them to recover. Throughout the procedure, the lizard's body temperature was maintained using a slide warmer. Respiration rate was monitored every 15 minutes and the lizard was kept hydrated by adding Plasma- Lyte solution on her body intermittently. We recommend referring to the video of the surgical procedure by Rasys et al.,2019. Once the lizard was recovered, we monitored her wound, health, and behavior for a week before introducing the male into the lizard cage. We calculated how many follicles were injected per lizard in a round of surgeries. We then combined 4-5 lizards with a similar number of injected follicles in a cage. Every surgery cage had 4-5 females and one male (Lizard Gene-Editing Handbook,2021).

Follicle train, length of egg collection, and egg husbandry

I estimated the screening time window for each lizard by creating a follicle train. The follicle train is the order in which a lizard will lay the ovarian follicles (Rasys et al., 2019). We made two main assumptions while creating the follicle train. First, in a lizard the egg laying will alternate between her two ovaries and second larger follicles will be laid sooner as they are later in development (Rasys et al., 2019). We also added two extra weeks to the screening window in order to be safe. For the details, we recommend referring to Lizard Gene-Editing Handbook (2021). We collected the eggs in this screening window every week for ZRS Cas12a experiments, HLEB Cas12a and HLEB Cas9 experiments. For ZRS Cas9 experiments, eggs were collected every day.

For the HLEB experiments and ZRS cas12a experiments, I transferred the eggs after 20 days from vermiculite to sponges until the time they were hatched. I cut the sponges around 3-5mm thick and rinsed them well. Each sponge was placed in a tissue culture plate (24 well plate or 6 well plates). The egg was nestled in the sponges with the yolk side facing down. The eggs were incubated at 28^0C till they hatched or were dissected. Eggs were monitored every day for health and moisture levels. In case of an egg death, I collected the tissue and processed it for genotyping.

Genotyping screening and Sanger sequencing

Tail tissue was collected from every embryo/hatchling. Genomic DNA was extracted. 50-100ng of DNA template was used to set up a 20µl PCR reaction. The PCR product was visualized on a 15% polyacrylamide gel. The electrophoresis was performed for 6.5 hours at 120mV. Any crispant with a unique pattern different from WT was then sequenced by Sanger sequencing to identify the nature of the mutation. The chromatograms were analyzed using the DECODR program (Bloh et al., 2021). The primer sequence for amplification is in table 3.2. For ZRS amplification, PCR conditions were 95^0C for 2 minutes,33 cycles of 95^0C for 30 sec;58^0C for 30sec;72^0C for 30 sec,72^0C for 5 minutes. For HLEB amplification conditions were 95^0C for 2 minutes,33 cycles of 95^0C for 30 sec;56^0C for 30sec;72^0C for 30 sec,72^0C for 5 minutes.

Fixing, dehydration, and storage of embryos/hatchlings

Embryos were dissected in 1X PBS and fixed in 4% PFA overnight at 4^0C in a 5ml Eppendorf tube on a rotating shaker. I euthanized the hatchlings according to the Menke lab animal use protocol (AUP). The hatchlings were then carefully spread on a Kim wipe in a petri dish with the limbs and the digits spread apart. I submerged the hatchlings in

100% MeOH and 4% PFA solution (1:3 ratio). In this way, the hatchlings were fixed overnight at room temperature. After fixing, the hatchlings/embryos were washed in 1X PBS followed by gradual serial dehydration in MeOH. The hatchlings and/ or embryos were stored in 100%MeOH at -20^{0}C.

Record-keeping and gene editing efficiency calculations

For every surgery cage, the egg collection record indicated the date of surgery, the date of egg lay, the date of dissection or hatching, the developmental stage, and phenotypic abnormalities, if any. Detailed records for every single egg were kept tracking the post-surgery weeks and generation of mutants. Gene editing efficiency was calculated as (number of mutants generated/total number of follicles injected) x 100.

Figures and Tables

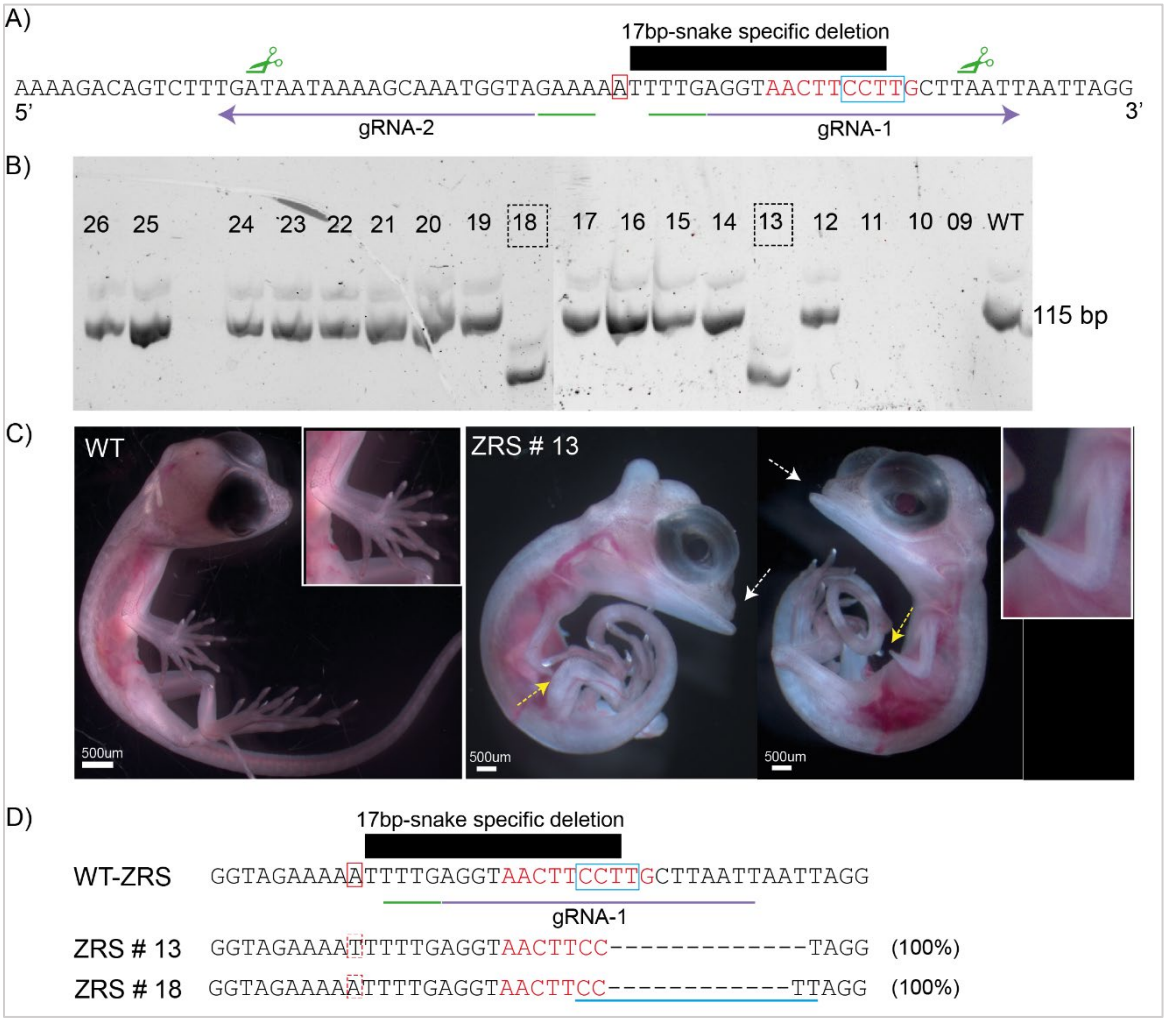

Figure 3.1: Gene editing of ZRS region in *Anolis sagrei* using CRISPR/Cas12a system using two gRNAs. (A) Schematic representation of partial ZRS region and gRNA design. The red rectangle around base A indicates naturally occurring SNP. The sequence of the 17 bp snake-specific deletion region is highlighted by a black rectangle. Purple arrows indicate gRNA. Arrowhead indicates the direction of the gRNAs. The sequence of the ETS1 binding site is represented in red colored letters. The core binding site is highlighted by a blue rectangle. Green line segments represent the TTTV PAM sites. Green scissors depict the potential cut site. (B) A representative polyacrylamide gel image for initial

genotype screening for mutants. The number represents a unique ID for each embryo/hatching collected post-surgery. The mutants are highlighted with a dashed rectangle around their unique ID number. (C) Images of Crispant ZRS#13 with severe limb phenotype. A stage- matched WT embryo image is on the left of the panel for reference. The yellow arrow points to the loss of digits on the forelimbs. The white arrow indicates abnormal jaw morphology. (D) Sanger sequencing results confirming the nature of the mutation in Cripsant ZRS #13 and ZRS #18. WT ZRS sequence is at the top for reference. The purple line segment indicates the position of gRNA-1. The sequence of the ETS1 binding site is represented in red colored letters. The core binding site is highlighted by a blue rectangle. The thin green line segment represents the (TTTV) PAM site. The number of black dashes indicates the number of deleted bases. The percentage values in the parentheses indicate the percentage of alleles detected with the particular mutation in a given PCR reaction. The restored ETS1 core binding site in ZRS #18 is highlighted by a blue line.

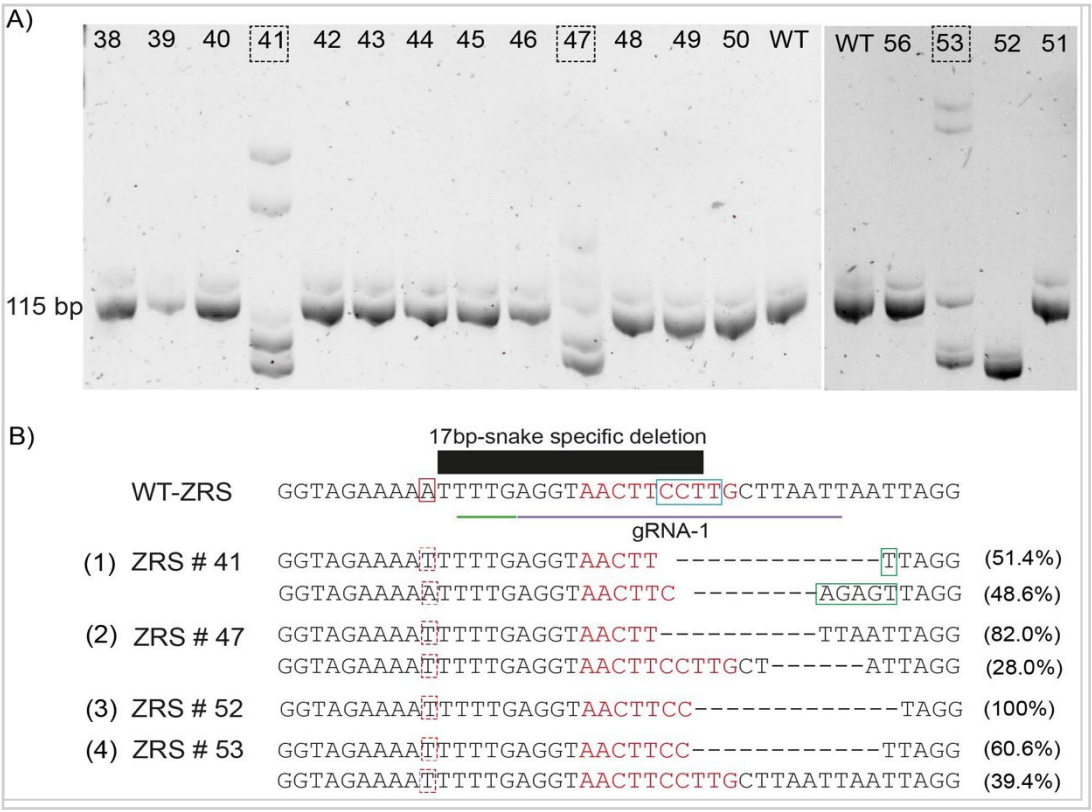

Figure 3.2: Gene editing of ZRS region in *Anolis sagrei* using CRISPR/Cas12a system using single gRNA. (A) A representative polyacrylamide gel image used for initial genotype screening for mutants. The number represents a unique ID for each embryo/hatching collected post-surgery. The mutants are highlighted with a dashed rectangle around their unique ID number. (B) Sanger sequencing results confirming the nature of the mutation in the cripsants. WT ZRS sequence is at the top for reference. The purple line segment indicates the position of gRNA-1. The sequence of the ETS1 binding site is represented in red colored letters. The core binding site is highlighted by a blue rectangle. The green line segment represents the (TTTV) PAM site. The number of black dashes indicates the number of deleted bases. The insertion of bases is highlighted by the green rectangles. The percentage values in the parentheses indicate the percentage of alleles detected with the particular mutation in a given PCR reaction.

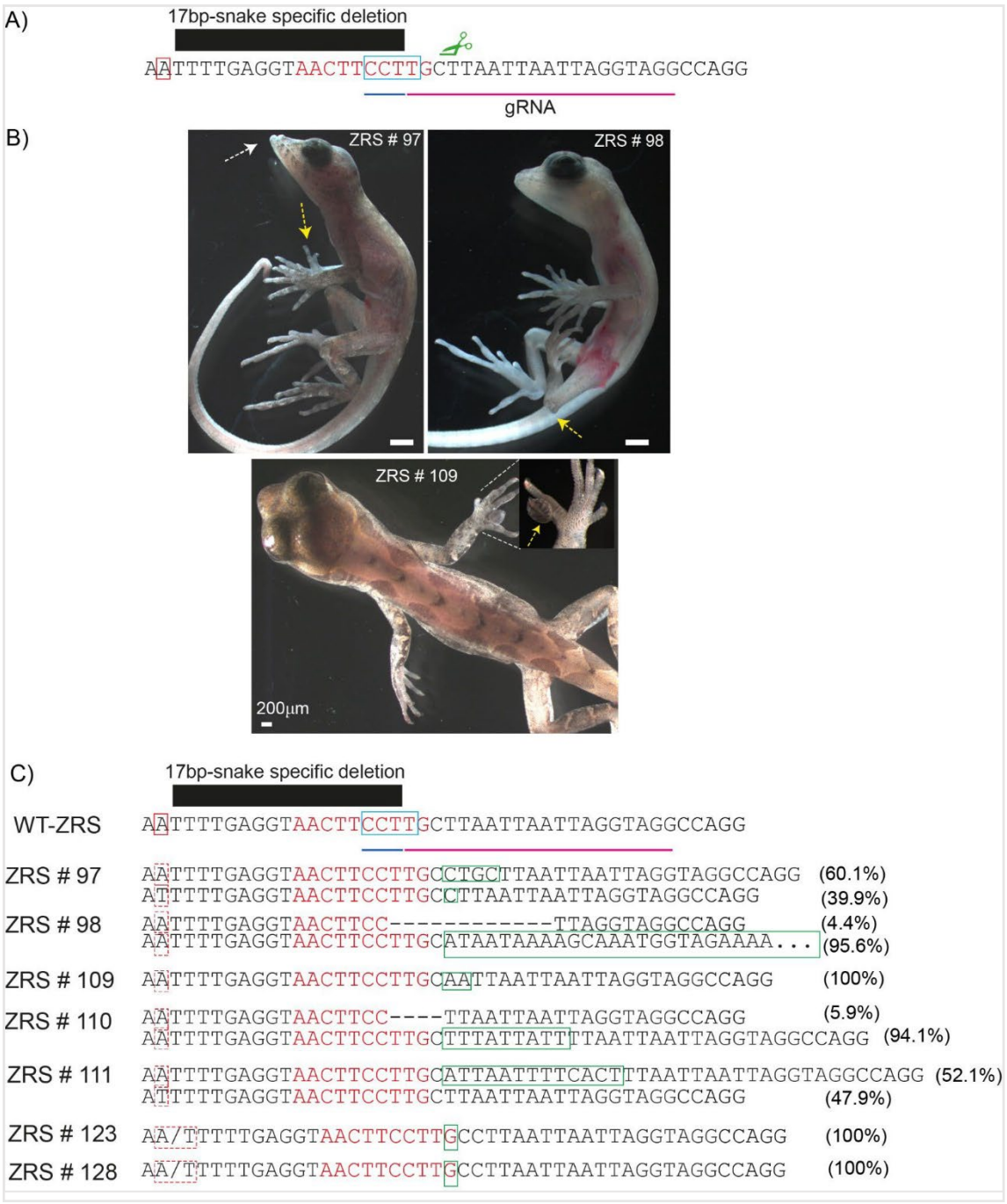

Figure 3.3: Gene editing of ZRS region in *Anolis sagrei* using CRISPR/Cas9 system

(A) Schematic representation of partial ZRS region and Cas9-gRNA design. The red rectangle around base A indicates naturally occurring SNP. The sequence of the 17 bp snake-specific deletion region is highlighted by a black rectangle. The pink line

segment indicates gRNA. The blue line segment represents the (NGG) PAM site. The sequence of the ETS1 binding site is represented in red colored letters. The core binding site is highlighted by a blue rectangle. Green scissors depict the potential cut site. (B) Images of crispant ZRS # 97,98 and 109 with abnormalities in limbs and craniofacial morphology. The yellow arrow points to the loss of digits on the forelimbs. The white arrow indicates abnormal jaw morphology. The scale bar =500µm unless mentioned otherwise (C) Sanger sequencing results confirming the nature of the mutation in the cripsants. The number of black dashes indicates the number of deleted bases. The insertion of bases is highlighted by the green rectangles. For crispant #98 the insertion size is 33bps and it is indicated by three black dots. The percentage values in the parentheses indicate the percentage of alleles detected with the particular mutation in a given PCR reaction. The naturally occurring SNPs are highlighted by a red rectangle.

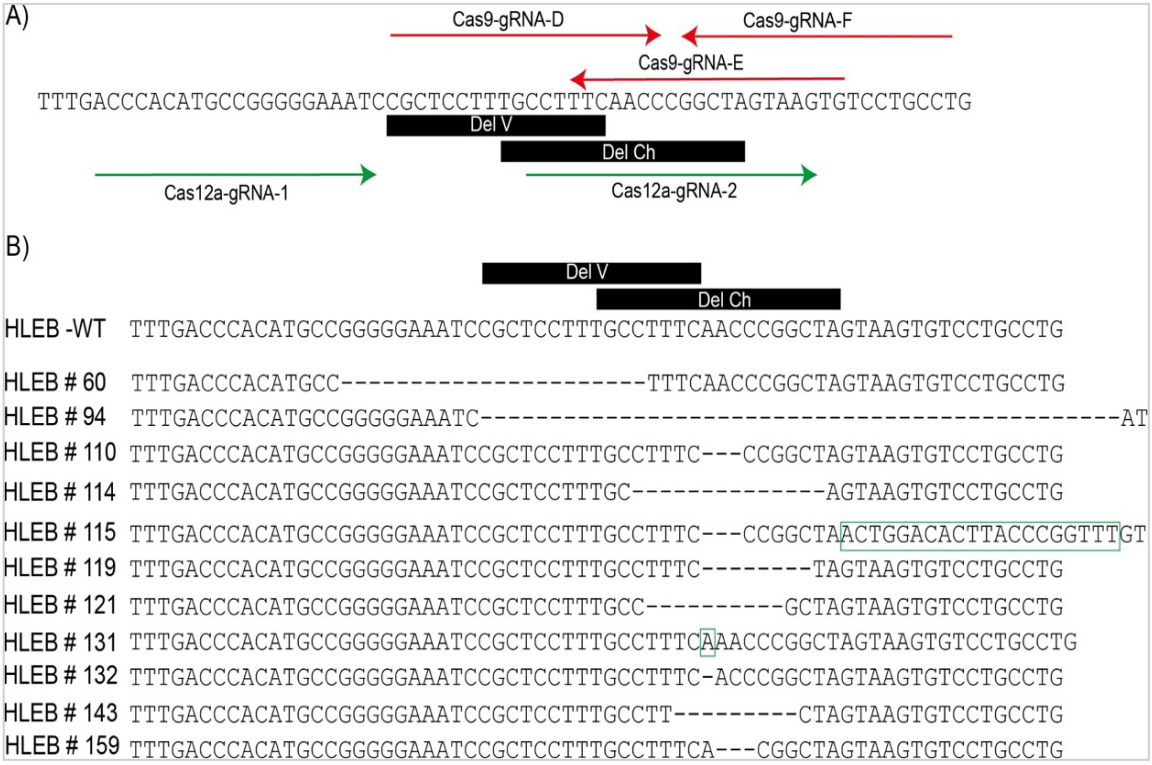

Figure 3.4: Gene editing of HLEB region in *Anolis sagrei* using CRISPR Cas12a and Cas9. (A) Schematic representation of partial HLEB region and sgRNA design for CRISPR/Cas9 and CRISPR /Cas12a experiments. The red arrow indicates the position of sg RNAs for the Cas9 system. The green arrows depict sgRNAs for the Cas12a system. The arrowhead represents the direction of the sgRNAs. The location of naturally occurring HLEB deletions in short limbed species is annotated by Del V and Del Ch. (B) Representation of sanger sequencing data showing indels in the cripsants. Black dashes indicate deletions and green rectangles represent the insertion of bases. HLEB 60 and 94 were generated using the cas12a system while the rest of the crispants were generated by the cas9 system. The position of Del V and Del Ch are also annotated in the figure.

Table 3.1: List and sequences of sgRNA

Loci	**CRISPR system**	**sgRNA name**	**Sequence**
ZRS	Cas12a	ZRS-gRNA-1	AGGTAACTTCCTTGCTTAATT
ZRS	Cas12a	ZRS-gRNA-1	TACCATTTGCTTTTATTATCA
ZRS	Cas9	ZRS-Cas9-gRNA	CCTACCTAATTAATTAAGCA
HLEB	Cas12a	HLEB-Cas12a-1	ACCCACATGCCGGGGGAAATC
HLEB	Cas12a	HLEB-Cas12a-2	CCTTTCAACCCGGCTAGTAAG
HLEB	Cas9	HLEB-sgRNA-D	CGCTSCTTTGCCTTTCAACC
HLEB	Cas9	HLEB-sgRNA-E	CACTTACTAGCCGGGTTGAA
HLEB	Cas9	HLEB-sgRNA-F	AGGCAGGACACTTACTAGCC

Table 3.2: Primer sequences for ZRS and HLEB mutant screening

Loci	Name	Sequence	Application
ZRS	ZRS-Screen-F1	CTTGGAGAACATCAAAAGACAGTC	PAGE
ZRS	ZRS-Screen-R1	CACAGACCTCTTCACTTCCAC	PAGE
ZRS	ZRS-Screen-F2	AGCAACATCCTGACCAATTATGC	Sequencing
ZRS	ZRS-Screen-R2	CTTTGTTCCTCCTCCATTGTGC	Sequencing
HELB	HLEB-Screen-F2	ATGCGGCTAATGTGTCAGGAC	Sequencing
HLEB	HLEB-Screen-R2	GAAGCGGAGATTAAAGCCAGTC	Sequencing

Table 3.3: Percentage efficiency of gene editing of ZRS region by Cas9 and Cas12a CRISPR system in *Anolis sagrei*

CRISPR/Cas system used	Number of follicles injected	Number of mutants generated	% Efficiency
Cas12a	74	6	8.1
Cas 9	46	7	15.2

Table 3.4: Analysis of CRISPR mediated HLEB mutants using amplicon-based NGS data

No	Cripsant ID	% of total indels
1)	HLEB # 60	99.7
2)	HLEB # 114	99.86
3)	HLEB # 119	99.82
4)	HLEB # 121	99.71
5)	HLEB # 131	97.65
6)	HLEB # 132	98.19
7)	HLEB # 138	43.57
8)	HLEB # 142	28.24
9)	HLEB # 143	66.98

Table 3.5: Percentage efficiency of gene editing of HLEB region by Cas9 and Cas12a CRISPR system in *Anolis sagrei*

CRISPR/Cas system used	Number of follicles injected	Number of mutants generated	% Efficiency
Cas12a	33	2	6.1
Cas 9	71	11	15.5

CHAPTER 4

FORMING AND MANAGING GROUPS IN UNDERGRADUATE LABORATORY CLASSES

Introduction

National science education policies promote collaboration as a key scientific practice (AAAS, 2010; NGSS, 2013; Olson & Riordan, 2012). Students engaged in collaboration demonstrate greater learning, achievement, attitudes toward learning, and persistence in STEM courses and programs (Lou et al., 1996; Metoyer, Miller, Mount, & Westmoreland, 2014; Springer et al., 1999). Laboratory courses, in addition to providing important opportunities for scientific investigation, also provide excellent opportunities to foster the development of the social skills necessary for effective collaboration (Corwin, Graham, & Dolan, 2015; Seifert, Fenster, Dilts, & Temple, 2009). However, instructors need to be proactive in forming groups to ensure effective collaboration (Johnson & Johnson, 1999; Kreijns, Kirschner, & Jochems, 2003), but there is a lack of consistent advice for instructors who wish to structure teams to ensure diversity in gender, ethnicity, or prior academic performance (Donovan, Connell, & Grunspan, 2018; Wilson, Brickman, & Brame, 2018).

Self-Selection vs Randomization in Forming Collaborative Groups

Students who are allowed to select their group members report positive outcomes that include: reported satisfaction (Bacon, Stewart, & Silver, 1999; Chapman et al., 2006;

Connerley & Mael, 2001; Myers, 2012); higher initial group cohesiveness, communication, enthusiasm, and confidence in each other (Chapman et al., 2006; Ciani, Summers, Easter, & Sheldon, 2008; Strong & Anderson, 1990); higher grades (Mahenthiran & Rouse, 2000); and greater ownership of group tasks (Mello, 1993). Students who are comfortable with their groups also demonstrate greater content mastery, with the greatest predictor of comfort being friendship status (Theobald, Eddy, Grunspan, Wiggins, & Crowe, 2017). However, researchers have demonstrated negative outcomes when students are allowed to select their groups (Feichtner & Davis, 1984) including: negative opinions of the course, instructors, projects, and classmates (Brickell, Porter, Reynolds, & Cosgrove, 1994) and poor test results in physical sciences laboratory classes (Lawrenz and Munch,1984). The negative perceptions of self-selected groups might derive from the fact that on their own, students often select group members that they already know or who are from similar cultural, ethnic, racial, and academic backgrounds (Chapman, Meuter, Toy, & Wright, 2006; S. Freeman, Theobald, Crowe, & Wenderoth, 2017; Jalajas & Sutton, 1984-1985; Rienties, Alcott, & Jindal-Snape, 2014). This creates homogeneous groups, a situation referred to as homophily in the field of social network analysis (McPherson, Smith-Lovin, & Cook, 2001). Homogeneous groups that lack gender and national diversity have been shown to exhibit lower cognitive complexity in their collaborative group work, lower performance, and lower quality ideas during collaboration (Curseu & Pluut, 2013; McLeod, Lobel, & Cox Jr, 1996; Watson, Kumar, & Michaelsen, 1993).

Science educators, who have been urged to promote diverse, equitable, and inclusive classrooms (Malcom, 2017; Ruedi, Feder, Thompson, & Conn, 2020), may question if homophily supports or perpetuates a cultural divide between students. Non-

white students express a greater preference for participating in collaborative learning experiences compared to White students (Cabrera et al., 2002). Students from historically underserved groups may achieve a greater sense of comfort when they can choose their group members. For example, LGBTQIA students report problems with assigned groups, preferring to be able to work with someone who would be accepting of their identities (Cooper & Brownell, 2016). Similarly, international students seek comfort from students with similar cultural backgrounds (Hendrickson, Rosen, & Aune, 2011).

Instructors who choose to assign student groups to enhance diversity must consider a host of characteristics including ethnicity, nationality, gender, age, sexual orientation, prior academic performance, and teamwork experience for which there may be a paucity of experimental evidence of the benefits or balancing these factors in group settings. Most studies categorize gender as a dichotomous variable that misses nuanced aspects of the personal and cultural construction of gender (Knaak, 2004). Even with that limitation, in some research settings gender composition was found to have no impact on the final outcomes of group work (Takeda & Homberg, 2014). While in other cases, for example, problem solving in Physics classrooms, homogeneous-gender groups and mixed gender groups perform better than groups with a single female (Heller & Hollabaugh, 1992). These researchers noted that male group members were more likely to dominate discussions in groups with only one female (Heller & Hollabaugh, 1992). This tendency for male members to receive more attention from their group members and exert stronger influence compared to female group members has been shown in multiple sociological settings (Carli, 2001), and that male team members'dominance could be alleviated through greater gender-balance.

A quasi-experimental study in a postgraduate event management program with 138 students tested an instructor intervention to randomize the groups for diversity with respect to gender and nationality. Interestingly, no difference was observed in students' experience between self-selected and randomized groups with respect to their team's cohesion and performance. However, more "knowledge spillovers" (unintentional, informal, and uncompensated transfer of knowledge among the interacting individuals) occurred when groups were randomized, and the authors concluded that randomizing groups could have positive effects on informal and formal interactions between the students from diverse backgrounds beyond the group. (Rienties, Alcott, & Jindal-Snape, 2014).

Theoretical Framework

Using a sociocultural perspective, Salvin posited that the level of social cohesiveness of a group determines the quality of the interactions between its members which results in greater collaboration and achievement (Salvin R., 2014). In this study, we were interested in examining how comfort, communication, and conflict arose in student collaborative lab groups. Prior research had demonstrated that when selecting their group members, students often choose peers from similar gender, ethnic, racial, and social backgrounds with whom they are more comfortable (Chapman et al., 2006; Freeman S. et al., 2017). In addition, while studying group dynamics and student learning in a large biology undergraduate classroom, Theobald and colleagues (2017) were able to confirm that friendship was the major predictor for students' perception of comfort while working in a group as well as a major contributor to learning. We posited that demographic factors would positively influence students' perception of comfort. Comfort within the group would positively influence how likely students were to communicate resulting in less social

tension in the group, higher satisfaction, and a positive effect on learning. We examined group formation and group conflict in an introductory inquiry-based biology laboratory classroom. Even though group work is an essential part of science laboratory classes, not a lot of evidence is available that explores the group dynamics in these settings. We wanted to address this knowledge gap. We did an exploratory study to understand when and what type of conflict arose in a laboratory classroom where around 50% of the final grade is from collaborative work. As an instructor, it is crucial to understand students' perceptions about the characteristics of a good or poor group member and how it factors into their decision of choosing group mates. The motivation behind this study was to suggest instructor intervention strategies for the formation of groups and resolving group conflict proactively.

Measures of Group Interactions

Productive group interactions foster intellectual debate while unproductive interactions often involve the unequal distribution of labor and interpersonal conflict (Aggarwal & O'Brien, 2008; Hall & Buzwell, 2012; Livingstone & Lynch, 2000; Pauli, Mohiyeddini, Bray, Michie, & Street, 2008; Shimazou & Aldrich, 2010). Unproductive interactions within a group over time can lead to dysfunctional groups. There are several recommended strategies to monitor and identify dysfunctional groups (Jehn & Mannix, 2001; Lejk & Wyvill, 2001). We utilized conflict identification items designed by Jehn and Mannix (2001) that measure three types of conflict: Task conflict which occurs when students debate conflicting ideas or points of view; process conflict which occurs when there is a disagreement about who should do what along with individual's responsibility about a specific duty; and relationship conflict that is emotional in nature and

involves disagreements about personal issues. Jehn and Mannix (2001) used the means for the three types of conflict to compare high and low performing student groups working on authentic consulting projects for local businesses in US business students. High performance teams demonstrated low but increasing levels of process conflict, low levels of relationship conflict, with a rise near project deadlines, and moderate levels of task conflict at the midpoint of group interaction. Taking a social capital perspective, Cursue and colleagues (2012) used structural equation modeling to test a path model of group cognition complexity and three conflict. They discovered that cognitive activation associated with task conflict affected collaboration. They found that communication between team members had a positive effect on the discussion of ideas (task conflict) in their structural equation model, while relationship conflict has a negative effect on the cognitive complexity of the final group assignment (Cursue P.L *et al*, 2012).

Social Network Analysis

Social interactions and relationships between students are key to monitoring group work. Social networks define groups of individuals and their relations within that group (Wasserman &Faust,1994). Social network analysis (SNA) is a useful tool for exploring relational data to uncover the implications of structures on individual or group behavior and/or attitudes (Carolan Brian V, 2014, chapter 1). A social network comprises actors or nodes (in our case actors are students) and connections between them, which are called ties or edges. There are four basic assumptions of Social Network Analysis - (1) Individuals and their actions are interdependent (2) Information is transmitted via relational ties between individuals (3) Relationship patterns (Social structures) can influence individual actions by providing opportunities for and /or constraints on individual behavior (4) Social

network models conceptualize relationship patterns among actors in a network (Carolan Brian V, 2014, chapter 1; Wasserman and Faust, 1994). SNA is grounded in systemic empirical data and relies on the use of graph theory, mathematical, statistical, and computational models to represent complex social interactions (Freeman L.C, 2004). SNA can be used to visualize student interactions, draw statistical inferences from the relational data, to understand the flow of information /attitudes within the actors, and to explore the learning outcomes of a classroom (Carolan Brian V, 2014, chapter 1).

Clustering observed as a structural property of a network can provide insight into relationship patterns. This clustering or homophily is defined as connections formed between two actors (nodes) in a network as a result of shared attributes or characteristics. These characteristics can be sociodemographic, behavioral, and/or intrapersonal (McPherson, Lovin, and Cook, 2001). There are two types of homophily: uniform and differential. Uniform homophily refers to the assortative mixing tendency of students to work in a group of students with the same ethnicity or gender. Differential homophily accounts for the fact that the assortative mixing tendencies for the formation of groups are different, depending on the friendship status of the individual students involved (Goodreau S.M et al.,2009; https://github.com/eehh-stanford/SNA-workshop). The social ties formed as a result of homophily inherently restrict information flow as the actors get locked in a fixed position in a network (Carolan Brian V, 2014). It is important from an instructor's point of view to be aware of patterns of homophily in a classroom which might restrict information and knowledge flow.

We utilized SNA in our study as a visual (qualitative) and statistical tool to understand group formation in an undergraduate laboratory classroom setting. Prior SNA

studies have revealed that a student's position within communication and interaction networks correlates with performance (Brewe and Brunn, 2013; Grunspan D et al., 2014) and that student's performance can be influenced by GPA, gender, attendance, and the number of ties within a network (Martin, Buchenorth et al., 2017). Network analysis tied to the statistical approach of exponential graph random models (ERGMs) has been used to investigate adolescent friendship social networks to explore the sociodemographic clustering in friendship networks and the processes generating it (Goodreau S.M et al., 2009). Additional data-driven SNA approaches have also been useful in an evidence-based revision of active learning curricula, comparing strategies for promoting peer interaction and group learning, and understanding group formation in a classroom (Martin, Buchenorth et al., 2017).

Overarching Goal of the Study. We wished to examine if there were differences in diversity, satisfaction, and performance of student groups who self-assembled after an initial period of enforced interaction compared to students who were allowed to self-assemble without enforced interaction. We tested this in a large inquiry-based introductory biology lab. In half of the laboratory sections, students were allowed to choose their group members from the very first lab session (unstructured labs). In the other half of the sections, students were randomly assigned to work with different students each week for the first four weeks, and then made their own choice about selecting groups when they began a group experiment (structured labs). We hypothesized that if students were allowed to self-select their group members they would first select their friends but then later select students that manifested traits perceived as "good" by their group members (Chapman et al., 2006).

We decided to investigate the following research questions:

1. How does enforcing interaction prior to group formation influence student selection of group mates?
2. Are there differences in positive (e.g. higher grades, higher communication frequency, increased satisfaction) or negative (higher task and relationship conflict) outcomes for groups that have enforced structured opportunities to select their members?
3. What do students do when they encounter poor group members?

Results

Question 1: *How does enforcing interaction prior to group formation influence student selection of group mates?*

Students in unstructured labs were given sole choice in whom they worked with from the start of the course, while students in structured labs had enforced exposure to different students in labs 1-4 before deciding on their formal groups in lab 5. We followed interaction patterns for all students throughout the first 4 weeks, and in week 5 and used ERGMs to understand the degree to which being a pre-class friend or similar gender drove the student's choice of group. Table 4.2 summarizes ERGM analysis results. The details of individual labs and *p* values can be found in Table S4.3. Out of the 26 labs we analyzed, only one lab had an equal proportion of male and female students. The remaining lab sections had female-to-male ratios of at least 1.5 (Table S4.2), so we considered differential homophily to ask if the male students were clustering together because of their gender, pre- class friend status, or both. We found that 2 unstructured lab sections and 3 structured lab sections showed students clustering together by gender and pre-class friend status (Table S4.3). However, we cannot conclude if gender might have a role in friendship status itself.

Nearly all the other groups in the lab sections with pre-class friends were always mixed gender groups (p<0.001). However, the number of pre-class friends and male students in any given lab is smaller and hence the estimated coefficient values are smaller.

As the number of URM students is small in our sample population, we analyzed group formation qualitatively for this variable. Table 4.3 summarizes our findings between structured and unstructured lab sections. The number of groups in both structured and unstructured labs, where clustering was observed as a result of pre-class friend status, URM status, or Asian status, were comparable. All the groups clustered together because of pre-class friend status were of mixed gender or mixed ethnicity (Table 4.3). Details about individual lab sections are provided in Table S4.4. In cases of isolated URM students where there was an absence of pre-class friends or other URM students in a group, same- gender groups were formed. A similar trend was found with Asian students. The number of groups with this trend was comparable between both structured and unstructured lab sections. We found a comparable number of groups (22 in unstructured and 15 in structured lab sections) without any distinguishing pattern of pre-class friend status, ethnicity, or gender. These groups had a mix of White, Asian, and URM ethnicity and a higher proportion of female students. The higher proportion of female students was expected, as in each lab section, there were more female students than male students (Table S4.3).

Both the ERGM results and qualitative analysis indicate that there is no significant difference between structured and unstructured lab sections when it comes to students' choice of group members. This is in accordance with our theoretical framework where we expect the choice of selection to be driven by pre- class friend status, ethnicity, and/or gender as students may perceive it as a more comfortable environment to work in.

Question 2: *Are there differences in positive (e.g. higher grades, higher communication frequency, increased satisfaction) or negative (higher task and relationship conflict) outcomes for groups that have enforced structured opportunities to select their members?*

We examined the differences in communication between students in structured and unstructured lab sections. We anticipated that groups would communicate more, particularly in weeks 5-7 when students discuss their experimental designs and perform experiments, and the overall reported communication frequency scores were higher during those weeks (between 9-11, Figure 4.3). The average communication frequency in unstructured labs in week 4 was higher than in structured sections (p=0.014; Figure 4.3a and Table S5). In unstructured lab sections, communication frequency was statistically higher in weeks 4, 5, 6, and 7 as compared to week 3 with p<0.05 (Table S4.5). In structured lab sections, the communication frequency score was statistically higher in weeks 5, 6, and 7 as compared to week 4 with p<0.05 (Table S4.5).

We next examined differences in reported task conflict and discussions between structured and unstructured groups in weeks 5-7. We expected higher task conflict and discussion scores associated with the high communication frequency. Task conflict items measure student perceptions of discussion and exchange of ideas, with a score of 5 indicating these discussions occur very often and a score of 1 indicating a discussion did not occur at all. In both the structured and unstructured labs, the average task conflict score and discussion scores were lower than 2 (Figure 4.3b, Table S4.5), indicating that students either did not report the differences in opinions and discussions among the group members or there was a pattern of less discussion. In both unstructured and structured sections, average task conflict scores were higher in weeks 5 and 6 as compared to week 7 (p<0.05)

(Table S4.5). Week 5 activities require students to design their experiment and during week students conduct the experiment for the first time. Hence, this result was not unexpected. Unstructured lab sections had higher task conflict scores in week 7 compared to structured labs $p<0.05$ (Table S4.5).

We then studied differences in relationship conflict in weeks 5-7. Relationship conflict items indicate more emotional exchange and tension within the group with a score of 1 indicating an absence of any relationship conflict. The average relationship conflict score in both structured and unstructured lab settings was lower than 1.5, indicating rare incidents of reported relationship conflict. There was no statistically significant difference observed between unstructured and structured lab settings. In week 6 unstructured labs had slightly higher relationship conflict scores than in weeks 5 and 7 ($p<0.05$) (Figure 4.3c, Table S4.5).

We next analyzed differences in satisfaction scores from weeks 5-7. As per our theoretical model, with higher communication frequencies and lower relationship conflict scores, we expected higher satisfaction scores. A satisfaction score of 5 indicates that students strongly agree that they feel satisfied working with that group in a given week. A satisfaction score of 1 indicates that students strongly disagree that they feel satisfied working with that group in a given week. Both the structured and unstructured labs had average satisfaction scores of more than 4. There was no significant difference between structured and unstructured lab settings. Over time, in structured labs, students reported higher satisfaction in week 7 than in week 5 (Figure 4.3d, Table S4.5). Since in structured labs, week 5 is the first week of students selecting their formal group members, this difference is expected. Overall students reported that they were satisfied with their team

members. To identify any social loafers in the groups, we asked students if there were any process conflicts associated with sharing the workload in their group. The average process conflicts score was lower than 1.5 in both lab settings. The lower score indicated an absence of conflicts over responsibilities pertaining to the group tasks from weeks 5 to 7. There was no difference observed between structured and unstructured labs. We did not find any evidence that the average process conflict score was different over time. There was no evidence of any difference in the final grades in both the structured and unstructured labs. The average group grades and individual grades were 94% (0.94) and 90%(0.90) respectively in both the structured and unstructured labs (Figure 3e, Table S5).There are a total of 3 students who failed the class and hence their grade is zero. 2 students are from structured labs and 1 is from unstructured labs.

Overall, groups that had enforced structured opportunities to select their members did not have any significant advantage in terms of positive and negative outcomes.

Question 3: *What do students do when they encounter poor group members during the group formation phase and does enforcing interaction prior to group formation influence student's response?*

We then explored students' responses towards encountering poor group members. We examined the frequency of reporting of poor group member behavior during the group formation phase (weeks 1 to 4) and the common group selection factors reported. The total number of students who received comments in structured labs were week 1 =282,

week 2=271, week 3=282, and week 4=276. The total number of students who received comments in unstructured labs were week 1 =291, week 2=290, week 3=284, and week 4=283. The overall trend indicated that more students received negative comments from

their peers in structured labs. The probable reason is that the overall reporting of negative comments seems higher in structured labs. There are less than 10% students in unstructured labs over the four weeks who received negative comments. In structured labs, more than 10% but less than 20% of students received negative comments. The differences observed between structured and unstructured labs in weeks 3 and 4 are statistically significant, $p<0.05$ (Figure 4.4a). We identified the 'group selection factors' commonly reported by students (Table S4.2). We used a cumulative approach and calculated the total number of students who received at least one negative comment in the category of behavioral traits in all the first four weeks. This distribution of students is represented as a Venn diagram in Figure 4.4b. In structured lab sections 100 students received a negative comment in the 'attention/interest' category, 81 students received a negative comment in the 'participation' category and 63 students received a negative comment in the 'preparedness' category. Hence in structured labs, students' perception of poor group member behavioral traits was ranked as first 'attention' followed by 'participation' in the class, and lastly 'preparedness' for the class. In unstructured lab sections 54 students received a negative comment in the 'attention/interest 'category, 52 students received a negative comment in the 'participation' category and 24 students received a negative comment in 'preparedness' category. Hence in unstructured labs, students' perception of poor group member behavioral traits was ranked as- equal for 'attention' and 'participation' in the class followed by 'preparedness' for the class (Figure 4.4b). In structured labs 9.6% students (16 students out of 166) while, in unstructured labs 6.45% (6 students out of 93) of students received all three negative behavioral trait comments from their peers. In structured labs, 37.34% (62 out of 166) students and in unstructured lab sections 33.33% (31 out of 93) students received at least

2 negative behavioral trait comments from their peers (Figure 4.4b). We then focused on the weekly distribution of the students who received negative comments. The results are represented in a Venn diagram in Figure 4.4c and 4.4d. We found a similar number of students in each structured and unstructured lab section, who received at least 2 negative comments from their group members about behavioral traits.

To understand the pattern and frequency of poor group member behavior we defined PPR students. The definition is described in the methods section in detail. We identified 3 PPR students out of a total of 323 in structured lab sections and 5 PPR students out of 320 students in unstructured lab sections. Their characterization is represented in Figure 4.5. We analyzed these 8 PPR students as case studies to understand the type of PPR behavior, group members' response to this behavior and the influence of PPR students on group satisfaction, conflict, and performance.

Case studies

Figure 4.5a and c represent the percentage of negative comments received by PPR students from week 1 to 4 in structured and unstructured labs respectively. We examined their shared workload score for those individual weeks (Figure 4.5b and d). The total number of negative comments received by PPR students were at least two each week, except for week 1. Thomas received only one comment and it was negative. The rest of his group members did not complete the survey for that week. We identified 3 types of behavior patterns and grouped the students accordingly. PPR group (I) students received consistently 100% negative comments along with below class average shared workload scores for consecutive weeks. There were only two students who had this pattern, Cesar, and Odin. For both of them, the shared workload score was 2 or below for the labs where

they received 100 % negative comments. For week 1, Cesar received 50% negative comments and Odin received 28.57% negative comments. For both of them, the shared workload score was below 3.5 for week 1. In all 4 weeks, Odin and Cesar have received a shared workload score below the class average for that week (Figure 4.5c). For the class average shared workload score please refer to Table S4.6. PPR- group (II) students consistently received negative comments for all the labs they attended along with lower than class average of shared workload scores for consecutive weeks. We identified 4 students with this behavior pattern. Athena, Olivia, and Caliban consistently received negative comments for the four weeks. They received 50 % or more negative comments for only two weeks. All three of them also received lower than the class average of shared workload scores for three consecutive weeks. Thomas did not attend a lab in week 4 and hence data from that week is missing for him. However, he received negative comments consistently in all the remaining three weeks and had a lower than class average of shared workload score for consecutive two weeks. PPR- group (III) students had not received any negative comments for at least a week. For the weeks they received negative comments, their shared workload score was below the class average. Two students fit into this category. Zeus received 0% negative comments for two consecutive weeks and Ophelia had 0% negative comments in one week. Both Zeus and Ophelia had a shared workload score more than the class average for those corresponding weeks. For both Zeus and Ophelia, the shared workload score for the weeks when they received more than 50% negative comments was 3 and 3.5, which was below the class average. Ophelia received 16 % negative comments for week two with the shared workload score below the class average of 4.6.

Both structured and unstructured lab sections had all three types of PPR students. We examined the response of the group members toward these PPR students. We explored the outcomes of students' encounters with PPR students during the group formation phase during the first four weeks. Figure 4.6 (a) and (b) represent network data from groups of students who worked with Cesar and Odin from weeks 1 to 4 along with their comments. In the unstructured lab section, all the group members who provided negative comments for Odin and specifically mentioned in the surveys that they do not want to work with him again, continued to work with Odin in week 5 when formal groups were formed. They worked with the same group members from day 1 and never changed their group irrespective of poor behavior from Odin (Figure 4.6 d). Cesar was in a structured lab section. As a result of randomization, he worked with different students for the first four weeks before finalizing the group in week 5. All the students who gave negative comments to Cesar reported in the surveys that they did not want to work with him again. In week 5 when formal groups were formed, none of these students chose to work with Cesar. Simon reported that he is a pre-class friend of Cesar. Neither Cesar nor Odin completed any surveys except the one in week 1.

We then analyzed PPR group (II) and PPR group (III) students. We found a pattern of behavior in both structured and unstructured lab sections. Every group member who gave negative comments to the PPR students, reported the desire to not work with them again. However, in unstructured lab sections, these students continued to work with PPR students (Table 4.3). In structured sections, the group members chose not to work with PPR students in week 5 (Table 4.3).

We observed an overall behavior pattern in unstructured labs. In unstructured lab sections, students formed a group in week 1 and then they continued to work with the same students throughout the semester even though they had an opportunity to change groups. There were some exceptions: 15 students (out of 330) changed the group from week 1 to 2. These students continued to work with the same group after week 2; 4 students (out of 330) changed the group from week 2 to 3. These students continued to work with the same group after week 3; 3 students (out of 330) who worked with the same group for the first four weeks changed their group in week 5. We do not have any specific information about why they changed their group. These students were not identified as PPR students as per our analysis.

We investigated if the PPR students had any influence on group satisfaction over weeks 5-7 and group performance. We focused on research articles and research proposal grades as discussed in the methods section. Table 4.4 summarizes the data. The average class grade for individual performance is 87.95%. Cesar, Ophelia, and Zeus have individual grades lower than the class average. The average class grade for group performance is 92.17%. Groups with Cesar, Thomas, and Ophelia have lower than the class average group grades. Apart from the group with Thomas, other groups have satisfaction scores below the class average.

Instructor's point of view

We interviewed 10 GLAs to know if they observed any difference between their structured and unstructured lab sections. 2 GLA's responses will not be included here as we excluded their sections from our data analysis as explained in the methods. For interview questions please refer to appendix B. 7 out of 8 GLAs had a very positive attitude

towards group work. Jack informed us that "*group work can be tricky, and I never liked it in school*". He mentioned that he understands the necessity of group work. He was aware of the problems with the group work. His overall outlook regarding intervention was to let students handle the issue by themselves. Other GLAs had a common theme when it came to interventions. All the other GLAs reported that if they are aware of the situation, they like to talk to the individual student and then to the group. Jasmine and Jade mentioned that they prefer it if instructors decide and form the group. According to them, it is less stressful for the students and Jade thought if students with similar ambitions are grouped , there will be fewer group dynamic issues. All the GLAs we interviewed agreed that they rely on the students to self-report the problem. Sometimes GLAs noticed problems with groups in real time during the class but they still depended on self report.

Each of the GLA we interviewed had at least one group complaining about their group behavior. The common themes were free riding/social loafing and communication issues. When asked about the intervention strategies used to address the issue, Jo and Juliette reported that they deducted points for the assignment as a response to social loafing. Jade resolved the problem in two groups by talking to individual students. Josephine informed that by the time she was made aware of the problem it was too late and nothing could be done to fix the problem. We do not know if any of these groups with issues had PPR students identified by us. The GLAs could not recall the names and exact situations at the end of the semester when we interviewed them.

When we asked GLAs if they noticed any differences between structured and unstructured labs, Justin, Juliette and Jasmine mentioned that students in their unstructured sections never changed groups and just "*sat with the same people*" – Justin, "*worked with*

the same people" – Juliette, or "*cliqued up right away and did not change throughout the semester*" – Jasmine. Josephine reported a similar behavior pattern we observed in unstructured lab sections. She said: " *I think it's good to have this experience (enforced initial interactions) with other people. Even if they (students) can choose, I feel like they do not feel comfortable to leave the group if there is a problem. For example, I sit with this group in the first week, and then in the second week I sit again with them. Then if I have a problem with anything, I won't leave because other groups are already made. I saw some of the groups not working well in the beginning but none of the students tried to leave the group*". When asked to clarify what 'does not work well' means, she reported the lack of communication, interaction, and engagement within the group.

Five GLAs reported that students in their structured sections were more interactive and cohesive in the later part of the semester. Juliette said "*Towards the end of the semester everyone knew each other from the randomized (structured) section. I think just because of forced socialization the randomized groups had, they really got to know each person rather than the (only) group that they chose.*" Josephine said "*Since they had an opportunity to know a little bit of the class, I felt like they were more comfortable in the class in terms of asking questions. I felt that they were more engaged*." Jasmine said, "*later in the semester when they had individual reports and groups for debate, they (students from structured) were more comfortable breaking up into different groups than their experiment group (formal group) for watershed debate (another assignment at the end of the semester with informal group work)*." Three GLAs did not observe any differences between the two sections.

Discussion

What influences a student's decision to work with a peer in the early phases of group formation?

Instructors receive inconsistent recommendations on how to structure groups to balance students' comfort, group cohesiveness, and confidence in each other with diversity and work styles and preparation. Our results indicate that, while forming groups, students tend to first select their friends then the people who share the same gender and/or similar racial/ethnic background. There were no apparent benefits to structuring groups in terms of preventing this homophily; Students with enforced exposure to different students in weeks 1-4 before deciding on their formal groups in week 5 had the same level of homophily as groups that were allowed to select their groupmates from the first week. One major limitation of our data was that it was collected in a predominantly White Institution with individual lab sections dominated by female students with low numbers of URM students. We found that even though students had a chance to work with everyone in the class, they preferred to form a formal group with their pre-class friends. This data is in accordance with the reports from Theobald E., et al (2017) and Premo J. et al (2022) where comfort and willingness to work with other students is associated with familiarity. We observed a significant problem with this process, however, because students who formed their own groups rarely changed members (<5%) even when they reported serious behavioral problems.

Davies (2009) recommended the use of instructor-designed rubrics for identifying characteristics of an ideal team member that were enlisted from students themselves. Influenced by this recommendation, we performed preliminary data exploration to

understand students' perception and how they defined good and bad group members (Figure S4.2 and Table S4.2). The top 3 responses were behavioral traits associated with interest and engagement (participation and preparedness), and these terms were used to generate "group selection factors." Along with the behavioral characteristics, concerns over free riding group members and unequal distribution of work were reported as poor group member behaviors. We found that when asked a categorical yes/no question about willingness to work with a particular group member in the future, students' responses were more forthcoming compared to their responses to survey items using a Likert scale to rate their peers. Unwillingness to work with a group member in future was associated with the participation and perception of unequal distribution of work members in the early phase of group formation. Premo J. and colleagues (2022) reported personal connections and contributions in the groups work were major predictors of student's willingness to work with a particular group member in future. Our data suggests a similar pattern of social predictors dictating student's choice of group members.

In general, students are reluctant to report group problems or to directly confront free-riders and /or social loafers (Brickman et al., 2021; Strong & Anderson, 1990). Our data indicate that few students report the presence of a poor group member through surveys, and fewer problems are reported in unstructured lab sections. In these sections, where students were allowed to choose their own groups from day 1, they stayed with a poor group member they identified rather than confront the issue and change groups. In structured sections, when students identified a poor group member, they were more likely to avoid working with that group member in their formal groups. Perhaps, the process of

randomization helped students to avoid the social pressure that is associated with refusing to work with another student during the early phases of group formation.

Limitation of Likert scale survey items to identify conflict and poor group members

We observed that students reported poor group member behavior using 'group selection factors' about a particular group member that directly contradicted the high scores they provided for group satisfaction, communication frequency and a low score for relationship conflict (Table S4). In addition, although students reported unequal distribution of work as the major concern about group work, they simultaneously provided high ratings for their peers for shared workload in both structured and unstructured lab sections. Survey items were less effective at identifying conflict or unequal distribution of workload. Our findings concur with what Brickman and colleagues (2021) found that students reported the presence of a problematic group member with a single 'yes/no answer' survey question more effectively than task and relationship conflict items from Jehn and Mannix (2001).

Persistently poorly rated students (PPR) and students' response towards them

Prior work showed that students who contribute more to the group, don't distinguish between strugglers, social loafers and free-riding students in their definition of a good or poor group member (Freeman & Greenacre, 2011). Free riders are the students who receive grades or rewards without putting in any effort. Social loafers are students who put in less effort because of a lack of identity or feeling of belongingness in a group (Davies, 2009). Social loafing can lead to free riding (Davies, 2009; Watkins, 2004) and strugglers are the students who fail to contribute to the group or class because they are behind in their understanding of the class material (Freeman & Greenacre, 2011).

Constructive penalties for free riding behavior range from anonymously giving poor grades to the free rider or complaining about them to the instructor. This generally spurs free riders into action and resolves group conflict. Destructive social behaviors range from excluding free riders from any type of communication, completely removing peer support and assigning tasks to the free riding students which is unsuitable for them (Freeman & Greenacre, 2011). These socially destructive behaviors affect the learning environment for a struggling student and hence they can perform poorly. These behaviors may also exasperate relationship conflicts within the group (Freeman & Greenacre, 2011). Understanding the reason why certain students do not contribute and mediating interventions are critical to supporting learning. Premo J. and colleagues (2022) reported that in a biology classroom students' definition of contribution and interaction in a group at the beginning of the semester changed dramatically at the end of the semester. We used responses to our group selection factors items in early phase of group formation to help identify and characterize the prevalence of persistently poorly rates (PPR) students. We identified three types of PPR students with distinctive behavior patterns: PPR (I) who failed to participate in any informal group work, demonstrated consistent poor performance, but were rare (less than 1% of students); PPR (II) who received consistent negative comments and poor shared workload for most labs from more than half of their groupmates; and PPR (III) who had sporadic negative comments and poor shared workload. PPR student behavior (II, and III) suggest that students may be identified by his/her peers as a poor group member for contributing intermittently. There are a variety of reasons why a student is not attentive in a class or is not prepared for that week: they had conflicting deadlines, personal issues, sleepless night, etc. Instructors were largely unaware of most of these

problems from observing groups in lab in early group formation. Surveys play a critical role in identifying issues and helping instructors intervene before student learning is affected. However, our data suggest that students in structured labs are more likely to label someone as bad group member in the early phase of group formation after only one class session. Instructors should take this into account and wait until they see several reports to identify persistently poor behavior patterns to determine if intervention is needed.

From an instructor's perspective, is it worth enforcing initial student interactions through randomization?

We found no benefit to initially structuring groups in terms of students reported, conflict scores (task, process, or relationship), satisfaction, or final group and individual grades. However, we did observe subtle differences in reported communication frequency over the period of 7 weeks in both structured and unstructured sections. These differences are associated with the length of time two students worked together and hence the familiarity among the group members. The majority of GLAs we interviewed informed us that the structured labs were more interactive, engaged, and cohesive at the end of the semester because of enforced interactions in the first four weeks. This effect was similar to the one reported by Rienties and colleagues (2014) where students who were in randomly assigned groups showed more "knowledge spillovers" outside their groups.

In addition, we did observe a tendency for students to avoid persistently poorly rated students in final group composition in structured labs. Social network analysis literature describes how relationships in social contexts generate a reputation cost for bad behavior and thus aid in collaboration (Burt et al., 2013). In an undergraduate laboratory or classroom setting, it is possible to observe the relational embedding and structural

embedding of the network. Relational embedding indicates two students with a relational history, such as friend or pre-class friend status. Structural embedding is associated with students who have many mutual informal or formal connections, such as the connections enforced in our structured labs. The probability of bad behavior being discovered is higher in a more connected student network (Burt et al., 2013). This may explain the higher reported negative behavior in structured labs and the increased tendency to identify and avoid PPR students.

Implications for Instructors

Instructors have an impact on shaping classroom peer ecology in terms of how students evaluate each other (Hendrickson et al., 2011). We found that students would rather stay with a problem group member that they are familiar with rather than confront the issue and change groups. This is where instructor-mediated intervention is needed to resolve group conflict and help struggling group members. In our study, GLAs intervened to resolve conflicts in some groups in later portions of the semester by simply addressing communication gaps between the group members. For example, the free riders in two groups were penalized by deducting their grade for the assignment. Following recommendations from Davies (2009) that ask students to define of good and poor group members might help create clearer expectations about group work before these problems accelerate. Administering our group selection survey could help serve as a guideline to identify struggling students in early group formation and help the instructor to observe, identify and mediate conflicts in real time. Likert survey items are not as effective as simple categorical 'Yes/No' and 'my fellow group member was not performing well and why' questions that can be a powerful strategy in early phases of group formation.

Methods

Instructional Context

We collected data from an inquiry-based introductory biology lab (Principles of Biology II Laboratory) at the University of Georgia in Spring 2019. In the first four weeks of the lab, informal group work is used to help students explore aquatic and terrestrial biodiversity field activities and introduce scientific processes and the writing-intensive curriculum (Figure 4.1). Formal student groups are formed during week five. For the following three weeks, groups agree on a biologically significant question, design a scientific experiment to answer that question, perform an introductory literature review, draft a research proposal, collect and present data, and communicate their findings in a research article. The formal group grade for these weeks comprises the submission of a research proposal and bibliography. Students present their data as a group and are graded as a group on this presentation. Assignments and grades were distributed so that 53% of points were derived from individual assignments and 46% from group assignments. After week 8 students are expected to perform data analysis and submit an individual research article as well. For the rest of the semester, students perform fieldwork and associated experiments as a class activity. They have at least 2 more weeks of informal group work where they can work with anyone they prefer. As the majority of the group grade is associated with formal group work, we anticipated to observe maximum group related conflicts in weeks 5-7. Hence, we collected data from weeks 1 to 7 along with the final grades. Each instructor/graduate laboratory assistant (GLA) taught two lab sections. For individual instructors, we randomized students in one of their lab sections and other lab sections students were allowed to self-select their group for the first four weeks. In this

way, we could control for any specific instructor variables that might have influenced student interaction.

Class roles were used to create unique surveys (Qualtrics, 2019) for each lab section and were administered to become available at the start of the lab so that students could complete the survey at the end of lab activities for that week. The data was collected from a total of 30 lab sections (15 structured and 15 unstructured). Student demographics were collected during the first weeks of lab (Table 4.1). Students who preferred not to provide their gender identity were given 'other' designation. We defined racial and ethnic URM status using NSF's definition of underrepresented minorities in Science and Engineering: this included students who identified themselves as Black, Hispanic, Native American, Alaskan natives and/or Pacific Islanders, and biracial. As expected for a predominantly White institution, we observed a low percentage of URM students (<20%). Subsequently, we combined the five classifications into a single URM status for statistical analysis (Table 4.1). We also collected demographic data on students who reported their ethnicity as Asian (Indian, Korean, Chinese, Filipino, and Japanese) (Table S4.1). Students who did not wish to disclose their ethnicity and/or who chose every single option from the drop-down menu in the survey were compiled together and given 'Other- ethnicity' status.

No statistically significant differences were observed between student demographics (gender and URM status) between structured and unstructured labs (gender: $p=0.4048$; URM: $p=0.226$, Pearson's chi-square test). The average self-reported GPA of structured labs was 3.48 out of 4 and unstructured labs was 3.50 out of 4. When we compared class ranks, we found that structured labs had more students in their junior year than unstructured labs. ($p =0.03469$; PearsonChi-squared test).

Laboratory Layout

Each laboratory section was taught by a single instructor and consisted of 6 Tables each with a maximum capacity of 4 students who worked together for each group activity. Each section had a maximum of 6 groups with 3 or 4 students per group for a possible enrollment cap of 24 students. (See schematic representation in Figure S4.1). To ensure that students were randomly assigned in the structured labs, name tags were generated with each Table number for all students in those sections.

Instruments

Survey questions were designed to gather both qualitative and quantitative characteristics that students used to select and rate group members. Surveys were emailed at the start of the lab meeting each week, and reminder emails were sent every two days to those who had not responded until the beginning of the next lab meeting. Students were asked to indicate all the students that they worked with that week and identify any who were pre-class friends, "someone the student considered as a friend before the term." See Appendix A for all survey items.

Qualitative Measures: To develop qualitative group selection items, we developed "Group Selection Factors" using student answers to open-ended questions during a previous semester. Responses from a total of 74 students (3 unstructured and 2 structured labs) over the period of the first four weeks of the labs provided pairs of traits students used to define a peer as a good or poor group member. The responses were coded by two researchers who first independently read 530 examples that students provided for good group member traits and 527 examples that students provided for poor group member traits. Each researcher created categories that defined the major factors that students identified to

classify group members and used an iterative process to refine those categories into three final classifications: cognitive traits, attributes, and behavioral traits (Figure S4.2). The most common trait that students provided to define poor and good group members was behavioral (50%). The three most common behavioral traits mentioned were used to create a "Group Selection Factor" item as well as an item that asked students to rank their group members depending upon their perception of sharing group workload (Table S4.2, Appendix A). We surveyed students using these items during the group formation phase (weeks 1-4). Students also indicated how much each member of the group participated in sharing the workload on a scale from very well to very poor. Both shared workload and identification of poor and/good group member traits were used to confirm if group selection factors were significantly different from our preliminary data analysis.

Quantitative measures of communication frequency within the group were assessed every week (from surveys 1-7). Additional quantitative measures of group dynamics were administered during weeks 5-7 and included prior validated items on group satisfaction and conflict (Jehn & Mannix, 2001; Curseu et al, 2012).

Instructor /Graduate Laboratory assistant (GLAs) interviews

We interviewed GLAs about their demographic information (Table 4.1c), teaching experience, their observations about the problems/conflicts with group work in their labs, the intervention strategies they employed to resolve identified conflict/problems, and their personal experience with conflicts in their research labs. The survey questions are in appendix 2. The interviews were recorded and then transcribed by researchers.

Data collection

The data collection theme is described in detail in figure 4.1.

Social Network Analysis to Identify Group Dynamics

Qualitative analysis- Visualization of the group formation data for labs 1-*4* was performed using R packages statent,ergm,ergm.count,ergm.rank,latentnet with the R3.6.1 version. Every laboratory section is confined by the physical space and time of the day so that students from one lab section cannot work with the students from other lab sections: thus, each laboratory section is considered to be an individual network with its boundary. Each lab has a maximum of 24 students. Each student was asked to self-report which 3 other students they worked with in each lab session. Ideally, each student can work with a maximum of 23 students over the period of 4 weeks. We plotted cumulative networks for both structured and unstructured lab sections over the period of the first 4 weeks. For weeks 1-4, directed binary social networks were plotted where 0 represents no interaction between the two students and 1 represents an interaction between two students. Binary networks indicate if two students worked together or not. The directionality indicates if a student self-reports that tie. Network densities and transitivities were calculated using modified code as a primer (Grunspan et al., 2014). We used cumulative data from weeks 1 through 4 to visualize the difference in the pattern of interactions in structured and unstructured labs. Because enforced randomization was used to ensure that students have a maximum number of chances to work with other students in the structured labs, we confirmed that we had one large network in these sections at the end of four weeks (representative section plotted in Figure 4.2). The network patterns were similar in 13 of the 15 unstructured sections. In 4 sections we saw no difference between structured and unstructured labs. Discussions with the GLAs for those lab sections revealed that they had inappropriately randomized their unstructured lab sections and allowed us to additionally

randomize the structured sections. So, for these 2 GLAs both their lab sections were randomized. Therefore, we removed a total of four lab sections (2 unstructured and 2 structured sections associated with these GLAs) from the further data analysis.

Statistical analysis-Exponential Random Graph Models (ERGMs) - We used the 'ergm' package for R in the 'statnet' package for statistical network analysis. This package provided tools for models called exponential family random graph models (ERGMs) or p-start models (Holland and Leinhardt, 1981;Wasserman and Pattison, 1996; Robins,Pattison,Kalish and Lusher, 2007a). This package predicts approximate maximum likelihood estimates (MLEs); stimulates random networks from a specified ERGM and performs graphical goodness of fit checks for the types described by Hunter, Goodreau and Handcock (2008) .We analyzed directed valued networks after formal groups were formed (week 5). In a valued network, 0 ties indicated no interaction; 1 tie indicated an interaction between two students; 2 ties represented interaction between two students who were pre-class friends. The likelihood of having a tie between two nodes (students) did not depend on the value of other ties in the network but depended only on the attributes of the nodes (students) involved. Hence, our network model was dydatic independent. Our reference/baseline distribution was binomial as our data was binomial. Our graph model was GLM style. We calculated differential homophily using gender and pre-class friend status. For ERGM analysis, we used the tutorial and code published by Pavel N Krivitsky, Carter T.Butts and The Statenet Development Team, 2016.

Qualitative methods used to identify persistently poorly rated students (PPR students) from group selection factors and shared workload scores

We asked students to score individual group members using a numerical Likert scale from 5 (very well) to 1(very poor) to indicate how well they shared the groups' workload each week. Shared workload scores were calculated for each student by averaging all scores they received from all their group members for the first four weeks and used to identify reported free-riding or social loafing behavior. In addition, we asked students to identify group members they would work with again, and for every group member they worked with for that week, they were asked to select positive or negative traits from a pre-populated list which contributed to their decision of working with /without them in subsequent weeks. Data was compiled for every student, and to normalize data, we calculated the total number of comments an individual received that week and then calculated the percentage of those comments which were negative and positive. With an average group size of four students, each student could receive a maximum of 9 positive or 9 negative comments. We determined the number of students receiving negative comments every week in both structured and unstructured labs as well as the type of negative comment received from the pre-populated list. Data visualization was used to identify natural clusters which helped us define a persistently poorly rated (PPR) student as any student who received greater than 50% negative comments for two or more weeks.

Statistical analysis

Descriptive Statistics - Student's responses to the survey questions were converted into numerical value on the Likert scale. For the satisfaction score the numerical values assigned were: strongly agree =5, agree=4, neutral=3, disagree=2, strongly disagree=1. For

task and relationship conflict scores, the numerical values assigned were: none =1, Rarely/little=2, Some=3, Often/much=4, very much/very often =5. For the shared workload score the numerical values assigned were very poor =1, poor =2, neutral =3, good =4, very good=5. For the communication frequencies, the -5 to+5 scale was converted to 0 to 11. A score of 6 indicates an average level of communication (Cursue P.L. et al., 2012). We used the Shapiro-Wilk test for checking the normality assumption for satisfaction, task conflict, relationship conflict, process conflict, discussion scores, communication frequencies, shared workload scores, and final grades. As the data is not normally distributed, non-parametric tests were used for data analysis.To compare the difference between structured and unstructured labs scores, the Wilcoxon rank sum test with a confidence interval of 0.95 was used. For comparing differences in satisfaction, conflict, communication frequencies, and shared workload scores over time in structured or unstructured labs, the Kruskal-Wallis rank sum test followed by an ad hoc kruskalmc test was used. All the analysis was performed in R using packages psych, clinfun, pastecs, pgirmess, remotes, and car.

To compare the frequency of reported poor group members between structured and unstructured labs, we performed a chi-squared test to identify any statistically significant difference. For this purpose, we used the total number of students and the total number of students who received at least one negative comment for a particular week.

PPR students' grade analysis- Descriptive statistical analysis of satisfaction and relationship conflict scores were calculated using Microsoft Excel for students whom we identified as PPR and their respective group members. We used one assignment - the individual research article- to determine the performance levels of PPR compared to their

respective group members. For group performance, we combined both the group research proposal and group presentation scores. These major writing assessments for the experiment the groups conducted during weeks 5-7 are the best qualitative measure to contrast individual with group performance.

Figures and Tables

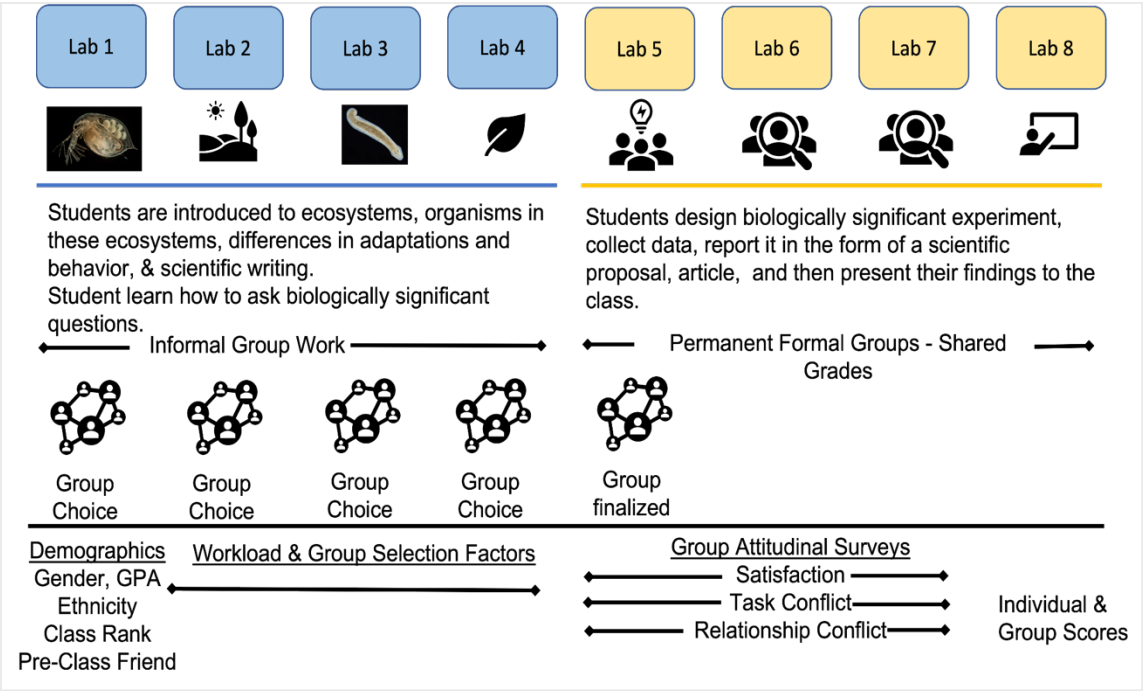

Figure 4.1 Data collection scheme. Lab numbers indicate the weeks when data was collected. Surveys for weeks 1-4 included questions that asked students to select the members that they worked with that week, indicate if they were pre-class friends, and rate their participation. Surveys in weeks 5-7 asked students to indicate satisfaction and conflict with their permanent group members. Individual grades and total group grades were then collected after week 8 at the end of the semester.

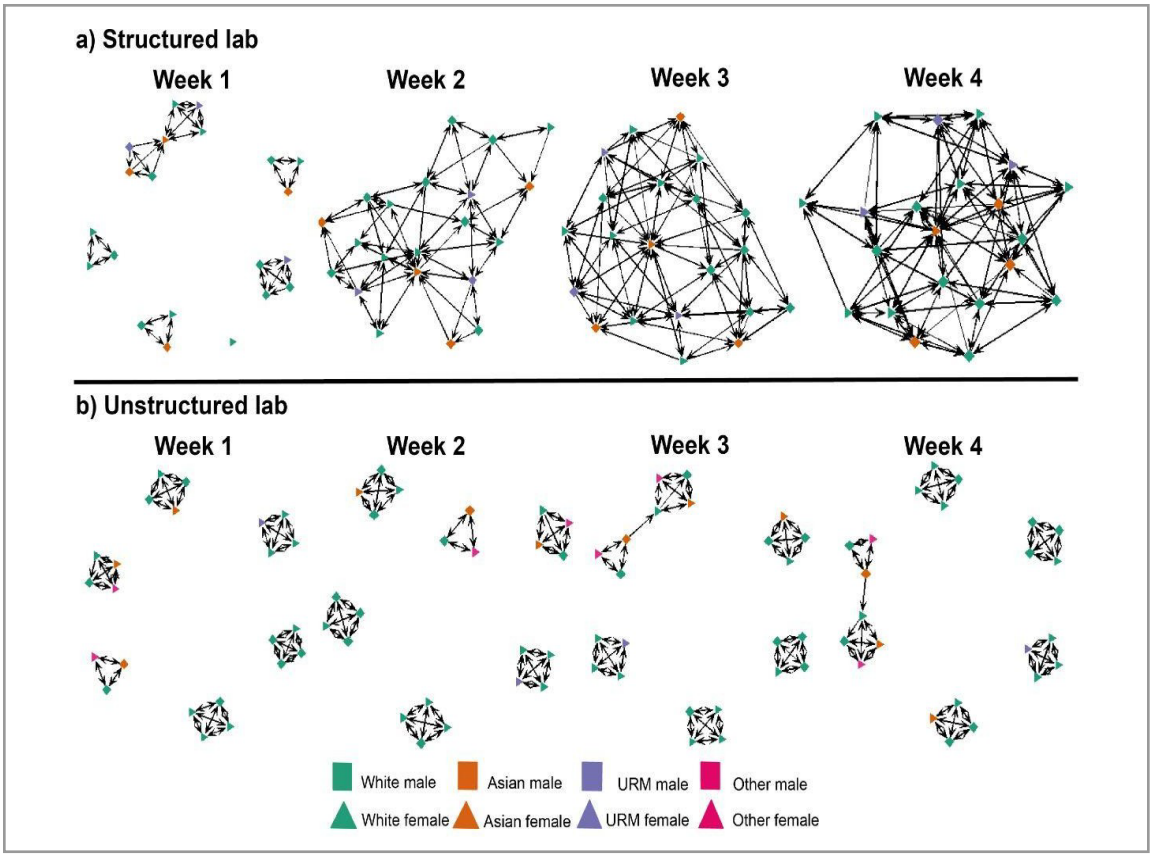

Figure 4.2: Representative examples of directed binary social networks for weeks 1 to 4 in structured and unstructured lab sections.

Each line represents a tie between two students (nodes of the network). The directionality of the tie is represented by the direction of the arrow. If the arrows are not bidirectional, it means only one student reported the interaction. The color of nodes indicates ethnicity. Green = White, Orange= Asians, Violet = URM, Pink = Other. Triangles represent female students and squares represent male students.

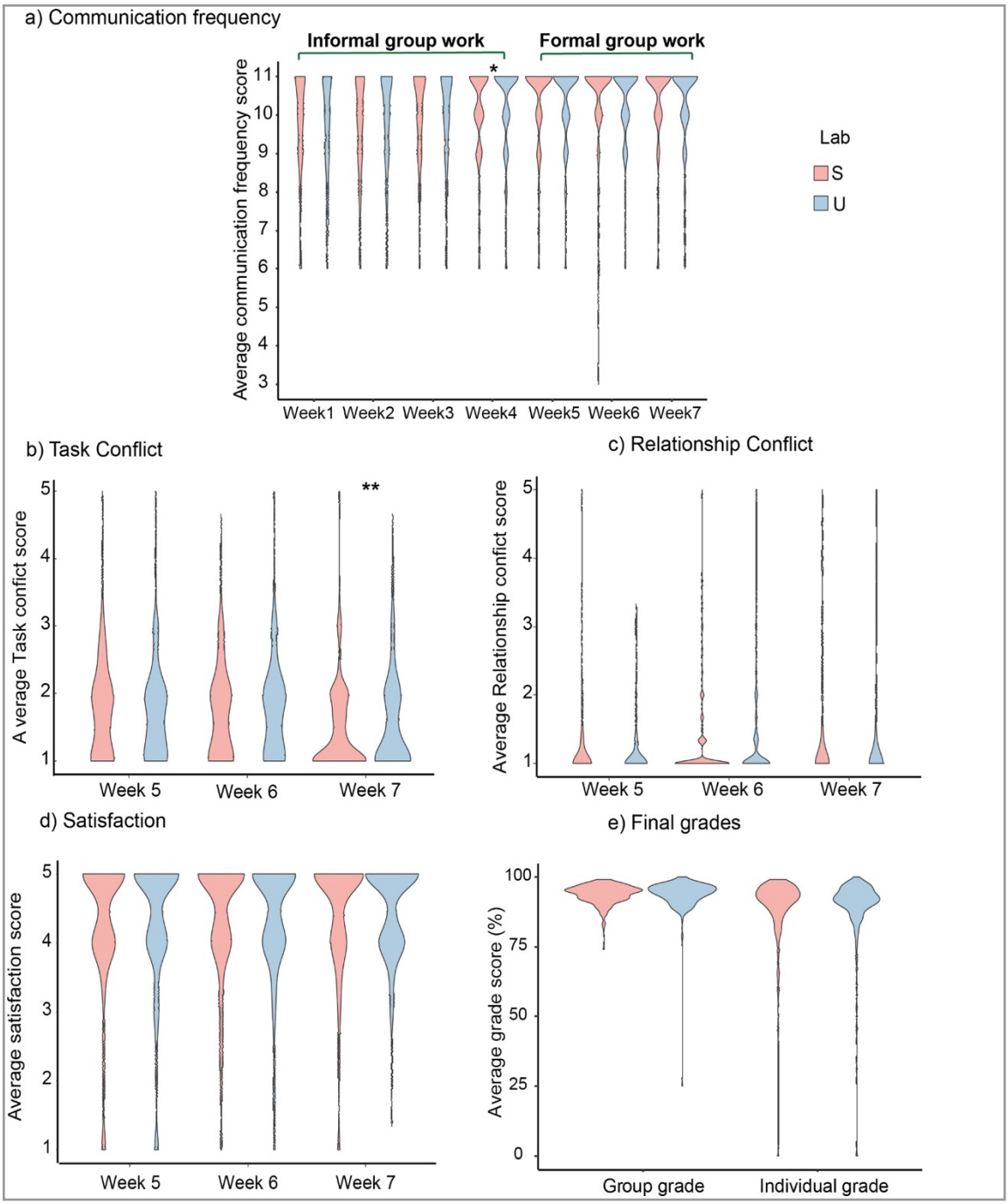

Figure 4.3: Differences in average scores between structured and unstructured in communication frequency, task conflict, relationship conflict, satisfaction and final grades. The data is collected from 13 unstructured and 13 structured lab sections. Pink color represents data from structured labs and blue color represents data from unstructured

labs. (a) Communication frequency reported from week 1 to week 7. *The* Y axis indicates the communication frequency reported by group members the and X axis is weeks. *In week 4 unstructured labs have a higher average communication frequency (10.30) than structured labs (10.07) with $p<0.05$. (b)Task conflict score from week 5 to 7 when formal groups were formed. The Y axis indicates the average task conflict score and the X axis is weeks.** In week 7 unstructured labs reported higher average task conflict value(1.58) than structured lab sections (1.45) with $p<0.05$. (c) Relationship conflict score from week 5 to 7 when formal groups were formed. The Y axis indicates the average relationship conflict score and X axis is weeks. (d)Satisfaction score reported from weeks 5 to 7 when formal groups were formed**.** Y axis indicates the average satisfaction score and X axis is weeks.(e) Final grades comprising group grades and individual grades. The Y axis indicates the average final grades in percentage and the X axis is the category of the grades.

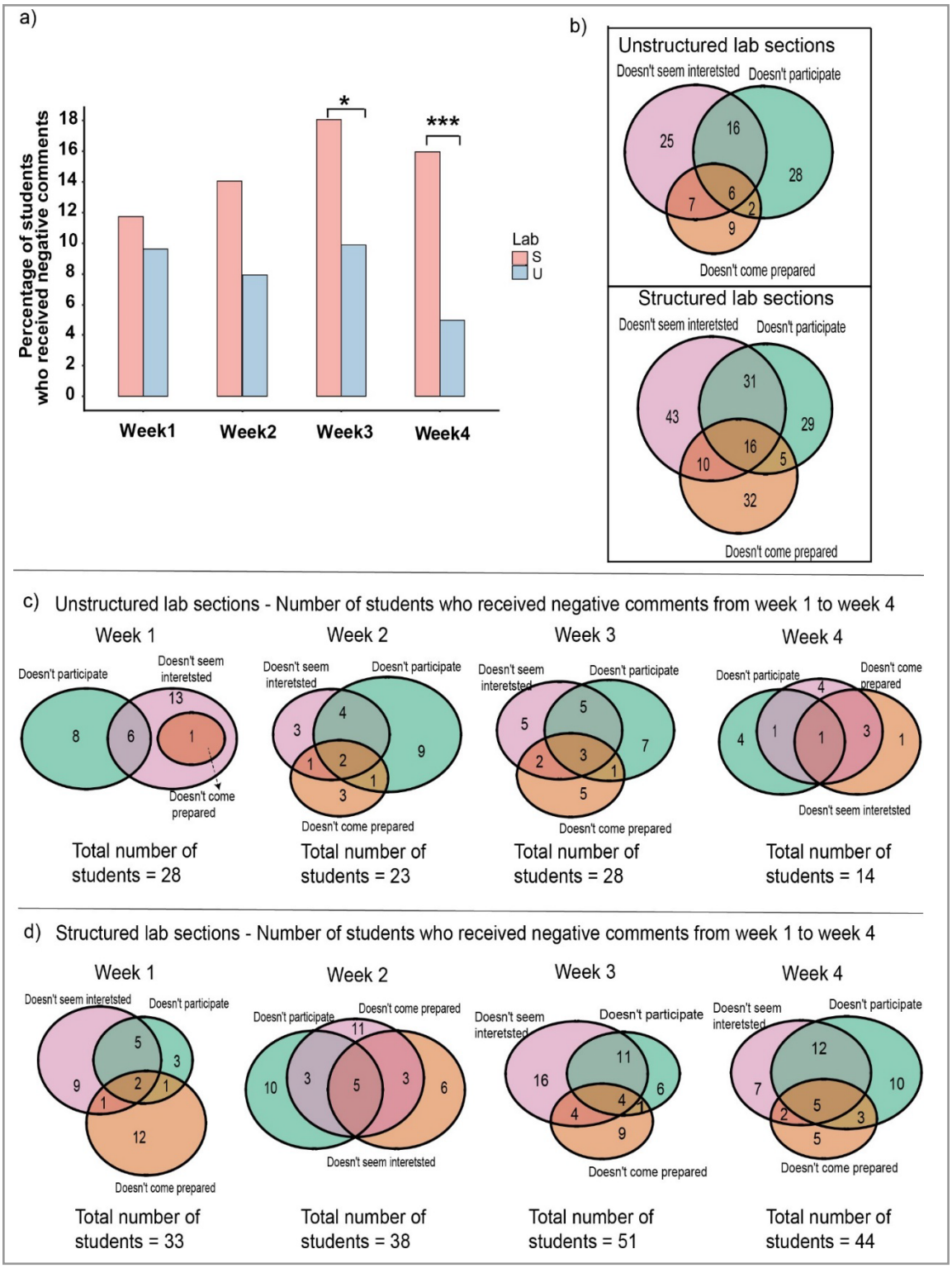

Figure 4.4: Report of poor group member behavior according to negative comments given by peers (group selection factors).

(a) The bar graph represents the percentage of students who received negative comments from their group members from week 1 to week 4 in both the structured and unstructured lab sections. Y axis indicates the percentage of students who received negative comments assigning them as poor group members. X axis represents weeks. Pink bars represent the data from structured labs and the blue bars indicate data from unstructured labs. * Pearson's chi-square test with Yates' continuity correction statistics X-squared = 5.4504, df = 1, p-value = 0.01956 **Pearson's chi-square test with Yates' continuity correction statistics X-squared = 13.726, df = 1, p-value = 0.0002115. (b) A Venn diagram representing the number of students in the three categories of group selection factors in structured and unstructured lab sections over the first four weeks of the lab classes . Each circle represents one category of group selection factor. The number in each circle is associated with the number of students who received that particular category of comment from their group members and the difference in circle size is indicative of that. (c) Venn diagrams of weekly data of students with negative comments in unstructured lab sections. (d) Venn diagrams of weekly data of students with negative comments in structured lab sections. Green = category "A student doesn't participate", pink = " A student doesn't seem interested", orange = "A student doesn't come prepared for the class".

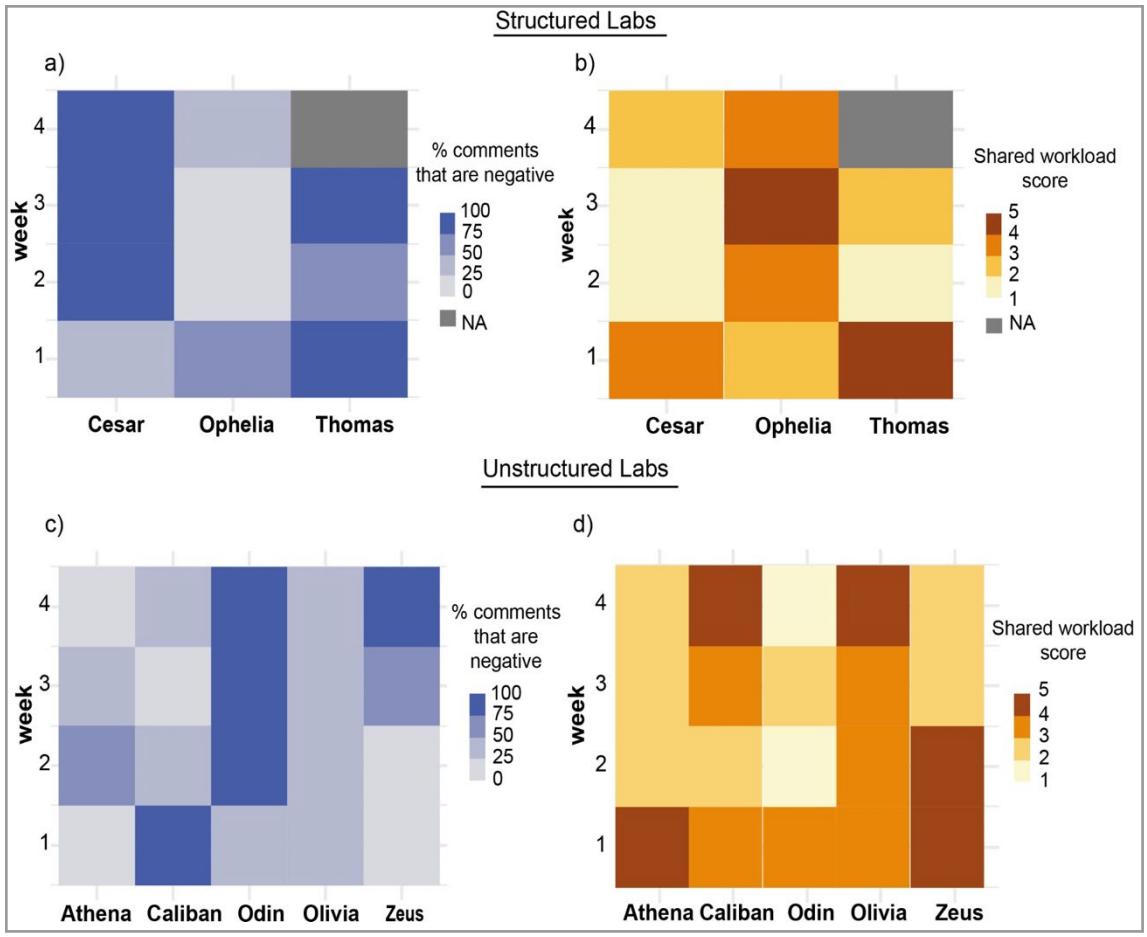

Fig 4.5: Identification /Characterization of PPR students. (a) and (c) A heatmap of the percentage of negative comments a student received from their group members that week in structured or unstructured lab sections. The color intensity is associated with the percentage and is indicated by a scale next to the heatmap. A gray square indicates that the student was either absent that week or did not receive any comments from his/her group member that week. A darker blue color indicates a higher percentage of negative comments. (b) and (d) A heatmap of the shared workload score of a student received from their group members in structured and unstructured lab sections. The maximum score is 5 and the minimum is 1. The color intensity is associated with the score received and is

depicted by a scale next to the heatmap. A gray square indicates that the student was either absent that week or did not receive any score from his/her group member that week.

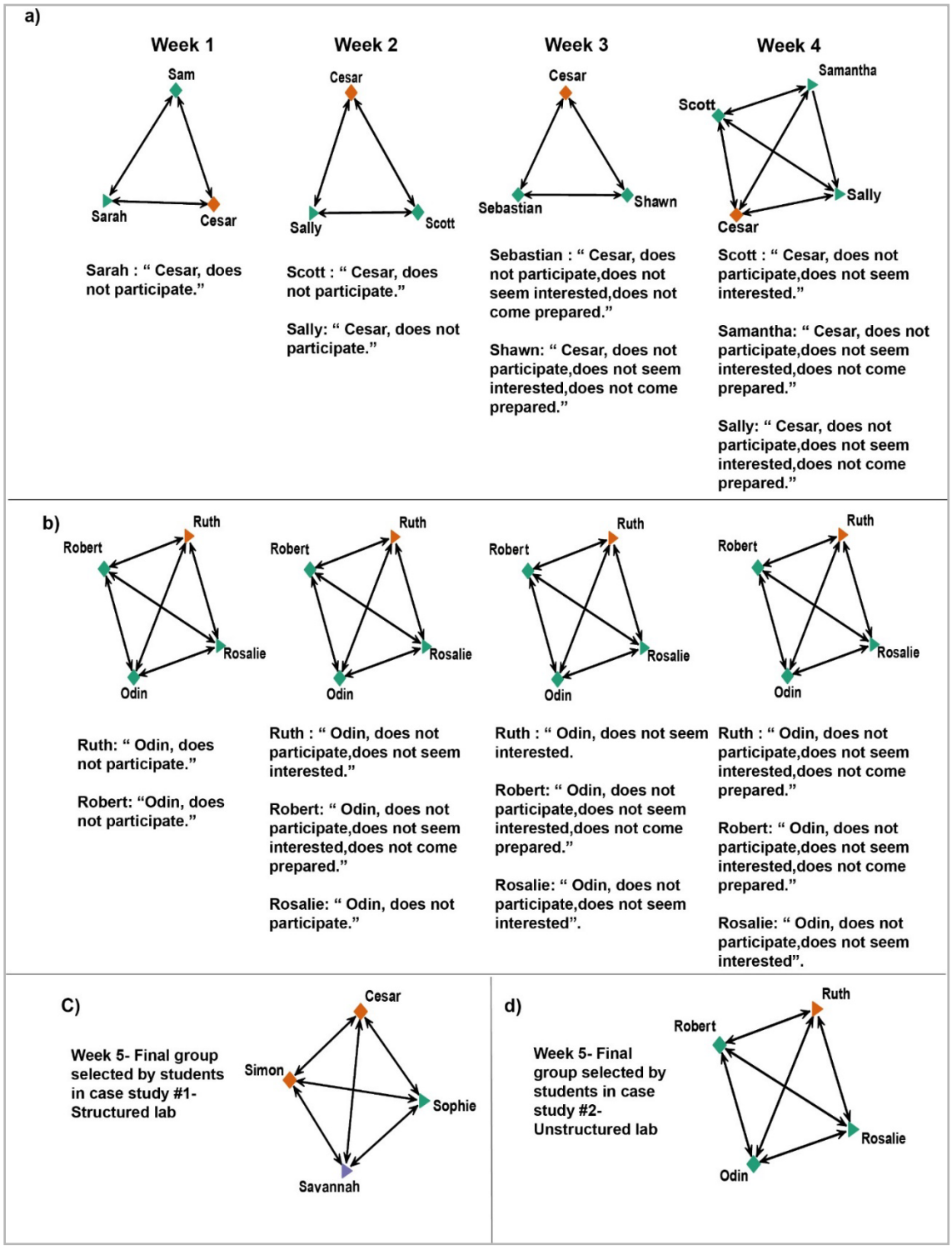

Figure 4.6: Case studies of the PPR group (I) students from structured and unstructured labs. Each network image represents the number of students in a group. Each line represents a tie between two students (nodes of the network). The directionality of the tie is represented by the direction of the arrow. If the arrows are not bidirectional, it means only one student reported the interaction. The color of nodes indicate ethnicity. Green = White, Orange= Asians, Violet = URM, Pink = Other. Triangles represent female students and squares represent male students. (a) Case study#1-Network data from a group of students who worked with Cesar from a structured lab section. (b)Case study#2-Network data from a group of students who worked with Odin in an unstructured lab section. (c) Case study#1- A network representing the final group selected by students in week 5 in a structured lab. (d) Case study#2- A network representing the final group selected by students in week 5 in an unstructured lab.

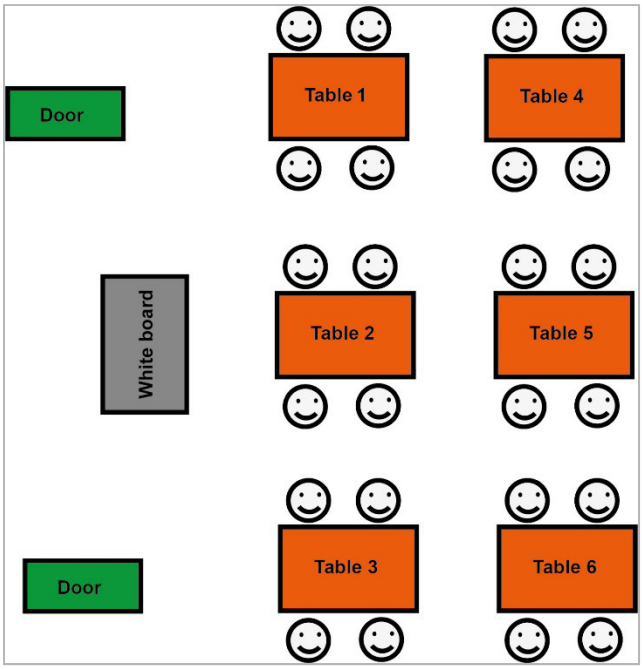

Figure S4.1: Laboratory layout for BIOL-1108 labs. Each section had a maximum of 24 students and one GLA.

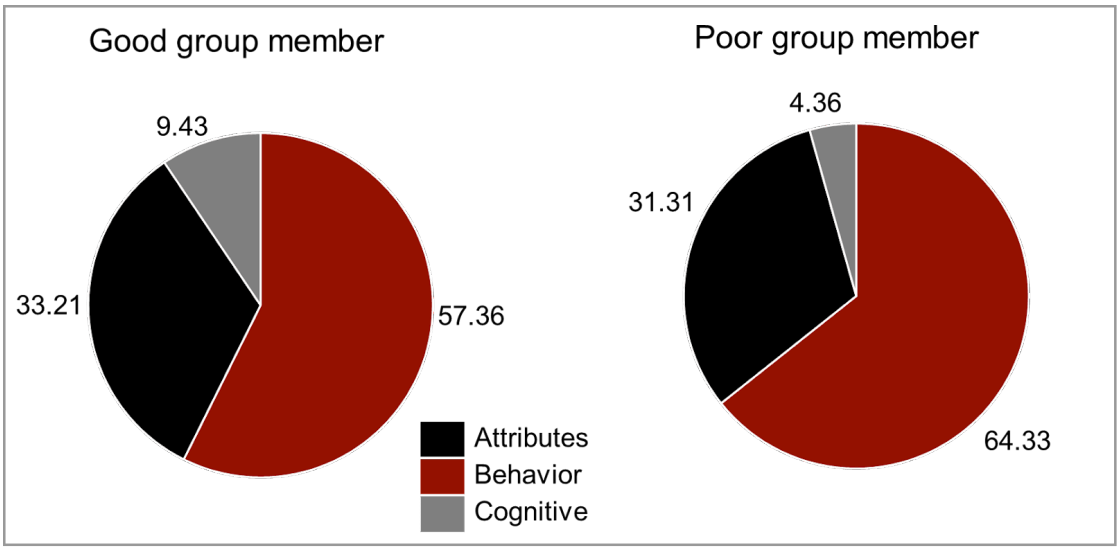

Figure S4.2: Preliminary qualitative data from Summer 2018 for survey item development about students' perceptions of good and poor group members.

Students defined "good" and "poor" as they perceived it without any definition or specification provided in the survey questions asked. The completion rate for all of the surveys was 84.8%. Good group member codes (n=530) and Poor group member codes (n=527) were calculated as the frequency of responses per code over the total number of responses for all four surveys (%).

Table 4.1: (a) Demographics of the students -gender and ethnicity

Labs	Total number of students	Gender			URM status		
		Female	Male	other	URM	Non-URM	other
Structured	323	207	113	3	49	256	18
Unstructured	330	224	105	1	64	254	12

Table 4.1: (b) Demographics of the students -class rank

Labs	Class Rank				
	Freshman	Sophomore	Junior *	Senior	Prefer not to answer
Structured	33	201	67	16	6
Unstructured	27	182	94	27	0

Table 4.1(c) Demographics of the lab instructors (GLA- graduate lab assistants)

Identifier/Pseudo Names	Previous teaching experience	Ethnicity	Gender
Jack	Yes	White	Male
Josephine	Yes	Latino/Hispanic	Female
Justin	Yes	White	Male
Jasmine	No	White	Female
Jordan	Yes	White	Male
Jo	Yes	White	Other
Juliette	Yes	White	Female
Joy	No	Biracial	Female
Jade	yes	African American/Black	Male
Julius	yes	White	Male
Jane	Yes	White	Female

4 instructors out of 15 did not consent to participate in the study. Jane discontinued teaching the course after week 5.

Table 4.2: Lab sections with statistically significant homophily

Labs	Total lab sections analyzed	Sections with differential homophily pre-class friend and same gender	Sections with differential homophily pre-class friend and different gender
Structured	13	5	12
Unstructured	13	2	13

Table 4.3: Qualitative analysis of the groups where homophily observed with respect to pre-class friend status and ethnicity

Labs	Total number of groups	Total number of groups with students who are pre- class friends (at least two students in a group)	Total number of groups with URM students working together with no pre-class friend status (at least two students in a group)	Total number of groups with Asian students working together with no pre-class friend status (at least two students in a group)
Structured	77	31	5	3
Unstructured	78	33	8	5

Table 4.4 Group's response to PPR students			
Sr.no	PPR student	PPR behavior group	Did the other group members continue working with the PPR student?
Unstructured Lab sections			
1	Odin	I	Yes
2	Athena	II	Yes
3	Caliban	II	Yes
4	Olivia	II	Yes
5	Zeus	III	Yes
Structured Lab sections			
6	Cesar	I	No
8	Thomas	II	No
7	Ophelia	III	No

Table 4.5 - Group satisfaction, Group performance and Individual performance of PPR students						
Sr.No	Student	Lab	Average group satisfaction score (out of 5)	Group grade (%)	Individual grade (%)	Average individual grade of other group members (%)
PPR group (I)						
1)	Cesar	Structured	3.85	90.00	76.73	82.60

2)	Odin	Unstructured	3.81	97.64	91.86	92.23
PPR group (II)						
3)	Thomas	Structured	4.86	90	88.43	86.08
4)	Athena	Unstructured	3.97	87.64	89.03	93.08
5)	Caliban	Unstructured	3.88	87.35	90.71	93.21
6)	Olivia	Unstructured	3.51	94.11	98.23	75.29*
PPR group (III)						
7)	Ophelia	Structured	3.51	97.64	55.29	91.06
8)	Zeus	Unstructured	3.25	95.29	84.80	93.01

*- one student did not submit an assignment hence, the group's individual grade average is low.

Table S4.1 - Demographics of students- Details of Ethnic/Racial distribution			
Ethnicity/Race		Number of students in Structured Labs	Number of students in Unstructured Labs
White (non-URM)		194	200
Asian (non- URM)	Asian Indian	41	20
	Chinese	6	10
	Korean	4	8
	Vietnamese	8	12
	Filipino	2	4
	Japanese	1	0

URM	African american/Black	32	44
	Hispanic/latinx	17	19
	Native american/Pacific islanders/ Alaska Natives	0	1

Table S4.2 - Survey item development- Top 3 behavioral traits

Behavioral trait category	Description for good group member	Description for poor group member
Attention/Interest	Pays attention	Does not seem interested in the class
Participation	Participates in the discussion and offer meaningful suggestions	Does not participate in the discussions / listen to others
Preparedness	Well prepared for class and knows material well in advance	Does not come to class prepared or does not know material in advance

Table S4.3: ERGM differential homophily coefficients and p values						
Unstructured lab sections - Formal group formation						
Lab ID	Total number of students and (♀/♂ ratio)	Likelihood Descriptions	exp(**β**)	probability	p value	# of groups with pre class friends out of total of 6
11	22 (♀/♂ ratio = 1.63)	a) 2 students are pre-class friends and are of different gender b)2 students are pre-class friends and are females c)2 students are pre-class friends and are males	0.0307 2.0932 3.0089	0.029 0.676 0.750	< 1e-04 *** 0.01605 * 0.00307 **	3
21	19 (♀/♂ ratio = 2.8)	a) 2 students are pre-class friends and are of different gender b)2 students are pre-class friends and are females c)2 students are pre-class friends and are males	0.0498 0.8712 1.4344	0.047 0.465 0.589	<1e-04 *** NS NS	2
31	24 (♀/♂ ratio = 3.8)	a) 2 students are pre-class friends and are of different gender b)2 students are pre-class friends and are females	0.0418 1.0811	0.040 0.519	< 1e-04 *** NS	3

		c)2 students are pre-class friends and are males	1.6770	0.626	NS	
41	19 (♀/♂ ratio = 5.33)	a) 2 students are pre-class friends and are of different gender	0.0355	0.0343	< 1e-04 ***	4
		b) 2 students are pre-class friends and are females	1.2604	0.557	NS	
		c) 2 students are pre-class friends and are males	1.5446	0.607	NS	
61	22 (♀/♂ ratio = 1.44)	a) 2 students are pre-class friends and are of different gender	0.0385	0.037	<1e-04 ***	2
		b) 2 students are pre-class friends and are females	1.4100	0.585	NS	
		c) 2 students are pre-class friends and are males	1.2378	0.553	NS	
91	22 (♀/♂ ratio = 2.14)	a) 2 students are pre-class friends and are of different gender	0.0478	0.045	< 1e-04 ***	1
		b) 2 students are pre-class friends and are females	1.0571	0.513	NS	
		c) 2 students are pre-class friends and are males	0.6792	0.404	NS	

101	24 (♀/♂ ratio = 5)	a) 2 students are pre-class friends and are of different gender b) 2 students are pre-class friends and are females c) 2 students are pre-class friends and are males	0.0633 0.8646 NA	0.059 0.463 NA	<1e-04 *** NS NA	4
121	24 (♀/♂ ratio = 1.6)	a) 2 students are pre-class friends and are of different gender b) 2 students are pre-class friends and are females c) 2 students are pre-class friends and are males	0.0211 3.0697 4.8919	0.020 0.754 0.830	<1e-04 *** <1e-04 *** <1e-04 ***	2
141	22 (♀/♂ ratio = 2.14)	a) 2 students are pre-class friends and are of different gender b) 2 students are pre-class friends and are females c) 2 students are pre-class friends and are males	0.0451 1.1982 1.1013	0.043 0.545 0.524	<1e-04 *** NS NS	2
151	23 (♀/♂ ratio = 1.55)	a) 2 students are pre-class friends and are of different gender	0.0494	0.047	< 1e-04 ***	3

		b) 2 students are pre-class friends and are females	1.2827	0.561	NS	
		c) 2 students are pre-class friends and are males	1.1611	0.537	NS	
171	24 (♀/♂ ratio = 2)	a) 2 students are pre-class friends and are of different gender	0.0520	0.049	< 1e-04 ***	4
		b) 2 students are pre-class friends and are females	0.8876	0.470	NS	
		c) 2 students are pre-class friends and are males	0.7082	0.414	NS	
181	20 (♀/♂ ratio = 1.86)	a) 2 students are pre-class friends and are of different gender	0.0446	0.042	<1e-04 ***	2
		b) 2 students are pre-class friends and are females	1.2014	0.545	NS	
		c) 2 students are pre-class friends and are males	0.7802	0.438	NS	
Structured Lab sections - Formal Group formation						
12	21 (♀/♂ ratio = 0.91)	a) 2 students are pre-class friends and are of different gender	0.0413	0.039	NS	2
		b)2 students are pre-class friends and are females	0.9855	0.496	NS	

		c)2 students are pre-class friends and are males	0.7632	0.432	NS	
22	20 (♀/♂ ratio = 3)	a) 2 students are pre-class friends and are of different gender	0.0363	0.035	<1e-04 ***	2
		b)2 students are pre-class friends and are females	1.1753	0.540	NS	
		c)2 students are pre-class friends and are males	3.1164	0.757	0.0183 *	
32	22 (♀/♂ ratio = 2.14)	a) 2 students are pre-class friends and are of different gender	0.0746	0.069	< 1e-04 ***	4
		b)2 students are pre-class friends and are females	0.7407	0.425	NS	
		c)2 students are pre-class friends and are males	0.9126	0.477	NS	
42	23 (♀/♂ ratio = 2.14)	a) 2 students are pre-class friends and are of different gender	0.0483	0.046	< 1e-04 ***	2
		b)2 students are pre-class friends and are females	0.9067	0.475	NS	
		c)2 students are pre-class friends and are males	0.9031	0.474	NS	
62	20	a) 2 students are pre-class friends	0.0525	0.049	<1e-04 ***	2

	(♀/♂ ratio = 1.71)	and are of different gender b)2 students are pre-class friends and are females c)2 students are pre-class friends and are males	 1.1262 0.8423	 0.529 0.457	 NS NS	
92	22 (♀/♂ ratio = 2.14)	a) 2 students are pre-class friends and are of different gender b)2 students are pre-class friends and are females c)2 students are pre-class friends and are males	0.0431 1.3179 1.3854	0.041 0.568 0.580	< 1e-04 *** NS NS	4
102	19 (♀/♂ ratio = 1.71)	a) 2 students are pre-class friends and are of different gender b)2 students are pre-class friends and are females c)2 students are pre-class friends and are males	0.0333 1.3395 1.2794	0.032 0.572 0.561	<1e-04 *** NS NS	2
122	22 (♀/♂ ratio = 3.2)	a) 2 students are pre-class friends and are of different gender b)2 students are pre-class friends and are females	0.0383 1.4997	0.036 0.599	< 1e-04 *** NS	3

		c)2 students are pre-class friends and are males	1.3660	0.577	NS	
132	18 (♀/♂ ratio = 5)	a) 2 students are pre-class friends and are of different gender	0.0681	0.063	<1e-04 ***	2
		b)2 students are pre-class friends and are females	0.9084	0.476	NS	
		c)2 students are pre-class friends and are males	1.7507	0.636	NS	
142	24 (♀/♂ ratio = 1.67)	a) 2 students are pre-class friends and are of different gender	0.0317	0.030	<1e-04 ***	3
		b)2 students are pre-class friends and are females	1.9101	0.656	0.0334 *	
		c)2 students are pre-class friends and are males	2.3857	0.704	0.0180 *	
152	23 (♀/♂ ratio = 1.88)	a) 2 students are pre-class friends and are of different gender	0.0291	0.028	< 1e-04 ***	2
		b)2 students are pre-class friends and are females	1.7971	0.642	0.0378 *	
		c)2 students are pre-class friends and are males	2.1571	0.683	NS	
182	21	a) 2 students are pre-class friends	0.0176	0.017	<1e-04 ***	3

	(♀/♂ ratio = 1.63)	and are of different gender b)2 students are pre-class friends and are females c)2 students are pre-class friends and are males	5.6802 2.5395	0.850 0.717	<1e-04 *** 0.0204 *	

Table S4.4: Qualitative analysis of the lab sections for homophily observed with respect to ethnicity in week 5 when permanent groups are formed

Labs ID	Total number of URM students	Total number of Asian students	Number of groups with URM students working together with no pre-class friend status (at least two students in a group)	Number of groups with Asian students working together with no pre-class friend status (at least two students in a group)
Unstructured lab sections				
11	4	1	1	0
21	7	3	2	0
31	5	2	1	0
41	2	3	0	1
61	5	5	1	1
91	6	2	1	1
101	6	4	0	0
121	5	3	0	0
131	4	2	1	0
141	5	2	0	0
151	1	3	0	0
171	4	7	1	2

181	4	3	0	0
Structured lab sections				
12	3	4	0	0
22	3	3	1	1
32	0	3	0	0
42	1	4	0	0
62	3	7	0	1
92	6	2	0	0
102	2	5	0	0
122	8	3	2	0
132	1	7	0	1
142	6	2	1	0
152	4	4	1	0
172	4	3	1	0
182	3	4	0	0

Table S4.5 – Descriptive statistics for satisfaction, discussion, conflict scores and final grade in structured and unstructured labs

Lab	**Week 5**			**Week 6**			**Week 7**		
	Mean	SD	Median	Mean	SD	Median	Mean	SD	Median
(a) Satisfaction score									
Structured	4.28	1.06	5	4.38	0.94	5	4.44 ✣	0.93	5
Unstructured	4.31	1.04	5	4.38	0.91	5	4.49	0.77	5
(b) Task conflict score									

Structured	1.74 ✣ ✣	0.79	2	1.72 ✣ ✣	0.78	2	1.45	0.69	1
Unstructured	1.73 ✣ ✣	0.78	2	1.78 ✣ ✣	0.87	2	**1.56** ***	0.77	1
(c) Relationship conflict score									
Structured	1.14	0.49	1	1.21	0.56	1	1.2	0.58	1
Unstructured	1.12	0.41	1	1.23 ✤	0.59	1	1.19	0.59	1
(d) Process conflict score									
Structured	1.40	0.68	1	1.41	0.65	1	1.28	0.56	1
Unstructured	1.38	0.66	1	1.54	0.83	1	1.42	0.81	1
(e) Discussion score									
Structured	1.79	1.23	1	1.84	1.22	1	1.75	1.25	1
Unstructured	1.67	1.23	1	1.85	1.28	1	1.85	1.35	1

*** Wilcoxon rank sum test with continuity correction W=241976, p =0.005 difference between structured and unstructured labs in week 7.

✣ Kruskal-Wallis chi-squared = 12.469, df = 2, p-value = 0.001961 with multiple comparisons with kruskalmc test. Diff = TRUE (for p=0.05)between week 5 and week 7

✣ ✣ Structured labs - Kruskal-Wallis chi-squared = 82.536, df = 2, p-value < 2.2e-16 with multiple comparisons with the kruskalmc test. Diff = TRUE (for p=0.05)between week 5 and week 7 and week 6 and week 7. Unstructured labs- Kruskal-Wallis chi-squared = 40.669, df = 2, p-value = 1.475e-09 with multiple comparisons with the

kruskalmc test. Diff = TRUE (for p=0.05) between week 5 and week 7 and week 6 and week 7.

✤ Kruskal-Wallis chi-squared = 20.481, df = 2, p-value = 3.569e-05 with multiple comparisons with kruskalmc test. Diff = TRUE (for p=0.05) between week 5 and week 6.

Table S4.5 (continued) - Mean, SD and median values for satisfaction, discussion, conflict scores, communication frequencies and final grade in structured and unstructured labs

(f) Final grades

Lab	**Group grades**			**Individual grades**		
	Mean	SD	Median	Mean	SD	Median
Structured	0.94	0.03	0.95	0.94	0.05	0.95
Unstructured	0.90	0.10	0.93	0.90	0.11	0.92

Table S4.6 – Descriptive statistics for communication frequencies in structured and unstructured labs over the period of 7 weeks

Weeks	**Structured Labs**			**Unstructured labs**		
	Mean	SD	Median	Mean	SD	Median
Week 1	9.88	1.33	10	9.77	1.42	10
Week 2	9.79	1.30	10	9.98 ✤	1.28	10
Week 3	10.03	1.24	10	10.02	1.32	10
Week 4	10.07	1.25	11	10.30 *	1.11	11
Week 5	10.38	1.12	11	10.36	1.09	11
Week 6	10.40	1.20	11	10.40	0.99	11
Week 7	10.41	1.12	11	10.42	0.94	11

✤ Wilcoxon rank sum test with continuity correction ,W = 31359, p-value = 0.04414

* Wilcoxon rank sum test with continuity correction , W = 27366, p-value = 0.01477

Shaded part of the table :

Structured labs - Kruskal-Wallis chi-squared = 100.87, df = 6, p-value < 2.2e-16 with multiple comparisons with the kruskalmc test. Diff = TRUE (for p=0.05) between week 4 and weeks 5,6, 7.

Unstructured labs - Kruskal-Wallis chi-squared = 70.35, df = 6, p-value = 3.466e-13 with multiple comparisons with the kruskalmc test. Diff = TRUE (for p=0.05) between week 3 and weeks 4,5,6, 7.

Table S4.7 – Descriptive statistics for shared workload in structured and unstructured labs over the period of first four weeks

Weeks	**Structured Labs**			**Unstructured labs**		
	Mean	SD	Median	Mean	SD	Median
Week 1	4.61	0.46	4.66	4.62	0.44	4.67
Week 2	4.57	0.58	4.67	4.63	0.57	5.00
Week 3	4.60	0.55	4.67	4.66	0.47	5.00
Week 4	4.61	0.59	5.00	4.72 * ✤	0.45	5.00

* Wilcoxon rank sum test with continuity correction W = 36236, p-value = 0.04552

✤ Unstructured labs- Kruskal-Wallis chi-squared = 11.511, df = 3, p-value = 0.009261 with multiple comparisons with the kruskalmc test. Diff = TRUE (for p=0.05) between week 1 and week 4.

CHAPTER 5

CONCLUSION AND FUTURE DIRECTIONS

Technology development and research impact

The major goal of this dissertation was the establishment and development of tools and reagents to increase the repertoire of the molecular toolbox for genetic and functional genomic studies in *Anolis sagrei.* Consistent with this goal I have developed an immortalized *Anolis sagrei* embryonic fibroblast cell line. This cell line grows robustly at 29^0 C and 5% CO2 . I have also optimized a molecular assay to manipulate shh signaling in this cell line. I then optimized CRISPR/Cas9 gene editing in these cells and successfully mutated the *ift88* gene. I have provided evidence that this cell line can be used robustly to create targeted mutations with around 40% efficiency. We have optimized the immunostaining experimental conditions for working with this cell line and have provided evidence that commercially available antibodies for α acetylated tubulin and ift88 show species cross-reactivity and can specifically stain the lizard proteins. This was important because lizard-specific antibodies are not commercially available. Hence, for future cilia-related studies, we now have two working antibodies with optimized experimental conditions.

To explore reptilian biology questions, this cell line itself has inherent advantages of speed and control of a cell culture system. For reptilian biologists who wish to perform

genetic studies but cannot do so *in vivo*, this cell line will be an easily accessible and robust tool. The cell line grows at lower temperatures than mammalian cell lines. This opens an opportunity to explore biology at lower temperatures. This is particularly important for researchers studying the biology of thermo regulation in the context of climate change. The cell line was derived from a male (XY) embryo and hence provides an important genetic tool to address the research questions about the biology of sex determination and the Y chromosome in reptiles. The cell line can be a versatile tool for studying infectious diseases and developing diagnostics for wildlife and captive reptiles. This will be a useful reagent for veterinary biomedical science research. For biomedical research, studying evolutionarily conserved pathways and gene functions in different species is crucial. Our cell line is a novel tool representing reptilian biology. We believe this tool will be used by evolutionary geneticists, developmental biologists, and molecular biologists for understanding human health questions from an evolutionary perspective.

We also established an *in ovo* experimental system to manipulate the shh signaling pathway using pharmaceutical compounds. We optimized a method for the uptake of chemical compounds by capillary action in *A.sagrei* eggs. Our method generated reproducible results in terms of digit morphology in response to shh pathway manipulations. The robustness, ease, and cost-effectiveness of this method provide a useful tool to effectively study the impact of environmental pollutants, and pharmaceutical chemicals on signaling pathways in reptiles using anoles as a model system in laboratory settings. This is important as this method provides a simple tool to understand ecotoxicological impacts on the embryological development of reptiles. The generated data

in turn can be used to strengthen the efforts for the conservation of reptiles in the human-altered ecological landscape.

Rasys et al.(2019) developed a method to perform gene editing in *Anolis sagrei* by microinjecting unfertilized oocytes. They used the CRISPR/Cas9 system for gene editing. To increase our repertoire of gene editing tools for genetic and functionality experiments in *Anolis sagrei* we wanted to further optimize the method they developed as well as try other CRISPR systems. The use of the Cas12a enzyme offers an opportunity to specifically target AT-rich regions increasing the flexibility of gene editing. Hence, we used and optimized the CRISPR/Cas12a system to create targeted mutations in two highly conserved enhancers ZRS and HLEB known to be crucial for limb development (Infante et al., 2018). I successfully generated mutants with indels in the targeted region in both cases using the Cas12a system. I also used the Cas9 system to create targeted mutations in the same region of ZRS and HLEB to understand the working gene editing efficiency of both Cas9 and Cas12a systems in lizards. We found that Cas12a gene editing efficacy is lower than Cas9 system. Optimizing and using the Cas12a system was important because it provided a flexible tool to create targeted mutations in the loci which are not easily accessible with the Cas9 system. Cas12a creates a staggered cut with an overhang of 5 nucleotides at the target strand (Zetsche et al., 2015). We can thus use Cas12a with a homology-directed repair (HDR) donor to create more precise targeted knock-out or knock-in mutations in anoles.

In lizards, tail regeneration is regulated by Ihh (Lozito & Tuan, 2016). The technological tools we have developed will further open an avenue to study tail regeneration and the role of Ihh in skeletal development. Targeted mutation in the ZRS

region in Newts indicated potentially different regulation of shh via ZRS in embryonic development and regeneration (Suzuki et al., 2018). In Xenopus, the hypomethylated state of ZRS in tadpoles is associated with the complete regenerative ability. In adults, however, the loss of regenerative ability is associated with the hypermethylated state of ZRS. The data is indicative of epigenetic regulation of shh expression or repression during limb patterning and regeneration (Yakushiji et al., 2007). As anoles can regenerate their tail, they are a good model system to study the biology of tissue regeneration in the context of epigenetic regulation of hedgehog signaling.

Sonic Hedgehog signaling in context to reptilian biology

Major findings - Conserved targets of shh signaling in reptiles and mammals and role of primary cilia

To study the conservation of the shh signaling targets in developing limbs of reptiles and mammals which diverged from each other ~325 mya, I used primary embryonic fibroblast cells from the limb buds. Being primary embryonic cells, biologically they were more representative of a limb bud than an immortalized cell line. I induced the shh signaling pathway using a pharmaceutical compound (SAG) to understand how many target genes of shh signaling pathways are shared between reptiles and mammals. There were a total of 133 shared upregulated genes and 20 downregulated genes between squamates and mammals. Among the upregulated DE genes, *gli2* was not upregulated in *A.sagrei* samples. The other known direct targets of the pathway *gli1*, *ptch1* and *ptch2* were upregulated and *Hip*1 was downregulated in both species. *Gas*1 and *Boc* were downregulated only in mice.

We induced the shh signaling pathway in the ASEC-1 cell line with SAG and performed RNAseq analysis to understand DE genes between SAG-treated and untreated cell lines. This analysis also provided evidence that *gli*2 was not differentially expressed as a response to Shh pathway induction.

The *ift88* mutant cell lines with no primary cilia (#28 and #30), were unresponsive to the SAG treatment (quantified by lack of relative *gli*1 expression). However, the basal *gli*1 expression in these cell lines is elevated as compared to WT clones with primary cilia indicating derepression of the shh signaling pathway. This depression is similar to what is observed in zebrafish lacking cilia that display strong ligand independent Hh derepression that cannot be further increased by Hh ligands (Huang & Schier, 2009). The derepression of the pathway observed in zebrafish is through the reduction in Gli repressor activity as a result of loss of cilia and ligand-independent activation *gli*1. Whereas in mammals *gli*1 and *gli*2 both are the activators of the pathway and *gli*3 has the repressor role. However, *gli*2 is the predominant activator of the pathway (Bai et al., 2002; Ding et al., 1998; Hui & Joyner, 1993; Matise et al., 1998; Mo et al., 1997; Wang et al., 2019). Genetic evidence in mouse embryos lacking cilia shows a pronounced loss of ligand-induced Hh pathway activation and mild ligand-independent (derepressed) activity (Chen et al., 2009; Jia et al., 2009). In these mutants, cilia dependent *gli*2 activation is lost which in turn reduces the activation of *gli*1 drastically. Hence the derepression observed in mice is through the loss of Gli activator function.

Our RNAseq data from primary limb cells and ASEC-1 shows that in reptiles *gli*1 is upregulated as a response to activation of the shh pathway and *gli*2 is not induced at all. Along with the data from *ift88* mutants showing de-repression of the pathway as a result

of loss of cilia, suggests the possibility that the role of *gli2* as a major activator of the shh signaling pathway is specific to mammalian lineage.

Future directions- To understand the role of the primary cilium and shh signaling in squamates

In vertebrates, primary cilia act as a specialized center for receiving the Hh signal and processing of Gli proteins. The ratio of Gli activators and Gli repressors decides the cell's graded response to the Hh signal. Hence, in vertebrates, primary cilia mediate Hh signaling by modulating Gli protein activities (Falkenstein & Vokes, 2014). Within the vertebrate lineage, the role of Gli proteins has diverged. In mice, *Gli*2 is a major activator of the pathway, and activation of *Gli*2 in mice depends on cilia (Eggenschwiler & Anderson, 2007; Santos & Reiter, 2014 ,Bai et al., 2002; Ding et al., 1998; Mo et al., 1997). In zebrafish, *gli*1 is the major activator of the pathway and *gli*2 can act as a minor activator or repressor (Huang & Schier, 2009; Tyurina et al., 2005). In chicken *GLI2* and *GLI3* can act as both activators and repressors (Yt et al., 2016). Our data indicates in squamates *gli*1 possibly acts as a major activator of the pathway.

To provide genetic evidence, we propose to systematically create targeted mutations in *gli*1, *gli*2, and *gli*3 in anoles using the ASEC-1 cell line. In support of our hypothesis and data from mice and zebrafish, we expect *gli*1 -/- mutant cells will not respond to shh or SAG stimulus which can be recorded as a change in *ptch*1 expression (Karlstrom et al., 2003; Lipinski et al., 2008). We also would like to perform RNAseq analysis on at least 2 *ift88* null mutant cell lines and 2 WT clones to understand DE genes in these cell lines in response to the lack of primary cilium. The primary cilium is known to be required for shh signaling and is proposed to be required for Wnt signaling and

PDGFRA signaling (Goetz & Anderson, 2010). Studying DE genes in WT cells and *ift88* null mutants which lack primary cilia will provide insight into direct and indirect targets of shh signaling along with the potential role of cilia in regulating other developmental signaling pathways. This can be useful to generate hypotheses which then can be functionally tested using the ASEC-1 cell line. To further explore and understand the role of Gli proteins, we propose to create *gli*1, *gli*2, and *gli*3 null mutant lizards and study embryonic neural patterning and limb development.

Future directions- To study limblessness and ZRS

Our data indicated that we successfully deleted regions of the limb specific shh enhancer ZRS. However phenotypic analysis is yet to be done. We propose to sanger sequence a larger PCR amplicon in ZRS #13 to understand the exact nature of the mutation which led to the severe phenotype. This would shed light on the discrepancies between the genotype and phenotype of mutants 52 and 13.

Studies in mice discovered that the functional activity of ZRS comprises two domains: one domain predominantly acts over short range and the other promotes long range chromosomal conformation changes associated with the gene activity. The enhancer composition identified multiple HoxD and ETS factors binding sites. The occupancy of these sites together defines and regulates shh expression in the ZPA region (Lettice et al., 2014). The sequence analysis of limbed vertebrates and limbless vertebrates narrowed down a 17bp snake-specific deletion in ZRS region (Kvon et al., 2016; Leal & Cohn, 2016). This 17 bp deletion overlaps with the ETS1 transcription factor binding site (Kvon et al., 2016). We tried to create targeted mutations in this ETS1 binding site as our approach with Cas12a and Cas9 did not generate any mutants with the deletion of this specific site, we

propose to use an HDR approach in the future to create a precise mutation changing the ETS1 binding site motif.

The loss of this particular E1 binding motif associated with the ETS1 factor in mice was not sufficient to alter the limb bud expression (Lettice et al.,2012). In boa, which is a basal snake, ZRS lacks both E0 and E1 ETS1 binding sites. However, transgenic mice experiments indicated boa ZRS was active in the limb buds. This suggests that the lack of these ETS1 factor binding sites is probably not the only reason for the evolution of limblessness(Kvon et al., 2016). A recent study of limbless glass lizard showed that the relevant mutations in ZRS are absent from this species suggesting a different evolutionary pathway for limbless ness (Roscito et al., 2022). The loss of HoxD13 binding sites in the ZRS region of python is implicated for the weak ZRS activity in python limbs(Leal & Cohn, 2016).

To understand limblessness in snakes we propose to first provide evidence of direct binding of ETS1 factors and HoxD13 factors in developing limbs of anoles by Chip Seq analysis. We then propose to create mutations in the ETS1 binding site using the CRISPR/Cas12a system. Depending upon the genotype of the mutations and severity of the phenotype, we propose to establish an ETS1 mutant lizard line. We then propose to use these mutants to create mutations in the HoxD13 binding site in anole ZRS. Taken together the double mutant for ETS1 and HoxD13 binding sites, may provide a functional test to understand potential evolutionary pathways for limblessness in snakes.

Group formation and identification of persistently poorly rated (PPR) students in an undergraduate introductory biology laboratory class

Major findings -Our data from studying group formation and conflicts in BIOL 1108L, suggests that there is not an inherent advantage between allowing students to self-select the groups from day 1 and enforcing social interaction through randomization of the students before forming the formal groups in context to group grades, individual performance, group satisfaction, and group conflicts. We found that even though students had a chance to work with everyone in the class, they preferred to form a formal group with their pre-class friends. This data is in accordance with the reports from Theobald E., et al (2017) and Premo J. et al (2022) where comfort and willingness to work with other students are associated with familiarity.

Influenced by the recommendations for the instructors by Davies W. M. (2009) for implementing group work in a classroom, we created 'group selection factors' by asking students their perception and definition of good and bad group members. Asking students their definition of good and poor group members might help with clear expectations about group work from fellow peers. This can also work as a guideline for the instructor to observe, identify and mediate conflicts in real time. The top 3 responses we report were behavioral traits associated with interest and engagement. Our data suggested that students mention behavioral characteristics as most critical in defining good and poor group members in the early phase of group formation.

We report that these 'group selection factors' worked as a more efficient tool than conflict items we used by Jehn and Mannix (2001) to identify persistently poorly rated (PPR) or problematic students in the early phase of group formation. Similar to Brickman

and colleagues (2021) we report that Likert survey items are not as effective as simple categorical 'Yes/No' and 'my fellow group member was not performing well and why' questions.

When students were allowed to choose their own groups from day 1, they stayed with a poor group member they identified rather than confront the issue and change groups. While in laboratory sections where we enforced interactions prior to the group formation students identified and avoided working with a PPR student. It is possible that the process of randomization helped students to avoid the social pressure that is associated with refusing to work with another student during the early phases of group formation.

Recommendation for instructors -From the instructor's point of view, we report enforcing interactions between the students before the formation of the formal group helped with knowledge spillover and cohesiveness of the whole classroom. This finding is similar to the reported finding by Rienties B and colleagues (2014). The probability of bad behavior being discovered in a more connected student network is higher which also generates a reputation cost for it (reviewed in Burt R.S, Kilduff M and Tasselli S,2013). We report the group selection factors we generated as a part of this study can be used as a guideline for the instructor to observe, identify and mediate poor group member behavior in real time. We recommend to the instructors that enforcing interactions prior to the group formation can be used as a strategy to keep free riders in check, providing students a choice to avoid working with a poor group member as well as promote a more cohesive classroom learning environment.

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Appendices

Appendix A – Survey questions for lab 1-7 (Chapter 4)

Lab 1

Thank you for taking this survey. It will help us improve the experience of BIOL1108 lab students by uncovering factors students use to create and manage in-class groups. There are no right or wrong answers for this. We will ask you similar questions a few times this summer. These data are incredibly valuable, so we truly appreciate your answers. Please know that your responses to the questions in this else before the end of the class. someone in this class that you would consider a friend now but not before this class, do not select them as a pre-class friend.

1. Please select the students with whom you worked in lab today. {List just contains the students in that lab section}

2. {Populated question with the answers from question 1} For each student that you worked with today, select those that you consider a pre-class friend: A student that you would consider a friend from BEFORE the term of this class. If you have met survey are confidential. All names will be re-coded for analysis. This information will never be used for any class purpose, grading purpose, or anything

3. {Populated question with the answers from question 1} For each of the students that you've worked with today, identify which you believe to be smart students: those that you believe are good at understanding the class material.

4. {Populated question with the answers from question 1} For each student that you worked with in lab today, rank how well you felt they shared the workload as a group member: shared workload includes discussing ideas, using equipment, recording data, presenting your group's ideas, asking relevant questions, etc.

	very poor	poor	moderate	good	very good
(Group Member 1)	o	o	o	o	o
(Group Member 2)	o	o	o	o	o
(Group Member 3)	o	o	o	o	o

5. Indicate the frequency or communication between members of your group in lab today.

-5 indicates extremely rare communication (several members did not talk at all)

0 indicates average level of communication

+5 indicates very frequent communication (all students communicated throughout lab)

6. Please provide at least two traits you used (or would use) to identify someone as a good group member:

7. Please provide at least two traits you used (or would use) to identify someone as a poor group member:

8. For each student that you worked with today indicate whom (if any) you would like to work with again. Why/ Why not.

9. What factored into your decision for the last question? {Populated with individual students}

a. Positive Options:

Well prepared for class & knows material well in advance,

Pays Attention

Participates in discussion and offers meaningful suggestions

b. Negative Options:

Does not come to class prepared or does not know material in advance

Does not seem interested in the class

Does not participate in the discussions / listen to others

{Demographics Questions}

In order to understand the different characteristics that you use to form groups, please provide some details about yourself in the questions below.

1. Please indicate your gender

o Male

o Female

o other _____

o prefer not to respond

2. With which race(s) and ethnicity/ies do you most closely identify? Please choose all that apply.

o African American or Black

o American Indian or Alaskan Native

o Asian Indian

o Chinese

o Filipino

o Japanese

o Korean

o Latina / Latino or Hispanic

o Native Hawaiian or Other Pacific Islander

o Vietnamese

o White

o Other - please

explain: _____

o Prefer not to respond

3. Please indicate your class standing in college.

o freshman (0-30 hours)

o sophomore (31-60 hours)

o junior (61-90 hours)

o senior (above 90 hours)

4. Please indicate your current cumulative college GPA (e.g 3.87 or 3.25).

_____

Labs 2-4

Thank you for taking this survey. It will help us improve the experience of BIOL1108 lab students by uncovering factors students use to create and manage in-class groups. There are no right or wrong answers for this. We will ask you similar questions a few times this summer. These data are incredibly valuable, so we truly appreciate your answers. Please know that your responses to the questions in this else before the end of the class. someone in this class that you would consider a friend now but not before this class, do not select them as a pre-class friend.

1. Please select the students with whom you worked in lab today. {List just contains the students in that lab section}

2. {Populated question with the answers from question 1} For each of the students that you've worked with today, identify which you believe to be smart students: those that you believe are good at understanding the class material.

3. {Populated question with the answers from question 1} For each student that you worked with in lab today, rank how well you felt they shared the workload as a group member: shared workload includes discussing ideas, using equipment, recording data, presenting your group's ideas, asking relevant questions, etc.

	very poor	poor	moderate	good	very good

(Group Member 1)	o	o	o	o	o
(Group Member 2)	o	o	o	o	o
(Group Member 3)	o	o	o	o	o

1. Indicate the frequency or communication between members of your group in lab today.

-5 indicates extremely rare communication (several members did not talk at all)

0 indicates average level of communication

+5 indicates very frequent communication (all students communicated throughout lab)

2. Please provide at least two traits you used (or would use) to identify someone as a good group member:

3. Please provide at least two traits you used (or would use) to identify someone as a poor group member:

4. For each student that you worked with today indicate whom (if any) you would like to work with again. Why/ Why not.

5. What factored in to your decision for the last question? {Populated with individual students}

a. Positive Options:

Well prepared for class & knows material well in advance,

Pays Attention

Participates in discussion and offers meaningful suggestions

b. Negative Options:

does not come to class prepared or does not know material in advance

does not seem interested in the class

Does not participate in the discussions / listen to others

Labs 5-7

1. Select the members of the group that you will be working with for the next few lab sessions: {List just contains the students in that lab section}

2. {Populated question with the answers from question 1} Please indicate which of these students was a pre-class friend.

3. In general, how would you rate your previous experience working in a group?

a. Extremely bad

b. Bad

c. Neutral

d. Good

e. Extremely Good

4. How do you feel your group worked today?

Satisfaction Items

5. I am satisfied with my present teammates

   Strongly agree, agree, neutral, disagree, strongly disagree

6. I am pleased with the way my teammates and I worked together today.

   Strongly agree, agree, neutral, disagree, strongly disagree

7. I am very satisfied working with this team.

   Strongly agree, agree, neutral, disagree, strongly disagree

8. What is your biggest concern working in a group? Please explain.

Conflict Items

Task Conflict

9. How much conflict of ideas is there in your work group?

None/Not at all, Little/Rarely, Some, Much/Often, Very Much/Very Often

10. How often do people in your work group have conflicting opinions about the project you are working on?

None/Not at all, Little/Rarely, Some, Much/Often, Very Much/Very Often

11. How often are there disagreements about who should do what in your work group?

None/Not at all, Little/Rarely, Some, Much/Often, Very Much/Very Often

Relationship Conflict

12. How much relationship tension is there in your work group?

None/Not at all, Little/Rarely, Some, Much/Often, Very Much/Very Often

13. How often do people get angry while working in your group?

None/Not at all, Little/Rarely, Some, Much/Often, Very Much/Very Often

14. How much emotional conflict is there in your work group?

None/Not at all, Little/Rarely, Some, Much/Often, Very Much/Very Often

Process Conflict

15. How frequently do you have disagreements within your work group about the task of the project you are working on?

None/Not at all, Little/Rarely, Some, Much/Often, Very Much/Very Often

Discussion of ideas

16. How often do you have open discussion about these issues in your group?

None/Not at all, Little/Rarely, Some, Much/Often, Very Much/Very Often

Appendix B -GLA interview questions

Q1) How was your overall experience during the semester? Do you have any comments or suggestions?

Q2) What were the problems you encountered?

Q3) Which sections had more interactions according to you? DO you have any idea why there might be a difference?

Q4) What is your opinion about group work? Do you think students should self assemble, or do you want to assign groups to the students?

Q5) What problems have you anticipated/experienced in group work as an instructor?

Q6) Did you observe any conflicts within the groups? Did students report the conflicts?

If Yes: How did you deal with it?

If not: In a hypothetical situation, how will you deal with it?

Q7) According to you, how did you promote / facilitate group discussions? Did it help students?

Q8) In your research lab, how do you deal with issues that come up when you and your lab mate disagree about ideas/direction of a project?

Q9) In your research lab, how do you deal with issues that come up with personalities of your lab mates?

Q10) How do you think your mentor finds about or solves the disagreement between your lab mates?

Q12) How does your research background help you to facilitate group discussions in the inquiry-based labs?

Q13) Do you have any other comments or suggestions about the study?