Retina Architecture: A Story of Networking Success and Failure

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Authorization to Submit Dissertation

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Abstract

Neural circuitry is a fascinating system that animals possess, giving rise to our unique capacity to react to changes in our environment. It is my passion to describe and explain this wonderful capacity to anyone who wishes to learn some aspect of the nervous system.

There are three stages during the development of the nervous system. First, a precise regulation of the birth and death of neuron progenitor cells initiates neural tissue development. Second, a specific differentiation and migration pattern of those surviving progenitor cells in our tissue forms the basis of neuronal architecture that will support normal physiological function. Finally, in a process unique to neurons, they establish, eliminate and maintain appropriate neurite contacts over time in order to comprehend information-flow in complex neural networks.

Genetics is the essence of developmental processes. Some genes reveal multiple regulatory roles and make them significant targets because of their potential clinical value. Down syndrome cell adhesion molecule (Dscam), one of the 33 genes associated with the Down syndrome critical region, contributes to neural cell death, cell spacing and neurite lamination. These roles make *Dscam* an ideal candidate for understanding the complex process of neural developmental.

In my studies, we developed and utilized the first ever gain-of-function *Dscam* allele in the mouse model and assayed its role in all aspects to complement existing loss-of-function studies. We also developed software to efficiently quantify neurite patterning. Our results indicated that *Dscam* is sufficient to drive cell death and is necessary for precise

lamination. We further demonstrated that *Dscam* and *Bax* are two faces of a double-safe mechanism in the neural circuitry to redundantly regulate cell populations and restrict their neurite targeting specificity. This type of functional redundancy protects us from severe neural developmental defects caused by genetic mutations, and also provides alternative insight into the treatment of genetic conditions.

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Dedication

To my parents Li Nian Nian, Li Shang Li, my host families: The Reyes, Neals, Tennesens and my fiancée Christine Schmidt.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

AC: Amacrine Cell

BPC: Bipolar Cells

DS: Down Syndrome

DSCR: Down Syndrome Critical Region

DSCAM: Down Syndrome Cell adhesion Molecule

HC: Horizontal Cell

INL: Inner Nuclear Layer

IPL: Inner Plexiform Layer

NNA/NNRA: Nearest Neighbor Analysis

OLM: Outer Limiting Membrane

ONL: Outer Nuclear Layer

OPL: Outer Plexiform Layer

RGCs: Retina Ganglion Cells

VD/CoV:Voronoi Domain Analysis

CHAPTER 1: Who We Are

Who are we?

We are the shapes seen in the mirror

We are the emotion nothing comes near

We are all mighty and clever, gone to space more than endeavor

We are all unique and weird, only birth and death are no different

Finally we know what we are, we are what we know

The Wonder

In the middle of a summer night, countless stars are scattered through space, and the vast universe makes its majestic creations on an abundant and magnificent scale. Our limited view cannot even comprehend all the information that exists within our perception. Our limited attention span forbids us from focusing on more than a few objects at a time. The rest of the information is simply lost in transition, compressed into a small fraction of a blurry memory.

Memories are everything to us, but on the massive scale of the universe, human memories become insignificant. Every great event that ever existed in our history is only a brief moment on earth. Earth is just a small planet in the Milky Way. Outside the Milky Way there are one hundred billion galaxies, each with more than one hundred billion stars and their planets revolving with one another. The magnitude of insignificance for who we are becomes a horrifying thought to our very egocentric nature, but it's a good reminder to use our time wisely. Perhaps we can seek the meaning of our lives on our little planet.

Most of human history is filled with wars and superstitions; science was not widely accepted until recent centuries. But, long before the concept of science existed, humans were already exploring the planet due to the pressure of survival and the curiosity and the wonder that lived within us. Those traits raise an extraordinary ability: we can use our complex brain to figure things out.

I used to have a deep wonder about the world. I loved astronomy and the mysterious darkness far away from the surface of the earth. I learned zodiacs and lunar years when I was a kid, and heard stories about ancient Greeks and Egyptians explaining the sky and gods, future and past. The nature of wonder, for me, was all about the outside world, before computers and internet dominated our views. People used to look up, really look up, into the skies for its beauty, not search on google for its images instead.

Looking up into the sky and observing the stars are perhaps the earliest scientific observation by mankind. This ability to recognize lights in the dark space provokes us to think about ourselves, our lives, and our stories. Different cultures made up different stories from the stars, deeply reflecting our thoughts on seeking who we are and where we come from. Those stories were linked with objects and events in people's lives, and the movement of stars was used to guide seasons, determine celebrations, and calculate timing for agriculture. The sky used to be an essential part of our lives. It represents our curiosity, imagination and primitive wisdom.

Technological advancement in the 21st century dramatically changed how we observe the sky and the world, and what is essential for our lives. Now, we are able to see the universe in magnificent detail without ever looking up by using satellite telescope. We

are able to find the most advanced knowledge by simply searching on the internet. We can spend 99 dollars on a chip from *23 and me* to sequence our genes and know the imperfections we are carrying. The essence of our modern lives cannot live without technology; this trend of technological focus in value even shifted the outcome of our higher education. Payscale inc conducted research which indicated a person with a bachelor degree in engineering earns four times the salary, with an astonishing $160k a year at mid-career, than a person with a degree in elementary education, social work, or special education. Engineering and IT degrees dominate the top paid careers, while humanity related degrees (education, art, and social services) stay in the very bottom of the income ladder.

The trend of career specialization may increase individual's pay check, but it also accelerates the segregation of society between career groups and creates a more severe income gap, and, consequently, a more polarized society with polarized talents. This is unhealthy for our society and culture as whole. Our sense of wonder is deeply bound with our instinct and is used to explore the universe we know of. That's why different talents are equally valuable and should command similar respect.

The first sense that enables us to explore the world and fulfill our wonder is the ability to see. It's interesting to think about how long it took us to understand this ability. Vision in short is an accumulation of many advantageous coincidences built upon one another through natural selection. Million years were used to cultivate this ability from photosensitivity to trichromatic vision. The outcome of vision is image; it is an accurate yet vague perception made of complex electrochemical and biochemical signals in our brain. We now know many details of how this system works, yet many more mechanisms remain

unknown. However, one thing we do understand is the origin of the visual signal-light, the fundamental particle created by the universe.

The Universe

Stars are sources of lights visible for our eyes. This type of light is created when gravitational forces created by the mass of the matter within the star violently crushes elements together. We call this process fusion reaction. Whenever a new element is created by fusion, energy is released as magnetic radiation, each with its own signature of small fluctuations in their wave length. They travel at three hundred million meters per second in space, defining what we know as speed of light. Those energy particles are named photons.

Light generated from a distant star will only have a tiny fraction that can ever be observed by mankind. Photons have to travel through the vast space-time continuum, escape gravity from other stars, planets, and even black holes, eventually arrive at our planet, and present themselves as the night sky. We are the lucky ones who are capable of perceiving one of the most fascinating events in the universe.

With light and our eyes, we can recognize water flowing and fish swimming. We can appreciate the colors of the rainbow and clouds. We even kept fire so the world would be visible at night. While we enjoy the wonderful sensation and satisfaction of the colorful world, we should know that compared to the full spectrum of the wavelength, we can merely perceive a tiny fraction of its beauty.

Dandelions perhaps remind you a sunny hill top where everything is covered by the color of yellow and white. The dandelion absorbs UV light at their distal petals and reflects

it at proximal region, creates a landing site which is invisible to human eyes but extremely helpful to insects[1]. Humans have physical limitations in color vision. We also have limits on how much information can directly perceive at a given time. To combat those inabilities and satisfy our wonder, we invented methods and instruments to explore the world beyond our perception. In the recent centuries, we started to engage the realm of scientific understanding of the universe, which based on measurable empirical data. Let wonder inspire us and let the human spirit live on.

The Visual System

Evolution gives us a brief history of how cells of simple organisms adapted and specialized in their ability to detect light. This ability helps them orientate in three dimensional space and escape from harm. A study published in Nature suggested this evolution of eyes happened somewhere during the Cambrian explosion period around 524 million years ago, and gave rise to the structure of complex eyes[2]. Mathematical calculation proved independent eye evolution is possible in as little as 364 thousand generations to transform from light sensitive cells into complex eyes[3]. With multiple independent adaptations to detecting light, some creatures are specialized to sense differences between specific wave lengths to get more information, and some can see a wider spectrum than others. Pigeons for example are hentachromatic, meaning they can see two sets of spectrums beyond human capacity[4].

Eyes detect the environment by trapping photons. When photon is trapped by a layer of neurons in our eyes, their unique electromagnetic energy will trigger a neuro-signal which relays to our brain and forms an image. We are only capable of visualizing photons that have

wave lengths from 380 to 750 nanometers. This range matches the colors of a rainbow. Black, however is not a color it's a perception for the lack of any visible color. Together, we have the spectrum that defines the beautiful world we see.

The biological structure of the eyes allows us to receive and process visual signals. To achieve this goal, eyes are composed of many different cells made up of different structures to protect and perfect our vision. The core of this detecting machinery is a structure named the **retina**. The word retina came from late 12th century Arabic *shabakiyyah* and later in 14th century translated into Latin refers to "a netlike layer". The retina in our eye is a system made of a massive layered neuron network. This complex network is responsible for collecting and processing information carried by photons.

Photon carries unique wave property and energy. It travels through space, escapes the gravity of other matters, by chance enters our cornea, and then is focused by our lens. Light passes through blood vessels and many layers of retina tissue before being absorbed by a group of specialized neurons called photoreceptors. Two types of photoreceptors working together give us the comprehensive images we perceive—rods, and cones. Cones are receptive to a very small spectrum of wavelengths, and consists several types. Each type of cone is sensitive to a narrow spectrum of wavelength—hence the color. Cones are more condensed in the center of human retina, a region known as Fovea. Rods are a group of receptors highly sensitive to a wide spectrum of light wavelength, and work well under dim light conditions. Rods form black and white images and the contrast of the image. They are spread throughout the retina and outnumbered cones by a factor of 20.

Once the visible wavelength is absorbed by photoreceptors, those cells can generate an electrical signal that passes down to the interneurons and converge onto retina ganglion cells (RGCs), eventually projecting into the visual cortex forming an image. The rest of the photons pass by photoreceptors uncaptured and are absorbed by a layer of pigment (RPE) in the very back of our eye. This is why everyone's pupil looks black in the center; because all light entering the eye is absorbed by biological structures.

A superficial description of the retina is that it's an organized structure made up of distinct layers. Each layer of the retina contains either a collective group of neuron cell bodies or their dendrites, axons, and the synapse connections between the two. The dendritic layers are like the internet used by neurons sending information to one another via electrical signals. Each group of cells contributes or responds to one particular function. For example, in the very bottom of the retina layer, one group of neurons is known as melanopsin sensitive retina ganglion cells. They are not photoreceptors, but they can sense light with a unique photopigment—melanopsin. What's more amazing is the light they receive does not form any images in our brain; instead, these cells detect the presence of the light and determinate whether it's day or night and promote our circadian rhythms. In recent years, the protein melatonin, which the neuron produces, has been used to treat sleep disorders.

The mechanism that stops the light in photoreceptor are protein compounds called opsins (melanopsin is just a member of that family[5].) Opsins are capable of transforming their molecular shapes once struck by photons. This transformation will stop cation channel activity through many complex steps, and put photoreceptors into a state called hyperpolarization. This state will change the rate of neurotransmitter released from

photoreceptors to the downstream interneurons, create a biological reaction in the cell, hence a signal is generated.

Interneurons are responsible for integration the specific information the photon carries, including intensity, brightness, contrast and direction. More complex information like movement and depth of objects are calculated by binocular vision, linking objects in relation to time to predict their movement and depth. This type of information is possible to perceive because our eyes are separated by a certain distance and our brain is powerful enough to calculate the difference between two images from two eyes and give a raw estimate of how far away the object is. Patterns and many other aspects of the visual function are also modulated by interneurons.

At the very end of the retina system, all information converges onto retina ganglion cells. Their long axons are information highways for vision, extending from back of the eye to the back of the brain and into the visual cortex to generate images and present to us the surrounding environment. This complex system starts with 120 million rods and 6 million cones receiving and processing electromagnetic information, and ends up with 2.5 million ganglion cells transferring data to the brain, a conversion ratio of 100:1[6].

The Age of Research

In early ages, people studied retinas from large animals like cows. The size of the tissue was much easier to handle without precision tools, but before the microscope was invented we could not clearly observe many details. Today, the overall organization of retina

is well understood with advanced microscopes and morphological staining. However, the biological mechanisms involved with visual system are still in the early age of discovery.

Diseases and conditions impacting human vision are very common. Some of those conditions we have already resolved by simple technology like glasses and eye drops. For other conditions we do not have a solution, such as diseases that cause blindness. The World Health Organization (WHO) estimate 285 million people have impaired vision worldwide, 39 million are blind, and 82% of them are at age 50 and above. While we are enjoy the colorful world, keep in mind that 1 in every 34 people is not that lucky.

Scientists and researchers are working hard to understand the nature of those diseases. The end goal is to find solutions for diseases that damage human vision. In the age of research, we employee empirical data supported by measurement and double blinded tests to prove any drug or technology that may help us overcome this obstacle.

One fact about research is it's never perfect and will never be. A hundred years from today when people look back at our cutting edge technology, they will be astonished by how primitive, inaccurate and wasteful our advanced technologies are. At same time without those incremental improvements we are working on, it's unlikely we will achieve a new height.

In the 21st century we have the freedom to browse any knowledge we wish to learn. This accessibility means a new age of self-learning and unrestricted discoveries in combined disciplines based on individual's interest. I have never learned mechanics, but whenever my car has issues, the first thing I do is to find a video instruction on YouTube that matches the problem and try to fix it by myself. All I need is tools, time and the will to figure things out.

The physical limit to knowledge does not exist anymore; what restrict new generation from utilizing knowledge are the mindset and the will to search and research.

The age of research does not apply only to science. As big data become common and communication becomes much more sophisticated, research can rapidly change and improve social structures for developing countries by connecting their people to developed countries on social media. Research can improve art and culture by easy access to new artistic works and events on website. Research can reduce paper and packing waste by creating ecofriendly shipping method and manufactures. In this age, research represents what we care, what we do and **who we are**.

CHAPTER 2: What, How and Why

In my years of research, my curiosity covered a wide spectrum of research interests. As a consequence, I moved rapidly from project to project. I cultured yeast, bacteria, and mammalian cells, worked on molecular biology and genetic design, and finally transitioned into mouse work and retina neuron research. Rapid learning and doing things without knowing why is a common stage for young scientists, and I would like to share my experience with you, including how to minimized the time wasted on this stage with a method popularize by Simon Sinek, the most viewed TEDx speaker, Understand What, How and Why.

What

What we do defines partially who we are. Our occupation is a domesticated training last at least 16 years of education to fit every person into a box with a description of technical skills he/she possesses. In this strange world, career system helps us realize that the goal of life is simple: find a stable, well-paying job with great benefit that lasts a life time, be well off, then send your offspring to the same loop.

This cycle may seem like harmony, but the mentality and methodology we apply in general career path is lethal to innovative and scientific minds. With a 7.3 billion world population, only new innovations and discoveries can overcome many sustainability issues, like nature resources and energy. Doing what we do cannot deliver solutions to those obstacles, it’s simply not sustainable.

Many historical examples demonstrated the dramatic changes we adapt to when cycle of doing what we do breaks. Agriculture removed the needs of gathering and hunting, starting the settlement of our kind. A population that settled at the wrong location was permanently erased from the history. Language and writing reduced the needs for mouth to ear learning (mouth to ear learning was the reason why the classroom was created. There was no better way to teach knowledge other than storytelling in a room). The ones that failed to adapt this technology stalled in the past. The industrial revolution dramatically improved productivity at the cost of individual and environmental health. Countries too slow for this change were locked permanently in the low economic ladder.

Today with rapid technological advancement and increasing focus on machine learning, more jobs will be replaced by robots and machines while more people will still be born, creating a decline in workforce demand. What can we do to face this future? One solution is to innovate and do what we never done before.

How

How to achieve innovation to benefit our world is a hard question. The knowledge of "How To", in contrast, is rather confusing and challenging.

There is much faulty information spawning in our ears in this information age, serving its own specific goals. For example, once people learn that starting a business is hard, existing businesses can prevent competitions from new start-ups. Every company is hiring people with working experience, but where is the experience come from if no one gets hired fresh out of school? It's a tactic used by old men to prevent youngsters from taking over their jobs by telling them "it's hard and you don't know what you are doing." For the new

generation this situation can be confusing, however, the solution is simple (but not easy): go ahead apply the job you wanted. It doesn't matter that they need 10 years of experience and you just graduated last month. Prove your ability and ask them right questions so that you can know if a company is the right place for you. Your ability to handle things during interview, the fearlessness and confidence you show, and the ability to take risks will make you stand out from the crowed.

If innovation is not used as a good deed it can become a burden for our society. A good example is the infamous self-serving financial industry. How does the industry achieve this goal? It is done by making simple tasks extremely difficult and complex, such as inventing rules within rules for investment and management of funds, bonds, and stocks. The financing industry developed a sophisticated trading system of all asset types as well as the cash market system, but by integrating it with a complex and difficult interface, few people can actually trade freely by themselves. By constantly adding more rules, fees, and formulas, eventually they achieved a monstrosity of self-serving industry where they invest public money in themselves to grow more money for the industry. With a financial bubble that periodically busts in every 10 or 20 years, the general public pays a huge price both physically and mentally for the losses the financial industry intentionally caused.

Scientific innovations should be carefully evaluated by their intention. Negative influences should be minimized and the methodology should be simple to understand and explain to general public. If the technology is difficult to explain in simple terms then we should generalize the principle to fit the audience's capability.

Methods are the explanation of "How To" in science, and every scientific paper is a required to detail all the procedures and conditions used to conduct the experiment. Many important discoveries could not have happened without sophisticated scientific method. Sulfa drug, for example, was discovered during WWII and was the first drug to combat bacterial-caused infections such as pneumonia, TB and meningitis. The discovery was made possible by determined scientists screening all known chemicals known to mankind for anti-microbial properties[7].

Sophisticated methodology is how we achieve great discoveries. To come up with great methodology requires logic and reasoning. More important is a determined motivation and drive to make it happen.

Why

"**Why"** is the action of seeking out the cause, is the motivation to do things right, is the passion of fundamental reasoning. If "what" and "how" are steps used to explain a phenomenon by empirical data and show solid evidence to the audience, "why" is the action taken before these steps. It is for convincing oneself (and maybe others) what he/she is going to do is valuable and sets up the determined mind to achieve certain goal.

When I started my Ph.D. career, I had some laboratory skills and I knew how to perform those skills in a right setting, but I did not equip myself with the mindset of why I was doing research in neuroscience. I was able to generate data and I was able to maintain my work, but I wasn't able to innovate and make my findings impactful. I was constantly adding new data to my results and planning new experiments to make my data set more

"complete". For a period of time, I was drowning in my own experimental design and didn't know where the end of the experiment was.

At the end of my second year, I spent most of my Christmas vacation in the lab. I didn't do much experimenting, but rather reviewed my notebook, organized the lab and wrote down my thoughts. When the New Year came, I put pieces together and saw the reason why I was doing that particular experiment—to prove a new neuronal mechanism, and my data was very positive. Soon after, our lab put the paper together and sent in for review.

I wish I had visualized an end goal of my project before I started, so that I could have remained focused and had a clear vision to follow with. Nonetheless, the process was a valuable experience. Mistakes were made and I learned them. Self-reflecting is a good way to understand "why" and makes me keep up with myself.

The reason why I do science is to promote critical thinking and inspire new ideas to serve as possible explanations to things we don't understand. I set the goal of my Ph.D. career as studying a particular gene that is associated with Down syndrome, and being the first one who comprehensively describes the gene's role in mammal neuronal development. It's pretty awesome.

CHAPTER 3: Heart of Biology

It took us tens of thousands years to create fire

It took us tens of thousands years more to extend its use

It took brightest people to discover its mechanism

It's essential to our survival yet we still don't know its full potential

It's hard to discover useful knowledge

It's harder to understand and use the knowledge in the right way

There is never a level called hardest, things will only get harder and harder

Conscious of Health

Have you ever stuck in a food storage freezer after night shift and everything went dark, air lock shuts down to preserve the temperature, and suddenly realizes that you are in negative 20 Celsius with no direction or orientation? My experience of this situation was largely my own fault—without knowing the schedule of power reset at my facility. It gave me a memory of having difficulty to control my anxiety. Fortunately, everything went back to normal before it starts damage my health.

As a child, I lived in extreme poverty, food insecurity, and horrible living conditions. I was diagnosed twice with terminal pneumonia before I was six. My bed was only few inches away from the ground and would flood whenever it rained. The only electrical unit was the lamp, and even then power shortages were still a daily event. When I was little older, middle school to high school age, I experienced the cruelty of a military style of education where kids are isolated from the rest of the world and guarded by soldiers. Many students

were punished physically until they passed out and insulted by teachers and other students when they failed to complete the 60-70 hours a week study routine. Mental break down and suicide happened on a regular basis, which was normal, and we were told “that’s how weak ones die in this competitive world.” My world was darkened and I didn’t believe there was a purpose to life.

When I finally reached my young adulthood, I witnessed social inequality at a higher level: severe government corruption and country-wide favoritism to the rich. I was once a famous college writer in my city and won many awards for my poems against the general social trends but, again, things quickly fade away when no one really cares about what the world has become. I tried, I fought, and I lost. There was nothing I could lose and I decided to start everything new. I liquefied everything I owned into cash and applied to foreign schools in Europe, Australia, New Zealand, Canada and the USA. I went through one month of intensive English training so I could talk in the embassy to apply for a student visa to study English aboard. Once I had my visa, I bought a one way ticket to the US. Eight years have passed; I have not yet returned home once. All I can appreciate is that I survived all monstrosities and can now focus on things where I can make an impact.

I experienced and had a deep memory of how mental and physical stress can influence individual’s health. It is wise to be conscious of your own health, know your body’s limit and read the signs of tiring, stress and discomfort. If you don’t take care of your body early, then it won’t take care of you later. After the prime age of our life, the world becomes smaller each day as your body loses mobility and sight.

A Brief History of Genetics

Sight perhaps is the highest priority sense in human perception; we wish to see the beautiful sunrise and sunset, the people we love and children's smile, the colorful paint and wonderful night sky.

The possibility of losing our sight due to different diseases and conditions motivates many scientists understand diseases and conditions that can harm our vision. We have the hope that once we understand the fundamental mechanisms behind those diseases, we can use the right tool to cure or prevent it from happening.

In the early ages, those studies were difficult to conduct and data were hard to interpret. There was no model to follow, no guide on how to perform a systematic study and dissection of human body was forbidden. As we moved forward, we started to understand the morphology and physiology of the eye, and we gained clarity on the keys to studying and treating vision diseases. In little bit more than 200 years we went from first reported cataract surgery in 1748 to today's 2mm wounds in eye surgery and advanced lens replacement[8]. More recently, studies moved from a structural level to a cellular and molecular level. This allows us to prepare for next generation of noninvasive treatment. One branch of knowledge has, since its birth, become a powerful tool to explain and describe disease and its fundamental mechanism. That is genetics—the systematic study of genes.

Genetics is a new word, but the idea behind it is not new at all. In ancient China, during a period known as Han dynasty (80B.C), people were well fed and wealthy, thanks to the rich land, and had advanced technology in agriculture and advanced material fabrication methods. However, due to lack of entertainment, people were bored. They tried exploring

new things to do and invented new hobbies to fill up their spare time. Scholars studied mice because the different variations of coat colors and the occasional appearance of albino mice made them somewhat popular. The first pet mice appeared as an international gift in 1654 when two pet mice were gifted to Japan from a Chinese priest[9, 10]. The domestication of mice in China existed far before the concept of genetics, and god knows how many other weird creatures like crickets, grasshoppers and cockroaches have been captured and bred for entertainment and other purposes.

Because of a lack of systematic documentation and mathematical interpretation, those inbred animals in the old Chinese dynasty did not contribute to any great scientific discoveries.

After nearly two thousand years, the science behind genetics finally emerged with evidence from Mendel, who described the rules of inheritance. His observations and underlying hypothesis became influential long after his death. In 1856, Gregor J Mendel decided to grow his famous garden pea in his back yard. He noticed that the flower color of his peas was somehow determinate by its parental flowers. With close examination and documentation for a mathematical significance, he hypothesized those colorations were determinate by copies of genes which can be "*dominate*" or "*recessive*" (yes he coined those terms). Unfortunately, at the time his results were largely ignored. The popular paradigm of inheritance in 1850s was blending inheritance, which stated that characteristics were simply blended and averaged out between parents. I am glad this statement was wrong, or everyone would end up with the same face.

Mendel's work was nothing but a nameless publication forgotten by history. It wasn't until Thomas Morgan integrated Gregor's model into *Drosophila* (fruit fly) in 1910, 50 years after Mendel's discovery made history. Morgan observed and examined countless flies to describe what in modern days is known as chromosomal theory of inheritance: that genes are linked on chromosome and give offspring a defined phenotype. For Morgan's great explanation and advancement of biology, he was awarded the Nobel Prize in Physiology or Medicine in 1933. Because of his discovery, *Drosophila* became one of the most popular animal models for genetic research to this day. This mile stone made people wonder if inheritance worked the same way in higher-order animals as in the flies. It started an era of mammal genomic research.

1990 was a special year for human history. We launched the Human Genome Project, officially starting the understanding of ourselves and what makes us who we are. While human research is unethical, mice quickly gained attention, becoming the mammal counterpart for research. Soon, mice became one of the core organisms in genetic studies and was preserved in the genetic archive.

Many human genetic conditions and diseases are studied in mice, and many drugs are also tested in them before moving on to human trials. This is because, on average, 85% of the mouse genome is identical to that of humans[11].

Why Genetics is Important

Among vast information, I can simplify the explanation into a simple story. All life on the planet earth is coded with four basic chemical structures, A T C G, known as four nitrogenous bases. The combination of four bases drive infinitive mathematical possibilities

spawn into a spectrum of complex molecules and proteins. Each complex code gives rise to one protein and we call that code a gene. All genes together we call it DNA—the template used to compose all live forms that ever existed on the planet earth.

Cells without a brain use DNA template to produce functional proteins and structures responsible for detect environment by mechanical force or chemical signal or even electrical/magnetic current. Those signals will activate different responses according the master mind of our genetic blueprint, decides where to move, how to grow, time to defense or time to die.

All diseases can be linked to one or more genes. Inherited diseases are directly caused by the defective genes that are passed on from parents to offspring. Defective genes were once normal but were mutated by the environmental damage we are exposed to in daily life. Those mutations sometimes cannot be fixed and will stay with the individual and are passed on with reproduction.

For example, breast cancer is the leading cancer among woman and second cause of cancer-related death among women (#1 is lung cancer, also the 2nd leading cancer). Genetics studies helped identified two gene markers to diagnose breast cancer: BRCA1 and BRCA2. They are two tumor suppressor genes present in humans, and mutations in those genes will increase by 20-25% the chance of an individual to develop breast cancer. Unfortunately the discovery of the two genes became a profitable business and was used by a single company for breast cancer testing under the protection of patents for many years[12].

Besides cancer, genes can regulate protein production and cause other conditions. Autoimmune diseases like Type 1 diabetes are caused by not enough insulin being made in

the pancreas. In this condition, our immune system mistakenly attacks our own cells as if they were pathogens. The exact mechanisms remain unclear, but the cellular recognition involved in this process is regulated through gene expression. A comprehensive genetic study in the future could lead to some clues to understanding and preventing such a condition.

There are also less obvious conditions related to our genetic background. Infectious diseases are triggered by pathogens or parasites. Our body produces immune cells to fight the infections in response to these foreign invaders. Some populations inherited genes that can handle certain diseases far better than others. This is because many pathogens and parasites use a unique cellular structure or protein as a channel to spread the infection, and some variations of the structure or protein can significantly reduce the risk of getting that disease.

For example, one of the most devastating infectious diseases in human history is malaria, a parasitic disease spread by mosquito bites. In 2013 alone, there were 200 million people infected by malaria and 584,000 deaths worldwide. The parasite can grow inside red blood cells and uses its resources to multiply, eventually rupturing the host cell and infecting surrounding neighbors. However, when a specific gene, HgbS, is mutated, it disrupts the round structure of the blood cell and makes it much difficult for parasite to attach to. Individuals with one copy of this mutated gene, therefore, are immune to malaria. On the other hand, two copies of this mutated gene will result blood cells completely deforming and usually is lethal to individuals. Study of this gene led to better diagnosis and gave insight on how some parasitic mechanisms works in human body.

Mutation occurs in every organism at all levels. Pathogens can become resistant to one or many antibiotics through rapid genetic mutation. This resistance reduces the available of treatments we have against infectious disease and is a global issue threatening the healthcare system.

Genetics is not only important for human health; it also has many applications in other areas. In gardening, flower colors are specifically bred through the laws of genetic heredity to achieve their beauty. Agriculture is also heavily reliant on genetics to increase production and resistant to insects and climate.

Genes are blueprints to build our body structure and maintain its functions. They are the ultimate keys to understanding and overcoming diseases and conditions. With the rapid advancement of technology like CRISPR and Cas technology, we are able to efficiently edit genes with high accuracy. We can now target anywhere in the genome, adding, removing and changing the DNA sequence of a given organism. In 2013, Editasmedicine was founded by a group of talented researchers and raised 43 million dollars of first round funding for editing human hematopoietic stem cells of bone marrow. This could lead to permanently curing cystic fibrosis and sickle-cell anemia. With such exciting technology, we now can take a faithful leap towards the next stage of biological discovery.

Model Species

There are more than 8 million species living on the planet earth, but only a tiny fraction of them are widely used as research models. In general, model animals share many commons traits. First, they are easy to breed and maintain. This gives scientific facilities the flexibility to arrange the use of space without extra effort. Breeding and maintaining a strain

of animal in a controlled condition can avoid wasting resources on capturing them from the wild and preventing spread of pathogens carried in the wild.

Second, model animals are small, easy to control and have little potential to harm researchers. Nobody wants to study something that could potentially kill them. In our scientific research history, Alexander Bogdanov successfully completed 11 transfusions on himself since 1924 but died on the last transfusion with malaria contaminated blood. Malcolm Casadaban, a professor at University of Chicago, died in 2009 by his own plague culture. Biological experimental safety indeed is a concern for the model we use.

Third, model animals raise minimal ethical issues. Ethics in research has become an important issue today. Only a few decades ago, the US and most advanced countries were conducting experiments using domesticated animals like dogs and cats. Even monkeys, at one time, they were very important research subjects because they have a close lineage to humans. With increasing awareness of animal rights and public concern about animal research, most countries now have set up tight regulations on animal research. To enforce those regulations, regulatory organizations conduct surveys and periodically visit research facilities for evaluation. Labs and organizations that fail to pass those evaluations can be permanently shut down.

Considering all the species on our planet, only about 20 of them fulfill the above conditions. The most famous ones are *E. coli* (bacteria), *C.elegans* (nematode), *Drosophila* (fruit fly), *D. rerio* (zebrafish) and *Mus musculus* (house mouse).

The Ultimate Human Disease Model

One important factor for an organism to be useful in genetic and molecular biology is knowing the sequence of an organism's genome. Not many species have been fully sequenced on the genomic level due the huge effort and heavy financial expenses required. For example, we started sequencing the human genome in 1990s, and it took us more than a decade and cost three billion dollars to complete. Calculating inflation, that's more than 4.8 billion dollars in 2015(CPI inflation calculator from bls.gov). Right after the Human Genome Project, the mouse genome was sequence in 2002. This genetic data base dramatically increased the value of mouse as a scientific research model.

With a genetic blueprint we can manipulate any gene we want. This allows us to systematically test genetic functions in the given organism and use the information to benefit society. One popular strategy in genetic manipulation is to modify genes in an organism. We call the collection of these organisms Genetically Modified Organisms (GMOs). It's a scary term in modern society due to lack of the fundamental biological understanding. The misconception can be blamed on few irresponsible industry practices, especially in the food industry. Nonetheless, genetic studies tell us the fundamental mechanisms of diseases, the cause of aging, and possible the answer to life. It is an unavoidable step on our scientific journey to understand life itself.

In my research project, I designed a genetically modified mouse to express a Down Syndrome (DS) gene. I used this new transgenic mouse strain as a model to study human genetic conditions and related neurological issues. Among all model organisms, the mouse is representative of mammals. It has surprisingly similar genetic information to humans.

From an evolutionary aspect, humans and mice diverged less than 75 million years ago[13]. Both encode around 30,000 functional genes; 85% of these coding areas are identical in humans and mice. An individual gene in mice and humans can have a similarity ranging from 60% all the way to 99.9%. With a more successful evolutionary advantage in reproduction and a bad reputation for carrying diseases and general "pest" status, the mouse is an ideal organism to study genes related human diseases.

In term of vision, human and mouse retinas have nearly identical structure and layers, with some minor differences. Mouse retinas lack the fovea, a region that acts as a focal point for color vision in humans. Therefore, mice do not have accurate color vision. This lack of sensitive color vision is caused by selective adaptation—because mice are active at night, their sense of smell is their dominate cue. Black and white vision is used to complement the sense of smell, and this visual pathway is controlled by rod photoreceptors.

We use the terminology scotopic vision to descript black and white vision. In Greek root, skotos means darkness and opia means condition of sight. Light stimuli generate an identical response in mouse and human's rods, indicating that the fewer cones in mice do not affect other physiological functions of the retinal circuitry. Therefore, it is ideal to use the mouse visual system as a model to study neurological defects caused by diseases and conditions. Plus, the retina is a well-organized structure and abnormalities are easy to observe. This will help us define whether certain genes directly cause morphological changes.

CHAPTER 4: Down Syndrome

Like all old stories, this one has a dark and stormy beginning. Before the emergence of Chromosomal and genetic data explaining neurological disorders like Down syndrome, mental illness was an undesirable trait and was not tolerated by society. Combined with the fear of the unknown causes of mental disorder with the political propaganda of the early 20th century, the eugenics movement became widespread across European countries and in the US. Individuals with mental disability were forcibly sterilized. Down syndrome was a major target for eugenics movement at the time. After the invention of karyotype technique in 1950s, scientists were finally able to screen chromosomes and discover the real cause of Down syndrome.

Down Syndrome, The Condition

It was the harsh winter of 2010, with a snow storm raging on the Palouse, an area located in the northwest of the United States. It's comprised of a vast region of eastern Washington and northern Idaho. The plateau of the Palouse was formed during the last glacial outwash 2.6 million years ago; rich organic compounds and minerals deposits made this area one of the most fertile on the earth. In the late 19th century, the forestry landscape was transformed into wheat land through industrial machines. Today it is the most important lentil-growing region in the US. The harsh snow reduced many insect populations and remained on the ground until spring next year, then it would slowly melted through the year to supply vegetation on this fertile land.

Besides being one of the most famous farm lands, there are also two land-grant universities located with 8 miles of each other: the only land-grant university in Washington, Washington State University, and the only land-grant university in Idaho, the University of Idaho. These two universities work closely together deliver high quality research that benefits the local and national scientific community.

One of the newly established labs at University of Idaho took a brave step towards genetic diseases research. The lab was focused on a particular troublesome condition, known as Down syndrome. This condition is not rare in humans; in fact, every 1 in 700 newborns is diagnosed with this condition[14]. Within 24 years, from 1979 to 2003, the Down syndrome birth rate increased by 31.1% and the trend is still on the rise[15]. The new lab had one goal: to figure out the mechanisms that contribute to neurological disability in Down's individuals.

Down syndrome individuals are known for their characteristic lower IQs, stunted growth and physical appearance including oval shaped eyes, a flat nose, small ears and tongue stuck out side of the mouth. Health-wise, Down's individuals have a high risk of early onset of heart diseases, vision and hearing impairment, early onset of Alzheimer's disease, and muscle weakness[16-19]. Those conditions are the result of triplication of Chromosome 21.

How the triplication event occurs is still unknown, but statistical data suggests a few correlations. The highest correlation factor is the mother's age. When a woman reaches 40 years of age, her chance of having a Down's baby sharply increased to 1 in 100[20], compared to 1in 1440 at age 20. Besides maternal age, 8% of Down's is caused by non-separation of chromosome in the father and 3% of the cases happened after fertilization by

two normal gametes[21]. 95% of Down's individuals have triplication in every cell of their body. With the other 5% cases, 2.5% are known as mosaic Down syndrome, in which only some cells in the body have the extra copies of chromosome 21 and other don't. Those individuals tend to have mild DS phenotypes because some cells are still normal. The other 2.5% Down's are known as translocation of the chromosome 21, which behave similarly to normal Down's. In this case, for unknown reason the long arm of the c21 is attached to another chromosome, usually chromosome 14[22].

This complex genetic disorder possesses many questions for scientists to answer. Most importantly, we don't have any method to treat or prevent this condition other than abortion of the fetus upon genetic screening.

Down Syndrome Critical Region

Down syndrome is not referred to as one condition with one characteristic phenotype, but a collective conditions caused by the presence of extra genetic material from human chromosome 21. This chromosome contains 20,000 to 25,000 genes and encodes 200-300 functional proteins[23]. Most individuals with Down's usually do not inherit the entire extra copy of chromosome 21, but rather a small fraction of it. As a result of this mechanism, Down individuals display very different mental and physical severity based on genetic dosage.

One region on chromosome 21 is known for its critical role in association with Down's symptoms—Down Syndrome Critical Region (DSCR)[24]. This is a region located on the long arm of chromosome 21, where 33 genes code for functional protein production. Those 33 genes define many characteristics of the Down syndrome phenotype. If this region

is not triplicated in individuals with Downs, they are less likely to have mental retardation issues, but minor problems such as joint hyperlaxity and hypoyonia[25, 26].

One of the most troubling symptoms of Downs is the lower IQ. This leads many parents today to screen for the condition during prenatal days and often fetus are terminated during early pregnancy. The lower IQ is believed to be associated with the neuron population changes in the brain. Brains with this condition tend to have fewer neurons, and fewer cells may directly influence's our capacity to perform simple tasks.

Studies suggest that a few genes on DSCR are linked with intellectual disability and cause neuron population defect. DYRK1A is one of those genes. This gene produces a protein kinase and is regulated by phosphorylation of tyrosine, an enzyme that changes chemical conformation and consequently influences molecular function in the brain. Studies done specifically on this gene in human individuals showed speech delay, seizures, autism, motor delay, feeding problems and poor weight gain[27]. A mouse study illustrated that DYRK1A's involvement can regulate cell survival, and imbalance of such a gene will lead to changes in neuron cell population[27, 28]. Recent studies done on DYRK1A suggest a role in activating T cell transcription factors and promoting beta cell proliferation and differentiation[29]. All the regulatory roles can indeed influence how neurons behave in down's individual.

Another problematic symptom is the early onset of heart disease and smooth muscle dysfunction. This is linked to DSCR1, the Down syndrome critical region gene 1, which regulates a subunit of Ca2+/calmodulin-dependent protein phosphatase, which is associated

with calcium mediated stress and damage[30]. This gene is highly expressed in cardiac tissue and the fetal brain in Downs, which might explain some aspects of the heart diseases.

Downs individuals are also largely reported have vision and hearing problems. Traditionally it's believed this is caused by loss of neurons occurs in the brain where in the region where visual and hearing information are processed. Such as visual cortex in occipital lobe and auditory cortex in the temporal lobe, is affected. Theoretically, any influence in those regions will lead to low visual cuing or hearing loss. However, this logical speculation has never been confirmed by solid data. Down's individuals with vision and hearing problems, with long term care, can have a quality life to an average person.

Symptoms like the high correlation of Down syndrome with testicular cancer are not well understood. Some suspect Down syndrome critical region gene 9 and 10 are specific genes that play a role in cancer because these two genes are only present in primates, especially in testicular and renal tissue[31, 32]. However, no proteins have ever been detected and no research has done on this topic.

Although strong evidence and a large body of studies suggest the importance of DSCR for Down syndrome phenotype, we have to admit that we don't know everything. We are still at a very primitive stage of understanding the complexity of life and disease. The dots we connected between few critical genes and their dosage to symptomatic conditions are inconspicuous and may not be true. One study has shown that the abnormal facial structure in Down's individual may not correlate with a particular gene's overexpression on the DSCR, but rather is the result of a more complex genetic homeostasis[33]. I agree with this idea; when a large structure is composed of many small structures, one change in one of

the small structures may not sufficient change the overall outlook of the large structure, but an accumulation of small changes eventually can lead to the alteration of the large structure. Therefore, it's possible that the large scale genetic homeostasis hypothesis can explain some aspects of the diseases and the conditions are simply an additive effect following the principle of emerging property.

Nonetheless studies on DSCR improved our understanding on some aspects of the disorder, and we should dig deeper and find out more about the truth carried by this gene region.

Down Syndrome Cell Adhesion Molecule

In 1997, Dr. Yamakawa and his crew were working at Cedars-Sinai Research Institute of UCLA. They were one of the first groups to try to understand what was contributing to the symptoms of Down syndrome. In doing so, they looked deep into the genomic scale of the condition and tried to tease out the genetic mechanisms involved in Down syndrome. cDNA cloning was performed in their lab to help identify genes on the chromosome 21, especially the long arm q22 region which showed high correlation with Down syndrome.

During this venture, a novel immunoglobulin protein family was isolated, and a new class of adhesion molecule was found. This novel molecule was named Down Syndrome Cell Adhesion Molecule (DSCAM) and it was actively expressed in the neural crest-derived tissues.

As more research was conducted, Dr. Yamakawa's group found that this family of genes was expressed during the development of the neuro tube, brain, and spinal cord, in both the central and peripheral nervous system[34]. The timing of gene expression suggested this family of gene could relate to neurogenesis. Defects in neuron neurogenesis will result in neuron imbalance and consequently contribute some aspects of the Down syndrome.

Two years after the first isolation of the DSCAM, the pioneer work on this gene was published on the top journal *Cell* by Dr. Zipursky and his lab at UCLA. His study illustrated that the DSCAM protein in fruit flies (*Drosophila*) is "extraordinarily diverse," with more than 38,000 alternative splicing forms that could be generated[35]. "Alternative splicing" means many versions of the same mRNA are made from the original genetic blueprint, each with some minor modifications. Those minor differences enable them to have slightly different functions in the same tissue. Each splice form of DSCAM is capable of neuron guidance in an isoform-specific manner.

Later in 2003 and 2004, his follow up experiment in *Drosophila* illustrated that the axonal targeting of olfactory neurons is controlled by the unique yet diverse DSCAM protein isoforms. In 2004, another article by Dr. Chess's group at MIT confirmed that *Drosophila*'s *Dscam* has 38,016 different splice forms[36]. For the next ten years, Dr. Zipursky's group continued working on *Dscam* in *Drosophila*. During this time, much collaboration happened. Findings illustrated *Dscam*'s unique ability in cell adhesion and repulsion, guiding neuron axons, promoting self-avoidance, helping target proper dendritic lamination and regulating developmental cell death (DCD). DCD in this context is also known as programed cell death during development.

Isoform-specific binding is an excellent strategy accomplishing the unique functional diversity in fruit fly. The diverse Dscam isoforms in insects is generated through competing intronic RNA secondary structures[37, 38]. Cell type-specific interactions exist between DSCAM expression cells and the same isoform could form bindings to promote or inhibit neural development.

Studies in humans showed this gene is highly relevant to our health. Alzheimer's individuals have an elevated DSCAM expression in the brain. This expression is associated with the core and periphery senile plaque formation. When neurons over produce this type of cell adhesion molecule, it could lead to protein aggregation; one of the mechanisms that cause mental retardation and memory loss[39].

The discovery of the *Dscam* gene had a significant impact in the field of neuroscience. It quickly became one of the hot topics in neuroscience and attracted lot of attention in the scientific community. New books were published and biological text books were updated to reflect the most recent findings. Soon after, a functionally and structurally deduced protein Dscam Like 1(DscamL1) also emerged and appeared to have a role as part of the functional neuron wiring circuits[40]. DscamL1 binds in homophilic fashion to other DscamL1 molecules.

Studies illustrated many functionalities of *Dscam*, but due to majority studies being done on *Drosophila*, we could only conclude that most of the known functions only exist in insects. From a historical perspective, the *Dscam* gene diverged very recently within the fly family. *Drosophila melanogaster* and *Drosophila simulans* are less than 10 million years apart[41]. The *Dscam* in flies and mosquitos are about 200 million years apart and 300

million years between flies and bees[42]. It's not hard to imagine that, with such a long time of diverging, Dscam in mammals is very different from Dscam in insects. Only 2 *Dscam* isoforms have ever been identified in mouse, and only three alternative transcripts have ever been described in human[40]. This is significantly less than the 38 thousand isoforms in *Drosophila*.

More detailed phylogenetic relationship in the *Dscam* transmembrane domain illustrated that human and mouse DSCAM and DSCAML1 protein composition is far away from the insect lineage group[43]. On amino acid level, human and mouse *Dscam* gene only have one amino acid difference, and the *DscamL1* gene is identical.

The *Dscam* gene is highly conserved among arthropods[44]. Some studies suggested both invertebrate *Dscam* and vertebrate *Dscam* are conserved in motif structures. One study suggested human *Dscam* and *DscamL1* is functionally similar to *Drosophila Dscam* TM1, an isoform group that primarily regulates dendrites instead of axons[43]. It is reasonable that the *Dscam* gene in mammal is much more conserved and regulates a uniformed role rather than the diverse functions seen in insects.

The high degree of similarity in protein structure between human and mouse *Dscam*, and the lack of *Dscam* splicing forms in mammals suggest a high research value to studying this gene in mice[45]. The result is much more likely to be used to interpret how the human *Dscam* analog behaves. We can also gain a considerable amount insight into how this gene contributes to neurological issues in actual Down syndrome individuals.

Tale of the Jackson Lab

Long winters in Maine turn the beautiful Mt. Desert Island into a frozen and isolated world. Without tourist attraction, many bars and shops on the island close. Snow slowly covers the entire Acadia National Park and turns it into a true white wonderland. However, one place on the island is forever busy and noisy—the Jackson Laboratory, located close to the Bar Harbor on the island.

Founded in 1929 by C.C. Little, president of University of Michigan and former president of University of Maine, the laboratory was dedicated in the memory of Roscoe B Jackson, the donor of the facility. This facility is the heart of the mouse genetics on the planet Earth. The non-profit organization distributed 2.5 million JAX mice in 2014 around the globe for use in education, research and clinical trials. Mice from JAX are linked to 26 Nobel Prize winners and countless drug discoveries. Without its existence, many medical improvements and scientific discoveries in the last 50 years simply could not have happened.

Some of these discoveries were more challenging than others, and the discovery of Down syndrome cell adhesion molecule belongs to this category. In 1981, a mouse mutation was identified in the Jackson Lab. The mutation colony had some strange phenotypes, including walking on their toes like a scared cat. The line also displayed deformed heads abnormally large in size, and in general a smaller body. With the lack of tools for genetic research in the 80s, scientists could not figure out what the problem was. Polymerase chain reaction (PCR) wasn’t even invented at this time, and genotyping and sequencing was still a secret in the black box. After documenting this strain as an unknown mutation, the busy facility soon forgot their existence.

Another naturally occurring mutation was identified in 1994 at the Jackson Lab. This new mutation was similar to the ones in 1981 but with less severe phenotypes. A different team was sent to investigate the origin of the mutation and to test if two mutations were somehow linked. Scientists used a complementation test to examine if the mutation occurred on the same chromosome. This was done by cross breeding two different mutation strains and observing their offsprings' phenotype. The resulting offspring suggested both mutations were located on the same chromosome, and further tests were able to narrow down the range of mutation on mouse chromosome 16. However, in the middle 90s we still lacked sophisticated technology for screening DNA mutations. Experimental procedures in this field were extremely difficult to perform and time consuming. With many obstacles and a lack of interesting findings, the study on this particular mutation was stalled once more.

Something was about to change. In 2005, 3 years after mouse genome was fully sequenced, and 24 years after the first mouse mutation with a crunched back was recognized in Jackson Lab, yet another new mutation was been identified from the massive colony of Jackson Laboratory. This time, one scientist would not let it go so easily.

Dr. Fuerst, a young passionate scientist who joined Jax not very long ago, was fascinated by this new mutation. He spent countless days working with the animals and observed detailed nerve tissues. He came up with a few hypotheses on how the mutation could occur: the crunched back could possibly be the result of Kyphosis, but, unlike in humans, this Kyphosis has a very early onset in the animal's life. It was possibly a developmental issue soon after birth. Skull deformation could be involved with hydrocephalus in the brain, and vestibular defect was too obvious to be ignored since the

animal was suffering body balancing issue. More intriguingly, the severe neuronal abnormalities appeared in the retina were never seen before.

Acting on his curiosity, Fuerst decided to identify this mutation on the genomic scale. Positional cloning, a method used to isolate a genetic sequence of interest by flanking out the locus near the point of interest, was performed soon after. Chromosome 16 was narrowed down to a few regions for this mutation. After months of searching for the candidate gene of mutation, a northern blot revealed a significant reduction in mRNA level of *Dscam.* Exon sequencing of this region further confirmed a 38 base pair deletion occurred in the mouse mutation. This was the first time ever that scientists identified a mammal genetic mutation in a cell adhesion molecule that was directly linked to a severe defect in the process of neural development.

With previous knowledge from Dr. Zipursky's studies in fly Dscam1 and how it influenced neurons in the fly brain and Dr. Yamakawa's evidence of *Dscam* expression in the mouse brain, Dr. Fuerst directed his study towards *Dscam*'s influence in mammal neuronal development[46]. He became the first person to illustrate that a *Dscam* mutation in mammals could change neuronal activity and cell population. The novel mutation identified in 2005 was named *Dscam* del 17, referring to the deletion that occurred in exon 17 of the *Dscam* gene.

Mice with this mutation have more neurons present in the retina at postnatal day 4 and disrupted neuron lamination. Tyrosine hydroxylase labeled dopaminergic amacrine cells (DACs) displayed severe aggregation of the neuron dendrites as well as disrupted cell spacing. The aggregation and spatial disruption of neuron mosaic was universal across *Dscam* expression neurons but not for non-*Dscam* expression neurons[47]. Neurons need a

well-organized structure and correct spacing to be functionally competent in collecting and exchanging information. This discovery was a strong evidence to prove that *Dscam* is a critical gene expressing protein that regulates the functional integrity and organization of the nervous system.

The publication was officially published in *Nature* magazine in 2008. The mutation's influence on the development and refinement of the nervous system in mammals was so clear, it heated up the field of neuroscience and got people wondering: what if the *Dscam* and mammal study was done a decade earlier? If we discovered how *Dscam* directly influences neurons and cause them to disorganize in the 90s, millions of funding could have nourished the field of neuroscience and led to a blooming biological industry working on possible cures for neurological conditions. Research on Alzheimer's, Autism, Huntington, Parkinson's and many other neurological diseases could have been funded to their full extent, with possible solutions already approved by this date.

Dscam del 17 was a significant finding, but many questions about this gene remain unanswered. To understand more about this mutation and DS gene, Dr. Fuerst looked for similar mouse strains existing in the facility. After some data mining, documents linked two mouse mutations once forgotten by time: the mysterious 1981 and 1994 mutations in the Jackson laboratory.

Following this, Fuerst first identified the 1981 mouse mutation. It was a four base pair duplication event on *Dscam* exon 19 that caused a frame shift mutation and stopped DSCAM protein production[48, 49]. This mutation had many similarities to the del 17 mouse. It displayed a significant increase in cell population, disorganization of the IPL and spatial mosaic disruption of neurons. More importantly, strong evidence indicated this cell

adhesion molecule was involved in regulating developmental cell death, which led to the phenotype of increased cell number in the retina. This mutation was the second *Dscam* mutation found in the Jackson Lab, so it was named *Dscam*^{2J}.

Dscam Defect in Mouse

In 2010 Dr. Fuerst established his own Lab, located at the University of Idaho. I was very lucky to be involved in the early process of identification and characterization of *Dscam*^{3J} mutation. *Dscam*^{3J} refers to the 1994 mutation, the third *Dscam* mutation found in Jackson Lab. This opportunity allowed me to explore and understand the *Dscam* gene's influence in mammal neurons. Sequencing of this mutation revealed *Dscam*^{3J} was a point mutation. Only one base pair was mutated, and it changed the amino acid at the 1018th base pair of the *Dscam gene* from arginine to proline [50] (Figure 1 A).

This tiny change in gene sequence had a huge consequence. To understand this change, we have to briefly touch on biochemistry. Arginine is an amino acid with a positive charge; proline on the other hand, due to its cyclic side chain structure, it has a rare rigidity acts frequently as a protein secondary structure disruptor to dramatically change the scaffolding direction of a protein.

This one base pair mutation altered the structure of the second fibronectin domain at the N-terminal of the DSCAM protein, making it more disorganized. Unlike the previous 2J mutation, the DSCAM protein in 3J was not completely truncated. Western Blot was used to assay protein content, and our results indicated the existence of the N-terminal DSCAM protein fragment with a decreased dosage in the 3J mutation. This fragmentation was not detected in the 2J mouse tissue because the protein was not produced at all. (Figure 1 C) In

general, the 3J mutation displayed similar phenotypes. Mice with this mutation walked on their toes and suffered from central and ventricular defects, but the degree of severity was reduced compare to the 2J and del17 mutations.

The 3J mutation retina was found to be very similar to the two other mutations in terms of cell numbers through histological analysis. However, the overall organization was not severely disrupted. Notice how the inner nuclear layer was evenly laminated in the 3J retina compared to 2J (Figure 2 A-C). In the whole mount retina, the 3J mutation had severely disrupted spatial and dendritic lamination of Melanopsin positive retina ganglion cells[51, 52] (RGCs). It also influenced *Dscam* expressing TH positive Dopaminergic amacrine cells (DACs) and bNOS positive amacrine cells, with less severity compared to the 2J mutation (Figure 2 D-L).

Cross sections of the retina were used for a systematic view of neuron lamination in the IPL for each cellular marker. The 3J mutation displayed less neurite lamination disruption in Cholinergic amacrine cells (ChAT), Dopaminergic amacrine cells (TH), bNOS positive retina ganglion cells and melanopsin positive retina ganglion cells compared to the 2J mutation in IPL (Figure 3 A-F). The bipolar cell markers PKCα and Syt2 illustrated that the 3J mutation targeted more specifically in IPL compared to the 2J mutation (Figure 3 G-L).

Protein expression was tested carefully using Western Blot Analysis (WBA) of cell cultures. Compared to the wild type cells, cells with $Dscam^{3J}$ gene only produced 14% as much DSCAM protein. Hence, the 3J mutation with altered second fibronectin domain had a significant impact on protein secretion. It seemed highly possible the structural changes

prevented the protein from being secreted into the environment. This speculation was tested by IHC staining, and we observed DSCAM to be concentrated in the cell body rather than diffused away like in the WT retina tissue. We concluded that the 3J mutation led to DSCAM protein alternation and prevented the secretion of diffusible protein in extracellular space (Figure 4).

Overall, the disrupted gene deletion in del 17, truncated protein production in the 2J mutation and altered 2nd fibronectin domain in the 3J mutation all led to similar neurological defects. However, more preserved protein structures like in 3J mutation could reduce the severity of the defects. These findings suggested that the DSCAM protein structure integrity and/or the protein dosage secreted from cell play a major role in regulating neural organizations.

Studies on Down syndrome cell adhesion molecule in mice illustrated the complexity of mammal nervous system. It provided evidence of how neurons interact with each other via cell adhesion molecules. It further illustrated that a single gene can regulate many different events cross the spectrum of neurological diseases, from synapse activity and guidance of neuron terminals, to neural development, to influencing the structure and organization of the neuronal circuitry. We planned future investigation of the *Dscam* gene to deepen this understanding of how elegant the process of neural development is in the mammalian nervous system.

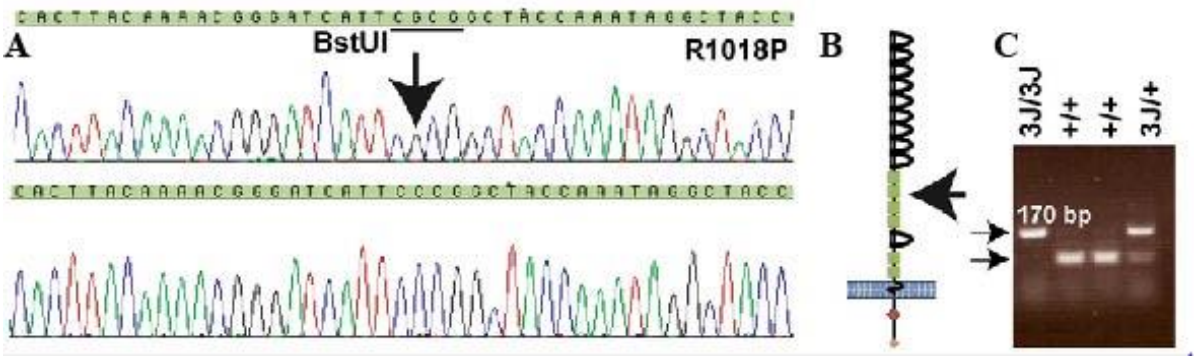

Figure 1 Amino Acid substitution R1018P is genetic basis of nm2122

A, After complementation tests with another *Dscam* mutant, NM992 (*Dscam2J*), failed, the *Dscam* open reading frame was sequenced and a single nucleotide substitution was found resulting in substitution of proline at 1018 in place of the wild type arginine. The nm2122 mutation will henceforth be referred to as Dscam3J. **B**, The Dscam3J mutation is located in the second fibronectin domain (arrow). **C**, The mutation destroys a recognition site for the enzyme BstUI, allowing wild type, heterozygous and homozygous mutants to be identified based on PCR of the mutation-containing region followed by subsequent restriction enzyme digest. The wild type allele is digested by BstUI resulting in two bands of close to equivalent size, while the mutant allele remains intact.

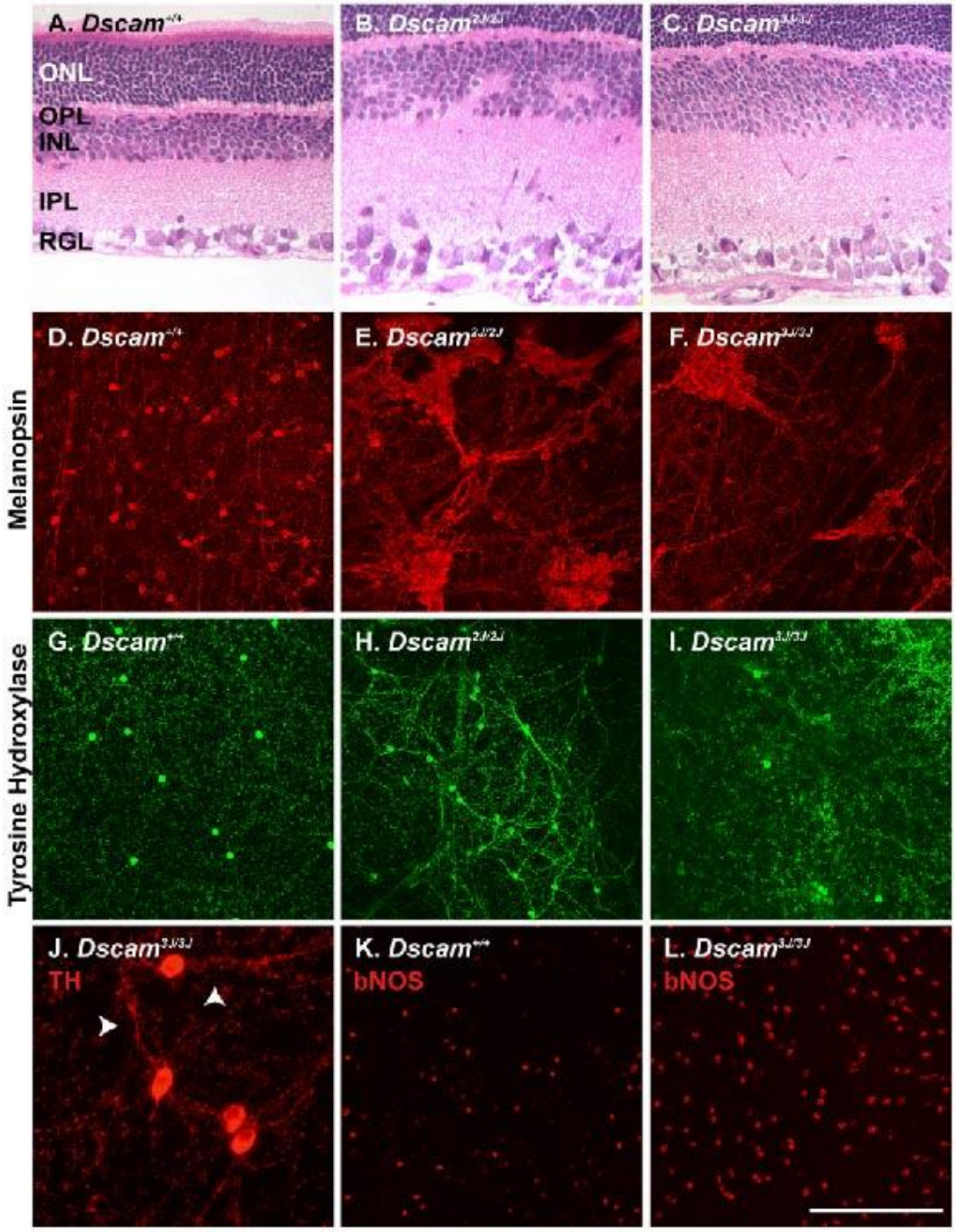

Figure 2 *Dscam*^{3J} mutation reproduces some aspect of *Dscam* null retina

A–C, Retinal sections from wild type, Dscam2J and Dscam3J mice were stained with hematoxylin and eosin. In the wild type retina (A) the three cellular layers are neatly stacked and the synapse containing plexiform layers do not intrude within the cellular layers. **B**, Cell number is increased in the Dscam2J mutant retina, with ectopic cells located in the inner plexiform layer, which projects into the inner nuclear layer. **C**, The Dscam3J retina is

hypercellular; however, cellular lamination is more neatly organized compared to the Dscam2J retina. **D–F**, Retinal ganglion cell spacing and arborization is disrupted in both the Dscam2Jand Dscam3J retina compared to wild type. **G–I**, Amacrine cell spacing and arborization is disrupted in the Dscam2J mutant retina but this degree of disruption is not observed in the Dscam3J mutant retina (I).J, Occasional loose fasciculation of Dscam3J dopaminergic cell neurites was observed (arrows). **K** and **L**, Wild type and Dscam3Jretinas were stained with antibodies to bNOS, to detect bNOS-positive amacrine cells. The number of bNOS positive amacrine cells is increased in the Dscam3J retina. The scale bar in (A-C) is equivalent to 132 µm. The scale bar in (I) is equivalent to 320 µm in D-I and K and L, and 100 µm in J.

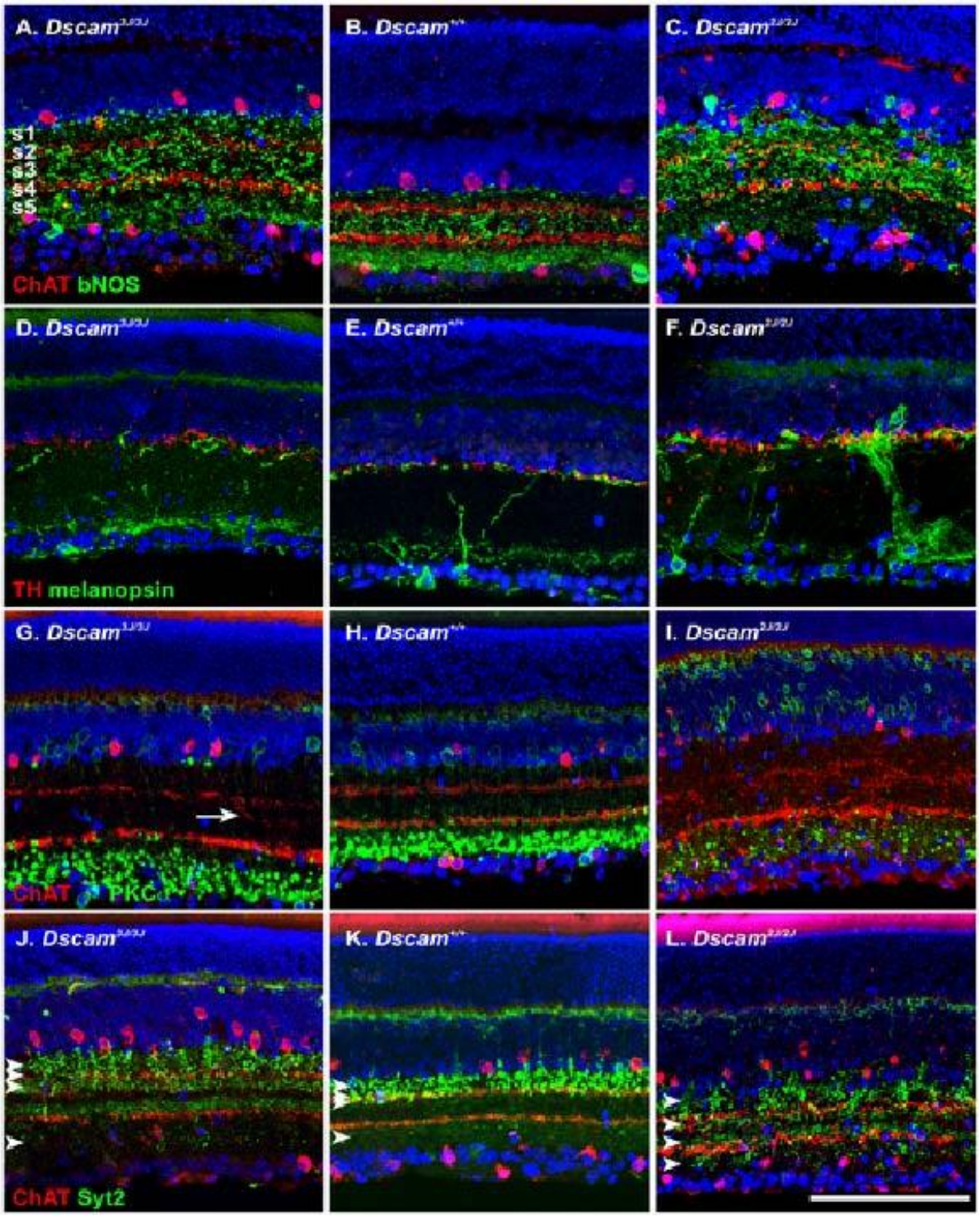

Figure 3 Neurite laminar specificity is preserved in Dscam3Jmutant retina

A–C, Wild type, Dscam2J and Dscam3J retinal sections were stained with antibodies to ChAT, a marker of starburst amacrine cells and bNOS, a marker of bNOS-amacrine cells (**A–C**). Wild type laminar specificity of bNOS-positive neurites in s1, s3 and s5 is roughly

preserved in the Dscam3J, but not Dscam2J retina. **D–F**, Wild type,Dscam2J and Dscam3J retinal sections were stained with antibodies to TH, a marker of dopaminergic amacrine cells and melanopsin, a marker of melanopsin-positive retinal ganglion cells. Although extensive fasciculation of neurites is observed, the laminar specificity pattern of cell types is maintained in mutant genotypes. **G–I**, Wild type, Dscam2J and Dscam3J retinal sections were stained with antibodies to ChAT, a marker of starburst amacrine cells and PKCα, a marker of rod bipolar cells. The gross disorganization of cholinergic amacrine cell neurites observed in the Dscam2J retina is not observed in the Dscam3J retina, although limited examples of disorganization do occur (E; arrow). **J–L**, Wild type, Dscam2J andDscam3J retinal sections were stained with antibodies to ChAT, a marker of starburst amacrine cells and Syt2, a marker of type 2 and type 6 cone bipolar cells (J-L). The wild type laminar specificity pattern of type 2 and type 6 cone bipolar cell axons is disrupted in theDscam2J retina (arrowheads). The scale bar in (L) is equivalent to 112.5 μm.

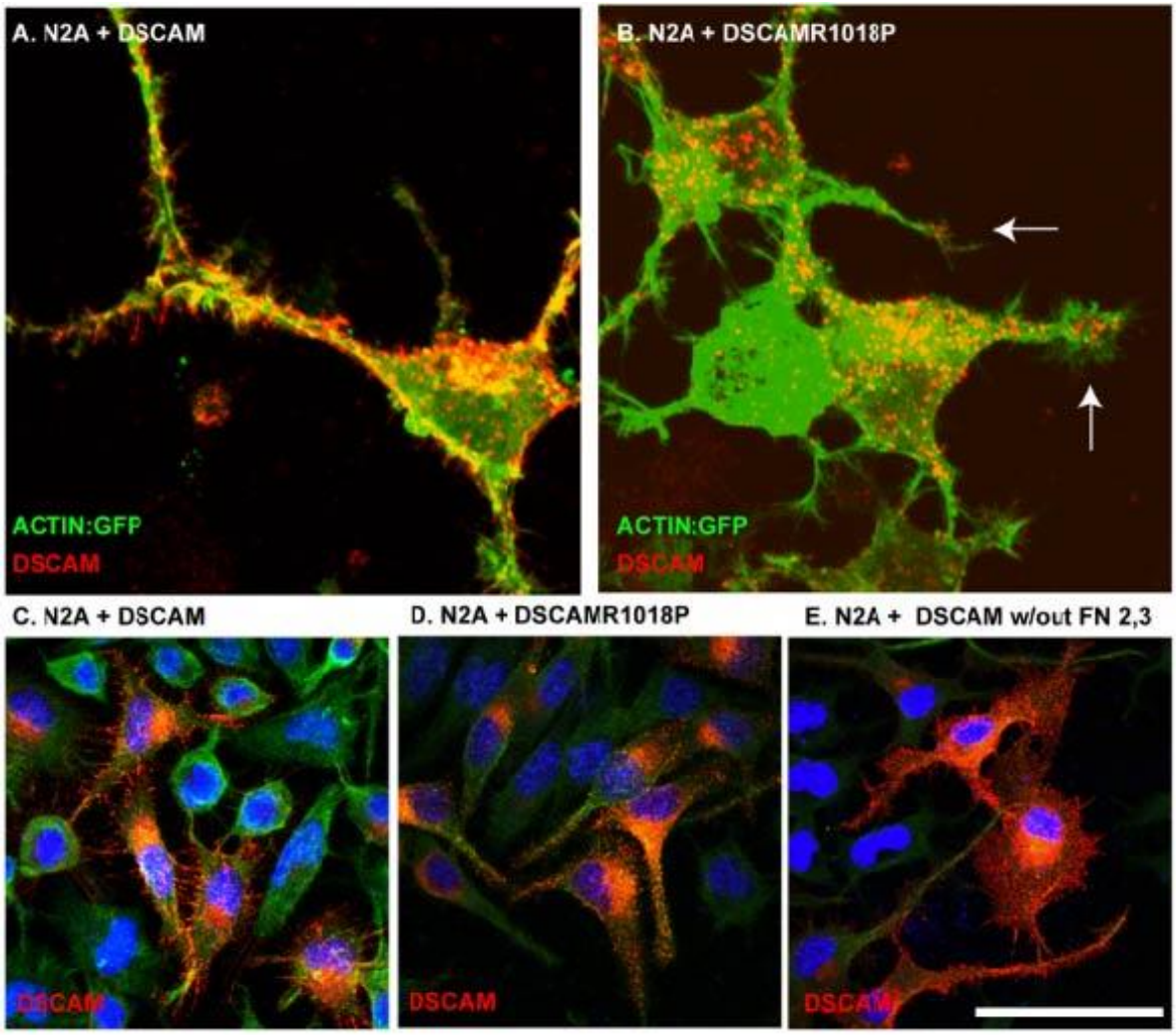

Figure 4 *Dscam*^{3J} mutation inhibits filopodial localization of DSCAM

A–C, Dscam expression constructs were transfected into N2A Cells. A, Full length canonical DSCAM is abundant in the golgi-ER, where it is translated and trafficked, along the membrane and at the tips of filopodia. **B**, DSCAMR1018P is translated and trafficked in and from the golgi-ER and appears in trafficking vesicles within growth cones but does not appear to be localized to the filopodia. **C**, The localization defect in DSCAMR1018P is not a result of disrupted fibronectin domain function, as deletion of the second and third fibronectin domain of DSCAM does not interfere with filopodial localization. **D** and **E**, DSCAM or DSCAMR1018P were transfected into N2A cells together with a plasmid expressing an actin-GFP fusion. DSCAM was easily visualized at the tips of actin filaments, whereas little DSCAMR1018Pwas observed at the end of such filaments. Scale bar in **E** is 12.16 µm in **A** and **B**, 36.5 µm in **C**-**E**.

CHAPTER 5: A Brave Step

On my first trip to the Yellow Stone National Park in 2012, I witnessed a warm wind melt Yellowstone Lake's ice shell in May within 2 days. Tiny geysers around lake shore spouting out steam of water mixed with hydrogen sulfide made this scene a blue diamond floating above clouds. One of the most predictable and visually pleasing geysers in the Yellow Stone is the Old Faithful. With the help of underground magma, ground water is heated and turned into powerful steam. It's like a giant underground pressure cookers powered by nature. High temperature boils the water and builds up pressure, then steam erupts into the air through vents connected to the ground. Nature can always turn its amazing geological mechanisms into powerful and beautiful art works that everyone can feel and see.

The science behind nature's greatest events inspires me to think deeply and rationally. The trip to the Yellow Stone was during my second semester of graduate school. My brain was filled with wonders like geysers, stars, light, visual systems and neurons. I was passionate to do something new and creative, so I started design and create the first transgenic mouse to overexpress *Dscam*.

Oh, Did I Mention Grad School?

During my passionate but mindless first year of graduate school, I reviewed *Dscam* papers and realized all of studies on the *Dscam* gene in mammals were only focused on *Dscam* loss of function because of model availability. No study had ever been done on its gain of function counterpart.

Published in PLOS ONE, R.Dee Schramm(co-first author), Shuai Li(co-first author), Belinda S. Harris, Ryan P. Rounds, Robert W Burgess, F. Marty Ytreberg, Peter G. Fuerst. A Novel Mouse Dscam Mutation Inhibits Localization and Shedding of DSCAM. Published: December 26, 2012 DOI: 10.1371/journal.pone.0052652

In genetics, loss of function and gain of function models are paired functional studies to demonstrate possible genetic functions. Loss of function is set up to understand what happens when a gene is missing or not functional, and gain of function is set up to test what happens when certain gene is expressed over normal dosage. The cohesive nature of the two functional studies confirms the necessary functions of a gene.

Using only loss of function studies for *Dscam* is unrealistic since what really happens in Down syndrome individuals is the triplication of *Dscam* gene, resulting in an over-production of its protein. The opposite occurred during the loss of function experiments that had been done on this gene. So why didn't people do the complementary experiment? The answer is that *Dscam* gain of function model did not exist. No mutation has been identified that produces excessive DSCAM protein. The closest candidate model available was a mouse strain named Ts65Dn, an overhaul of the triplication of mouse chromosome 16, which contains many genes orthologous to those on human chromosome 21. This mouse strain was and still is a widely used model for Down syndrome and related mental disorders, but it is not the best model for this disorder.

Ts65Dn yielded important results for Down syndrome. It illustrated the overall changes that occur in animals following triplication of the mouse chromosome 16. It proved a link between the amyloid proteins of Alzheimer's disease and autism[53-56]. Fundamental physiological studies also used the Ts65Dn mouse strain, like electrophysiology study on GABA neurotransmitter[57]. However, this genetic manipulation could not answer the precise question of how each gene influences the nerve system's function that I was interested in. Since there was no gain of function mouse strain to support existing *Dscam*

loss of function data, Dr. Fuerst and I decided to create one—a new transgenic mouse line that overexpressed the *Dscam* gene. This would be the first time a mammal reflected the real condition of *Dscam* expression in Down syndrome cases, and we could either prove or disprove previous findings related to the disease solely based on *Dscam* mutations.

We Create

On a fine winter afternoon in 2011, with a black sharpie and a white board, Dr. Fuerst and I started to sketch the genetic construct of this exciting new transgenic animal. The process was accomplished with many rectangular boxes, lines, gene names, plasmid names, restriction enzyme sites and some crazy drawing. It was like a classical science fiction movie scene where scientists are solving an extremely difficult task all standing in front of a white broad, and within 20 seconds and with intense back ground music, the problem was solved. The difference was that, in reality, there was no music and it took us more than couple month to get everything validated.

In transgenic research, one of the most used methods for designing conditional expression of a gene is the loxp-cre technique. This technique is a DNA-site specific recombination using special enzymes called recombinases. Cre is one such enzyme. It works like a scissor targeting the Loxp DNA sequences (discovered from bacteriophage) in the genome and cutting out the sequence in between two Loxp sites. Next DNA ligase, which acts like a glue, quickly rejoins the pieces together and the DNA sequence in between the two Loxp site is removed.

The *Dscam* gain of function plasmid is constructed with pCAGIG plasmid (addgene plasmid 11159,) because it is a mammalian expression vector with a cytomegalo virus

enhancer, a Chicken beta-actin promoter and a Globulin acceptor to maximize the expression efficiency. Additionally, it contains an IRES-GFP, which is an internal ribosomal entry site that contains a green fluorescent protein from *Aequorea Victoria*(this is the famous Nobel prize winning jelly fish florescent protein.) The plasmid can be easily cultured in DH5alpha *E. coli* strain with the help of the Ampicillin resistant site of the vector. A tandem dimer red florescent protein amplified from Thy1-Brainbow-2.1R plasmid (addgene, 18727) with a stop codon placed before the *Dscam* coding sequence (a gift from Daniel Voyats and Robert Burgess).

In a simple illustration (Figure 5 A), this is how the transgene works: once the flox-cre-recombination technique is used, it removes the red fluorescent protein and the stop codon, allowing the overexpression of *Dscam* gene and a green fluorescent reporter in the targeted tissue. Where the gene is expressed depends on which cre line we use. We can directly observe the green fluorescent reporter in the tissue under microscope.

After generating the gene allele into the plasmid, we tested our initial construct in a cell culture experiment. (Figure 5 B) Our results indicated that once cre was activated, cells were able to turn to green from red, and DSCAM protein expression was detected in those green cells. Our results validated the design concept and proved the viability of the plasmid.

Mice, Mice, and More Mice

Two founder mice were generated from 150 injections at the University of Washington transgenic core. Breeding was challenging, but eventually things started to move towards the right direction. One founder carrying the gain of function transgene was

used to breed all of the colonies in my study to ensure the consistency of my experiment, and this strain now is available through Jackson laboratory (mouse stock #025543).

I remember being very excited and happy when the first litter was born in the cage I was taking care of. I took small tissue samples from their toes and looked under the fluorescent microscope. After little bit of adjustment, some tissues glowed brightly in the red channel under the fluorescent microscope. “It worked,” I thought to myself, “what a wonderful world.” That was a cheerful moment, knowing that the fundamental basis of my next experiment was working as it was supposed to. Later, it was emotionally hard to take those animals for study because I literally created them and had been taking care of them for so long, but science never stops because individual’s emotion.

To tease out the functionality of *Dscam*, more systematic tests and mouse strains were needed. Upon the arrival of the gain of function mice, we ordered different cre lines to activate the overexpression of *Dscam* in controlled neuron tissues. This tissue specific strategy would allow us to identify *Dscam*’s role in different tissues and tease out the important circuitry abnormalities linked with cell type-specific *Dscam* interactions.

The first cre line we used to recombine the flox transgene was pax6a cre. Pax6 is an important gene required for eye development[58]. Knocking out this gene will lead to extremely under developed eyes in humans, mice, zebra fish and fruit flies. This particular cre line, for some unknown reason, expresses cre-recombination restricted to a middle dorsal-ventral mosaic fashion within the retina. WT tissue, in this case, would be in the central dorsal ventral area of the retina surrounded by the activated cre tissue. Therefore, it

was a viable strategy to observe the effect of *Dscam* gene over expression on the retina's circuitry, and its influence on adjacent wild type neighbors.

The second cre line we used was brn3bcre. This line activates the transgene in a `group of retina ganglion cells which expresses brn3b (aka POU4F2)[59, 60]. Knocking out this gene will result in an axon defect in those cell types and loss of transdifferentiation, which means loss of the ability to switch cell types. We did further study on this particular cre line and find other useful applications, including its unique high incident of germline deletion in the male animal. This allowed us to easily producing ectopic expression of DSCAM in whole animals.

The third cre line was GFAP cre. GFAP stand for Glial fibrillary acidic protein, a protein making up the intermediate filaments in the CNS glia cell populations. This particular cre line targets Müller glia cells, which are non-neurons believed play many roles in maintaining neuron function and secreting factors help shape the nervous system. By studying this cell population, we could test the influence of overexpression of *Dscam* on non-neurons.

We also used other transgenic lines to support our study. The most important one was a *Bax* gene knock out mouse, in which the developmental cell death process in the retina was directly influenced. The *Bax* conditional knock out line and a *Bak* gobal knock out line were also used to illustrate the tangible relationship between *Dscam* and the cell death process. A third line, THGFP is another fluorescent tagged transgenic animal. It brightly labels type 2 TH (dopaminergic amacrine) cells in the retina with a clear cellular

morphology. By observing and analyzing their stratification patterns, we could gain some insight on how those cells were influenced by *Dscam* dosage.

Before the arrival of the new *Dscam* Gain of Function ($Dscam^{GoF}$) mice, we maintained a large colony of different *Dscam* Loss of Function ($Dscam^{LoF}$) mice from Jax lab, including $Dscam^{2j}$ $Dscam^{3j}$ and $Dscam^{del17}$. We could use those mice to create different *Dscam* gene dosages for our study to understand the differences between less protein and a complete lack of the protein.

We had all the mice we needed to carry out a full functional study. We could target different neuron populations by crossing a founder with different cre lines. Each cre had a target that would illustrate a particular cell population of interest and how this population was influenced by the overexpression of *Dscam* gene. We could also tell whether or not each combination altered the retina circuity. By combining the gain of function allele with known cell death genes like *Bax* and *Bak*, I could presenting compelling evidence on *Dscam's* involvement with the regulation of cell death.

Validation of the Transgenic Animal

During its cold winters, Moscow, Idaho, is usually covered by heavy snow. With the everlasting subfreezing temperature, the town offers mainly snow sports during this time of the year. I used to hike in the mountains with my friends and their dogs around the end of February every year. Deep in the Moscow Mountains, the silent white world would occasionally be disrupted by a flip of wings from snow owls, and then the action would quickly disappear like nothing ever happened. I could notice the change of the climate in April when the slightly warmer wind from Pacific Ocean climbed over the Cascade Range

in the east Washington, then finally arrived on the Palouse. Within a week or two, the area give up the cold weather and vegetation turn into a shining green color, followed by the arrival flocks of migratory birds and the hatching of Canadian geese; the Palouse suddenly became a paradise for life.

It was 2013, the winter of my second year of graduate study. I clearly remember my activities besides staying at home and hiking in the mountains: working in the lab. Daily routines included collecting tissues from mice of different ages and different strains, then staining them up with antibodies and to spot the differences, like the game we all used to play when we were kids. Sometimes I could find things that were obvious and interesting in a morphological sense, but, other times, phenotypes weren't quite clearly distinguished from the norm. The occasional excitement of new discovery was a great feeling and kept me motivated for my experiment.

Before I went too far, scientific validation needed to take place. The cell culture experiment demonstrated that our vector, a carrier for our DNA of interest, was valid. This vector later was implanted into an animal embryo through many complex procedures. I had to validate that our genetic design worked in animal tissue just like in the cell cultures. The retina in particular need to be double validated because it was our tissue of interest.

To test the expression of the transgene in animal tissue, I assayed the expression of red fluorescent protein (RFP) in the mouse retina through developmental time points. The RFP was part of the transgene, and its presence is an indicator that our transgene was activated and expressed. My data suggested that RFP was activated throughout the retina upon birth in our new transgenic animal. This universal expression of transgene continued

until 11 days of age. A high level of RFP expression was then condensed into the inner nuclear layer (INL) of retina, Müller glia and some lineages of photoreceptors. At adulthood, the RFP transgene expression became more evenly distributed between the inner plexiform layer (IPL) and outer plexiform layer (OPL) (Figure 6)

Next, to test the presence of DSCAM protein, I performed DSCAM antibody staining and intensity analysis (Figure 7 A top). All tissues and images were kept at same parameters. We compared DSCAM antibody staining in *Dscam*^{GOF}, wild type and *Dscam*^{LOF}. *Dscam*^{GOF} retinas did show an increase in antibody intensity of the IPL at 12 days of age. This indicated there was an increase in DSCAM protein expression in the gain of function retina. To further validate this statement, I collected different tissues from germline activated *Dscam*^{GOF} mice and analyzed them by Western Blot Assay—an assay that measures protein concentration and size using antibodies and conjugated horse radish peroxide. The result can be visualized by light sensitive film exposure (Figure 7 A Bottom). My results indicated our *Dscam*^{GOF} mice had much more DSCAM protein present in all tissues tested, including the cerebellum, cortex and heart. This was a good validation because in wild type mouse the protein DSCAM is absent in the heart.

Protein function-wise, in the previous *Dscam* loss of function study we observed induced aggregation of TH positive Dopaminergic amacrine neuron dendrites. At same time, clusters of melanopsin-positive retina ganglion cells were also observed. To restore the genetic dosage, we overexpressed the *Dscam* gene in the loss of function mice. The results demonstrated the aggregation of dendrites and cell bodies was resolved upon activation of the *Dscam*^{GoF} gene in LOF tissue. The rescue of *Dscam*^{LoF} confirmed that our new

Dscam^{GoF} transgene line produces functional protein and was an excellent model to study DSCAM protein in the nervous system.

An Overview of Findings

It's hard to take the brave first step. It is harder to stay on the right path afterwards. Once the *Dscam*^{GoF} mice colony was successfully maintained in our animal facility, we were ready to characterize the retina circuitry of the new mice. We set up an overall characterization analysis to keep us on the track amidst the vast information we could obtained through the new transgenic animal.

The overview analysis was done based on histological staining. The gold standard for histological analysis is Hematoxylin and Eosin stain. Once successfully stained, a permanent purple color would label the cell nucleus and a light pink color would label cytosolic region of the cell. Initial gross morphology indicated (Figure 7 B-D) that the retina of *Dscam*^{GoF} was thinner than wild type and with less cells in the inner nuclear layer (INL) and retina ganglion cells (RGCs). This was the first time we observed the opposite phenotype to *Dscam*^{LOF}, and it supported previous findings on the *Dscam* gene's role in regulating cell number.

With fewer neurons in the *Dscam*^{GOF} retina, many signal transduction pathways were likely severely impaired. Knowing that the mechanism reducing cell number was critical to the disease pathomorphology, I planned a full investigation on cell death and cell birth to tease out possible molecular mechanisms.

The reduction of thickness in the IPL of *Dscam*^{GOF} retina could have been partially contributed to by the reduction in cell numbers. Fewer neuron cells would drive fewer dendrites and axons; consequently less space would be needed for the IPL. However, we also observed irregular dendrites from the IPL projecting into the ONL. This indicated a cause besides cell number, as neuron projection and stratification were also altered by the overexpression of *Dscam* gene. Additionally, protrusion of photoreceptors into space normally occupied by the inner and outer segments was also observed (Figure 7 C figure arrows). The cluster of cell somas is mostly rod photoreceptors, with a small number of cones. This is possibly due to fewer cones being present in the mouse retina.

To systematically analyze how *Dscam* influence each cell type within circuitry, we planned to use immunohistochemistry to mark specific cell types. Immunohistochemistry is a staining process that uses species-specific antibodies to bind targeted proteins. The target protein could be expressed anywhere from the cell membrane to intracellular organelles to synaptic terminals. This advanced technique offers an in depth analysis based on cell morphology and the distribution of the protein content.

Besides changes in the retina, there were other interesting phenotypes upon activation of the transgene in the germ line. One noticeable effect on the overall health of the mice was that *Dscam*^{GOF} animals had a lower birth weight than WT. *Dscam*^{GOF} animals also experienced delayed development after birth, based on the growth of fur or other characteristics used to age mouse pups according to The Jackson Laboratory's aging criteria. The weight and size difference between wild type and *Dscam*^{GOF} male mice was steady to about 1 month of age (Figure 8 A). Once mice reach sexual maturation, wild type males and

females become different in size. Male *Dscam*^{GOF} adults tended to stay small compared to their wild type male litter mates, while female *Dscam*^{GOF} at P60 did not display a statistical difference to their female litter mates (Figure 8 B) and it is possibly due to pregnancy. Elder females caged separately were significantly smaller and had less body fat compared to WT, unfortunately not enough mice were collected at elder age for statistical analysis. In large litters, individual mice with *Dscam*^{GOF} germline overexpression tended to be severely smaller in size. This is possibly caused by competition for food resources.

Another interesting fact is that adult *Dscam*^{GOF} animals, upon dissection, had significantly less body fat than litter mates of the same age. I suspect this is caused by hyperactivity. Once the mice gained motility (as early as 4 days old), the *Dscam*^{GOF} animals displayed a high frequency of consistent circular motion (Supplemental video) while their littermates spent most of their time sleeping, eating and grooming each other with short periods of exploration. Clinically, animals displaying circular motion are due to infection in the inner ear, mutation of hair cells or a defect in the semicircular canal. These defects can disrupt an animal's ability to detect direction and give rise to their consistent circular motion as the animals try to walk straight.

We took a small detour to check out the morphology in the inner ear, but we failed to find significant results to explain such a phenotype and none of our evidence indicated any inner ear defect. While the neurological relevance remained interesting, to stay on focus we had to stop our investigation of this part of the animal.

In the beginning of the breeding setup, we had a difficult time maintaining the gain of function strain on the germline deleted background. Pups of mothers with germline

overexpression experienced a high fetal mortality rate. After continued observation, I found that most fatalities were due to their problematic feeding. Mothers were moving in a circular motion and constantly stepping on their young. Since males do not carry feeding duties, we decided to use only male *Dscam* germline overexpression animals to breed with wild type females. This change improved the survival of overall number of pups.

Histological analysis gave us a big picture of retina abnormalities, including reduced retina thickness, especially in the IPL, INL and RGCs, and displaced photoreceptors. $Dscam^{GOF}$ animals overall were smaller and experienced a slow growth rate with problems in sensing directions and possible hyperactivities. It led us to propose a more detailed assay of retina circuitry by using immunohistochemistry to look into cell morphology in a cell type-specific manner.

Decide a Path

The large amount of information from overall observation and comparison was exciting. Dr. Fuerst and I made a list of analyses to systematically test each interesting observation.

First, to confirm the histological data, a more detailed histological analysis was scheduled. We planned multiple time points for this study. From embryonic stage to infancy, and juvenile to adult, different ages would help us determine the developmental influence *Dscam* had on retina circuitry.

Since a decreased cell number was found in the $Dscam^{GOF}$ retina, which is complementary to all previous finding in $Dscam^{LOF}$ animal, it was not hard to believe

Dscam was involved in regulating cell numbers, but the mechanism remained unclear. To solve this question, I decided test the rate of cell birth and cell death by using specific markers in $Dscam^{GOF}$, $Dscam^{LOF}$ and WT retinas. Additionally, to tease out the role of cell death in the fundamental mechanisms, *Dscam* mice were crossed with *Bax* knock outs to eliminate developmental cell death process. The experiments and findings are in the Chapter 6 of this book.

Second, we planned a neuron spatial distribution analysis complemented with cell vertical organization and horizontal organization analysis. In a three dimensional world, an analysis with height, width and depth would help us gain an insight of how different neurons are spaced and how the Dscam gene influences this spatial pattern.

$Dscam^{LOF}$ retinas had increased cell spatial organization. We first tested if cell organization in $Dscam^{GOF}$ was decreased. Since the cell number differed between those genotypes, we had to internally control for a standard cell number to ensure the spatial distribution was not caused by the difference in cell number in the first place. This was achieved by crossing $Dscam^{GOF}$ onto a $Bax^{-/-}$ background. Both vertical and horizontal organizations were analyzed and the results can be found in the first few sections of Chapter 7 of this book.

Third, we planned a systematic analysis of retina circuity by cell type-specific antibodies. This would help us tease out *Dscam's* influence on different cells groups, including groups which naturally express *Dscam* and the groups which don't. It could also provide evidence of interactions between groups and how this type of interaction promotes changes in the retina circuitry.

In *Dscam*^{LOF} retinas, neurons displayed aggregation of the dendrites, and hence lost cell type-specific avoidance. We hypothesized that, opposite to *Dscam*^{LOF}, an extravagated avoidance would be present in the *Dscam*^{GOF} mice. Since abnormal lamination in the IPL was also found in an early histological analysis of the *Dscam*^{GOF} retina, we analyzed lamination markers and quantified many lamination defects to tease out how those defects were associated in a *Dscam*^{GOF} animal. This would explain a very fundamental question of the neuroscience: how neurite lamination is regulated. The result can be found in the last few sections of Chapter 7 of this book.

Fourth, we planned a synaptic terminals assay to explore how neurons interact with their partners under altered morphology using the out-placed photoreceptors. By testing synaptic terminal markers and using electron micrographs we could explain what happened when abnormal cells interacted with their normal partners.

Our electron micrographs were very detailed images acquired by a professional service on the scale of 7 nanometers per pixel. Put into perspective, a cell membrane is 15 nanometers in thickness, twice as thick as the resolution allowed to observe. Some of the terminals were misplaced with the photoreceptors. Those terminals, in some studies, were believed to be part of the pathology that needed to be treated. We carefully reconstructed those misplaced synaptic terminals using advanced 3D reconstruction software and measured several parameters, then tested their morphology in comparison to wild type.

Additionally, over time we found those intrusions were recovered. We thought this could be an indicator of recovery and/or regeneration within central nervous system in mammals. If that statement held true, the traditional dogma that mammals have very limited

recovery and regeneration ability would have to be reconsidered. The result can be found in Chapter 8 of this book.

With a plan in hand, we started to test each aim and expand the research based on the results we got. With the variety of phenotypes and the systematic results, we were able to gain tremendous insight into how *Dscam* influences neurons on cellular as well as a systematic level. The dynamics of biochemical functions never ceased to amaze me, and I learned that the morphology of the entire retina circuitry is capable of being altered by a single gene yet can maintain integrity through functional redundant genes and proteins.

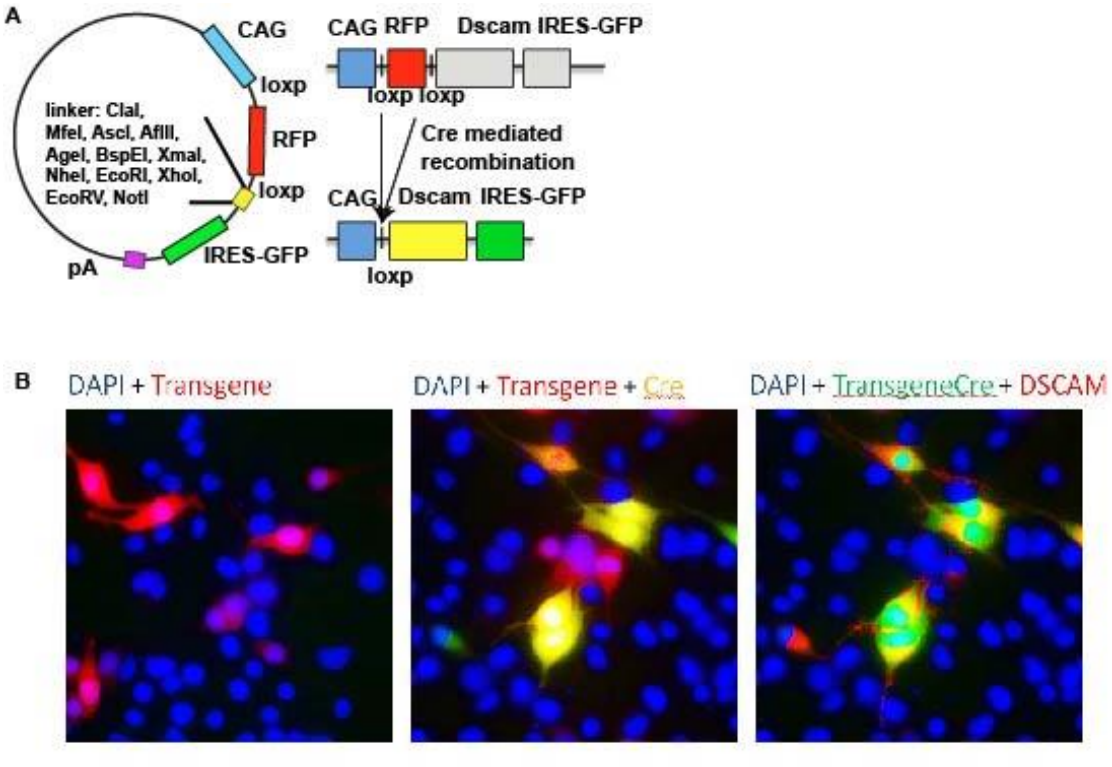

Figure 5 *Dscam* gain-of-function allele

A, A conditional gain-of-function allele of mouse *Dscam* was generated. **B**, Cell transfection of *Dscam* and *Cre* indicated successful activation and expression of the DSCAM protein.

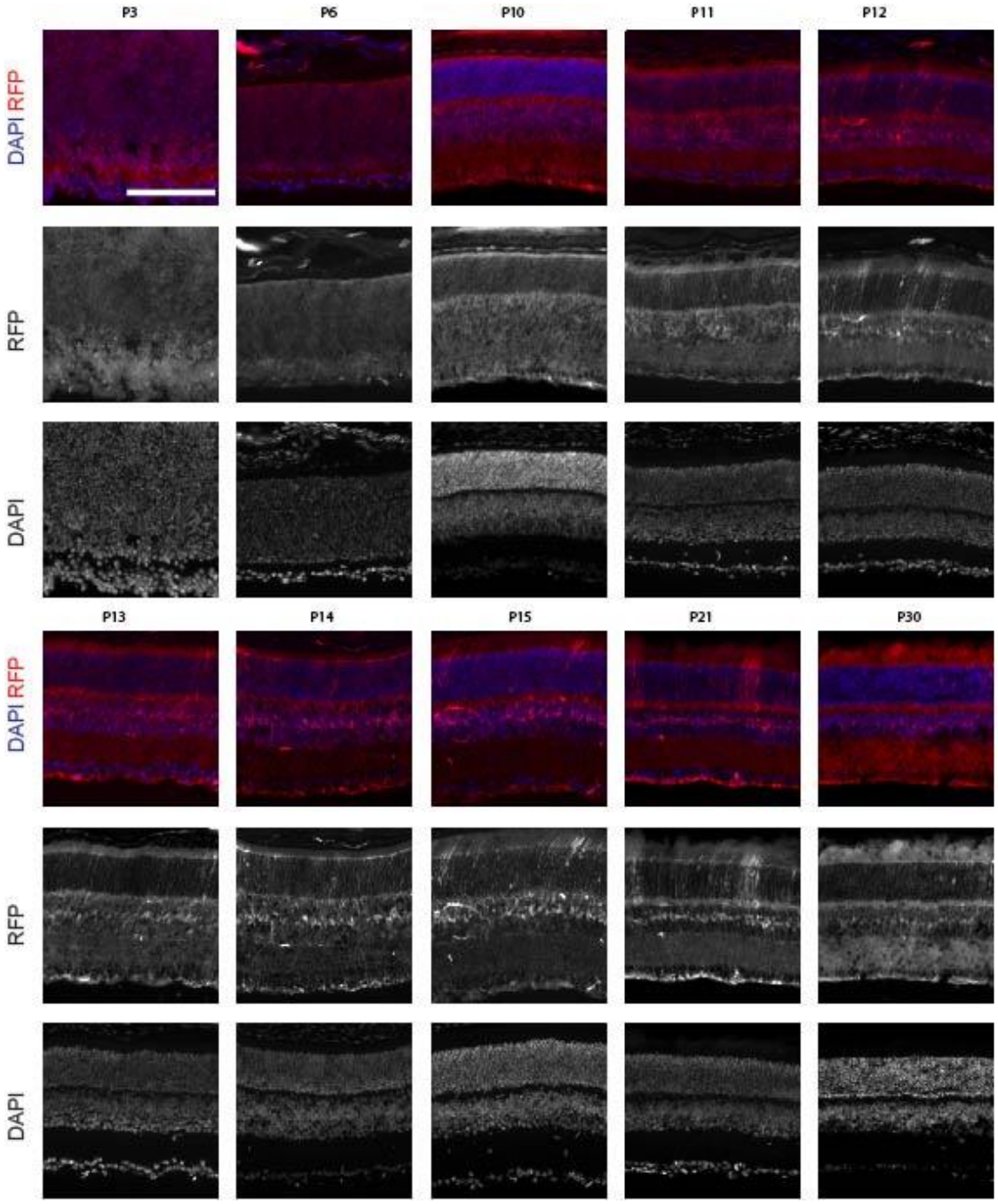

Figure 6 Transgene expressions through developmental age

The RFP is expressed throughout the retina in early age then intensively expressed in the interneuron from P10 and peaked at P15-P20. Scale bar is 200µm through all images.

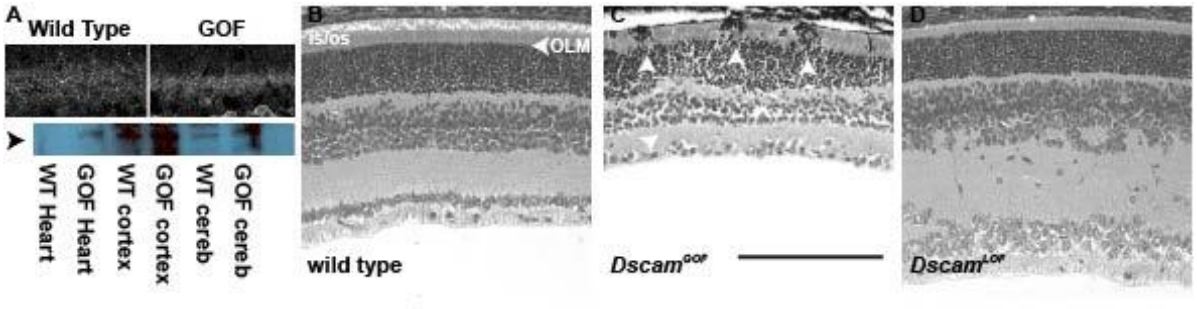

Figure 7 *Dscam* expression in the retina

A, Top, Immunofluorescence of the retinal IPL Where IPL is more intensively concentrated with DSCAM protein. Bottom, Western blot analysis of DSCAM protein in tissue and tissue lysates from wild-type and *Dscam*^{GOF} mice. **B-D**, H&E stained sections of wild-type and *Dscam*^{GOF} retina. Arrowheads in C indicate location of ectopic rod photoreceptors in the *Dscam*^{GOF} retina. Scale bar in C is 100µm in B-D.

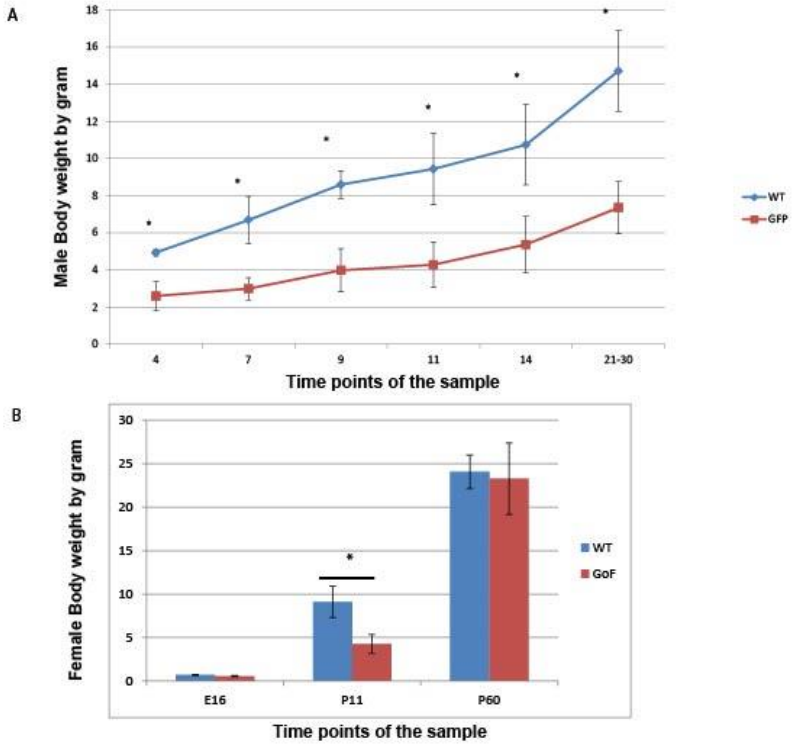

Figure 8 Mice weight associated with age and gender

A, During early development, male *Dscam*^{GoF} are significantly smaller in weight. **B**, Same effect was observed in female but at 2 month of age, female weight was not significantly different compared to wild type and is possibly due to undocumented pregnancy. (n>13 for male weight tracking and n>8 for female weight tracking, student-t test)

CHAPTER 6: Cell Death, the Good, the Bad and The Ugly

The word “death” usually reminds people of something unpleasant, degrading, sad, and opposed to life. In a beautiful ordinary world, nothing is immortal. All beings progress through the cycle of life—starting with birth, followed by growth and maturation, and hopefully reproduction, then death. Death in many cases is not a dark counterpart of life but rather a collaborative partner who improve the quality of life.

Deal With Death

How does death make life better? To understand this concept, let’s look at our body first. We are made of trillions of cells, and there are 10 times more bacteria that are not from our bodies but living on or within us at any given moment. All those cells have to go through life stages, as we know, but they are just microscopic in size, and we don’t notice them that much. Most cells in our bodies will grow, mature, duplicate, and die, but some neurons do stay alive without duplicating for the length of our life time and slowly degrade as we aging.

In our life span, cells are very likely to encounter and accumulate errors. When an error occurs as a result of mutation in some critical region of our gene, our bodies may not functional normally. For example, some genetic mutations can stall programed cell death in our body, resulting an uncontrollable growth of immortal cells. Those immortal cells would constantly grow and recruit all nutrients to themselves, starving the surrounding tissues. This uncontrollable growth of a tissue we generally call a tumor. It is highly possible at some

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point, tumor will loses its identity as a part of one tissue and start to spread all over the body. By then, we have a devastating problem known as cancer.

Cells go through cell death; it a normal process that happens every day, every moment, in order to repair and regenerate our body. The biological term for this regulated death is called “apoptosis”. This process is so vital for normal development of an organism that, without it, we wouldn’t be what we are.

We can easily find evidence of apoptosis in our bodies. All animals have evolutionary ancestors that lived in the ocean before they adapted to land. This is the why every animal’s embryo is bathed in a “liquid” environment-to mimic the ancient environment. In humans, we call this liquid where the fetus develops “amniotic fluid”. In the early stage of the embryonic development, we all have webbed fingers and toes. As development proceeds, the webs between appendages start disappear. This disappearance is caused by cells within that part of the tissue undergoing apoptosis. When apoptosis of those tissues is complete, we are then born with separated appendages.

If the developmental cell death didn’t occur or some steps went wrong during apoptosis, it would result a condition known as syndactyly, meaning webbed toes or fingers. The same apoptotic process happens during the development of the eyelid, which results eyelid separation and allows us to see. Interestingly enough, Down syndrome is also associated with syndactyly and polydactyly, even though specific mechanisms are still unknown[61].

While most of the cells in our body have to go through cell death, we still have those neurons that stay with us for the rest of our lives. Their consistent activities using chemical

electronic signals enable us to think, to remember and to be human. We hope those cells stay healthy during our existence. In classical literature, it is believed that neurons in our brain will not regenerate once damaged. Trauma, toxins, drug abuse, and lack of brain exercise all can induce neuron degeneration and the consequences are typically memory loss, decreased ability to think and hallucination[62].

Developmental cell death helps us grow and define many region of our body, but how exactly do our cells know what to do? How does each little microscopic organism without a brain knows what condition it is in? Do they even ask themselves “to be or not to be”? It took years and many experts from several different fields of science to explain what we know, and there is still much more that we don’t know.

Major Players in Cell Death

Many genes are involved in regulating the complex cell death system, including the famous p53 gene, Bcl family genes, caspases, PKC and cell stress signal genes like cJun and JNKs[63, 64]. Apoptosis can be triggered by many different internal or external cues, but the result, however, is always mitochondria death. Right after the degradation of mitochondria, a cascade of events leads to the release of cytochrome C, and, consequently, activation of Caspase 9. Caspases 9 then activates other caspases to cause DNA fragmentation and the formation of apoptotic bodies. Those apoptotic bodies contain pieces of old cell waiting for immune cells to clean up and recycle the cellular material.

In neuronal development, the exact mechanisms of how cells are selected to die are still unknown. Strong evidence suggests that neurons and neuro-progenitor cells have to compete for certain neurotrophic factors in order to stay alive[65]. Neurotrophic factors like neurotrophins[66, 67], class of nerve growth factors (NGF)[68], can sustain the survival of the cell when it experiences high levels of environmental stress. Others suggest developmental cell death (DCD) can also be regulated intrinsically by lineage, position and timing of the cell[69, 70].

Since my research involves developmental cell death, I will first introduce the well-known gene family *Bcl-2*(B-cell leukemia-2). This family regulates cell death by receiving stimuli from the environment. When a threshold is reached, the Bcl family is activated and directs cells to die.

One of the *Bcl-2* family members is the *Bax* gene, which is a pro-apoptotic molecule that induces cell death. *Bax* previously showed activation in response to IL-7 (a trophic factor) withdraw[71]. The activation of the BAX protein complex can initiate mitochondria membrane pore formation through a cytochrome c/IAPcomplex[72, 73]. Mitochondria are the energy factories of a cell; their death will certainly shut down the cell once for all. The release of cytochrome C from mitochondria membrane will further trigger escalating events leading to the attraction of macrophages to the apoptotic site.

The functionality of members of the *Bcl-2* family is depends on the presence of the *Bcl-2* homolog and BH3 domains or the lack of the BH4 domain[74]. Those domains are complex protein structures that act like an internet domains. Each website can only be visited when the right domain is typed into the browser. In terms of protein structure, only

when certain domain are present can the interaction between proteins can be executed and hence trigger cellular changes. Pro-apoptotic members in this gene family lack the BH4 domain, including the famous *Bax* and its counterpart *Bak*. Anti-apoptotic members in *Bcl-2* family can bind with others in the family to overcome their pro-apoptotic properties related to BH3 and BH4 domains.

From peripheral motor neurons to central hippocampus neurons, *Bax* is sufficient and necessary for the naturally occurring developmental neuron cell death[75]. Recent studies suggest *Bax* also plays a role in regulating homeostasis in neuronal ca^{2+} level[76]. This opened up a wide range of functionalities that this classical cell death gene could be involved with. Now we have a general understanding how *Bax* is involved in developmental cell death, but we are far away from knowing all of the mechanisms *Bax* is involved with in this process. Furthermore, *Bax* is not the only gene regulates programed cell death.

The second apoptotic family besides *Bcl-2* is the caspases family downstream of the apoptotic *Bax* gene. They are the executors of cell apoptosis. The discovery of caspases goes back to the study of *C.elegans*. One particular gene, CED3, in *C.elegans* has a homolog related to mammalian interleukin-1β converting enzyme (ICE or caspase-1) which did not show significant role in regulating cell death. However, the discovery of CED3 consequently led to the discovery of its large family of proteases—caspases[77]. This family of proteases can disassemble cells by a set of complementary methods: from promoting apoptosis to structural disassembling to protein cleavage. This process will stall the cellular function and disintegrate it from the inside. With a little help from immune cells, apoptotic cells will have all their parts recycled and used for other machineries in our bodies.

The third family is PUMA, the gene upstream of the *Bax* pathway. PUMA, in some text book known as BBC3 in humans, stands for p53 upregulated modulator of apoptosis. P53 is a gene famous for its ability to control tumors; hence it gained a nickname of "the tumor suppressor gene". PUMA is regulated by P53 and is a BCL-2 family binding component. It is able to free *Bax* and *Bak* from their dimer form, enabling them to further propagate the cell death signal[78]. Knock out of this gene will keep the *Bax* and *Bak* complex in its dimer form and lead to less cell death[79].

Bax and *Bak* are functionally redundant; only when both genes are K.O will a severe phenotype be produced in mammal tissue[80]. This level of regulatory redundancy is to ensure system stability when it encounters mutations on one of the genes. When this happened, a gene from the same family can fully or partially take over its role and maintain a degree of stability.

The genes listed above are a fraction of the genes involved in the complex cell death system. The complexity and redundancy is important to our system because different genes carrying similar functions is essentially a double safe mechanism to our bodies. Without this double safe mechanism, more diseases could be induced by a single genetic mutation.

Cystic fibrosis and sickle cell anemia are examples of diseases caused by single gene mutation. In cystic fibrosis, a single genetic mutation alters protein structure, preventing the function of the protein as a chloride pump[81]. Failure to pump out chloride ions in lung tissue can produce thick mucus and clog the air way and lead to death. Sickle cells are simply the result a structural change due to a mutation occurring on an important hemoglobin gene. This genetic mutation produces a long and inflexible protein chain,

causing red blood cells to turn into an inflexible sickle shape, reducing oxygen exchange and potentially clogging capillaries. These are examples of a lack of functional redundant genes and how devastating a disease we can have because of a single genetic error.

Developmental Cell Death in Retina

Like every other part of the body, Developmental Cell Death (DCD) also occurs in the retina. Among developmental biologists, there are some who have specialized in mouse study. They identified three waves of DCD in the mouse retina. The 'first' wave was described by Strongin in 1981, who studied embryonic neurogenesis. He observed cell death occurring apart from that associated with closure of the fetal fissure[81]. At this developmental stage, cells are not specialized into neurons yet, so mostly they are neuro-progenitors. Strongin proposed that the reason behind this wave of cell death is to clean up progenitor cells that migrated into inappropriate places.

The next wave of cell death in mouse is believed to be a result of competition for neurotrophic factors occurring five to six days after the birth of RGCs[82]. This wave of cell death generally refers to the official first wave of retinal DCD because there is observable excessive cell death in the retina from postnatal day 0 to 5. The last wave of cell death in mice is believed to be the result of retina refinement. In this stage, neurons are signaled to die when inappropriate synapses are made. This happens between postnatal day 8 to 15, and while the exact mechanisms are still unclear[83]. Published data illustrates that many cell adhesion molecules are involved in this process, helping signal and trigger DCD in cells making the inappropriate synapses[49].

The mouse is not the only animal to have systematic developmental neuron cell death in the retina. Chicks[84-86], frogs[87] and zebrafish[88] all display two to three distinct waves of cell death. Similar to mice, cell death in other animals is accompanied by the developmental process and maturation of retina ganglion cells, interneurons and photoreceptors.

The popular hypothesis of neurotrophic factors for retina cell death has been demonstrated in chick. Brain-derived neurotrophic factor (BDNF) regulates retinocollicular projections by the elimination of retina ganglion cells with the wrong projections[89]. Neurotrophins, one of the neuron growth factors (NGF), is capable of activating tumor necrosis factor P75 to cause neuron death[86].

In mouse, different retina layers are controlled by different cellular mechanisms. *Bax* plays an important role in regulating inner retina cell death[82, 90]. More importantly, Paul Hahn in 2000 with his group from University of Pennsylvania illustrated with double knock out mouse retinas that *Bax* and *Bak* null had an additive effect in cell death compared to single *Bax* null. Double null dramatically increased the thickness of the retina and reduced cell death occurring during development[91]. This experiment illustrated that the retina is an elegant circuity in the central nervous system and relies heavily on complex genetic regulations, such as the ability to maintain integrity through functionally redundant genes.

Bax gene in mouse retina cell death pathway is particularly interesting to me because knock out of this gene share many common characteristics with previously studied *Dscam* loss of function models. In $Dscam^{2J}$ and $Dscam^{3J}$ mutations, the retina had an increase in cell number; they also had an increase in overall retina thickness[50]. In contrast,

overexpression of *Dscam* resulted in the opposite phenotype compared to the *Dscam* mutations. Retinas from *Dscam*^{GoF} were thinner with much fewer cells occupying the RGC and INL. All those phenotypes suggested a close relationship between *Dscam* and cell death. By crossing *Bax* with *Dscam* gain of function or loss of function mice, we could further test if *Dscam* regulates cell death through *Bax*, the well-known cell death regulator.

Systematic Analysis of *Dscam* and *Bax* Regulated Cell Death

We assayed cell numbers through a developmental time point study of the *Dscam*^{GOF} retina. This could help us understand what histological defects were associated with *Dscam*^{GOF} animals. The following animal ages were selected to further support this part of the experiment: animals at embryonic stage (E15 and E18), right after birth (P0, P4), young adults (P12, P18), adults (1 month of age, 2 month of age) and old adults (from 4 month up to 1 year of age).

Overall morphological analysis in cell number revealed that wild type and *Dscam*^{GOF} retinas were similar before birth[92]. This suggested that *Dscam* overexpression was not sufficient to make many morphological changes before they normally become competent to do so (Figure 9 A and B). Shortly after birth, a decrease in the number of inner retinal cells became apparent in the *Dscam*^{GOF} retina compared to wild type, as seen by a decreased thickness in the RGL, IPL and INL (Figure 9 C and D). Photoreceptor intrusion occurred soon after animals opened their eyes (Figure 9 E and F) and the retina in *Dscam*^{GOF} maintained a reduced inner neuron population in old adult. Interestingly the photoreceptor intrusion was mostly recovered in the old adults (Figure 9 G and H).

The significant decrease in cell number lead to an interesting question: did *Dscam*^{GOF} directly influence cell death? If the answer is yes, it would be the first time an experiment proved all previous findings linking the *Dscam* gene to cell death in *Dscam*^{LOF} model. To answer this question, I needed to examine the correlation between cell numbers and possible cell death pathways. Therefore, I employed six genetic combinations and quantified cell numbers in the retina by nuclear staining (DAPI or Drag5) at P18 days of age. Those genetic crosses were set to tease out any possible mechanisms associated with *Bax* influencing the cell death pathway in the retina: *Dscam*$^{LOF/+}$, *Dscam*^{LOF}, *Bax*$^{-/-}$, *Dscam*^{LOF}*/Bax*$^{-/-}$, *Dscam*^{GOF} and *Dscam*^{GOF}*/Bax*$^{-/-}$.

First, in heterozygous *Dscam*$^{LOF/+}$ there was an increased cell population present in the IPL. This phenotype was consistent in *Dscam* homozygous and *Bax* $^{-/-}$ retina. Both *Dscam* homozygous and *Bax*$^{-/-}$ showed a thicker retina, and in *Dscam*^{LOF}*/Bax*$^{-/-}$ the retina was thicker than any of the single gene knock outs, and retina organization for each cell layer was severely disrupted (Figure 10 A-G). *Dscam*^{GOF} however, had a significant reduction in retina thickness with much fewer cells present. Interestingly, when *Dscam*^{GOF} was crossed with *Bax*$^{-/-}$, the overall organization was restored and the retina thickness was relatively normal.

A retina's organization can heavily influence the functionality of the system. Therefore, we investigated the disruption in each retina layer. One of the abnormalities was the presence of cells in the inner plexiform layer (IPL). The IPL plays an important role in the exchange of synaptic signals from interneurons to the retinal ganglion layer. Normally the synaptic space is only occupied by neurites; however, with reduction and/or elimination

of BAX and/or DSCAM protein, those intercellular spaces were occupied by misplaced neuron bodies (Figure 10 C-E red arrow heads).

This defect suggested there could be a failure in cell migration or a failure in the retina neuron refinement process. Statistical data indicated loss of *Dscam* or *Bax* led to a significant increase in the number of neurons located in the IPL. Elimination of both genes led to a significant additive effect and the accumulation of more neurons in the IPL than single gene knock out. Meanwhile, crossing $Dscam^{GOF}$ to $Bax^{-/-}$ rescued the misplaced neurons phenotype in the IPL (Figure 10 H). This revealed that DSCAM and BAX are functionally similar proteins in regard to regulating cells in the IPL, eliminating those misplaced neurons during early retinal refinement.

Different retina layers are occupied by different types of neurons. 3 of the layers are nuclear layers: the Outer Nuclear Layer (ONL), Inner Nuclear Layer (INL) and Retina Ganglion Cell layer (RGC). The ONL is where photoreceptors are located. The INL is where interneurons are located, including bipolar cells, horizontal cells and amacrine cells. The RGC is mainly composed of RGCs and some groups of amacrine cells. We assayed cell number in the three nuclear layers of the retina. Our statistical data indicated no significant difference in ONL cell number when comparing any of these genotypes to wild type (Figure 10 I, ONL; wild type cell number values are provided in the figure legend. Misplaced photoreceptors in the $Dscam^{GOF}$ subretinal space were not included in counts). This was consistent with the observation that *Bax* does not regulate the outer retina[90].

However, in the inner retina a significant difference was detected. We found an increase in cell number in the $Dscam^{LOF}$/ $Bax^{-/-}$ retina compared to the $Dscam^{LOF}$ or $Bax^{-/-}$

retina (INL). Further statistical data indicated a decrease in cell number in both the INL and RGC was found when comparing *Dscam*^{GOF}/*Bax*$^{-/-}$ retina to *Bax*$^{-/-}$ retina (Figure 10 I, INL and RGL). Our results indicated that eliminating either *Dscam* or *Bax* gene would reduce cell death and result in higher cell numbers in the inner retina, and eliminating both genes would grant an additive effect. Knock out one gene but overexpression of another restored the cell balance, which suggested that *Dscam* and *Bax* gene can regulate cell death independently in a dosage dependent manner. That different genes regulate similar functions strongly suggests that the nervous system has many built-in double safe mechanisms to protect us from single gene mutations.

A significant rescue in cell number was found when comparing the *Dscam*^{GOF}/*Bax*$^{-/-}$ retina to the *Dscam*^{GOF} retina (Figure 10 I). This suggested that although *Dscam* can influence cell number independently of *Bax*, *Bax*-dependent cell death is still the main driver of *Dscam*-mediated changes in cell number in the *Dscam*^{GOF} retina, so when we implant *Bax* gene under the overexpression of DSCAM, *Bax* can prevent DSCAM induced cell death. These results are consistent with findings that DSCAM promotes cell death by acting through BAX and at least one other cell death pathway, and this additional pathway is sufficient to decrease disorganization by eliminating misplaced cells.

Too Much Death or Too Little Birth?

After we validated cell numbers in the retina, one question remained uncertain for the *Dscam*^{GOF} retina: the true mechanism of reducing cell number. There are three possibilities for a reduced cell number in the retina: an increase in cell death, a decrease in cell birth, and the combination of both, which could be more complicated and much harder

to iron out. Therefore, to follow up with my cell number analysis, I tested whether or not cell birth took part in this numerical change. Phosphate histone 3 is a biomarker for cells goes through cell division, which can be used to identify the birth of new cells. TUNEL is the gold standard for determining which cells are experiencing cell death. It is an immunohistochemistry reagent which labels DNA fragmentation during apoptosis. By complementing TUNEL with H3 in different developmental stages, we were able to identify the cause of reduced cell number.

Out results indicated cell birth was not different between wild type and *Dscam*^{GOF} at any given age at inner retina (Figure 11 A B and E). However, cell death became extremely potent in *Dscam*^{GOF} retina after birth (Figure 11 C D and E).

This result confirmed that *Dscam* was involved in the developmental cell death process rather than cell birth and can have a significant impact on related human health. Beside the analysis done based on retina layers, I also performed cell type based tests. My results further confirmed that most cell types in the inner retina go through cell death when *Dscam* is overexpressed, except for Dopaminergic Amacrine cell type I. This cell type also remains the same population in Loss of function study (table 1).

Through our comprehensive analysis and tests we concluded *Dscam* was sufficient to drive cell death. This cell death mechanism was accomplished with *Bax,* in a cell subtype specific population of the inner retina. Both genes regulate one cell death pathway like a double safe mechanism. In other cell populations, *Dscam* was involved with a non- *Bax* gene to direct cell death. Outer retina cell number was not influenced by *Dscam* or *Bax,* indicating outer retina circuitry is independent from *Dscam* and *Bax* in the developing retina.

This type of genetic redundancy dramatically improved the stability of the neuron circuitry. Instead of calling it redundancy, I would recommend using the term ***built in double safe mechanisms*** of the nervous system.

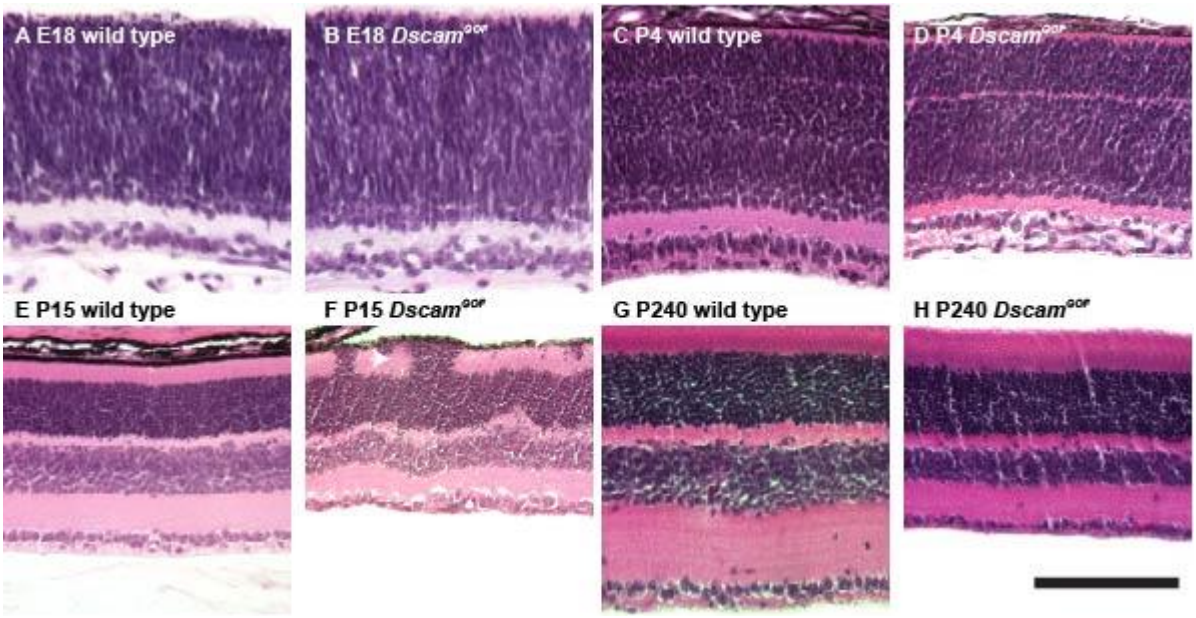

Figure 9 DSCAM promotes developmental cell death

Wild type and *Dscam*^{GOF} retina sections were stained with hematoxylin and eosin. **A** and **B**, Differences between the wild type and *Dscam*^{GOF} retina were not detected at embryonic stages. **C** and **D**, After P1, differences between the wild type and *Dscam*^{GOF} retina become apparent, most prominently a decrease in the number of cells in the inner retina and a decreased thickness of the IPL (shown at P4). **E** and **F,** Aggregates of rods are observed by P11 in the outer retina, where the outer segments of photoreceptors would normally develop (shown at P15; arrow). **G** and **H**, Sections of wild type and *Dscam*^{GOF} retina at eight months. Ectopic localization of photoreceptor nuclei resolve in the *Dscam*^{GOF} retina over time. Scale bar in **H** is 120μm in all images.

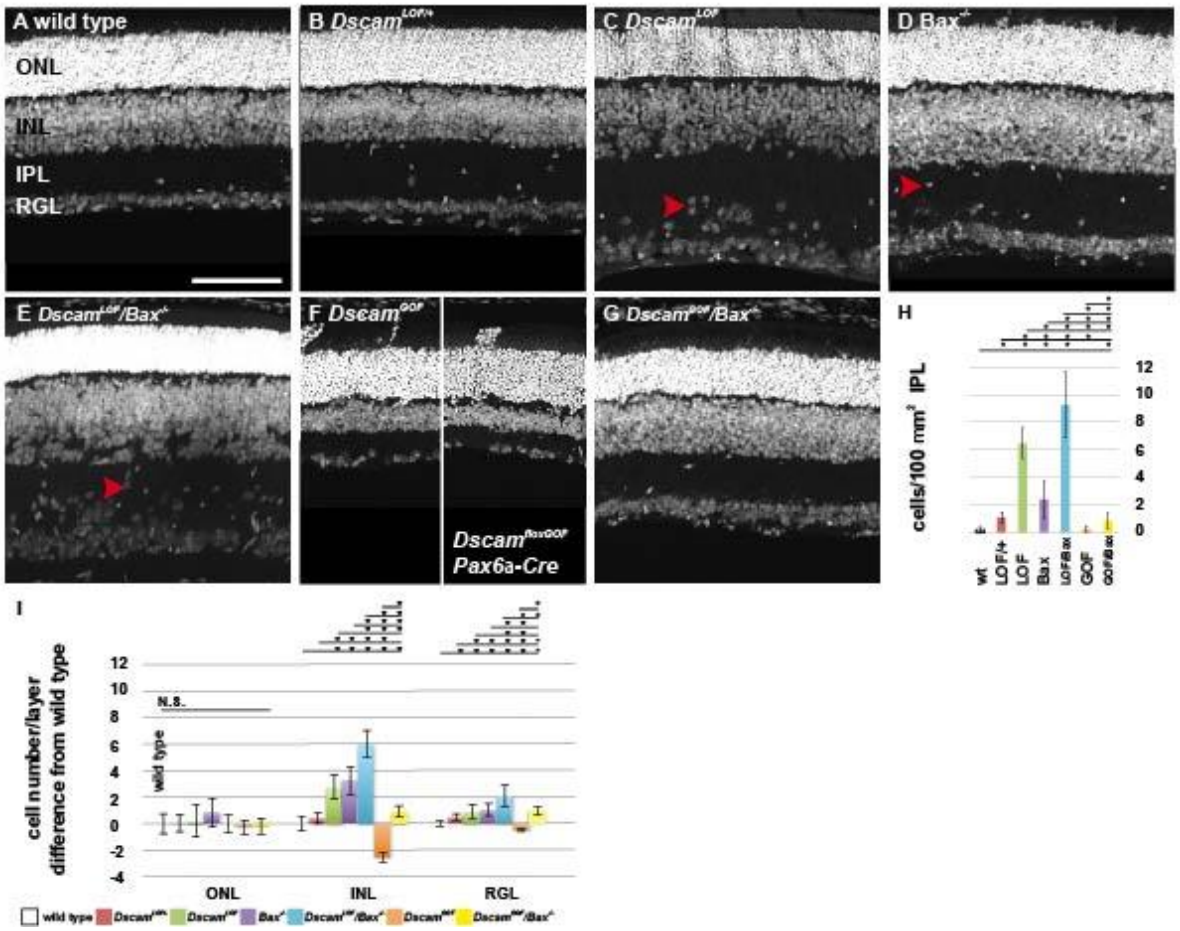

Figure 10 DSCAM regulates cell number in Bax-dependent and -independent manners

A–G, DRAQ5 stained retina sections: wild-type (A), *Dscam*$^{LOF/+}$ (B), *Dscam*^{LOF} (C), *Bax*$^{-/-}$ (D), *Dscam*^{LOF}/*Bax*$^{-/-}$ (E), *Dscam*^{GOF} (also depicts *Dscam*^{floxGOF} targeted with Pax6_-Cre; F), *Dscam*^{GOF} / *Bax*$^{-/-}$ (G). **H**, Quantification of displaced neurons in the IPL. **I**, Quantification of cell number in retinal layers. Values are plotted as average difference from wild-type. Wild-type cell number in ONL: 10.5±0.08, INL: 6.64±0.53, RGL:1.17±0.18. RGL: retinal ganglion layer. P18 N>3 for all genotypes. Scale bars: (in **A**) **A–G**, 100µm. Mean_SD; *p≤0.01 (Student's t test).

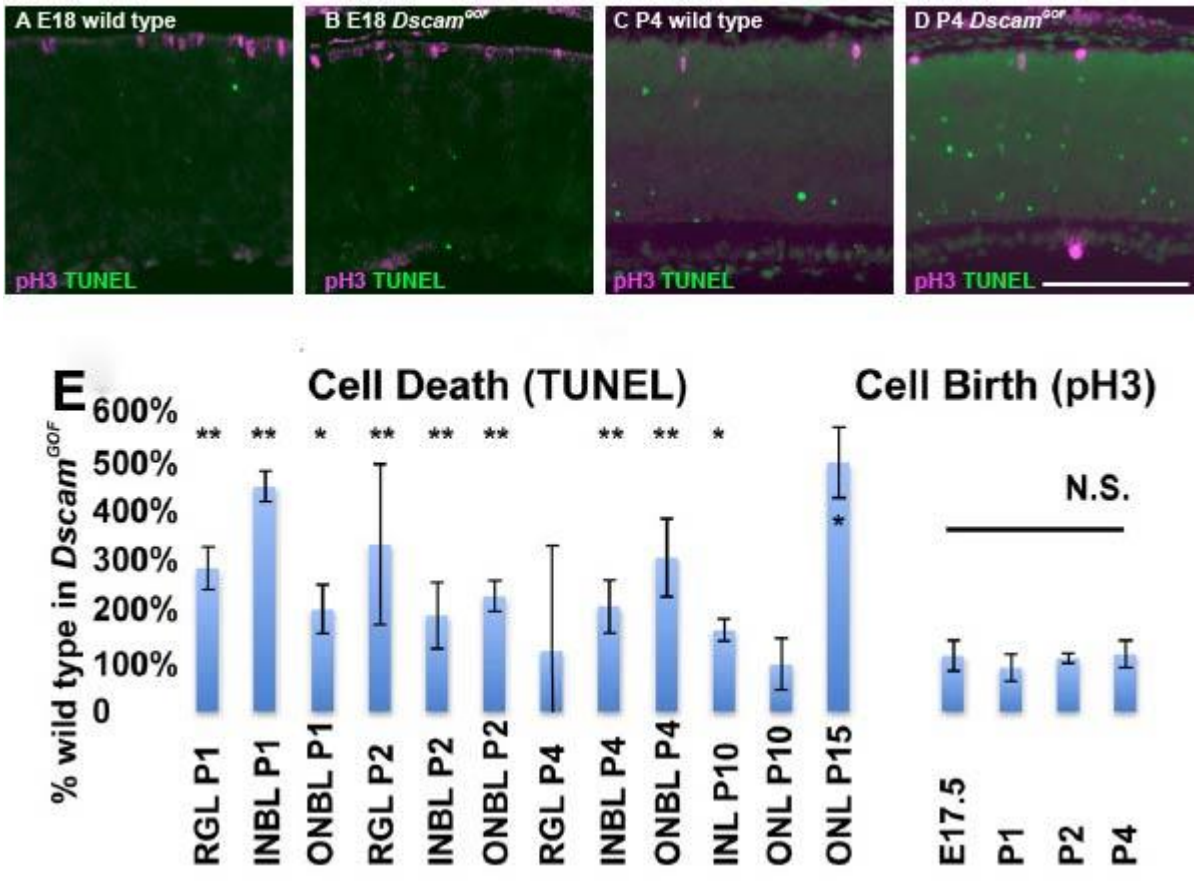

Figure 11 Tunel Analysis

A-D, Sections of wild type and *Dscam*^{GOF} retina were stained with antibodies to phospho-histone H3 to detect mitotic cells, and with TUNEL staining, to detect apoptotic cells. **A** and **B**, Only rare examples of apoptosis were observed during embryonic stages and no significant difference in the number of mitotic cells was detected. **C** and **D**, An increase in the number of apoptotic cells was observed in the *Dscam*^{GOF} retina compared to wild type. Cell death in the *Dscam*^{GOF} retina was observed in cell populations at similar time points to those at which cells undergo DCD in the wild type retina. The scale bar in (**D**) is equivalent to 100 µm.

Table 1 Cell death and birth in the retina

Table 1 Cell Death and Birth				
Age	TUNEL+ % WT	p-value	pH3+ % WT	p-value
E17.5	N.N.O.	N.A.	111%±30%	0.48
RGL P1	284%±42%	**0.0007****	87%±27%	0.33
INL P1	446%±30%	**2.1E-6****	N.A.	N.A.
ONL P1	203%±48%	**0.04***	N.A.	N.A.
RGL P2	331±159%	**2.9E-6****	106%±10%	0.49
INL P2	190±65%	**3.22E-5****	N.A.	N.A.
ONL P2	229±30%	**7.9E-8****	N.A.	N.A.
RGL P4	121±66%	0.37	114±27%	0.22
INL P4	208±52%	**2.0E-6****	N.A.	N.A.
ONL P4	305±77%	**0.005****	N.A.	N.A.
RGL P10	N.N.O	N.A.	N.N.O.	N.A.
INL P10	162±22%	**0.05***	N.A.	N.A.
ONL P10	94±51%	0.85	N.A.	N.A.
RGL P15	N.N.O	N.A.	N.N.O.	N.A.
INL P15	N.N.O	N.A.	N.A.	N.A.
ONL P15	493±70%	**0.02***	N.A.	N.A.

N.N.O. Negligible Number Observed N.A. Not Applicable
* Significant ** Highly Significant
The number of apoptotic and mitotic cells were counted in at least four sections from three or more retinas and expressed as the percent of cells being born or dying compared to wild type. P-values were generated by comparing the number of mitotic or apoptotic cells per length or volume of retina using a Student's *t*-test. Volume was used in order to normalize for the thinner *Dscam*^{GOF} retina at later time points.

CHAPTER 7: What is More Important Than Cell Death?

Salesmen prioritize wealthy individuals, making sure capture the biggest wallet. Athletes focus on physical training to win a competition. Prioritizing matters based on relevance and importance directly impacts our productivity and allows us to quickly approach goals. From a biological perspective, survival is the top priority. Cell death, of course, plays an important role in animals' development, fitness and survival. What if there are other elements that are more critical than cell death for animals' survival? To find out the answer, we will take a short trip to uncover the fundamental principle of form and function in neurological circuitry.

Form Follows Function

There is an explanation behind every physical form and chemical property. Those innate characteristics are determined by fundamental principles of the universe. Those principles can only be understood through detailed observation and abstract thinking. We call those carefully examined thoughts concepts, and complex concepts are always built on the simple ones such as the relationship between form and function.

For example, before humans understood gravity, we could not comprehend the idea of the earth not being flat. The surface of earth was thought to serve a single function that reflects the egocentricity of human nature: it allows all creatures to walk on it; therefore, it must be flat. With the advancement of the science, we finally realized the shape of the earth has nothing to do with us at all; its form simply reflects the property of the gravity.

Stars and planets are almost spherical because the gravity from other matters push and pull them from all directions in the universe. Wheels are the most efficient shape for

vehicles because at any given moment it only has to overcome a small friction from a minimal surface area that adhesion to the ground. Feathers are soft, light weight and water resistant; those are properties enable birds to fly. Form follows function— this is a concept well tested and proven by time.

Synapses are neurological connections between neurons. They occur at tree branch-like structures called dendrites and axons, which are the extension of cellular cytoplasm of the neuron. The right branching and connection pattern can maximize the speed of neurological signal and generate correct responses to stimuli. Without a clear organization and the right connection between neurons, we would not be able to see, to think, to feel as human beings.

Many psychiatric and neurologic diseases are companied by synaptic abnormality. Fragile X syndrome, for example, is an inherited genetic condition resulting in mental retardation, and most individuals meet the criteria for autism. Studies indicate that Fragile X individuals have an increased number of brain dendritic spines which display an immature structural phenotype[93, 94].

Autism individuals were found to have genetic mutations that changed the cell adhesion system of Neuroligins and Neurexins[95, 96]. This system is required for processing complex signals in synaptic neurotransmission. Failure of this system promotes the likelihood of developing cognitive diseases[97].

Alzheimer’s became a hot topic in the last few decades as the aging population becoming a significant social issue, and this aging trend will likely continue to rise over the next several decades. Alzheimer’s is caused by the accumulation of proteins between

synapses. This accumulation eventually leads to synaptic failure and neuron degradation[98]. Other disorders like Down syndrome can also cause neuron degeneration; individuals with Down's suffer a 40% reduction in synapse connections in their brain neurons compared to healthy individuals[99]. These examples illustrate the importance of synaptic activity and it's critical role in normal neurological function.

Synapses in the retina are the reason we can see, and the retina architecture has been formed over billions of years of evolution. The complexity is hard to describe with my limited knowledge, but in general the system is very sophisticated. If a wrong connection is established between two neurons, one of the contacts will either retract or the cell will be signaled to die. This process of dendritic retraction during development is known as neuron refinement. In the retina, two synaptic layers contribute to a majority of information exchanges, known as IPL and OPL. Without developmental refinement of dendritic projections, those layers become a terrible traffic disaster for visual signals.

Spacing Follows Horizontal Organization

The connectivity and organization of many neurons is influenced by DSCAM. In previous fruit fly studies, *Dscam* was found to be required for specific dendritic pairing and avoidance. This mechanism is made possible by a complex controlling system composed of more than 38,000 splice forms of *Dscam* in the fly's genome[100]. Without *Dscam*, fly dendrites failed to avoid themselves, resulting in neurite aggregation and disrupted spacing. In mouse, *Dscam* only has two isoforms. Without the diverse isoforms like its homolog in insects, the *Dscam* gene act as a self-recognition module, with help from the DscamL1 molecule and possibly other adhesion proteins, rather than contributing to specific dendritic

paring in the nervous system[47, 101, 102]. This type of recognition and avoidance causes neurons within a network to form a well-organized spatial distribution (neuron spacing).

Neuron spacing is one characteristic used to analyze abnormality of a neuro-circuitry. In general, a dense neuron distribution throughout the retina space indicates small dendritic coverage but high information processing volume. A scattered distribution indicates one type of neuron has to cover a large area with their dendrites and it's more likely that each neuron handles less information density. This spatial distribution helps us to understand the different neuron types and their active roles in the neurological circuitry such as lateral inhibition, lateral propagation and filed sampling.

Neuron spacing is critical in the visual system because photoreceptor spatial distribution directly influences our ability to see. During the day time, images are focused and clean in the center of our visual field, but blurry in peripheral. Cones, one of the two types of photoreceptors, are sensitive to color wavelength and concentrated in the middle of the retina in a region called fovea. The fovea gives us a high cue of color vision during daylight, when light source is abundant. At night we can see the stars and moon with high clarity even in the peripheral of the visual field. This effect is due to rod photoreceptors, which is sensitive to small amount of light and scattered throughout the retina to give us good night vision across the entire vision field.

Mice through millennia of evolution have adapted underground living habits and are mainly active at night to avoid predators. Smelling and hearing have become their dominate sensation. Vision, on the other hand, is adapted to a dim environment due to the time of their active period, and their retina is mainly composed of rods. However, due to the shared

lineage of mammal visual systems, the downstream signal transduction is almost identical between humans and mice.

Dscam mutations in the mouse retina result in a severe aggregation of neurons and alters normal mosaic spacing, including that of dopaminergic amacrine cells and RGC[47, 101]. This spatial disruption is both somatic and dendritic. Opposite to $Dscam^{LOF}$, in the $Dscam^{GOF}$ study, the retina indeed also showed a disruption in spacing. However, both $Dscam^{LOF}$ and $Dscam^{GOF}$ have a defect in cell number, and previously this factor was impossible to control. However, crossing $Dscam^{GOF}$ on $Bax^{-/-}$ background could stabilize cell numbers in the retina. We could then control neuron population internally, and achieve an elegant experimental design unlike any other.

With this phenomenal genetic cross, we could testify that cell spatial organization is directly influenced by the *Dscam* gene without a change in cell numbers within the retina circuitry. Previously we found through DRP, NNA and VD analysis, that starbust amacrine cell (SAC) in $Dscam^{GOF}$ retina had a decreased spatial organization in correlation to cell number (Figure 12 A and B). *Bax* null retinas increased this organization (Figure 12 C), and once cell number was maintained at the wild type level by $Dscam^{GOF}$ $Bax^{-/-}$, expressing extra dosage of DSCAM protein failed to induce the same spacing defect (Figure 12 D and E)

Next we assayed horizontal cells—a type of neuron that plays a major role in lateral inhibition of the visual signaling. Its population remained stable in both WT and $Dscam^{GOF}$ animals. We failed to see an increase in spatial correlation of cell somas; instead we found a small decrease in NNRI. HC dendrites also maintained a normal overlap compared to the

wild type (Figure 12 F, G and H). Because SAC and HC do not express *Dscam* naturally, we conclude that ectopic expression of *Dscam* in those cells is not sufficient to change cell spacing and organization.

What about the cells that do naturally express *Dscam*? What would happen to their spatial organization when extra dosage of *Dscam* protein was induced? We tested type 1 DAC (TH), which express and require *Dscam*, and AII ACs, which express and require *Dscam* L1 for their normal spacing and arborization [47, 101]. In term of dendrites, in $Dscam^{LOF}$ we observed aggregation. Based on this observation *Dscam* was believed to serve as avoidance in the dendritic function, but in $Dscam^{GOF}$ we failed to induce over avoidance in AII ACs. Their synaptic contacts were still continued between Type 1 DAC and AII ACs in the $Dscam^{GOF}$ retina. Therefore, even though *Dscam* is required for normal spacing and dendritic arborization, additional dosage of *Dscam* does not prevent cell type—specific interactions or promote avoidance (Figure 12 I-M)

Interestingly, *Bax* is a known requirement for normal spacing for type 1 DACs, but by crossing $Bax^{-/-}$ with $Dscam^{GOF}$ the spacing defect was not rescued[52]. Instead, both $Bax^{-/-}$ and $Bax^{-/-}$ $Dscam^{GOF}$ shared similar NNRI to random distributions. AII AC did not alter spatial organization even they have a reduced cell number induced by *Dscam* (Figure 12 N-P). All of our data suggested that the possible roles in $Dscam^{LOF}$ study do not have the opposite defect in $Dscam^{GOF}$. Extra dosage of *Dscam* does not induce horizontal spatial disruption— it's only a secondary effect due to the change in cell number.

Migration Follows Vertical Organization

Upon the birth of neuro-progenitor cells in the early stages of the embryo, cells with a particular fate will enter a migration path through the retina to the right location. This migration process is critical for functional circuitry to form. A failure in this process would potentially influence the function of the entire retina.

With the help of more than 30 antibodies and many months of work, I was able to systematically analyze the development of different neuron types in different genetic crosses over time. The analysis included their pattern of migration, stratification and synaptic formation.

Under the influence of the ideology "no phenotype no paper," my third year of grad school was spent identifying interesting abnormalities in the retina. One part of this analysis was a 3 time point immunofluorescent study. The data revealed an intriguing defect that was not documented previously, but was clearly associated with *Dscam*. Time points were set at postnatal day 10, 15 and adult (8 months of age). At P10, some neuro-circuitries have not completely developed, but the retina layers are clearly separated. P15 is the time by which most neuron circuitries are developed, and in adults the retina circuitry is matured and the aging process is already taking place. I will describe those findings in sequential orientation from outer retina to inner retina.

Cone arrestin is a cone photoreceptor marker. At P10, in both WT and $Dscam^{GOF}$, we can see that cones are still migrating to their corresponding location (Figure 13A and B). When the mouse reaches P15, most cones are located in the right layer in the WT retina. In the $Dscam^{GOF}$ retina, many neuron somas are stuck near the OPL (Figure 13 C and D). In

old adults, cones in both WT and *Dscam*^{GOF} become relatively normal again (Figure 13 E and F). PNA labels cone pedicles at OPL, an area where cone axons communicate with downstream bipolar cells. In *Dscam*^{GOF} retinas, there was a clear mis-localization at the young age. This was possibly due to the delayed retina development. The older *Dscam*^{GOF} retina did not display disrupted pedicles, and I speculate that this is due to the process of retinal refinement. Both staining suggested a level of delayed development and illustrated a potential of neural plasticity in mammals that has not been seen before.

As we previously saw in H and E staining, there were out-placed photoreceptors in the *Dscam*^{GOF} retina. Since the cones did not show a severe displacement, I selected the rod cell marker recoverin (it also labels dime type 2 bps) to further tease out the abnormality. The recoverin stain indicated that at P10 the outer limiting membrane (OLM) in both genotype is normal (Figure 14 A and B), but by day 15 rod somas severely intrude through OLM. Additionally, a few bipolar cells, labeled with Chx10, were out placed in the ONL (Figure 14 C and D). However, in adults, this phenotype was strangely resolved, and, overall, fewer bipolar interneurons were present in the *Dscam*^{GOF} retina (Figure 14 E and F, table 2). This could be the result of the refinement process or a degree of plasticity that occurs once neurons migrated to the wrong place.

After noticing the change in bipolar cell population in the previous Chx10 staining, we further assayed sub-type specific bipolar cell number and morphology. PkaRIIb is one of the markers we used; it labels type 3B bipolar cells, which naturally expresses *Dscam*. We observed a reduced cell number in this population in *Dscam*^{GOF} retina throughout the age points, and this reduction was not recovered in adults (Figure 15, Table 2). Type 3b bipolar

cells that survived did not project their axons deep into the IPL next to RGCs like their wild type litter mate controls (Figure 15 C-F arrow heads). This bipolar cell phenotype suggested that overexpression of DSCAM protein in *Dscam* expressing interneurons could lead to a defect in axonal targeting.

Another BP marker is Pkcα, which labels rod bipolar cells that do not express *Dscam* naturally. This cell type's dendrites have the tendency to project towards the ONL in $Dscam^{GoF}$ retina (Figure 16 A-F, Table 2), and a strange axon terminal patterning was observed at old age that formed holes instead of an even distribution like in the WT (Figure 16 G-H). We did not investigate the physiology correlated this particular phenotype, but it would be additional evidence address the issue of non- *Dscam* neurons being influenced through non-autonomous interaction with their neighbors.

Calbindin is a horizontal cell marker labeling horizontal cell also labels some amacrine cells. At the early age, the amacrine cells did not brightly express calbindin compared to litter mate controls. The most severe intrusion of the horizontal cell dendrites occurred by age 15 days old, then slowly resolved with aging. (Figure 17 and table 2) This was not the only population that altered its neuron targeting upon OLM leakage. Markers like TH, PSD95 and dystroglycan all showed the same trend where synaptic connections followed the misplaced photoreceptors. This result suggested that *Dscam*, besides regulating cell death in the inner retina, also plays important role in maintaining retina integrity during a critical period (P10-young adult) and is capable of influencing cell migration and neuron stratification in the outer retina. After this critical and rapid developmental period in the retina, *Dscam* may not be essential to the maintenance and plasticity of the nervous system.

The separation of development and maintenance in the nervous system could lead to the new insight to understand how our nervous system is regulated.

The consistent observation regarding reduced IPL in *Dscam*^{GOF} could be a secondary effect due to lose of neuron population in the first place. Many neurons are secreting or accumulating neuronal factors to attract dendrites and/or guide axons. It's my pure speculation with less neurons present in the inner retina, neural factors become less available. Compensating the functionality required for axon/dendritic stratification, a plexiform layer is scaled down to maintain certain level of neuronal factor concentration. This could explain the *Dscam*^{LOF} had more axons/dendrites projecting into the IPL and result a thicker retina. Even though many neuronal factors are not directly linked with *Dscam*, it's logical to believe their secretion is directly influenced by the number of cells available to produce them.

Stratification Follows Double Safe Mechanism

It is comforting to know that after early development we still have a chance to repair our nervous system. This level of maintenance is not influenced by early developmental genes. Down's syndrome is not lethal and many individuals with this condition are alive and suffer relatively mild symptoms. This is possible because our bodies are protected by many genetic mechanisms, some of which act like double safe mechanisms. This is to help avoid severe damage and failure of the organism caused by a single genetic failure. There are two ways to accomplish this goal: first multiple genes regulate one pathway and second, different pathways with similar biological functionality.

Bax and *Dscam* are two genes that redundantly regulate neuron lamination. Traditionally the IPL lamination pattern was described and analyzed as five conceptual "layers" by Cajal, who pioneered the convention of subdividing the retina's IPL (Figure 18 A). This method of dividing the IPL into strata S1-S5 became a widely accepted method to help distinguish different neuron populations based on the strata their neurites occupy. Later, some researchers adopted a 10 strata model[103][103][103][103][103][103][103][103][103][103][103][103][103][103][103][103][103][102].

In a WT retina those stratum have clear boundaries and consists of its own target, but in *Bax* and *Dscam* deficient mice, the boundaries became unclear, which likely indicated genetic functions in regulating neurite patterning.

In previous study, we found abnormalities in the IPL of both $Dscam^{GOF}$ and $Dscam^{LOF}$. The $Dscam^{GOF}$ IPL was thin, and substratum could not be distinguished clearly. TH, bNOS and vglut 3 are three amacrine cells markers which showed a reduction in lamination spacing of the IPL compared to WT control (Figure 18 B-E). When we induced $Dscam^{LOF}$ into the $Bax^{-/-}$background, the IPL was thick and severely disorganized compared to single null retinas. The dendrites and axons did not follow a well-defined boundary for their cell types.

In order to quantify the comprehensive genetic influence on IPL lamination pattern in single mutant and double mutant, we designed a uniformed method with empirical formulas to define sub-layers and boundaries without subjective decisions that relied on

experience. Each dendrite sub-layer distribution was calculated through intensity distribution analysis and averaged across samples (Figure 18 F).

bNOS lamination looked disrupted in *Bax* $^{-/-}$, but my data suggests there was no significant intensity difference in distribution between *Bax*$^{-/-}$ and WT. *Dscam*^{LOF}, *Dscam*^{LOF} *Bax*$^{-/-}$, and *Dscam*^{GOF} *Bax*$^{-/-}$ all showed a significant difference in bNOS dendrite distribution (Figure 18 G. * in the graph where the differences are).

Type 1 DAC (TH) had a significant defect in projecting dendrites into the INL space for *Bax*, *Dscam*^{LOF}, double K.O and *Dscam*^{GOF} *Bax*$^{-/-}$ genotypes. The double K.O had a severely disrupted overall organization in the off strata. *Dscam*^{GOF} *Bax*$^{-/-}$, however, was able to reduce disruption severity compared to the double K.O in S1 and S2 layer (figure 18 H).

Finally, in wild type, vglut3 positive AC dendrites are limited between two distinct ChAT bands. ChAT positive neurites specifically occupy the S2 and S4 layers of the IPL. This restriction was not true in *Bax* $^{-/-}$, where we observed ectopic dendrites in the S1 layer, and *Dscam*^{LOF}, where we found multiple disruptions in the retina. The disruption of ectopic dendrites were rescued by *Dscam*^{GOF}, but the S2, S3, S4 layers remains less organized. This illustrated that neurite restriction requires the collaboration of both *Dscam* and *Bax* (Figure 18 I).

Our data revealed that multiple types of neurons in mouse retina require DSCAM for proper lamination in the IPL, where this protein is normally expressed. *Bax* regulates this neurite lamination to a degree, but in the absence of both *Bax* and *Dscam* genes, neurites lose their identity and fail to target the right circuitry. Eliminating *Bax* but overexpressing *Dscam* can help reduce the severity of the phenotype, but not enough to rescue the entire circuitry and form a normal distribution. This suggests both *Dscam* and *Bax* are involved in

a similar lamination targeting system and are able to cover each other's function to a degree so that the IPL won't completely lose its integrity when one of the two genes become defective. At same time, they are unique enough to be distinct from one another and to create their own restrictions on neurite targeting. This is an ideal example of a double safe mechanism in mammal nervous system, yet illustrates target-specific independence within the double safe mechanism.

Software Follows the Need

The previous analysis was made possible by automating analysis of intensity into one simple custom made program, following the principle that every scientist should attempt to make scientific data more accurate and the scientific method more sophisticated.

The current research standard for image analysis is ImageJ, a free program funded by NIH. It is small and compact, but powerful enough to manipulate images and has more than enough analytic functions to read out the images. Those built-in functions are important for multichannel imaging analysis, and we integrated those functions with our algorithm of IPL analysis to generate a new software tool for neuroscientists. This software significantly reduces the work load involved in quantifying retinal lamination and automates demarcation of laminar depth, thereby removing biases introduced by different genetic backgrounds and human error.

I started designing and coding the software in the early 2015 with help from Brenda Hanley and, later, Michael Woodfin contributes to polishing and updating. The overall program flow chart demonstrates how each function is operated upon the selection of Region of Interest (ROI) (Figure 19).

The program is named IPLaminator. It's used as a tool to rapidly analyze neurite stratification patterns in the retina and other neural tissues (Figure 20). A range of user options allows researchers to segment the IPL based on fixed points, such as the neurites of cholinergic amacrine cells, or to define a number of segments into which the IPL will be divided. Options to analyze tissues such as cortex were also added. Statistical analysis of the output then allows a quantitative value to be assigned to differences in laminar patterning observed in different models, genotypes or across developmental time. The latest version can be found at http://isoptera.lcsc.edu/NeuronAnalysis/.

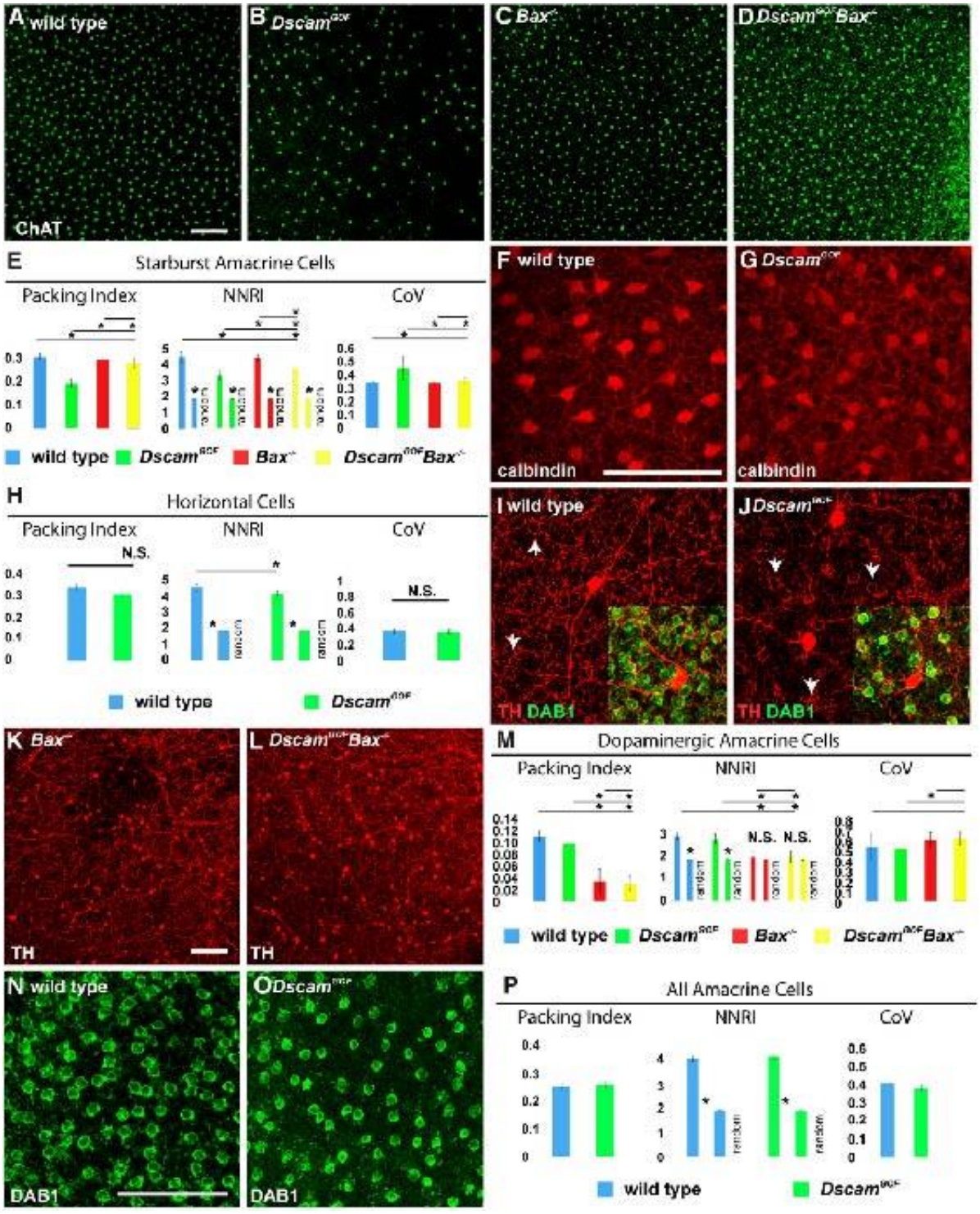

Figure 12 DSCAM is not sufficient to increase spacing or avoidance in the mouse retina

A–D, Confocal images of OFF SACs. **E**, Quantification of DRP (based on packing index), NNA(based on NNRI), and VD analysis (based on CoV) for wild-type, *Dscam*^{GOF}, *Bax*$^{-/-}$, and *Dscam*^{GOF} / *Bax*$^{-/-}$ SACs. **F**, **G**, Confocal images of horizontal cells in the wild-type and *Dscam*^{GOF} retina. **H**, Quantification of packing index, NNRI, and CoV for wild-type and *Dscam*^{GOF} horizontal cells. **I, J**, Confocal image of type 1 dopaminergic amacrine and AII amacrine cells. Type 1 DACs wrap dendrites around AII amacrine cells in wild-type and *Dscam*^{GOF} retinas (arrowheads). **K, L**, Confocal image of type 1 DACs in *Bax*$^{-/-}$ and *Dscam*^{GOF} /*Bax*$^{-/-}$ retinas. **M**, Quantification of packing index, NNRI, and CoV forwild-type, *Dscam*^{GOF}, *Bax*$^{-/-}$, and *Dscam*^{GOF} / *Bax*$^{-/-}$ type 1 DACs.N,O, Confocal images of AII ACs in wild-type and *Dscam*^{GOF} retinas.P, Quantification of packing index, NNRI, and CoV for wild-type and *Dscam*^{GOF} AII ACs. P28 –P42. N>4 for all genotypes. Scale bars: (in A) A–D, 100µm; (in F) F, G, I, J, 100µm; (in K) K, L, 100µm; (in N) N, O, 100µm. Mean SD; *p_0.05 (Student's t test).

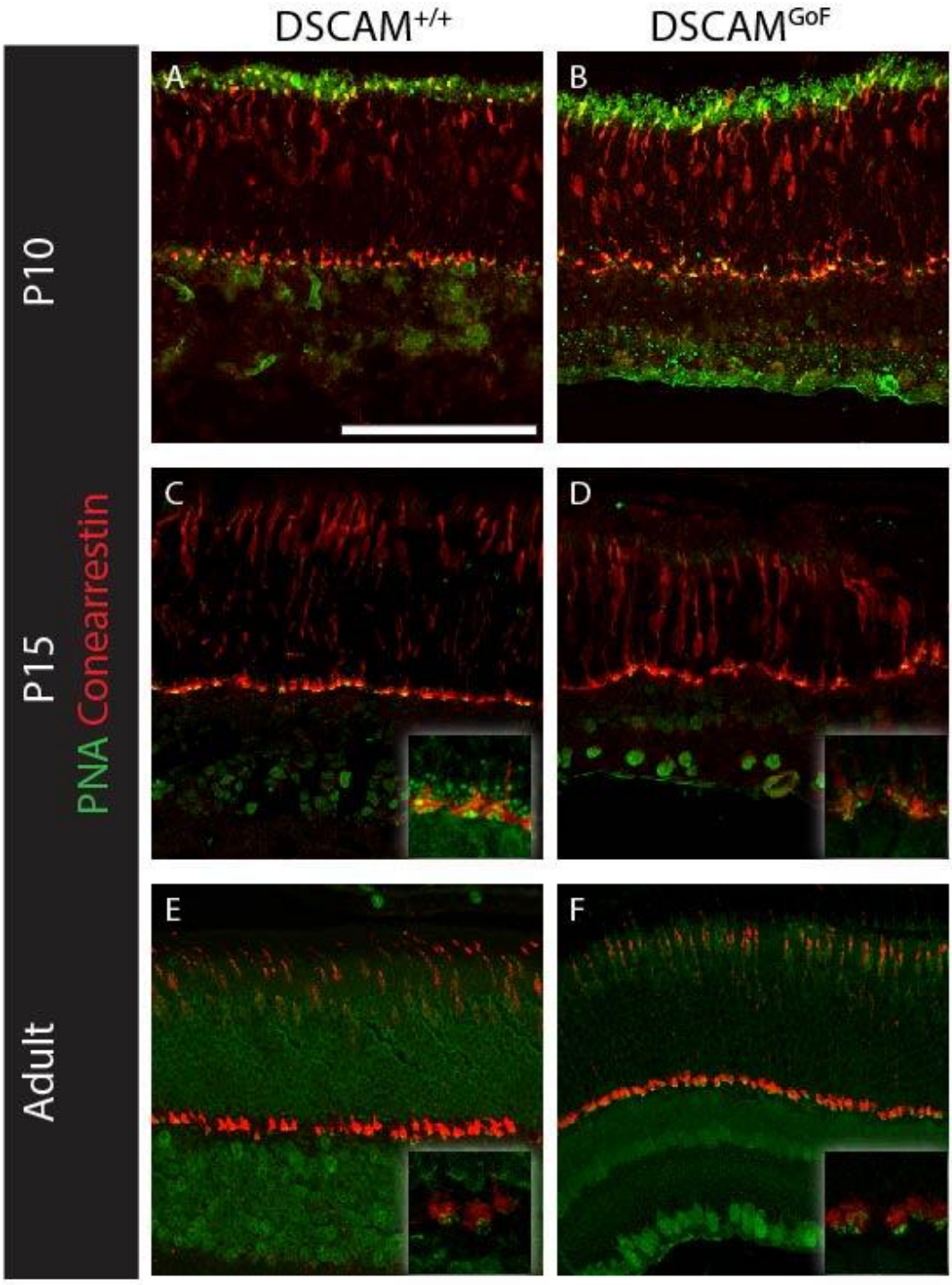

Figure 13 Cone organization was altered during early development

Young (A-B), Juvenile (C-D) and Adult (E-F) retinas stained with PNA and Cone arrestin showed cones were spatially altered during early developmental stage but recovered at older age. Cone population was not influenced. Scale bar in A is 200μm.

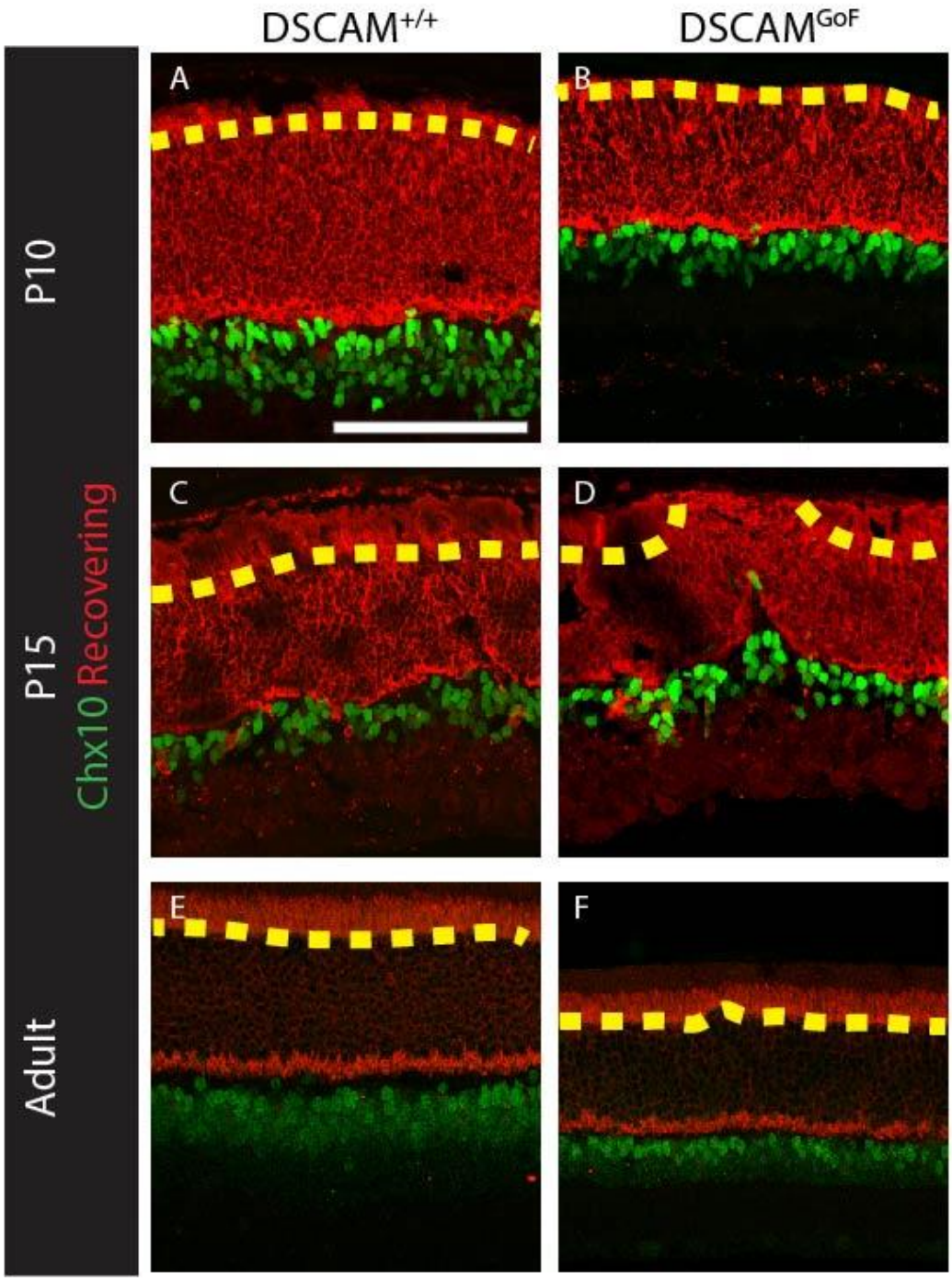

Figure 14 Rods and bipolar cell organization was influenced during early development

Young (**A-B**), Juvenile (**C-D**) and Adult (**E-F**) retina stained with Chx10 and Recoverin. Rod photoreceptor and bipolar cell intrusion were observed at age P15 and recovered at adult age in the *Dscam*^{GoF}. The population of the bipolar cell was not recovered. Scale bar in A is equivalent to 200µm.

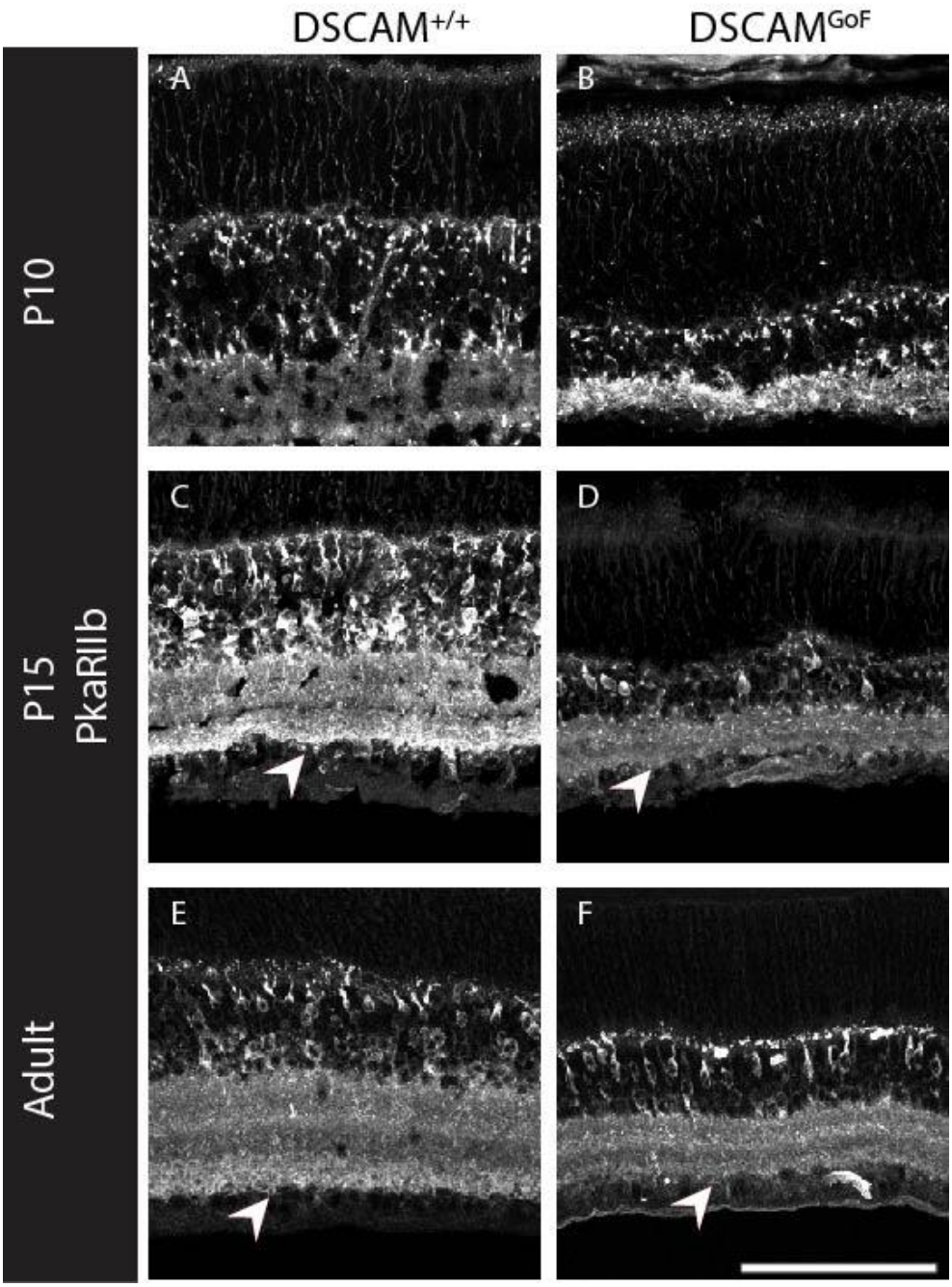

Figure 15 Type 3B bipolar cell population was reduced in *Dscam*^{GoF} retina

Young (**A-B**), Juvenile (**C-D**) and Adult (**E-F**) retinas stained with PkaRIIb. Type 3B bipolar cell population was significantly reduced in *Dscam*^{GoF} retina and the axon projection depth was also altered. Less stain intensity is detected in IPL basal layer through all ages. Scale bar in **F** is equivalent to 200µm.

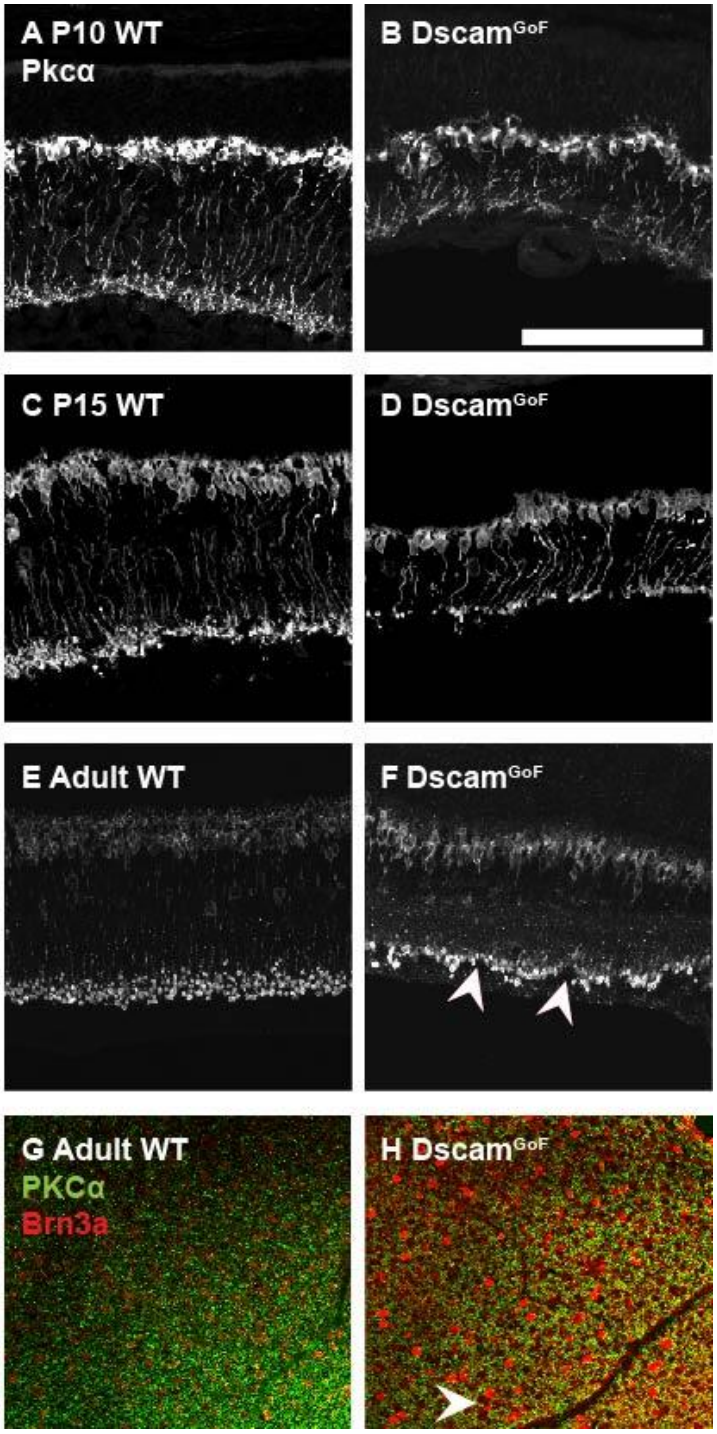

Figure 16 Rod bipolar cell abnormality in the *Dscam*^{GOF} retina

Young (**A-B**), Juvenile (**C-D**) and Adult (**E-F**) retinas stained with PKCα. P10 and P15 retina showed reduced axon length related with decrease IPL thickness (**A-D**). Cross section and whole mount of the aged retina indicated *Dscam*^{GOF} could alter the spatial patterning of the axonal projection. Scale bar in **B** is equivalent to 200μm in all images.

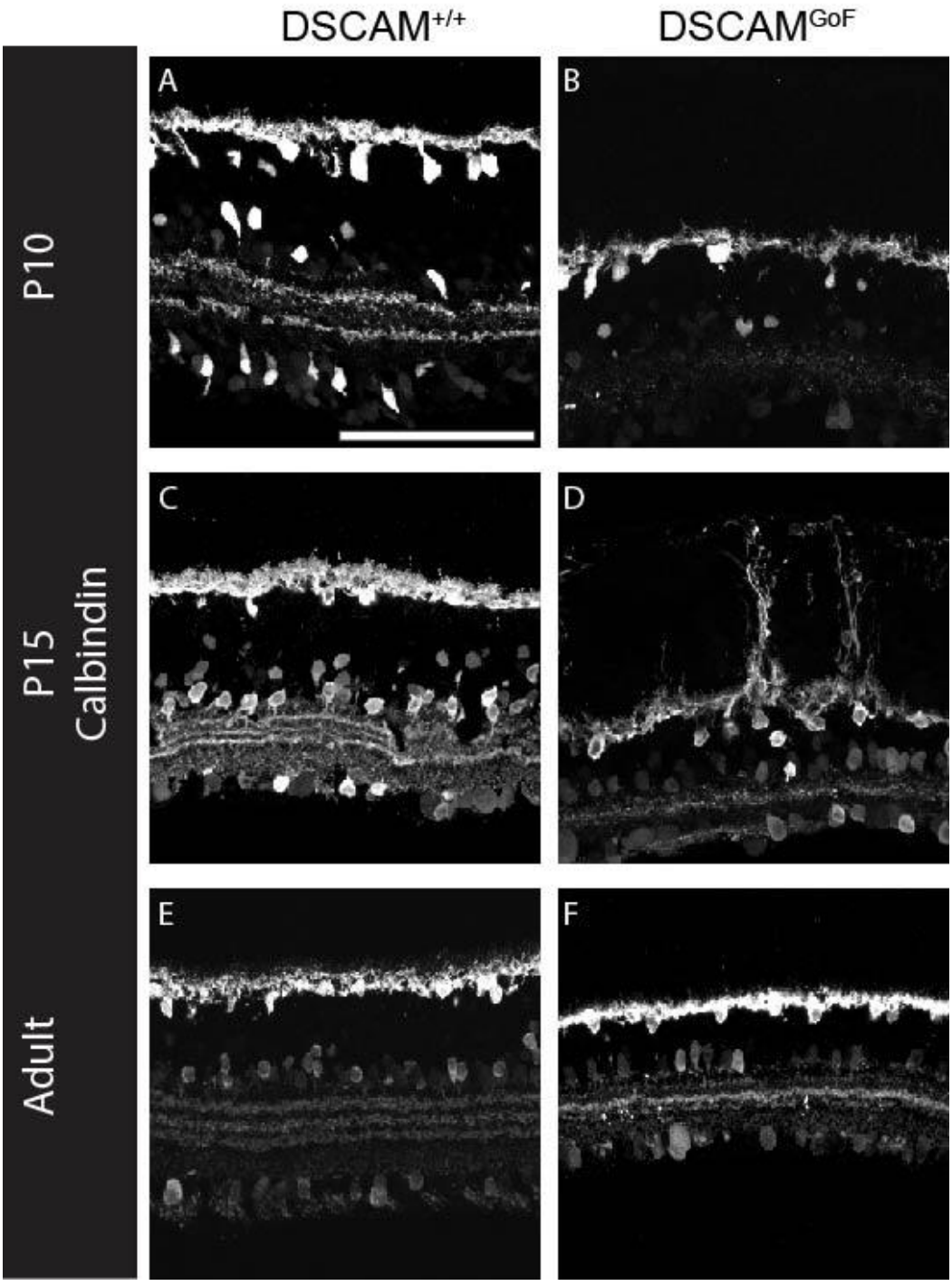

Figure 17 Horizontal cell dendritic outgrowths

Young (**A-B**), Juvenile (**C-D**) and Adult (**E-F**) retinas stained with calbindin. The outgrowth of dendrite started after P10 and was extremely obvious at age P15. This phenotype however also slowly recovered as mouse aging. Scale bar in **A** is equivalent to 200µm.

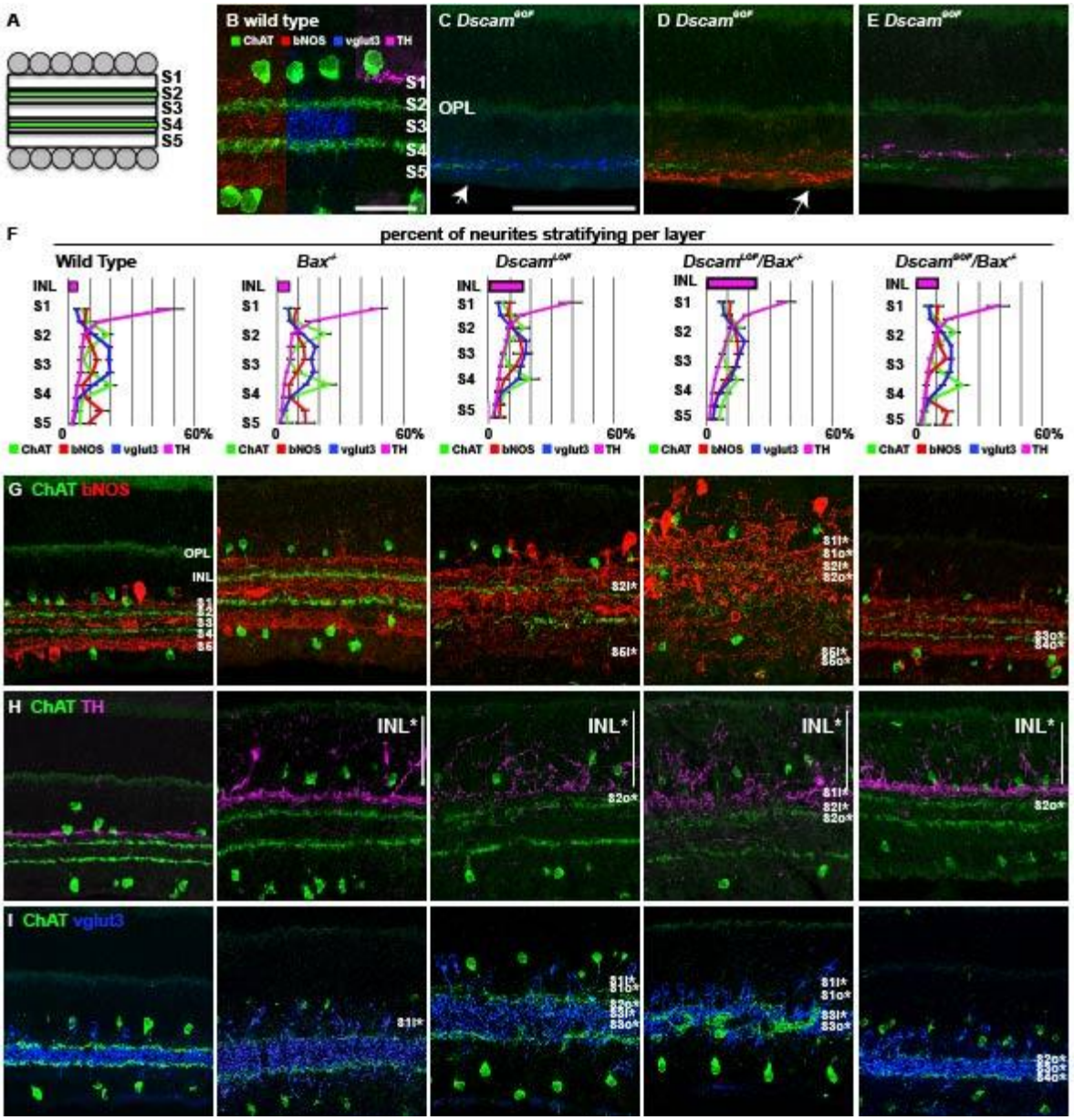

Figure 18 *Dscam* is necessary for IPL lamination

A, Cartoon depiction of the retinal inner plexiform layer (IPL) divided into five strata, S1-S5. Location of starburst amacrine cell (SAC) dendrites are shown in green. **B**, Location of assayed dendrites in the wild type retina. **C**-**E**, Lamination of SAC, bNOS+ AC, vglut3+ AC and type 1 DAC dendrites in the $Dscam^{GOF}$ retina. **C**, A single SAC band was observed in most of the $Dscam^{GOF}$ retina (arrow). **D**, Dendrites projected to regions of the RGL not occupied by cell bodies (arrow). **F**, Plot of percent dendrites laminating in S1-S5 in the wild type, $Bax^{-/-}$, $Dscam^{LOF}$, $Dscam^{LOF}/Bax^{-/-}$ and $Dscam^{GOF}/Bax^{-/-}$ genotypes. Each stratum was divided into an inner and outer half for plotting and quantifying percent lamination. Asterisks in panels **G**-**I** denote a Mann Whitney U-test p-value <0.01 compared to wild type. **G**, Lamination of bNOS+ amacrine cell dendrites. **H**, Lamination of type 1 dopaminergic

amacrine cell (DAC) neurites. **I**, Lamination of vglut3+ AC dendrites. Abbreviations: TH: tyrosine hydroxylase, INL: inner nuclear layer. P18, N3≥for all genotypes. Scale bar: (in B) B= 25 µm (in C) C-E, G-I= 100 µm. Mean±SD; *p≤0.01 (Mann Whitney U-test).

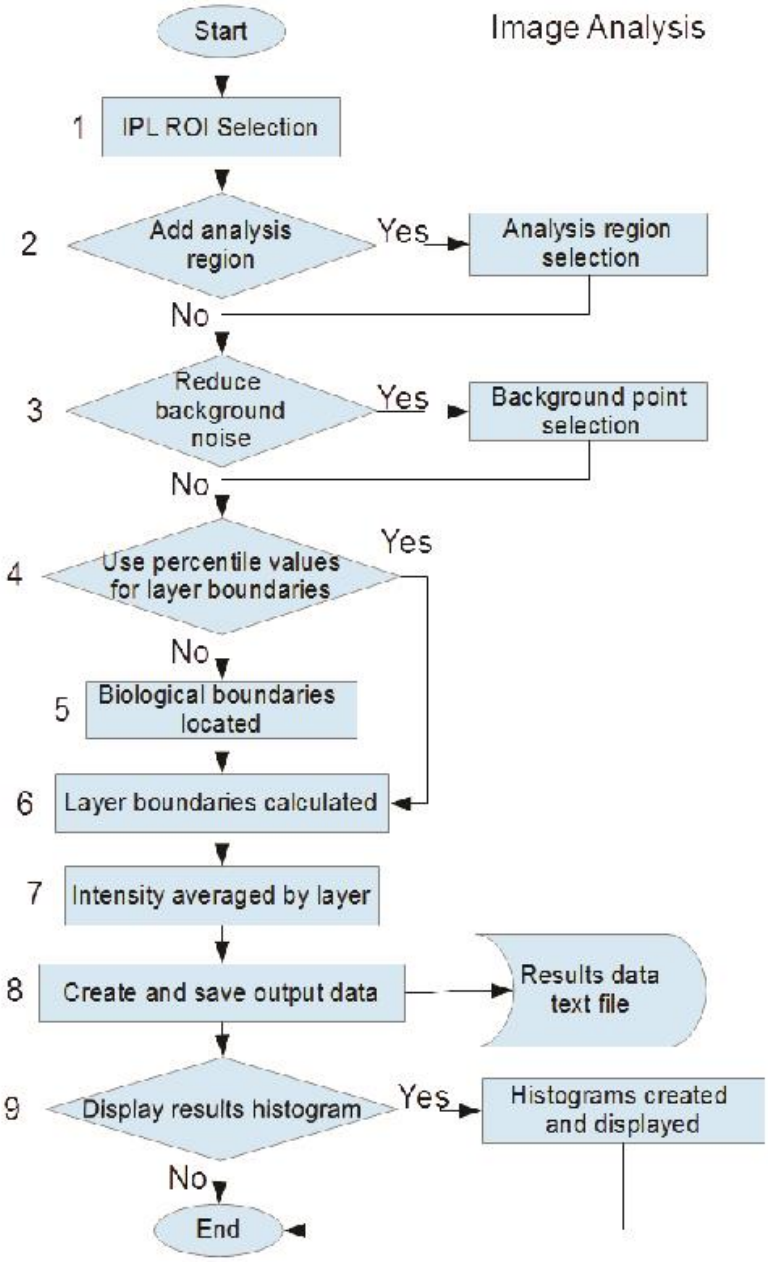

Figure 19 Retina layer analysis software flow chart

The overall flow chart illustrated how the program is structured and how functions are executed.

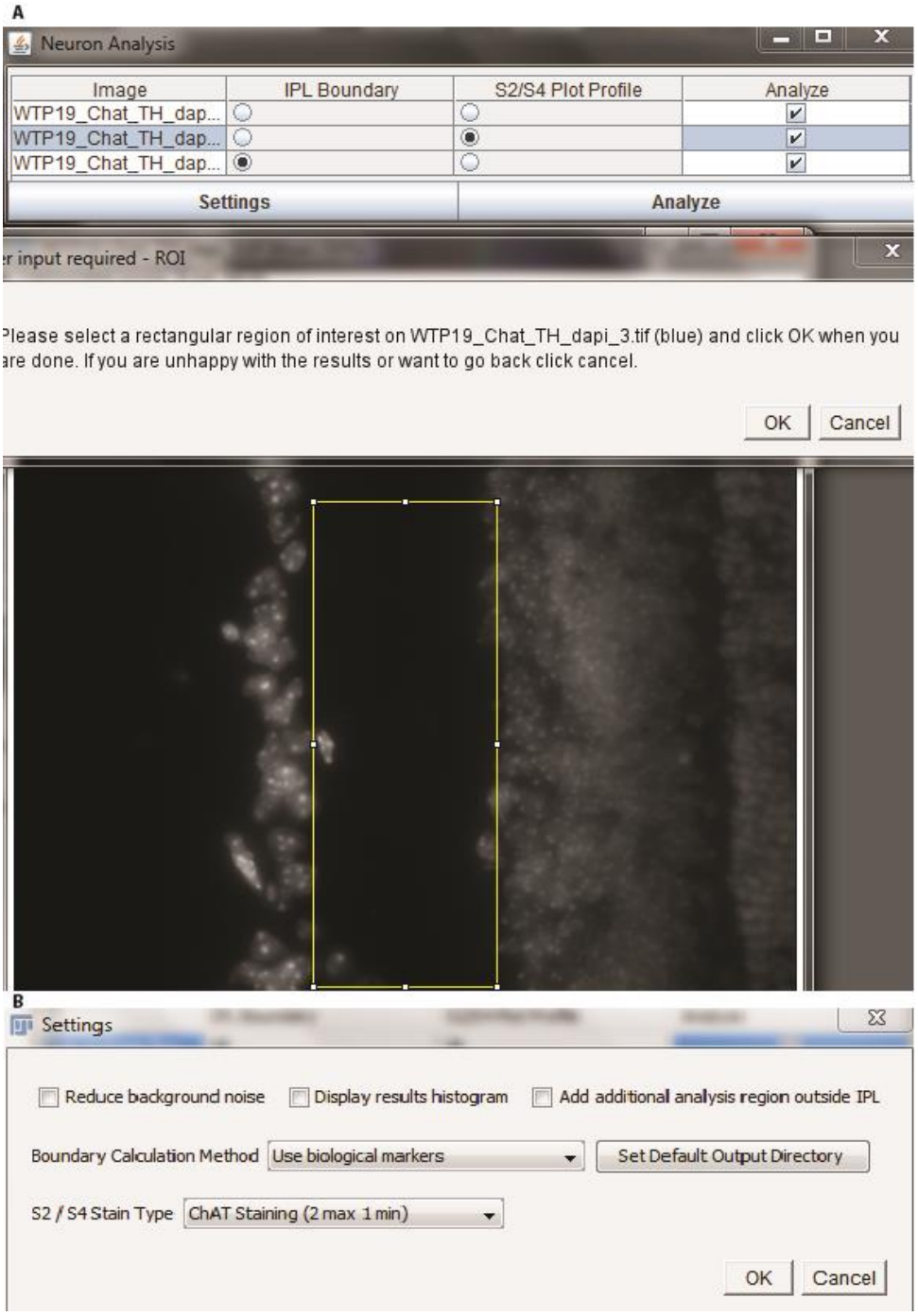

Figure 20 Program user interface and sample of area selection

This program allows user to efficiently collecting data without mind numbing process that has been used for over a hundred years.

Table 2 Cell number by type

*Significant **Highly Significant		
Cell Type	% vs. WT	p-value
INL Amacrine Cells (Pax6-positive)	47±7	**6.1E-11****
Bipolar Cells (Chx10-positive)	56±12	**2.5E-9****
Horizontal Cells	135±20	**0.045***
SACs	40±21	**2.3E-05****
DA Cells	116±10	0.1
Müller Glia	92±9	**0.015***
Cones	100±7	0.2
RGCs E15	83±16	0.2
RGCs E18	120±7	0.06
RGCs P15	30±23	**5.3E-10****
Type 3A CBCs	27.7±11	**5.9E-6****
Type 3B CBCs	27.8±12	**4.6E-10****

The number of cells in the *Dscam*^{GOF} and wild type retina were counted in at least three retinas and expressed as the percent of cells in the *Dscam*^{GOF} retina compared to wild type. Cell number per length or volume of the retina, as described in the methods section, were used to conduct paired Student's *t*-tests.

CHAPTER 8: Neural Circuitry Maintenance

Cell death and neuronal organization are both critical for our nervous system. These events happen in the early stages of development, and as animals aging they will face a different challenge: long-term maintenance of the nervous system. High quality of maintenance can stabilize the integrity of the neural circuitry and protect it from aging and diseases.

There are few maintenance mechanisms existing in the nervous system. First is the systematic refinement during early development which is responsible for neurite lamination. Second is a repair mechanism which is activated by variety of stimuli, especially during cellular damage. When the damage is so severe that repair becomes impossible, our system will try to activate regeneration. Regeneration is a hot topic in science, but the research in this area is still young and regenerative ability of nervous system differs across tissues and species.

Refinement

Systematic refinement of the nervous system can start as soon as dendrites begin to explore surrounding areas. The difference between dendrite refinement and dendrite lamination is that refinement happens during lamination and causes the retraction of incorrect lamination so the proper dendritic projection to the right synaptic partner can be established.

TH positive Dopaminergic Amacrine cells (Type 2 DACs) express *Dscam* naturally, and when we reduce or knock out *Dscam* expression the cell population displays a very

consistent phenotype: lack of refinement during early dendritic exploration. The severity was *Dscam* dosage dependent. When type 2 DACs start projecting their dendrites, they extend into S1 and S3 of the IPL during an early exploration stage. The S3 layer is where dendrites are stabilized once the connection is established for type 2 DACs. On the other hand, dendrites of type 2 DACs in the S1 layer are short-lived following a brief exploration (Figure 21 A-C).

Whole mount $Dscam^{LOF}$ retinas illustrated that these consistent unrefined dendrites from type 2 DACs are stuck in the S1 layer in young adults at age P15. Statistical analysis indicated an increase in cell density and abnormality in spatial organization of the type 2 DACs (Figure 21 E-M).

We further assayed dendritic morphology through developmental time points used in the early study. In the early age, type 2 DACs projected dendrites into the S1 layer in both wild type and $Dscam^{LOF/+}$ retina which demonstrates that early exploration was not influenced by the loss of *Dscam* gene (Figure 22 A B arrows). But two days later, at 5 days of age, we saw that an excessive amount of cells had dendrites persisting in S1 layer in $Dscam^{LOF/+}$ retina, where the same layer dendrites in wild type had started to disappear and undergo the refinement process (Figure 22 C D arrows). By 10 days of age, type 2 DACs completed the refinement process and S1 layer contained no observable dendrites in WT. $Dscam^{LOF/+}$ retina, on the other hand, had a significant amount of dendrites projecting into S1 layer, which indicated that the refinement of dendrites had not yet taken place (Figure 22 E-G).

Our results suggested that *Dscam* promotes the refinement of Type 2 DACs shortly after 3 days of age. This refinement process is accomplished by reducing the stability of the dendrites in a *Dscam* dosage dependent manner: the more *Dscam*, the less stable the dendrites (figure 22 H).

Repair and Regenerate

Refinement is a process which makes circuity functional and sufficient for its role. It happens during early developmental stage. Repair mechanisms, however, are constantly fixing emerging problems, such as damage occurring on the cellular and tissue levels. Repair takes place throughout our lifetime.

One significant abnormality of the $Dscam^{GoF}$ retina is the intrusion of photoreceptor somas to the outer segment layer crossing over the outer limiting membrane (OLM) (figure 23 A and B). This is a boundary composed from Müller glia end feet, and is part of the non-neuron system in the retina. The membrane acts as a net and holds all photoreceptors in place. Only the inner and outer segments of the photoreceptors can pass through and attach to the supporting pigment cells. This way, a clear organization can be formed and photon sensitive proteins in the photoreceptors can be excited by light efficiently.

The intruding photoreceptors were likely the result of a disrupted OLM-caused leakage (Figure 23 C-G). Whole mount images revealed giant holes in the OLM of $Dscam^{GoF}$ retina (Figure 23 H). We tested Müller Glia morphology using Glutamine Synthase, and found that leakage occurred at age P15 but not at P10. This time point is precisely correlated to the maturation of photoreceptors. Whether the leakage allows cell somas to migrate outside of OLM or the somas push through the OLM (causing the hole)

remains unknown (Figure 24 A-D). A GFAP cre-mediated gain of function study indicated that overexpression of *Dscam* in the Müller Glia doesn't result in OLM leakage[92]. A dramatic recovery of the photoreceptor intrusion was observed in adults, this suggests that the supportive cells are repairing themselves (Figure 24 E and F).

The intrusion of photoreceptors appeared as early as postnatal day 12 and varied in time by one to two days depending on the number of mice in a given litter and individual size of the $Dscam^{GOF}$ mouse. We illustrated in the previous chapters that the intrusion of photoreceptors would cause a severe disruption of vertical organization. This degree of disruption in a well-organized neural structure is believed to represent irreversible damage in mammalian central nerve system (CNS). The evidence of self-recovery in the retinal neuron circuitry upon disruption of organization that we observed provided new insight on our own neurological repair limits.

The reason for mammals having very limited neuronal regeneration compare to other spices is still unknown. Within mammals, regenerative ability differs drastically between the central nerve system (CNS) and the peripheral nervous system (PNS). The complexity of the CNS and the increased myelination of the PNS could explain part of the picture; however, the full picture is still missing many pieces.

Rethink

Today, microchip implantation, photoreceptor replacement, gene therapy and other strategies are used to treat blindness. Some of them show promising results. Antibodies against VEGF in particular are already used to treat age-related macular degeneration and diabetic retinopathy[104, 105]. Molecular biology and genetics are the keys to the future of

disease treatment, and we have many possible proteins and genes we can discover the functionality of through animal research.

Some animals evolved with extraordinary self-regenerative capabilities. For example, starfish can grow back an entire arm and everything within it. Vertebrates like zebra fish are able to regenerate photoreceptors in the eye after ablation without ever going blind. A gecko can grow back its own tail after bailing out on predators, and salamanders can regenerate a variety of structures, including their entire limbs. In mammals, a Murphy Roth's large mouse is capable of extraordinarily scar-less regeneration, including the regeneration of appendages[106]. Studies in the past 10 years have confirmed genes like P21 are directly linked to the mammal tissue regeneration[107, 108]. Surprisingly, research also has illustrated that inhibition of p21 activated kinase-PKA can rescue partial mental retardation phenotype in fragile X syndrome animal models[109].

We are working with exciting discoveries. Neurological study of genetic influence could lead to many applications that revolutionize how we treat, and even prevent, diseases. Hopefully, in the distant future we will have a renovated health care system based on molecular biology and genetics to that can deliver solutions without the need to worry about side effects or long term medical administration.

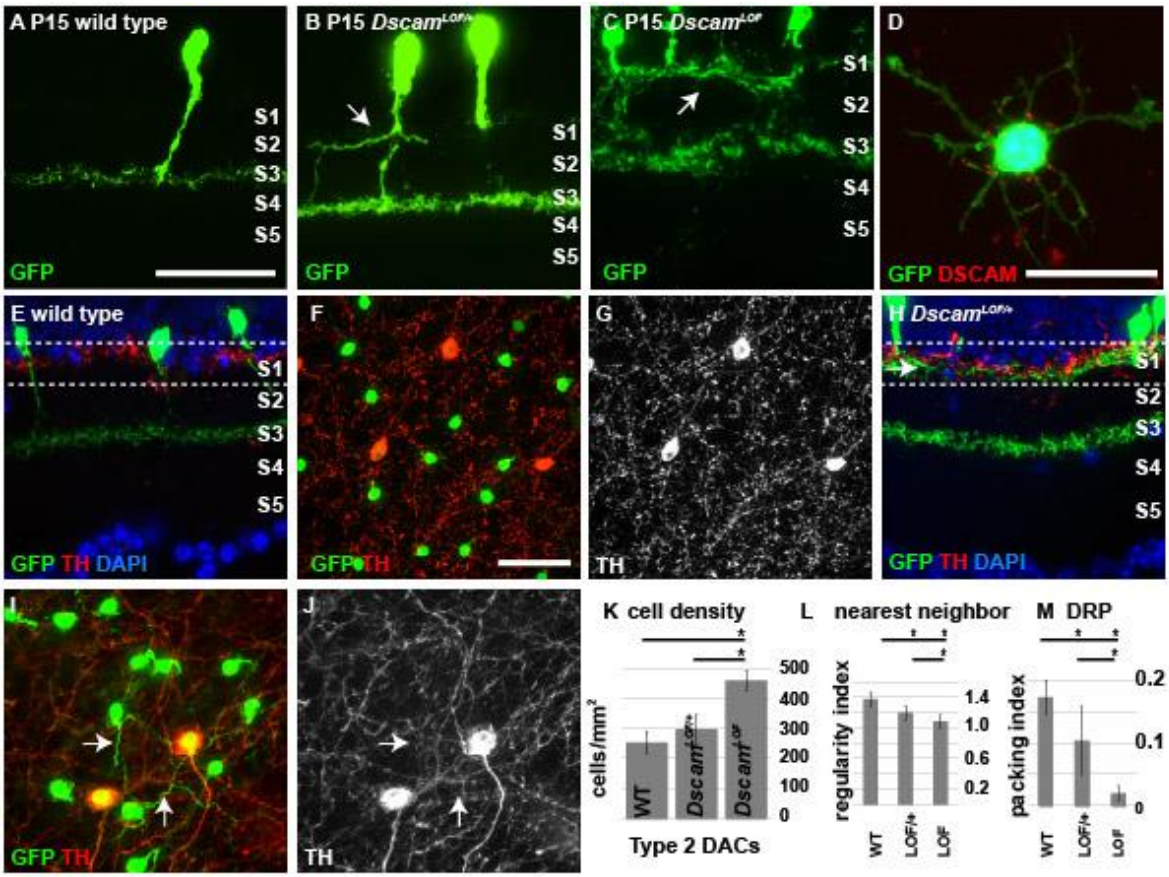

Figure 21 Type 2 DAC lamination defects are dependent on *Dscam* dosage

A-**C**, Type 2 dopaminergic amacrine cells (DACs) labeled with a green fluorescent protein (GFP) expressing transgene. **A**, Type 2 DACs project dendrites to S3 at P15 in the wild type retina. **B** and **C**, Type 2 DACs in the *Dscam*$^{LOF/+}$ or *Dscam*^{LOF} retina project dendrites to both S1 and S3 (arrows; **B** and **C**). **D**, Type 2 DAC *in vitro* labeled with an antibody to DSCAM. DSCAM protein was localized to dendrites. **E**, Section of wild type retina with type 1 and 2 DACs labeled (type 1=dim GFP and tyrosine hydroxylase (TH), type 2= GFP only). Dotted lines show the depth of confocal images in **F** and **G**. **F** and **G**, Type 1 and 2 DACs in a confocal section of layer S1 of the wild type retina. Dendrites in S1 are those of type 1 DACs. **H**, Section of *Dscam*$^{LOF/+}$ retina with type 1 and 2 DACs labeled (type 1=dim GFP and TH, type 2= GFP only). Dotted lines show the depth of confocal images in **I** and **J**. Dendrites of type 2 DACs laminate in S1 of the *Dscam*$^{LOF/+}$ retina as evidenced by the lack of TH staining in bright GFP-positive dendrites (arrows; **I** and **J**). **K**, Quantification of type

2 DAC cell number in the wild type, *Dscam*$^{LOF/+}$ and *Dscam*^{LOF} retinas. Cell density was significantly increased in the *Dscam*^{LOF} retina compared to wild type and *Dscam*$^{LOF/+}$ retina. Significant differences were not detected comparing the wild type and *Dscam*$^{LOF/+}$ retina. **L**, Nearest neighbor (NN) analysis of type 2 DACs. NN regularity significantly decreased as *Dscam* dosage decreased. **M**, Density recovery profile (DRP) analysis of type 2 DACs. Packing index values significantly decreased as *Dscam* dosage decreased. Abbreviations: GFP: green fluorescent protein, TH: tyrosine hydroxylase, DRP: density recovery profile. Postnatal day 15: N≥10: A-C, E and H, N=3: D, N≥4: F, G, I, J. Scale bar: (in A) A-C, E, H=50 µm, (in D)=25 µm, (in F) F, G, I and J= 50 µm. Mean±SD; *p≤0.05 (Student's *t*-test).

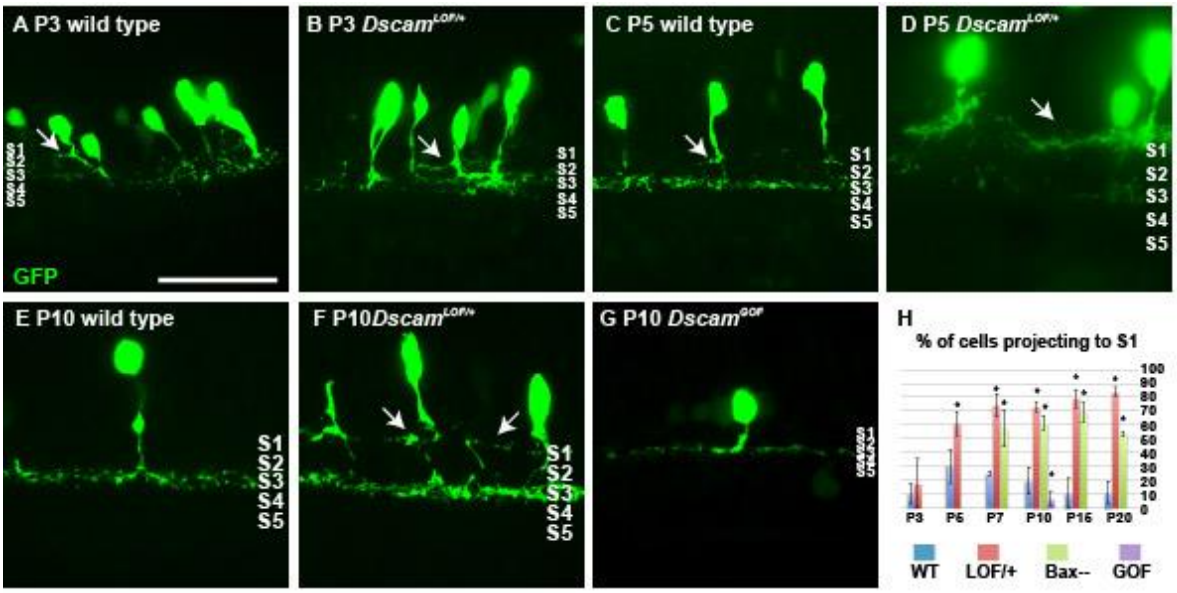

Figure 22 DSCAM prevents stabilization of exploring dendrites

A-G, Type 2 dopaminergic amacrine cells (DACs). **A** and **B**, Type 2 DACs in the wild type and *Dscam*$^{LOF/+}$ retina at postnatal day 3 (P3). A statistically similar number of type 2 DACs project into S1 comparing the wild type and *Dscam*$^{LOF/+}$ retina (arrows; **A**, **B** and **H**; P3). **C** and **D**, Type 2 DACs in the wild type and *Dscam*$^{LOF/+}$ retina at P5. A statistically significant increase in the number of type 2 DACs that project dendrites into S1 of the *Dscam*$^{LOF/+}$ retina was observed as compared to controls (arrows; **C**, **D** and **H**; P5). **E-G**, Type 2 DACs in the wild type, *Dscam*$^{LOF/+}$ and *Dscam*^{GOF} retina at P10. A statistically significant increase in the number of type 2 DACs project dendrites into S1 of the *Dscam*$^{LOF/+}$ retina was observed compared to wild type or *Dscam*^{GOF} cells. A statistically significant decrease in the number of type 2 DACs projecting dendrites into S1 was observed in the *Dscam*$^{GOF/+}$ retina compared to wild type or *Dscam*$^{LOF/+}$ cells (**E**, **G**; arrow and **H**; P10). **H**, Quantification of type 2 DAC projections into S1. Abbreviations: GFP: green fluorescent protein. N≥3 P3, P5, P10, P15. N=2 WT and *Bax*$^{-/-}$ P7 and P20. Scale bar (in A) A-G=50 µm. Mean±SD; *p≤0.05 (Student's *t*-test).

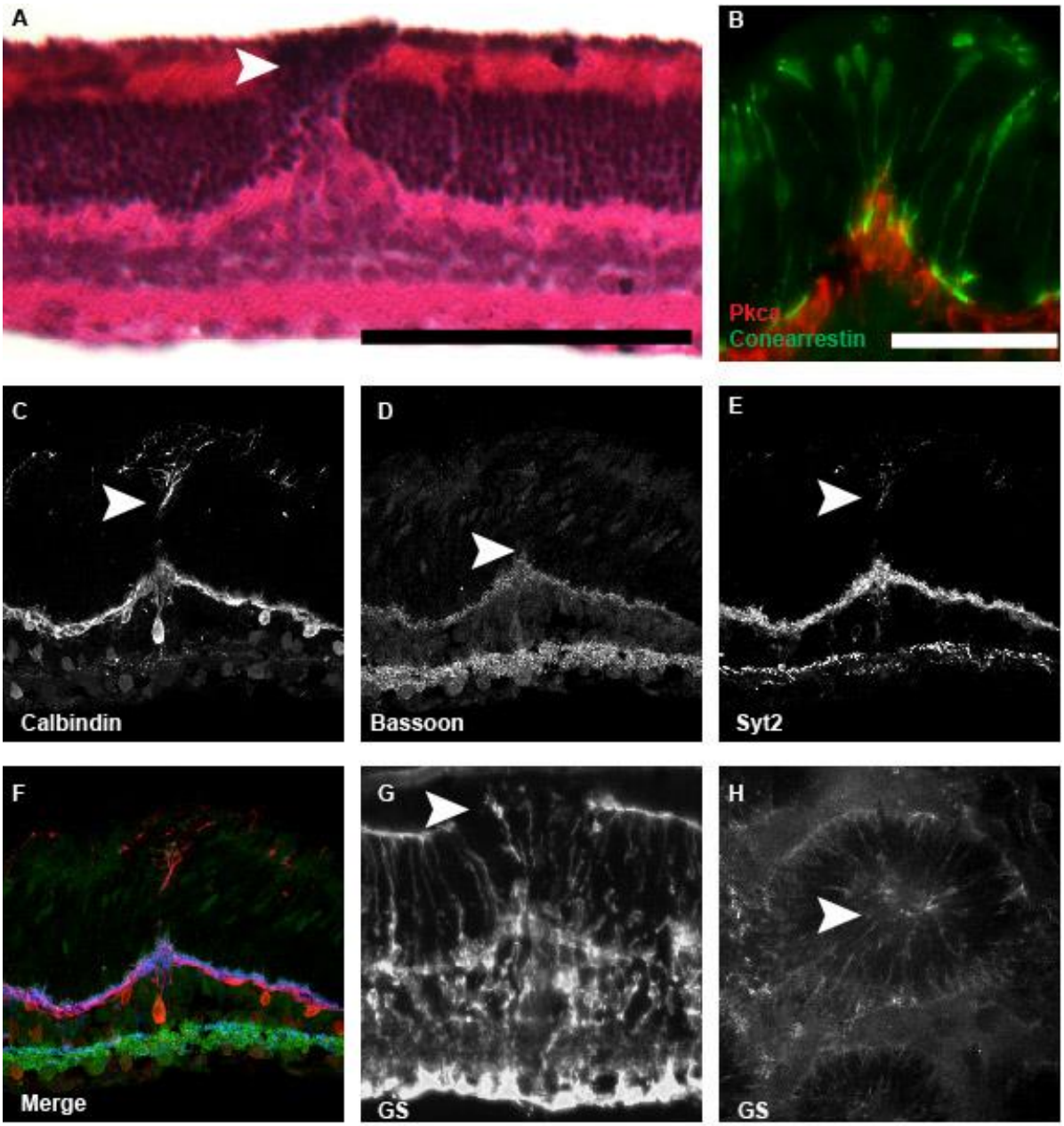

Figure 23 OLM leakages and the subsequently shifting the basal boundary of the ONL

A. H&E staining of a P30 days *Dscam*^{GoF} retina indicated the photoreceptor soma was placed outside of the OLM and the basal layer of that region was pulled up towards the ONL. **B**. Cone and bipolar cell staining indicated the out placed photoreceptors were composed of both cones and rods. **C-F**. Synapses were out placed follow this abnormality. **G**. GS staining indicated a clear leakage in OML. **H**. Whole mount *Dscam*^{GoF} retina GS staining indicated the leakage was always in a hollow circle. Scale bar in **A** is 98um. Scale bar in **B** is 60 μm in **B**,106.5um in **C-E**,80um in **G**, and 100um in **H**.

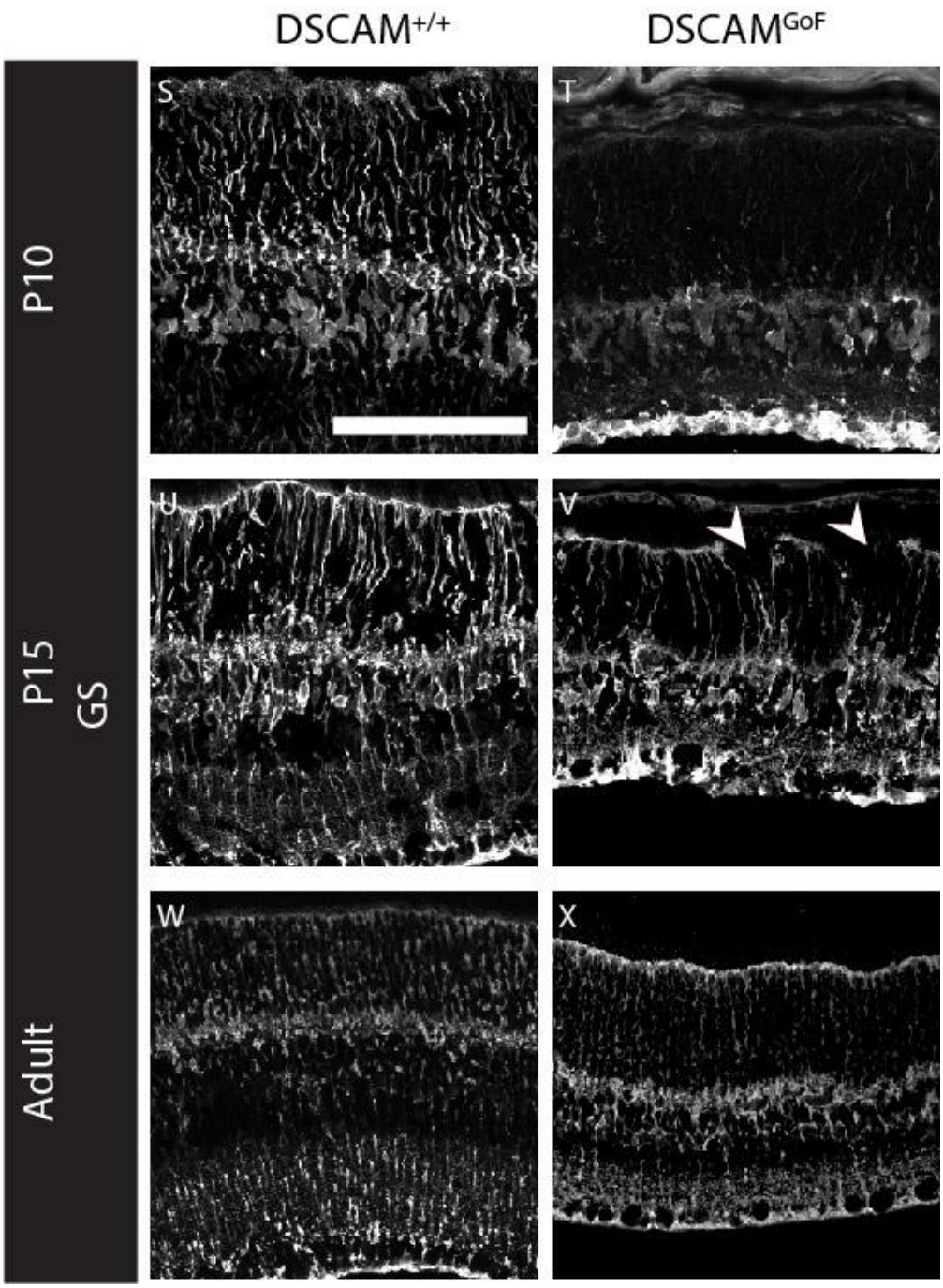

Figure 24 Developmental time study of the OLM leakage

Young (**A-B**), Juvenile (**C-D**) and Adult (**E-F**) retinas stained with calbindin GS. The OLM was evidentially normal at P10 in the *Dscam*^{GoF} retina but severely disrupted at age P15, just a few days after rod maturation. This phenotype later was recovered in 8 month old retina, which indicated a degree of plasticity a constant repair of the outer retina abnormality. Scale bar in **A** is equivalent to 200µm.

CHAPTER 9: Where to Go From Here

"Twenty years from now you will be more disappointed by the things that you didn't do than by the ones you did do. So throw off the bowlines. Sail away from the safe harbor. Catch the trade winds in your sails. Explore. Dream. Discover." –P.S I Love You[110]

It is amazing to see the world transformed within few decades by computers. To see the dramatic improvement in life expectancy with antibiotics and vaccines. A few decisions we made transformed the life we used to live. As the world becomes more complex and dynamic, it opens up vast options for us to choose from. We have to be wise with these options, because every seed we plant will begin to bear fruit and affect the quality of our lives as we get older, for better or worse.

Future of the Neurological Disorders

One seed human society has planted is a growing population. As we age, neurological disorders become common and it is threaten the stability of our world. Alzheimer's, for example, is one of the most common neurological disorders, affecting 5.1 million people in the US population. It can easily erase hours, days and years of memory away from peoples' lives. They don't know where they are, what they are doing, or even who they are. Their family and friends unavoidably suffer from emotional loss.

Down syndrome and Autism are common neurological conditions resulting in intellectual disability. Down syndrome incidence has been on the rise in the last few decades, and in 2010 1 in 68 newborns had Autism. Both conditions put emotional and physical hardship on countless families. With intellectual disability and physical abnormality,

individuals with these conditions require constant personal care, yet, as with Alzheimer's, no treatment is available to this date.

78.6 million US individuals are obese today[111]. That is 34.9% of the total population. Additional, 21.9 million individuals have diabetes--roughly 9.3% of the total population. Those two groups are at a high risk of developing peripheral neuropathy, a condition which causes the loss of sensation and control for hands, feet and peripheral nerve system in general[112]. Unfortunately, vision is a part of the central nervous system that can also be impacted by a condition known as diabetic retinopathy, which causes impaired vision and blindness.

Amyotrophic lateral sclerosis (ALS), a neurological disorder that was popularized by the Ice Bucket Challenge, is a condition caused by muscle neuron degradation, resulting in the loss of muscle control and leading to paralysis. The famous theoretical physicist Stephen Hawking has this condition.

An epidemic of neurological disorders is unavoidable in our near future. We have to work on solutions to prepare this epidemic, so our future generations won't regret the path we didn't take. I imagine the solutions require a collaboration of neuroscientists, engineers, doctors and policy makers working together to investigate innovative methods and products to test and prevent neurological disorders. Additionally, the general public should be aware of this situation and have a fundamental understanding of these conditions and diseases through public education sessions.

Future of the Science and Education

Scott Young is a self-motivated learner. Without attending MIT, he completed a 4 year computer science curriculum in less than a year using the internet and other limited resources. He called it the MIT Challenge in his TEDx Talk. The future of the science and education is no longer a pipeline dedicated to degrees and publications. Fast paced self-learning and constant adaptation to better methodology in the near future will become the key to driving innovation and productivity for scientific advancement.

Whether learning a solid technical skill set or an abstract concept, the internet can acquire majority of the necessary information in an instant. Increasing the transparency of the internet revealed the reliability of different techniques people tried and minimized the risk of learning something that isn't true or useful. This accessibility of information is forcing the scientific community and educational system to accept the fact that people can create great innovations, make scientific discoveries and gain an extraordinary scientific education without years of formal training.

Youtube is one successful example of this phenomenon. Many passionate young individuals started to set up their own daily show about cool scientific experiments and innovative explanations of the science shown. Michael Stevens is one such example. He popularized the scientific/philosophical channel Vsauce, and now has 9 million subscriptions with more than 800 million viewers. As a double major science degree holder and a general educator/youtuber, at age 28 he has the influence even Nobel Prize scientists can't compete with. He's successfully proved that people are curious about science and want to learn but in a different way compared to conventional education.

The future of the science and education is to serve innovation, bridge disciplines, create values, transform talent and support an efficient system to speed up this process.

Future of the World

Information does not only influence science and education. Information technology (IT) also drives the world economy and how we live in daily life. Improvement in IT changes how we manage companies, how we hire employees and drives new business models to match new technology. New information promotes software and hardware development to deliver information more efficiently.

Technology is the key to the future; it changes the world and the careers it needs. Ice making removed the need for ice cutters. Automating processes removed entry level jobs in every business sector. Wikipedia removed the need for encyclopedia. Events like this have happened over and over in our history. A recent example is the way the internet changed the newspaper and magazine industry. A detailed description can be found in the documentary film *Stripped* by Dave Kellett.

In summary, before 1820 only people with real talent and skill could draw very good portrait for important occasions. The invention of photography brought an end to this career, but it improved our way of documenting life events. Some artists adapted this change and shifted their focus from documentation of real life events to pure entertainment. They made up stories that were unreal and expressed their ideas with imagination. This successful adaptation led to individual wealth and a booming industry in the next a hundred years accompanied by popularization of magazine and newspapers. That was the golden time for

column artists, cartoon artist and comic artists, included Walt Disney. Things took another sharp turn in 2000s when internet became popular. There were more than 160 newspapers and 230 magazines closed between 2008 and 2010, and Sunday comic page of the newspaper became a history. During this time, most column artists lost their jobs, but some transitioned their work from print to online. Those quick adaptors suddenly became the stars of the internet entertainment industry and were followed by a large number of audiences. Soon, with online advertising fees and vendor sales, those artists exceeded their former glory.

Being able to adapt in a dynamic environment is a key to thriving in the future world. Technology is unpredictable and will bring big changes to the world, some can be devastating to many careers, but it also bring great opportunities for people who are able to quickly adapt and react to those changes. Highly skilled or talented individuals, especially in science, may not be immediate victims but should take caution regarding those changes and put away the pride when the waves come.

Information will become more diverse and technical areas will become specialized and hard to grasp. The future world will need individuals and organizations to cross disciplines, utilize what we know and turn knowledge into applicable products benefiting society and the planet as a whole.

I go from here, starting today, knowing the future will have the need to resolve diseases, knowing that science will become more complex and branched, knowing the world needs integration and innovation. I wish myself a good venture during my blink of existence in the universe; make a dent for neurological disease prevention and treatment.

Methods

Mouse Strains and Handling

Dscam^{GOF} and all other mouse line used were described in Chapter 5. Both *Dscam*^{FD} and 2J alleles are referred to as Dscam loss-of-function (*Dscam*^{LOF}) to contrast them with the gain-of-function allele (*Dscam*^{GOF}). *Dscam*^{LOF} refers to homozygous mutants, while heterozygotes are referred to as *Dscam*$^{LOF/+}$. *Bax* null mice were acquired from The Jackson Laboratory[113]. TH-GFP mice express GFP brightly in type 2 dopaminergic amacrine cells (DACs) and more dimly in type 1 DACs[114]. TH-GFP mice were a kind gift from Dr. Suzy Appleyard, Washington State University. All mice were housed on a 12-hour light:dark cycle and fed ad libitum. Mice used in this study were maintained on a mixed C57Bl/6J, C3H/HeJ and 129/P background except for mice carrying the Dscam2J allele, which are carried on an inbred C3H/HeJ background. The defective allele of Pde6b was crossed out of the C3H/HeJ mice. Mice of either sex were utilized for analysis in all experiments. All procedures performed on mice used in this study were approved by the University of Idaho Animal Care and Use Committee or by the Cleveland Clinic Institutional Animal Care and Use Committee and were in accordance with the Institute for Laboratory Animal Research Guide for Care and Use of Laboratory Animals.

Genotyping

Tissue biopsies were boiled in sodium hydroxide and neutralized in Tris Cl pH 5.0. PCR was performed using one shot master mix supplemented with primers. Genotyping has previously been described for all strains[47]. *Dscam*^{GOF} mice were genotyped by the presence of RFP (carries transgene), GFP (carries recombined transgene) or no fluorescent protein (does not carry the transgene).

Immunocytochemistry and Immunohistochemistry

Mice were perfused with PBS, retinas were hemisected and fixed in 4% PFA for either 30 minutes at room temperature (worked for all staining except for DSCAM), or for 50 minutes on ice (worked for all staining but is suboptimal for staining of some cytoplasmic proteins). Tissues to be embedded in wax were fixed in 1:3 acetic acid:methanol for 2-12 hours (paraffin sections gave equivalent antibody staining except for DSCAM, melanopsin and vglut3 staining). Tissue was stained as previously described[115].

List of Antibodies:

Mouse anti-DSCAM (R&D Technologies; MAB36661; 1:25),

Rabbit anti-cone arrestin (Millipore; AB15282; 1:5,000),

Rabbit anti-Dab1 (generous gift of Brian Howell, 1:500),

Goat anti-ChAT (Millipore; AB144P; 1:400),

Rabbit anti-calbindin (Swant; CB38a; 1:1,000),

Rabbit anti-bNOS (Sigma Aldrich; NZ280; 1:15,000),

Mouse anti-synaptotagmin2 (ZIRC; ZNP1; 1:500),

Mouse anti-PKCα (Santa Cruz Biotechnology; sc8393; 1:500),

Mouse anti-dystroglycan (DSHB, 1:500),

Mouse anti-PKARIIβ (BD biosciences; P54720; 1:1,000),

Rabbit anti-TH (Chemicon; 1:500),

Mouse anti-GS (BD Transduction Laboratories; 610517; 1:2,000),

Mouse anti-PSD95 (NeuroMab; 75-028; 1:200),

Rabbit anti-melanopsin (generous gift of Ignacio Provencio, 1:5,000),

Mouse anti-neurofilament Smi-32 (Covance, 1:1000),

Mouse anti-tyrosine hydroxylase (Nova Castra, 1:50),

Guinea pig anti-vglut3 (Millipore 1:5,000 Cell Signaling Technology)

Rabbit anti-phospho histone 3 (Cell Signaling Technology 1:500).

Dapi reagent was mixed into the second wash after incubation with secondary antibodies at a dilution of 1:50,000 of a 1 mg/ml stock.

Draq5 (Cell Signaling Technology) was used at a 1,000 dilution incorporated with secondary antibodies.

Peanut lectin (PNA): PNA, conjugated to alexa 488, cy3 or alexa 647, was incorporated into the secondary antibody incubation at a dilution of 1:1000 of the manufacturer's recommended concentration (Invitrogen).

TUNEL staining: Sections were stained with Roche's in situ cell death kit.

Secondary Antibodies: Secondary antibodies were acquired from Jackson Immuno Research and used at a concentration of 1:1000.

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Appendix A: Protocol Approval from Animal Care and Use Committee from the University of Idaho

University of Idaho
Institutional Animal Care and Use Committee

Date: Wednesday, January 14, 2015
To: Peter Fuerst
From: University of Idaho
Institutional Animal Care and Use Committee
Re: Protocol 2013-69
Role of Cell adhesion molecules in neural patterning

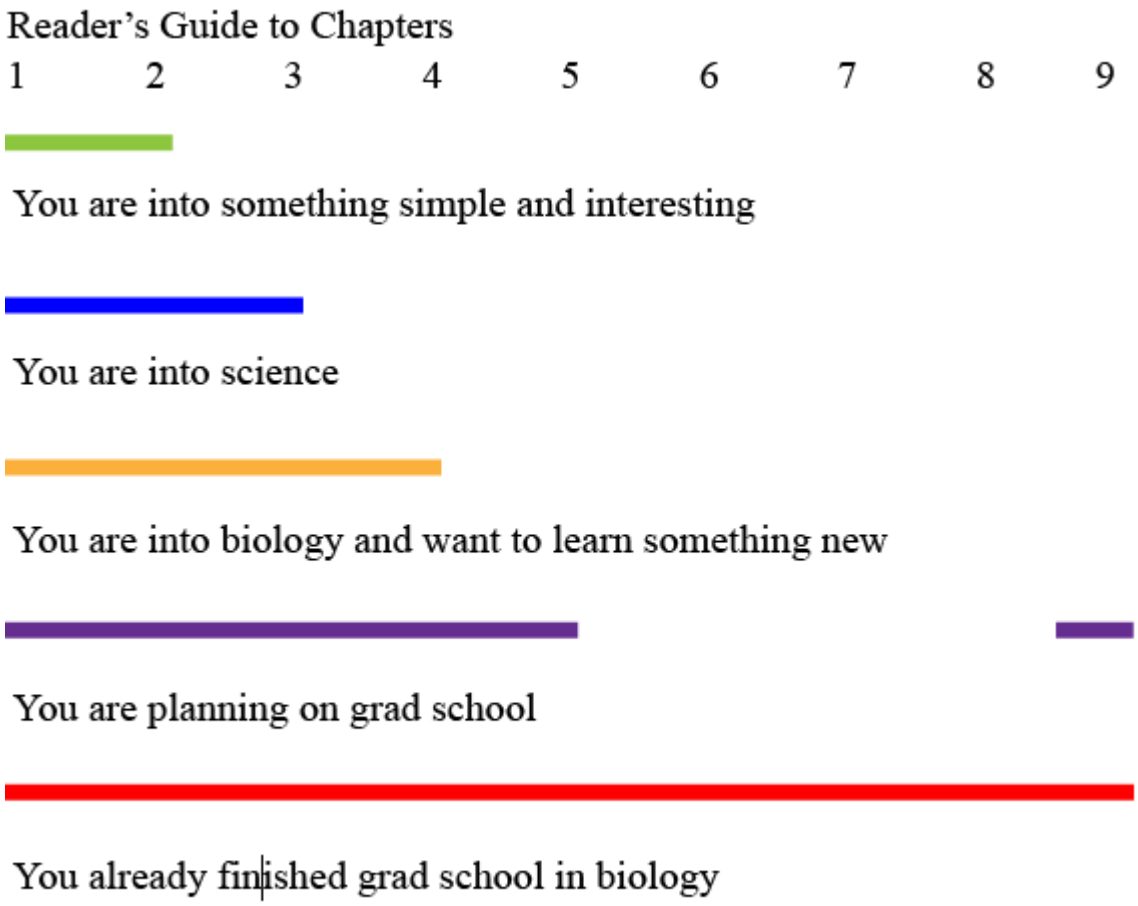
Your requested renewal of the animal care and use protocol shown above was reviewed and approved by the Institutional Animal Care and Use Committee on Wednesday, January 14, 2015.

This protocol was originally submitted for review on: Saturday, May 11, 2013
The original approval date for this protocol is: Thursday, June 20, 2013
This approval will remain in affect until: Thursday, January 14, 2016
The protocol may be continued by annual updates until: Monday, June 20, 2016

Federal laws and guidelines require that institutional animal care and use committees review ongoing projects annually. For the first two years after initial approval of the protocol you will be asked to submit an annual update form describing any changes in procedures or personnel. The committee may, at its discretion, extend approval for the project in yearly increments until the third anniversary of the original approval of the project. At that time, the protocol must be replaced by an entirely new submission.

Barrie Robison, IACUC Chair

Appendix B: How to Read This Book



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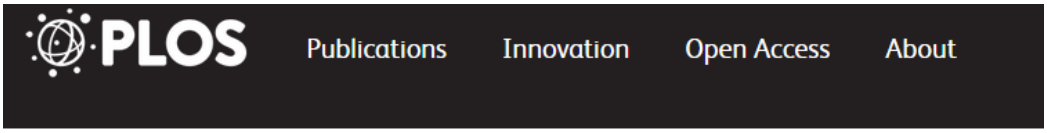

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