Role of Anaplastic Lymphoma Kinase (Alk) in *Drosophila* hematopoietic niche maintenance

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CERTIFICATE OF EXAMINATION

This is to certify that the dissertation titled “Role of Anaplastic Lymphoma Kinase in *Drosophila* hematopoietic niche maintenance” submitted by Aarathy R G (Ms12057) for the partial fulfillment of B.S.-M.S. dual degree programme of the Institute, has been examined by the thesis committee duly appointed by Institute. The committee finds the work done by the candidate satisfactory and recommends that the report be accepted.

Prof.Anand Kumar Bachhawat Dr. Kavita Babu Dr.Lolitika Mandal

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Dated :April 21, 2017

DECLARATION

The work presented in this dissertation has been carried out by me under the supervision of Dr.Lolitika Mandal at the Indian Institute of Science Education and Research Mohali.

This work has not been submitted in part or in full for a degree, a diploma, or a fellowship to any other university or institution. Whenever contributions of others are involved, every effort is made to indicate this clearly, with due acknowledgement of collaborative research and discussions. This thesis is a bonafide record of original work done by me and all sources listed within have been detailed in the bibliography.

Aarathy R G

(candidate)

-

Dated: 21-04-2017

In my capacity as a supervisor of the candidate's project work, I certify that the above statements by the candidate are true to the best of my knowledge.

Dr.Lolitika Mandal

(superviosor)

List of Figures

TABLE OF CONTENTS

ABSTRACT

Anaplastic Lymphoma Kinase(Alk) is a receptor tyrosine kinase protein with oncogenic potential. It is found in diverse activated forms in different cancer conditions including cancers in the blood system. However the physiological function of this protein is not well studied in mammalian system due to some constraints such as lack of information of a well defined ligand. In this study, we used the larval hematopoietic organ of *Drosophila* –the lymph gland as a model to understand the physiological role of Alk in the hematopoietic system. During this study, knocking down Alk from the hematopoietic niche resulted in an increase in niche cell number revealing that Alk functions as a negative regulator of hematopoietic niche cell number. However the loss of Alk from the niche did not affect the functionality of niche which implies that Alk solely regulate the proliferation of the niche cells. Through further analyses we were able to show that Alk could be involved in regulating cell cycle progression from G2 phase of cell cycle. These informations could shed light on the physiological significance of Alk in hematopoietic system. Moreover, we could establish a model that can be used to unravel the mechanistic basis of Alk signaling in normal situation which would further enable to better understand the alterations leading to oncogenesis.

CHAPTER 1

INTRODUCTION

I.1*Drosophila: a brief introduction*

Drosophila melanogaster, widely known as the common fruit fly is a really helpful and amazing model system for genetic and developmental studies because of the availability of large library of mutants, a large repertoire of genetic tools and the ease of phenotype analyses. In addition, the short lifecycle, ease of rearing in laboratory conditions and fully sequenced (**1**) small genomes makes *Drosophila* a convenient model system to handle.

Drosophila is a holometabolous insect, having distinct developmental stages as-egg, larva, pupa and adult. A mature adult female lays eggs at a rate of 50-70 per day. Development of *Drosophila* is dependent on temperature. At 25^{O}C, the eggs hatches after 24 hours and the first instar larva emerges out of it. The emerged larva feeds on the provided food and after 24 hours, undergo moulting to form a bigger second instar larva. The second instar larva continues to feed on the provided food and undergoes another moulting after next 24 hours to form the third instar larva which also feeds on food but later moves out of it to a relatively dry region to start pupation after about 30 hours. The pupal stage lasts for 3-4 days during which the metamorphosis takes place by replacing most of the larval structures with adult structures. The adult fly which emerges from the pupal has a life span of about 6 weeks. (*Figure :I.1*)

Drosophila melanogaster has a pair of sex chromosome and 3 pairs of autosomes, constituting a total of four pairs of chromosomes. The 4th pair is known as the dot chromosome which just contributes approximately 2 % of fly genome. Male fruit flies do not show meiotic recombination. *Drosophila* researchers make use of modified chromosomes called balancer chromosomes inorder to keep recessive lethal mutations in stock, preventing meiotic recombination and eventual loss. Balancer chromosomes have multiple inverted sequences which makes homologous recombination lethal. These chromosomes carry recessive lethal mutations which prevent them from becoming

homozygous and they also carry dominant visible markers which help in tracing them through generations.

DROSOPHILA LIFE CYCLE

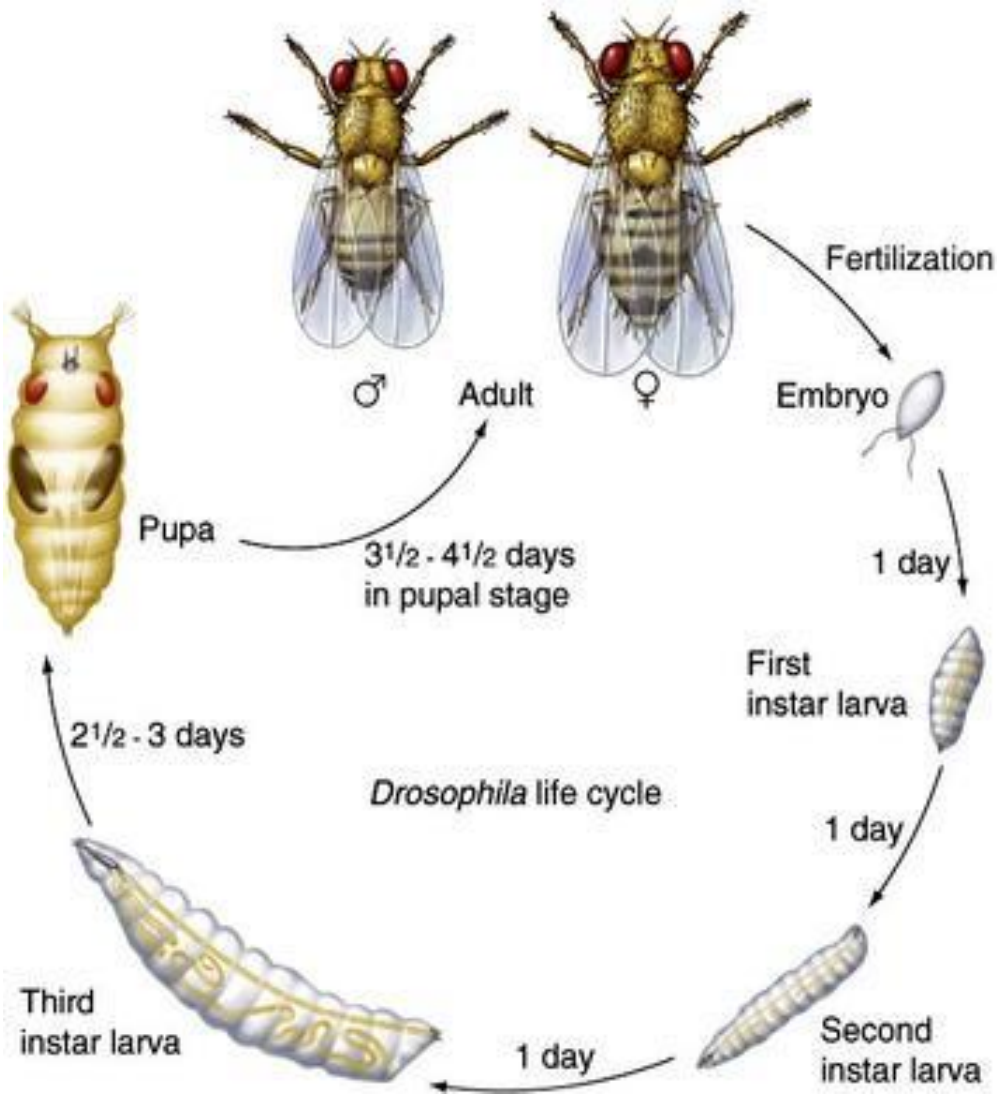


Figure (I.): Drosophila life cycle.

(http://www.creative-diagnostics.com/Drosophila.htm)

Comparative genome studies have shown that 60% of fly genes share similarity to human genes(**2**).Significant conservation of genes involved in development, aging, diseases including cancers and neurodegenerative disorders between the fruit-flies and humans makes flies a good model system to study these aspects.

I.2Drosophila: a model to understand blood cell development

Hematopoiesis or the process of blood formation is one such developmental process that is well studied in the fruit flies. *Drosophila* has been used as an efficient invertebrate model system to study various aspects of blood development. The low variability in cell types and less complexity in developmental processes makes it easier to dissect out the progressive developmental events and underlying pathways and molecular players in *Drosophila* model system. Significant degree of conservation is present between *Drosophila* hematopoiesis and the vertebrate counterpart not only in terms of the transcription factors (*table I.1*) and signaling pathway molecules but also in terms of the two waves of hematopoiesis that occurs during the development. Moreover, in both vertebrates and *Drosophila,* the blood cell lineage initiates from a common precursor for endothelial and blood cell lineage (**3**).

As in mammals, *Drosophila* hematopoiesis occurs in two waves- primitive and definitive (*Fig I.2*). In *Drosophila,* the primitive wave arises in the head mesoderm region during embryonic stage 5. It is similar to the mammalian primitive hematopoietic wave which sets in during the early embryonic stage in the extra-embryonic yolk sack. The primitive hematopoiesis is initiated with the specification of blood cells by a zinc finger transcription factor Serpent (srp) (member of GATA family) which is orthologous to the GATA factors involved in the blood cell specification of vertebrates (**3**)(**4)**. The definitive hematopoiesis in *Drosophila* occurs in the lymph gland which is similar to the mammalian definitive hematopoietic wave that arises in the Aorta-Gonad-Mesonephros (AGM).

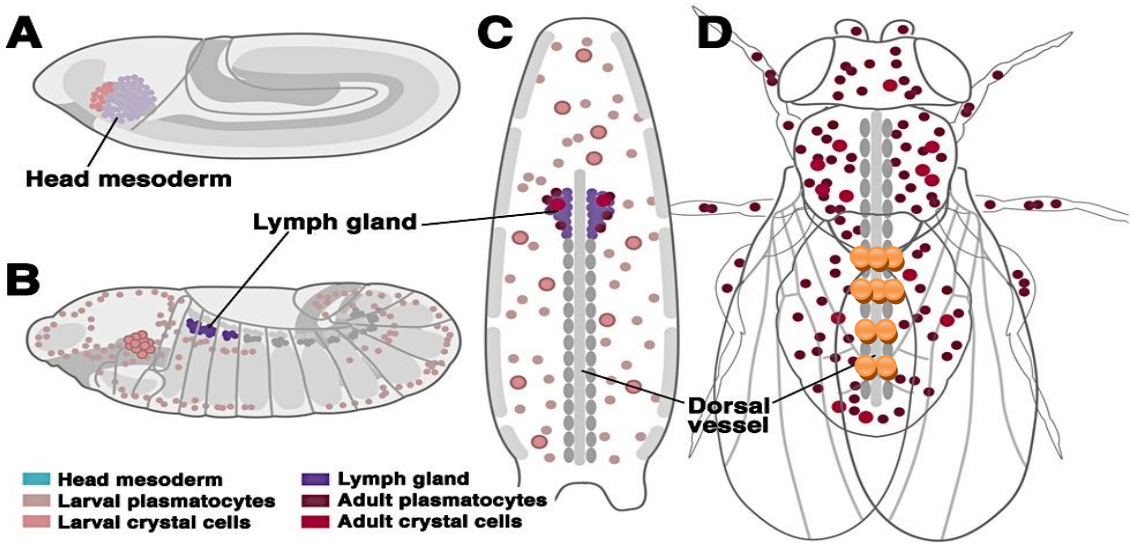


Figure I.2: Drosophila hematopoiesis (Modified from Cory J Evans et al., 2007).

A. Emryonic head- mesoderm, site of primitive hematopoiesis

B. Genesis of larval hematopoietic organ in the dorsal region of embryonic cardiogenic mesoderm.

C. Lymph gland

D. Hemocytes populating the hemolymph of adult fly

Drosophila	**Mammals**
Serpent	GATA
U shaped	Friend of GATA
Lozenge	Runx
Cut	Cux
Dorsal/Cactus	NFkB/IkB
Collier	Early B cell factor
Notch/Serrate	Notch/Jagged
Hop/Stat92E	JAK/STAT
Toll/18-Wheeler	Toll like receptors
Dpp	BMP4

Table I.1: comparative account of transcription factors and signaling molecules relevant for hematopoiesis in *Drosophila and vertebrates*

In contrast to the large variety of blood cell types present in vertebrates, only three blood cell types are found in *Drosophila.* - The macrophage like plasmatocytes, the melanising crystal cells and the large encapsulating lamellocytes. These cell types are similar to the myeloid lineage blood cells in vertebrates. The plasmatocytes (8-10µm) are macrophage like cells that are primarily involved in phagocytosis of foreign particles, microbes and tissue debris. The crystal cells(10-12)µm are so named for the presence of crystalline inclusions of prophenol oxidase enzymes are majorly involved in the melanisation, which is an insect specific immune response mechanism against pathogens. During melanisation, the phenol oxidases catalyzes the conversion of phenols to quinons which further polymerizes to form melanin that can sequester invading microbes to wound sites. Lamellocytes (15-40µm) are rare cell types that are generally activated during parasitic invasions. They are involved in encapsulation and neutralization of invasive objects that are too large for plasmatocytes to phagocytise.

I.3Lymph gland-the larval hematopoietic organ

The lymph gland which starts forming at the embryonic stage grows and matures through the initial larval stages to reach the mature multi-lobed structure in third instar larva (*FigureI.3*). The primary lobes of the lymph gland are much well characterized. It has three major regions- the cortical zone (CZ), the medullary zone (MZ) and the Posterior signaling centre (PSC) (FigureI.3). The outer cortical zone is composed of the differentiated cells. The inner medullary zone is homed by the stem like progenitor cells and the posterior signaling centre is composed of the niche cells which functions as a microenvironment for the maintenance of progenitor cells (**5**).The progenitor cells (the pro-hemocytes) are capable of self renewal and differentiation into different cell types- the plasmatocytes, crystal cells (and lamellocytes during pathogen infection). These different cell types can be identified based on the expression of specific molecular markers.

The lymph gland is formed initially during embryonic stage from embryonic thoracic segments T1, T2 andT3. Three odd skipped-expressing (odd skipped-a zinc finger transcription factor) clusters from these embryonic segments come together to form the primordial lymph gland having two primary lobes composed of approximately 20

primitive hemocytes.**(3)** By the end of second instar stage, the cells in primary lobe would proliferate to form about 200 cells in each lobe and further continues to proliferate and to reach around 2000 cells per primary lobe in a mature third instar lymph gland. This larval hematopoietic organ gets ruptures during the first 12 hours of pupation and the hemocytes are released into circulation.

The multi-potent progenitor cells that home the medullary zone of the lymph gland are the source of the functional differentiated cell types. Hence the maintenance and regulation of the progenitor population is crucial. This maintenance is highly dependent on the PSC/niche cells. The niche cells are specified during early embryogenesis by the expression of the homeobox protein antennapedia (antp). It have been shown that signaling mediated from niche through secreted Hedgehog and the JAK/STAT pathway **(6)(7)** are necessary for maintenance of the pro-hemocytes.

The niche cells are thus required for proper maintenance and homeostasis of the lymph gland. In a wild type scenario, the 4-5 niche cells at the embryonic stage increase in number to about 45-50 cells in a mature third instar lymph gland in the primary lobe. But this is in contrast to the prohemocytes that multiplies at a much higher rate. This is indicative of a tight regulation in the proliferation of niche cells. The maintenance of the hematopoietic niche is worth attention since they are regulating the maintenance of progenitor cells.

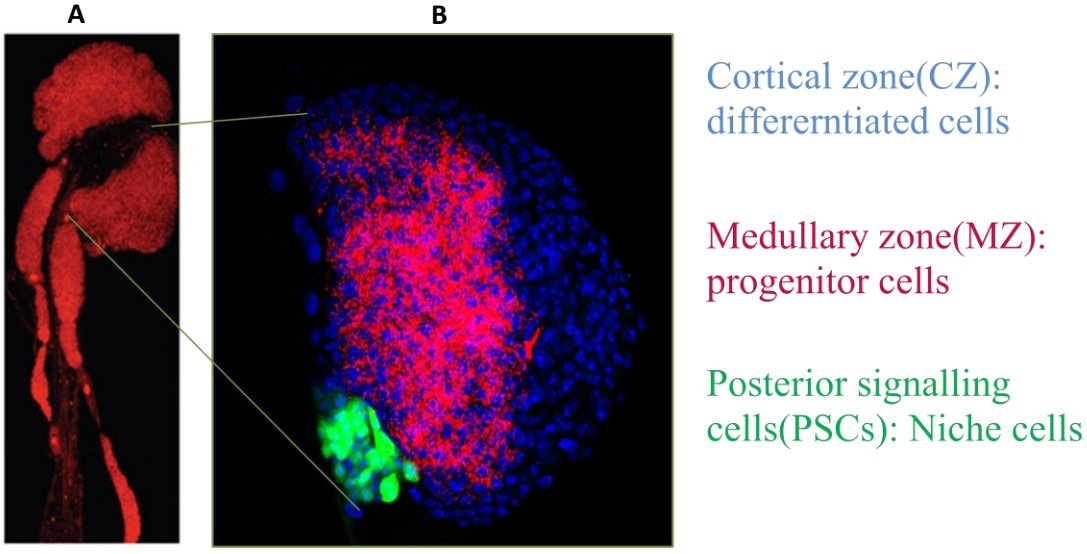


Figure I.3: Larval lymph gland

A: Third instar larval lymph gland with multiple lobes

B: Primary lobe divided into three different regions

I.4Genetic tools used in this study:

I.4.1Gal4-UAS system

Gal4 –UAS system, introduced to scientific world by Robert Perimon (in 1993), is one of the elegant genetic tool that has been most commonly used in fly system for tissue specific gene expression or gene knock down, especially to understand their developmental roles. In this technique, two different transgenic parental lines are used- an activator and an effector line. In the activator line, yeast transcription factor Gal4 is inserted downstream to a tissue specific promoter- the driver (The promoter of a gene which is specifically expressed in the tissue in which we intend to express our gene of interest). The effector line has our gene of interest fused next to the UAS sequence which is the binding site for the Gal4 protein. These two lines are crossed .In the F1 progeny, Gal4 protein is expressed according to the expression pattern of the driver. This Gal4 goes and binds the UAS sequence and express our gene of interest. Thus our gene of interest also follows the expression pattern of the driver. *Figure I.4.*If a reporter gene like a GFP is fused downstream to the UAS sequence, this line can thus be used as an enhancer-trap

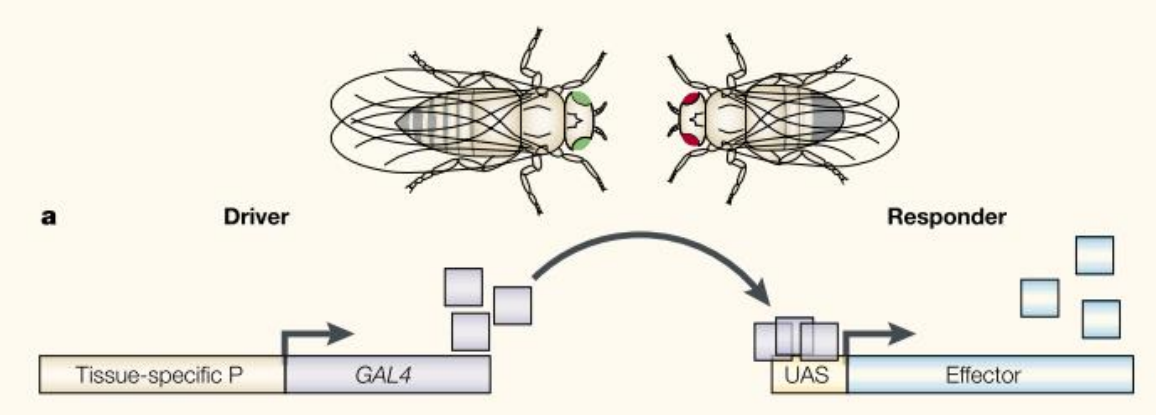


Figure I.4: Gal4-UAS system (http://hawaiireedlab.com/wpress/?p=477)

An additional temporal regulation over this system can be achieved by using a temperature sensitive gal80 (ts gals80) protein along with this system. The ts gal80 binds

to the activation domain of gal4 protein and prevents it from binding to the UAS sequence at $18^{O}C$ whereas at higher temperature (about $25^{O}C$ and above) the gal80 remains inactive. In such a system, the Gal4 protein shows maximum expression at $29^{O}C$.

I.4.2RNA interfertence

RNA interference was first observed by Fire and Mello in *Caenorhabditis elegans.* They observed that double stranded RNA (ds RNA) is a source of sequence specific post transcriptional inhibition of gene expression.

RNA interference is a biological process in which RNA oligo-nucleotides inhibits gene expression in a sequence specific manner by interfering with the messenger RNA (mRNA), either by blocking translation or by degrading the mRNA. It is natural process evolved in various biological systems in different contexts. For instance RNAi is evolved as a mechanism to fight against pathogen invasion in plants. In some animals, RNAi functions to lower down the expression of certain developmental genes.

In RNA interference pathway, double stranded RNAs are cleaved along their length by an enzyme called Dicer into duplex oligonucleotides which are 19-22 nucleotides long. These oligo-nucleotides are known as small interfering RNAs (si RNAs). These double stranded si RNAs further associates with RNA Induced Silencing Complexes (RISCs) which are large protein complex having argonaute proteins at their catalytic core. The si RNAs will get unwound inside the RISC complexes and their sense strands will be degraded. The RISC complex is directed to the target mRNA by the antisense strand of the si RNA which gets retained. It anneals to the complementary region in the target mRNA and triggers endo-nuclease activity of the RISC complex to degrade it.

The RNA interference is now extensively used for genetic and developmental studies in several model organisms including *Drosophila.* One way of introducing artificial RNA interference is to develop transgenic RNAi lines by inserting DNA coding for short hair pin RNA (sh RNA- single stranded RNA molecule with two sequences which are complementary to each other are separated by a non-complementary sequence so that the complementary sequences fold on to each other to form a hairpin-like structure). These sh RNAs are further processed through endogenous RNA interference pathway which finally

lead to formation of si RNA that can specifically interfere with the mRNA of our interest and knockdown the expression of corresponding protein. A schematic diagram of such an induced RNAi is depicted in *Figure I.5*

In the fruit flies, the RNA interference have been extensively used along with the Gal4-UAS system to specifically knock down a gene of interest in a tissue specific manner. The use of tsgal80 enables an additional temporal regulation over this system. This system can be exploited to unravel tissue specific role of various genes.

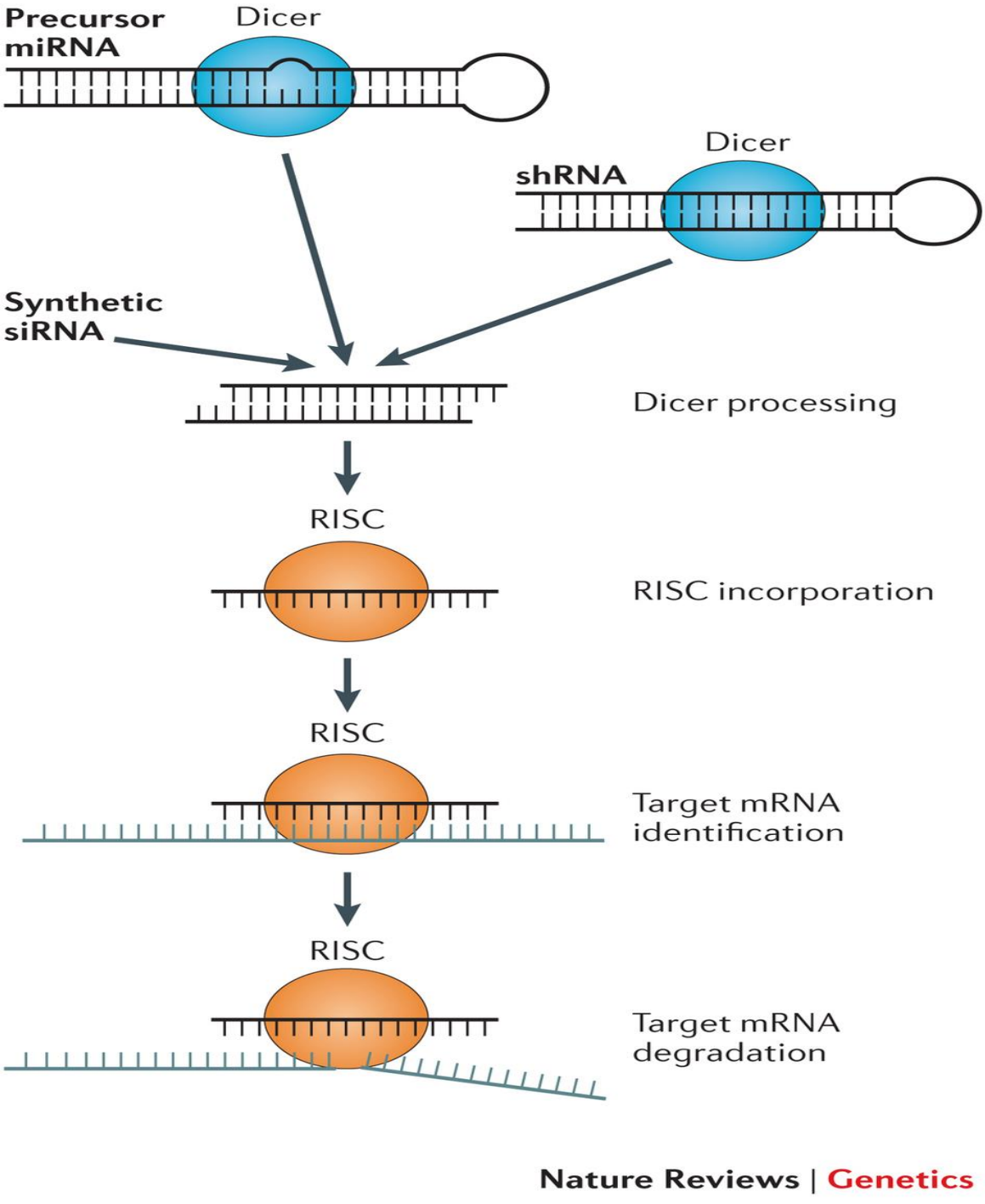


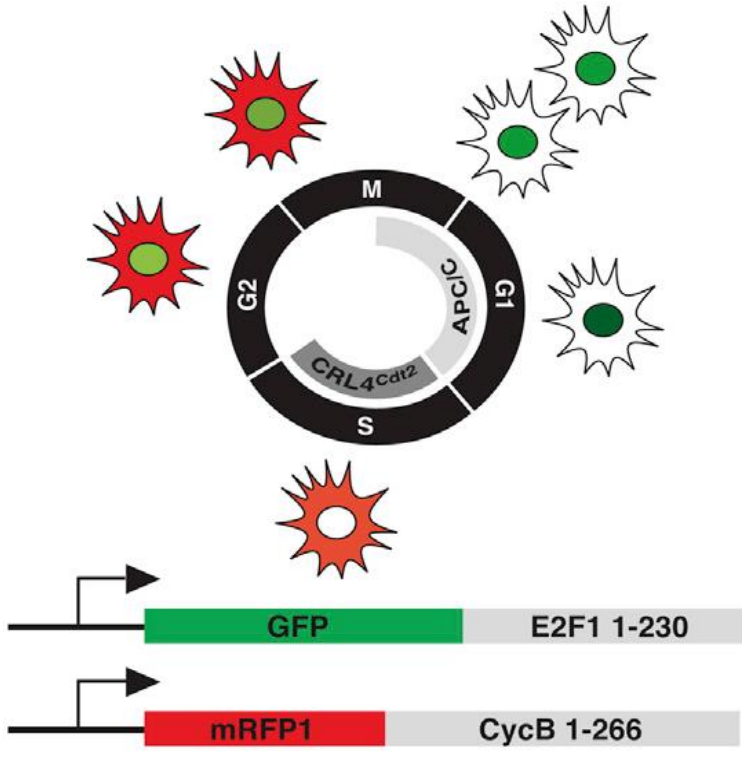
Figure I.5: RNA interference pathway

(Anders Wittrup & Judy Lieberman 2015)

I.4.3 Fly FUCCI

Fly FUCCI (fluorescence ubiquitination based cell cycle indicator) is a powerful genetic tool that can be used to understand the cell cycle status of individual cells. FUCCI allows the distinction of G1, S and G2 phases of cell cycle.

When a normal cell, From mid-mitosis through G1 phase, Cyclin B is tagetted to degradation by an ubiquitin ligase APC/C by recognizing a degron in the Cyclin B protein. Similarly the E2F1 protein is targeted to degradation by the ubiquitin ligase $CRL4^{Cdt2}$ on the onset of S phase. FUCCI relies on this wherein an RFP tagged degron of Cyclin B1 and GFP tagged degron of E2F1 are expressed continuously in desired cell types with the use of Gal4-UAS system. In G2 phase due to the presence of both the fluorescent probes, cells will fluoresce yellow. From mid mitosis through G1, mRFP1-$CycB_{1–266}$ is targeted to degradation by the APC/C complex due to which the cells will fluoresce green whereas in S phase GFP-$E2F_{11–230}$ is targeted to degradation $CRL4^{Cdt2}$ due to which the cells will fluoresce red *Figure I.6*



(Norman Zielke et al (2014))

Figure I.6: FLY FUCCI

MATERIALS AND METHODS

II.1 Fly stocks

Flies were raised on standard agar-corn flour yeast medium and were generally kept in 25^{O}C. These vials were transferred in to 29^{O}C based on the developmental time window in which the Gal4 needs to be activated since Gal4 shows maximum activity at 29^{O}C. Around 20 females and 15 males were used for crosses in vials.

Mutant lines

Fly line	Feature	Source
Alk^{1}/cyo actin GFP	• Truncated mutant of Alk (homozygous) • Present in second chromosome	Dr. Ruth Palmer
Jeb ^{k05844}/cyo actin GFP	• Lac Z insertion in the promoter of Jeb (homozygous) • Present in second chromosome	BDSC

Table II.1: Mutant fly lines

UAS RNAi lines

The different UAS-RNAi fly lines used in this study were generated under Transgenic RNAi Project (TRiP) of Harvard University, USA. They were obtained from Bloomington Stock Centre, Indiana, USA.

Fly line	Feature	Source
UAS Alk RNAi	• Expresses dsRNA for RNAi of Alk under UAS control • Inserted chromosome: 3	Bloomington Drosophila stock centre(BDSC)(27518)
UAS Tramtrack RNAi	• Expresses dsRNA for RNAi of tramtrack under UAS control • Inserted chromosome: 3	Bloomington Drosophila stock centre(36748)
UAS Dscam RNAi	• Expresses dsRNA for RNAi of Dscam under UAS control • Inserted chromosome: 3	Bloomington Drosophila stock centre(38945)
UAS Dystroglycan RNAi	• Expresses dsRNA for RNAi of Dystroglycan under UAS control • Inserted chromosome: 3	Bloomington Drosophila stock centre(34895)
UAS String RNAi	• Expresses dsRNA for RNAi of string under UAS control • Inserted chromosome: 3	Bloomington Drosophila stock centre(34831)

Table II.2:UAS RNAi fly lines

UAS line

Fly line	Feature	Source
UAS Alk^{DN}	• Dominant negative mutant of Alk • Present in second chromosome	Dr. Ruth Palmer
w^{1118}; P{UAS-GFP.E2f1.1-230}32 P{UAS-mRFP1.NLS.CycB.1-266}19/CyO, P{en1}wg^{en11}; MKRS/TM6B, Tb^{1}	Fly-FUCCI line	BDSC (55121)

Table II.3: UAS fly lines

Reporter fly line

Fly line	Feature	Source
w*; P{TRE-EGFP}attP16	Reporter of JNK pathway	BDSC

Table II.4: Reporter fly line

II.2mmunocytochemistry assay

Late third instar larvae from synchronous culture were dissected in 1x PBS on ice, followed by fixation of the tissues in 4% para-formaldehyde for 40 minutes at room temperature on shaker. After fixation tissues were washed three times in 0.3% PBT. This was followed by blocking in 10% NGS (Normal Goat Serum) for 40 minutes. Once blocking is done, samples were incubated in primary antibody overnight (18 hours) at 4^{O}C. After incubation, the samples were washed 4 times, 15 minutes each using 0.3%PBT. Then blocking was done using 10% NGS (for 40 minutes). After this samples were incubated with secondary antibody overnight at 4^{O}C. After secondary incubation, the samples were washed using 0.3% PBT for 4 times, 15 minutes each and they were incubated with DAPI solution for 1 hour. After DAPI treatment samples were quickly washed in PBS two times, 5 minutes each and mounted in Vectashield (Vector Labs).

Note: For wingless staining 0.1% tween 20 was used instead of 0.3% PBT, 1%BSA was used as block.

II.3Fly FUCCI analysis

Late third instar larvae from synchronous culture were dissected in 1x PBS on ice, followed by fixation of the tissues in 4% para formaldehyde for 40 minutes at room temperature on shaker. After fixation tissues were washed three times in 0.3% PBT followed by 40 minutes DAPI incubation. After DAPI treatment samples were quickly washed in PBS two times, 5 minutes each and mounted in Vectashield.

II.4Antibodies used

Different primary antibodies used in this study are listed below in *table II.5*, *table* II.6

Antibody	Developed in	Source	Details
Anti-antennapedia	Mouse	DHSB, Iowa	Working dilutuion 1:10
Anti –wingless	Mouse	DHSB, Iowa	Working dilution 1:3
Anti-P1	Mouse	Generously gifted Prof Ando	Working dilution 1:40
Anti-hedgehog	Rabbit	Generously Gifted by Prof.Stephen Cohen	Working dilution 1:500

TableII.5: List of primary antibodies.

Secondary antibodies for immunostaining

Different secondary antibodies used in this study are listed in *table II.6.*

Secondary antibody	Source	Details
Cy^{TM3}-Conjugated affinipure donkey anti rabbit IgG(H+L)	Jacksons Immuno Research Laboratories, USA # 711-165-152	• conjugated with cyanine Cy^{TM3} dye (absorption maxima/ emission maxima is 550 nm/ 570 nm) • working dilution: 1:700 • Detect primary antibodies raised in rabbit.
Cy^{TM3}-Conjugated affinipure goatanti mouseIgG(H+L)	Jacksons Immuno Research Laboratories, USA # 711-165-152	• conjugated with cyanine Cy^{TM3} dye (absorption maxima/ emission maxima is 550 nm/ 570 nm) • working dilution: 1:700 • Detect primary antibodies raised in rabbit.

Table II.6: List of secondary antibodies used.

II.5 Genotype of fly lines analyzed

For secondary screening of selected genes for confirmation of RNAi phenotype got through primary screen	
Driver fly	**Genotype generated/ used for the study**
Antp >Gal4	*w; tubgal80 ^{ts}/+; AntpGal4,UAS GFP/UAS Alk RNAi*
	w; tubgal80 ^{ts}/+; AntpGal4,UAS GFP/UAS Dscam RNAi
	w; tubgal80 ^{ts}/+; AntpGal4,UAS GFP/UAS Dystroglycan RNAi
	w; tubgal80 ^{ts}/+; AntpGal4,UAS GFP/UAS String RNAi
	w; tubgal80 ^{ts}/+; AntpGal4,UAS GFP/UAS Tramtrack RNAi
	w; tubgal80 ^{ts}/+; AntpGal4,UAS GFP/+
To understand the role of Alk in niche maintenance	
Driver	**Genotype generated/used**
Antp >Gal4	*w; tubgal80 ^{ts}/UAS Alk DN; AntpGal4,UAS GFP/+*
To understand the cell cycle status of niche cells	
Driver	**Genotype generated/used**
Antp >Gal4	*w; UAS fucci/UAS Alk DN; AntpGal4/+*
Antp>Gal4	*w; UAS fucci/+; AntpGal4,UAS RFP/+*

To understand the signaling pathway employed by Alk in niche regulation	
Driver	**Genotype generated/used**
Antp>Gal4	*w; UAS Alk ^{DN}/TRE GFP; AntpGal4,UAS RFP/+*
	w; +/TRE GFP; AntpGal4,UAS RFP/+

TableII.7: Genotype of flies analyzed in the study

Note: Antennapedia (*Antp*) is a homeotic gene that specifies the hematopoietic niche cells. The Gal4 is expressed under the P2 promoter of this gene.

II.6 Stains used

DAPI (4',6-diamidino-2-phenylindole dihydrochloride, Molecular Probe, India # ZB1130). This is a blue fluorescent dye that binds to A-T rich region in dsDNA.It is generally used to stain nuclei in live as well as fixed tissues. Its absorption maxima/ emission maxima are 351nm/461nm. The working dilution is 1µg/ml.Tissues after secondary antibody treatment were washed thrice for 5 minutes each with 1X PBS. Then, tissues were incubated in DAPI solution (1µg/ml) in 1X PBS for 30 minutes at room temperature. After incubation, tissues were washed with 1X PBS and observed after mounting in vectasheild.

II.7 Imaging

Images of the mounted samples were taken by using confocal microscope(Zeiss LSM780).The images were further processes by using Image J(NIH software)

Magnification: (40xoptical magnification)x(0.9digital zoom). For zoomed image (63x optical magnification)x(1.3digital zoom)

II.8 Cell counting

Following image processing in Image J software, the niche cell number was counted manually by going through the Z stack.

II.9 Statistical analysis

Data are expressed as mean±standard deviation (SD). Statistical analysis was performed using two-tailed Student's t-test. Significance was accepted with P values of <0.01%; <0.001% and <0.0001%, mentioned as *, **, *** respectively.

RESULTS AND DISCUSSION

Understanding the novel functions of known genes and identification of novel genes involved in physiological functions are key to advancements in understanding developmental systems. Genome wide RNAi screen is a powerful method that can be used to address these interesting questions. It has been widely used over the past few decades in different models.

In our lab, a genome wide RNAi based screen was employed to unravel the genes that are involved in the regulation of niche cell maintenance. Using Gal4 UAS system, individual genes were knock down specifically from the niche employing a niche specific driver and the resultant niche cell number was counted based on the GFP expression (Antp>Gal4,UAS GFP X UAS gene x RNAi). The schematic representation is depicted in *figure III* .1)

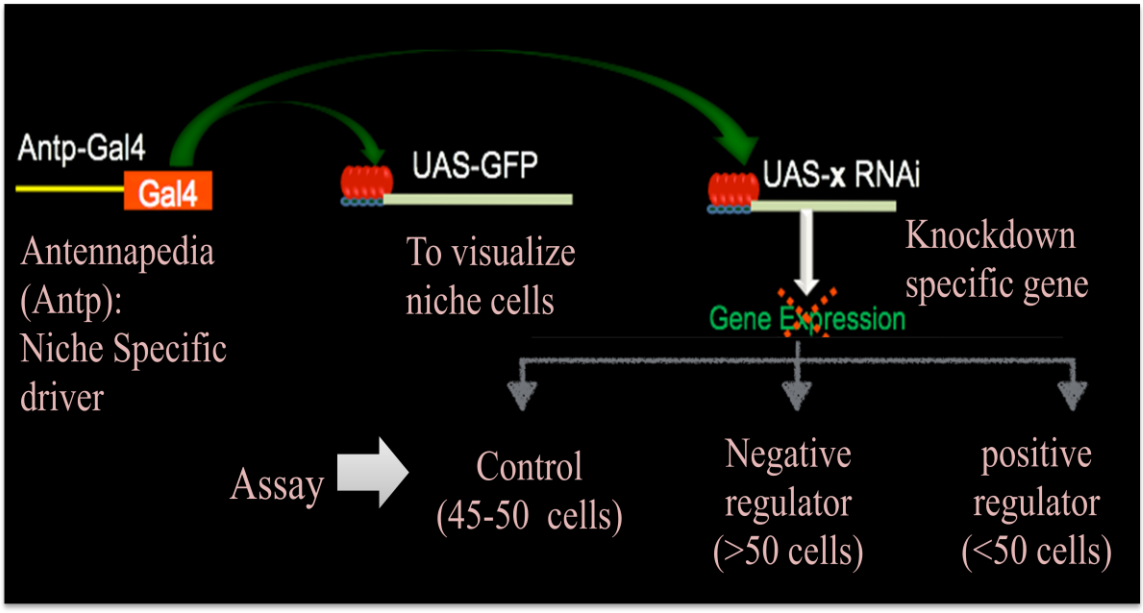


Figure III.1: Genome wide RNAi screen to unravel regulators of niche cell maintenance

Several genes were identified as potential regulators of niche maintenance through the primary screen. When I joined the laboratory, I was assigned the task to confirm the phenotype obtained from RNAi of some selected genes from this initial screen. I thus performed a secondary screen for these selected candidate genes to further validate the phenotype. For secondary screening, immunostaining against the niche specific protein Antennapedia was done to identify and quantify niche cell number rather than quantification based on GFP expression. As part of the secondary screen, the candidate genes were knock down individually from the niche cells by employing the Gal4-UAS technique. Flies harbouring Antp>Gal4UASGFP, were crossed to the responder flies which had the UAS sequence proximal to the RNAi of our gene of interest, whose function we wanted to attenuate from the hematopoietic niche. The crosses were maintained in vials at 25^{o}C and immediately after synchronous egg collection; the vials were shifted to 29^{o}C and reared till dissection.

III.1 Secondary screening of selected genes for validation of RNAi phenotypes

The larvae were dissected at late third instar stages and the tissues were processed for Antennapedia staining. The image was taken in confocal microscope (Zeiss LSM780) and processed using image J software. The number of niche cells was counted manually by going through the Z stacks with the help of image J software. The phenotypes observed are shown in *Figure III.2*

Secondary screening of selected candidates for validation of RNAi phenotypes

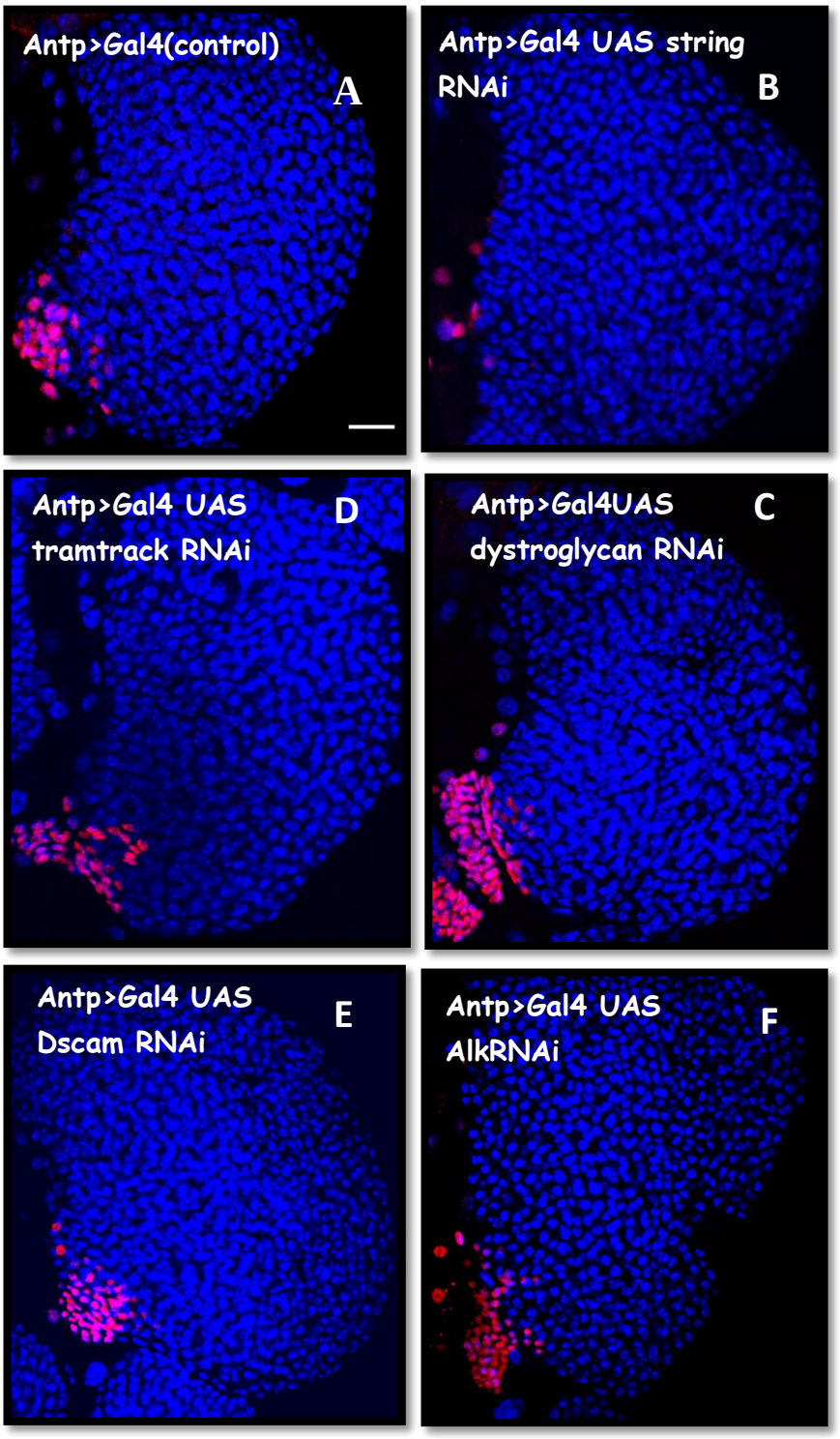


Figure III.2: Validation of RNAi phenotypes using Antennapedia staining

The list of genes that I have selected and the results obtained are summarized in (*Table III.1*)

Genes screened	Phenotype when knock down from niche using RNAi	Role in niche cell number regulation
Dystroglycan	Increase in niche cell number	Negative regulator
Tramtrack	No significant change	No role
String	Decrease in niche cell number	Positive regulator
Dscam	No significant change	No role
Anaplastic Lymphoma Kinase	Increase in niche cell number	Negative regulator

Table III.1: selected genes screened for validation of RNAi phenotype

Note:

Dystroglycan: A transmembrane protein majorly involved in connecting extracellular matrix proteins to cytoskeleton.

Dscam: Homologue of human Down syndrome cell adhesion molecule, belonging to the immunoglobulin superfamiy.

Tramtrack: Transcriptional repressor proteins. Involved in cell specification, cell cycle regulation and cell proliferation

String: Drosophila homologue of cdc 25 which is a protein phosphatase involved in removing inhibitory phosphorylation of cdk1, allowing it to function together with Cyclin B1, leading to G2-M transition of cell cycle.

Anaplastic lymphoma kinase: A transmembrane receptor tyrosine kinase.

From the genes screened as part of secondary screen, I chose Anaplastic lymphoma kinase (Alk) for further characterization.

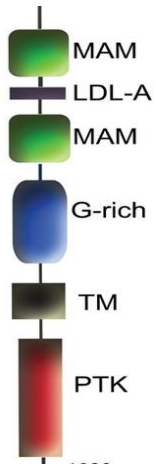
III.2 Anaplastic lymphoma kinase (ALK)

Anaplastic lymphoma kinase (ALK) is a Receptor Tyrosine Kinase protein (RTK) that belongs to the insulin receptor super family and it shows close relation to the orphan receptor-Leukocyte Tyrosine Kinase (LTK). ALK was first identified as a fusion protein with nucleophosmin (NPM-ALK) resulting from chromosome (2;5) translocation in Anaplastic large cell lymphoma.

ALK has a multi domain structure *Figure III.3*. The extracellular region of ALK is composed of two MAM domains, an LDLa domain and a glycine rich domain. Followed by the extracellular domains, there is a transmembrane region and cytoplasmic Protein Tyrosine Kinase Domain.

ALK in mammals

ALK is found to be activated in several cancers including anaplastic large cell lymphoma (ALCL), diffuse large B cell lymphoma (DLBCL), non-small-cell lung cancer (NSCLC, inflammatory myofibroblastic tumour (IMT), ovarian cancer and breast cancer. In multiple cancers, the kinase domain of ALK is found to be fused to various proteins, leading to immediate dimerization, rendering them to constitutively active forms. In some cancers such as neuroblastoma, NSCLC and Anaplastic Thyroid Cancer (ATC), point mutations especially in the PTK domain are also reported. Alk is found to be activating diverse downstream signaling pathways in different cancer contexts. This include the RAS–MAPK-ERK pathway**)**, phospholipase Cγ (PLCγ) pathway, Janus kinase -signal transducer and activator of transcription (JAK-STAT) and PI3k-Akt pathway, Jun N terminal kinase (JNK) pathway (**8)** (**9)** (**10**). However the physiological role of ALK in mammals is still not dissected out properly. This is mainly due to the lack of information about a well defined ligand for ALK in the mammalian system. Information about downstream signaling pathways in mammalian ALK is obtained from activated oncogenic forms of ALK and these might be altered functionality developed in context of cancer. Therefore other model systems such as *Drosophila, C. elegans* and Zebra fish which shows significant conservation for the ALK protein with the mammalian counterpart can be used for dissecting out the physiological role of this protein.



(***Modified from*** Christian ***C. Lee et al***)

Figure III.3 : Anaplastic Lymphoma Kinase(ALK)

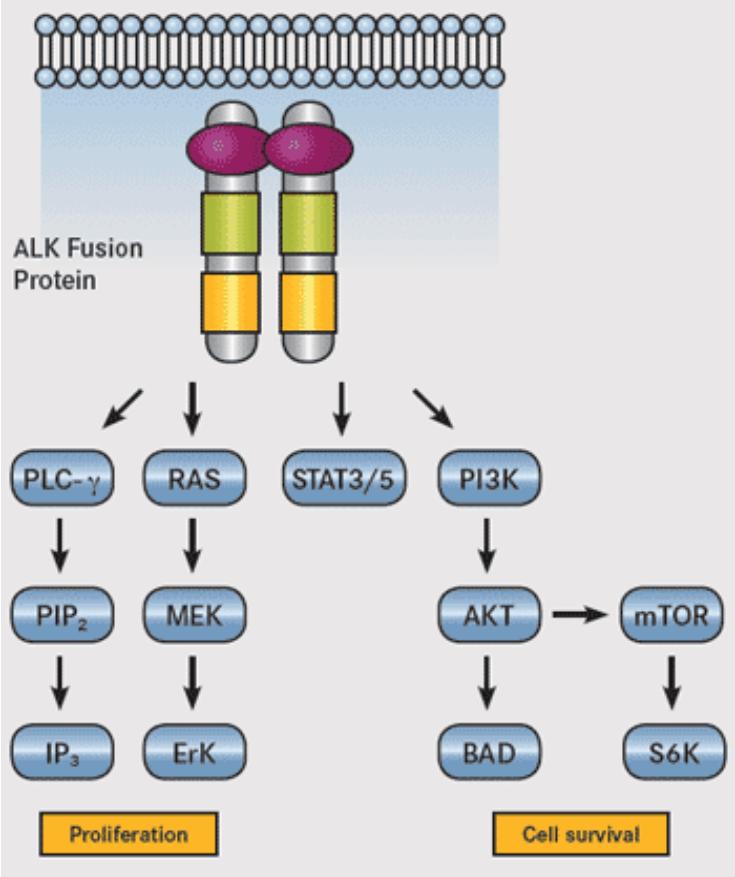
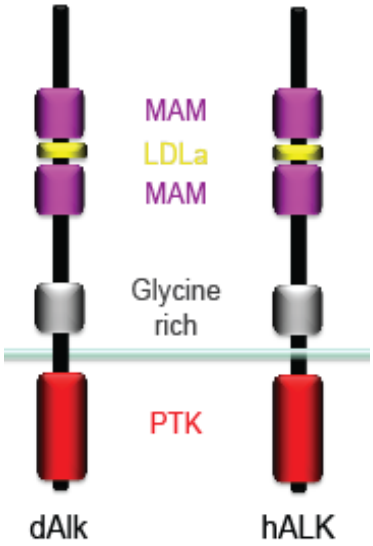


Figure III.4: Activated Alk signalingin cancer

(www.pinterest.com/onclive/medical-illustrations-and-graphs)

Anaplastic lymphoma kinase (Alk) in *Drosophila*

Anaplastic lymphoma kinase (Alk) was first discovered in *Drosophila* in 2001(**9**). It shows significant similarity to the mammalian ALK in its domain architecture (*Figure III.5).* In addition, the ligand of Alk is already discovered in *Drosophila*. The ligand, known as the Jelly Belly (jeb) is a protein of about 50 kDa. Jeb-Alk signaling is studied in different developmental contexts in *Drosophila.*



(Jikui Guan et al, elife 2015)

Figure III.5: conservation between human and Drosophila Alk

It is found that Jeb/Alk signaling is required at somatic-visceral mesoderm junction during embryonic stage for specification of visceral muscle cells. (**10**) Alk signaling is also implicated in the context of eye development (for axonal targeting) (**11**)and in establishment of functional synaptic connectivity in developing motor circuit (**13**). Alk is also reported to be involved in sparing the growth of Central Nervous System (CNS) during nutrient restricted conditions (**14**). Alk protects growth of neurablast against the low level of amino acid and insulin like peptides during nutrient restriction (NR). During NR, Alk suppresses growth requirement of amino acid sensing through slimfast/Rheb/TOR complex by leading to phosphorylation of 4-EBP, independent of TOR C1 and by activating PI3K, overriding the requirement for Insulin Receptor (InR).

Mutations in Alk

Mutations were introduced in *Drosophila* Alk for inferring more about its function. The mutations can be divided into three major classes- Truncations, point mutations in extracellular domain and point mutations in intra cellular PTK domain.

The major mutants developed in *Drosophila* by a designer mutation in Alk 53 locus followed by an Ethymethane sulphonate(EMS) screen are depicted in (*Figure III.6*) through schematic indications.(**15**)

In addition to these, a dominant negative mutant of Alk having the extracellular and transmembrane domains followed by a residual intracellular domain is also used in *Drosophila.*

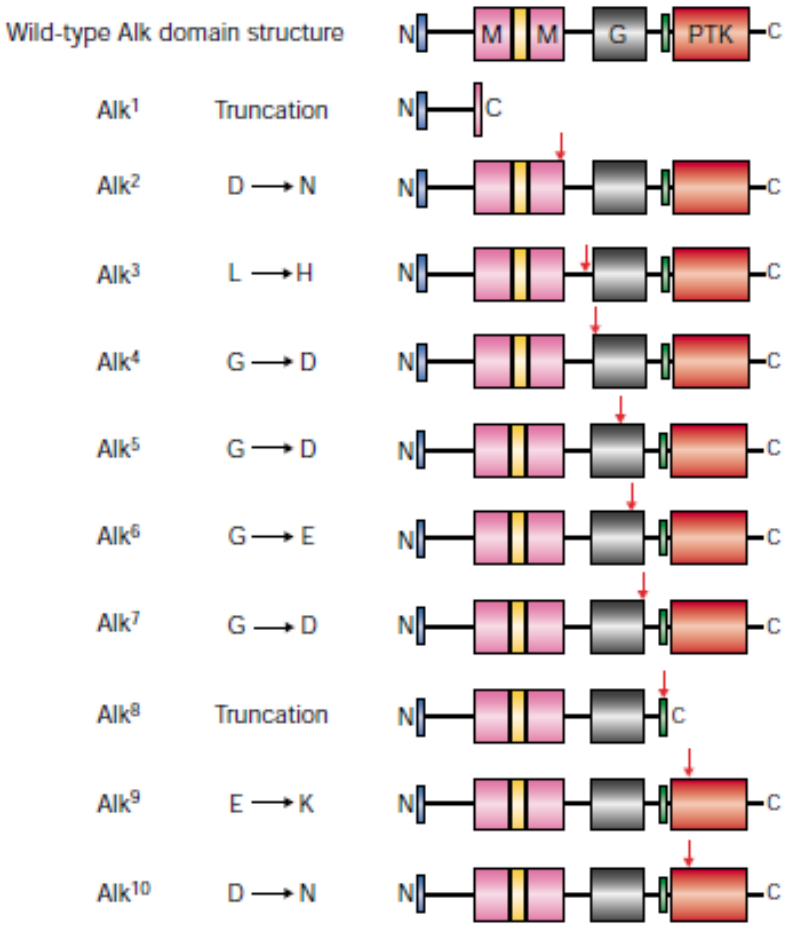


Figure III.6 : Mutations in Alk **(**Christina E Loren et al; 2003 ***)***

Objectives

- Confirm the phenotype observed through RNAi of Alk by expressing a dominant negative mutant version of Alk in niche and further analyze the status of niche cells in the background of a classical loss of fuction mutant of Alk.
- Understand the role played by Alk in regulating proliferation
- Characterize the pathway/pathways employed by Alk in the niche cell regulation

III.3 Anaplastic Lymphoma kinase negatively regulates niche cell number

For further confirmation of the phenotype obtained through RNAi, A dominant negative mutant form of Anaplastic lymphoma kinase (Alk ^{DN})was expressed in the niche using Gal4 UAS system and the phenotype was analyzed. For that, flies harbouring Antp>Gal4UASGFP, were crossed to the responder flies which had the UAS sequence proximal to gene encoding Alk ^{DN} and the progeny larvae were dissected in late third instar stage. The results obtained are shown in *Figure III.7*

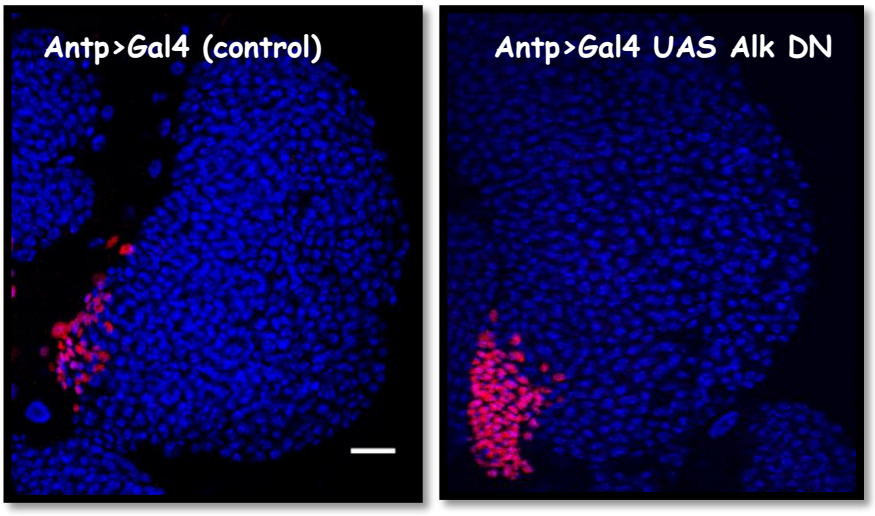


Figure III.7: Niche cell number in control vs Alk ^{DN}

(Scale bar:20µm)

The data clearly indicates that there is a drastic increase in the niche cell number when Alk is knock down from the niche using dominant negative mutant form of the protein.

The experiment was repeated (n=10) and the results obtained are summarized in the graph- **figure III.8.**

Average niche cell number in control : 45 ±7

Average niche cell number in Alk^{DN} background: 102 ± 18

`A comparison of niche cell number in control Vs Alk knockdown codition

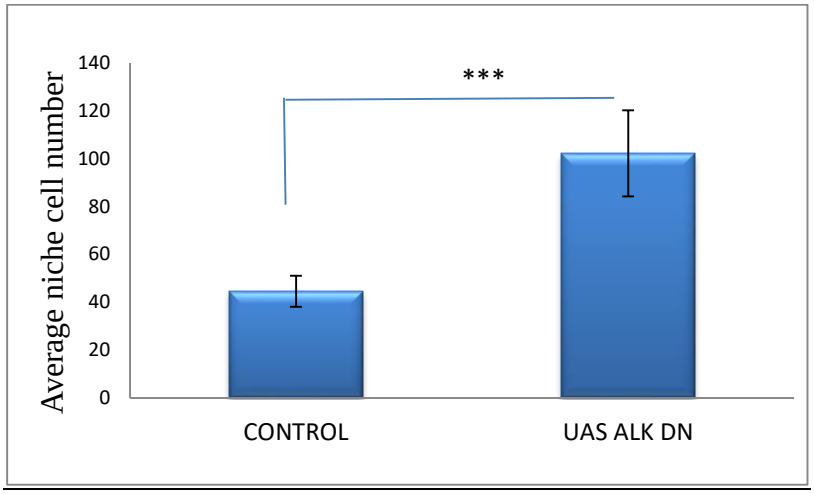


Figure III.8: graph summarizing the difference in niche cell number in control Vs Alk^{DN} background

There was a significant increase in the the niche cell number when Alk was knock down from the niche using the dominant negative mutant.

Analysis of classical loss of function mutants of the receptor Alk and ligand Jeb

Upon knocking down Alk from the niche using RNA interferece as well as by expressing dominant negative mutant specifically in niche, a hyperplastic niche was observed. We next analyzed the niche cell number in a classical loss of function mutants of Alk (Alk^{1}) as well its ligand Jeb ($Jeb^{k05844)}$.

Alk^{1} and ($Jeb^{k05844)}$ are lethal in homozygous condition. Hence we crossed w; Alk^{1}/cyo and w ;Jeb^{k05844};cyo actin GFP;+/+ with wild type Oregon R flies to obtain w; Alk^{1}/+; +/+ and w; Jeb^{k05844}/+; +/+ respectively. Interstingly, heterozygos condition yielded a phenotype.

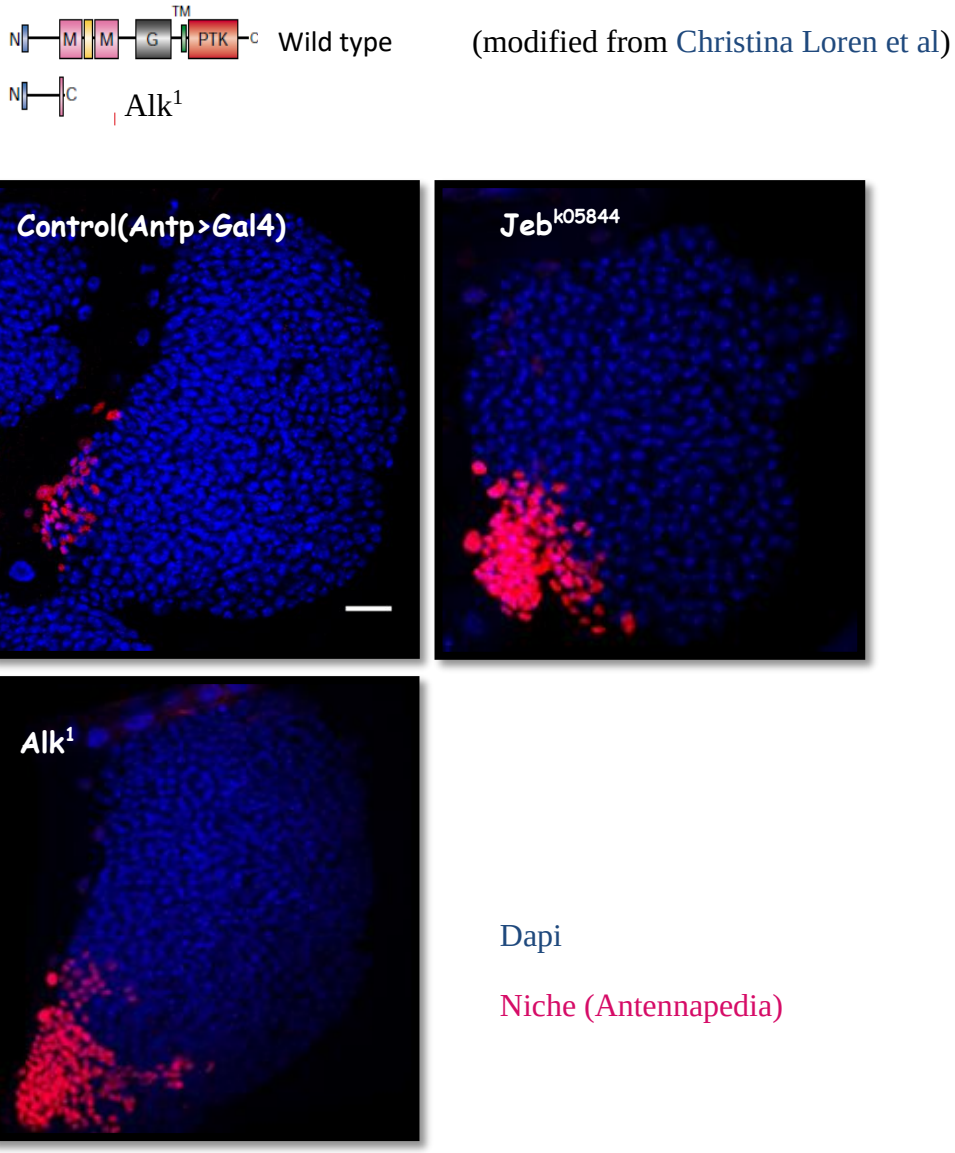


Figure III.9: Increased niche cell number in classical loss of function mutants of Alk and ligand Jeb .(Scale:20µ)

The data clearly shows that there is significant increase in niche cell number in the background of Jeb^{k05844} and Alk^{1} as compared to the control.

Inference: Alk functions as a negative regulator of niche cell number. Owing to this, when Alk is knock down from the niche, a hyperplastic niche is resulted. Loss of ligand Jeb also gives a similar phenotype indicating that anterograde Jeb-Alk signaling is involved in regulating niche cell maintenance.

III.4 Analysis of the niche functionality

Anaplastic lymphoma kinase(Alk) is found to be negatively regulating niche cell number. Due to this, during loss of Alk, niche cell number increases significantly. Further we tried to analyze whether this hyperplastic niche is indeed fuctional.

Hedgehog signaling from niche is known to be required in the maintenance of progenitor cells.(**7**). Hence an increase in Hedgehog protein was expected in the hyperplastic niche if it was functional.

Inorder to analyze this flies harbouring Antp>Gal4, UASGFP, were crossed to the responder flies which had the UAS sequence proximal to gene encoding Alk ^{DN}. After dissection of the larvae, tissues were processed for Hedgehog immuno staining. The results are shown in *figure III.10*

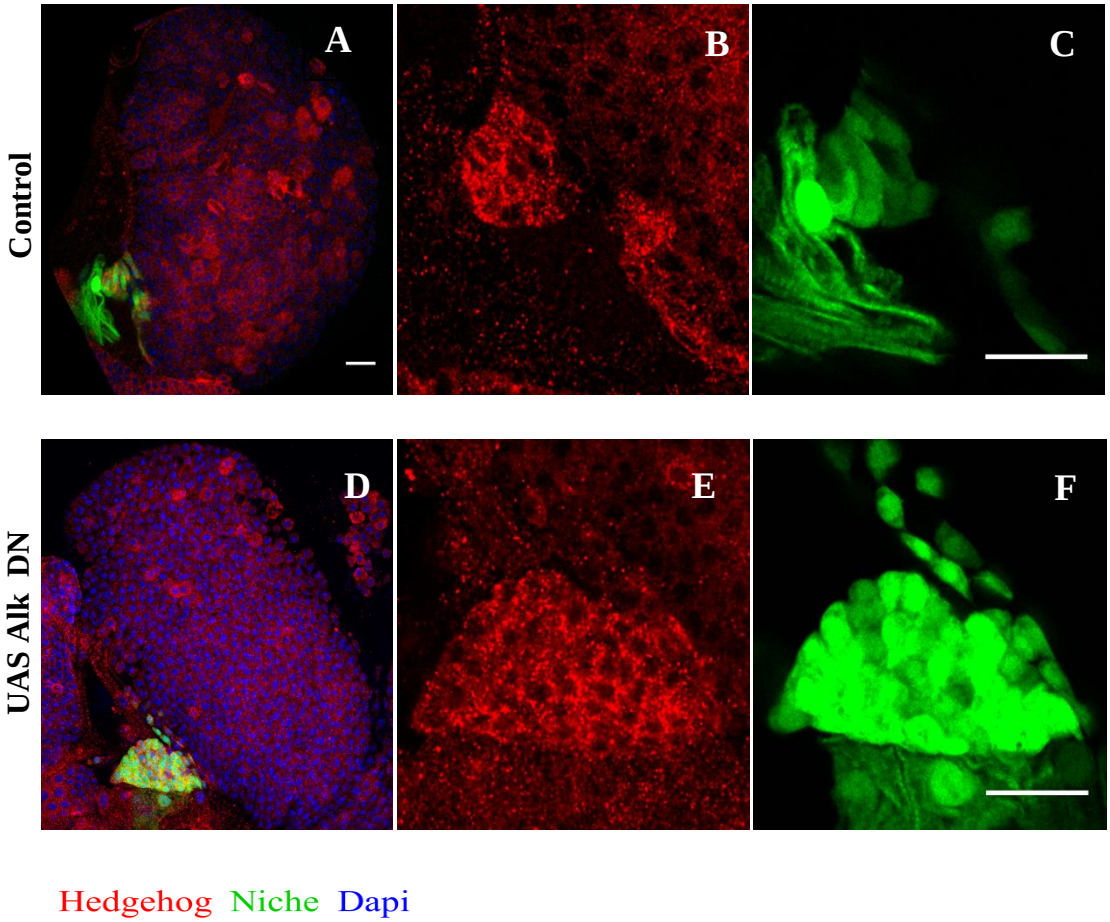


Figure III.10: Hedgehog staining in Control Vs Alk ^{DN} background

(Scale:20µm). Hedgehod(red) is expressed in antennapedia expressing(green: *Antp>gal4, UAS GFP*)**A-C:(***Antp>gal4, UAS GFP* X oregon R). **D-F:** (*Antp>gal4, UAS GFP* X UAS Alk^{DN}).**B:**magnified image of **A** showing hedgehog expression(red). **E:** magnified image of **D** showing hedgehog expression(red)**C,F:**magnified image showing antennapedia expressing(green) niche in control and Alk^{DN} respectively.

There is a significant increase in the Hedgehog protein in the hyperplastic niche expressing Alk^{DN} as compared to the control.

III.5 Analysis of progenitor maintenance

In order to confirm that the hyperplastic niche is functional, status of progenitors and differentiated cells were analyzed by performing immunostaining against cell specific markers.

To analyze the status of progenitors and differntiated cells, flies harbouring Antp>Gal4UASGFP, were crossed to the responder flies which had the UAS sequence proximal to gene encoding Alk ^{DN}. For observing the status of differentiated cells, the tissues were stained for P1 which specifically marks the plasmatocytes which constitutes the major population of differentiated cells.

The results obtained are shown in *Figure III.11*

Control

UAS Alk^{DN}

P1

Antp

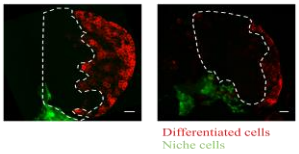


Figure III.11: **Status of progenitors and differentiated cells**(scale:20µm) The progenitors expressing P1(red) and the niche cells expressing antennapedia(green). **A**, :(*Antp>gal4, UAS GFP* X oregon R) **B:** (*Antp>gal4, UAS GFP* X UASAlk ^{DN}).

III.6 Analysis of cell cycle status

Since the niche cells were hyperplastic in Alk knockdown background as compared to the control, we inferred that Alk might be involved in regulating some stage of cell cycle progression. Inorder to test this hypothesis, cell cycle status of niche cells were analyzed and compared between control and Alk^{DN} mutant.

Flies harbouring UAS-GFP.E2f1.$_{1-230}$,UAS-mRFP1.NLS.CycB.$_{1266}$}/CyO; Antp>Gal4 were crossed with responder lines harbouring UAS sequence upstream to Alk^{DN} or control Oregon R flies.

After dissection , tissues were processed for DAPI staining and were imaged and analyzed. Cells in each stage of cell cycle were counted manually.

G1:cells appear green

S:cells appear red

G2 cells appear yellow

Note: Cells in mitotic stage were identified through pattern of DAPI staining.

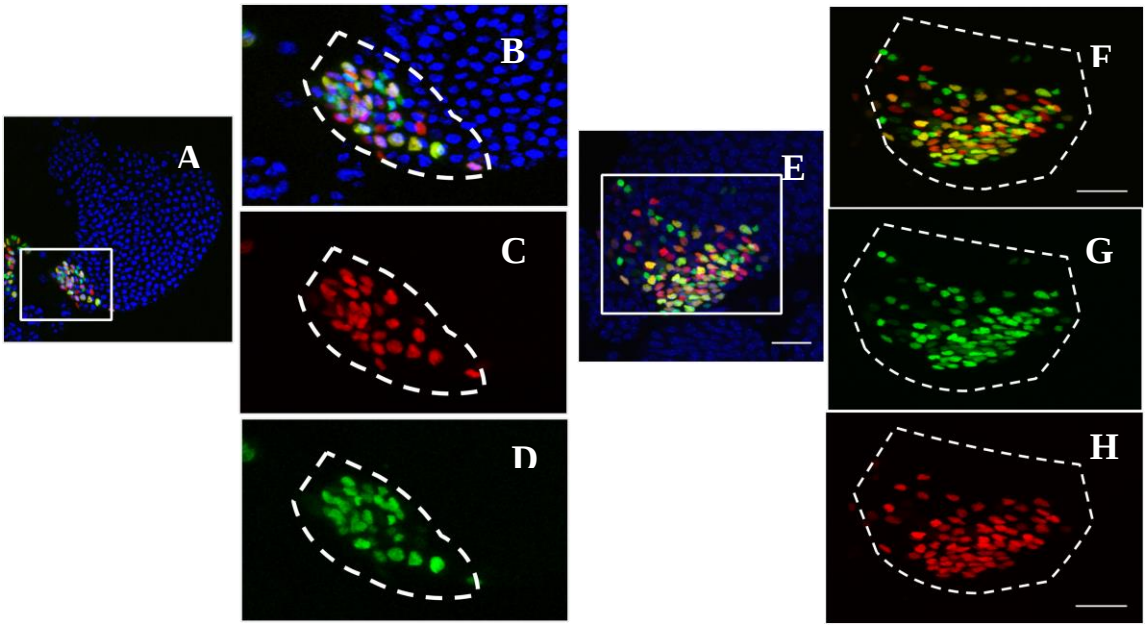


Figure III.12 : FUCCI analysis in control vs Alk^{DN}

(Scale bar:20µm) **.A,B-D:** niche cells(fucci/cyo;Antp>Gal4 X ore R).**B:** magnified image of **A.** Cells in G2 phase(yellow) expressing both mRFP1.NLS.CycB.$_{1-266}$ and GFP.E2f1.$_{1-230}$, S phase(red) expressing (mRFP1.NLS.CycB.$_{1-266}$) alone, G1phase(green) expressing (GFP.E2f1.$_{1-230}$).**C:**cells expressing mRFP1.NLS.CycB.$_{1-266.}$ **D:**cells expressing GFP.E2f1.$_{1-230.}$

E: niche cells(fucci/cyo;Antp>Gal4 X UAS Alk^{DN}). **F:** magnified image of **E**.Cells in G2 phase(yellow) expressing both mRFP1.NLS.CycB.$_{1-266}$ and GFP.E2f1.$_{1-230}$, cells in S phase(red) expressing (mRFP1.NLS.CycB.$_{1-266}$), cells in G1 phase(green)expressing (GFP.E2f1.$_{1-230}$) **G:**cells expressing mRFP1.NLS.CycB.$_{1-266.}$ **H:**cells expressing GFP.E2f1.$_{1-23}$.

The results obtained though comparison of cell cycle status of niche cells in control Vs Alk^{DN} are summarized in the pie chart *figure III.13*

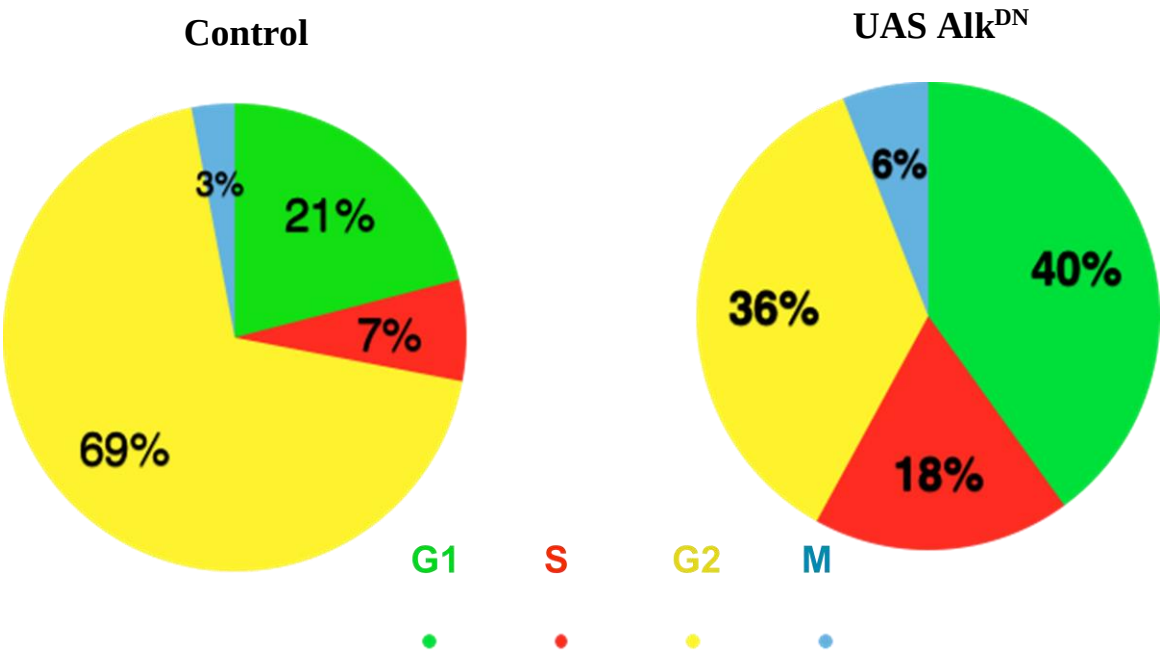


Figure III.13: Cell cycle status of niche cells in control Vs Alk^{DN}

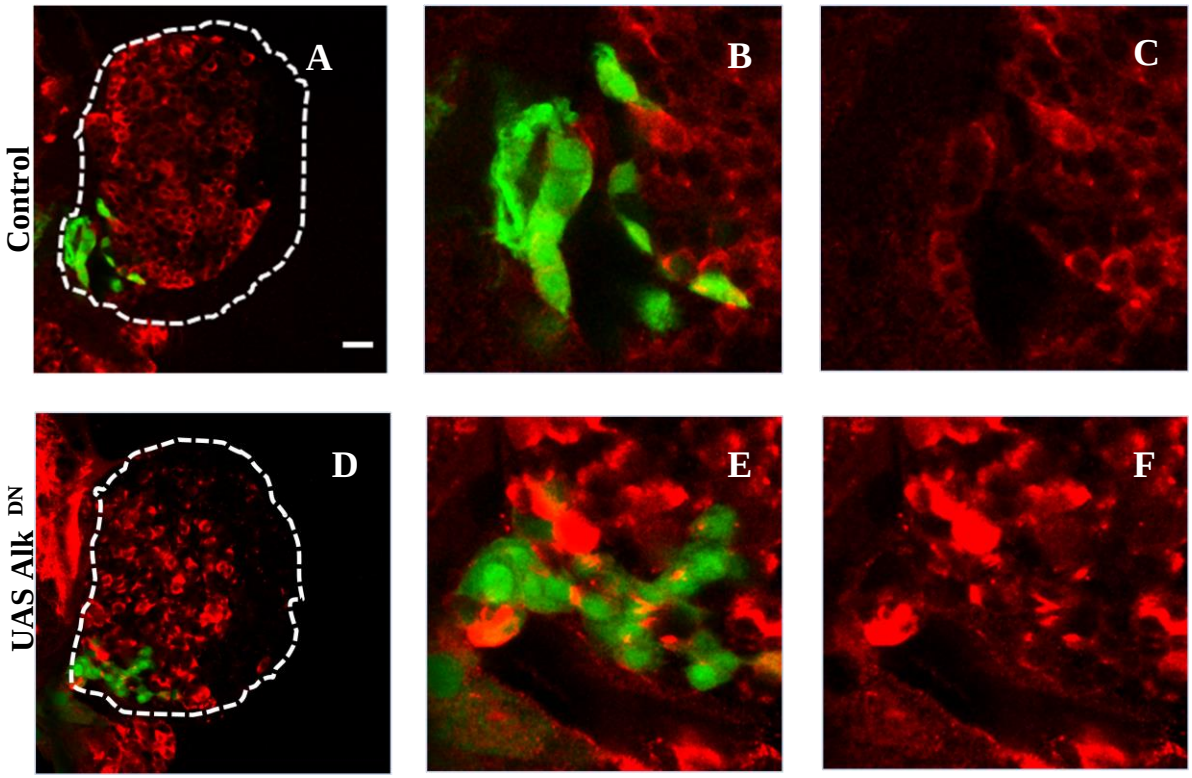
Most of the cells seem to be arrested at G2 phase of cell cycle in control whereas the cells in G2 has significantly decreased in Alk^{DN}. There is a corresponding increase in M, G1 and S phase also.

Inference : Majority of niche cells are in G2 phase of cell cycle. They seem to be getting arrested in G2 phase in a control scenario. This arrest might be getting released when Alk is knockdown from the niche. This indicates that Alk is having a role in regulating cell cycle progression from G2 phase i.e Alk might be involved in arresting the cells in G2. it would be because of this arrest getting released and cells actively resuming cell cycle progression, there is corresponding increase in cells at G1 phase (longest phase in cell cycle).

III.7 Inquiring downstream pathway

The data from the cell cycle analysis were indicative of Alk getting involved in regulating cell cycle progression, by somehow arresting the cells in G2 phase of cell cycle. We further wanted to analyze the underlying pathway employed for this regulation. For that we picked up two candidate pathways -wingless pathway and JNK pathway that could potentially be involved in/being regulated through Alk signaling .Wingless was previously reported to be involved in proliferation of niche cells.(**16**) Hence we suspected that wingless might be getting regulated by Alk. In order to inquire this possibility, flies harbouring Antp>Gal4UASGFP, were crossed to the responder flies which had the UAS sequence proximal to gene encoding Alk ^{DN}. The larvae were dissected and the tissues were processed for wingless staining.

The results are shown in *figure III.14*



Antennapedia wingless

Figure III.14: wingless staining in control Vs Alk^{DN}.

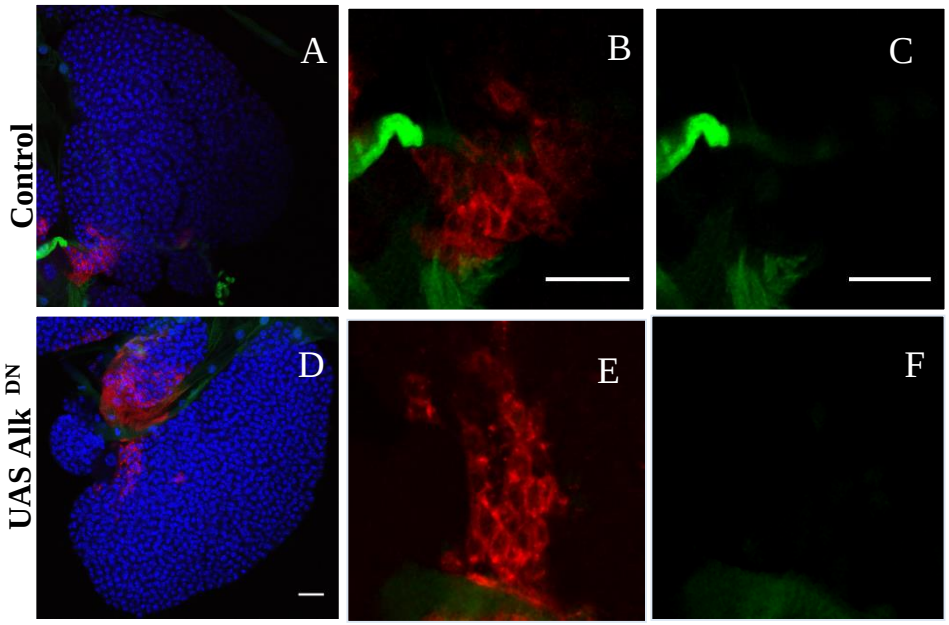
(Scale bar:20µm).**A-C:** wingless staining in control (*Antp>Gal4, UAS GFP X Oregon R*).**B**: magnified image of A showing wingless staining(red) in niche cells(green).**C:**magnified image showing wingless staining(red) in niche cells.

D-F: wingless staining in Alk knockdown (from niche)background (*Antp>Gal4, UAS GFP X UAS Alk^{DN}*).**E**: magnified image of **D** showing wingless staining(red) in niche cells(green).**F:**magnified image showing wingless staining(red) in niche cells

The data suggests there is no significant difference in wingless protein in control Vs Alk^{DN}.

Inference : Alk is not regulating the niche cell number through wingless signaling pathway .Moreover, This result is consistant with our previous inference that the progenitor maintenance is not compramised when Alk is knockdown from the niche since the wingless sigal in Alk^{DN} is comparable that in control scenario.

Further we analyzed the JNK signaling pathway which is widely involved in cell proliferation and also reported to be activated by Alk in cancer conditions(**9)**. We used the signaling pathway reporter-TRE-GFP. TRE (Tetradecanoylphorbol Acetate response element) is a response element in which AP-1 trascription factor dimers binds and initiates transcription, upon activation of JNK pathway. Hence in this case GFP will be expressed whenever JNK pathway is activated.



Dapi Niche TRE GFP

Figure III.15: Analysis of JNK pathway reporter in control Vs Alk^{DN}

(Scale bar:20µm). **A-C:** TRE GFP in control (*TRE GFP;Antp>Gal4, UAS RFP X ore R*). **B**: magnified image of **A** showing niche(red) and TRE GFP(green)expression in the tissue.**C:**magnified image showing TRE GFP expression.

D-F: TRE GFP in Alk^{DN} (*TRE GFP;Antp>Gal4, UAS RFP X UAS Alk^{DN}*).**E**: magnified image of **D** showing niche(red) and TRE GFP(green)expression in the tissue .**F:**magnified image showing TRE GFPexpression .

The data shows that there is no significant change in TRE GFP expression in control vs Alk^{DN}.

Inference: Alk is not regulating the niche cell number through JNK signaling pathway.

III.9Discussion

Anaplastic lymphoma kinase (Alk) is found to be activated in several cancer conditions including blood cancer in humans. Still the physiological role of this protein remains enigmatic in mammalian system. The lack of information of a defined ligand in mammals is one of the reasons hindering proper characterization of this protein in mammals. However, Alk shows considerable conservation to invertebrate counterpart including *Drosophila* Alk. Moreover, the ligand of Alk is already identified in *Drosophila*, known as the Jelly Belly protein or Jeb. Hence using *Drospophila* as model system, we are attempting to dissect out the role of Alk in hematopoietic system.

We could identify that Alk negatively regulates niche cell number in the *Drosophila* hematopoietic system. Since, a hyperplastic niche was resulted on knocking down Alk from niche, we inferred that it could be regulating some stage of cell cycle. To test this possibility, the cell cycle status of niche cells upon knockdown of Alk was analyzed. This led to the observation that in comparison to the control scenario where majority of cells were found to be in G2 phase, the number of cells in G2 phase decreased and got distributed over other stages (especially G1) of cell cycle when Alk was knock down. This clearly suggest that Alk is involved in regulation of G2 phase cell cycle progression.

Further we tried to find out the pathway that could be leading to the hyperplastic niche during loss of Alk since this information can throw light on how Alk leads to the G2 arrest of niche cells in normal scenario. We analyzed the status of two potential pathways- wingless and JNK (basket in *Drosophila*). Interestingly both of these pathways were unaffected when Alk was knock down from the niche reflecting that some other signaling pathway is involved in hematopoietic niche regulation by Alk.

Another potential pathway that could be involved in this signaling event could be ERK pathway which is shown to be involved in certain phosphorylation events that can lead to cell proliferation. This include phosphorylation of ribosomal protein S6 kinase (p70S6K), S6 ribosomal protein (S6RP) which leads to stimulation of translation.(**14**), phosphorylation of eukaryotic initiation factor 4E-binding protein 1 (EIF4EBP1)which leads to the removal of inhibitory action of this protein over the elongation factor of

translation, phosphorylation and consequent activation of an enzyme known as CAD which is involved in catalyzing an important step in pyramidine synthesis. ERK is referred to be a possible downstream player in Alk signaling in *Drosophila* visceral mesoderm specification in embryonic stage (**11**). It is reported to be activated by Alk signaling and leading to increased cell proliferation in cancer conditions (**9**) (**17**)(**18)**. However in our case, knock down of Alk is leading to increase in proliferation. The ERK pathway status can be investigated in niche cells when Alk is downregulated inorder to understand in what way Alk is affecting this pathway in a physiological situation rather than in cancer.

We have found that Alk is involved in regulation of G2-M transition. It will be very interesting to check the status of Cdc25 (string in *Drosophila)* as well as cyclin B. String is a protein phosphatase that is involved in removing inhibitory phosphorylation of cdk1 which then get activated and associate with cyclin B1, enabling cell to enter the mitotic stage of cell cycle(**19**) (**20**). Hence, it is plausible that Alk signaling is involved in regulating the activity of this protein to have control over the G2-M transition.

CONCLUSION AND FUTURE PERSPECTIVE

IV.1Conclusion

Through this study, we could dissect out that Alk functions as a negative regulator of the hematopoietic niche cell number. Interestingly, it is not affecting the functionality of niche. Hence the role of Alk would be solely in regulating the proliferation of niche cells. It is inferred from this study that Alk negatively regulates cell cycle progression of niche cells at the G2 phase of cell cycle. However, the underlying mechanism of this control still needs to be unravelled.

Eventhough this study could not nail down to the mechanistic basis of regulation of cell proliferation by Alk, it could establish a model to unravel the physiological role of Alk in the blood system, bipassing its tumorigenic nature.

IV.2Future perspective

It is important to understand the signaling pathway regulated by Alk in order to well characterize the functional significance of this protein. To understand the signaling pathway employed in niche cell regulation by Alk, it would be worth addressing the potential downstream effectos of Alk signaling such as RAS-ERK pathway, JAK-STAT pathway(**21**),PI3K-Akt pathway (**22**) which are identified as downstream targets of activated forms of Alk during cancer conditions. Analyzing the status of these pathways in this system not only contributes to the understanding of mechanistic basis of niche cell regulation by Alk, but it can also give insight into whether Alk is involved in signaling through these pathways (which are other wise stimulated through its activated forms in cancer conditions) in normal physiological system as well and to see how it is different from signaling in cancer scenario.

From the FUCCI analysis, it is evident that Alk is playing some role in regulating ell cycle progression at G2 phase. The mechanistic basis still needs to be unravelled.

Whether Alk plays any role in regulating the cdc25(string) for getting control over the G2-M transition can be investigated.

To better understand the Alk function, it is also important to characterize the source of its ligand. It would be intriguing to know whether Alk signaling is cell autonomous in nature or whether signaling inputs from nearby cells are also involved.

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