Computational modelling of protein-protein and cell-cell interaction across multiple scales

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NANYANG
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**COMPUTATIONAL MODELLING OF PROTEIN-
PROTEIN AND CELL-CELL INTERACTION ACROSS
MULTIPLE SCALES**

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INTERDISCIPLINARY GRADUATE SCHOOL
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2018

COMPUTATIONAL MODELLING OF PROTEIN-PROTEIN AND CELL-CELL INTERACTION ACROSS MULTIPLE SCALES

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**Interdisciplinary Graduate School
NTU Institute for Health Technologies**

A thesis submitted to the
Nanyang Technological University
in partial fulfilment of the requirement
for the degree of
Doctor of Philosophy

2018

Declaration of Authorship

I, KOON Yen Ling, declare that this thesis titled, “Computation Modelling of Protein-Protein and Cell-Cell Interaction Across Multiple Scales” and the work presented in it are my own. I confirm that:

- This work was done wholly or mainly while in candidature for a research degree at this University.
- Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated.
- Where I have consulted the published work of others, this is always clearly attributed.
- Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work.
- I have acknowledged all main sources of help.
- Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself.

Signed:

Date: 17/01/2018

Nanyang Technological University

Abstract

Interdisciplinary Graduate School

Doctor of Philosophy

**Computational Modelling of Protein-Protein and Cell-Cell Interaction Across
Multiple Scales**

by KOON Yen Ling

To gain a deeper insight into the inner workings of biology, computational modelling is performed for three projects spanning three different scales: namely at the molecular or protein scale, at the intracellular scale and at the intercellular level.

1) The first project is conducted at the molecular or protein level where we have uncovered Chk1 as a novel interactor of POPX2 through our bioinformatics analysis pipeline. Our bioinformatics analysis pipeline is a combination of two separate strategies: 1. Prediction of POPX2 substrates by finding proteins with close phylogeny relation to known substrates of POPX2 and PP2C domain, 2. Prediction of POPX2 substrates by curation of known interactors of proteins sharing homology to POPX2 using STRING database.

2) The second project is performed at the intracellular level. By constructing a reaction-advection-diffusion model, we have found that to achieve optimum signalling efficiency of proteins transported by scaffold proteins and motor proteins, an optimum concentration of scaffold proteins as well as an optimum speed of motor proteins is necessary.

3) The final project investigates the intercellular realm where we explore how differential regulation of angiogenesis leads to different sprouting angiogenesis patterns. Specifically, we conclude that by considering two commonly neglected mechanisms, namely intracellular Notch heterogeneity as well as tension modulation of rate constants, experimentally observed sprouting patterns can be recapitulated.

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Contents

Declaration of Authorship	iii
Abstract	vi
Acknowledgements	vii
1 Prologue	1
1.1 Computational modelling of protein-protein and cell-cell interaction	1
1.1.1 Relevance of computational modelling in biology	1
1.1.2 Biology exhibits different scales	2
1.2 Biology at the molecular level	3
1.2.1 Brief description of biology at the molecular level	3
1.2.2 Methods to probe protein-protein interaction	3
1.2.3 A brief introduction to POPX2	9
1.3 Biology at the intracellular level	11
1.3.1 Brief description of microtubules and associated proteins . .	12
1.3.2 Importance of microtubule-based transport in physiology .	17
1.3.3 Computational modelling of microtubules and motor pro- tein motion along microtubules	18
1.4 Biology at the intercellular level	22
1.4.1 Description of biology at the intercellular level	22
1.4.2 Description of angiogenesis	23
1.4.3 Sprouting angiogenesis models	30
2 Discovery of a novel interaction between POPX2 and Chk1	33
2.1 Introduction	33

2.2	Results	35
2.2.1	Bioinformatics analysis to search for potential POPX2 sub- strates	35
2.2.2	Prediction of POPX2 substrates by relation to known sub- strates of POPX2 and PP2C domain	36
2.2.3	Prediction of POPX2 substrates by curation of known in- teractors of proteins sharing homology to POPX2	38
2.3	Experimental validation of interaction between Chk1 and POPX2 .	39
2.4	Discussion	41
2.5	Methods	42
2.5.1	Computational prediction analysis	42
2.5.2	Experimental procedures	43
	Cell culture	43
	Cell lysis and western blot	43
	siRNA and plasmid transfection	43
	Glutathione S-transferase (GST) and flag pull-down assay and immunoprecipitation	44
	Real-time quantitative PCR	44
	Fluorescence-activated cell sorting (FACS)	45
3	Computational modeling reveals optimal strategy for kinase transport by microtubules to nerve terminals	49
3.1	Introduction	49
3.2	Methods	53
3.3	Results	60
3.3.1	Scaffolded cytoskeletal transport can result in a higher JNK* activation than diffusion	60
3.3.2	Increase in speed of cytoskeletal transport does not always lead to an increase in signaling rate	61
3.3.3	An optimal scaffold protein concentration and optimal mo- tor speed exist for which signal amplification is maximal. . .	64

3.3.4	Optimum scaffold protein concentration and optimal cytoskeletal transport speed depend on signaling parameters .	67
3.4	Discussion	70
3.5	Conclusion	74
4	An enhanced Delta-Notch lateral inhibition model incorporating intracellular Notch heterogeneity and tension-dependent rate of Delta-Notch binding to explain cellular patterning during sprouting angiogenesis	77
4.1	Introduction	78
4.2	Materials and methods	84
4.2.1	Experimental procedures	84
	Image quantification	85
4.2.2	Detailed explanation of the enhanced Delta-Notch lateral inhibition model	85
	Classical lateral inhibition model	85
	Lateral inhibition model with intracellular Notch heterogeneity	87
	Lateral inhibition model with intracellular Notch heterogeneity and tension-dependent rate of Delta-Notch binding	89
4.2.3	Derivation of steady state solutions for lateral inhibition models	91
4.3	Results	93
4.3.1	Tip-stalk patterns with more than one stalk cell in between tip cells recovered in lateral inhibition model with intracellular Notch heterogeneity	93
4.3.2	Lateral inhibition with intracellular heterogeneity and tension-dependent rate of Delta-Notch binding yields a large number of tip-stalk patterns with more than one stalk cell in between tip cells	102
4.3.3	Existence of intermediate cell states <i>in vitro</i>	104

4.4	Discussion	108
4.5	Conclusion	112
5	Conclusion	115
5.1	Future work	116
	Bibliography	117

List of Figures

1.1	Four molecules of life	4
1.2	Co-immunoprecipitation steps	5
1.3	Illustration of yeast two-hybrid assay	6
1.4	Procedures in affinity purification coupled to mass spectrometry . .	7
1.5	Organelles in a mammalian cell	12
1.6	Actin and microtubule as seen under the microscope	13
1.7	Cross-section of a microtubule	14
1.8	Functions of scaffold proteins	17
1.9	Cellular organization of the digestive system	23
1.10	Angiogenesis in cancer	24
1.11	Delta and Notch transmembrane proteins on the cell membrane . .	27
2.1	Schematic of the bioinformatics analysis	36
2.2	Alignment between PP2C _{3UJG} and PP2C _{POPX2}	37
2.3	Phylogenetic tree of known substrates of PP2C and proteins containing Pkinase domain	46
2.4	POPX2 interacts with Chk1 in vitro when expressed exogenously and endogenously	47
2.5	POPX2 interferes with cell cycle by increasing Cyclin E expression .	48
3.1	Schematic of model	56
3.2	Kymograph of JNK* activity	61
3.3	Signaling rate for various values of f and p	62
3.4	Sharp changes in signalling rate between region 3 and region 4 . . .	65
3.5	Signal amplification for various values of f and p	66

3.6	Values of f and p for which signal amplification is optimum depend on signaling parameters	69
3.7	Phase diagram to summarize the possible strategies utilized by the cell	72
4.1	Schematic of Delta-Notch Lateral Inhibition	80
4.2	Lateral Inhibition Models	82
4.3	Delta and Notch Levels in Lateral Inhibition with Intracellular Notch Heterogeneity	95
4.4	Effect of diffusion on two-cell spacing for lateral inhibition with intracellular Notch heterogeneity	97
4.5	Delta and Notch Levels in Lateral Inhibition with Tension Modulation of Rate Constants	101
4.6	Delta and Notch Levels in Lateral Inhibition with Intracellular Notch Heterogeneity and Tension Modulation of Rate Constants	103
4.7	Summary of tip-stalk patterns observed under different conditions of W and h	105
4.8	Four-cell and Six-cell Spacing in Lateral Inhibition with Intracellular Notch Heterogeneity and Tension-Dependent Rate of Delta-Notch Binding	106
4.9	Varying fluorescent levels are exhibited by cells depending on their position from the tip cells	107

List of Tables

2.1	Interactors of proteins sharing homology to POPX2	39
3.1	List of reactions and their corresponding rate constants	55
3.2	Diffusion coefficients and initial distribution of all species modeled	58

Chapter 1

Prologue

1.1 Computational modelling of protein-protein and cell-cell interaction

1.1.1 Relevance of computational modelling in biology

Complexity is inherent in biology, where even the simplest lifeform is characterized by numerous interconnected components. These components have to work together holistically in order for proper functioning of the organism. Such intertwined complexity makes it extremely difficult to unravel the functions of certain components. Such high interconnectivity is exemplified in the yeast protein-protein interaction network uncovered by Schwikowski which consists of over 1500 interacting proteins with 2358 interactions (Schwikowski, Uetz, and Fields, 2000). Should these complicated systems breakdown, the underlying cause may also be difficult to uncover. Furthermore, due to the high connectivity of biological networks, experiments to elucidate the role of certain biological modules may be problematic to perform without perturbing other unrelated pathways. To this end, computational modelling can aid in illuminating biological phenomenon where perturbation studies or prediction analysis can be conducted independently in silico. Successful examples of how computational modelling have helped in understanding biological phenomena can be observed in (Lai and Chiam, 2011), where computational models of cell migration have led to prediction of the various phases involved in cell motility such as protrusions, retractions and ruffling. A model to elucidate the dynamics of signalling proteins

involved in circular dorsal ruffle formation has also shown that circular dorsal ruffles propagates like an excitable wave, similar to that in nerve signal transmission (Zeng et al., 2011). Other successful uses of computational modelling in biology includes in the field of drug discovery (Sliwoski et al., 2014; Kumar et al., 2006; Katsila et al., 2016) as well as in disease detection and classification (Iliyasu and Fatichah, 2017; Haleem et al., 2017; Kosakovsky Pond et al., 2009; Shen, Olshen, and Ladanyi, 2009). Such in silico findings would allow biologists to better grasp the mechanisms behind biological processes and shed light behind the workings of more complicated biological systems.

1.1.2 Biology exhibits different scales

Often when modelling a biological phenomenon, a suitable spatial scale which is appropriate for observation is chosen. Selecting the modelling scale is crucial in biology as biological organization span across many scales. These scales cover several magnitudes ranging from the molecular scale to that of a living organism. At the molecular level, biology consists of proteins and lipids ranging from 1nm to 10nm in size. At the micron scale, larger structures are observed. For instance, many subcellular structures within the eukaryotic cell span several microns in length. Mitochondria typically are between 0.5 to 10 μ m while the average diameter of a human nucleus is around 6 μ m. Certain bacteria also belong to the micron scale such as *Escherichia coli* which has a length of roughly 2 μ m. Beyond the micron scale, subcellular structures are organized into cells. These cells are further organized into tissues, and tissues organized into organs. Finally, organs coordinate their functions to make up the organism itself.

In this thesis, I am going to focus on the following three scales in biology to understand the complexities of protein-protein and cell-cell interaction. In Chapter 2 of this thesis, modelling will be performed at the molecular scale in which prediction of protein-protein interaction is conducted. Next in Chapter 3 of the thesis, the realm of intracellular scale is explored where signaling dynamics along the microtubule is simulated. Lastly, in Chapter 4 of the thesis, intercellular communication during sprouting angiogenesis is modelled. A brief

introduction of computational modelling in the aforementioned biological scales are elaborated in the subsequent sections of the Prologue. Altogether, the aforementioned three chapters will allow us to get a better understanding of biological processes involved at different scales.

1.2 Biology at the molecular level

1.2.1 Brief description of biology at the molecular level

Proteins, lipids, polysaccharides and nucleic acid constitute the four major building blocks of life illustrated in Fig 1.1. Of all these organic molecules, proteins are the most abundant, making up about half of a cell's dry mass. The importance of proteins is evident from the many roles it plays within the body. Most of all cellular processes are directly or indirectly regulated by proteins. These processes include DNA transcription and translation, cellular metabolism, cellular replication as well as cell death. Proteins regulate these processes through highly specific interactions with other proteins, i.e. through protein-protein interactions (PPIs). These interactions if aberrant can lead to disastrous consequences such as Alzheimer's disease and cancer. It has become increasingly clear that diseases are often not caused by abnormality within a single gene, but due to the dysfunctional interactions between proteins. As such, major efforts are underway to characterize and understand these PPIs in the hope of unravelling the molecular basis of diseases.

1.2.2 Methods to probe protein-protein interaction

The importance of PPIs has led to the development of many experimental methods to probe and identify these interactions. Co-immunoprecipitation (Co-IP) remains the gold standard assay for identifying PPIs when executed with endogenous proteins. In Co-IP, a specific antibody is used to sieve out the desired protein of interest along with its interacting partners. The interacting partners are subsequently identified using western blot. These steps for Co-IP is depicted in Fig 1.2.

FIGURE 1.1: Proteins, lipids, polysaccharides and nucleic acids form the four building blocks of life. (Obtained from Wikipedia Commons)

Although Co-IP is accurate, it is labour-intensive and thus not suitable for large scale PPI screening. To circumvent this limitation, other high throughput methods can be used. Of these, the most widely adopted are yeast two-hybrid (Y2H) screening and affinity purification coupled to mass spectrometry (AP-MS). In Y2H screening, two plasmids are simultaneously transfected into yeast cells. The first plasmid or bait contains the gene of the protein of interest along with the DNA-binding domain of a transcription factor. The second plasmid or prey contains the gene sequence of the query protein fused to the activation domain of the transcription factor. Should the protein of interest interact with the query protein, the bait and prey will complex to form a proper transcription factor. Interactions between proteins can thus be confirmed from expression of reporter genes. An illustration of Y2H is shown in Fig 1.3. However, Y2H method suffers from low specificity as well as poor detection of transient PPIs (Rajagopala et al.,

FIGURE 1.2: Co-immunoprecipitation steps. (Obtained from Wikipedia Commons)

2012).

In affinity purification coupled to mass spectrometry, the tagged protein is purified together with its interacting partners, i.e. affinity purification. The PPIs are later identified by mass spectrometry (Jones and Thornton, 1996). Steps for AP-MS can be seen in Fig 1.4. Like Y2H, AP-MS harbour difficulty identifying interactions which are transient and dynamic which impede discovery of biologically meaningful interaction (Lee et al., 2017; Kaake, Wang, and Huang, 2010).

Computational tools are thus helpful for PPI discovery especially for interactions that may not be easily detected through conventional experimental means. Predictions from these computational methods can be verified at a later stage using conventional experimental tools such as co-immunoprecipitation. These

FIGURE 1.3: Illustration of yeast two-hybrid assay. Prey and bait proteins interact resulting in association of the DNA-binding domain (BD) and activating domain (AD) of Gal4 to the upstream activating sequence (UAS) and subsequent transcription of reporter gene in yeast two-hybrid. (Obtained from Wikipedia Commons)

computational methods will be summarised in the following and include methods utilizing 1) genomic context, 2) sequence information, 3) structure information as well as 4) text mining techniques for probing PPIs. One of the earliest PPI prediction method is based on genomic context using gene neighbouring or gene co-localization information (Dandekar et al., 1998; Tamames et al., 1997; Overbeek et al., 1999). Genes performing similar functions are often located in close proximity to one another in the prokaryotic genome. For instance, an operon is a segment of DNA containing many genes of similar functions allowing for transcription into a single mRNA. Hence, if the gene coding regions of two proteins are found as neighbours in several genomes, these two proteins are likely to be functionally related. This concept is embedded in ProFunc, a web

FIGURE 1.4: Procedures in affinity purification coupled to mass spectrometry. In this figure, proteins in two different cell conditions are first tagged using different isotopes. Next, mass spectrometry is used for protein identification. (Obtained from Wikipedia Commons)

server for protein function prediction where the function of an unknown protein is deduced from the function of its genomic neighbours (Laskowski, Watson, and Thornton, 2005). This method however has low coverage for PPI prediction due to the paucity of complete genomes (Panchenko et al., 2008). Also, this method is more appropriate for prokaryotes since gene proximity does not equate to co-functionality in eukaryotes and identified gene clusters in eukaryotes are found to be sparse and not adjacently located (Yi, Sze, and Thon, 2007). Gene fusion or the rosetta stone method is another protein-protein interaction prediction tool based on genomic context. Certain genes although separate in some organisms, become fused in other organisms. For instance, topoisomerase

II is the result of the fusion between *Escherichia coli* DNA gyrase Gyr A and Gyr B subunits (Wang, 1985). Such a fusion event indicates the functional relationship between these separate proteins which might associate to form a protein complex. Gene fusion methods are extremely reliable methods for PPI prediction (Henry et al., 2016). Unfortunately, gene fusions are rarely observed and this method may not be informative for most proteins (Panchenko et al., 2008). Sequence-based computational prediction of PPIs involves collecting sequence information of interactors in known interactions and comparing those sequences against the desired protein of interest. Some sequence-based approaches infer interaction using conserved sequence signatures found in curated interactions (Sprinzak and Margalit, 2001; Fang et al., 2005). These methods using sequence signatures have also been combined with machine learning techniques like support vector machines to boost accuracy (Keskin, Tuncbag, and Gursoy, 2016; Shen et al., 2007; Martin, Roe, and Faulon, 2005). Amino acid combination frequencies in interacting as well as non-interacting protein pairs has also been used to deduce interaction like in “Universal In Silico Predictor of Protein-Protein Interactions” (UNISPPI) where interaction is learnt using a decision tree model (Valente et al., 2013). Other sequence-based methods infer interaction based on known interaction of orthologous proteins in other organisms (Matthews et al., 2001). Using this method of expanding known orthologous protein pairs, interologs for various species can be inferred and has led to the prediction of more than thirty thousand human PPIs (Lee et al., 2008). Protein domains have also been used to surmise protein-protein interactions. A domain-based probabilistic classification method based on domain pairing information from interacting and non-interacting pairs predicts PPI with 90.27% specificity (Li, Tan, and Ng, 2005; Corpet et al., 2000). In methods using protein structure information to predict PPI, two proteins are predicted to interact if they are structurally similar to proteins that have already been found to interact. Homology models can be used if the three dimensional models of the proteins are not present. Using three-dimensional structural information for PPI prediction

has been reported to achieve higher accuracy and coverage than prediction methods not relying on structure (Zhang et al., 2012). Many PPI prediction tools which make use of structural information are freely available on the Internet. PrePPI (<http://bhapp.c2b2.columbia.edu/PrePPI>) is a PPI interaction database incorporating structural, functional, evolutionary and expression information into its prediction (Zhang et al., 2013a). iWRAP (<http://cb.csail.mit.edu/cb/iwrap/>) integrates structural information by threading of protein sequences to structural templates of protein-protein interfaces following which a boosting classifier based on the putative interaction surface is used for interaction prediction (Hosur et al., 2011). Similarly, in InterPreTS (<http://www.russelllab.org/cgi-bin/tools/interprets.pl>), proteins are searched for their homologues within a database of known interacting structures. Prediction of the interaction between a protein pair is then computed based on the degree to which interfacial residues are preserved as compared to their homologs (Aloy and Russell, 2003). With publications increasing at an exponential rate, biomedical literature mining approaches have become increasing popular tools to scour through the expanding literature (Pautasso, 2012). Text mining and literature mining algorithms has also been used for PPI prediction. In these approaches, natural language processing (Bandy, Milward, and McQuay, 2009; Li et al., 2015; Ramani et al., 2005) or rule based methods (Lee et al., 2013; Subramani et al., 2015) are used to detect protein-protein interactions. Machine learning classifiers can also identify these interactions by learning from the text pattern (Fang et al., 2005; Huang et al., 2004; He and Lin, 2009). Unfortunately, questions remain regarding the reliability of such text mining techniques (Jaeger et al., 2008; Zahiri, Bozorgmehr, and Masoudi-Nejad, 2013). Nonetheless, the ever increasing amount of published data may make such methods more accurate in the future.

1.2.3 A brief introduction to POPX2

POPX2 (Partner of PIX 2) is a phosphatase belonging to the protein phosphatase 2C (PP2C) family, one of the four main classes of serine/threonine-specific protein phosphatases in mammals (Yamamoto et al., 1999; Weiss, Tanowitz, and

Kirchhoff, 2011). POPX1 or Partner of PIX 1 is first isolated from a two-hybrid screen using full-length α PIX as bait (Koh et al., 2002). Subsequently, POPX2 is identified in GenBank as a protein which has similar homology with POPX1 (Koh et al., 2002). POPX2 shares extensive homology with POPX1 with approximately 66% protein sequence similarity within the core phosphatase domain (Koh et al., 2002). POPX1 is mainly expressed in the brains and testis whereas POPX2 is ubiquitously expressed in most human tissues.

Following the discovery of POPX2, efforts have been underway to understand the role of POPX2 as a phosphatase. To date, two kinases have been identified to be substrates of POPX2: PAK1 and CaMKII. P21-activated kinase 1 (PAK1) is dephosphorylated by POPX2 at Thr422, an essential regulatory site within the kinase activation loop, leading to PAK1's deactivation (Koh et al., 2002; Ishida, Kameshita, and Fujisawa, 1998). The inhibition of actin stress fiber breakdown observed during POPX2 overexpression has been suggested to be a consequence of PAK1's inactivation (Koh et al., 2002). The second kinase is calcium-calmodulin kinase (CaMKII) where it is dephosphorylated by POPX2 at the autophosphorylation site of Thr286 leading to CaMKII's inactivation (Susila et al., 2010).

Recently, POPX2 is emerging as an important regulator of cancer metastasis. In a study which compared the expression of POPX2 amongst several breast cancer cell lines, POPX2 expression is found to be positively correlated with invasiveness (Zhang et al., 2013b). POPX2 levels are highest in invasive MDA-MB-231 cell lines while the lowest POPX2 expression is seen in non-invasive MCF7 breast cancer (Zhang et al., 2013b; Zhang et al., 2017). POPX2 could possibly be modulating invasiveness by regulating motility as POPX2 overexpression is found to cause an increase in motile behaviour within MDA-MB-231 and MCF7 cell lines (Zhang et al., 2013b; Zhang et al., 2017). Clearly, POPX2 appears to promote tumour progression through enhancement of cell motility and invasiveness. However, reports suggesting POPX2's role as a tumor suppressor is also emerging. POPX2 silencing is observed to result in larger and more numerous tumour nodules at metastatic sites (Zhang et al., 2017). Such enlargement of

tumour nodules is due to an increase in exosome secretion leading to induction of various pro-angiogenic cytokines following POPX2 silencing (Zhang et al., 2017). All in all, these studies illustrate POPX2 playing multiple roles in regulating tumor progression including modulating both motility as well as exosome secretion.

In this thesis, we seek to further deepen our understanding of POPX2's role in cancer via identification of other POPX2 substrates through computational prediction approaches. Potential substrates predicted to interact with POPX2 are then validated through experimental means including co-immunoprecipitation. Phosphatases are defined by their promiscuity, capable of interacting with tens or hundreds of multiple substrates (Rowland, Harrison, and Deeds, 2015). This is exemplified by the PP2C family of phosphatases which exhibits broad substrate specificity (Lennarz and Lane, 2013). Thus, it is highly probable that other substrates whose phosphorylation status is regulated by POPX2 exist. In chapter 2 of this thesis, we embark on this endeavour of searching for other POPX2's substrate using computational prediction in order to unravel POPX2's role in cancer development. Having introduced biology at the molecular or protein level using POPX2 as an example, in the next section, we explore another realm of biology: Biology at the intracellular level.

1.3 Biology at the intracellular level

At the intracellular level, cells are organized into organelles and structures. These organelles include endoplasmic reticulum, Golgi apparatus, nucleus and mitochondria. Each of these organelles serves a specific purpose necessary for survival of the cell. For example, the endoplasmic reticulum is responsible for proper folding of protein molecules as well as the transportation of these folded proteins to the Golgi apparatus. The mitochondria on the other hand are the sites of respiration, providing for the cell's adenosine triphosphate (ATP) needs. These organelles and their roles are depicted in Fig 1.5.

FIGURE 1.5: Organelles in a mammalian cell. (Obtained from Wikipedia Commons)

The cytoskeleton is also an important structure within the cell. The purpose of the cytoskeleton is to help organize cellular organelles as well as give a cell its shape. The cytoskeleton is a complicated meshwork of filaments, both actin filaments and intermediate filaments, as well as microtubules (Fletcher and Mullins, 2010). The microtubule cytoskeleton will be a focus of study in Chapter Three of this thesis. Fig 1.6 shows how these actin filaments and microtubules look under the microscope.

1.3.1 Brief description of microtubules and associated proteins

Microtubules are polymers of tubulin arranged to form a long hollow cylinder. In eukaryotes, microtubules are formed from polymerization of α - and β -tubulin dimers (Weisenberg, 1972). During the polymerization process, the β -subunit of a particular dimer associates with the α -subunit of another dimer to form a long

FIGURE 1.6: Actin and microtubule as seen under the microscope
(Obtained from Wikipedia Commons)

linear tubulin row called protofilament. Due to this “end-to-end” polymerization, these protofilaments exhibit polarity i.e. if a particular end of a protofilament ends with the α -subunit, the β -subunit will be exposed on the opposite end. Conventionally, the end with the α -subunit is assigned the negative end (-) while the end with the exposed β -subunit is the positive end (+). Only protofilaments of same polarity can bundle together leading to polarity also being exhibited by the microtubule. The cross-section of the microtubule is seen in Fig 1.7. The polarity observed in microtubules has important biological consequences: while elongation of microtubules can happen at both the negative and positive ends, elongation at the positive end is considerably faster (Walker et al., 1988). Lateral association of protofilaments lead to the semi-helical structure of the microtubule. Most often, microtubules are composed of thirteen protofilaments although exceptions where microtubules with more or less protofilaments have been observed (Chrétien et al., 1992).

Microtubules usually grow at specialized structures within the cell termed as MTOCs or microtubule-organising centres. γ -tubulin associate with numerous other proteins to form the γ -tubulin ring complex within the MTOC. This complex which exhibits a 13-fold symmetry serves as the scaffold for polymerization of α - and β -tubulin dimers (Kollman et al., 2010). The complex also acts

FIGURE 1.7: Cross-section of a microtubule. (Obtained from Wikipedia Commons)

as a cap preventing depolymerisation at the negative end of the microtubule while growth persists along the positive direction (Desai and Mitchison, 1997). As such, the microtubule network can grow to be extensive: radiating from the MTOC beside the nucleus and ends eventually at the plasma membrane. In this microtubule network, the negative ends of the microtubules are embedded in the MTOC while the positive ends lie close to the plasma membrane (Thadani-Mulero, Nanus, and Giannakakou, 2012).

The polarity within the microtubule network is exploited by motor proteins for directional cargo trafficking within the cell (Gundersen and Cook, 1999; Babich and Burkhardt, 2011). Two major classes of motor proteins capable of motion on microtubules exist. The first is kinesin which moves cargo towards the positive end of the microtubule (i.e. movement towards plasma membrane, aka. anterograde transport) while the second is dynein which moves towards the negative end (MTOC) or retrograde transport. Kinesin is a tetrameric molecule consisting of two heavy chains which make up the motor subunits as well as two light chains for cargo binding. A similar structure exists in dynein where the motor subunits are formed from two heavy chains. These heavy chains however are

connected to a variable number of light and intermediate chains and are responsible for cargo transport in dynein. Motor proteins serve to transport cargo such as vesicles or organelles which are too large to diffuse to their destination. Motor proteins carry out their role of cellular transport by attaching to their cargo and walking unidirectionally along the microtubule using energy derived from ATP hydrolysis (Schnitzer and Block, 1997; Schmidt and Carter, 2016).

Besides motor proteins, a variety of microtubule-associated proteins (MAPs) are also known to modulate cargo transport along microtubule. These MAPs can be separated into two main categories. The first category is made up of MAPs that are smaller than 62kDa and are termed tau proteins. Native tau proteins are highly soluble and assist in microtubule stabilization and assembly (Cleveland, Hwo, and Kirschner, 1977). Unfortunately, aggregation of tau proteins to form paired helical filaments and neurofibrillary tangles can occur (Wang and Mandelkow, 2016) as observed in neurodegenerative diseases such as Alzheimer's diseases (Kolarova et al., 2012), Huntington's disease (Zerr and Bähr, 2016), frontotemporal dementia (Iqbal et al., 2010) as well as Parkinson's (Lei et al., 2010). The second class of MAPs consists of proteins with a larger molecular weight varying between 200 to 1000kDa. Depending on the type of MAPs, they can exert either a stabilising or destabilizing effect on the microtubules. For example, MAP2 binds cooperatively to microtubules and increase its stability. Similarly, MAP4 which has been implicated in cell division (Samora et al., 2011) is also associated with stabilization of microtubules (Kremer, Haystead, and Macara, 2005). Microtubule-destabilizing MAPs such as katanin has been postulated to preferentially sever older microtubules (Sharma et al., 2007). Spastin, the protein mutated in hereditary spastic paraplegias is also involved in destabilizing microtubules. Both katanin and spastin appear to target older, post-translationally modified microtubules. Mice which has lower spastin expression exhibit axon swellings with accumulation of deetyrosinated, stable microtubules (Tarrade et al., 2006).

Various cargoes are transported by kinesin and dynein along microtubules. One such example is scaffold proteins. Some scaffold proteins are themselves

also MAPs such as MAP2 and MAP1B (Lim and Halpain, 2000; Riederer, 2007). Scaffold proteins are important regulators of signalling cascades. They do so by binding to multiple proteins participating in the same signalling pathway. They accomplish this act by containing numerous protein-protein interaction domains most commonly the PDZ and SH3 (Src homology 3) domains (Pawson and Nash, 2003; Good, Zalatan, and Lim, 2011). By virtue of their binding to signalling proteins, scaffold proteins can increase the local concentration of these signalling components thus increasing the rate of reaction. Also, by concentrating components of a particular signalling pathway, unnecessary reaction with other proteins can be prevented. Furthermore, by binding to activated proteins, scaffold proteins can also prevent the inactivation of these activated molecules (Shaw and Filbert, 2009b). This is especially important in the presence of high phosphatase activity (Locasale, Shaw, and Chakraborty, 2007). Some signalling proteins require several interaction events involving different proteins in order for their complete activation. Scaffold proteins can enhance this distributive process by binding to all of these components at the same time, converting the reaction to a processive process (Levchenko, Bruck, and Sternberg, 2000; Carpenter, 2000). Some scaffold proteins also exhibit catalytic properties by inducing allosteric changes in the signalling protein following binding to the scaffold protein (Burack and Shaw, 2000). These properties of scaffold proteins are illustrated in Fig 1.8. Besides these generic functions of scaffold proteins, certain scaffold proteins are also involved in other more specialized functions such as the PDZ scaffolds par-3 and par-6 which aids in asymmetry creation and cleavage separation in *C. elegans* (Kemphues et al., 1988; Watts et al., 1996). ZO-1, another PDZ scaffold is associated with regulating the barrier properties of tight junctions (Stevenson et al., 1986). Altogether, these evidence suggest that scaffolds perform essential roles in various different pathways and that their dysfunction may cause dire outcomes. This is exemplified in breast cancer where the scaffold protein GAB2 is frequently amplified and has been reported to enhance proliferation and metastasis (Bentires-Alj et al., 2006).

FIGURE 1.8: Functions of scaffold proteins. Scaffold proteins can tether signalling components leading to increase in their local concentration. They can also bind to multiple kinases simultaneously allowing coordination of positive and negative feedback. Scaffold proteins also assist in localizing proteins to specific cellular regions. Lastly, scaffold proteins protect phosphorylated signaling components from activity of phosphatases. (Obtained from Wikipedia Commons)

1.3.2 Importance of microtubule-based transport in physiology

Microtubule-based transport is particularly important in neurons due to their extreme lengths. For instance, the longest axon within the human has been recorded to be up to one metre long (Franker and Hoogenraad, 2013). Furthermore, many neurological diseases manifest due to mutation of proteins involved in microtubule transport. Mutations in kinesin KIF5A is observed in spastic paraplegia while cytoplasmic dynein mutations lead to a Huntington's disease-like phenotype (Eschbach et al., 2011). In neurodegenerative diseases such as Alzheimer's or Huntington's disease, disruption of proper cargo transport is perceived as the lead cause leading to aggregation of proteins and organelles within the axons (De Vos et al., 2008; Millicamps and Julien, 2013). As such, many studies are ongoing to probe the regulation of microtubule transport to understand how their dysfunction causes diseases. To that end, computational modelling has

contributed a significant role in understanding the dynamics of these transport proteins.

1.3.3 Computational modelling of microtubules and motor protein motion along microtubules

Computational modelling has been useful in the understanding of microtubule dynamics due to the dynamic nature of microtubules as well as the complexity of the microtubule network and its regulations.

Dynamic instability is a key organizational feature of microtubules (Desai and Mitchison, 1997). Dynamic instability refers to the concurrent assembly and disassembly of the microtubules at their ends, such that the microtubule can transit between the growing and shrinking phases stochastically (Kirschner and Mitchison, 1986). This can occur to all microtubules either nucleated from the MTOC or formed randomly within the cytoplasm. Spontaneous nucleation of microtubules within the cytoplasm may play a compensatory role in ensuring the formation of microtubules in the absence of γ -tubulin or MTOC (Job, Valiron, and Oakley, 2003). Such stochastic properties of microtubules can be regulated by force (Janson and Dogterom, 2004; Janson, Dood, and Dogterom, 2003). This allows the microtubules to adjust their length according to cell size since microtubules collapse upon contact of any cellular structures such as cell membrane. A key question in dynamic instability is how microtubules switch between the slow growth and rapid shrinkage phases. This switch is termed as microtubule catastrophe (Gardner, Zanic, and Howard, 2013). The typical saw-tooth growth and shortening phases of microtubule has been replicated numerically using a lateral cap model where tubulin GTP molecules is only present at the terminal layer of the microtubule (Bayley, Schilstra, and Martin, 1989). By accounting for the experimentally derived lifetimes of growing microtubules, mathematical modelling suggests that catastrophe occurs only after three protofilaments have stopped growing (Bowne-Anderson et al., 2013). Mathematical modelling have also shed light on how dynamic instability influences microtubule dynamic in small compartments. By allowing the concentration of free tubulin to be limiting,

such as in small compartments like in neuronal growth cones, it is found that microtubules in such conditions have shortened growth and shrinkage times (Janulevicius, Pelt, and Ooyen, 2006).

Theoretical models have also attempted to understand molecular transport of motor proteins along microtubules. Molecular motors are either modelled as singular entities and simulated using Monte-Carlo simulations, or they can be modelled as a population using differential equations dependent on their binding and unbinding rates (Karsenti, Nédélec, and Surrey, 2006). A three-dimensional Monte Carlo simulation of motors has found that thermal fluctuations could lead to decrease in motor velocity (Erickson et al., 2011). Also, the presence of cargo on the motor would enhance binding of motor to the microtubule since the cargo would prevent the motor-cargo complex from diffusing away from the microtubule (Erickson et al., 2011). Monte Carlo simulations have also shown how mutual exclusion of motors lead to traffic jams along the microtubule. Mutual exclusion occurs because no two motors can bind to the same microtubule spot. Thus when a motor is bound on the microtubule at a particular position, the motor excludes another motor from binding at the same position (Lipowsky, Klumpp, and Nieuwenhuizen, 2001). Effects of mutual exclusion is found to be more prevalent at high motor concentrations (Klumpp, Nieuwenhuizen, and Lipowsky, 2005). Computational modelling is also useful in elucidating the cooperativity of cargo transport by more than one molecular motor (Klumpp and Lipowsky, 2005). Such multi-motor modelling is more relevant physiologically because cargo particles can be pulled by several molecular motors at any one instance, and can even exhibit ‘tug of war’ when oppositely directed molecular motors bind to the same cargo (Hancock, 2014).

Microtubule modelling is also extensively applied to understand the mitotic spindle (Mogilner et al., 2006). These models typically address a particular process of the cell cycle and till date, the entire spindle morphology has yet to be modelled throughout the whole cell cycle (Karsenti, Nédélec, and Surrey, 2006). The dynamic instability mechanism has been found to be an efficient capture mechanism for chromosome capture during prophase resulting in the “search

and capture” mechanism (Holy and Leibler, 1994). Separately in (Wollman et al., 2005), mathematical modelling shows that a spatial gradient which enhances microtubule dynamics towards the chromosome, termed the biased search-and-capture may lead to a more efficient chromosome capture mechanism. Mechanistic models for pole formation during mitosis have also been proposed suggesting that self-organised spindle patterns can form spontaneously in the presence of cross-linking motor Eg5, chromokinesin and dynein (Schaffner and José, 2006; Chakravarty, Howard, and Compton, 2004). Motor protein transport modelling have also been extended to chromosome transport during anaphase where the motor motion is modelled as a Brownian ratchet with an opposing load (Raj and Peskin, 2006). Using this model, the experimental finding that chromosome speed is uncorrelated to chromosome length can be replicated and this result is attributed to the flexibility of the chromosome (Raj and Peskin, 2006).

Computational models are also useful in understanding the intricacies of scaffold proteins. A quantitative model of the mitogen-activated protein kinase (MAPK) cascade with a generic scaffold protein has shown that scaffolds can be used to achieve both signal specificity and signal amplification (Levchenko, Bruck, and Sternberg, 2000). However, an optimum concentration of scaffold is necessary for maximal signal propagation (Levchenko, Bruck, and Sternberg, 2000). This observation of optimal scaffold levels has been verified experimentally using the Ste5 scaffold where the signal output was shown to display an optimum concentration depending on the scaffold concentration (Chapman and Asthagiri, 2009). Such complex behaviours of scaffolds are also supported by mathematical modelling. Using Monte Carlo simulations, it was found that scaffold proteins can both amplify and dampen signals under different environments: amplifying signals at high phosphatase activity while suppressing signals at low phosphatase activity (Locasale, Shaw, and Chakraborty, 2007). Dynamical modelling of scaffold proteins are also used to understand the effect of KSR and MP1 scaffold proteins on the EGFR-ERK signalling where scaffolding has been postulated to fine-tune ligand sensitivity (Huang et al., 2011). Similar approaches are also conducted to model the yeast pheromone pathway

where equations representing signal propagation following pheromone stimulation are modelled (Kofahl and Klipp, 2004). Crucial in the model is Ste5 scaffold which enables the graded response of yeast cells to changing concentrations of the pheromone (Kofahl and Klipp, 2004). Mathematical modelling of scaffold binding in cell signalling also shows that scaffold protein is crucial to the establishment of bistability in cell signaling (Chan et al., 2012).

In this thesis, we will be modelling the signalling dynamics with scaffold proteins as they are transported along microtubules with reference to the c-Jun NH2-terminal kinase (JNK) signalling cascade. MAPK signalling pathway is an evolutionary conserved pathway important for the relaying and integration of extracellular stimuli into cellular responses. These cellular responses include apoptosis, proliferation as well as differentiation (Weston and Davis, 2002b). The JNK pathway is one of the major MAPK pathways involved in stress activation (Wagner and Nebreda, 2009). Besides the JNK pathway, there are two other MAPK signalling pathways namely the ERK and p38 pathways. What separates these three groups of MAPKs are their phosphorylation motifs. ERK, p38 and JNK kinases contain different dual phosphorylation motifs, respectively they are Thr-Glu-Tyr and Thr-Gly-Tyr and Thr-Pro-Tyr (Davis, 2000). Environmental and genotoxic stresses lead to activation of JNKs causing changes in survival, migration, differentiation (Karin and Gallagher, 2005; Kyriakis and Avruch, 2001).

In the JNK signalling cascade, receptors activated upon stress exposure first lead to activation of MAP4K, which in turn causes activation of MAP3K. The activated MAP3Ks then transmit these signals to MAP2Ks. Ultimately, JNK which is a MAPK is activated by MAP2K. Activated JNK proceeds to phosphorylate the transcription factor c-JUN leading to changes in transcriptional activity (Smeal et al., 1991; Pulverer et al., 1991). c-JUN modulates cell cycle progression by ensuring sufficient cyclin D1 kinase activity (Wisdom, Johnson, and Moore, 1999) as well as countering the pro-apoptotic behaviour of p13 in hepatocellular carcinoma (Eferl et al., 2003). Scaffold proteins play an important role in regulating the JNK pathway via JIP1 which binds JNK, MAPKK7 as well as mixed lineage kinase (MLK) (Whitmarsh et al., 2001; Yasuda et al., 1999). MAPKK7 is a

MAP2K while MLK is a MAP3K. Upon stress exposure, JIP1 concentrates in the soma along with activated forms of JNK and c-Jun. Following mutation of the *Jip1* gene, JNK activation caused by exposure to stress becomes inhibited. Thus, JIP1 scaffold is a crucial regulator of JNK signal transduction (Whitmarsh et al., 2001; Yasuda et al., 1999). JIP1 has a role in modulating cargo transport due to its association with kinesin-1, KIF5. Association between JIP1 and kinesin has been identified in a yeast two-hybrid screen where kinesin light chain is used as bait (Verhey et al., 2001). Furthermore, inhibition of KIF5 also leads to inhibition of MAP3K localization. These suggest that scaffolds are also able to traffic signalling molecules while modulating cellular response. Unfortunately till date, no holistic modelling has been performed to explore the interplay between cargo transport and the effects of scaffolding on signal transduction. These aspects will be thoroughly explored in chapter three of the thesis.

1.4 Biology at the intercellular level

1.4.1 Description of biology at the intercellular level

Beyond the cellular level, cells are organized into tissues and tissue into organs. This is observed in Fig 1.9 which describes the cell organization in the digestive system. In order for cells to coordinate their roles in these macrostructures, intercellular communication is necessary. Intercellular signalling can be divided into four main types: autocrine, paracrine, endocrine and juxtacrine. Autocrine signalling refers to signalling where a cell secretes messengers such as a growth factor leading to its own activation. In paracrine signalling, a chemical signal secreted by a cell leads to activation of neighbouring cells. This is contrasted with endocrine signalling where hormones secreted by specialized group of cells travel through the circulatory system eliciting changes in other parts of the body. Lastly, juxtacrine signalling refers to contact-dependent signalling where signalling occurs between contacting neighbouring cells. This can occur in many forms, 1) through the release of chemicals into the extracellular space, 2) via transport systems such as nanotubes and cytonemes or 3) through

ligand-receptor interactions during cell-cell contact. In this section, we are going to focus on intercellular communication involved during angiogenesis and illustrate how a group of homogeneous endothelial cells is capable of differentiating into different roles simply through juxtacrine signalling.

FIGURE 1.9: Cellular organization of the digestive system. Organs are formed from tissues and tissues from cells. In order to achieve such complexity, intercellular communication is necessary. (Obtained from Wikipedia Commons)

1.4.2 Description of angiogenesis

Angiogenesis refers to the development of new blood vasculature from existent blood vessels (Birbrair et al., 2015; Birbrair et al., 2014). Such a definition distinguishes angiogenesis from vasculogenesis where vascular endothelial cells emerge from mesodermal progenitor cells (Risau and Flamme, 1995). Vasculogenesis is responsible for blood vessel formation during the embryo stages (Flamme, Frölich, and Risau, 1997), while angiogenesis takes over the formation of most if not all blood vessels at later stages of an organism's life (Flamme, Frölich, and Risau, 1997).

Angiogenesis is an essential physiological process for proper growth and development. For example, during the wound healing process, angiogenesis is necessary for the formation of the granulation tissue (Tonnesen, Feng, and Clark, 2000b). Granulation tissue refers to the newly formed connective tissue and blood vessels that develop within the wound as the wound heals. Vascularization of the wound is necessary to transport nutrients and leukocytes into the recovering tissue as well as removal of metabolic waste. Angiogenesis is also important in the development of a malignant tumour and is regarded as one of the hallmarks of cancer (Hanahan and Weinberg, 2000; Hanahan and Weinberg, 2011). In the seminal paper by Douglas Hanahan and Robert Weinberg, six hallmarks characterize the transition of normal cells to malignant cells (Hanahan and Weinberg, 2000). One of these hallmarks include sustained angiogenesis which is defined as the ability of cancer cells to stimulate the growth of blood vessels towards the tumour thus providing the growing tumour with a nutrient supply (Hanahan and Weinberg, 2000). Sustained angiogenesis is important for continual growth of a tumour since diffusion becomes less efficient when the tumour becomes bigger. In order to ensure a constant blood supply, cancer cells activate non-cancerous cells to grow blood vessels by decreasing the concentration of angiogenic inhibitors and increasing angiogenic factors to stimulate blood vessel formation in the tumour vicinity (Santin et al., 1999; Bergers and Benjamin, 2003). This is depicted in Fig 1.10.

FIGURE 1.10: Angiogenesis in cancer. Cancer cells stimulate the growth of blood vessels towards it thus ensuring a constant oxygen and nutrient supply. (Obtained from Wikipedia Commons)

Two forms of angiogenesis have been identified. They are sprouting angiogenesis and intussusceptive angiogenesis. Both of these angiogenic processes are present *in utero* and are ongoing throughout the organism's life. Between these two angiogenic processes, sprouting angiogenesis is better characterized since it is discovered nearly two hundred years ago, while intussusceptive angiogenesis is only recently uncovered by Burri in the last couple of decades (Burri and Tarek, 1990; Caduff, Fischer, and Burri, 1986).

As its name suggests, sprouting angiogenesis consists of the formation of blood vessel sprouts made from endothelial cells. These sprouts typically grow in response to an angiogenic stimulus such as vascular endothelial growth factor (VEGF). Thus, tissues which are previously void of blood vasculature can have blood vessels growing within them via sprouting angiogenesis. This is in contrast with intussusceptive angiogenesis where blood vessels are formed from splitting of existing blood vessels. Many steps are involved in sprouting angiogenesis. First, the capillary basement membrane will undergo enzymatic degradation. Next, endothelial cells will proliferate and migrate towards the angiogenic source. In tissues that are poorly perfused, the hypoxic conditions trigger parenchymal cells such as myocytes and astrocytes to secrete VEGF (Villegas, Lange-Sperandio, and Tufro, 2005). The VEGF gradient thus serves as guidance for endothelial cell migration. As the endothelial cells migrate, blood vessel tubes will be formed aka. tubulogenesis. Tubes that are formed can also undergo fusion as well as pruning. Further maturation of the nascent blood vessel sprout requires pericyte attachment and production of new extracellular material around the blood vessel (Bergers and Song, 2005; Senger and Davis, 2011). Within the blood vessel sprout, two cell types can be distinguished, namely tip cells and stalk cells. The endothelial tip cell is responsible for directional migration towards the angiogenic source (Gerhardt, 2008; Horowitz and Simons, 2008). Tip cells contain filopodia which are long and thin protrusions. These protrusions produce enzymes that aid in extracellular matrix degradation. In addition, filopodia also possess VEGF receptors which enable tip cells to discern

the VEGF concentration gradient. Upon attachment of filopodia onto the underlying substrate, actin filament contraction within the filopodia will enable the tip cell to be pulled forward (Adair and Montani, 2010). Trailing the tip cells are the endothelial stalk cells which proliferate leading to elongation of the capillary sprout. Lumen formation begins in the stalk cell as vacuoles develop and fuse. Eventually, the stalk cells become the trunk of the blood vessel. Should tip cells from two separate sprouts meet, these tip cells will coalesce leading to a continuous lumen through which oxygenated blood can circulate. With a proper blood circulation, tissues are able to obtain the oxygen they require and as oxygen levels increase to normal, VEGF levels also return to its basal state.

The Delta-Notch signalling pathway is an essential pathway controlling the proportion of tip cells and stalk cells in sprouting angiogenesis. In the Delta-Notch signalling pathway, the Delta-like-4 (DLL4) ligand binds to the receptor, Notch on neighbouring cells resulting in Notch's activation. Since both Delta and Notch are transmembrane proteins, activation of the Notch receptor can only occur upon cell-cell contact. Exposure to VEGF leads to an increase in production of DLL4 within tip cells. Subsequently, Notch is activated in the neighbouring cells upon DLL4 binding. Notch activation within the neighbouring cells results in repression in production of VEGF receptors. Thus the neighbouring cells are less responsive to VEGF and express less DLL4. Ultimately, the neighbouring cell has high Notch but low DLL4 levels and eventually takes on the stalk cell fate while the tip cell expresses low Notch activity and high DLL4 expression. The Delta-Notch signalling pathway thus ensures that the neighbours of the tip cell do not take on the tip cell fate but instead become the stalk cell. Tip cells are most likely to be formed from endothelial cells that are exposed to the highest concentration of VEGF (Suchting et al., 2007; Carmeliet et al., 2009; Horowitz and Simons, 2008). The DLL4 and Notch proteins are depicted in Fig 1.11.

The Notch signalling pathway is present in most metazoans and is highly conserved (Artavanis-Tsakonas, Rand, and Lake, 1999). Research on Notch began as early as 1914 when John S. Dexter noticed the presence of notches on *Drosophila* wings. The genes responsible for these notches are then isolated in

FIGURE 1.11: Delta and Notch transmembrane proteins on the cell membrane. Upon cell-cell contact, Delta activates Notch on the neighbouring cell. (Obtained from Wikipedia Commons)

1917 by Thomas Morgan. Since then, other participants of the Notch signalling pathway have been identified. In mammals, four notch receptors are present consisting of Notch1-4 as well as five transmembrane ligands, Delta-like ligand 1,3 and 4 as well as two Jagged proteins (Sasnauskien et al., 2014). Following ligand binding to Notch, ADAM10, an ADAM metalloproteinase cleaves the extracellular portion of Notch (Tetering et al., 2009). Next, a second enzyme, γ -secretase cleaves the intracellular portion of Notch releasing the intracellular domain of the Notch protein or the NICD (Kopan, 2012). The NICD then travels to the nucleus and activates the transcription factor CSL eliciting gene expression regulation (Lubman et al., 2007). Notch regulates various pathways including stem cell maintenance (Es et al., 2005; Gaiano, Nye, and Fishell, 2000) and cell fate determination in different organs (Kiernan, 2013; Milner and Bigas, 1999).

Activated Notch has also been reported to be pro-oncogenic by promoting cell survival and cell proliferation (Guo et al., 2009; Shelly, Fuchs, and Miele, 1999; Jundt et al., 2002).

The Delta-Notch signalling pathway can also be described as a lateral inhibition mechanism (Appel, Givan, and Eisen, 2001). Lateral inhibition is first observed in neurobiology where an excited neuron triggers its neighbours to reduce their activity. Such inhibition prevents the lateral spread of action potentials along the neurons allowing a sharp contrast to be created. Lateral inhibition is found to be important in vision in 1865 through the discovery of Mach bands. Now, lateral inhibition is also found to be pervasive in other non-neurogenic systems such as in the selection of tip and stalk cell during angiogenesis.

At present, the Delta-Notch pathway is still largely not understood. Nonetheless, some aspects of the pathway are clear. Firstly, adequate levels of VEGF are necessary for proper blood vasculature formation. Decreasing VEGF expression by half lead to observable defects in the vasculature and is embryonically lethal (Ferrara et al., 1996; Carmeliet et al., 1996). In cancer where VEGF expression is abnormally high, excess tip cells numbers have also been reported where the tumour vasculature was observed to be leaky and disorganized (Milner and Bigas, 1999).

In the second type of angiogenesis which is intussusceptive angiogenesis, new blood vessels are formed from splitting of an existing vessel into two. Intussusceptive angiogenesis is also commonly referred to as splitting angiogenesis. Unlike sprouting angiogenesis, intussusceptive angiogenesis is faster and more efficient. This is because endothelial cell migration and proliferation is unnecessary and only reorganization of existing endothelial cells is required. Even though intussusceptive angiogenesis takes place throughout an organism's life, it is especially important during the embryonic stages where resources are limited and yet growth is rapid. Besides forming new capillaries where capillaries are already present, intussusceptive angiogenesis is also necessary for pruning of larger blood vessels (Burri, Hlushchuk, and Djonov, 2004).

Dysfunctional angiogenesis is responsible for the progression of many diseases such as diabetes and cancer. At present, many angiogenic stimulators or inhibitors are either available or undergoing clinical trials for therapeutic treatment. For example, Avastin or bevacizumab is an anti-VEGF monoclonal antibody which is approved by the FDA for use in metastatic colorectal (Hurwitz et al., 2004), non-small-cell lung cancers (Sandler et al., 2006) and renal cell cancers (Rini, 2007). Besides Avastin, Nexavar (sorafenib) and Sutent (sunitinib) have also been approved by FDA (Bergers and Hanahan, 2008). The latter two drugs acts as kinase inhibitors targeting the VEGF receptor tyrosine kinases. Despite the clinical milestone achieved with these drugs proving that angiogenesis can be targeted with successful therapeutic outcomes, these drugs do not persist to improve outcome in most patients (Jain, 2005; Saltz et al., 2007; Kindler et al., 2010). Initially, modest results are observed with the use of such drugs such as tumour stasis or even shrinkage (Miller, Sweeney, and Sledge, 2005). Unfortunately in most cases, the tumour develops resistance and eventually resumes its growth and cancer progresses (Miller, Sweeney, and Sledge, 2005). Thus the search for other potential angiogenic inhibitors continues. Recently, inhibitors targeting the Notch signalling pathway is being explored for cancer treatment as evidence suggesting that Notch signalling is dysregulated in tumour angiogenesis accumulates (Takebe, Nguyen, and Yang, 2014; Li and Harris, 2005; Nickoloff, Osborne, and Miele, 2003). Furthermore, Notch has also been implicated in cancer stem cells maintenance suggesting its oncogenic potential (Pannuti et al., 2010; Takebe et al., 2011). Notch's role in cancer is first observed in T-cell acute lymphoblastic leukaemia (T-ALL) where Notch1 was observed to be truncated with a missing extracellular subunit (Reynolds, Smith, and Sklar, 1987; Ellisen et al., 1991). Since then, mutations resulting in gain-of-function in Notch1 is found in half of surveyed T-ALL cases (Weng et al., 2004). Notch misregulation is also observed in various solid tumours such as breast, lung, melanoma, cervical as well as prostate (Ranganathan, Weaver, and Capobianco, 2011; Reedijk et al., 2005; Santagata et al., 2004; Collins, Kleeberger, and Ball, 2004; Hendrix et al.,

2002; Zagouras et al., 1995). In about 40% of breast cancer and 30% of lung cancer, Numb, a regulator of the Notch signalling pathway is lost (Stylianou, Clarke, and Brennan, 2006). High expression levels of Notch1 is also observed in a quarter of human breast tumor and the high levels are reported to be associated with lower overall survival (Ellisen et al., 1991). Notch signalling is also implicated in epithelial-mesenchymal transition during tumour metastasis (Ranganathan, Weaver, and Capobianco, 2011; Reedijk et al., 2005). Currently, various drugs targeting different aspects of Notch signalling trials are in various phases of clinical trials (Sandler et al., 2006). γ -secretase inhibitors which inhibit Notch activation by blocking the gamma-secretase enzymes are already in early clinical trials: Merck's GSI MK-0752 targets patients suffering from leukaemia, lymphoma and breast cancer (Purow, 2012). Antibodies blocking Delta-like-ligand-4 is also currently being explored for use in anti-angiogenic treatments. Since inhibition of Notch activity prevents tip cell formation and suppresses sprouting, usage of specific antibodies against DLL4 is observed to increase tip cell number, leading to formation of chaotic and aberrant tumour vasculature, and ultimately tumour regression (Ridgway et al., 2006; Noguera-Troise et al., 2006; Hellström et al., 2007).

1.4.3 Sprouting angiogenesis models

Till date, many models seek to explain and predict the angiogenesis phenomenon. These models can be broadly divided into discrete models or continuum models.

In discrete modelling, cells are modelled as discrete units obeying specified laws and move in a discretized manner. Such discrete stochastic modelling allow the system to replicate the digitized nature of tip cell numbers as well as capture small scale features (Scianna, Bell, and Preziosi, 2013). Phenomenon like branching and anastomosis can also be more accurately reproduced using discrete modelling. Anastomosis refers to connections between blood vessel branches and is difficult to replicate in continuum modelling. The anastomosis phenomenon can be observed in (Bauer, Jackson, and Jiang, 2007) where the Cellular Potts model is used to model endothelial migration and division. From their simulation, the

authors observed that branching and anastomosis occur due to inhomogeneity in the underlying substrate (Bauer, Jackson, and Jiang, 2007). Agent-based models are also used extensively to model angiogenesis. In agent-based models, each cell or cell portion is represented by an agent which follows logic-based rules (Logsdon et al., 2014). This is exemplified in (Bentley, Gerhardt, and Bates, 2008), where portions of endothelial cells are modelled as agents which can retract and extend in response to their DLL4 and VEGF levels. The results in (Bentley, Gerhardt, and Bates, 2008) show that oscillations in DLL4 and VEGF levels within a blood vessel will occur when VEGF concentration is high. Similarly in (Walpole et al., 2015), an agent-based model of sprouting angiogenesis is used to study sprout initiation frequency whose sprouting frequency and sprouting location predictions matches experimental time-lapsed images.

Typical continuum models of angiogenesis attempt to model angiogenesis by simulating the migration of endothelial cells towards higher concentration of angiogenic factors leading to vasculature formation. The first attempt of continuum modelling in angiogenesis is by Deakin (Deakin, 1976). Since then, other similar continuum angiogenesis models have been developed (Flegg et al., 2015; Olsen et al., 1997; Pettet et al., 1996). Most of these models utilize advection-reaction-diffusion equations to calculate endothelial cell densities as well as chemoattractant concentration (Scianna, Bell, and Preziosi, 2013). In (Balding and McElwain, 1985), a distinction is made between tip cells and stalk cells such that only tip cells respond to VEGF. Numerous numerical results are produced for different parameter values in (Balding and McElwain, 1985) which can be tested and verified experimentally.

Continuum models have also been used to model smaller sub-processes within angiogenesis such as the Delta-Notch signalling pathway. In this case, chemical concentrations of Notch and Delta are monitored within each cell to as to predict the tip and stalk cell positions. In (Collier et al., 1996), a general mathematical model derived from experimental observations is used to construct the Delta-Notch signalling pathway model. The model embodies the following properties: Notch activation leads to Delta inhibition and Notch can only be activated

by Delta on neighbouring cells. Based on this simple model, a double negative feedback loop is generated between neighbouring cell resulting in the salt-and-pepper pattern where consecutive tip cells are separated by one stalk cell. Similarly in (Giurumescu, Sternberg, and Asthagiri, 2006), fate segregation within *Caenorhabditis elegans* vulva is modelled in which the double negative feedback loop is shown to be essential in fate segregation. Similarly, in (Koizumi, Iwasa, and Hirashima, 2012), a continuum model is also used to model the Delta and Notch levels expression in *Drosophila* trachea endothelial cells. In (Koizumi, Iwasa, and Hirashima, 2012), the authors found that besides the widely known signal amplification property of lateral inhibition, lateral inhibition can also dampen signal differences under certain parameters. This has been suggested to allow for greater robustness in the tip cell selection mechanism. Last but not least, in (Chen et al., 2014), a diffusion term that accounts for Notch diffusion between neighbouring cells is added into the canonical Delta-Notch lateral inhibition model to account for long-range patterning. As a result, more tip-stalk patterns can be recovered including those with multiple stalk cells in between tip cells. In Chapter 5 of this thesis, we explore how other mechanisms specifically, Notch heterogeneity and tension modulation of rate constants integrate with the Delta-Notch signalling pathway to result in an expansion of possible tip-stalk patterns.

Chapter 2

Discovery of a novel interaction between POPX2 and Chk1

This chapter was co-written with graduate student, Kim Purum in preparation for manuscript submission. Specifically, I carried out the bioinformatic analysis and wrote the computational methods and bioinformatics results. Kim Purum conducted the experimental validation and wrote the experimental aspects of the methods and results. Both Kim Purum and I contributed equally in writing of the Introduction. The results in this chapter will also be a part of Kim Purum's thesis.

2.1 Introduction

POPX2 (Partner of PIX 2) is a phosphatase belonging to the protein phosphatase 2C (PP2C) family, one of the four main classes of serine/threonine-specific protein phosphatases in mammals (Yamamoto et al., 1999; Weiss, Tanowitz, and Kirchhoff, 2011). POPX2 shares extensive homology with POPX1 with approximately 66% protein sequence similarity within the core phosphatase domain (Koh et al., 2002). POPX1 is mainly expressed in the brains and testis whereas POPX2 is ubiquitously expressed in most human tissues. POPX2's role as a phosphatase is exemplified in its regulation of P21-activated kinase 1 (PAK1) and calcium-calmodulin kinase (CaMKII) (Harvey, Banga, and Ozer, 2004). POPX2 has been found to be a negative regulator of PAK1 by dephosphorylating PAK1 at Thr422, a crucial regulatory site within the kinase activation loop (Koh et al., 2002; Chong

et al., 2001). In agreement with the negative regulation of PAK1, overexpression of POPX2 leads to inhibition of actin stress fibers breakdown (Koh et al., 2002). POPX2 has also been reported to dephosphorylate CaMKII at the autophosphorylation site of Thr286 leading to CaMKII's inactivation (Ishida, Kameshita, and Fujisawa, 1998).

Recently, POPX2 has gained prominence due to its roles in cancer metastasis. The invasiveness of breast cancer cells has been found to be positively correlated with POPX2: POPX2 levels are highest in invasive MDA-MB-231 cell lines while non-invasive MCF7 breast cancer cell lines express low amounts of POPX2 (Susila et al., 2010). In addition, POPX2 overexpression leads to increase in motility of MDA-MB-231 and MCF7 cell lines, possibly by modulating mitogen-activated protein kinases (MAPK) signalling (Susila et al., 2010; Zhang et al., 2013b). Evidently, POPX2 can enhance tumor progression via promotion of cell motility and invasiveness. This is contrasted with POPX2's role in late metastasis where larger and more numerous tumour nodules are observed at metastatic sites following POPX2 silencing (Zhang et al., 2017). POPX2 silencing appears to increase angiogenesis and consequently metastasis by increasing exosome secretion causing induction of various pro-angiogenic cytokines (Zhang et al., 2017). These studies point towards POPX2 as a multi-faceted regulator of cancer metastasis modulating multiple biological signalling programmes including MAPK signalling and exosome cytokine secretion as well as exhibiting discrepant roles at various stages of cancer development.

In this study, we seek to further unravel POPX2's role in cancer by identifying other substrates of POPX2 through bioinformatics analysis and validating prospective candidates through experimentation. Phosphatases are typically promiscuous, having tens or hundreds of multiple substrates (Rowland, Harrison, and Deeds, 2015). The PP2C family of phosphatases is no exception and is characterized by broad substrate specificity (Lennarz and Lane, 2013). Thus it is highly likely that POPX2 may also regulate the phosphorylation status of many other unknown substrates. Bioinformatics analysis will allow us to make a more informed conjecture about likely POPX2 interactors and help to narrow

the pool of possible substrates. The predicted substrates can then be tested using co-immunoprecipitation.

Through our work, we uncover a novel interaction between POPX2 and Checkpoint Kinase 1(Chk1). Chk1, a serine/threonine kinase executes a crucial role in the coordination of DNA damage response (McNeely, Beckmann, and Bence Lin, 2014). Upon genotoxic stress, Chk1 is phosphorylated and becomes activated (Patil, Pabla, and Dong, 2013). Activated Chk1 causes arrest of cell cycle, activation of DNA repair pathways as well as induction of apoptosis under severe DNA damage (Harper and Elledge, 2007; Dai and Grant, 2010; Chen and Poon, 2008). Chk1 has been postulated to cause chemotherapy resistance due to the ability of tumour cells to withstand higher levels of DNA damage under increased Chk1 levels (Liang et al., 2009). The discovered interaction between POPX2 and Chk1 once again reinforces POPX2's importance in tumour progression where we demonstrate that POPX2 is also implicated in the DNA damage response pathway by dephosphorylating Chk1.

2.2 Results

2.2.1 Bioinformatics analysis to search for potential POPX2 substrates

We conduct a two-pronged bioinformatics analysis to uncover novel POPX2 substrates. False positive rate is kept low since only substrates that are picked out by both strategies are used as leads for experimental validation. The two-pronged approach for prediction of POPX2 substrates is illustrated in Fig 2.1 and enumerated below:

1. Prediction of POPX2 substrates by relation to known substrates of POPX2 and PP2C domain
2. Prediction of POPX2 substrates by curation of known interactors of proteins sharing homology to POPX2

FIGURE 2.1: Schematic of the bioinformatics analysis. The analysis is split into two parts. The first part involves looking for potential substrates by comparing similarity between known substrates of POPX2 and proteins containing the PP2C domain, as shown by the pink dotted box. The second part involves curation of known interactors of proteins exhibiting similar homology as POPX2, as shown by the green dotted box

2.2.2 Prediction of POPX2 substrates by relation to known substrates of POPX2 and PP2C domain

Proteins evolve via shuffling of functional domains; the same domain can be observed in various dissimilar proteins (Shoemaker, Panchenko, and Bryant, 2006). These domains mediate protein-protein interaction (PPI) and many domain pairs are maintained in evolution across different organisms (Itzhaki et al., 2006). Thus, information about domain-domain interactions (DDI) can be used to infer protein interactions reliably (Wojcik and Schächter, 2001; Rao et al., 2014).

Using NCBI Conserved Domains search, POPX2 is found to contain a PP2C domain, PF00481, between amino acids 155 to 406 (E-value: 1.28×10^{-86}) (Marchler-Bauer and Bryant, 2004). The domain PP2C has been reported to interact with the Pkinase domain as observed in the protein data bank (PDB) structure, 3UJG (Finn et al., 2014). Examination of 3UJG suggests that the DDI between PP2C and

Pkinase is by docking of the kinase activation loop into the active site of PP2C following which, the serine site within the activation loop is dephosphorylated (Soon et al., 2012).

An alignment is performed between the PP2C domain of POPX2 (here and so forth termed as PP2C_{POPX2}) and the PP2C domain in PP2C in 3UJG (here and so forth termed as PP2C_{3UJG}) as seen in Fig 2.2. Active site residues between PP2C_{POPX2} and PP2C_{3UJG} are well conserved suggesting that PP2C_{POPX2} may also interact with other Pkinase domains via a similar mechanism. This is further substantiated by the fact that known substrates of POPX2 namely PAK1 and CaMKII do contain the Pkinase domain and PAK1 has been observed to be dephosphorylated by POPX2 at the activation loop region. Thus, we narrow our search for potential substrates of POPX2 by only concentrating on proteins containing the Pkinase domain.

FIGURE 2.2: Alignment between PP2C_{3UJG} and PP2C_{POPX2}. The red boxes, both thin and thick, indicate the active site regions and the thick red box demarcates the histidine and aspartic acid residues which are likely to be responsible for catalysis.

There are approximately 20000 protein-coding genes in the human genome (Ezkur-dia et al., 2014; Clamp et al., 2007). To reduce the pool of proteins for initial screening, we leverage on previous proteomic data performed on POPX2- knock-down MDA-MB-231 breast cancer cells (Zhang et al., 2013b). In (Zhang et al., 2013b), proteins within POPX2-knowndown and wild-type cells are identified using liquid chromatography–mass spectrometry. A drawback of mass spectrometry is that it preferentially identifies more abundant proteins. However, this limitation proves to be beneficial since important substrates of POPX2 should be present in sufficient amounts to elicit any phenotypic effect. The mass spectrometry data thus enables us to sieve out proteins that are present in overtly low levels.

Proteins identified in (Zhang et al., 2013b) are filtered for Pkinase domains. Out of the 2146 proteins, only 44 proteins contain Pkinase. Phylogenetic analysis is then performed on the Pkinase domains of these 44 proteins to identify their relation with known substrates of PP2C including the Pkinase domain in 3UJG as well as Pkinase in PAK1 and CaMKII. The phylogenetic tree is plotted in Fig 2.3. From the phylogenetic analysis, Pkinase domain of Chk1 is observed to be closely related to that in CaMKII and in 3UJG. Chk1 is a regulator of cell cycle and apoptosis during DNA damage. In addition, proteins involved in the cell cycle pathway are previously found to be differentially expressed in POPX2-knockdown cells (Zhang et al., 2013b). These evidences strongly suggest Chk1 as a likely candidate for POPX2 interaction.

2.2.3 Prediction of POPX2 substrates by curation of known interactors of proteins sharing homology to POPX2

In the second part of the analysis, we identify potential substrates of POPX2 by consolidating known interactors of POPX2 homologs. Proteins with close sequence similarity to POPX2 are isolated from BLAST. Unsurprisingly, isolated proteins are members of the PPM1 family like PPM1A and PPM1M.

For each of the PPM1 member, we identify their known interactors using STRING (Szklarczyk et al., 2015). Homologous proteins are likely to share similar

TABLE 2.1: Interactors of proteins sharing homology to POPX2

Substrate	PPM1D	PPM1K	PPM1N	PPM1B	PPM1A	PPM1G	PPM1L	PPM1M	PPM1E	counts
RIPK4	0	1	1	0	0	1	1	0	1	5
MAP3K7	0	1	0	1	0	0	1	0	1	4
MAP3K9	0	1	1	0	0	0	0	0	1	3
CDK9	0	0	0	1	1	0	0	0	0	2
STK11	0	0	0	0	1	0	0	0	1	2
ZAK	0	1	0	0	0	0	0	0	1	2
LYN	0	0	1	0	0	0	0	0	0	1
CHK1	1	0	0	0	0	0	0	0	0	1
CHK2	1	0	0	0	0	0	0	0	0	1
MAPK11	0	0	0	0	0	0	1	0	0	1
MAPK12	0	0	0	0	0	0	1	0	0	1
MAPK14	0	0	0	0	0	0	1	0	0	1
MYH14	0	0	0	0	0	0	0	1	0	1
SMAD1	0	0	0	0	1	0	0	0	0	1
SMAD2	0	0	0	0	1	0	0	0	0	1
SMAD3	0	0	0	0	1	0	0	0	0	1
SMAD5	0	0	0	0	1	0	0	0	0	1
STAT6	0	0	0	0	0	1	0	0	0	1
STMN2	0	0	0	0	0	1	0	0	0	1

interactions, hence, interacting partners of the partner of any particular protein will be enriched by its homologs (Espadaler et al., 2005). This implies that known interactors of POPX2's homologs may also be substrates of POPX2. The table 2.1 summarises the proteins that have been reported to interact with PPM1 family. Once again, Chk1 appears in the list where it is previously been reported to interact with PPM1D. PPM1D binds to Chk1 leading to dephosphorylation of Chk1 at Ser345 with a subsequent reduction of checkpoint activity (Lu, Nannenga, and Donehower, 2005).

Based on the bioinformatics analysis detailed above, Chk1 appears to be a plausible substrate capable of interacting with POPX2. In the ensuing section below, we show experimental validation of interaction between Chk1 and POPX2.

2.3 Experimental validation of interaction between Chk1 and POPX2

In this section, we seek to confirm the prediction from the bioinformatics analysis by validating the interaction between POPX2 and Chk1.

Firstly, Chk1-Flag is found to co-precipitate with GST-POPX2 following co-transfection of Chk1-Flag and GST-POPX2 in HEK293 cells (Fig 2.4A). In addition, after Chk1-Flag is pulled down using Flag-beads, GST-POPX2 is present in the pulldown as illustrated in Fig 2.4B. These results indicate that Chk1-Flag do interact with GST-POPX2. Furthermore, endogeneous Chk1 also co-precipitated

with GST-POPX2 after GST-POPX2 is transfected into HEK293 cells (Fig 2.4C, lane 2) . However, this interaction between endogenous Chk1 and GST-POPX2 is lost when cells are treated with Hydroxyurea (HU). HU is a DNA replication inhibitor which depletes deoxyribonucleotide and causes DNA double stranded breaks near replication forks (Koc et al., 2004). Abrogation of interaction between Chk1 and POPX2 in the presence of HU might be due to changes in configuration of Chk1 induced by HU treatment (Fig 2.4C, lane 3). HU has been reported to lead to Chk1's phosphorylation at S317, S345 and S296 upon HU treatment (Wilsker et al., 2008; Liu et al., 2000). Altogether, these experiments demonstrate the interaction between exogenous POPX2 and exogenous Chk1. To confirm the endogenous interaction of POPX2 and Chk1, endogenous Chk1 is precipitated using anti-Chk1 and POPX2 can be detected in the Chk1 pulldown (Fig 2.4D). Similarly, Chk1 is also found to precipitate together with endogenous POPX2 in POPX2-IP in HEK293 cells (Fig 2.4E). Therefore, we have confirmed the interaction between POPX2 and Chk1 both *in vitro* and *in vivo*.

Since POPX2 interacts with Chk1, a regulator of cell cycle, and cell cycle related genes has been found to be separately enriched in the transcriptome and proteome of MDA-MB-231 POPX2 knockdown cells through KEGG pathway enrichment analysis (Zhang et al., 2013b), we further investigate how POPX2 may interfere with cell cycle progression.

We examine the proportion of cells at various stages of cell cycle following a double nocodazole block. Nocodazole prevents microtubule polymerization and cells treated with a double nocodazole block are arrested at the G2/M phase and cannot complete mitosis. A double nocodazole block thus ensures that all the cells are synchronized in the same cell cycle phase following nocodazole release. POPX2 knockdown cells have a higher proportion of cells in S phase compared to control cells (43.7% vs 35.85%) as observed in Fig 2.5A. To verify the above result, expression of cyclin E is monitored in both POPX2 knockdown cells as well in control cells. Cyclin E is used as a marker since expression of cyclin E increases as the cell progresses through late G1 to S phase. In both POPX2 knockdown cell lines, cyclin E levels increases 4 hours after nocodazole release

(Fig 2.5B). This is contrasted with control cells where cyclin E levels are observed to increase only after 10 hours after nocodazole release. Furthermore, cyclin E transcript levels are elevated in POPX2 knockdown cells as compared to control (Fig 2.5C). These results suggest that POPX2 interfere with cell cycle progression by increasing expression of cyclin E resulting in a shorter time to reach S phase and a larger proportion of cells in S phase.

2.4 Discussion

Identifying protein–protein interactions (PPIs) is central in molecular biology due to the imperative role proteins play in cells (Keskin, Tuncbag, and Gursoy, 2016). Many PPI tools are available as open source websites. These PPI tools however give us less than ideal results. For example, both InterPreTS (Aloy and Russell, 2003) as well as Interactome3d (Mosca, Céol, and Aloy, 2013) are unable to deduce any interaction between POPX2 and known substrates of POPX2 such as PAK1 and CaMKII. Presumably, such methods may not perform well due to insufficient training as a consequence of finite time and computational resources. The vast possibilities of proteins that POPX2 may interact with in the human genome may also result in false negatives. We circumvent such a problem by using only proteins that are picked out initially from mass spectrometry thus reducing our sample size from about 20000 to only 2146. Furthermore, we attempt to uncover interactors of POPX2 using a novel two-pronged bioinformatics strategy. In the first strategy, potential POPX2 substrates are chosen based on curated domain-domain interactions as well as relation to known POPX2 substrates. The second strategy involves discovering substrates of POPX2 from known interactors of the PPM1 family (the alias of POPX2 is PPM1F). Both of these strategies are guided by evolutionary principles. Domain-domain interactions that mediate protein interactions have been found to be maintained across evolution (Itzhaki et al., 2006). Homologous proteins are also likely to share similar interactions (Espadaler et al., 2005). Through our bioinformatics analysis, we predict Chk1 to be a novel interactor of POPX2. Chk1’s possibility of interacting

with POPX2 is further supported from previous pathway analysis which found that cell cycle pathway proteins are differentially enriched upon POPX2 knock-down (Zhang et al., 2013b). These evidences strongly suggest Chk1 as a likely candidate for POPX2 interaction. which is validated subsequently through *in vitro* and *in vivo* experimentation.

In this study, we discover that POPX2 knockdown cells exhibited irregular cell cycle progression with increased proportion of cells present in S phase and also possess elevated cyclin E levels. Interestingly, cyclin E levels are also modulated by the MAPK kinases by regulating glycogen synthase kinase (GSK) (McCubrey et al., 2014; Diehl et al., 1998). Since, we have previously shown that POPX2 decrease MAPK signaling through inhibiting GSK, these evidence suggest that POPX2 may affect cyclin E expression through regulating GSK and MAPK (Zhang et al., 2013b).

Following discovery of Chk1 as a novel interactor of POPX2, we tested other protein-protein interaction tools available on the internet to check if they are capable of picking out this interaction. Unfortunately, to our best knowledge, no PPI tools are able to detect the interaction between Chk1 and POPX2. Hence, our two-pronged protein-protein interaction prediction protocol is more superior than current methods presumably due to the usage of prior information of known interactors. In future, we would like to extend our protein-protein interaction protocol for interaction prediction of other proteins.

2.5 Methods

2.5.1 Computational prediction analysis

Identification of protein domains is performed using NCBI conserved domain search (Marchler-Bauer and Bryant, 2004). Domain-domain interaction search is implemented using iPfam database (Finn et al., 2014). Alignment between PP2C_{POPX2} and PP2C_{3UJG} is done using the alignment programme in Modeller (Eswar et al., 2007). Phylogenetic analysis is conducted using Randomized Axelerated Maximum Likelihood (RAxML) (Stamatakis, 2014).

2.5.2 Experimental procedures

Cell culture

MDA-MB-231 (Control, shRNA#1, shRNA#2) were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 1g/L glucose and 10% fetal bovine serum (FBS). HEK293 were cultured in DMEM supplemented with 4.5g/L glucose and 10% FBS. All mammalian cell lines were incubated at 37°C with 5% CO₂.

Cell lysis and western blot

Cells were washed with 1X PBS and lysed with protein lysis buffer (50 mM HEPES [pH 7.5], 300 mM NaCl, 1 mM MgCl₂, 1 mM EGTA, 10 mM β -glycerophosphate, 10 mM NaF, 1 mM sodium vanadate, 5% glycerol, 5 mM DTT, 0.5% TritonX-100) supplemented with Protease inhibitor and PhosSTOP (Roche). Cell lysates were centrifuged at 140,00rpm for 10 mins at 4°C. Protein concentration was measured by Bio-Rad protein assay kit. Equal amount of protein lysates in final 1X SDS sample buffer were heated at 100°C for 5 mins to denature proteins. Proteins were loaded in SDS-PAGE gels, transferred onto nitrocellulose membranes and blocked in 5% skimmed milk in 1X TBST for 1 hour. Membranes were incubated with primary antibodies at recommended dilutions overnight at 4°C and incubated with secondary antibodies at 1:4000 dilution for 1 hour at RT. Protein bands were detected using chemiluminescence.

siRNA and plasmid transfection

HEK293 cell was transfected with plasmid 1 to 2 μ g or 50 μ M siRNA with lipofectamin 2000 according to manufacturer's protocol and diluted in opti-MEM. After 24 to 48 hours, cells were harvested with IP buffer (150 mM NaCl, 1% Triton-X in 1X PBS).

Glutathione S-transferase (GST) and flag pull-down assay and immunoprecipitation

HEK 293 cells were transfected with respective plasmids and lysed with IP buffer as described above. 40 μ g of total lysate was used for input and the remaining supernatant was incubate with 50 μ g of Glutathione Sepharose 4B(GE) beads or Flag M2 affinity gel (Sigma). GST and Flag beads were washed thrice with IP buffer and incubated with cell lysates at 4°C overnight. Proteins pull downed with beads were loaded into SDS-PAGE gels and western blot was carried out as described earlier.

For immunoprecipitation assay, HEK293 cells were lysed with IP buffer and centrifuged at 14,000 rpm for 10 mins. For pre-clearing, the supernatant was incubated with protein G magnetic beads (Milipore) at 4 °C for 2 hours under constant rotation. The pre-cleared supernatant was then incubated with respective antibodies at 4 °C overnight. Lysate-antibody mixture was incubated with protein G magnetic beads at 4 °C for 4 hours. The beads were washed thrice with IP buffer and processed by western blot.

Real-time quantitative PCR

Total RNA was extracted using RNeasy Mini Kit (Qiagen) and 2 μ g RNA were used for cDNA synthesis using SuperScript VILO cDNA synthesis Kit (Invitrogen). qPCR reaction reagents include 1:10 diluted synthesized DNA, SYBR Green PCR master mix, forward primer, reverse primer and DEPC water. Each target gene contains three technical and biological replicates with no template control (NTC) and GAPDH was used as the internal standard. Step One Plus real time PCR machine (Applied Biosystems) was used for analysis and relative gene expression level was normalized with GAPDH expression level.

Fluorescence-activated cell sorting (FACS)

Cells were fixed with ice-cold 70% ethanol at 4°C overnight, centrifuged at 13000 rpm for 5 mins and washed with 1X PBS twice. Fixed cells were stained with Propidium Iodide for 1 hour at room temperature. Stained samples were analyzed with X-20 Fortessa (BD Biosciences) using FACSDiva software and FlowJo.

FIGURE 2.3: Phylogenetic tree of known substrates of PP2C and proteins containing Pkinase domain. Chk1, also known as CHEK1 is labelled in pink while CaMKII, PAK1 and 3UJG is labelled in blue. PAK1 is labelled in a different shade of blue since it is part of a different branch. The scale bar refers to the number of amino acids changes per site.

FIGURE 2.4: POPX2 interacts with Chk1 in vitro when expressed exogenously or endogenously. (A) GST or GST-POPX2 was co-expressed with Chk1-Flag in HEK293 cells and cell lysates were incubated with GST-beads at 4°C for overnight. Chk1-Flag was co-immunoprecipitated with GST-POPX2. Lane1, GST + Chk1-Flag; 2, GST-POPX2 + Chk1-Flag. (B) Chk1-Flag or Flag-CaMKII, (positive control) was pulled down using Flag-beads and GST-POPX2 was observed using anti-GST. Lane1, GST-POPX2 + Chk1-Flag; 2, GST-POPX2 + Flag- CaMKII. (C) GST or GST-POPX2 was transfected in HEK293 cells and isolated using GST-beads. Endogenous Chk1 was observed with GST-POPX2 using anti-Chk1 antibodies, but no co-precipitate was observed under HU treatment. Lane1, GST + Chk1-Flag; 2, GST-POPX2 + Chk1-Flag; 3, GST-POPX2 + Chk1-Flag +100 μ M HU. (D) Endogenous Chk1 was isolated using anti-Chk1 antibodies and endogenous POPX2 was co-immunoprecipitated with Chk1. Lanes 1 and 2, input (40 μ g); 3, IgG, a negative control; 4, Chk1 antibody (1 μ g); 5, Chk1 antibody (2 μ g). (E) Endogenous Chk1 was co-immunoprecipitated with isolated POPX2 using anti-POPX2 antibodies. Lane 1, input (40 μ g); 2, IgG, a negative control; 3, POPX2 antibody (1 μ g); 4, POPX2 antibody (2 μ g)

FIGURE 2.5: POPX2 interferes with cell cycle by increasing Cyclin E expression. (A) The percentage of cells in S phase in control (35.85%) and POPX2 KD (43.7%) cells. (B) Control and POPX2 KD cells were harvested after nocodazole block from 0 hr to 16 hr. Cyclin E represents the different stages of cells and POPX2 indicates POPX2 knockdown efficiency in KD1 and KD2. GAPDH was used as a loading control. (C) Cyclin E mRNA expression was determined by real-time PCR in control and POPX2 KD cells.

Chapter 3

Computational modeling reveals optimal strategy for kinase transport by microtubules to nerve terminals

This chapter has been published in PLOS ONE (Koon, Koh, and Chiam, 2014).

3.1 Introduction

Computational modeling of the dynamics of intracellular signaling pathways is an area of active research. Chemical equations such as the law of mass action or other higher-order reactions have been used to simulate the various intermolecular interactions involved in signaling pathways (Klipp and Liebermeister, 2006; Aldridge et al., 2006). Such equations can be solved analytically or numerically, and their steady state values can be further analyzed to gain deeper insights into the functions of the signaling pathways. Unfortunately, such analysis, when performed with the inherent assumption of the cell as a homogeneous mixture or as a well-stirred reactor, neglect the heterogeneous environment within a cell. The importance of such heterogeneity has been increasingly exemplified by evidence supporting the spatial localization of signaling proteins in a cell as an important contributor to the cell's proper functioning (Kholodenko, 2006; Bray,

1998). To this end, models have been extended to include compartmentalization to account for interactions happening in non-interacting compartments such as the membrane, cytoplasm, and nucleus (Lipniacki et al., 2004; Banks et al., 2003). Other models added diffusion to their reaction equations to include for molecular diffusion (Kondo and Miura, 2010; Chiam et al., 2006). Yet other models account for more specific forms of spatial variation such as subdiffusion to mimic the motion of proteins in a dense and crowded cytosol (Yuste, Abad, and Lindenberg, 2010; Haugh, 2009; Chiu and Chiam, 2008). However, an aspect of signaling that contributes to spatial variation has to date not been well studied: the assisted transport of signaling proteins by cytoskeletal-associated motor proteins. Even though, computational studies concerning motor proteins in transport have been investigated in aspects of vesicle transport (Klann, Koeppel, and Reuss, 2012) and heterogeneity matter distribution (Neri, Kern, and Parmeggiani, 2013b; Neri, Kern, and Parmeggiani, 2013a), studies exploring the interplay between cytoskeletal transport and signalling is lacking. This manner of transport and the significance it plays in signaling will be the focal point of this article.

Such transport of proteins and organelles is especially important in neuronal cells. Most axonal proteins are synthesized within the neuronal cell body and mechanisms need to be in place to direct these proteins to the growing axon tips (Gallant, 2000). The complexity of transport is magnified by the sheer length of the distance involved in axonal transport. Axons of sciatic nerve cells have been reported to achieve lengths of more than one meter. Studies examining the molecular components of axonal transport have uncovered two classes of motor proteins that exist to transport cargo proteins along the cytoskeleton. Kinesin mainly governs anterograde axonal transport and transport mitochondria, transport vesicles and synaptic precursors from the cell body towards the synapse (Hirokawa, 1998; Hirokawa et al., 2009). On the other hand, dynein regulates retrograde axonal transport by carrying used components from the neurite tips back to the cell body for degradation and recycling (Schnapp and Reese, 1989). These proteins govern two different modes of transport, namely, fast axonal transport

and slow axonal transport. Membrane-spanning proteins or proteins possessing anchoring domains are packaged into vesicles and transported via fast axonal transport achieving rates of $0.5\text{--}4\ \mu\text{m/s}$. Slow axonal transport moves cytoskeletal and cytosolic proteins at average rates of $0.01\text{--}0.1\ \mu\text{m/s}$ (Hirokawa and Take-mura, 2005; Brown, 2003; Roy et al., 2005).

Often, proteins that are transported by motor proteins are also bound to scaffold proteins. Scaffold proteins have been known to interact and/or bind with various players of a signaling pathway and to tether them into complexes. In doing so, they regulate signal transduction and aid in localization of signaling cascades to specific parts of the cell. Signal activation by irrelevant stimuli can also be prevented, thus providing the cell with spatial and temporal control of signaling (Good, Zalatan, and Lim, 2011). Computational models have shown that scaffold proteins are capable of amplifying signals for a limited range of scaffold protein concentrations (Levchenko, Bruck, and Sternberg, 2000; Shaw and Filbert, 2009a). The biphasic dependence of signaling activity on the concentration of the scaffold protein has been verified experimentally for the prototypical scaffold protein, Ste5, in yeast cells (Chapman and Asthagiri, 2009). However, it is unclear if such biphasic behavior of scaffold proteins exists in the presence of cytoskeletal transport.

One specific example of a signaling cascade that makes use of both scaffold proteins and motor proteins is the JNK (c-JUN NH₂-terminal kinase) signaling pathway. The JNK group of mitogen-activated protein (MAP) kinases modulate a number of cellular processes in mammalian cells such as early embryonic development, apoptosis, oncogenic transformation and the immune response (Davis, 2000) and can be activated by environmental stress or inflammatory cytokines (Ip and Davis, 1998). The JNK signaling module consists of various components including the mixed-lineage kinase (MLK) groups of MAP kinase kinase kinases (MAPKKKs), MAP kinase kinases (MAPKKs) like MAP kinase kinase 4 (MKK4) and MAP kinase kinase 7 (MKK7), and the MAP kinase, JNK. The JIP (JNK-interacting protein) group of scaffold proteins facilitate the signal transduction of the JNK signaling cascade by interacting with components

of the JNK signaling pathway (including MLK, MKK7, and JNK) (Whitmarsh et al., 1998; Morrison and Davis, 2003; Yasuda et al., 1999). The JIP proteins have been demonstrated to be differentially located within cells. It accumulates in the growth cones at the tips of extended neurites (Whitmarsh et al., 2001; Meyer, Liu, and Margolis, 1999; Kelkar et al., 2000) as well as within cell surface projections of cultured cells (Yasuda et al., 1999). Prominent localization of JIP1 in synapses has been identified via immunocytochemical analysis of the brain (Pellet et al., 2000). Specific localization of the JNK signaling cascade to the cell periphery appears to play a crucial role in its function since subcellular organization of JIP1 is altered following stress exposure and disruption of the *Jip1* gene in mice prevented JNK activation (Whitmarsh et al., 2001). Local activation of JNK primarily within axons is also induced during nerve injury. Activated JNK and adaptor protein Sunday Driver (syd, also known as JIP3) are then transported retrogradely, bringing about the idea that a mobile axonal JNK-syd complex may generate a transport-dependent axonal damage surveillance system (Cavalli et al., 2005).

JIP localization to the cell periphery could be modulated via its association to kinesin. In fact, JIP1, JIP2 and JIP3 have been identified as binding partners to kinesin using yeast two-hybrid procedure with kinesin light chain as bait (Verhey et al., 2001). Constructs of kinesin-1 or KIF5 that inhibit neurite tip localization of JIP also inhibit localization of MAPKKK scaffolded by JIP (Verhey et al., 2001). These support the notion that the JIP scaffold is preloaded with its kinase cascade prior to reaching its final destination of transport, differing from the conventional view that signaling molecules assemble on scaffolds at their final destination. The findings also reinforce the idea that signaling scaffolds, in addition to juxtaposing kinases in a cascade, are capable of carrying information about the trafficking and localization of the cascade (Blacque, Cevik, and Kaplan, 2008). Many kinesin superfamily proteins (KIFs) have been reported to reach speeds ranging from 0.2 to 1.5 $\mu\text{m/s}$, which are consistent with the speed of fast axonal transport *in vivo* (Hirokawa, 1998; Hirokawa and Takemura, 2005).

In this article, using the JNK signaling pathway as a model system, we study how the activity of JNK is being modified by being scaffolded via JIP1 and, in

addition, transported to a distant part of the cell along the cytoskeleton via KIF5. Therefore, we seek to understand how the combined effects of both scaffolding and cytoskeletal transport modify signaling activity compared to the case if JNK is to diffuse to the distant part of the cell without scaffolding or cytoskeletal transport. We model the activation of JNK that is being scaffolded by JIP1 and then transported along the cytoskeleton via KIF5 by a set of reaction-diffusion-advection equations, and investigate how signaling rate and signal amplification are modified by the presence of scaffold and motor proteins. In Section II, we describe our model as well as the algorithm used to simulate the scheme effectively. Results of the simulations will be presented in Section III and discussed in Section IV. Finally, in Section V, we present our conclusions.

3.2 Methods

In our model, the signaling protein JNK exists in either an inactive (unphosphorylated) or active (phosphorylated) state, denoted by JNK and JNK*, respectively. The activation of JNK is catalyzed by an upstream activated kinase MKK7 and the inactivation from JNK* to JNK is catalyzed by a phosphatase M3/6. Both the activation enzyme MKK7 and the inactive signaling protein JNK can bind to the scaffold protein JIP1. The scaffold protein JIP1 is assumed to possess catalytic properties such that the rate of activation of JNK by MKK7 is higher within the scaffold than that outside of the scaffold. Enhancement of catalysis within the scaffold has been observed experimentally where the prototypical scaffold Ste5 unlocks the Fus3 MAP kinase for activation by Ste7 MAPKK, thereby increasing the phosphorylation rate (Good et al., 2009). The scaffold protein, bare or complexed with either MKK7 or JNK or both, can bind to the motor protein KIF5. The motor protein and its cargo, i.e., the kinase-scaffold complex, are then transported through the cytosol along the microtubule cytoskeleton. Proteins that are not bound to the motor protein traverse the cytosol by diffusion, with a diffusion coefficient that is inversely proportional to the square root of their relative masses. The various molecular species JNK, JNK*, MKK7, M3/6, JIP1, and KIF5

and their interactions are depicted in Figure 3.1 with Table 3.1 containing the list of reactions and their rate constants.

In the JNK signaling cascade, three kinases are successively activated under stimulus. The cascade starts with activation of MAPKKKs such as MLK3 which go on to phosphorylate and activate the MAPKKs MKK4 and MKK7 which finally phosphorylate and activate JNK (Weston and Davis, 2002a; Davis, 2000). However, in our model, we only consider the final two kinases in the cascade, i.e., the activation of JNK by activated MKK7. By focusing on the last step of activation in the signaling pathway, we believe that the complicated reaction dynamics involved in activating JNK can be abridged, thus providing a clearer analysis regarding the behavior of the JNK signaling cascade making use of scaffold proteins for recruitment and motor proteins for transport. In (Levchenko, Bruck, and Sternberg, 2000), simulations are compared between a simplified model in which a scaffold only can bind to two proteins and a complete model in which the scaffold can bind to all three members of the MAPK cascade. (The simplified model in (Levchenko, Bruck, and Sternberg, 2000) is similar to our model in which each scaffold can only hold two kinases.) Overall, the conclusions and observations between the simplified model and the complete model in (Levchenko, Bruck, and Sternberg, 2000) matches except that the reaction is inhibited at much lower scaffold concentrations in the complete model. This is because a higher membership scaffold produces more nonfunctional complexes. Thus, discoveries made using a simplified model, like our model, are still viable although exact concentrations at which certain trends are observed may be slightly altered. Association of M3/6 with JIP1 has also been neglected since only a small proportion of JIP1 is complexed with M3/6 in resting neuronal cells (Willoughby et al., 2003). In the model, we also assumed that binding of JIP1 to KIF5 is sufficient for activation of motor even though both JIP1 and fasciculation and elongation protein ζ 1 (FEZ1) are necessary for KIF5 activity (Blasius et al., 2007). Furthermore, we are concerned with the delivery of JNK to the cell periphery and thus neglect reactions involving JNK at the nerve terminals.

In our deterministic model, we solve for the set of reaction-diffusion-advection

TABLE 3.1: List of reactions and their corresponding rate constants

Reactions	Rate Constants [†]
$\text{JNK} + \text{MKK7} \xrightleftharpoons[k_{b1}]{k_{f1}} \text{JNK-MKK7} \xrightarrow{k_{cat1}} \text{JNK}^* + \text{MKK7}$	$k_{f1} = 1.0/\mu\text{M}$ $k_{b1} = 1.0/\text{s}$ $k_{cat1} = 0.1/\text{s}$
$\text{JIP1-JNK} + \text{MKK7} \xrightleftharpoons[k_{b2}]{k_{f2}} \text{JIP1-JNK-MKK7} \xrightarrow{k_{cat2}} \text{JIP1} + \text{JNK}^* + \text{MKK7}$	$k_{f2} = 1.0/\mu\text{M s}$ $k_{b2} = 1.0/\text{s}$ $k_{cat2} = 0.4/\text{s}$
$\text{JIP1-MKK7} + \text{JNK} \xrightleftharpoons[k_{b3}]{k_{f3}} \text{JIP1-JNK-MKK7} \xrightarrow{k_{cat3}} \text{JIP1} + \text{JNK}^* + \text{MKK7}$	$k_{f3} = 1.0/\mu\text{M s}$ $k_{b3} = 1.0/\text{s}$ $k_{cat3} = k_{cat2} = 0.4/\text{s}$
$\text{JNK}^* + \text{M3/6} \xrightleftharpoons[k_{b4}]{k_{f4}} \text{JNK}^*\text{-M3/6} \xrightarrow{k_{cat4}} \text{JNK}^* + \text{M3/6}$	$k_{f4} = 1.0/\mu\text{M s}$ $k_{b4} = 1.0/\text{s}$ $k_{cat4} = 0.1/\text{s}$
$\text{KIF5-JIP1-JNK} + \text{MKK7} \xrightleftharpoons[k_{b5}]{k_{f5}} \text{KIF5-JIP1-JNK-MKK7}$ $\xrightarrow{k_{cat5}} \text{KIF5-JIP1} + \text{JNK}^* + \text{MKK7}$	$k_{f5} = 1.0/\mu\text{M}$ $k_{b5} = 1.0/\text{s}$ $k_{cat5} = 0.4/\text{s}$
$\text{KIF5-JIP1-MKK7} + \text{JNK} \xrightleftharpoons[k_{b6}]{k_{f6}} \text{KIF5-JIP1-JNK-MKK7}$ $\xrightarrow{k_{cat6}} \text{KIF5-JIP1} + \text{JNK}^* + \text{MKK7}$	$k_{f6} = 1.0/\mu\text{M s}$ $k_{b6} = 1.0/\text{s}$ $k_{cat6} = 0.4/\text{s}$
$\text{JNK} + \text{JIP1} \xrightleftharpoons[u1]{b1} \text{JIP1-JNK}$	$b1 = 0.1/\mu\text{M s}$ $u1 = 0.1/\text{s}$
$\text{MKK7} + \text{JIP1} \xrightleftharpoons[u2]{b2} \text{JIP1-MKK7}$	$b2 = 0.1/\mu\text{M s}$ $u2 = 0.1/\text{s}$
$\text{JNK} + \text{KIF5-JIP1} \xrightleftharpoons[u3]{b3} \text{KIF5-JIP1-MKK7}$	$b3 = 0.5/\mu\text{M s}$ $u3 = 0.1/\text{s}$
$\text{MKK7} + \text{KIF5-JIP1} \xrightleftharpoons[u4]{b4} \text{KIF5-JIP1-MKK7}$	$b4 = 0.5/\mu\text{M s}$ $u4 = 0.1/\text{s}$
$\text{KIF5} + \text{JIP1} \xrightleftharpoons[u5]{b5} \text{KIF5-JIP1}$	$b5 = 0.5/\mu\text{M s}$ $u5 = 0.1/\text{s}$
$\text{KIF5} + \text{JIP1-JNK} \xrightleftharpoons[u6]{b6} \text{KIF5-JIP1-JNK}$	$b6 = 0.5/\mu\text{M s}$ $u6 = 0.1/\text{s}$
$\text{KIF5} + \text{JIP1-MKK7} \xrightleftharpoons[u7]{b7} \text{KIF5-JIP1-MKK7}$	$b7 = 0.5/\mu\text{M s}$ $u7 = 0.1/\text{s}$

[†]Values of rate constants were chosen to correspond to estimates in (Levchenko, Bruck, and Sternberg, 2000) which were in turn obtained from (Bray and Lay, 1997). In (Bray and Lay, 1997), rate constants are calculated from the strengths of individual bonds such as the number of hydrogen bonds shared by two interacting proteins.

FIGURE 3.1: Schematic of model. (a) Schematic of a cell showing assisted-transport of proteins involved in the JNK signaling cascade, namely JNK and MKK7, by KIF5 (motor) via association with JIP1 (scaffold) from the cell body towards the cell periphery such as neurite tips. KIF5-bound proteins are transported along the microtubule track as depicted by the black arrow, indicating concerted direction of movement towards neurite tips. Proteins not bound to KIF5 diffuse as illustrated by the jagged black arrow. (b) Reactions modeled in the JNK signaling cascade. JIP1 serves as the scaffold for the recruitment of JNK and MKK7. It can be transported along microtubule tracks by the motor KIF5. Red arrows denote reactions with Michaelis-Menten kinetics. Green arrows denote reactions modeled using mass action kinetics.

equations in Table 3.1 in a radial slice of the cell, i.e., a one-dimensional domain, $0 \leq x \leq L$, with the cell centre and cell periphery located at $x = 0$ and $x = L$, respectively. This one dimensional space is then discretized into discrete mesh elements each of size ΔL where the set of reactions in Table 3.1 take place at each discrete mesh element. In this article, we have used a discrete mesh of 200 elements where $L = 100\mu\text{m}$ (and therefore $\Delta L = 0.5\mu\text{m}$).

Species that are not bound to KIF5 move by diffusion only. They follow the Neumann boundary condition $\partial S/\partial x = 0$ at $x = 0$ and $x = L$ where S refers to the species in question. Species transported along the cytoskeleton, namely those that are motor protein-associated, follow the Dirichlet boundary condition $S = 0$ at $x = 0$. The motor protein KIF5, when not bound to cargo, is assumed to be immobile since KIF5 is present in a folded conformation that results in autoinhibition of the N-terminal motor domain by C-terminal tail domains in the absence of cargo (Verhey et al., 1998; Verhey and Hammond, 2009).

The initial distribution of all the species with the exception of M3/6 is assumed to follow a Gaussian distribution centered at $x = 0$. We used a standard deviation of $0.158\mu\text{m}$ for KIF5 and $0.5\mu\text{m}$ for the other species. On the other hand, M3/6 is assumed to be homogeneously dispersed throughout the domain with a uniform concentration of $1.0\mu\text{M}$. The initial distribution of proteins is listed in Table 3.2. Diffusion coefficients of the species are also listed in Table 3.2. We aimed to model the dynamics of signalling proteins as they are transported to the cell periphery in the presence of motor proteins. Ideally, at the start of the simulation, the motor proteins should all be located at the cell body. Unfortunately, a localized increase in concentration of motor proteins tend to result in computational irregularities and errors. Hence, a smaller standard deviation of $0.158\mu\text{m}$ was chosen to ensure that most of the motor proteins are still situated at the cell body while allowing efficient simulation.

We solve the set of reaction-diffusion-advection equations numerically using the Forward-Time Central-Space (FTCS) scheme for the diffusion equations and the second order Lax-Wendroff scheme for the advection equations. From our simulations, we answer the following questions. First, how does the activated

TABLE 3.2: Diffusion coefficients and initial distribution of all species modeled

Molecular Species	Initial Distribution (μM)	Diffusion Coefficient Notation	Diffusion Coefficient [⊕] ($\mu\text{m}^2/\text{s}$)
JNK	$\text{JNK}(x) = 10 \exp(-x^2/2(0.5)^2)$	D_{JNK}	10
JIP1	$\text{JIP1}(x) = (0 \text{ to } 20) \exp(-x^2/2(0.5)^2)$	D_{JIP1}	10
MKK7	$\text{MKK7}(x) = 1.6 \exp(-x^2/2(0.5)^2)$	D_{MKK7}	10
JNK*	$\text{JNK}^*(x) = 0$	D_{JNK^*}	10
M3/6	$\text{M3/6}(x) = 1.0$	$D_{\text{M3/6}}$	10
JIP1-JNK	$\text{JIP1-JNK}(x) = 0$	$D_{\text{JIP1-JNK}}$	10
JNK-MKK7	$\text{JNK-MKK7}(x) = 0$	$D_{\text{JNK-MKK7}}$	7.07
JIP1-MKK7	$\text{JIP1-MKK7}(x) = 0$	$D_{\text{JIP1-MKK7}}$	10
JIP1-JNK-MKK7	$\text{JIP1-JNK-MKK7}(x) = 0$	$D_{\text{JIP1-JNK-MKK7}}$	5.77
JNK*-M3/6	$\text{JNK-M3/6}^*(x) = 0$	$D_{\text{JNK}^*-\text{M3/6}}$	10
KIF5	$\text{KIF5}(x) = 10 \exp(-x^2/2(0.16)^2)$	-	-
KIF5-JIP1	$\text{KIF5-JIP1}(x) = 0$	-	-
KIF5-JIP1-JNK	$\text{KIF5-JIP1-JNK}(x) = 0$	-	-
KIF5-JIP1-MKK7	$\text{KIF5-JIP1-MKK7}(x) = 0$	-	-
KIF5-JIP1-JNK-MKK7	$\text{KIF5-JIP1-JNK-MKK7}(x) = 0$	-	-

[⊕] Diffusion coefficients were chosen to be similar to estimates in (Moran, Phillips, and Milo, 2010).

kinase JNK* accumulate at the periphery of the cell, $x = L$, from a source of inactivated kinase JNK initially clustered at the centre of the cell, $x = 0$. Two possible mechanisms could happen: the inactive kinase JNK diffuses around the cytosol, depending on chance encounters with the upstream kinase MKK7 to become activated. The activated kinase JNK* then also diffuses until it reaches its destination. The aforementioned mechanism would rely entirely on diffusion without any dependence on motor proteins. In the second mechanism, cytoskeletal transport via motor proteins are involved. The inactive kinase JNK, while undergoing diffusion around the cytosol, chances upon and associates with the scaffold JIP1. In some cases, the scaffold will already have associated with MKK7, and so, the kinase complexed with these scaffolds will be activated. The scaffold complex diffuses and can encounter and bind to the motor protein KIF5. The whole motor protein and cargo complex is then transported along the microtubule cytoskeleton to their destination.

Next, we want to understand the relative importance between protein diffusion and cytoskeletal transport in the arrival and accumulation of JNK* at the cell

periphery. Specifically, we are interested in how two parameters, the concentration of scaffold protein JIP1 and the speed v of motor protein KIF5, modify signaling activity. These two parameters can be expressed in dimensionless forms, f and p respectively, where f is the ratio of the initial concentration of JIP1 to the initial concentration of JNK,

$$f = \frac{\int_{x=0}^{x=L} [\text{JIP1}_{t=0}] dx}{\int_{x=0}^{x=L} [\text{JNK}_{t=0}] dx}, \quad (3.1)$$

and p the ratio of the rate of advection to the rate of diffusion,

$$p = \frac{vL}{D_{\text{JNK}}}, \quad (3.2)$$

where D_{JNK} is the diffusion coefficient of JNK. We can also view p as the inverse ratio of the time of transport by motor proteins to the time of transport by diffusion to the same distance.

We shall now proceed to quantify the transport activities more carefully. In particular, we define and make use of two metrics, namely, signaling rate and signal amplification. Signaling rate, R is defined to be the inverse of the time needed for the JNK^* to reach its maximum concentration at the cell periphery,

$$R(f, p) = \frac{1}{t_{\max}} \quad (3.3)$$

where $t_{\max}$ is the time at which maximum signaling activity is achieved at the cell periphery, $x = L$. Next, signal amplification, $A(f, p)$, is defined to be the ratio of the maximum concentration of JNK^* achieved at the cell periphery over time for a particular value of f and p to the maximum concentration of JNK^* when there are no scaffold and motor proteins present, also at the cell periphery. Signal amplification measures the extent to which signaling activity is enhanced by the combined effect of scaffolding and cytoskeletal transport,

$$A(f, P) = \frac{\max[\text{JNK}^*(f, p)_{x=L}]}{\max[\text{JNK}^*(f = 0, p = 0)_{x=L}]}. \quad (3.4)$$

3.3 Results

3.3.1 Scaffolded cytoskeletal transport can result in a higher JNK* activation than diffusion

We first solve for the purely diffusive scenario where $f = p = 0$, i.e., no scaffolds are present and no motor proteins are present (or more accurately, motor proteins are present but are stationary), and transport takes place by diffusion only. We next compare this control scenario to the scenario when both f and p are not equal to 0, i.e., when there is scaffolding and transport by the motor proteins on the cytoskeleton. Space-time kymographs of the level of JNK* concentration are shown in Figure 3.2(a) and (b) for two scenarios, respectively. In panel (a), as time progresses, JNK* moves about purely by diffusion. Compare this to the scenario in panel (b), where there are both associations to JIP1 and cytoskeletal transport by KIF5. Thus, JNK* activity moves at a constant speed towards the cell periphery. Maximum value of JNK* attained at the cell periphery is $0.0645 \mu\text{M}$ which is more than that achieved by diffusion alone ($0.0389 \mu\text{M}$). At maximum signaling activity at the cell periphery, JNK* is also observed to be localized to the periphery for motor proteins-assisted transport whereas JNK* is spread across the entire cell length for the purely diffusive case. This is supported by (Verhey et al., 2001) where localization of dual leucine zipper kinase (DLK), a member of the MLK family of kinases, is abolished when kinesin is inhibited. Furthermore, when kinesin is not inhibited, a higher concentration of DLK is observed at the neurite tip compared to the case when kinesin is inhibited (Verhey et al., 2001). Furthermore, in panel (b), JNK* attained its maximum value after 490 seconds whereas in panel (a), JNK* requires a far longer time of 3300 seconds to reach maximum value. These results suggest that scaffolded cytoskeletal transport can indeed result in a higher level of JNK* activation at the cell periphery than diffusion alone. This is supported in (Kholodenko, 2003) where the combined effect of small protein diffusion coefficients and rapid dephosphorylation leads to hampering of information transfer and it is suggested

that assembling protein kinases on a scaffold and using motor proteins to transport these signaling complexes can lead to a more efficient way of delivery.

FIGURE 3.2: Kymograph of JNK* activity. Kymograph plots of JNK* activity (red=low, yellow/white=high) for (a) $f = p = 0$ and (b) $f = 0.75, p = 2.5$. Comparison between (a) and (b) reveals that JNK that is scaffolded and transported on the cytoskeleton (case (b)) can result in delivery of JNK and activation to JNK* at the cell periphery more efficiently than relying on diffusion alone (case (a)). Maximum value of JNK* attained at the cell periphery in (b) is $0.0645 \mu\text{M}$ which is more than that achieved by diffusion alone ($0.0389 \mu\text{M}$). Also, in (b), JNK* at the cell periphery attains its maximum value at 490 seconds whereas diffusion alone requires 3300 seconds.

3.3.2 Increase in speed of cytoskeletal transport does not always lead to an increase in signaling rate

If we fix $p = 2.5$ and vary f , we see that the signaling rate increases as JIP1 concentration or f increases; see Figure 3.3(a). KIF5 motors are capable of motion only when it is associated with JIP1. When JIP1 concentration increases, more KIF5 motors are activated. An increase in activated KIF5 will lead to the delivery of more associated kinases to the cell periphery, leading to an increase in signaling rate.

Similarly, if we now fix $f = 0.5$ and vary p , we see that signaling rate increases with p . An increase in p will lead to an increase in the transport of any JNK or MKK7 bound to KIF5 via JIP1. Kinases can be delivered to the cell periphery at a shorter time at larger p values leading to improved signaling rate.

FIGURE 3.3: Signaling rate for various values of f and p . (a) Signaling rate, R , for fixed $p = 2.5$ increases with increasing f . (b) Signaling rate for fixed $f = 0.5$ increases with increasing p . (c) Signaling rate for a range of f and p . Four distinct regions can be distinguished (labeled 1 to 4) and demarcated by black dashed lines. Region 1 is defined by low values of p and Region 2 by low values of f . Signaling rate is low in Regions 1 and 2. In moderate values of f and p lies Region 3 where signaling rate is high and increases with both f and p . Region 4 lies beyond Region 3 and is characterized by low signaling rate even at high values of f and p . The blue dashed lines denote the cases illustrated in (a) and (b).

Intuitively, one would expect that an increase in the motor speed (or equivalently, p) will always result in an improvement in the signaling rate. However, as we show in Figure 3.3(c), such is not the case. The signaling rate is not observed to be monotonically increasing with p but instead dependent on both f and p . In fact, we can identify four distinct regions as denoted in Figure 3.3(c):

1. At low values of p (meaning transport by motor proteins is slower than transport by diffusion, and denoted by Region 1), the signaling rate is low regardless of the value of f or scaffold concentration. This is attributed to the slow movement of KIF5. Slow movement of KIF5 will lead to the slow delivery of associated kinases to the cell periphery causing the signaling rate to be low.
2. At low values of f (no JIP1 scaffold proteins, and denoted by Region 2), JNK and MKK7 are not transported by KIF5 in the absence of JIP1 since JIP1 scaffolds are required as a linker to bind JNK and MKK7 to KIF5. Thus, transport of JNK and MKK7 to the cell periphery will depend only on diffusion, resulting in a low signaling rate.

3. For moderate values of f and p (denoted by Region 3), the signaling rate increases with increases in both f and p . Due to the inability of KIF5 to move unless associated with cargo, increasing concentration of JIP1 will lead to an increase in cargoes capable of activating motion in KIF5, thus improving signaling rate. Increasing p also improves signaling rate since KIF5 motors can transport its associated JNK and MKK7 at a faster speed to the cell periphery. The two cases of fixing $p = 2.5$ and varying p and fixing $f = 0.5$ and varying p discussed above both lie within Region 3.
4. For high values of both f and p (denoted by Region 4), the signaling rate actually decreases to a low value. This can be explained as follows. When the cell contains a large amount of JIP1 scaffolds, most of them will predominantly be empty instead of being bound to JNK or MKK7 since there are insufficient kinases to bind to all the scaffolds. Majority of the cargoes loaded and transported by KIF5 would therefore be empty scaffolds. With a large majority of KIF5 bound to empty scaffolds, transport of JNK and MKK7 would have to depend on diffusion. Even so, a small percentage of these scaffolds would be loaded with one of the kinases but scaffolds loaded with both JNK and MKK7 would be rare. In such a situation, formation of JNK* would again, predominantly be contributed from free JNK and free MKK7 since JIP1-JNK and JIP1-MKK7 cannot react. Since free JNK and free MKK7 move via diffusion, formation of JNK* is thus determined by the speed of diffusion leading to poor signaling rate.

An interesting feature here is that the boundary demarcating high signaling rates (Region 3) from low signaling rates (Region 4) depends on both f and p . This would mean that for the cell to achieve high signaling rate at high speeds, low f is required, and, vice versa, a low value of p is needed to attain high signaling rate at high values of f . At low f , JIP1 would predominantly be in the form complexed with its kinases, either JNK, MKK7 or both. Thus, KIF5 will associate with JNK and MKK7 at low f and can transport these kinases towards the cell periphery even at high p . At high f , KIF5 would largely be associated with empty

JIP1 without JNK and MKK7. In such a situation, if speed of cytoskeletal transport is faster than the speed of binding of JNK and MKK7 to KIF5-JIP1, JNK and MKK7 kinases would not be bound to KIF5 and have to rely on diffusion to reach the cell periphery. Thus in order to achieve high signaling rate at large f , low p is necessary. In summary, Region 3 is the region where cytoskeletal transport is able to deliver kinases to the cell periphery and Region 4 is the region where cytoskeletal transport, though present, is ineffective in transporting kinases and kinases move to the cell periphery by diffusion. A sharp change in signaling rate occurs between Region 3 and Region 4. This is because in Region 3, rate is governed by speed of cytoskeletal transport while in region 4, signalling rate is dependent upon speed of diffusion as motor proteins are either moving too quickly for kinase binding, or motor proteins are transporting empty or single-kinase scaffolds which cannot convert JNK to JNK*. As a result, a sharp change exists since speed of cytoskeletal transport is a lot faster than speed of diffusion. This change is illustrated in Figure 3.4. The boundary separating Regions 3 and 4 can be adjusted by modifying the strength of binding of free kinases with KIF5-JIP1. Indeed, the boundary between Region 3 and Region 4 is shifted upwards in the presence of stronger binding. (Data not shown.) Increasing binding strength of JNK and MKK7 to KIF5-JIP1 thus serves to increase association of kinases to KIF5 allowing for high signaling rates at fast cytoskeletal transport speed.

3.3.3 An optimal scaffold protein concentration and optimal motor speed exist for which signal amplification is maximal.

Next, we look at the signal amplification, $A(f, p)$, for different values of p and f . If we fix $p = 2.5$ and vary f , we see that the signal amplification $A > 1$ when $f > 0$; see Figure 3.5(a). As the concentration of JIP1 or f increases, signal amplification increases. However, there exists a maximum for signal amplification A at $f = 0.75$. Beyond $f = 0.75$, if the concentration of JIP1 is increased further, signal amplification decreases. In this case, continued increase in scaffolds result in dilution of kinases lowering signaling activity. Thus, there exists an optimal

FIGURE 3.4: Sharp changes in signalling rate between region 3 and region 4. (a) Signaling rate, R , for fixed $p = 7.0$ experience a sharp change as f increases. (b) Signaling rate for fixed $f = 1.0$ experience a sharp change as p increases.

scaffold concentration where amplification of signaling activity is maximal. This observation, when there is cytoskeletal transport, $p > 0$, is consistent with the stationary case obtained by previous authors (Levchenko, Bruck, and Sternberg, 2000; Chapman and Asthagiri, 2009). We have now demonstrated that this result is still true even in the presence of cytoskeletal transport. Similar profiles are observed for other values of p in Figure 3.5(b).

Similarly, if we now fix $f = 0.5$ and vary p , we see that there is signal amplification, $A > 1$, when $p > 0$. This amplification increases with increasing p ; see Figure 3.5(b). At small values of p , an increase in p will lead to an increase in the transport of any JNK or MKK7 bound to KIF5 via JIP1. Bound kinases can be delivered to the cell periphery in a shorter time thus less time is available for dephosphorylation events which inactivate JNK*. A maximum value of signal amplification occurs when almost all the kinases are scaffold-bound and the corresponding complexes are attached to the motor proteins moving towards the cell periphery. As p is increased further, signal amplification decreases. In this case, only a few JNK and MMK7 associated scaffold complexes will be actively transported since the speed of translocation of the motor proteins is faster than that of kinase/scaffold-motor binding. Thus, there exists an optimal cytoskeletal transport speed where amplification of signaling activity is maximal.

Similar profiles are observed for other values of f and p as observed in Figure

FIGURE 3.5: Signal amplification for various values of f and p . (a) Signal amplification, A , for fixed $p = 2.5$ and varying f . (b) Signal amplification for fixed $f = 0.5$ and varying p . In both cases, there exists a maximum value of A and hence an optimal value of f and p to attain this maximum. (c) Signal amplification for a range of f and p . Highest value of signal amplification was attained at moderate levels of f and p . Four distinct regions can be identified (labeled 1 to 4) and demarcated by black dashed lines. Region 1 is defined by low values of p and Region 2 by low values of f . Signal amplification is low in Regions 1 and 2. In moderate values of f and p lie Region 3 where signal amplification is increased and exhibits a biphasic behaviour in both f and p . Region 4 lies beyond Region 3 and is characterized by low signal amplification even at high values of f and p . The blue dashed lines denotes the cases illustrate in (a) and (b).

3.5(c). Likewise for the signaling rate plot in Figure 3.3(c), four distinct regions can be distinguished from Figure 3.5(c). Low amplification is observed in Region 1 and Region 2 defined by low values of p and low values of f respectively. Signal amplification increases and displays a biphasic behavior with respect to f and p in Region 3 at moderate values of f and p . The biphasic behaviour can be observed in 3.5(a) and (b) in which signal amplification increases initially as f and p increases, but after the maximum signal amplification is attained, further increase in f and p leads to a reduction of signal amplification. Drop in signal amplification at high f is due to dilution of kinases since most kinases are located singly on the scaffold and effective conversion of JNK to JNK* cannot occur unless both JNK and MKK7 are on the scaffolds. Reduction in signal amplification at high p is due to speed of motor proteins being faster than that of kinase/scaffold-motor binding. Lastly, Region 4 lies beyond Region 3 at high f and p . Signal amplification is low within Region 4. A smooth transition occurs

between Region 3 and Region 4. At high f and p values, amount of kinases carried by KIF5 decreases with increase in f and p . Consequently, amount of kinases that reaches the cell periphery by diffusion increases as f and p increases. Thus amplification changes gradually from Region 3 to Region 4 as observed in Figure 3.5(a) and (b). This is because drop in kinase delivery by cytoskeletal transport is compensated by slow increases of kinase delivery from diffusion as f and p increase. This is contrasted with Figure 3.4 where signalling rate increases sharp changes between Region 3 and Region 4.

3.3.4 Optimum scaffold protein concentration and optimal cytoskeletal transport speed depend on signaling parameters

We seek to understand how the values of scaffold concentration f and motor speed p which gives optimal signaling rate and signal amplification depend on the state of the cell.

Increasing concentration of M3/6 increases the value of f necessary for maximum signal amplification as shown in Figure 3.6(a). Signaling pathways are often inactivated by enzymes that reverse the activation state and/or induce the degradation of signaling components. Scaffolds have been proposed to prevent activated signaling molecules from inactivation and/or degradation. Mathematical modeling has shown that kinases in a cascade without scaffolds have a higher probability of being dephosphorylated by phosphatases before they are even able to phosphorylate downstream targets (Hirokawa, Niwa, and Tanaka, 2010). Therefore, in the presence of higher concentration of M3/6, more JIP1 scaffolds are needed to sequester JNK and MKK7 to increase the incidence of the forward reaction leading to a higher value of optimum f required for signal amplification.

A higher concentration of KIF5 motor protein increases the value of p for maximum signal amplification as shown in Figure 3.6(b). Since KIF5 can only be activated when it is cargo-bound, when more KIF5 is present, more JIP1 and kinases complexed with JIP1 can bind to KIF5 prior to KIF5 movement along the cytoskeleton. Cytoskeletal transport can thus take place at a higher speed

since more kinases are being bound to KIF5 at a higher concentration of KIF5. The following example serves to better illustrate the above statement. When low amounts of KIF5 are present, it is essential that KIF5 is complexed with JIP1 as well as the kinases before it translocates along the cytoskeleton. Thus, to ensure that there is sufficient time for binding of JIP1 as well as the kinases to KIF5, a low motor protein speed is essential. However, when large amounts of KIF5 are present, the equilibrium will be shifted such that most of the KIF5 would be already complexed to JIP1 as well as the kinases. Since more kinases are loaded onto KIF5 at high levels of KIF5, less time is necessary for binding to occur and cytoskeletal transport can occur at a higher speed. Unfortunately, crowding may occur when excessive motor proteins are present, since binding sites on the microtubules are finite and limited. Unfortunately, in our simulations, we did not take into account the effect of the number of microtubules and assume it to be in such abundance that they need not be considered. A future project which includes the effect of limited microtubules may thus shed more light on whether higher concentrations of KIF5 is effective and at what regime does this increase in effectiveness gets abrogated due to crowding.

Next, we look at how JNK concentration modifies the values of f and p to yield optimal signaling. An increase in the amount of JNK implies that a higher concentration of scaffolds can be present before dilution of kinases occur leading to an increase in optimum f as seen in Figure 3.6(c). Unlike optimum f , optimum p decreases as concentration of JNK increases as observed in Figure 3.6(d). At higher JNK concentration, cytoskeletal transport speed needs to be reduced to ensure that more JNK is bound onto KIF5 before KIF5 translocate along the cytoskeleton.

Thus, one can foresee a scenario where the cell upregulates JIP1 scaffolds and KIF5 motors when JNK concentration is increased at the cell body. Increasing the amount of JIP1 scaffolds serves to increase amplification of JNK* at the cell periphery while increasing KIF5 serves to increase the optimal speed of transport of associated kinases for faster delivery. JIP1 is observed to be upregulated with an increase in phosphorylation of JNK when GLUT1 (glucose transporter1)

FIGURE 3.6: Values of f and p for which signal amplification is optimum depend on signaling parameters. The value of f for which signal amplification is optimum increases with (a) increasing M3/6 concentration, and (c) increasing JNK concentration. The value of p for which signal amplification is optimum increases with (b) increasing KIF5 concentration. (d) However, this value of p decreases with increasing JNK concentration.

is overexpressed (Zhou et al., 2005). Genetic experiments performed in *C. elegans* also suggest that axonal transport depending on KIF5 is upregulated by the JNK pathway (Byrd et al., 2001; Sakamoto et al., 2005; Hirokawa et al., 2009; Horiuchi et al., 2007). Thus, it may be plausible that the JNK pathway may indeed upregulate both JIP1 and KIF5. On the other hand, there are reports that suggest that KIF5 can be phosphorylated by JNK which, upon phosphorylation, has a lower binding affinity to microtubules (Morfini et al., 2006; Stagi et al., 2006). This may be the root cause in spinal and bulbar muscular atrophy where JNK

has been found to be abnormally activated leading to inhibition of fast axonal transport (Morfini et al., 2006). Thus, more work remains to be done to determine how the JNK pathway interacts with its binding partners such as JIP1 and KIF5.

3.4 Discussion

The combination of scaffolding by JIP1 and transport by motor protein KIF5 can be summarized as follows. At low JIP1 scaffold concentration, few JNK are recruited to JIP1 for subsequent phosphorylation and transport by KIF5. Thus, majority of the JNK* reach the axon terminals by free diffusion, resulting in low signaling rate and signal amplification. However, at high JIP1 concentrations, JNK and MKK7 are spread out too widely amongst the scaffold proteins, leading to ineffective phosphorylation and a corresponding suppression of phosphorylation activity in the entire system. Active transport of JNK* still occurs, although scaffold-assisted phosphorylation is now suppressed.

On the other hand, at low KIF5 speed, both forms of JNK (activated or unactivated) and MKK7 diffuse freely along the axons, such that they are far beyond encounter distance from KIF5 which are concentrated near the cell body. Under such circumstances, signaling proceeds via free diffusion coupled with limited active transport, resulting in low signaling rate and signal amplification. At high KIF5 speed, however, motor proteins translocate along the cytoskeleton before the kinases can bind onto the motor. Here, we witness the other extreme case whereby free diffusion coupled with limited active transport prevails.

Finally, an ideal scenario should comprise an optimum JIP1 concentration to concentrate both JNK and MKK7 effectively and an optimum KIF5 cytoskeletal transport speed, such that most of the corresponding scaffold complexes are recruited by the motor proteins and actively transported along the axons. Such a scenario is observed at the maxima region in the phase diagrams of signal amplification and lies within the high signaling rate region of the signaling rate plot.

This is depicted in Region 3 of Figure 3.7(a) and Figure 3.7(b). Region 3 is characterized by moderate scaffold concentration and moderate motor transport speed. Highest JNK* signaling rate and largest JNK* signal amplification is contained within Region 3. In this region, the kinases are scaffold-bound and the corresponding complexes are attached to the motor proteins moving towards the cell periphery. In Region 1 defined by low p , the kinases may be bound to scaffold but the speed of KIF5 is too slow for efficient transport. In Region 2, scaffolds are low in abundance since f is small. Transport of kinases to cell periphery relies on slow diffusion since the JIP1 scaffolds are absent to serve as linkers between kinases and KIF5. Region 4 is the region at high f or high p . At high f , most of the scaffolds are empty and motor proteins will be transporting empty scaffolds instead of scaffolds loaded with kinases. At high p , KIF5 is moving too quickly and there is insufficient time for kinase binding. In both cases, kinases diffuse to the cell periphery instead of being transported along the cytoskeleton.

Even though our model is constructed specifically for the analysis of the JNK signaling cascade, we believe that our model is generic enough to be applied to other signaling pathways that also make use of scaffold proteins and cytoskeletal transport. Features extracted in our model such as the biphasic behavior in scaffold concentration and cytoskeletal transport speed should be universal features in other motor proteins-assisted scaffolded signaling complexes. In recent years, an increasing number of scaffold proteins that associate with motor proteins have been uncovered. A yeast-two-hybrid screen to identify proteins that interact with the KIF1C C-terminal domain identified proteins of the 14-3-3 family as binding partners (Dorner et al., 1999). The 14-3-3 family of proteins serves as scaffolds for a variety of signaling proteins such as phosphatases, kinases and transmembrane receptors. Costal2 (Cos2), a scaffold protein of the Hedgehog signal transduction pathway which recruits other signaling components, has also been reported to exhibit motility, thus functioning as a kinesin-like protein (Farzan et al., 2008). Cos2 is required for phosphorylation of Cubitus interruptus, Ci (Zhang et al., 2005). Cos2 immunocomplexes contain protein kinase A (PKA), glycogen synthase kinase 3 (GSK3) and casein kinase I (CKI) (Zhang et al., 2005). Amyloid

FIGURE 3.7: Phase diagram to summarize the possible strategies utilized by the cell. (a) In the first strategy denoted in Region 1 where p is low, motor proteins are moving too slowly for efficient transport. Scaffold proteins are few in the second strategy (Region 2, low f) and thus proteins are unable to hitch onto motor proteins and have to rely on slow diffusion to reach the cell periphery. The optimum strategy is the third strategy denoted by Region 3 and involves moderate scaffold concentration and moderate motor speed. In the last strategy denoted by Region 4, proteins are not bound to motor proteins either because motor proteins are moving too quickly for binding to occur or scaffolds are in such abundance that binding between proteins and motors will preferentially be involving empty scaffolds. (b) Schematic illustrating how the proteins are moving in each of the four strategies.

precursor protein (APP) has also been reported to bind to JIP proteins where the phosphorylation of APP by JNK was enhanced by the presence of the scaffold JIP *in vitro* and in cultured cells (Inomata et al., 2003; Taru et al., 2002; Matsuda et al., 2001). These findings support the notion that preassembled signal transduction cascades or transducisome are recruited to downstream motors in order to drive the regulated movement of attached cargo (Klopfenstein, Vale, and Rogers, 2000; Schnapp, 2003; Burack and Shaw, 2000; Tsunoda et al., 1997). Thus, the model developed in this article can be used to study various signaling cascades and can potentially be used for in-depth analysis of other signaling complexes that remains to be discovered in the future.

The role of JIP1 in modulating the JNK pathway has been well studied. JIP1

was originally assumed to be an inhibitor of JNK. JIP1 has been shown to suppress signal transduction of the JNK pathway by competing with substrates that interact with JNK. JIP1 overexpression has also been proposed to be cytoplasmic anchor for JNK as overexpression of JIP1 caused retention of JNK in the cytoplasm (Dickens et al., 1997). Recent discovery however reveals JIP1's role as a crucial scaffold protein for the MAP kinase cascade (Whitmarsh et al., 1998). In this article, we have elucidated another role of JIP1 in regulating the dynamics of the JNK pathway. By binding both motor proteins and members of the JNK signaling cascade, JIP1 serves to enable cytoskeletal-assisted transport of JNK* allowing for greater signaling rate and signal amplification.

Understanding cytoskeletal-assisted protein transport is important, because axonal and cell body accumulation of organelles and proteins is a histological feature in many human neurodegenerative disease. Examples include polyQ aggregates in Huntington disease, synuclein in Lewy bodies found in Parkinson's disease, amyloid beta and tau protein deposits in Alzheimers disease. These observations suggest that defects in axonal transport may contribute to neuronal inclusions and plaques (De Vos et al., 2008). However, current research on neurodegenerative diseases is primarily focused on axonal transport defects, such as mutation of motor proteins, destabilization of microtubules, disruption of motor-cargo protein interactions and mitochondria dysfunction (leading to insufficient ATP supply for motor proteins). There has been little effort made to quantify axonal transport performance as a function of the intrinsic properties of the axon transport machinery components. Previous studies exploring motor proteins in transport investigated its role in vesicle transport (Klann, Koeppl, and Reuss, 2012). Motor proteins were found to improve the recycling of SNARE protein and to result in cell polarization. Advances regarding motor proteins were also made in terms of its contribution to density heterogeneity where it was found that the transport of motor protein can lead to a spontaneous distribution of matter and that these heterogeneities can be controlled via various factors such as the topology of the cytoskeletal network (Neri, Kern, and Parmeggiani, 2013b; Neri,

Kern, and Parmeggiani, 2013a). In this article, we have shown that axonal transport performance changes with altered transport component concentrations and transport speeds. Such findings are important because it has been shown that differential activation time of JNK results in different induction of gene expression. Cell survival is promoted should JNK activation be early and transient. Prolonged JNK activation however leads to apoptosis (Ventura et al., 2006). Regulation of JNK temporal dynamics is thus critical to elicit an appropriate cellular response.

Finally, we discuss how the two parameters f and p used in this article can be varied experimentally. To vary f experimentally, JIP1 scaffolds can be up or down-regulated. While it is not easy to modify motor speed, we note that we only need to vary motor speed with respect to diffusion. Thus, an easier way to vary p is to vary protein diffusivity by introducing dextran beads into the cytosol. Acetylation of microtubules could be another option to vary p since it has been shown that hyper-acetylation of all microtubules in the central nervous system cell line Cath.a-differentiated (CAD) results in targeting of JIP1 to all neurite tips, nullifying the usual selectivity of its transport resulting in greater directed motion (Bulinski, 2007). Tau protein implicated in Alzheimer's disease can also be introduced into the cell to inhibit kinesin transport since tau impedes antero-grade transport (Ebneth et al., 1998; Trinczek et al., 1999; Stamer et al., 2002). This may be due to tau's effect on decreasing the attachment ability of kinesin to microtubules (Seitz et al., 2002; Dixit et al., 2008) and/or decreasing the traveling distance of kinesin (Vershinin et al., 2007).

3.5 Conclusion

We have studied computationally the various strategies that JNK may be transported from the cell body to the cell periphery. We have shown that binding to a scaffold JIP1 and then having the whole protein-scaffold cargo being transported by motor proteins KIF5 along the cytoskeleton is superior to relying on transport by protein diffusion, but only in a limited range of JIP1 concentration and KIF5

motor speed. We defined two metrics to quantify transport, namely signaling rate and signal amplification. It is only possible to achieve maximum amplification at a specific range of JIP1 concentration and KIF5 motor speed. These findings are summarized in Figure 3.7 which highlights the necessity of an optimum speed and scaffold concentration to achieve maximum signaling effectiveness.

Chapter 4

An enhanced Delta-Notch lateral inhibition model incorporating intracellular Notch heterogeneity and tension-dependent rate of Delta-Notch binding to explain cellular patterning during sprouting angiogenesis

This chapter is co-written with graduate student Muhammad Bakhait Rahmat and has been submitted to Scientific Reports. Specifically, I carried out the computational modelling, experimental analysis and writing of all of the manuscript sections excluding the experimental protocols. Muhammad Bakhait Rahmat conducted the experimental validation and wrote the experimental methods. The results in this chapter will not be a part of Muhammad Bakhait Rahmat's thesis.

4.1 Introduction

Angiogenesis is the physiological process that describes the formation of new blood vessels from preexisting blood vasculature (Birbrair et al., 2014). This definition of angiogenesis distinguishes it from vasculogenesis which is the de novo creation of blood vessels from precursor angioblast cells (Patan, 2004). Proper angiogenesis is vital during growth and development as well as in wound healing (Breier, 2000; Tonnesen, Feng, and Clark, 2000a). Diseases due to dysregulation of angiogenesis include diabetes, amyotrophic lateral sclerosis, Crohn disease and many more (Carmeliet, 2003; Kota et al., 2012). Even though angiogenesis plays an imperative role in sustaining and maintaining life, it is also critical in the advancement of tumour malignancy leading to the development of angiogenic inhibitors for cancer therapeutics (Vasudev and Reynolds, 2014). Such diverse and essential roles angiogenesis plays in bodily processes necessitates its deeper understanding. In this chapter, we seek to study angiogenic patterning in the light of two commonly overlooked mechanisms: intracellular Notch heterogeneity and tension-dependent rate of Delta-Notch binding. We discover that by considering the above two mechanisms, we are able to recapitulate angiogenic patterning observed in vivo and in vitro which is unachievable with classical angiogenic models alone.

During cancer development as the tumour matures in size, the cells deep within the tumour become farther from the blood vessels. These tumour cells thus receive less of the oxygen and nutrients it requires to survive. Under oxygen deprivation and nutrient starvation, the tumour cells secrete angiogenic factors that stimulate the growth of blood vessels into the tumour (Marjon, Bobrovnikova-Marjon, and Abcouwer, 2004). This form of angiogenesis in which new blood vessels form from extant endothelial cells is termed as sprouting angiogenesis (Hinsbergh and Koolwijk, 2008). New growth of the blood vasculature is important for the continual proliferation of tumour cells and metastasis by providing cancer cells with a sufficient reservoir of oxygen and nutrients and allowing for removal of their waste byproducts (Nishida et al., 2006).

As the endothelial cells of the blood vessel grow towards the angiogenic source, not all cells adopt the same cellular phenotype. Two distinct phenotypes are undertaken by the endothelial cells in the nascent blood vessel sprout, namely the tip cell phenotype and the stalk cell phenotype (Blanco and Gerhardt, 2013; Geudens and Gerhardt, 2011). Tip cells are defined by their long fingerlike protrusions termed as filopodia which bring about motile behaviour. These cells migrate towards the angiogenic source upon stimulation by chemotactic factors (Gerhardt et al., 2003). The second type of cells known as stalk cells trail behind the tip cells in the growing sprout. The stalk cells support the growth of the new vessel by virtue of their proliferative capacity. In addition, stalk cells ensure stability and integrity of the young sprout by forming adherent and tight junctions (Blanco and Gerhardt, 2013).

How an endothelial cell chooses to become the tip cell or the stalk cell is through the process termed as lateral inhibition (Geudens and Gerhardt, 2011; De Smet et al., 2009). In essence, lateral inhibition prevents the neighbours of a tip cell from taking on the same fate as itself. One of the more commonly known angiogenic factor is the vascular endothelial growth factor, VEGF (Hoebe et al., 2004). VEGF binds to VEGF-receptor (VEGFR) on the surfaces of endothelial cells thereby activating VEGFR. Activated VEGFR go on to increase expression of Delta-like ligand 4, here and so forth termed as Delta. Delta is a transmembrane ligand which binds to the transmembrane receptor, Notch of its neighbouring cell. Upon ligand binding, Notch of the neighbouring cell becomes activated. This triggers a cascade of signalling activity ultimately culminating in the downregulation of VEGFR and Delta within the neighbouring cell (Thurston and Kitajewski, 2008; Herbert and Stainier, 2011; Roca and Adams, 2007). The aforementioned signalling activities are depicted in Fig 4.1. As a result, a high Delta cell which has low Notch activity will have a low Delta, high Notch cell as its neighbour. Tip cells are characterized by a high Delta, low Notch expression while stalk cells are defined by a low Delta, high Notch expression. Lateral inhibition thus prevents the neighbours of a tip cell from attaining the same tip cell fate. Such regulation is of marked importance. If all cells take on the tip cell

fate, the blood vessel will disintegrate and fall apart. On the other hand, if all cells become stalk cells, the blood vessel can only grow in diameter and not in length (Gerhardt, 2008). Lateral inhibition thus tunes the number of tip cells and stalk cells for optimal growth and cohesion of the blood vessel.

FIGURE 4.1: Schematic of Delta-Notch Lateral Inhibition. Tumour cells secrete angiogenic factors such as vascular endothelial growth factor (VEGF). VEGF binds to VEGF-receptor (VEGFR) on the surfaces of endothelial cells leading to the activation of VEGFR. Activated VEGFR causes upregulation of transmembrane ligand, Delta. Delta ligand binds to the transmembrane receptor, Notch of its neighbouring cell. Upon Delta ligand binding, Notch of the neighbouring cell becomes activated and inhibits VEGFR and Delta expression.

Classical lateral inhibition models predict a salt-and-pepper pattern amongst the endothelial cells where a pair of tip cells are separated exactly by one stalk cell as illustrated in Fig 4.2A (Carrier et al., 2012; Collier et al., 1996). However, other forms of angiogenic patterns where tip cells are separated by more than one stalk cell have been observed both in vitro and in vivo. For example, the existence of two stalk cells and three stalk cells spaced between tip cells can be seen from whole mounts of mouse retinas (Toro et al., 2010; Aspalter et al.,

2015). Several lateral inhibition models incorporating only Delta and Notch has been proposed to explain such patterns. Most of these models serve to increase the number of cellular states possible such that the cells are no longer limited to a high Delta, low Notch state and a low Delta high Notch state. To accomplish this, Collier et al. proposed a two-dimensional grid system where each hexagonal cell is connected to six of its neighbours (Collier et al., 1996). The increased number of cell-cell contacts allow for creation of cells with moderate Delta and Notch levels. These moderate cells allow for a larger number of stalk cells spaced between tip cells (Collier et al., 1996). Similarly, Cohen et al. proposed a modified lateral inhibition model where each grid cell contacts both its direct neighbours as well as their neighbours' neighbours to account for bristle spacing observed within the *Drosophila* dorsal thorax. In the latter model, the increase in cell contacts are brought about by the presence of dynamic filopodia (Cohen et al., 2010). Lastly, Chen et al. invoked a mechanism involving a nearest-neighbour Notch gradient to reproduce patterning of epidermal sensory neurons within *Ciona intestinalis* grid lattices (Chen et al., 2014). Even though these models can resolve the patterns observed in their respective cell types, they have their shortcomings in explaining tip-stalk patterns observed in sprouting angiogenesis. Angiogenic sprouts are typically one-dimensional in nature which defies the two-dimensional system of Collier et al.'s. Cohen's model requires interaction between the filopodia of the tip cell and the stalk cell. This is contrasted during angiogenesis where the filopodia of tip cells serves to guide the angiogenic sprout towards the direction of migration (Gerhardt et al., 2003) and are typically not observed to interact with the lagging stalk cells. Lastly, the Notch gradient term in Chen et al. has so far not being observed experimentally.

Most models that seek to represent Delta-Notch signalling makes the inherent assumption that Delta and Notch levels are homogeneous within the cell. Notch being a large transmembrane receptor, has been shown previously to exhibit differential levels within the cell, i.e. found in heterogeneous concentrations at different spatial locations within a cell (Lai, 2004). Hence, we wonder if inclusion of differential localization of Notch into the classical lateral inhibition model

FIGURE 4.2: Lateral Inhibition Models. (A) Classical Delta-Notch lateral inhibition models describe the following reactions: Delta of one cell binds to Notch of the neighbouring cell, and Notch inhibits Delta expression within the same cell. Computational models of classical Delta-Notch lateral inhibition yield the salt-and-pepper pattern where tip cells are separated exactly by one stalk cell. (B) Schematic of lateral inhibition model with intracellular Notch heterogeneity. (C) Schematic of lateral inhibition model with intracellular Notch heterogeneity and tension-dependent rate of Delta-Notch binding. In lateral inhibition model with intracellular Notch heterogeneity and tension-dependent rate of Delta-Notch binding, rate constants are a function of the adherent Delta-Notch pairs.

will serve to reproduce the various angiogenic patterns observed. We discover that by considering intracellular Notch heterogeneity, more cellular states can be attained. With these additional states, we are able to recreate a limited set of angiogenic patterns where tip cells are separated by more than one stalk cell. Certain types of patterning are however never observed such as the case where three stalk cells are found between a pair of tip cells. This is due to inherent symmetry within the system of cells that invariably reduces a three-stalk cell spacing pattern to a one-stalk cell spacing pattern (will be discussed in detail in Results). As such, additional mechanisms that serve to break the symmetry within an array of cells are needed to reproduce the repertoire of tip-stalk patterning during angiogenesis.

Mechanical forces have been shown to modulate responses between ligand-receptor binding (Bose et al., 2010; Spillmann, Lomakina, and Waugh, 2004). The importance of forces in tuning adhesion between neutrophils and ligand-presenting endothelial cell is a significant primal discovery in the understanding of leukocyte-endothelial interaction (Spillmann, Lomakina, and Waugh, 2004). Bond formation rates were found to increase with increasing contact stress. Increasing impingement forces situated at the cell front may thus be a crucial regulator of cell motility transitions from rolling, to strong integrin adhesions (Spillmann, Lomakina, and Waugh, 2004). Similar force modulation has also been observed in Delta-Notch signalling where the rate of ligand-receptor binding is dependent on the adhesive strength between the cells (Ahimou et al., 2004). Intercellular adhesion or tension impacts signalling by modifying the binding reaction between ligand and receptor. It is currently unknown how adhesion or tension between cells regulate Delta-Notch signalling and therefore tip-stalk patterning. We seek to examine if tension-dependent rate of Delta-Notch binding can be the symmetry-breaking mechanism that is necessary on top of intracellular Notch heterogeneity to recreate tip-stalk patterns seen during angiogenesis.

In this chapter, we aim to elucidate the role of intracellular Notch heterogeneity and tension-dependent rate of Delta-Notch binding, two commonly overlooked mechanisms, on Delta-Notch signalling. These mechanisms would be

pervasive in all cells expressing Delta and Notch. Unfortunately, it is currently unknown how the mechanisms affect Delta's and Notch's regulation. We investigate if addition of intracellular Notch heterogeneity and tension-dependent rate of Delta-Notch binding into the classical lateral inhibition model will serve to explain and recapitulate the various forms of tip-stalk patterning observed during sprouting angiogenesis.

The chapter will be structured as follows. The next section, Methods, details the experimental procedures as well as the description of modifications made to the classical lateral inhibition model. In the subsequent section, Results, we present our findings of the modified model with intracellular Notch heterogeneity, modified model with tension-dependent rate of Delta-Notch binding as well as enhanced model with intracellular Notch heterogeneity and tension-dependent rate of Delta-Notch binding. We argue that both conditions of intracellular Notch heterogeneity and tension-dependent rate of Delta-Notch binding are required to reproduce the diverse forms of patterning observed experimentally. We also present the experimental results where we show the existence of the intermediate cell type, a crucial prediction of our enhanced model. Last but not least, we conclude by discussing the possible significant impacts of our findings.

4.2 Materials and methods

4.2.1 Experimental procedures

HUVEC cells (passage 5-7, ATCC®) are seeded on coverslips coated with matrigel in 60mm dish for 4 hours. Upon formation of capillary tubes, cells are fixed with paraformaldehyde for 15 minutes and wash thrice with phosphate-buffered saline (PBS). Cells are then blocked with blocking buffer (1x PBS/5%, Fetal bovine serum/0.3%, Triton-X) for 1 hour and incubated with CD34 primary antibody (1:100, [EP373Y] (ab81289), Abcam®), DAPI and rhodamine overnight. DAPI stains for the cell nucleus while rhodamine stains for the actin cytoskeleton. Cells are maintained in Endothelial Growth Media, EGM-2 Bulletkit (CC-3162 and CC-4176, Lonza®) throughout the experiment. Images are taken at

20 $\times$ and 40 $\times$ magnification with an Axio observer Z1.

Image quantification

Each cell is segmented and ImageJ is used to quantify the fluorescence intensity. Cell fluorescence intensities are normalized such that one corresponds to the brightest cell and zero corresponds to the dimmest cell in each image. The normalized cell intensities are then combined across all images and compared.

4.2.2 Detailed explanation of the enhanced Delta-Notch lateral inhibition model

In this section, we first describe the classical lateral inhibition model before introducing the modifications. This section will be organized as follows:

1. Classical Lateral Inhibition Model
2. Lateral Inhibition Model with Intracellular Notch Heterogeneity
3. Lateral Inhibition Model with Intracellular Notch Heterogeneity and Tension-Dependent Rate of Delta-Notch Binding

Assumptions and simplifications of each modification are listed in their respective subsection. We end off the section with details on how the models are resolved computationally. In all of the equations listed below, we consider a linear one-dimensional array of cells with periodic boundary conditions. Periodic boundary conditions are used so that a long array of cells can be modelled with a tractable number of equations.

Classical lateral inhibition model

Classical Delta-Notch lateral inhibition models take the following form: 1) Delta ligand expression is inhibited by high levels of intracellular activated Notch, and 2) Notch receptor is activated after binding of Delta expressed on neighbouring cells. At the same time, both Delta and Notch proteins undergo first order decay (Collier et al., 1996; Sprinzak et al., 2010; Koizumi, Iwasa, and Hirashima,

2012). This feedback loop amongst neighbouring cells is embodied in the following system of equations for a linear periodic one-dimensional array of cells up to cell N , $(1...j...N)$:

$$\frac{d\tilde{D}_j}{dt} = -k_D\tilde{D}_j + \frac{B_0}{1 + (\frac{\tilde{N}_j}{k})^2} \quad (4.1)$$

$$\frac{d\tilde{N}_j}{dt} = -k_N\tilde{N}_j + k_F \frac{(\tilde{D}_{j-1} + \tilde{D}_{j+1})}{2} (\tilde{N}_0 - \tilde{N}_j) \quad (4.2)$$

The first equation, Eq(4.1) describes the rate of change of Delta, $\tilde{D}$, in cell j at any time t . The rate of change of Delta is a combination of effects arising from Delta's decay, contributed by the first term on the right hand side of the equation, and inhibition from activated Notch contributed by the second term. k_D represents the decay coefficient for Delta, B_0 denotes the maximum expression rate of Delta and k refers to Delta's inhibitory coefficient which is the concentration of activated Notch necessary to result in half maximal Delta expression. Since the inhibitory effect of activated Notch on Delta's expression has been shown to follow the Hill dynamics, the Hill equation is used to model the interaction between activated Notch and Delta with a Hill coefficient of 2 (Giurumescu, Sternberg, and Asthagiri, 2006). Separately, another group experimentally validated that activation of Notch by Delta follows a Hill equation with a Hill coefficient of 2 after rounding to the nearest whole number (Sprinzak et al., 2010).

Similarly, the second equation, Eq(4.2) describes the rate of change of activated Notch, $\tilde{N}$ in cell j which is a summation of effects brought about by decay (first term on the right hand side) as well as activation by Delta from neighbouring cells, $\tilde{D}_{j+1}$ and $\tilde{D}_{j-1}$. k_N denotes the decay coefficient of activated Notch while k_F is the rate constant of the binding reaction between Delta and inactive Notch where $\tilde{N}_0$ represents the total amount of activated and inactivated forms of Notch.

In nondimensional form, the system of equation reduces to the following:

$$\frac{dD_j}{d\tau} = -D_j + \frac{b_0}{1 + (\frac{N_j}{K})^2} \quad (4.3)$$

$$\frac{dN_j}{d\tau} = -k_d N_j + k_f (D_{j-1} + D_{j+1})(1 - N_j) \quad (4.4)$$

where $t = \frac{\tau}{k_D}$, $\tilde{D}_j = D_0 D_j$, $D_0 = \frac{k_d}{B_0}$, $\tilde{N}_j = N_0 N_j$, $b_0 = \frac{B_0}{k_D D_0}$, $K = \frac{k}{N_0}$, $k_f = \frac{K_f D_0}{2k_D}$ and $k_d = \frac{k_N}{k_D}$.

In general, a lower K and higher k_f implies greater nonlinearity within the system of equations. A low K signifies a low concentration of activated Notch necessary for maximal inhibition of Delta while a high k_f indicates a low level of Delta necessary for activation of the neighbouring Notch receptor.

Lateral inhibition model with intracellular Notch heterogeneity

Modelling the above system of equations namely Eq(4.3) and Eq(4.4) on a one-dimensional grid of cells will obtain the salt-and-pepper steady state configuration where tip cells are regularly spaced by one stalk cell. Inherent in this system of equations is the assumption that Notch levels are homogeneous throughout the cell. However, Notch is a transmembrane protein and its large molecular size would result in low diffusivity. Furthermore, Notch is activated by Delta expressed on its neighbours and should the Delta levels of the neighbours differ, the amount of Notch that is activated within different parts of the cell may also be different. As such, Notch protein may not necessarily be homogeneous throughout the cell. Intracellular heterogeneity has previously been implicated in *Drosophila* bristle formation via the planar cell polarity pathway (Schamberg et al., 2010; Amonlirdviman et al., 2005). In (Schamberg et al., 2010), a negative feedback loop couples adjacent sides of neighbouring cells. If the negative feedback loop is sufficiently strong, individual cells will polarize and exhibit disparate concentration of proteins along different regions of the cell. Ultimately, this leads to an entire cell sheet possessing polarity. It is currently unknown how intracellular heterogeneity will affect patterning during sprouting angiogenesis. To include intracellular heterogeneity of activated Notch levels into lateral inhibition, we modify the above system of equations to account for differential levels of activated Notch within the cell. For simplicity, we adopted a similar approach in (Schamberg et al., 2010) and modelled each cell as having two sides where $N_{l,j}$

and $N_{r,j}$ represents fraction of activated Notch on the left and right side of cell j respectively.

Notch is activated by Delta expressed on neighbouring cells. Thus depending on the concentration of Delta in its neighbours, a cell may possess different levels of activated Notch where the side of the cell with a higher Delta neighbour will have higher activated Notch. Activated Notch translocates to the nucleus to result in downregulation of Delta. As a result, Delta levels are controlled by gene expression induced by the overall levels of activated Notch within the cell and thus is unlikely to exhibit deviating levels intracellularly. Hence, in the following modified lateral inhibition model, we only consider intracellular Notch heterogeneity.

Fig 4.2B depicts the schematic for lateral inhibition after considering for intracellular Notch heterogeneity with the system of equations listed below.

$$\frac{dD_j}{d\tau} = -D_j + \frac{b_0}{1 + \left(\frac{N_{l,j} + N_{r,j}}{\frac{2}{K}}\right)^2} \quad (4.5)$$

$$\frac{dN_{l,j}}{d\tau} = -k_d N_{l,j} + k_f (D_{j-1})(1 - N_{l,j}) + W(N_{r,j} - N_{l,j}) \quad (4.6)$$

$$\frac{dN_{r,j}}{d\tau} = -k_d N_{r,j} + k_f (D_{j+1})(1 - N_{r,j}) + W(N_{l,j} - N_{r,j}) \quad (4.7)$$

where $W = F_{Notch}/(L^2 k_D N_0)$ in which F_{Notch} is the diffusion coefficient of Notch and L is the length of the cell.

Like the classical lateral inhibition model, Eq(4.5) describes how decay of Delta and Notch inhibition affects the rate of change of Delta. A slight difference exists between Eq(4.5) and Eq(4.3). In Eq(4.5), Delta is inhibited by the average levels of Notch within the cell. This is however unnecessary in Eq(4.3) due to the assumption of Notch homogeneity. At the same time, the original equation for Notch, Eq(4.4) is split into two separate equations, one for each side of the cell where Eq(4.6) and Eq(4.7) describes the rate of change of activated Notch on the left and right side of the cell respectively. As in (Schamberg et al., 2010), a separate term that accounts for the exchange of Notch between the left and right side of the cells is introduced into the right hand side of Eq(4.6) and Eq(4.7). This

exchange is characterized by a diffusion term, W . When W goes to infinity representing extremely fast diffusion, activated Notch levels on the left and right side of the cell equilibrates and the classical lateral inhibition model is recovered.

In order to determine the validity of the assumption of Notch heterogeneity, time taken for diffusion of Notch was compared against time taken for reaction. Mean diffusion coefficient of Notch has been determined to be in the order of $0.076\mu\text{m}^2/\text{s}$ using quantum dots (Farlow et al., 2013). This slow diffusion coefficient can be attributed to the large molecular size of Notch as well as its transmembrane property which greatly limits its diffusion rate. The diffusion rate of Notch translates into a mean diffusion time of 5000s in a cell of $10\mu\text{m}$ radii (Moran, Phillips, and Milo, 2010). Comparatively, this diffusion time is slow in comparison to typical ligand binding reactions undertaken during Notch activation upon Delta binding where a prototypical ligand induced conformation change takes 1ms (Shamir et al., 2016).

Lateral inhibition model with intracellular Notch heterogeneity and tension-dependent rate of Delta-Notch binding

Receptor-ligand binding reactions have been known to be dependent on the distance between receptor and ligand (Erdmann and Schwarz, 2007; Hu, Lipowsky, and Weikl, 2013). Intuitively, if the receptor and ligand are too far apart, successful binding cannot take place. On the other hand, if they are too close, steric hindrance may interfere with binding. This dependence on receptor-ligand distance is incorporated in Bell's model where adhesion between cells and substratum is modelled by allowing the bond association and dissociation rates between the cell receptor and substrate ligand to vary as an exponential function of their bond length (Bell, 1978). Notch activation has also been shown to be dependent on receptor-ligand distance. By utilizing atomic force microscopy on live cells, Ahimou et al. established that the intercellular adhesive force affects the rate of Notch signalling where Delta pulling promotes Notch activation (Ahimou et al., 2004). Furthermore, the Notch pathway has recently been implicated in tension-regulation of cells where components of the Notch signalling pathway

are postulated to respond to low mechanical tension resulting in the inhibition of h2-calponin expression (Jiang et al., 2014). Therefore, there is prevailing evidence that suggests Delta-Notch signalling is influenced by intercellular tension. Unfortunately, to our best knowledge, it is not known how mechanical forces impacts Delta-Notch signalling within a system of cells and consequently how this tension contributes to tip-stalk patterning.

To account for how bond stress affects bond strain and ultimately the rate constants between adhesive molecules, Dembo and coworkers introduced a set of constitutive laws to calculate the chemical kinetics of the adhesion molecules (Dembo et al., 1988). The laws were then applied to study rolling velocity of granulocytes in venules as well as adhesion of red cells mediated by lectin (Dembo et al., 1988). As such, we modified the rate constant, k_f within Eq(4.6) and Eq(4.7) by allowing it to vary according to Dembo's model such that

$$k_{f,j} = k_{f0} \exp\left(-\frac{\sigma_{ts}(x_{m,j} - \lambda)^2}{2k_b T}\right) \quad (4.8)$$

In Eq(4.8), $k_{f,j}$ varies as an exponential function of the distance between neighbouring cells $x_{m,j}$ and the optimum distances between neighbouring cells, λ . Here, $k_{f,j}$ represents the rate constant of the Notch activation reaction between cells j and $j + 1$, $x_{m,j}$ denotes the intercellular distance between cell j and $j + 1$, k_{f0} represents the initial reaction rates, k_b is the Boltzmann constant, σ_{ts} is the hookean spring constant while T is the temperature. Eq(4.8) dictates that at optimum bond length between receptor and ligand, the rate constant will be the highest while deviations away from the optimum bond length will cause the rate constant to decrease.

Distances between neighbouring cells are calculated by considering the number of adherent Delta and Notch pairs at the cell-cell interface and allowing the intercellular distance to be a monotonically decreasing function of adherent molecules.

$$x_{m,j} = F(N_{r,j}, D_{j+1}, N_{j+1}, D_j) \quad (4.9)$$

We use the following function for F , where

$$F(N_{r,j}, D_{j+1}, N_{j+1}, D_j) = \frac{1}{\max([(1 - N_{r,j}), D_{j+1}]) + \max([(1 - N_{l,j+1}), D_j])}$$

Grouping constants together result in the final form of equation that describes $k_{f,j}$

$$k_{f,j} = k_{f0} \exp(-h(F(N_{r,j}, D_{j+1}, N_{j+1}, D_j) - \lambda)^2) \quad (4.10)$$

where h characterizes how the rate constants vary as a function of number of adherent Delta-Notch pairs. $h > 0$ implies tension-dependent rate of Delta-Notch binding while $h = 0$ suggests that the binding rate constants are independent of number of Delta-Notch adherent pairs. Fig 4.2C outlines the simplified representation for lateral inhibition with both intracellular Notch heterogeneity and tension-dependent rate of Delta-Notch binding.

To summarise, the classical lateral inhibition model comprises of Eq(4.3) and Eq(4.4) while the enhanced lateral inhibition model consists of Eq(4.5) to Eq(4.10).

These equations are then solved using Matlab to find the roots to the coupled system of equations at steady state. From here on, we denote n -cell spacing as n number of stalk cells between the tip cells and tip cells are defined as cells with the highest Delta concentration while stalk cells consists of cells with lower Delta concentration.

4.2.3 Derivation of steady state solutions for lateral inhibition models

We present how we derived the solution for the 2-cell spacing case analytically when W and h are kept at 0. When W and h are not zero, the matlab function *fsolve* is used to probe for existence of solutions.

We first consider a linear periodic array of cells and assume the existence of a 2-cell spacing pattern. Our purpose then is to determine if solutions exists for the 2-cell spacing case and if the solutions are stable. In a 2-cell spacing pattern, Delta levels vary from the first cell to the fourth cell in this order: D_1, D_2, D_3, D_4 . As 2-cell spacing is assumed, $D_1 = D_4$ and the order can be rewritten as : $D_1, D_2,$

D_3, D_1 . Since it is unlikely for Delta-Notch lateral inhibition to cause left-right asymmetry in an array of cells, we limit our solutions to cases where left-right symmetry exists. In other words, the order of Delta should be identical going leftwards from the first cell to the fourth cell, and rightwards from the fourth cell back to the first cell. With this taken into consideration, $D_2 = D_3$, and the Delta order now reads: D_1, D_2, D_2, D_1 . Further simplifying $N_{l,1}, N_{r,1}, N_{l,3}$ and $N_{r,3}$ using Eq(4.6) and Eq(4.7) yields $N_{l,1} = N_{r,1} = N_1, N_{l,3} = N_{r,2}$ and $N_{r,3} = N_{l,2}$. Finally, the system of equations reduces to the following:

$$\frac{dD_1}{d\tau} = -D_1 + \frac{b_0}{1 + (\frac{N_1}{K})^2} \quad (4.11)$$

$$\frac{dN_1}{d\tau} = -k_d N_1 + k_f D_2 (1 - N_1) \quad (4.12)$$

$$\frac{dD_2}{d\tau} = -D_2 + \frac{b_0}{1 + (\frac{N_1 + N_{l,2}}{2K})^2} \quad (4.13)$$

$$\frac{dN_{l,2}}{d\tau} = -k_d N_{l,2} + k_f D_1 (1 - N_{l,2}) \quad (4.14)$$

At steady state, rate of change reduces to 0 resulting in the following equalities. The subscript, ss, is used to denote steady state quantities.

$$D_{1,ss} = \frac{b_0}{1 + (\frac{N_{1,ss}}{K})^2}$$

$$N_{1,ss} = \frac{k_f D_{2,ss}}{k_d + k_f D_{2,ss}}$$

$$D_{2,ss} = \frac{b_0}{1 + (\frac{N_{1,ss} + N_{l,2,ss}}{2K})^2}$$

$$N_{l,2,ss} = \frac{k_f D_{1,ss}}{k_d + k_f D_{1,ss}}$$

Substituting $N_{l,2,ss}$ and $D_{2,ss}$, we get

$$D_{1,ss} = \frac{b_0}{1 + (\frac{N_{1,ss}}{K})^2} = Y(N_{1,ss})$$

$$D_{1,ss} = \frac{k_d(2K\sqrt{\frac{b_0k_f(1-N_{1,ss})}{k_dN_{1,ss}}} - 1 - N_{1,ss})}{k_f(1 - 2K\sqrt{\frac{b_0k_f(1-N_{1,ss})}{k_dN_{1,ss}}} - 1 - N_{1,ss})} = Z(N_{1,ss})$$

Hence, depending on the values of the parameters used, the 2-cell spacing scenario exists when $Y(N_{1,ss}) = Z(N_{1,ss})$.

Stability analysis is then performed after the roots has been obtained to check for stability. The Jacobian matrix of the system of equations Eq 4.11, Eq 4.12, Eq 4.13 and Eq 4.14 is computed using the roots computed at steady state. Roots are stable if and only if the sign of real part of eigenvalues of the Jacobian matrix is negative. Only stable steady states are reported in the Results section.

For larger cell spacings in which analytical solutions are not possible, random initial conditions are used and the matlab function *fsolve* is used to solve for solutions. Like before, solutions are checked for stability and only stable steady state solutions are reported.

4.3 Results

4.3.1 Tip-stalk patterns with more than one stalk cell in between tip cells recovered in lateral inhibition model with intracellular Notch heterogeneity

In this section, we first present the results where we consider intracellular Notch heterogeneity in the lateral inhibition model without tension-dependent rate of Delta-Notch binding, i.e. $h = 0$ in Eq(4.10). (Since $h = 0$, for all cells, $k_{f,j} = k_{f0}$). However, we have shown that in most classical lateral inhibition models, Notch heterogeneity is often not studied (Collier et al., 1996; Sprinzak et al., 2010; Koizumi, Iwasa, and Hirashima, 2012). Thus Notch is normally modelled as a uniform entity throughout the cell. However, we have shown that Notch being a large transmembrane protein with a low diffusion coefficient may likely exhibit disparate levels throughout the cell. It is currently unknown how a heterogeneous intracellular concentration of Notch will affect tip-stalk patterning.

Without Notch heterogeneity, the classical lateral inhibition model yields two forms of patterning. The first patterning is made up of a uniform array of cells each consisting of identical Delta and Notch levels. In the first pattern, no clear tip or stalk cell is present since all cells are identical. This is contrasted with the second form of patterning which produces the salt-and-pepper configuration, where the cells adopt an alternating arrangement of tip and stalk cells. Tip cells are defined by high Delta expression while stalk cells of high Notch. The first pattern where a single cellular phenotype is observed can be attributed to the existence of a lone stable steady state which becomes unstable at increasing non-linearity leading to its eventual instability. On the other hand, two stable states emerge in this nonlinear regime. These two stable states correspond to the high Delta low Notch state and the low Delta high Notch state responsible for the salt-and-pepper configuration. Furthermore, since every high Notch cell will have a high Delta cell as neighbour, the maximum number of cell spacing the classical lateral inhibition model can achieve is only the one-cell spacing pattern.

When we consider intracellular Notch heterogeneity with lateral inhibition, we similarly recover the cellular states responsible for the salt-and-pepper configuration, namely, the high Delta low Notch state and the low Delta high Notch state. Correspondingly, like the classical lateral inhibition case, the salt-and-pepper patterning or the one-cell spacing pattern is observed at high nonlinearity characterized by high k_{f0} and low K , as depicted in Fig 4.3H. A high k_{f0} in Eq(4.6) and Eq(4.7) indicates a lower level of Delta necessary for activation of the neighbouring Notch receptor. On the other hand, a low K in Eq(4.5) signifies a lower concentration of activated Notch necessary for maximal inhibition of Delta.

We also uncover other forms of patterning previously unseen in classical lateral inhibition models such as the two-cell spacing. Parameter space for two-cell spacing is demarcated in Fig 4.3I. These forms of patterning exist due to the creation of additional cellular states when Notch is allowed to exhibit intracellular heterogeneity. For example, a stable cellular state with moderate Delta levels, with high Notch on one side and low Notch on the other can exist when we consider

FIGURE 4.3: Delta and Notch Levels in Lateral Inhibition with Intracellular Notch Heterogeneity. Delta levels (A), Notch-left levels (B) and Notch-right levels (C) plotted against cell number for zero-cell spacing at $h = 0$, $W = 0$, $b_0 = 0.5$, $K = 1$, $k_{f0} = 0.1$ and $k_d = 1$. (D) Parameter space of K vs k_{f0} where one-cell spacing is observed at $h = 0$, $W = 0$, $b_0 = 0.8$ and $k_d = 0.1$. Delta levels (E), Notch-left levels (F) and Notch-right levels (G) plotted against cell number for one-cell spacing at $h = 0$, $W = 0$, $b_0 = 0.8$, $K = 0.01$, $k_{f0} = 0.1$ and $k_d = 1$. (H) Parameter space of K vs k_{f0} where one-cell spacing is observed at $h = 0$, $W = 0$, $b_0 = 0.8$ and $k_d = 0.1$. Delta levels (I), Notch-left levels (J) and Notch-right levels (K) plotted against cell number for two-cell spacing at $h = 0$, $W = 0$, $b_0 = 0.8$, $K = 0.01$, $k_{f0} = 0.3$ and $k_d = 1$. (L) Parameter space of K vs k_{f0} where two-cell spacing is observed at $h = 0$, $W = 0$, $b_0 = 0.8$ and $k_d = 0.1$. For cell 2, 3, 5 and 6, the Notch-left levels and Notch-right levels are different.

Notch heterogeneity in lateral inhibition. Examination of the two-cell spacing case in Fig 4.3J-K reveals that the two stalk cells spaced between the tip cells exhibit the aforementioned characteristics: high Notch on one side and low Notch on the other.

Next, we examine how patterning is affected by intracellular diffusion by varying the value of W . Since the Notch-left levels and the Notch-right levels are identical in the zero-cell spacing and the one-cell spacing, parameter space

for zero-cell spacing and one-cell spacing is independent of diffusion. The same is not true for the two-cell spacing pattern. We plotted the parameter regimes where the two-cell spacing can be identified under a range of W in Fig 4.4. Intriguingly, we observe that as long as diffusion remains finite, it is always possible to have a stable steady state solution for the two-cell spacing. For convenience of analysis, we limit our solutions to cases where left-right symmetry exists in a linear array of cells. Due to symmetry, the modified system of equations reduces to the following at steady state,

$$N_{r,2} = N_{l,2} + \frac{k_d N_{l,2} - k_f(D_1)(1 - N_{l,2})}{S} = N_{l,2} + \alpha$$

where α denotes the difference between $N_{l,2}$ and $N_{r,2}$. α goes to zero only when W goes to infinity.

Substituting α yields

$$D_1 = \frac{k_d N_{l,2} - S\alpha}{k_f(1 - N_{l,2})}$$

$$D_2 = \frac{k_d N_{l,2} + S\alpha + \alpha k_d}{k_f(1 - N_{l,2} - \alpha)}$$

Hence, D_2 will be different from D_1 so long as α does not become zero. This means that as long as W is finite, the 2-cell spacing pattern exists regardless of the value of W .

Even though we observe more tip-stalk patterns after we consider intracellular Notch heterogeneity, some cell spacings are never seen such as the three-cell spacing. We illustrate why below.

For three-cell spacing, Delta levels vary from the first cell to the fifth cell in this order: D_1, D_2, D_3, D_4, D_5 . Since 3-cell spacing is assumed, $D_1 = D_5$ and the order can be rewritten as : D_1, D_2, D_3, D_4, D_1 . By limiting our solutions to cases where left-right symmetry exists such that the order of Delta is identical going leftwards from the first cell to the fifth cell, and rightwards from the fifth cell back to the first cell, D_2 is thus equivalent to D_4 , and the Delta order now reads: D_1, D_2, D_3, D_2, D_1 . Thus, the equations for the three-cell spacing case at

FIGURE 4.4: Effect of diffusion on two-cell spacing for lateral inhibition with intracellular Notch heterogeneity. Delta levels (A), Notch-left levels (B) and Notch-right levels (C) plotted against cell number for two-cell spacing at $h = 0$, $W = 0$, $b_0 = 0.9$, $K = 0.025$, $k_{f0} = 0.3$ and $k_d = 0.2$. (D) Parameter space of K vs k_{f0} where two-cell spacing is observed at $h = 0$, $W = 0$, $b_0 = 0.9$ and $k_d = 0.2$. Delta levels (E), Notch-left levels (F) and Notch-right levels (G) plotted against cell number for two-cell spacing at $h = 0$, $W = 3$, $b_0 = 0.9$, $K = 0.025$, $k_{f0} = 0.3$ and $k_d = 0.2$. (H) Parameter space of K vs k_{f0} where two-cell spacing is observed at $h = 0$, $W = 3$, $b_0 = 0.9$ and $k_d = 0.2$. Delta levels (I), Notch-left levels (J) and Notch-right levels (K) plotted against cell number for two-cell spacing at $h = 0$, $W = 50$, $b_0 = 0.9$, $K = 0.025$, $k_{f0} = 0.3$ and $k_d = 0.2$. (L) Parameter space of K vs k_{f0} where two-cell spacing is observed at $h = 0$, $W = 50$, $b_0 = 0.9$ and $k_d = 0.2$.

steady state is as follows:

$$0 = -k_d N_1 + k_f (D_2)(1 - N_1)$$

$$0 = -k_d N_3 + k_f (D_2)(1 - N_3)$$

Rearranging:

$$N_1 = \frac{k_f D_2}{k_d + k_f D_2}$$

$$N_3 = \frac{k_f D_2}{k_d + k_f D_2}$$

Hence, N_1 is equal to N_3 , and consequently, D_1 and D_3 are identical to each other. Now the order becomes D_1, D_2, D_1, D_2, D_1 , which is in fact the one-cell spacing. As such, the three-cell spacing will never be observed in lateral inhibition if we only consider intracellular Notch heterogeneity. This is because, the three-cell spacing case will be reduced to the one-cell spacing pattern after symmetry considerations.

All in all, by considering intracellular Notch heterogeneity, an aspect that is commonly neglected in most lateral inhibition models, we are able to reproduce more forms of tip-stalk patterning than that observed from classical lateral inhibition models. And this is so because a greater number of cellular states are possible when Notch is allowed to vary within the cell. Nonetheless, some patterns such as the three-cell spacing cannot exist due to implicit symmetry constraints. As such, we look for other potential mechanisms that may possibly be the symmetry-breaking mechanism required for reproducing three-cell spacing. The next section details our examination of how intercellular tension impacts Delta-Notch signalling by modifying the rate constants of the Delta-Notch binding reaction.

Tip-stalk patterns with more than one stalk cell in between tip cells recovered in lateral inhibition model with tension-dependent rate of Delta-Notch binding

Delta and Notch being a transmembrane receptor-ligand pair, has being implicated in cell-cell adhesion. When Notch or Delta function is reduced, cell adhesion is observed to decrease (Murata et al., 2010; Celis and Garcia-Bellido, 1994; Goode et al., 1996; Renaud and Simpson, 2001). Moreover, when cells exclusively expressing Notch are mixed with cells solely expressing Delta, large cell aggregates are observed (Fehon et al., 1990). This implies that Notch and Delta proteins besides playing a role in signalling, may also perform adhesive functions. At the same time, atomic force microscopy experiments have shown

that Delta pulling accelerates Notch signalling (Ahimou et al., 2004). This together with analysis of the Notch receptor structure has established a model for processing of Notch. In this model, pulling of the ligand-receptor pair triggers the receptor to unfold thereby unmasking an ADAM (a disintegrin and metalloprotease) cleavage site (Andersson, Sandberg, and Lendahl, 2011). Cleavage of the ADAM cleavage site by members of the ADAM/TACE (tumour necrosis factor- α -converting enzyme) family of metalloproteases is necessary for Notch activation (Brou et al., 2000; Canault et al., 2010; Tian et al., 2008; Tousseyn et al., 2009). Clearly, Delta and Notch proteins affect intercellular adhesion, and this adhesive strength in turn affects Delta-Notch signalling. Delta-Notch adhesive properties would require their respective membrane anchorage. This is contrasted with their signalling functions which would necessitate their proteolytic cleavage counteracting their adhesive roles. It is however unknown how this paradoxical interplay affects tip-stalk patterning within a system of cells. In this section, we sought to investigate how intercellular adhesion affects Notch signalling. For ease of analysis, intercellular adhesion is first considered without intracellular Notch heterogeneity, i.e. $W = \infty$. First, we use the number of adherent Delta-Notch pairs to calculate the intercellular distance between neighbouring cells. Next, using the intercellular distance calculated, the rate constant of Notch activation can be determined using Dembo's laws which subsequently allow for elucidation of the Delta and Notch levels for each cell (Dembo et al., 1988).

We observe that simply considering tension-dependent rate of Delta-Notch binding in lateral inhibition, we are able to generate a limited range of tip-stalk patterning. We illustrate why with the two-cell spacing patterning. The following are the system of equations for the two-cell spacing pattern at steady state when $W = \infty$.

$$0 = -k_d N_2 + k_{f,1}(D_1)(1 - N_2)$$

$$0 = -k_d N_1 + k_{f,2}(D_2)(1 - N_1)$$

Hence, by accounting for tension-dependent rate of Delta-Notch binding, a steady state two-cell spacing can be obtained provided the following constraint is fulfilled.

$$k_{f,1}(D_1) = k_{f,2}(D_2)$$

Examining the equations for N_1 and N_2 and solving for k_d yields the following expression for k_d .

$$k_d = \frac{k_{f1}D_2(1 - N_1)}{N_1} = \frac{k_{f1}D_1(1 - N_2)}{N_2}$$

Substituting for D_1 and D_2 reduces to the following equality

$$\frac{1 - N_1}{N_1} [1 + (\frac{N_1}{K})^2] = \frac{1 - N_2}{N_2} [1 + (\frac{N_2}{K})^2]$$

A trivial solution will be when $N_1 = N_2$. Such will be the case of zero-cell spacing where all cells are identical to each other. However, nontrivial solutions exist when the function $M(x) = (1 - x)/(x)[1 + (x/K)^2]$ is a many-to-one function. Solving for the stationary points in M suggests nontrivial solutions are present only when multiple positive roots exists for the function M' where $M'(x) = -1/x^2 + 1/K^2 - 2x/K^2 = 0$. This limits the parameter space in which the two-cell spacing is observed such that they can only exist if $K < 0.1924$. Furthermore, due to the necessity to fulfil the above equality, two-cell spacing is rarely observed in parameter space and only occur under a set of very narrow parameters. Similar arguments can be made for three-cell spacing. Examples of two-cell spacing and three-cell spacing are depicted in Fig 4.5A-B and Fig 4.5C-D respectively. Three-cell spacing is the maximum spacing that can be observed under tension modulation since the steady state solutions for the four-cell spacing is unstable and the function $M'(x)$ limits the solution to a maximum of four-cell spacing.

In summary, lateral inhibition with tension-dependent rate of Delta-Notch binding allows us to reproduce some patterns but these spacings are not widely observed and only occur under a peculiar set of parameters. Nonetheless, we still

FIGURE 4.5: Delta and Notch Levels in Lateral Inhibition with Tension Modulation of Rate Constants. Delta levels (A) and Notch levels (B) plotted against cell number for two-cell spacing at $\lambda = 30$, $h = 0.0052$, $W = \infty$, $b_0 = 0.9$, $K = 0.1$, $k_{f0} = 3.719$ and $k_d = 0.0225$. Delta levels (C) and Notch levels (D) plotted against cell number for three-cell spacing at $\lambda = 10$, $h = 0.0761$, $W = \infty$, $b_0 = 0.9$, $K = 0.05$, $k_{f0} = 38.6324$ and $k_d = 0.4021$.

manage to recover the three-cell spacing pattern which is previously not possible when we consider lateral inhibition with intracellular Notch heterogeneity. This proves that tension-dependent rate of Delta-Notch binding may be viable as the symmetry breaking mechanism necessary on top of intracellular Notch heterogeneity for creation of more tip-stalk patterns.

4.3.2 Lateral inhibition with intracellular heterogeneity and tension-dependent rate of Delta-Notch binding yields a large number of tip-stalk patterns with more than one stalk cell in between tip cells

Now that we have established the effects of considering intracellular Notch heterogeneity as well as tension-dependent rate of Delta-Notch binding into the classical lateral inhibition model individually, in this section, we will go on to investigate the interplay between these two mechanisms with lateral inhibition. Specifically, we seek to understand if the inclusion of these two mechanisms will allow generation of a wider variety of spacing patterns. To recap, classical lateral inhibition can only produce two types of tip-stalk patterns: a homogeneous pattern in which all cells are identical to each other, and a heterogeneous salt-and-pepper formation. Lateral inhibition with intracellular Notch heterogeneity allows more forms of pattern to be observed, but some forms are never recovered such as the three-cell spacing due to symmetry restrictions. On the other hand, if tension-dependent rate of Delta-Notch binding is singly considered without intracellular Notch heterogeneity, two-cell and three-cell spacing can be recovered only under very strict parameter considerations.

Here, we present the results for the modified lateral inhibition model with intracellular Notch heterogeneity and tension-dependent rate of Delta-Notch binding in Fig 4.6. We observe that the various patterns: zero-cell spacing, one-cell spacing, two-cell spacing and three-cell spacing can be attained under a large range of parameter values.

At the same time, we observe parameters where the spacings can co-exist with each other indicating the existence of a multistable steady state system. Identification of parameters where the various cell spacings can be observed may be useful in guiding experimentalists in the future as they seek to design their desired blood vasculature density. Different blood vasculature density are required for distinct biological functions. For example, during the wound healing process, a dense blood vasculature, i.e. small cell spacing, is preferred to

FIGURE 4.6: Delta and Notch Levels in Lateral Inhibition with Intracellular Notch Heterogeneity and Tension Modulation of Rate Constants. Delta levels (A), Notch-left levels (B) and Notch-right levels (C) plotted against cell number for zero-cell spacing at $\lambda = 10$, $h = 0.076$, $W = 0$, $b_0 = 0.9$, $K = 0.1$, $k_{f0} = 40$ and $k_d = 0.4$. (D) Parameter space of K vs k_{f0} where zero-cell spacing is observed at $\lambda = 10$, $h = 0.076$, $W = 0$, $b_0 = 0.9$ and $k_d = 0.4$. Delta levels (E), Notch-left levels (F) and Notch-right levels (G) plotted against cell number for one-cell spacing at $\lambda = 10$, $h = 0.076$, $W = 0$, $b_0 = 0.9$, $K = 0.07$, $k_{f0} = 40$ and $k_d = 0.4$. (H) Parameter space of K vs k_{f0} where one-cell spacing is observed at $\lambda = 10$, $h = 0.076$, $W = 0$, $b_0 = 0.9$ and $k_d = 0.4$. Delta levels (I), Notch-left levels (J) and Notch-right levels (K) plotted against cell number for two-cell spacing at $\lambda = 10$, $h = 0.076$, $W = 0$, $b_0 = 0.9$, $K = 0.01$, $k_{f0} = 40$ and $k_d = 0.4$. (L) Parameter space of K vs k_{f0} where two-cell spacing is observed at $\lambda = 10$, $h = 0.076$, $W = 0$, $b_0 = 0.9$ and $k_d = 0.4$. Delta levels (M), Notch-left levels (N) and Notch-right levels (O) plotted against cell number for three-cell spacing at $\lambda = 10$, $h = 0.076$, $W = 0$, $b_0 = 0.9$, $K = 0.01$, $k_{f0} = 35$ and $k_d = 0.4$. (P) Parameter space of K vs k_{f0} where three-cell spacing is observed at $\lambda = 10$, $h = 0.076$, $W = 0$, $b_0 = 0.9$ and $k_d = 0.4$.

ensure sufficient perfusion of the wounded tissue with essential materials for regrowth of the tissues. This is contrasted with tumour angiogenesis where blood vasculature can be designed to be sparser, i.e. larger cell spacing, so that the tumour becomes starved of the oxygen and nutrients it requires thus inhibiting the tumour's growth. Knowledge of the parameter space where different spacing patterns can be easily found may allow experimentalists or tissue engineers to modify their cellular parameters such that their system of cells lie predominantly within their designed spacings.

Lastly, we end this section with a summary of the patterns possible under different modifications to the classical lateral inhibition model in Fig 4.7. It can be observed from the figure that both intracellular Notch heterogeneity and tension-dependent rate of Delta-Notch binding are necessary to recreate the various forms of tip-stalk patterns observed during angiogenesis such as two-cell spacing and three-cell spacing. Examples of higher-order spacings such as four-cell and six-cell spacing that can be created from the modified lateral inhibition model with intracellular Notch heterogeneity and tension-dependent rate of Delta-Notch binding is illustrated in Fig 4.8.

4.3.3 Existence of intermediate cell states *in vitro*

An important prediction in our enhanced Delta-Notch lateral inhibition model is the existence of intermediate cell states, i.e., cells that exhibit moderate Delta or Notch levels thus manifesting both tip cell and stalk cell traits. These intermediate cell states are necessary as additional building blocks besides the canonical high Delta low Notch tip cell and the low Delta high Notch stalk cell to create higher order spacings. To probe the existence of such intermediates, we perform the endothelial tube formation assay using Human Umbilical Vein Endothelial Cells (HUVECs) and stained for CD34. In the endothelial tube formation assay, structures resembling blood capillaries are formed in the presence of angiogenic signals available within the conditioned media (DeCicco-Skinner et al., 2014). Upon tube formation, the cells are stained with CD34, a known tip

Spacings		Tension Modulation of Rate Constants	
		$h = 0$	$h > 0$
Intracellular Heterogeneity	$W = \infty$	1	1 2 (rare) 3 (rare)
	$W < \infty$	1 2 4 etc.	1 2 3 4 etc.

FIGURE 4.7: Summary of tip-stalk patterns observed under different conditions of W and h . When $W = \infty$ and $h = 0$, the classical lateral inhibition model is recovered which yields exclusively the zero-cell spacing and the one-cell spacing. When $W < \infty$ and $h = 0$, this corresponds to modified lateral inhibition model with intracellular Notch heterogeneity. More tip-stalk patterns are observed such as the two-cell spacing but due to symmetry restraints, three-cell spacing is never observed. On the other hand, when $W = \infty$ and $h > 0$ which corresponds to modified lateral inhibition model with tension dependent rate of Delta-Notch binding, two-cell spacing and three-cell spacing are observed. Unfortunately, these spacings occur under very narrow parameter ranges rendering its rarity. Lastly, when $W < \infty$ and $h > 0$, which is the case of enhanced lateral inhibition model with intracellular Notch heterogeneity and tension-dependent rate of Delta-Notch binding, we recover the one-cell spacing, two-cell spacing, three-cell spacing etc. at wide parameter ranges.

cell marker (Siemerink et al., 2012). The CD34 antibody thus serves as a proxy for Delta and allows us to identify tip cells within the population.

It can be observed from the stained images in Figure 4.9A that the fluorescence intensities vary greatly amongst the cells. Strikingly, there are cells that are more brightly stained than others. Since CD34 is a tip cell marker, cells that stain most

FIGURE 4.8: Delta levels (A), Notch-left levels (B) and Notch-right levels (C) plotted against cell number for four-cell spacing at $\lambda = 10$, $h = 0.076$, $W = 0$, $b_0 = 0.9$, $K = 0.04$, $k_{f0} = 40$ and $k_d = 0.4$. Delta levels (D), Notch-left levels (E) and Notch-right levels (F) plotted against cell number for four-cell spacing at $\lambda = 10$, $h = 0.076$, $W = 0$, $b_0 = 0.9$, $K = 0.04$, $k_{f0} = 45$ and $k_d = 0.4$. Delta levels (G), Notch-left levels (H) and Notch-right levels (I) plotted against cell number for six-cell spacing at $\lambda = 10$, $h = 0.078$, $W = 0$, $b_0 = 0.9$, $K = 0.04$, $k_{f0} = 45$ and $k_d = 0.4$

strongly for CD34 are tip cells (Siemerink et al., 2012). Furthermore, if we quantify the fluorescence intensities of the cells based on their position from the tip cell, it can be observed that depending on their positions, cells exhibit significantly different fluorescence. This is even so if we compare fluorescence intensities of cells one-cell position away from the tip cell and cells that are two-cell position away from the tip cell as shown in Figure 4.9C. Fluorescence intensities of cells one cell away from the tip cell are significantly higher than the fluorescence intensities of cells two cells away from the tip cell. This result thus suggests that

we can distinguish at least three different cell types that occur during sprouting angiogenesis. The first cell type is the cell that is stained most intensely for CD34 which we know as the tip cell. The second cell type, the stalk cell which stains the weakest for CD34. And lastly, an intermediate cell type that exhibits moderate staining. Since the validity of the model hinges on the presence of the intermediate cell, the identification of the hybrid cell thus lends evidential weight to the legitimacy of the model

FIGURE 4.9: Varying fluorescent levels are exhibited by cells depending on their position from the tip cells. (A) Stained images of HUVEC using CD34 marker (green) and DAPI (blue). Scale bar represents $50\mu\text{m}$. (B) Blown up image of the red box marked in (A). The white arrow is pointing to a cell with a substantially brighter fluorescence as compared to its neighbours. Since CD34 is a tip cell marker, the white arrow is pointing to a tip cell. The red and blue arrows are pointing to cells one-cell and two-cells away from the tip cell respectively. Scale bar represents $50\mu\text{m}$. (C). Normalized intensities of the cells are plotted as a function of their position from the tip cell. Error bars denote standard errors. $p < 0.001$ represented by *** and $p < 0.01$ represented by **. (D). HUVEC cell staining images using CD34 marker (green), DAPI (blue) and rhodamine (red). White arrows are pointing to cells that have an intense fluorescence stain for CD34. Scale bar represents $100\mu\text{m}$. (E). Skeletonized image for (D) where red circles indicate tip cells and yellow circles represent intermediate cells and stalk cells. Physical connections between cells are represented by blue lines. The red and purple dashed lines indicate the presence of three-cell and six-cell spacing respectively.

In addition, we observe high order spacings such as six-cell spacing in many

of the stained images. An example is shown in Fig 4.9D. Such high order spacings implicate that tension modulation of rate constants is insufficient to supplement the classical lateral inhibition model to recapitulate spacings in nature. Observation of the three-cell spacing scenario also renders lateral inhibition with intracellular Notch heterogeneity inadequate in reproducing tip-stalk spacings. Based on these experimental observations, we conclude that nature operates in the regime where $W < \infty$ and $h > 0$, and that both intracellular Notch heterogeneity and tension modulation of rate constants are necessary to reproduce the myriad of tip-stalk spacings observed.

4.4 Discussion

Lateral inhibition is a universal mechanism invoked in many organisms for cell fate selection. In *Drosophila*, lateral inhibition is involved in selection for neuroblast cells within the neuroectoderm. Lateral inhibition ensures that the neighbouring cells around the potential neuroblast cell takes on the epidermoblast fate (Kunisch, Haenlin, and Campos-Ortega, 1994). Similarly in *Ciona intestilis*, lateral inhibition ensures that the epidermal sensory neurons are scattered at regular intervals along the peripheral nervous system (Pasini et al., 2006). Lateral inhibition is also at work in vulval development of *Caenorhabditis elegans* in which six vulval precursor cells specialized into the primary cell, secondary cell or the tertiary cell (Chen and Greenwald, 2004). Despite the differences in cell type in which lateral inhibition is at play, the fundamental concept of how lateral inhibition achieve its selection is the same; through a feedback mechanism that ensures that the neighbours do not take on the same fate as the chosen cell, i.e. the selected cell represses the cell fate choice of its neighbours.

In angiogenesis, a similar cell fate selection is undertaken by endothelial cells. Lateral inhibition in angiogenesis results in endothelial cells taking one of two fates. The first cell fate is the tip cell fate where tip cells respond to chemotactic gradient, migrate and possess filopodia. The second cell is the stalk cell which serves to proliferate and support the growth of the nascent endothelial

stalk. Stalk cells also keep the endothelial vessel intact by forming strong adherent and tight junctions. Such fate segregation within the endothelial cells is of utmost importance. If all cells are to become tip cells, the capillary sprout will disintegrate due to lack of cell-cell adhesion contributed by the stalk cells. On the other hand, if all cells take on the stalk cell fate, the sprout will not elongate but simply increase in diameter. Lateral inhibition in angiogenesis thus ensures an optimal tip-stalk cell distribution for ideal growth and cohesion of the blood vessel. Lateral inhibition in angiogenesis is undertaken by the Delta-Notch signalling mechanism. In a nutshell, the tip cell characterized by high Delta ligand expression activate the Notch receptor of its neighbour. Upon Delta ligand binding, Notch of the neighbour becomes activated culminating in the inhibition of Delta expression within the neighbour. Since the neighbouring cell has low Delta expression, Notch within the tip cell is not activated and Delta expression of the tip cell remains high. Ultimately, lateral inhibition ensures that the neighbours of the tip cell have low Delta expression thus taking on the stalk cell fate while the Delta expression of the tip cell persists to be high maintaining the tip cell fate. Lateral inhibition in angiogenesis thus serves to repress the tip cell fate amongst the neighbours of the tip cells. Classical lateral inhibition models typically embody this feedback feature of Delta-Notch signalling and produce the commonly observed salt-and-pepper configuration where tip cells are separated exactly by one stalk cell. In vivo and in vitro experiments have however revealed a repertoire of spacing patterns not limited to the salt-and-pepper configuration such as two and three stalk cells between the tip cells. Various groups have attempted to explain these alternative forms of patterning using models incorporating only Delta and Notch. Collier et al. used a two-dimensional cell system (Collier et al., 1996), Cohen et al. implicated interaction between filopodia and stalk cells (Cohen et al., 2010) while Chen et al. introduced a nearest neighbour Notch gradient term (Chen et al., 2014). However, these mechanisms may not be applicable during sprouting angiogenesis since the sprouts formed are typically one-dimensional in nature which defies Collier et al.'s system, filopodia rarely interact with lagging stalk cells in contrast with Cohen et al.'s and the

Notch gradient term in Chen et al. has not been observed. Hence, in this chapter, we seek to uncover a ubiquitous lateral inhibition mechanism for recapitulating patterning observed during sprouting angiogenesis.

Classical lateral inhibition models are limited to the salt-and-pepper configuration since only two cellular states are possible: a high Delta low Notch state and a low Delta high Notch state. In order to create more angiogenic patterns, the number of states possible must be expanded. We look to pervasive mechanisms in nature that are capable of expanding the number of states. Intracellular protein heterogeneity have been previously implicated in *Drosophila* bristle formation via the planar cell polarity mechanism (Amonlirdviman et al., 2005). Furthermore, Notch being a large transmembrane protein with a low diffusivity may exhibit varying concentrations within the cell. Hence, we investigate if lateral inhibition with intracellular Notch heterogeneity is capable of increasing the number of possible cellular states thereby allowing us to generate more forms of angiogenic patterns. We observe that with the inclusion of intracellular Notch heterogeneity, more cellular states are possible. For example, a cell possessing moderate Delta expression with high Notch on one side of the cell and low Notch on the other. With these additional states, we are indeed able to generate a greater variety of patterns not seen in previous classical lateral inhibition models such as the two-cell spacing pattern. However, we do notice that some forms of angiogenic patterns are never observed, such as the three-cell spacing pattern due to the constraint of symmetry. Hence, we look for other mechanisms that can be in play in addition to intracellular Notch heterogeneity for generation of tip-stalk patterns. Specifically, we require a mechanism that can potentially break the symmetry within a linear array of cells to make possible three-cell spacing.

Most lateral inhibition models assume a fixed rate constant amongst the cells.

However, it has been shown that tension can actually modulate the Notch signalling pathway and Notch may actually act as a mechanosensitive sensor (Ahimou et al., 2004; Jiang et al., 2014). As such, we investigate if addition of intracellular Notch heterogeneity and tension-dependent rate of Delta-Notch binding into the classical lateral inhibition model may allow us to recover the various forms of patterning observed during sprouting angiogenesis. The enhanced model with intracellular Notch heterogeneity and tension-dependent rate of Delta-Notch binding is capable of recapitulating the many forms of patterning observed such as the zero-cell spacing, one-cell spacing, two-cell spacing as well as the three-cell spacing case under a wide range of parameter values.

Next, we plotted the parameter space at which the different forms of angiogenic patternings are observed. Elucidation of the parameter space at which the spacings are observed can guide experimentalists in the design of blood vasculature. Should one desire to create very dense blood vasculature such as in the case of wound healing, one may choose to design the blood vessels such that they belong to the regime where the one-cell spacing case is predominantly observed. However, if one desires to create sparser blood vessels such as in tumour angiogenesis, parameters where the higher cell spacing are observed can be used instead. Molecular modulators of the cell density in angiogenesis would be a very good line for future investigation.

In recent years, hybrid cells types of various kinds have been postulated and identified. These include the hybrid epithelial/mesenchymal phenotypes (Jolly et al., 2015; Garg, 2017) in which co-expression of epithelial and mesenchymal signatures was strongly correlated with poor survival amongst patients suffering from breast cancer (Grosse-Wilde et al., 2015). Such hybrid cell states are also observed in small cell lung cancer. In addition to the known neuroendocrine/epithelial state (NE) and the no-neuroendocrine/mesenchymal-like (ML) state, a third cell state expressing both markers of NE and ML differentiation was discovered in small cell lung cancer (Udyavar et al., 2017). Such hybrid phenotypes have important physiological consequences. For instance, it was found that in small cell lung cancer, NE and ML cell populations evolved to the hybrid

cellular phenotype after cytotoxic treatment (Udyavar et al., 2017). Also, circulating tumour cells in patients with metastatic non-small cell lung cancer were observed to manifest a hybrid epithelial/mesenchymal phenotype (Lecharpentier et al., 2011). Clearly, hybrid cell types have important clinical consequences in therapeutic settings. In this chapter, we identify the existence of a hybrid cell type that exhibit intermediary tip cell and stalk cell characteristics. The identification was based on an immunofluorescence stain for CD34, a known tip cell marker (Siemerink et al., 2012). In the experiment, three distinct cell populations can be distinguished based on their fluorescence levels and position from the tip cells. The three cell types are the canonical tip and stalk cell, as well as the hybrid cell. The identification of this hybrid cell type is crucial to the validation of our model and is necessary as an additional building block complementing the tip cell and the stalk cell in the construction of large cell spacings. The discovery of the intermediate tip/stalk phenotype thus provides credible evidential support to the plausability of the model.

4.5 Conclusion

In conclusion, current lateral inhibition models involving only Delta and Notch are inadequate in explaining tip-stalk patterning in sprouting angiogenesis. In this chapter, we seek to uncover a general mechanism that is able to recapitulate cellular patterning observed by invoking mechanisms commonly neglected: intracellular Notch heterogeneity and tension-dependent rate of Delta-Notch binding. Such mechanisms do not require specific expression of particular genes and thus are universal across all cell types expressing Delta and Notch. We demonstrate that these two mechanisms are sufficient and necessary in recreating the rich behaviour of tip-stalk patterning observed. Furthermore, we also demarcate the parameter space for each tip-stalk pattern which may serve to guide experimentalists in the future when they seek to design their desired blood vasculature. Last but not least, we identify the existence of an intermediate cell type, a key prediction of our enhanced model thus substantiating the validity of the model

as well as its prediction.

Chapter 5

Conclusion

In this thesis, a few novel biological discoveries have been uncovered. These discoveries which were found using computational modelling and span multiple scales in biology are as follows:

1. At the molecular or protein level, we have uncovered Chk1 as a novel interactor of POPX2 through our bioinformatics analysis pipeline. This finding indicates that POPX2 interferes with the cell cycle process and may be a reason behind higher POPX2 levels among more invasive breast cancer cell lines (Susila et al., 2010).
2. At the intracellular level, we have found that to achieve optimum signalling efficiency of scaffolded proteins that are transported by motor proteins, an optimum concentration of scaffold proteins as well as an optimum speed of motor proteins is necessary.
3. At the intercellular level, we explore how differential regulation of angiogenesis leads to different sprouting angiogenesis patterns. We found that both intracellular Notch heterogeneity and tension-dependent rate of Delta-Notch binding are sufficient and necessary in recreating the rich behaviour of tip-stalk patterning observed.

All in all, by performing computational modelling of protein-protein and cell-cell interaction, we have discovered new biological information as well as gain a deeper understanding of processes involved in several biological scales. We hope

that this information will be useful to researchers in the future as they unravel the inner workings of the cell.

5.1 Future work

Future work includes discovery of more interactors of POPX2 and using all of these interactors to gain a bigger picture of how dysregulation of POPX2 may lead to disastrous consequences such as misregulated angiogenesis (Zhang et al., 2017). Our bioinformatics analysis pipeline can also be extended to look for substrates of other proteins and a webserver may potentially be built on our algorithm to provide a platform for users from all over the world to look for interactors of their desired proteins.

We would also like to test our computational predictions in Chapter 3 by varying protein diffusivity through introduction of dextran beads into the cytosol. Acetylation of microtubules could be another option to vary motor protein speed since it has been shown that hyper-acetylation of all microtubules in the central nervous system cell line Cath.a-differentiated (CAD) results in targeting of JIP1 to all neurite tips, nullifying the usual selectivity of its transport resulting in greater directed motion (Bulinski, 2007). Tau protein implicated in Alzheimer's disease can also be introduced into the cell to inhibit kinesin transport since tau impedes anterograde transport (Ebneth et al., 1998; Trinczek et al., 1999; Stamer et al., 2002). This may be due to tau's effect on decreasing the attachment ability of kinesin to microtubules (Seitz et al., 2002; Dixit et al., 2008) and/or decreasing the traveling distance of kinesin (Vershinin et al., 2007).

Lastly, we hope to be able to extend our angiogenesis model in Chapter 4 by including in the effects that pharmaceutical drugs have on angiogenic pathway signalling. The extended angiogenesis model can then be a testbed to probe the efficiency of different drugs in modulating angiogenesis, of which preliminary results can be validated experimentally.

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