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Literature Review of IFT Mechanisms and Functions in the Immune Synapse and Protein Family Tektin

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LITERATURE REVIEW OF IFT MECHANISMS AND FUNCTIONS IN THE IMMUNE SYNAPSE AND PROTEIN FAMILY TEKTIN

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ABSTRACT

Cilia are microtubule based organelles that serve as specialized projections for the cell. There are two distinct forms of cilia—motile and non-motile. Motile cilia have responsibilities in cell locomotion and patterned beating, as well as the movement of fluid over cell surfaces. Non-motile or primary cilia, function in sensory perception for the cell often sensing extracellular signals and transmits signals from the cilium to the cytoplasm in order to control gene expression and cell behavior. Cilia are comprised of many proteins, but a main focus of this thesis are tektin proteins. Tektin is a family of proteins that provide structural support and are closely related to intermediate filaments and nuclear lamins.

In order for both variations of cilia to develop, a system known as intraflagellar transport (IFT) must be functional. IFT is a motor-dependent cargo transport that is crucial for ciliary elongation, maintenance, and assembly of cilia. IFT is a coupled-bidirectional system that is comprised of two subcomplexes—IFT-A complex and IFT-B complex. The IFT-A complex primarily acts within retrograde transport to maintain the inward movement of materials, where as the IFT-B complex is involved with anterograde transport or outward movement of material during elongation and maintenance of cilia. Surprisingly, IFT has been found in cells that lack the presence of cilia. This can be seen in the formation of immune synapses of cytotoxic T-cells, which occurs at the same site where a primary cilium would develop. Although cytotoxic T-cells have not shown any projected appendages, there is a small bump present in the membrane that closely resembles the beginning formations of a primary cilium. This has been classified as a "frustrated cilium", where IFT proteins have been isolated and studied. This thesis reviews some of the recent literature on the tektin protein family and the functions of IFT proteins in both cilia and the immune synapse.

TABLE OF CONTENTS

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CILIARY STRUCTURE AND COMPONENTS

Cilia are microtubule based organelles that serve as specialized projections for the cell. Although historically, the term “flagella” has been used when characterizing longer organelles in fewer numbers, structurally cilia and flagella are very similar (Moran et al. 2014). Throughout the rest of this thesis, the terms cilia and flagella will be used according recent standards for each organism. All cilia have three main regions that contribute to the integrity of the structure, consisting of the basal body, the transition zone, and the axoneme, which is surrounded by a distinct ciliary membrane. The underlying structure is composed of microtubules, with multiple protein extensions and accessory proteins. Microtubules are hollow tubes composed of tubulin proteins that are involved with organization in eukaryotic cells (Alberts et al. 2014).

The basal body is essential to the stability of cilia, and acts as an anchorage site for the appendage. The location of the basal body allows it to serve as an organizing center for intraflagellar transport (IFT) and the cytoskeleton of the cell (Wingfield, 2018). The basal body is a cylindrical structure derived from a centriole that is composed of nine symmetrically arranged triplet microtubules—microtubule-A, microtubule-B, and microtubule-C (Prevo et al. 2017).

The transition zone is located at the distal portion of the basal body at the end microtubule-C and the start of the central pair. This structure is characterized by linkers between the microtubules and plasma membrane of the cell, which forms a ciliary gate. The cellular gateway regulates the entry of proteins to the cilia and contributes to the specific protein composition of the projection to ensure proper function. The transition zone links the basal body to the axoneme and provides a physical barrier between the

ciliary membrane and the plasma membrane. This barrier serves to prevent rapid exchange between the two membranes further supporting the lipid and protein integrity of the structure (Wingfield, 2018). Lastly, the axoneme is the only extracellular portion of the cilium and is assembled and maintained by intraflagellar transport. The axoneme is the microtubule-based core of the cilium (Ishikawa et al. 2017). This structure is crucial for cilia motility and beating. When mutations arise in the formation or functionality of this structure it can be a contributing factor to various diseases. The axoneme is present in both motile and non-motile cilia, but the formation of the microtubules varies (Branche et al. 2006).

MOTILE AND NON-MOTILE CILIA

Motile cilia have responsibilities in cell locomotion and patterned beating, as well as the movement of fluid over cell surfaces. Examples of motile cilia can be found in the respiratory epithelial lining within the respiratory tract, or airway. This allows for mucus, dirt, or bacteria to be swept out of the respiratory tract and lungs. In females, motile cilia are present in fallopian tubes to move the ovum from the ovaries to the uterus. Motile cilia are most often arranged in a "9 + 2" configuration consisting of nine symmetrically constructed microtubule-doublets connected by nexin links. Important additions to the axoneme that are present in motile cilia include outer and inner dynein arms and radial spokes that interact to aid in movement (Prevo et al. 2017).

Non-motile cilia, also referred to as primary cilia, are arranged in a "9 + 0" because the structure lacks the central pair of microtubules that are present within motile cilia. Additionally, non-motile cilia do not have outer and inner dynein motor components. This type of cilia serves in sensing extracellular signals and transmits signals from the

cilium to the cytoplasm in order to control gene expression and cell behavior (Prevo et al. 2017). Primary cilia are utilized in multiple signaling pathways during development, such as hedgehog signaling, Wnt signaling, notch signaling, and others. Non-motile cilia can be found in various mammalian cells, including, but not limited to, epithelial, endothelial, connective tissue, and neurons. A prime example of non-motile cilia is within the retina of the eye, where the cilia detect and transmit light signals (Wheway et al. 2018).

INTRAFLAGELLAR TRANSPORT MACHINERY

Intraflagellar transport is a motor-dependent cargo transport that is crucial for ciliary elongation, maintenance, and assembly of cilia, which is powered by kinesin-2 and dynein motor proteins (Prevo et al. 2017). This is a bidirectional transport system that has retrograde and anterograde components. Retrograde trafficking directs the movement of proteins from the tip of the cilia to the base, whereas anterograde trafficking is responsible for the movement of proteins from the base of the cilia to the tip. A major component of IFT machinery is the BBSome, which is present alongside axonemal microtubules. This component is responsible for the removal of cargo membrane proteins as it binds to the IFT-A/IFT-B particles machinery and is moved anterogradely and retrogradely when associated with the IFT particles (Lechtreck et al. 2009). The BBSome is linked to the ciliary membrane by a GTPase, which is required for proper function. Additionally, the BBSome is predicted to act as an adaptor when involved with the exporting of signaling proteins in the cilia (Prevo et al. 2017).

As described above, IFT is a bidirectional system consisting of the IFT-A complex and the IFT-B complex. The IFT-A complex has traditionally been more closely associated with retrograde trafficking, which runs primarily on the A-tubules of cilia. This complex

contains six subunits, IFT144, IFT140, IFT139, IFT122, IFT121, and IFT43, whereas IFT-B has sixteen subunits, IFT172, IFT88, IFT81, IFT80, IFT74, IFT70, IFT57, IFT56, IFT54, IFT52, IFT46, IFT38, IFT27, IFT25, IFT22, and IFT20. Since there are many more subunits present in the IFT-B complex, it is further split into two categories, IFT-B1 and IFT-B2, separating the core and peripheral subunits, respectively. The IFT-B complex has been found to mediate the anterograde trafficking that runs along the B-tubules. Both of these IFT trains are composed of multimeric proteins, having more than one polypeptide strand, that are similar to intracellular transport vesicles (Nozaki et al. 2019; Prevo et al. 2017).

IFT-A/IFT-B RELATIONSHIP

The IFT-A complex primarily acts within retrograde transport to maintain the inward movement of materials and is coupled with the anterograde transport or outward movement of the IFT-B complex during elongation and maintenance of cilia. IFT proteins are present within the matrix during these processes, which is the space between the membrane and the axoneme. Based on previously stated studies, IFT-A subunits contribute to the integrity of the complex, and result in stumpy, swollen flagella when key subunits are missing from the complex. Additionally, when subunits are lacking in the IFT-A complex the localization is affected and leads to malfunctions of both retrograde and anterograde transport. Though IFT-A is responsible for retrograde transport, it also has the ability to pull flagellar precursors from the cytoplasmic pool similar to the functions of the IFT-B complex (Zhu et al. 2017).

The IFT-B complex actively participates in anterograde transport of materials, and recently extensive studies have been conducted to investigate the roles of individual

subunits that construct the complex. In 2019, Huet et al. studied the functions of subunits IFT25 and IFT27 in trypanosomes, a member of the unicellular parasitic protozoa, *Trypanosoma*. The expression and movement of IFT25 could be seen when utilizing indirect immunofluorescence assay (IFA), an analytic test to show the presence of antibodies, with rabbit polyclonal antibody against GFP. By analyzing fluorescent signals and IFT movements, IFT25 was classified as a genuine component of the IFT-B complex that was located within the matrix of the flagella and had a heavy concentration at the base. Additionally, IFA showed that IFT25 contributed in anterograde and retrograde trafficking speeds similar to other IFT proteins. To further support that the subunit IFT25 is a component of the IFT-B complex in trypanosomes, immunoprecipitation was used to reveal association with other IFT-B proteins (Zhu et al. 2017; Huet et al. 2019).

In the absence of IFT25 both the IFT-A and IFT-B complexes were negatively affected. In the IFT-B complex, recycling of materials were inhibited, and the cells were immotile and lacked signaling in the flagellar region. Issues occurring within the IFT-B complex caused an uncoupling of the system and inhibited access of the IFT-A complex in the flagella. Without the IFT-A and IFT-B complexes interacting together, the cilia presented disorganized axonemes, defects in fibers and sheaths, as well as improper localization of vesicles (Huet et al. 2019; Zhu et al. 2017).

After establishing the localization and classification of IFT25, a study was conducted on the primary roles of the protein during flagellar assembly. A cell line was generated using RNA interference on IFT25, called IFT25^{RNAi}. This cell population resulted in a major growth defect when lacking IFT25, and after forty-eight hours results in cells with small, stumpy flagella. Additionally, the microtubule doublets were disorganized with

accumulation of materials on the distal portion of the flagella. Based on the previous results, IFT25 has a prominent role in both anterograde and retrograde transport, as well as formation of cilia (Huet et al. 2019). By contrasting cell line IFT25^{RNAi} in induced conditions with TdT::IFT140, a red fluorescent protein fused with IFT140, a western blot was constructed to reveal the accumulation of phosphorylated proteins. A western blot is analysis test to determine if a specific protein is present within a mixture of various proteins. During induced conditions it was determined in the absence of protein IFT25 recycling of the IFT-B complex was prevented because there was a large accumulation of IFT172. In the non-induced cell line protein trafficking functioned normally, but in the induced cell line the cells were immotile and had no signal in the flagellar region. Another result of this transformation included the inhibited access of the IFT-A complex in the flagella (Huet et al. 2019).

TEKTIN STRUCTURE AND FUNCTION

Tektin is another family of proteins present in cilia and flagella that provide structural support. Tektins display extensive coiled coils and are composed of long alpha helices. They are also closely related to intermediate filaments and nuclear lamins. Similar to intermediate filaments, Tektins have an amino and carboxy-terminal head and tail domains on either side of the coiled coil rod domain. Most importantly, Tektins serve as specific markers for ciliary and flagellar axonemes, basal bodies, and centrioles. Mutations within the proteins have been known to cause defects in sperm flagella and cause structural and motility issues within cilia (Amos, 2008).

Tektins were originally isolated from sea urchin sperm flagella. This protein family is also present in many different species such as Deuterostomes, Ecdysozoans, Spiralians,

Nonbilaterians, and unicellular organisms. Currently, Tektin-1 to Tektin-5 have been identified in vertebrates, however, most organisms do not possess all Tektin proteins at once. In sea urchins, Tektin-A, Tektin-B, and Tektin-C, which are orthologous to Tektin-4, Tektin-2, and Tektin-1, respectively, have been identified. From these isolated proteins, it has been determined that Tektins form long filaments that localize at the axoneme of the cilia. Each of these long filaments have three Tektin dimers; a dimer is a molecule made of two identical molecules joined together. Two of the proposed dimers are thought to be constructed with one Tektin-A and Tektin-B molecule in each, which is known as a heterodimer. A heterodimer is a protein comprised of two different or non-identical proteins, where as a homodimer is a protein consisting of two identical proteins joined together. The third dimer is thought to be formed by two Tektin-C molecules that together form one homodimer. When in the microtubule-doublet these dimers line the inside of microtubule-A, and based on the location and formation of Tektin, it is possible that they also serve as potential "rulers" in the cell to determine the length of cilia (Bastin et al. 2019).

TEKTIN-1 AND IFT-A INTERACTIONS IN CILIOPATHIES

In 2018, Ryan et al. conducted a study aiming to determine the contribution of genes *WDR19* and *TEKT1* in ciliopathies and to characterize the tektin-1 protein. The individual of interest was a female child suffering from Mainzer-Saldino syndrome (MZSDS), which causes renal, retinal, and skeletal issues, as well as lung infections and airway ciliary dyskinesia. The characterization of her symptoms have been classified as both primary and motile ciliary defects. To further investigate, researchers mapped ciliary related genes including ciliopathy genes, genes that encoded known ciliary proteins, and

genes encoding paralogues or ciliary protein family members. By using targeted exome sequencing, biallelic variations were found in gene *TEKT1* encoding for protein tektin-1 that is predicted to be a contributor in ciliary dyskinesia, and gene *WDR19*, which encodes for IFT-A subunit IFT144. The purpose of the study was to identify the contributions of each gene to the clinical manifestations in the patient (Ryan et al. 2018).

For the *TEKT1* gene, there were heterozygous variants identified in the affected individual. The first corresponds to a nonsense variant inherited from the mother and is predicted to result in a shortened protein and a loss of the last two C-terminal coiled-coils. The second is a missense event inherited from the father that has been classified as a disease-causing mutation by affecting the Lys311 residue. This amino acid is highly conserved in the Tektin family members and is important to the stabilization of tektins between the third and fourth coiled-coils. Due to the possible variants present in the affected patient, researchers aimed to understand the distribution and characterization of tektin-1 because of the tektin family's responsibilities in cell motility. To determine tektin-1 distribution within the patient, nasal brushing was used to collect ciliated cells. In control cells obtained from lung sections of a patient without MZSDS, *TEKT1* immunofluorescence staining was revealed at the apical region and tektin-1 was present at the axoneme of the motile cilia where it could possibly contribute formation or structural support. In contrast to the control cells, the protein tektin-1 was completely absent within the cilia of the patient with MZSDS (Ryan et al. 2018).

To test the effects of subcellular localization in other axonemal proteins in the absence of tektin-1, RSPH1 (radial spoke) and DNALI1 (inner dynein arms) were used as markers to study distribution of proteins. Both RSPH1 and DNALI1 co-localized normally in the

presence of tektin-1 and in its absence, showing that loss of tektin-1 does not lead to global defects in ciliary composition. Additionally, transmission electron microscopy was used to contrast the structure of the axoneme in control and affected cells taken from bronchial cells. In multi-ciliated cells collected from the individual revealed cilia that were sparse and misoriented, and some basal bodies were located deeper in the cytoplasm instead of docked at the apical plasma membrane. This supports that ciliary motility defects in patient cells are associated with loss of tektin-1 in cilia (Ryan et al. 2018).

As previously stated, the affected individual possessed a compound heterozygote meaning two mutant alleles were present. The maternal allele led to a deletion of genomic DNA from the 5'untranslated region to intron 4 resulting in no protein being produced. However, in the paternal allele there was a missense variation in exon 32 of *WDR19*, which allowed for protein production unlike the mutation in the maternal allele. This missense variation has been found to be pathogenic in other individuals presenting the same variation. Immunofluorescence with acetylated α-tubulin was used to study *WDR19* during ciliogenesis, and it was determined that mutations in *WDR19* directly impair the functions of IFT-A subunit IFT144 in primary cilia. The affected individual showed a decreased rate of ciliogenesis in fibroblasts and shorter cilia. In control fibroblasts, with normal *WDR19*, IFT144 accumulated at the cilium base and at a single portion of the ciliary distal tip. In fibroblasts with the mutation, IFT144 was absent from the tip. Additionally, when the ciliary membrane was stained it revealed a misshaped ciliary tip and accumulation of the IFT-B subunit IFT46, which indicates retrograde transport defects. The decreased expression of IFT144 and its mis-localization from the

ciliary tip is most likely the cause of defects in IFT-B retrograde transport observed in the affected cells (Ryan et al. 2018).

To observe the interactions between *WDR19* and *TEKT1* genes, three morphants were developed in zebrafish embryos. The first morphant consisted of a complete knockdown of *WDR19*, which resulted in zebrafish that had a shortened and curved body axis, microphthalmia (eye abnormality), and hydrocephalus (fluid buildup in the brain). The second morphant lacked *TEKT1* and presented situs inversus (reversed organ positions) and kidney cysts. The last morphant was a knockdown of both *WDR19* and *TEKT1*. The double knockdown morphants revealed phenotypes from both previous morphants including body curvature, microphthalmia, hydrocephalus, and laterality and otolith defects. Additionally, a dramatic loss of cilia was observed that did not occur in either of before mentioned single knockdowns. The phenotypes previously observed and the loss of cilia in zebrafish embryos reveal a synergic relationship between tektin-1 and WDR19 that have both primary and motile cilia defects (Ryan et al. 2018).

IFT-A TRAFFICKING COMPONENTS IN CILIOGENESIS

Little information is known about the materials transported by IFT-A complex and its function within cilia as compared to the extensive studies that have been conducted on the IFT-B complex. In 2017, Bing Zhu et al. conducted a study exploring the roles of IFT-A subunits (IFT43, IFT139, IFT121, IFT122, and IFT140) during ciliogenesis, while observing the effects of various IFT-A mutants in organisms such as the mouse, fly, worm, and *Chlamydomonas*. The main objectives of this study was to determine the IFT-A components that contributed to the integrity and stability of the complex and identify any additional roles of the IFT-A complex (Zhu et al. 2017).

Null mutants of subunits IFT43 and IFT139 were developed to determine if their loss would negatively affect the stability of other contributing IFT-A components. In IFT139, the null mutant resulted in cells that were aflagellate or presented stumpy flagella, as well as a reduction of other IFT-A subunits. Similarly, the loss of IFT43 caused a decrease in other IFT-A components, lack of flagella, and some short flagella. In this study, IFT43 was determined to have more profound effects within the complex and cellular protein levels, but both are needed for complete stability of the complex. These results support that having stability of IFT-A protein levels are crucial for the integrity of the complex. Additionally, IFT43 was found responsible for the localization of the IFT-A complex in the cell body. This was determined by contrasting the IFT43 null mutant with the IFT43Δ136 mutant, which has a partial deletion of the IFT43 conserved domain. In the IFT43 null mutant, the supporting cellular proteins involved with the IFT-A complex were diminished and the localization of IFT54 was altered, however, this did not occur when observing the IFT43Δ136 mutant (Zhu et al. 2017).

The IFT-A complex was thought to regulate retrograde direction in IFT but based on results of this study it is predicted that the complex also plays a role in anterograde IFT and transport of ciliary precursors. By generating null mutants of IFT43 and IFT140, flagellar regrowth could be evaluated. In these mutants, a delay occurred in flagellar regeneration when the new protein synthesis was blocked. Also, when elongation is induced by LiCl flagellar precursors are meant to be taken and used from the cytoplasmic pool of proteins, but this did not occur in IFT43 and IFT140 mutants. This finding supported the idea that IFT-A plays a role in transport of flagellar precursors from the cytoplasmic pool to the flagellar base, similar to IFT-B complex (Zhu et al. 2017).

IMMUNE SYNAPSE OF T-CELLS

The immune synapse is a stable contact surface between the T-cell and an antigen presenting cell (APC), a classification of cells that mediate cellular response by presenting antigens for recognition purposes (Finetti et al. 2011). This interface is comprised of two concentric rings known as central and peripheral supramolecular activation clusters, which characterize the T-cell receptor (TCR). The central supramolecular activation cluster (cSMAC) is thought to be a site for receptor internalization and degradation, which is necessary for long-term T-cell response regulation. Additionally, the peripheral supramolecular activation cluster, (pSMAC) forms a sealing ring in order to prevent leakage of cytolytic granule contents. These units together initiate the priming responses of the T-cell receiving information and effector functions of sending information. The immune synapse also allows for polarized delivery of granules to target cells by T-helper cells, cytotoxic lymphocytes, and regulatory T-cells in order to fight virally infected cells (Dustin et al. 2010).

The immunological synapse resembles neurological synapses and structures in regions of the plasma membrane known to be focal points for endocytosis and exocytosis during cilium formation and cytokinesis (Griffiths et al. 2010). The immune synapse forms at the same site as centrosomal docking at the plasma membrane, which has been previously characterized in primary cilia formation. In fully formed primary cilia the appendages of the mother centriole and the plasma membrane share physical links. In the process of cytokinesis, the centrosome also makes contact with the plasma membrane following the separation of the centrioles and the relocation of the mother centriole to the bridge. Contrastingly in the immunological synapse, the centrioles reposition at the synapse,

however, there is no identified preference for the mother or daughter to make contact at the plasma membrane. This may be a contributing factor as to why these cells form a frustrated cilium at the synapse, which is characterized as a bump in the membrane where the centriole contacts the plasma membrane (Griffiths et al. 2010). Though a complete cilium is not fully formed in immune T-cells, it was thought that IFT proteins may be found to localize at the synapse to aid in the beginning processes of formation and polarization.

SIMILARITIES BETWEEN FRUSTRATED AND PRIMARY CILIA IN T-CELLS

Although hematopoietic cells (stem cells that form other blood cells) lack a primary cilium, the position of the centriole and Golgi apparatus are located where a ciliary base would be present in non-hematopoietic cells with a primary cilium. Studies using electron micrographs of cytotoxic T-cells have not shown any projected appendages, however, a small bump in the membrane over the centriole location has been identified. Even though there is a complete lack of a functional, protruding cilium, this bump within the membrane where the centriole contacts the plasma membrane has been titled as a "frustrated cilium" (Finetti et al. 2011). Along with the similarities in centriole positioning, the Golgi apparatus, needed for polarization in the immunological synapse, has been identified near the plasma membrane at the site of a frustrated cilium formation like within primary cilia (Griffiths et al. 2010).

Despite T-cells not having proper cilia, the centriole and Golgi apparatus structures allow the cell to have functional properties of cilia at the synapse. This specific positioning is critical for the growth of the cilium and assembly of the synapse. The location of these

two structures also serve as signaling platforms in the cell because of a specialized membrane with a diverse protein composition to enrich the receptors and signaling mediators. Lastly, the cilia sites and immune synapse are found to have high levels of vesicular trafficking and targeted exocytosis, which is microtubular dependent and provides movement of proteins to and from the centriole and basal body (Finetti et, al, 2011).

IFT20 PROTEIN NECESSARY FOR POLARIZATION AND RECYCLING OF TCR/CD3 COMPLEX AT IMMUNE SYNAPSE

The study of "Intraflagellar Transport is Required for Polarized Recycling of the TCR/CD3 Complex to the Immune Synapse", investigated the expression of IFT proteins in lymphoid (peripheral blood lymphocytes, Jurkat T-lymphoma cells) and myeloid (monocytes, monocyte-derived DC) cells lacking primary cilia. Though IFT is governed by IFT particles and motor proteins in ciliary assembly of most eukaryotic cells, protein IFT20 was found to be necessary for the polarization of TCR/CD3 complexes in T-lymphocytes. The conducted research identified novel IFT proteins in the regulation of immune synapse assembly for T-cells as well as determining additional roles for IFT outside of ciliogenesis (Finetti et al. 2009).

Prior to the immune synapse assembling, the T-cell must be activated in order to form the interface with the antigen-presenting cell (APC). The interaction of the TCR by peptide antigen association with the major histocompatibility complex (MHC) initiates the formation of the immune synapse, which is critical for T-cell signaling. During the emerging of the immune synapse the microtubule organizing center (MTOC) is reoriented towards the APC, which is necessary for targeted delivery of signaling

molecules. One of the most important signaling molecules to be delivered to the immune synapse is the TCR/CD3 complex through polarized recycling. This cluster complex is responsible for the activation of cytotoxic T-cells and T-helper cells (Finetti et al. 2009).

To determine if any IFT proteins were found during activation of the immune synapse at the MTOC, immunofluorescence was used to analyze both human and mouse cells for the presence of IFT20. In Jurkat and human peripheral blood lymphocytes (PBL) IFT20 was found to localize in the cytosolic region centered around the MTOC during duplication in prophase, however, in metaphase and anaphase IFT20 staining was undetectable apart from two small structures near the MTOCs. IFT20 was found to reassemble during late telophase and cytokinesis, and then surround the MTOC. Additionally, the movement pattern of IFT20 localization is strikingly similar to that of the Golgi with the co-staining of Jurkat cells, which showed about 60% colocalization of IFT20 with the cis-Golgi. Upon induced fragmentation with treatment of brefeldin-A, IFT20 was also found to colocalize to smaller extents with the trans-Golgi network, recycling endosomes, early endosomes, and lysosomes (Finetti et al. 2009).

As previously stated, the formation of the immune synapse is dependent on the reorientation of the MTOC and the Golgi. To determine the role of IFT20 in this formation event, the gene coding for IFT20 was stably knocked down in Jurkat cells via RNA interference. In the generated IFT20-KD cells the MTOC and Golgi successfully translocated to the immune synapse, but immunostaining showed the TCR clustering was impaired. In the control cells with IFT20 TCR clustering was ~70%, where as in IFT20-KD cells displayed ~40% clustering. This experiment was replicated again, however, immunostaining of the CD3 occurred. The results were similar in that the IFT20-KD cells

revealed much less clustering than the control cells. These findings support the idea that IFT20 is needed for the trafficking of TCR/CD3 to the immune synapse during formation, but not for the translocation of the MTOC or Golgi (Finetti et al. 2009).

The assembly of the TCR/CD3 complex begins in the ER, undergoes modifications in the Golgi, and is finally transported to the cell surface. The TCR/CD3 complex after exposure on the surface goes through many rounds through the constitutive recycling pathway, which is an exocytosis pathway involving a di-leucine motif on the CD3γ cytosolic tail where it is targeted for degradation. To investigate the potential role of IFT20 in this process phorbol esters (PBDu) were used to activate a pathway dependent on PKC-mediated activation of the CD3γ di-leucine motif leading to TCR recycling. Both the control cells and previously generated IFT20-KD cells were treated with PBDu for one hour each resulting in ~40% TCR internalization. PBDu was then removed to allow CD3 recovery and was analyzed using flow cytometry. The results revealed that IFT20-KD cells there was no increase in surface CD3, but within the control cells surface CD3 was completely recovered. To further support the proposed role of IFT20 in the recycling of the TCR/CD3 complex, control and IFT20-KD cells were incubated at 20°C for sixteen hours in order to block trafficking and then for four hours at 37°C. When the temperature was increased to 37°C the control cells were able to recover surface CD3, but this was not possible in IFT20-KD cells. This evidence supports that IFT20 is not needed for the internalization of the complete TCR/CD3 complex, but vital to the recycling of the TCR/CD3 complex to the cell surface through an exocytosis pathway (Finetti et al. 2009).

This experiment also focused on recycling endosomes because they have been found to polarize the immune synapse by increasing TCR/CD3 levels. In control cells recycling endosomes appeared normal and the immune synapse was polarized. Surprisingly, in IFT20-KD cells recycling endosomes were unsuccessful in polarizing the immune synapse. This was replicated in peripheral T-cells with IFT20 knocked down. This data indicates that IFT20 is also required for the polarized TCR/CD3 recycling (Finetti et al. 2009).

The last portion of the research focused on the interaction between other IFT proteins with IFT20. As previously discussed, IFT20 is a member of the IFT-B complex, and immunoblotting blotting was used to determine expression of other IFT-B complex components. Human and mouse cells expressed IFT88 and IFT57, and a motor protein involved in anterograde IFT known as kinesin Kif3a. Immunofluorescence showed that IFT57 colocalized to IFT20 in resting cells near the TCR and the immune synapse. Additionally, proteins IFT20, IFT88, and IFT57 were found to form a complex, and during analysis with coimmunoprecipitation the presence of the complex increased in TCR engagement. Surprisingly, in IFT20-KD cells interactions with the TCR/CD3 complex and proteins IFT88 and IFT57 were impaired. IFT57 was also not transported to the immune synapse in IFT20-KD cells. The loss of IFT protein recruitment and the impaired interactions with the TCR/CD3 complex because of the knockdown of IFT20, supports that IFT20 drives the assembly of other IFT proteins in T-cells during TCR engagement and activation (Finetti et al. 2009).

IFT20, IFT57, AND IFT52 ASSOCIATION WITH EARLY ENDOSOMES RECYCLING PATHWAYS

Another extensive study was conducted by Finetti et al. in 2014, to determine further roles of IFT and IFT particles in areas of receptor cycling, networks with Rab GTPase and endosome association, and IFT20 interactions with other IFT proteins. The major role of IFT20 has been classified as marking membrane proteins in primary cilia and aiding in their delivery to the cilia and displays vesicular localization. IFT20 has not been the only IFT protein to have vesicular localization, both IFT57 and IFT52 also localize in a similar region (Finetti et al. 2009). In this study, IFT20, IFT57, and IFT52 were analyzed to understand their potential roles in coordinating the pathway used to recycle receptors at the immune synapse (Finetti et al. 2014).

In the polarized recycling pathway, receptors are sorted from $Rab5^+$ early endosomes and are later directed to the cell surface in $Rab4^+$ endosomes, or $Rab11^+$ pericentrosomal compartments for a longer route to the cell surface. Rab5, Rab4, and Rab11 are all classified as early and recycling endosome proteins. As described above, the protein IFT20 has already been identified as a contributor in the recycling of TCR receptors during immune synapse formation, however, by using confocal microscopy on Jurkat T-cells and peripheral T-cells transfected with Rab-GFP colocalization in the recycling pathway of endosomes was determined (Finetti et al. 2009). A majority of the IFT20 protein pool was revealed to be highly associated with early and recycling endosomes supporting that IFT20 is prevalent in regulating endosome recycling with Rab-based trafficking machinery. To assess IFT20 interactions with Rab GTPases, Rab4, Rab5, and Rab11, co-immunoprecipitation with Jurkat T-cells expressing GFP-tagged IFT20 was

analyzed. Minimal interaction of IFT20 was found with Rab11 and Rab4, but it had significant interaction with Rab5 (Finetti et al. 2014).

Rab5 has previously been identified to co-precipitate with the TCR in activated control cells. This was determined through immunoblot with anti-CD3 antibodies, and in IFT20-KD cells interaction between Rab5 and TCRs were impaired. The result of this impaired interaction supports that IFT20 could be implicated during the coupling of Rab5 to TCRs that are internalized at the cell surface once reaching early endosomes (Finetti et al. 2009). To examine if IFT20 is implicated during directed internalization, IFT20-KD cells were tested for colocalization of internalized TCRs with Rab5. The prediction was an expected block leading to the build-up of recycling TCRs in the Rab5 compartment without IFT20. In IFT20-KD cells there was an increased colocalization of TCR with Rab5, which supports that IFT20 is responsible for directing the transit of internalized TCRs to recycling endosomes (Finetti et al. 2014).

IFT20 is necessary for the recycling of the TCR/CD3 complex in the immune synapse and has been predicted to be involved with further recycling pathways for TCR, TfR, and CXCR4 receptors because of its association with different Rab networks. The TCR receptor mainly associates with the $Rab11^{+}$ and $Rab4^{+}$ endosomes. TfR and CXCR4 are both chemokine receptors that recycle through both $Rab4^{-}$ and $Rab11^{-}$ dependent routes. IFT20-KD and control cells were studied with flow cytometry using fluorochrome-labeled secondary antibodies. IFT20-KD cells resulted in impairment of TCR and TfR recycling, however, no changes occurred in CXCR4 recycling. A second experiment was conducted to study the recovery of surface receptors, and again IFT20-KD cells were unable to recover TCR and TfR receptors, but CXCR4 receptors were recovered without

issue. These results support that IFT20 is essential to the recycling of TCR and TfR receptors, but not for CXCR4 receptors. As a complimentary experiment, IFT was restored in IFT20-KD cells. This completely rescued TCR and TfR receptors and eliminated all recycling defects such as accumulation in recycling pathways (Finetti et al. 2014).

As discussed in the previous section, the complex IFT20-IFT57-IFT88 was discovered to increase in response to TCR stimulation and IFT20 expression was required (Finetti et al. 2009). Based on how IFT20 has the capability to lead and incorporate other IFT proteins, a screening was conducted to evaluate the expression of all known IFT proteins during endosome recycling in Jurkat cells and HEK293 ciliated cells as a control. IFT-B complex proteins IFT57 and IFT52 involvement in receptor recycling were focused on. Immunofluorescence directed at IFT57 proteins showed vesicular overlapping in Jurkat T-cells transfected with Rab—GFP, and strong colocalization with the centrosome. Since IFT57 was not found to localize with the Golgi, but instead have vesicular patterns it indicates IFT57 being restricted to post-Golgi compartments (Finetti et al. 2014).

By determining the location of IFT57, further steps were then taken to investigate the possible implications of knocking down this IFT protein in receptor recycling. This was done by using RNA interference to generate about a 40% IFT57 knock down in Jurkat cells. Flow cytometric analysis showed that a deficiency in IFT57 led to defects in recycling and polarization at the immune synapse in TCR and TfR, but not CXCR4, similar to IFT20-KD cells. These defects were confirmed by imaging internalized receptors, and in IFT57 there was heavy endosomal accumulation of TCR and TfR. The assembly of a functional immune synapse was not possible in IFT57-KD cells, which was

evaluated via staining of the APC. To evaluate the interactions between IFT57 and IFT20, IFT20-KD cells had IFT57 knocked down about 50%, however, the experiment did not lead to a more severe recycling defect. Although the recycling defects occurred at the same magnitude, with reduced IFT57 presence, IFT20 was not able to polarize to the immune synapse. This suggests that IFT20 and IFT57 need to coordinate for the proper assembly of the immune synapse (Finetti et al. 2014).

Lastly, a similar experiment was conducted with a knock down of IFT52 to analyze receptor recycling. This knock down also resulted in impaired TCR and TfR, but not CXCR4 recycling just like IFT20-KD and IFT57-KD. In IFT52-KD cells the defect in TfR recycling was to a lesser extent than in IFT57-KD and IFT20-KD, but deficiency in IFT52 also resulted in impaired TCR polarization at the immune synapse. The results of IFT20-KD, IFT57-KD, and IFT52-KD cells support that IFT proteins in the IFT system function in the same endosome recycling pathway, and that they must work together for the complete polarization to the immune system in TCR and TfR recycling (Finetti et al. 2014).

SIGNIFICANCE OF IFT PROTEINS RECYCLING IN T-CELLS

IFT20 is a prime contributor to the recycling of TCR and TfR that interacts with both Rab-GTPases and IFT proteins; IFT57 and IFT52. The absence of IFT20 was found to create a severe blockage and build-up of recycling TCRs in the Rab5 compartment, which supports the hypothesis that IFT20 is responsible for directing the transit of internalized TCRs to recycling endosomes. Similarly, IFT20-KD cells were unable to recover TCR and TfR receptors, but when IFT20 was restored complete recovery for both receptors occurred and there were no longer recycling defects in the pathways. This

shows that IFT20 is a key component necessary for proper recycling and receptor recovery (Finetti et al. 2014).

Additionally, deficiency of IFT57 and IFT52 proteins also lead to defects in recycling and polarization to the immune synapse of TCR and TfR. In IFT20-KD cells with deficient levels of IFT57 recycling defects were not made worse, but IFT20 was not able to polarize to the immune synapse. This revealed that IFT20 and IFT57 must be able to associate together for the proper assembly of the immune synapse. The last IFT protein to be studied was IFT52, which also led to impairments of TCR and TfR recycling pathways. When compared to the loss of IFT57, there were more negative effects to the formation of the immune synapse. The findings from this research showed that multiple IFT proteins function in the same endosome recycling pathway for a role other than ciliogenesis, and aid in the generation of critical structures in T-cell lymphocytes (Finetti et al. 2014).

CONCLUSIVE SUMMARY

The microtubule based organelles, cilia, are categorized into two subgroups—motile and non-motile, which are responsible for sensory and motility aspects of the cell. Non-motile cilia serve in controlling key signalizing pathways in development, sensing extracellular signals, and transmission of signals from the cilium to the cytoplasm to control gene expression and cell behavior. Motile cilia are present on specialized cells for fluid displacement and control cell locomotion. These two subgroups can be differentiated based on the structural organization of the axonemes. In non-motile cilia, the axonemes have nine pairs of peripheral microtubule-doublets with no microtubule central pair, which results in the “9 + 0” arrangement. Motile cilia are composed of nine pairs of

peripheral microtubule-doublets with a microtubule central pair providing a “9 + 2” arrangement (Ryan et al. 2018; Prevo et al. 2017). Cilia are formed by the movement of proteins by intraflagellar transport (IFT), which is a motor dependent cargo system that is crucial in the assembly, maintenance, and elongation of cilia (Lechtreck et al. 2009).

The IFT system has two subcomplexes—IFT-A and IFT-B. The IFT-A was thought to regulate retrograde direction in IFT, but further studies showed that it also plays a role in anterograde IFT and transport of ciliary precursors. The IFT-B complex has responsibilities in anterograde, outward movement, of traffic and materials during elongation and maintenance of cilia (Prevo et al. 2017). These complexes form a coupled system and oftentimes acts in both directions—retrograde and anterograde trafficking. When uncoupled by the loss of essential IFT proteins, the complexes are unable to form fully functional cilia, resulting in disorganized axonemes, defects in fibers and fiber sheaths, and vesicles in the lumen of the flagella (Huet et al. 2019). Deregulation of the IFT system is not the only mode of interrupting proper formation of the cilia. When mutations occur in tektin proteins the structural integrity of the cilia is also compromised (Ryan et al. 2018).

Apart from the commonly known motile and non-motile forms of cilia, some cells such as cytotoxic T-cells present frustrated cilia. This category of cilia do not have any projected appendages but form a small bump in the plasma membrane over the location of the centrosomal docking region in primary cilia formation. Instead of T-cells forming a cilium in this area the immune synapse is developed, however, IFT proteins have been found to localize here to aid in the completion and polarization of the synapse (Griffiths et al. 2010).

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