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The Identification of Notch1 Functional Domains Responsible for its Physical Interaction with PKC θ

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THE IDENTIFICATION OF NOTCH1 FUNCTIONAL DOMAINS RESPONSIBLE FOR ITS PHYSICAL
INTERACTION WITH PKC θ

A Thesis Presented

By

WESLEY D. ROSSITER

Submitted to the Graduate School of the
University of Massachusetts Amherst in partial fulfillment
of the requirements for the degree of

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Molecular And Cellular Biology

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ABSTRACT

THE IDENTIFICATION OF NOTCH1 FUNCTIONAL DOMAINS RESPONSIBLE FOR ITS PHYSICAL INTERACTION WITH PKC θ

FEBRUARY 2016

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The adaptive immune system is a complex network of cells that protect the body from invasion by foreign pathogens. Crucial to the function of the adaptive immune system is the activation, proliferation and differentiation of T cells in response to foreign pathogen presentation by antigen presenting cells. T cell activation is driven through different signaling pathways that are dependent on phosphorylation of substrates by kinases. In the PLC pathway that activates the *il2* gene program, Protein Kinase C- θ (PKC θ) and Notch1 localize to the immunological synapse and help drive the signaling cascade that leads to robust T cell activation. It has been previously shown that PKC θ and Notch1, both interact with the CBM complex at the immunological synapse. Additionally, PKC θ and Notch1 both have specific cytoplasmic and nuclear functions that help drive the *il2* gene program. Here, we demonstrate the localization of PKC θ and Notch1 constructs transfected into HEK 293 cells. The use of deletion constructs of Notch1 was intended to inform us of what functional domain of Notch1 was responsible for the interaction with PKC θ , however no direct interaction was demonstrated with the PKC θ and Notch1 constructs used in these experiments. We hypothesize that this is likely due to the inactive form of PKC θ found in our construct, or a result of the cell type used in these experiments.

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CHAPTER 1

THE ROLE OF NOTCH AND PKC θ SIGNALING IN THE DEVELOPMENT, ACTIVATION AND POLARIZATION OF THE ADAPTIVE IMMUNE RESPONSE

1.1. Adaptive Immune Response

1.1.1. Introduction

The adaptive immune system is an extremely complex network of specific cells and processes that protect the body against invasion from foreign pathogens. Crucial to the function of the adaptive immune system, also called the acquired immune system, is the ability to maintain self-tolerance while maintaining surveillance to foreign invaders. The adaptive immune system can be divided into two components, humoral immunity and cell-mediated immunity. The key lymphocyte in the cell-mediated component of the adaptive immune system is the T cell. T cells undergo a stringent selection process to produce a functional antigen-binding receptor known as the T-cell receptor (TCR). T cells expressing the TCR recognize a specific antigen bound to a membrane protein of an antigen-presenting cell (APC) called major histocompatibility complex (MHC) molecules.

The development of T cells begins in the bone marrow, where hemapotoietic stem cells (HSCs) continuously self-renew until their differentiation into specific cell types is initiated through a vast array of signals that drive the HSCs towards a particular fate. In the bone marrow, several types of cells are responsible for directing HSC development including osteoblasts, endothelial cells, reticular cells, and sympathetic neurons (Wang et al., 2011). Micro-niches within the bone marrow play an important role in HSC development by providing different environmental signals to HSCs. In the endosteal niche HSCs continuously self-renew, whereas HSCs in the vascular niche begin to differentiate or circulate to other tissues (Morrison et al., 2008). T-cell precursors mobilize from the bone marrow and travel through the circulatory system to the thymus where they assume the name thymocytes. Once inside the thymus, thymocytes begin to mature into T cells expressing unique

antigen-specific receptors, TCRs. At this stage in development thymocytes express both CD4 and CD8 receptors and are referred to as double positive T cells. Positive selection is the first stage of thymocyte development in the thymus following the generation of their TCRs, in which thymocytes with an intermediate affinity for the self MHC-peptide complexes presented by thymic epithelial cells are positively selected. Thymocytes with too low an affinity for the self MHC-peptide complexes die from neglect in the thymus (Klien et al., 2009). The second stage of selection ensures the TCR on developing thymocytes does not bind self MHC-peptide complexes with too high affinity, in a process called negative selection. Thymocytes that react too strongly to the self MHC-peptide complexes are induced to die, preventing immune-mediated destruction of healthy, normal tissue. Mature thymocytes that have the appropriate affinity for the self MHC-peptide complexes express either a CD4 or CD8 receptor and are referred to as single positive mature thymocytes. These cells circulate in the peripheral blood and become activated to expand and differentiate further following the presentation of a foreign peptide by an APC.

1.1.2 T Cell Activation and Polarization

T cells that have undergone selection in the thymus and enter the periphery are called naïve T cells until they encounter an antigen. Naïve T cells circulate through secondary lymphoid organs such as the spleen or lymph nodes until they become activated through the engagement of their TCR through the presentation of an antigen loaded onto a self MHC molecule that is recognized by the T cells' CD4 or CD8 receptor. Complete activation of T cells requires a co-stimulatory signal and the presence of stimulatory molecules called cytokines. The CD28 co-stimulatory receptor on T cells is responsible for providing a secondary signal that drives robust activation when it binds the co-stimulatory ligand CD80(B7-1)/CD86(B7-2) expressed on the surface of Dendritic cells (DCs) (Nunes et al., 1996). Following the engagement of the TCR together with co-stimulatory signals, T cells initiate a signaling cascade characterized by several phosphorylation events and localization of proteins at the cell membrane that ultimately drive an alteration in the transcriptional program of

the cell. The phospholipase C (PLC) pathway in T cells activates the gene program for interleukin 2(IL-2), a cytokine that helps drive T cell proliferation (Schwartz, 1990). The transcription factors necessary to drive the *IL2* gene program are activated protein-1 (AP-1), nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B), and nuclear factor of activated T cells (NFAT). These transcription factors are only able to localize to the *IL2* gene following a signaling cascade initiated by a sustained TCR engagement and phosphorylation events at the immunological synapse. After 24-48 hours following successful TCR engagement, T cells up-regulate the transcription of *IL2 α* , the high affinity IL-2 receptor (CD25), and pro-survival genes.

The polarization of activated T cells depends on the signals received from cytokines present during the activation. T cells come in a variety of subsets, divided into two groups based on their recognition of MHC class I or II. Cytotoxic CD8⁺ T cells (CTLs) express the CD8 receptor that recognizes MHC class I molecules. The MHC class I molecule is ubiquitously expressed by cells to prevent CTL-mediated destruction of normal cells, as they are constantly presenting self-antigen which does not elicit an immune response. Aberrant cells lacking MHC class I molecules will be properly identified and eradicated by natural killer cells. The second subtype of T cells is CD4⁺ T helper cells that express the CD4 receptor. The CD4 receptor recognizes MHC class II molecules on the surface of APCs. CD4⁺ T cells can be further subdivided into different classes characterized by the panels of cytokines they secrete, which provide specific signals to CD8⁺ T cells and B cells subject to a milieu of cytokines. The CD4⁺ T cells polarize into five main types (T_{H1}, T_{H2}, T_{H17}, T_{REG}, and T_{FH}) aided by the polarizing cytokines produced by the APCs during TCR engagement (Zhu & Paul, 2010). The driving force behind T_H subset polarization is the interaction between the pathogen and the pattern recognition receptors (PRRs) on the APC. Master gene regulators are activated to respond to different types of pathogens, resulting in the production of specific polarizing cytokines.

1.2 PKC

1.2.1 PKC Family and Structure

Upon activation, the Protein Kinase C (PKC) family specifically phosphorylates serine and threonine residues on target substrates. The PKC isoenzymes are classified into three categories based on their mechanism of activation: the conventional PKC family (α , β , and γ), the novel PKC family (δ , ϵ , η , and θ), and the atypical PKC family (λ and ζ). Activation of conventional PKC family members requires calcium and diacylglycerol (DAG), whereas activation of novel PKC family members is calcium-independent (Koivunen et al., 2006). Activation of atypical PKC family members is independent of calcium and of DAG.

PKC θ has been shown to play a critical role in T cell activation and its structure is relatively conserved across other members of the novel PKC family. The overall structure contains an NH₂ terminus, C2-like domain, pseudosubstrate (PS) domain, DAG binding domain (C1), unique V3 domain and kinase domain, depicted in Figure 1.1 (Xu et al., 2004). In the inactive form of PKC θ , the PS domain remains bound to the kinase domain preventing PKC θ from phosphorylating substrates.

1.2.2 PKC θ Signaling

In the PLC signaling pathway of T cells, PLC γ 1 breaks down phosphatidylinositol 4,5-bisphosphate PIP₂, generating IP₃ and DAG. DAG induces a conformational change in PKC θ prior to binding PKC θ , creating the active form of PKC θ . Critical to PKC θ activation is phosphorylation of a tyrosine residue in the C2-like domain by the kinase Lck, however it remains unclear at which stage this event takes place during PKC θ activation (Cartwright et al., 2011). Fully activated PKC θ bound to DAG is recruited to the membrane at the immunological synapse, where PKC θ phosphorylates a series of adaptor proteins (Carma1 and TAK1 complex). The phosphorylation of the scaffolding protein, Carma1, activates the CBM complex (Carma1, Bcl10, and MALT-1), which then recruits TRAF6, an ubiquitin ligase. TRAF6 ubiquitinates the I κ B Kinase IKK complex (composed of NEMO, IKK α , and IKK β), leading to the subsequent destruction of the NF- κ B inhibitor, I κ B (Sun et al.,

2004). Active NF- κ B translocates to the nucleus driving the transcription of the *IL2* gene. Importantly, PKC θ loss-of-function experiments have shown that T cell development remains normal, while effector T cells failed to produce normal responses (Sun et al., 2000). At the transcriptional level, *IL2* expression is impaired as a result of damped signaling through the NF- κ B, AP-1 and NFAT pathways (Hayashi et al., 2007). Recently, PKC θ has been shown to mediate T cell gene expression in the nucleus in a NF- κ B-dependent manner (Sutcliffe et al., 2012). This novel function of PKC θ raises many questions about the signaling events at the immunological synapse and the interacting proteins that aid in the translocation of PKC θ into the nucleus.

1.3. Notch

1.3.1 Notch Family and Structure

The Notch family of proteins plays a role in cell fate decisions across a variety of cell types, and is especially critical to T cell activation, proliferation, and differentiation. The Notch family was named after its discovery in *Drosophila melanogaster* in 1914, after mutations in the gene caused a 'notched' wing phenotype (Morgan et al., 1917). There are four paralogs of mammalian Notch proteins (Notch1-4). Notch proteins belong to the family of type I transmembrane receptors consisting of 36 ligand binding epidermal growth factor (EGF)-like tandem repeats and 3 LIN-12/Notch (LIN) repeats in the extracellular domain. The intracellular domain contains an RBPjk-binding (RAM) domain, six tandem ankyrin (ANK) repeats, a transcriptional activation domain (TAD) and a proline/glutamate/serine/threonine-rich (PEST) sequence, depicted in Figure 1.2 (Kopan et al., 2009). Generation of a fully mature Notch protein requires post-translational cleavage of the S1 site before forming a non-covalently linked heterodimer through the association of two extracytoplasmic membrane regions at the N- and C-terminus of the protein.

1.3.2 Notch Activation and Signaling

There are two families of Notch ligands capable of binding the Notch receptor and initiating Notch signaling, Jagged (1,2) and Delta-like (1,3, & 4). After binding a Notch ligand, the extracellular

domain of the Notch receptor is cleaved by TNF- α -converting enzyme (TACE) at S2. Fully activated, intracellular Notch (Notch^{IC}) requires a third cleavage by gamma-secretase at S3, at which point Notch translocates from the cell wall to the nucleus, depicted in Figure 1.3 (LaVoie et al., 2003). In the nucleus, Notch^{IC} acts as a molecular switch, initiating gene transcription by converting the repressor CSL (CBF-1, SuH, Lag-1) into an activator (Osborne & Minter, 2007). CSL then recruits a variety of co-activators to change the gene program of the cell. CBF1/RBP-J is critical to DNA-binding in the canonical Notch signaling pathway.

Non-canonical Notch signaling proceeds independently of CSL, by activating the transcription factor NF- κ B (Perumalsamy et al., 2009). In the non-canonical pathway, Notch1^{IC} interacts with the kinase mammalian target of rapamycin (mTOR) and rapamycin independent companion of mTOR (Rictor) which results in the activation of the kinase Akt/PKB independent of CBF1/RBP-J.

1.3.3 T Cell Activation and Polarization

The Notch1 paralog plays an important role in T cell development, activation, and differentiation. The role of Notch signaling in T cell activation was first identified after stimulation with anti-CD3 and anti-CD28 antibodies resulted in increased levels of Notch^{IC}. This discovery led to the identification of Notch1 as an important player in T cell polarization, implicated in the development of T_H1, T_H2, T_H17, and T_{REG} cells. The production of the polarizing and effector cytokine for T_H1 cells, IFN- γ , decreased when Notch was inhibited through the regulation of the transcription factor T-bet. Notch activation has been implicated in polarization of multiple other families of T helper cells through a variety of other pathways, demonstrating a conserved and important role in polarization.

Notch plays an important role in the development and activation of CD8⁺ T cells by altering the gene program responsible for the expression of granzyme B and perforin. By inhibiting Notch1, the transcription factor eomesodermin (Eomes), responsible for granzyme B and perforin

production decreased (Cho, et al., 2009). Further research demonstrated that the inhibition of Notch1 resulted in impaired cytotoxic activity by CTLs. The decreased function of CTLs resulting from Notch1 inhibition indicate the critical role Notch plays in the activation and development of a robust immune response to foreign pathogens.

1.4. Notch1 and PKC θ

1.4.1 Interaction

Currently, information on the direct interaction between Notch1 and PKC θ is limited. However, using microscopy and co-immunoprecipitation, Notch1 and PKC θ have been shown to co-localize at the immunological synapse following T cell activation and both proteins physically interact with the CBM complex (Shin *et al.*, 2014). Using stimulated Jurkat T cells, Shin *et al.* was able to demonstrate an association between PKC θ and Notch1 by immunoprecipitating Notch1 and probing for PKC θ . The interaction was believed to be a result of both proteins interacting with the CBM complex.

1.4.2 Notch1 and PKC θ Regulate T cell Activation

One of the most critical transcriptional changes that occurs in stimulated T cells, is the increased expression of the *IL2* gene driven by the NF- κ B signaling pathway. It has been previously shown that Notch1 and PKC θ play direct roles in the activation of NF- κ B. The mechanism by which NF- κ B is activated by PKC θ is well characterized; however, the function of Notch1 in the pathway is not. It appears that Notch1 may act as a scaffold protein that supports the localization and formation of the CBM complex that requires phosphorylation by PKC θ to induce complete activation of the complex (Figure 1.4). The primary function of the CBM complex is to activate IKK (I κ B kinase) complex through phosphorylation and ubiquitination. The active IKK complex phosphorylates the Inhibitor of NF- κ B (I κ B) resulting in I κ B becoming inactive. Inactivation of I κ B releases NF- κ B, allowing it to translocate to the nucleus and initiate gene activation. The ability of Notch1 to bind Carma1 paralleled with PKC θ 's direct phosphorylation of the CBM complex suggests

a synergistic role of PKC θ and Notch1 in T cell activation. Studies have also demonstrated that both Notch1 and PKC θ are required for the initial formation of the CBM complex furthering the notion that these proteins are critical to driving the activation of T cells mediated by NF- κ B. Additionally, the recent discovery of a nuclear function of PKC θ in the NF- κ B-mediated gene expression further supports the notion of an interplay between Notch1 and PKC θ , outside of their role in the formation of the CBM complex.

1.5 Aim of the study

1.5.1 Rationale

The activation and polarization of T cells is not only a crucial aspect of providing protection against foreign pathogens, but has therapeutic implications across several disease categories including cancer and autoimmune disease. Notch1 and PKC θ regulate the maturation and activation of CD8⁺ T cells as well as the activation and polarization of CD4⁺ T cells through the activation of the transcription factor NF- κ B. Outside of their unique functions in the assembly of the CBM complex, no direct interaction between Notch1 and PKC θ has been demonstrated that would identify their relationship in time and space within T cells, which appears to be seamlessly intertwined during T cell activation.

1.5.2 Hypothesis

In this study, we hypothesize that a conserved domain on Notch1 is responsible for interacting with PKC θ in the activation of T cells.

1.5.3 Experimental Approach

Previous studies that demonstrated Notch1 and PKC θ interacting were performed in T cell lines that contained the proteins that make up the CBM complex. In order to demonstrate a direct interaction, without the formation of the CBM complex, a HEK 293 cell line was selected to control for a non-direct interaction. The HEK 293 cell line does not express Notch1 and PKC θ , thus these proteins were expressed only after successful transfection of plasmid DNA encoding Notch1 and

PKC θ plasmids. The HEK 293 cell line also has the advantage of lacking all members of the CBM complex, which may result in the indirect interaction of Notch and PKC θ . The Notch1^{IC} construct contains an N-terminus GFP tag and a C-terminus Nuclear Export Signal (NES) (**LALKLAGLDL**) and Myc tag (EQKLISEEDL) in a pEGFP-C1 vector. The PKC θ construct was tagged on the C-terminus with FLAG in a pCMV6-Entry vector. After a direct interaction between Notch1 and PKC θ is established, Notch1 deletion constructs (Figure 1.5) generated by Dr. Hyun Mu Shin will be used to identify the conserved region on Notch1 responsible for binding PKC θ .

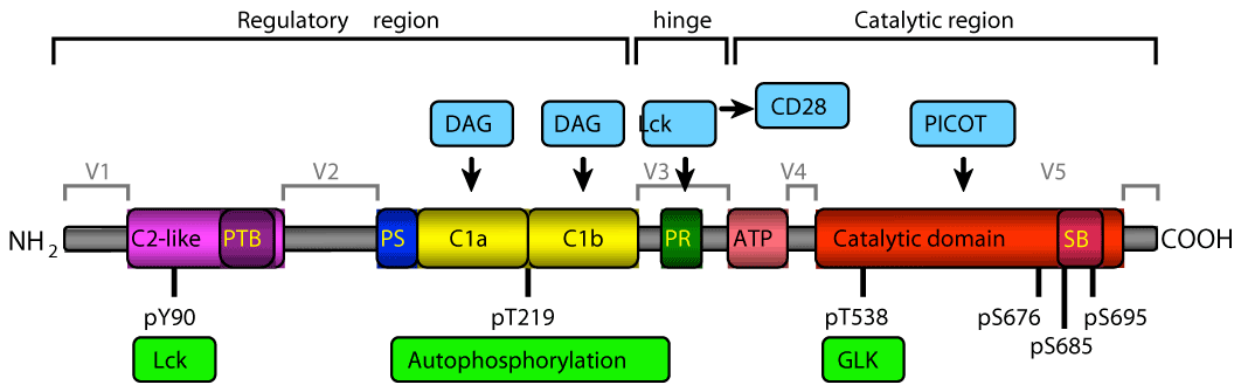


Figure 1.1: PKCθ Structure and Phosphorylation Sites

A diagram showing the domains PKCθ, containing phosphorylation sites and the catalytic region responsible for PKCθ's kinase activity. Lck phosphorylates a tyrosine residue within the C2 domain upon T cell activation. The C1 domain binds DAG, allowing the conformational change in PKCθ to form active PKCθ. (Isakov et al. J Clin Cell Immunol 2012)

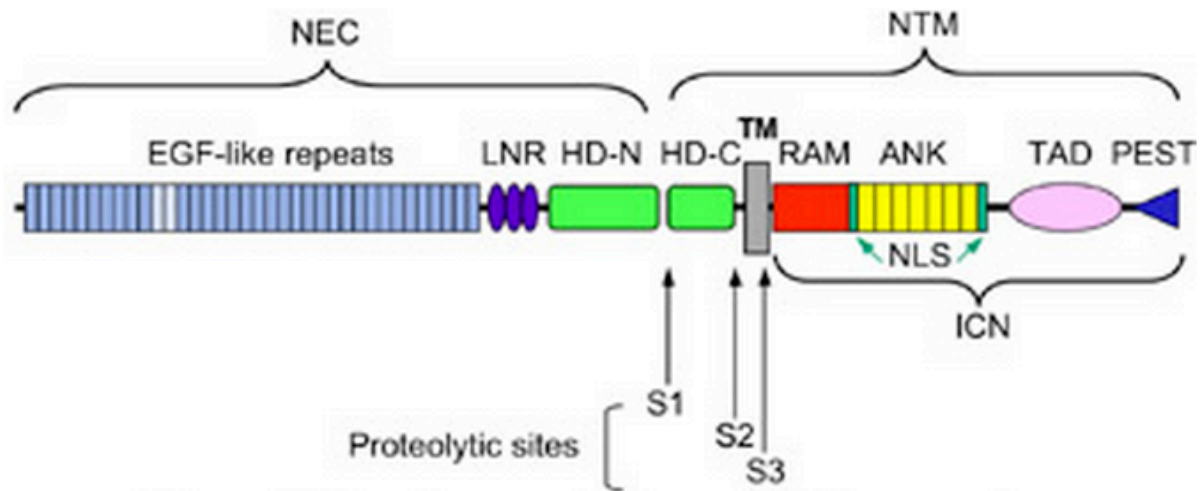


Figure 1.2: Notch1 Receptor Domain organization

The mammalian Notch1 receptor contains epidermal growth-factor-like repeats (EGF-like repeats). The EGF-like repeats bind to signaling ligands (Jagged and Delta-like). The 7 Ankyrin (Ank) repeats bind NF- κ B during T cell activation. (Blacklow et al., 2015)

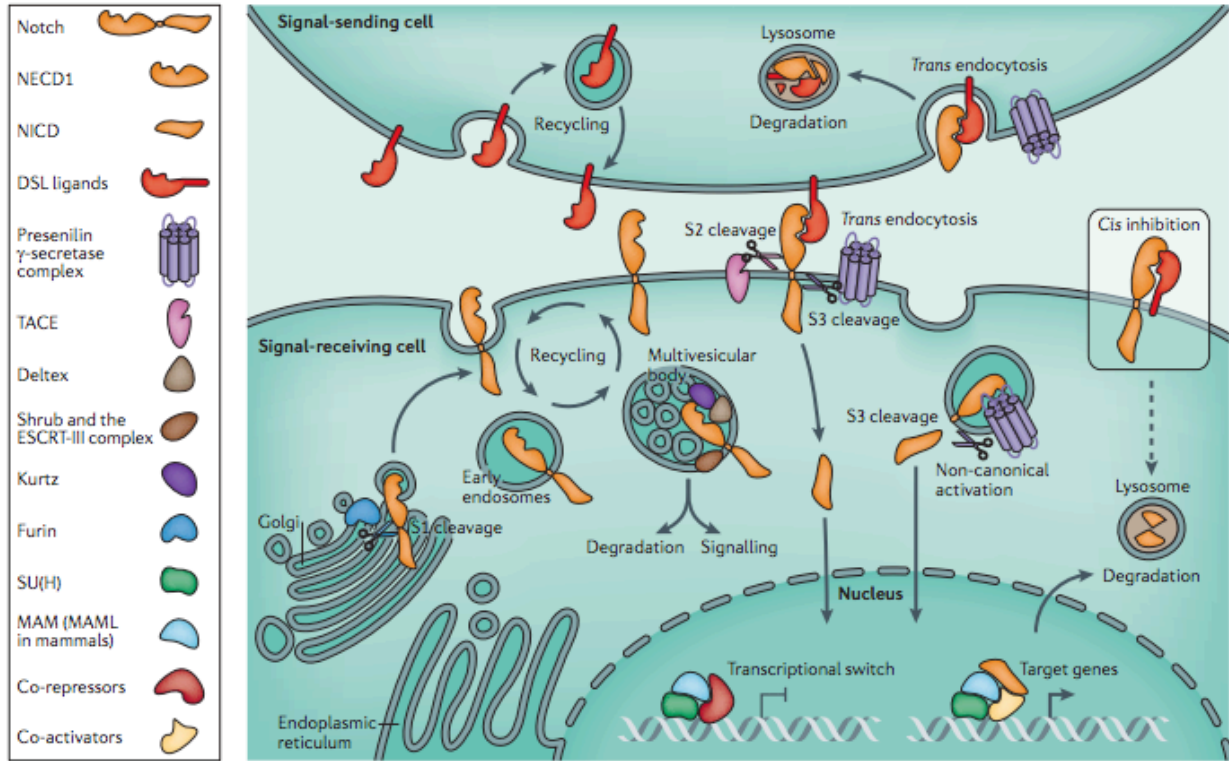


Figure 1.3 Notch1 Cleavages and Transcriptional Activation

Notch1 is cleaved after engagement by a Notch1 ligand, in a process mediated by TACE and gamma-secretase. Notch1 translocates to the nucleus where it binds CSL after cleavage. In the nucleus, Notch1 activates downstream targets that drive transcription. (Artavanis-Tsakonas et al., 2012)

NOTCH1

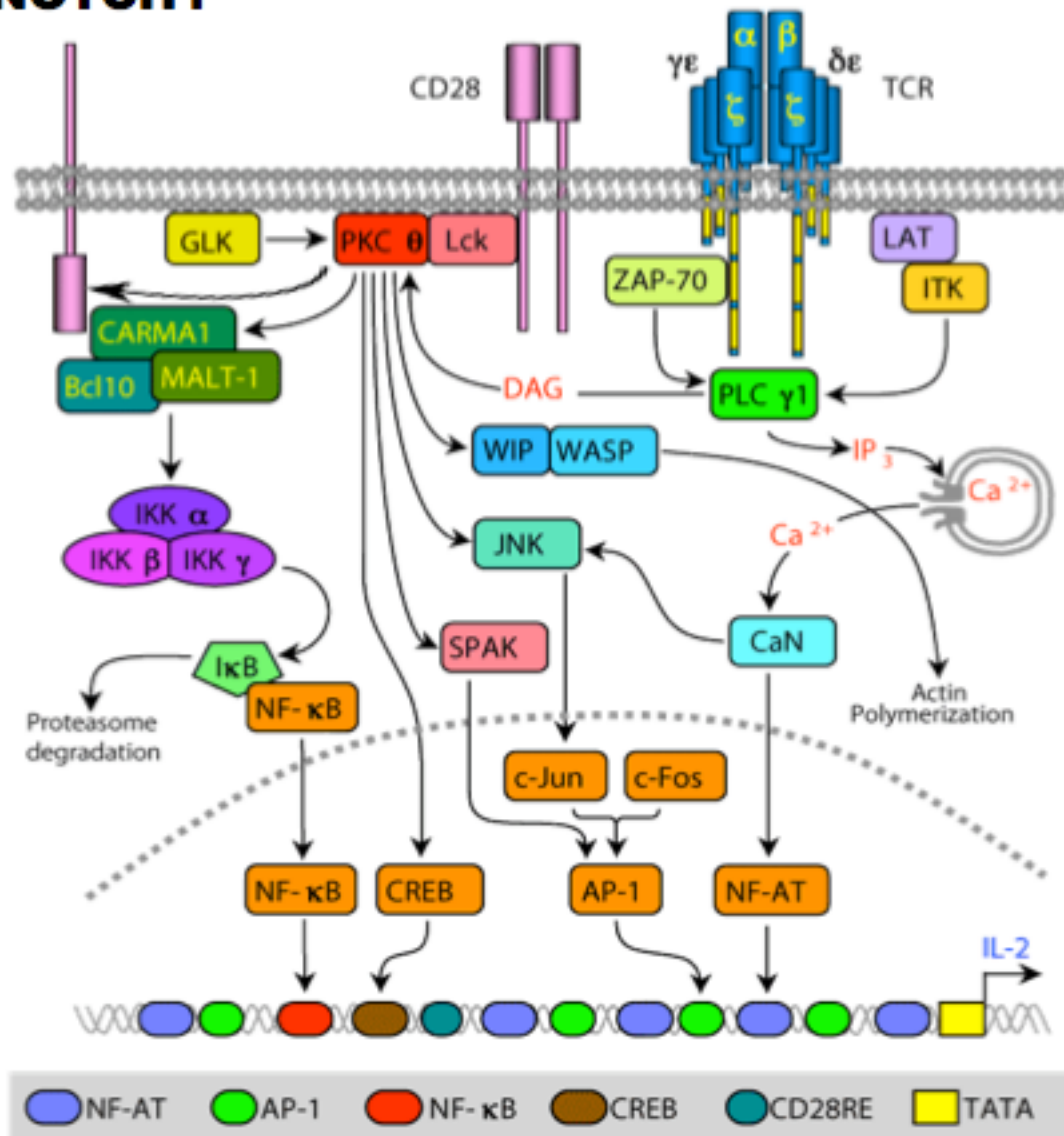


Figure 1.4: Notch1 and PKCθ signaling pathway

Following engagement of the T cell receptor and CD28 receptor, a series of phosphorylation events recruits PKCθ to the immunological synapse. Active PKCθ leads to the activation of the IKK complex, driving the degradation of the IκB complex. The release subsequent release of NF-κB drives the *il2* gene program. (Modified from Isakov et al. J Clin Cell Immunol 2012)

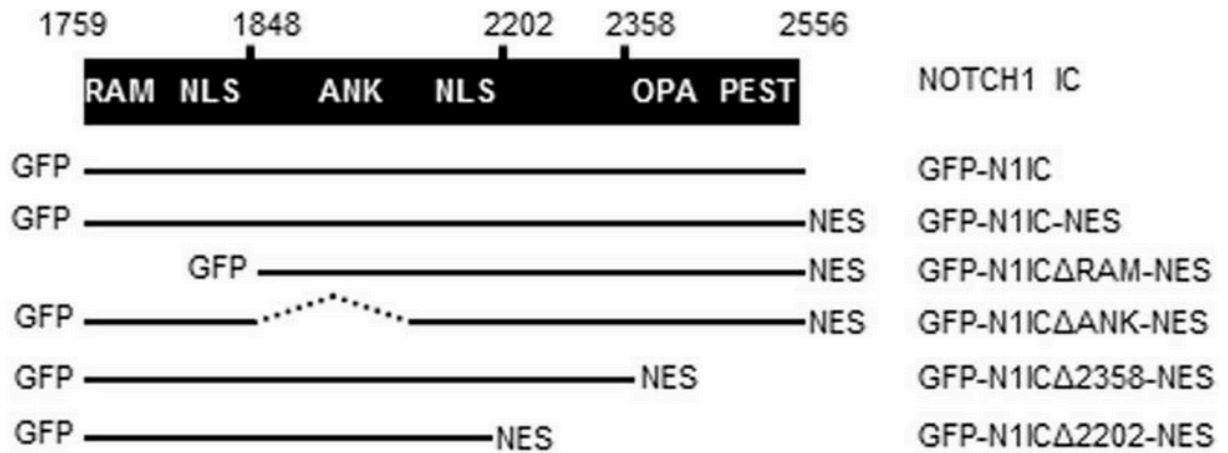


Figure 1.5 Notch1 Deletion Constructs

A diagram showing the deletion constructs of EGFP-Notch1. The Δ symbol represents the deletion of specific functional domains of Notch1 including RAM, Ank, the cDNA from 2358-2556, and the cDNA from 2202-2556. (Shin et al., 2014)

CHAPTER 2

RESULTS

2.1. Transfection Optimization

HEK 293 cells were cultured at 37°C, 5% CO₂ until they were 60-80% confluent. Single transfections of plasmid DNA were performed to confirm expression of Notch1-GFP fusion constructs and the Flag-tagged PKCθ construct. At 12h intervals, the cells expressing GFP were imaged using fluorescence microscopy to determine transfection efficiency (Figure 2.1). Visual estimates indicated peak GFP expression (up to 90%) for the transfected HEK 293 cells at 48 hours (Figure 2.2). After the 48h time point GFP expression gradually decreased due to cell death and cell division.

Transfection both of Notch1-GFP fusion constructs and the Flag-tagged PKCθ construct was performed using a 1:1 ratio of plasmid DNA from both constructs. After initial Western Blots showed a higher expression of the Notch1 construct and almost no expression of the PKCθ construct, the levels of plasmid DNA for each construct were optimized by changing the ratio of plasmid DNA of each construct as well as the amount of Xtremegene used in the transfection. By using a 2:1 ratio of the PKCθ plasmid to the Notch1 plasmid, a lower level of GFP-expression was seen using fluorescence microscopy but Western blot analysis was better able to detect both Notch1 and PKCθ in the whole cell lysates. It was noted that there was increased cell death during transfection experiments in which both plasmid were used compared to single transfection.

2.2. Localization of Notch1 and PKCθ

Isolation of nuclear and cytoplasmic lysates was performed using a commercially available kit (Thermo Scientific). The flag-tagged PKCθ transfected into HEK 293 cells was seen exclusively in the cytoplasm of the cell shown in Figure 2.3. The Notch1 constructs were found in both the nucleus and the cytoplasm. Notch1^{IC} localized to the nucleus which is where the naturally occurring Notch1^{IC} protein localizes following cleavage from the transmembrane domain upon T cell

activation. Notch1^{IC}-NES was primarily found in the cytoplasm as a result of the Nuclear Export Sequence tag.

2.3. Co-immunoprecipitation of Notch1 and PKC θ

HEK 293 cells transfected with a plasmid encoding EGFP-Notch1^{IC}-NES and a plasmid encoding flag-tagged PKC θ were harvested 48 hours post-transfection. Considerable cell death was observed following the two-plasmid transfection, with lower transfection efficiency than transfection of single plasmids measured using fluorescence microscopy. Lysates from the two-plasmid transfection were run on a Western Blot and both proteins could be detected using their tags (GFP and Flag). The results of the co-ip experiments were inconclusive (Figure 2.4). Notch1 and PKC θ co-ip attempt shown in Figure 5. The anti-GFP antibody was able to bind protein; however, it appears to be nonspecific. The anti-FLAG antibody did not bind any protein that was visible on the western blot further suggesting the bands seen on the anti-GFP blot were a result of nonspecific binding.

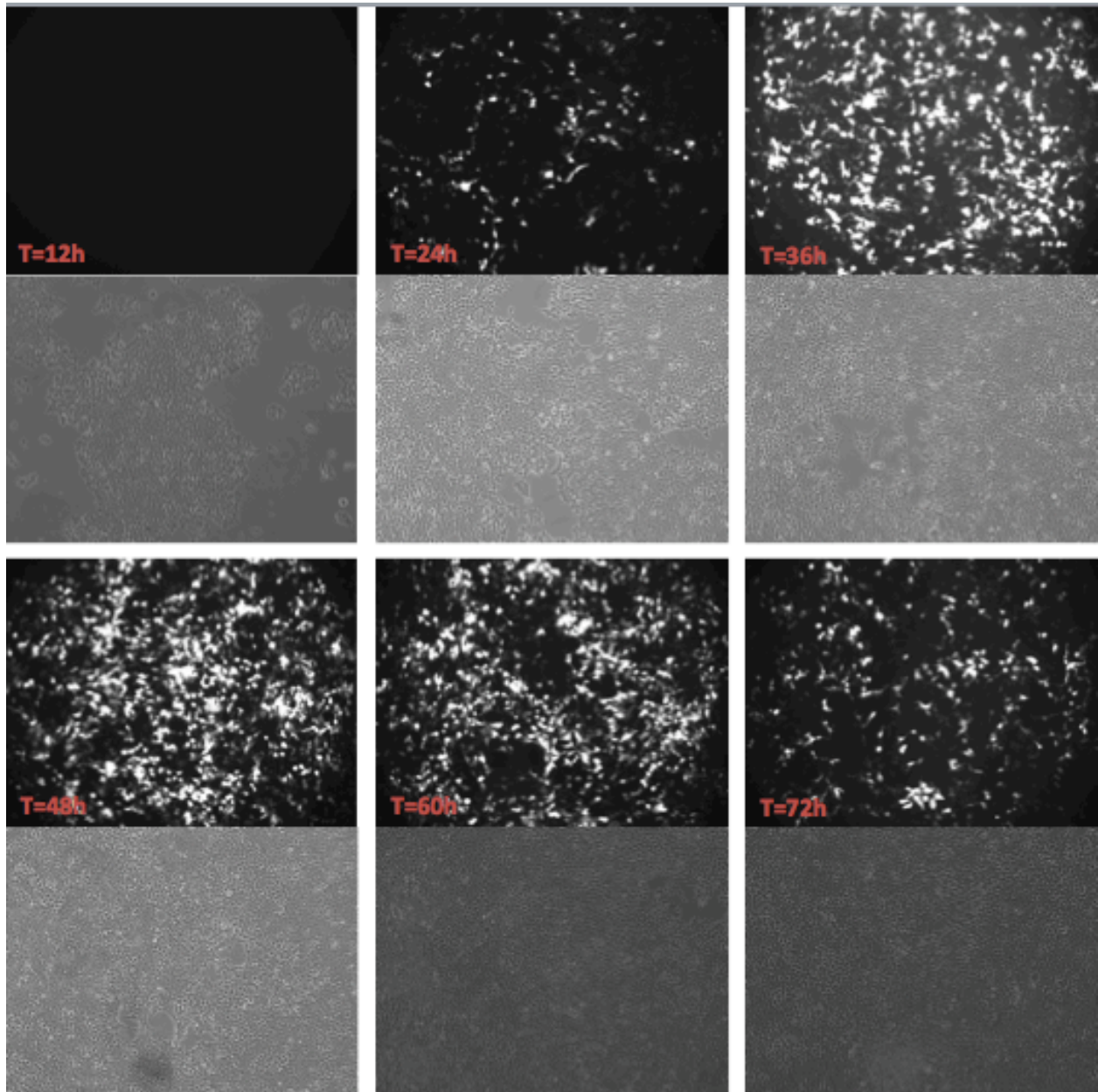


Figure 2.1: Peak EGFP expression following transfection in HEK 293 cell line at 48 hours. HEK293 cells imaged following transfection of plasmid DNA encoding EGFP. The cells were imaged at 12 hour intervals using fluorescent microscopy and phase microscopy. The EGFP expression is visible at 24 hours (upper panel) with the phase image (lower panel) showing the total number of cells present. As the time interval increases, the percentage of cells expressing EGFP increases.

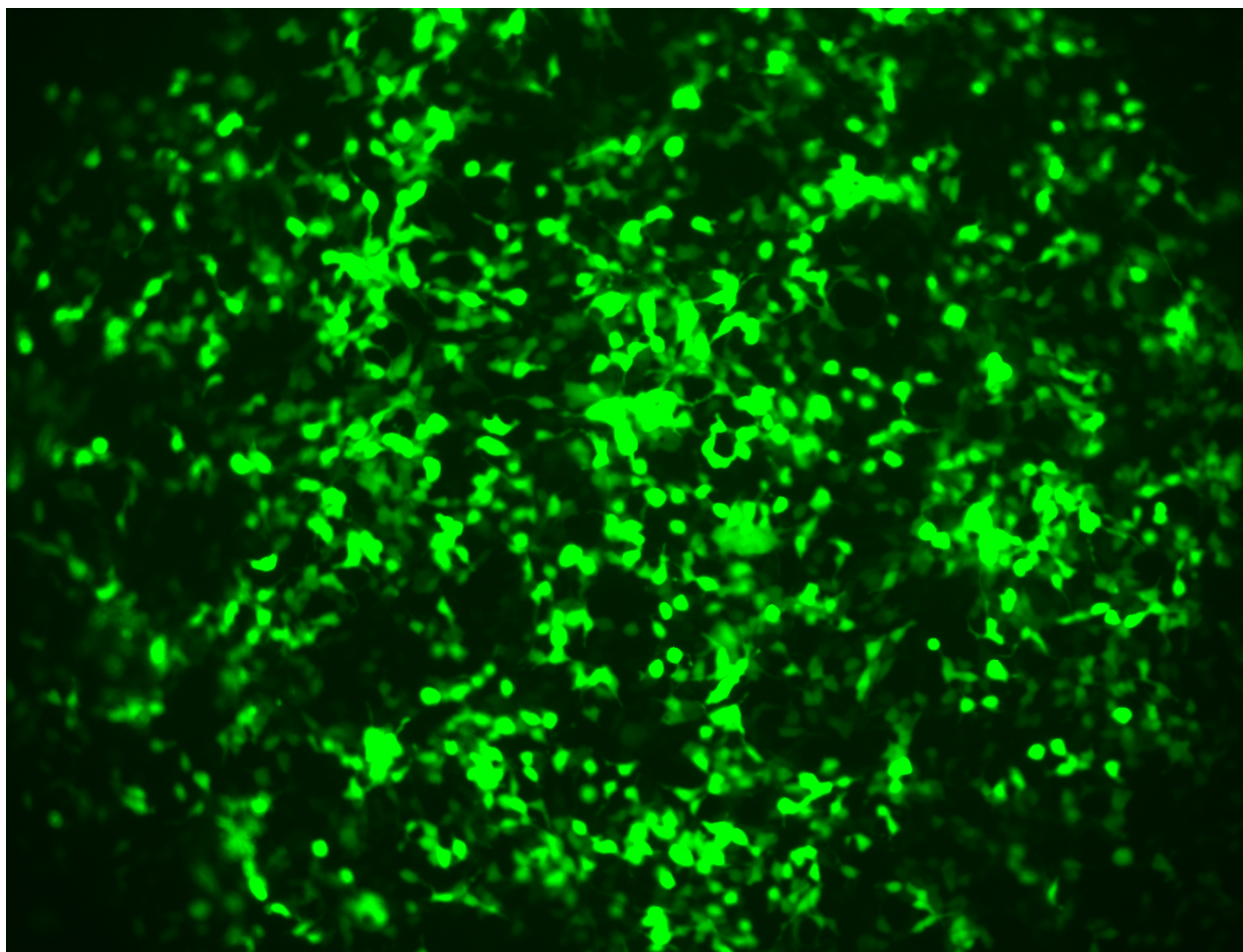


Figure 2.2: EGFP expression imaged using 10x FITC filtered fluorescent microscopy at 48 hours after transfection, illustrating robust EGFP expression and effective transfection of plasmid DNA.

HEK293 cells imaged following transfection of plasmid DNA encoding EGFP with a FITC-filtered image showing peak EGFP expression captured using fluorescent microscopy.

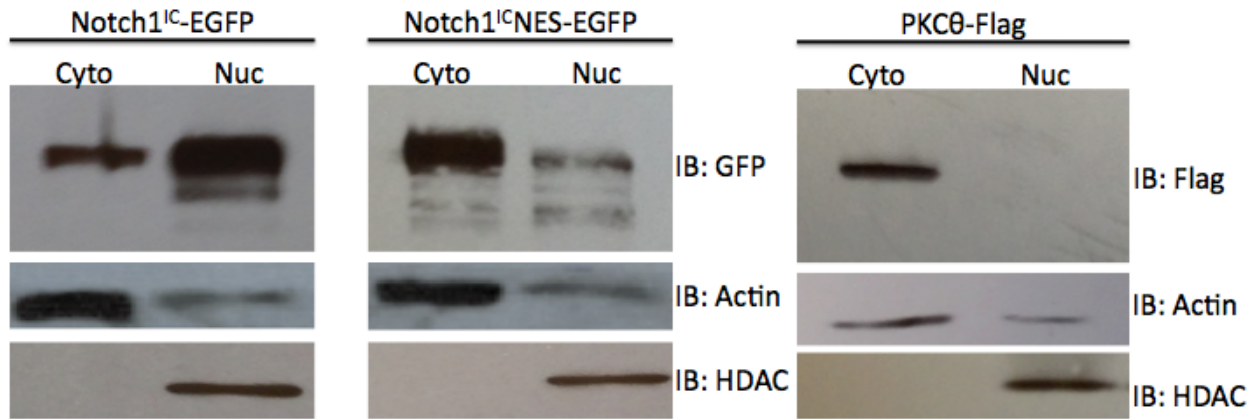


Figure 2.3: Nuclear and cytoplasmic fractionation in HEK 293 cell line show PKCθ remaining in the cytoplasm, while Notch1 localizes to the nucleus unless tagged with a Nuclear Export Signal (NES).

Western blot analysis of localization of Notch1 constructs and Flag-tagged PKCθ. HEK293 cells were transfected and lysates were collected 48 hours after transfection. Lysates collected from the transfected cell cultures were probed for Actin and HDAC as controls.

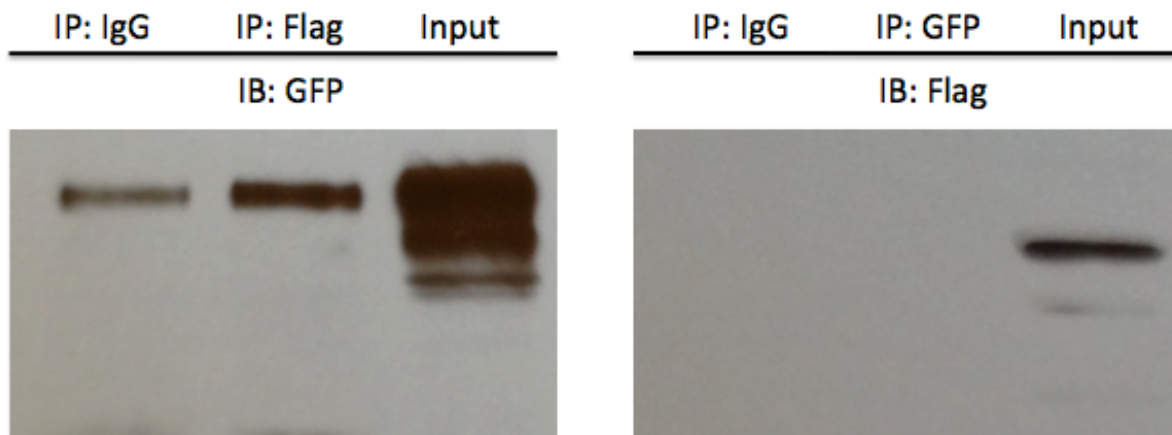


Figure 2.4: Co-IP of Notch1 and PKC θ constructs in HEK 293 cell line failed to demonstrate a direct interaction.

Lysates from HEK293 cells transfected with plasmids encoding EGFP-Notch1 and Flag-tagged PKC θ were co-immunoprecipitated for western blot analysis. Lysates co-immunoprecipitated using IgG were used as a negative control. Whole cell lysates were used as a positive control.

CHAPTER 3

DISCUSSION

The roles of Notch1 and PKC θ signaling in the development, activation, and polarization of the adaptive immune response are undoubtedly intertwined and synergistic in many T cell responses. Through the use of a HEK 293 cell line we attempted to dissect a very specific and direct interaction that is maintained independent of the CBM complex and engagement in the TCR. There are a multitude of reasons why the CO-IP experiments performed failed to demonstrate a direct interaction.

The human PKC θ construct that was used to transfect the HEK293 cells is not phosphorylated at the Thr538 residue, which, increasingly, appears to be critically important to proper PKC θ activity in T cell activation. The phosphorylated, active form of PKC θ is crucial to formation of the CBM complex, and suggests that the Notch1-PKC θ interaction we were seeking to identify is dependent upon a conformational change in PKC θ as a result of its phosphorylation.

HEK 293 cells lack TCRs and, thus, the immunological synapse where Notch1 and PKC θ may co-localize in T cells. In this experimental design, Notch1 and PKC θ may have failed to interact simply because they were not recruited to a conserved physical location in the cell to form a close interaction. Several scaffold proteins are critical to formation of the CBM complex in T cells, which might be cause for the lack of interaction in the HEK293 cell line. The Notch1 construct may have been cycling in and out of the nucleus while PKC θ remained in the cytoplasm as the nuclear and cytoplasmic lysates run on a western blot suggest.

The issue with the two-plasmid transfection is also a potential problem that resulted in the failure to show a direct interaction between PKC θ and Notch1. Both plasmids use the CMV promoter that was effective in single plasmid transfections; however, it is a very strong promoter so the cell may become exhausted and die when both proteins begin to be synthesized inside the cell. Our possibility is that the cells successfully transfected with both plasmids quickly died before

the 48 hour time point, and the remaining cells used in the Co-IP experiment were only expressing either the Notch1 construct or the PKC θ construct. It appeared that for unknown reasons the plasmid DNA encoding Notch1 was much more readily taken up and expressed in the cells, as measured by western blot. This difference in uptake of the plasmids during transfection may have resulted in Notch1 out-competing PKC θ resulting in only Notch1 expression in the HEK293s. Finally, the GFP antibody used to detect the fusion protein was extremely detectable on the western, with what appeared to be some non-specific binding that may have resulted in issues with the CO-IP as well.

CHAPTER 4

SUMMARY AND FUTURE DIRECTIONS

There are a lot of questions that remain unanswered stemming from the use of the HEK293 cell line to identify a direct Notch1-PKC θ interaction. A single transfection of the Notch1 construct and the PKC θ construct separately into a Jurkat cell line would provide insight as to whether the immunological synapse plays an important role in providing scaffolding to hold Notch1 and PKC θ in the same physical location of the cell to help mediate their interaction. Having the endogenous kinases and members of the CBM complex would also help support the interaction. Additionally, generation of a phosphorylated PKC θ construct would provide insight as to the requirements of PKC θ 's phosphorylation state in driving potential interactions that were not observed in transfection experiments in HEK293s. Using flow cytometry to sort of double positive (PKC θ and Notch1) HEK293 cells would ensure that the CO-IP experiments were performed with the proper cell population, as well as answer the question about the potential for the transfection of two plasmids to cause cell death.

CHAPTER 5

MATERIALS AND METHODS

5.1. Cell Culture

Human Embryonic Kidney (HEK) 293 cells were cultured in RPMI 1640 with 1% L-glutamate, 0.5% Na pyruvate, 1% penicillin-streptomycin and 10% FBS. The HEK 293 cells were incubated at 37°C, 5% CO₂.

5.2. Plasmid DNA

All Notch plasmids were generated by Hyun Mu Shin. The human PKC θ plasmid was purchased from OriGene (PRKCQ Human cDNA ORF Clone).

5.3. Competent Cells

XL1 Blue *Escherichia coli* cells were grown overnight in 50 mL of fresh LB media in a 200 mL conical flask to an OD₆₀₀ of 0.2-.0.5. The culture was pelleted and resuspended in 5mL of chilled TSS buffer (10% PEG 8000, 20mM MgCl₂, 5% DMSO, and LB media: filter sterilized). 100 μ L aliquots were added to chilled Eppendorf tubes and stored at -80°C for bacterial transformation.

5.4. Bacterial Transformation

Competent XL1 Blue cells were removed from -80°C and thawed on ice. 100 ng of plasmid DNA was added to 2 mL Eppendorf tubes containing 100 μ L of XL1 blue cells and incubated on ice for 20-30 minutes. The competent cell/DNA mixture was heat shocked in a 42°C water bath for 45 seconds, and then incubated on ice for 2 minutes. 500 μ L of LB media was added to the competent cell/DNA mixture. The Eppendorf tube was placed in a 37°C shaking incubator for 45 minutes. 200 μ L of the mixture was plated onto a 10 cm LB agar plate containing 50 μ g/mL kanamycin and incubated at 37°C overnight.

5.5. Mini-prep and Maxi-prep

All mini-preps were performed using QIAprep Spin Mini-prep Kit according to the manufacturer's specifications (Qiagen). All maxipreps were performed using Biotool's Maxi-prep Kit according to

the manufacturer's specifications (Biotool). All DNA concentrations were measured using Biodrop according to manufacturer's specifications (Biodrop).

5.6. Sequencing and Sequence Analysis

All sequencing of plasmid DNA was done by Genewiz using the primers outlined in Table 1. Analysis of the sequences was performed using the UGENE analysis software.

5.7. Transfection

HEK 293 Cells were transfected using Xtremegene according the manufacturers' specifications. Cells were grown in 10 cm cell culture plates until they were 65-80% confluent. 10 µg of plasmid DNA and 10 µL of Xtremegene were added to 1 mL of serum-free media and incubated for 30 minutes before being added drop wise to the HEK 293 cell culture. Cells were harvested 48 hours post transfection.

Transfection efficiency was monitored using fluorescence microscopy.

5.8. Protein Extraction

Whole cell lysates were collected using Lysis buffer (50 mM Hepes pH 7.8, 250 mM NaCl, 1% Nonidet P-40, 2 mM EDTA and protease inhibitors). Nuclear and cytoplasmic lysates were collected using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific) according to manufacturer's specifications. Protein concentrations were measured using a BCA Assay according to manufacturer's specifications (Thermo Scientific).

5.9. Western Blot and Co-Immunoprecipitation

Western blots were performed by adding 20-50 µg of protein to a 10% SDS-polyacrylamide gel and separated using electrophoresis. The protein was transferred onto polyvinylidenedifluoride (PVDF) membranes using a wet transfer (Millipore). The membranes were probed using antibodies outlined in Table 2. Horseradish peroxidase-conjugated secondary antibodies were used in western blot experiments. Protein bands were detected with enhanced chemiluminescence (ECL, Thermo

Scientific). Membranes were blocked using either PBS or TBS with 0.1% Tween-20 and 3-5% dry fat-free milk powder.

Following protein quantification using a BCA assay, 1 mg of whole cell lysate was incubated with 5-10 $\mu\text{g}/\text{mL}$ of primary antibody for 2-10 hours, 4°C. After washing lysates/antibody mixture, the supernatant was transferred to an Eppendorf tube containing 60 μL of protein A/G sepharose beads. The beads and supernatant was place on a wheel at 4°C. Laemmli sample buffer was added and the tubes were spun down after a 5-minute incubation at 95°C to disassociate the bead/antibody interaction. The supernatant was collected containing only the proteins that bound to the sepharose beads.

Table 1: Antibodies

Antigen	Species	Source
Notch1	Rabbit	Santa Cruz
PKC θ	Rabbit	Sigma
GFP	Rabbit	Sigma
Flag	Mouse	Santa Cruz
β -Actin	Mouse	Sigma
HDAC1	Rabbit	Cell Signaling

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