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# Ikaros Deficiency Leads To An Imbalance in Effector and Regulatory T Cell Homeostasis in Murine Pancreatic Cancer

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Ikaros Deficiency Leads To An Imbalance in Effector and Regulatory T Cell  
Homeostasis in Murine Pancreatic Cancer

by

Nadine D. Nelson

A dissertation submitted in partial fulfillment  
of the requirements for the degree of  
Doctor of Philosophy  
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Keywords: Transcription Factor, CK2, PP1, Ubiquitination, Apigenin

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## DEDICATION

I dedicate this dissertation to my grandmother, Waveney Nelson, and my parents, Hewley and Patricia Nelson. To my grandmother, thank you for being the perfect example of poise, strength and humility. To my parents, thank you for being an unwavering source of strength, encouragement and support, and for always believing in my dreams.

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## ABSTRACT

Pancreatic cancer is one of the deadliest cancers with a five-year survival rate of 6%. Pancreatic cancer is resistant to conventional chemotherapy and is usually diagnosed at late stages. Current treatment options have minimal effects in extending patients' lives beyond 10 months. One significant limitation in developing treatments to combat pancreatic cancer is its immunosuppressive microenvironment. Pancreatic cancer secretes factors that activate immunosuppressive cells, such as regulatory T cells (Tregs). These Tregs suppress effector CD4<sup>+</sup> and CD8<sup>+</sup> T cell anti-tumor immune responses. Therefore, novel treatment options to reduce Treg-mediated immune suppression and increase the numbers and functions of CD4<sup>+</sup> and CD8<sup>+</sup> T cells are paramount to enhance anti-tumor immunity in pancreatic cancer tumor-bearing (TB) hosts.

The alternatively spliced transcription factor Ikaros is essential for lymphocyte development and is considered a tumor suppressor in T cells. Ikaros' protein stability and function are regulated by its phosphorylation and dephosphorylation by protein kinase CK2 and phosphatase 1 (PP1), respectively. Mutations and functional inactivation of Ikaros have mainly been investigated in T cell leukemias and lymphomas. In this dissertation, we investigated the role of Ikaros in regulating T cell homeostasis in murine pancreatic cancer.

In this study, we report that Ikaros proteins are degraded by the ubiquitin-proteasome pathway in response to factors produced by murine pancreatic cancer cells. Our results further suggest that an increase in CK2 activity leads to Ikaros' degradation and disrupts its localization to pericentromeric heterochromatin in our murine pancreatic TB model. This loss of Ikaros expression is accompanied by an imbalance in T cell homeostasis. More specifically, we observe a significant decrease in effector CD4<sup>+</sup> and CD8<sup>+</sup> T cells but an increase in Treg percentages in TB and spontaneous pancreatic cancer models. T-cell specific defects in Ikaros protein expression were also observed in TB CD3<sup>+</sup> T cells. Apigenin, a natural plant flavonoid and CK2 inhibitor, restored expression of some Ikaros isoforms in our TB model. Apigenin also displayed immunological benefits evident by enhanced anti-tumor immunity in TB mice. These data provide mechanistic and functional evidence that pharmacological inhibition of CK2 can regulate Ikaros expression and identifies the possible involvement of Ikaros in regulating T cell immune responses in murine pancreatic cancer.

## CHAPTER ONE

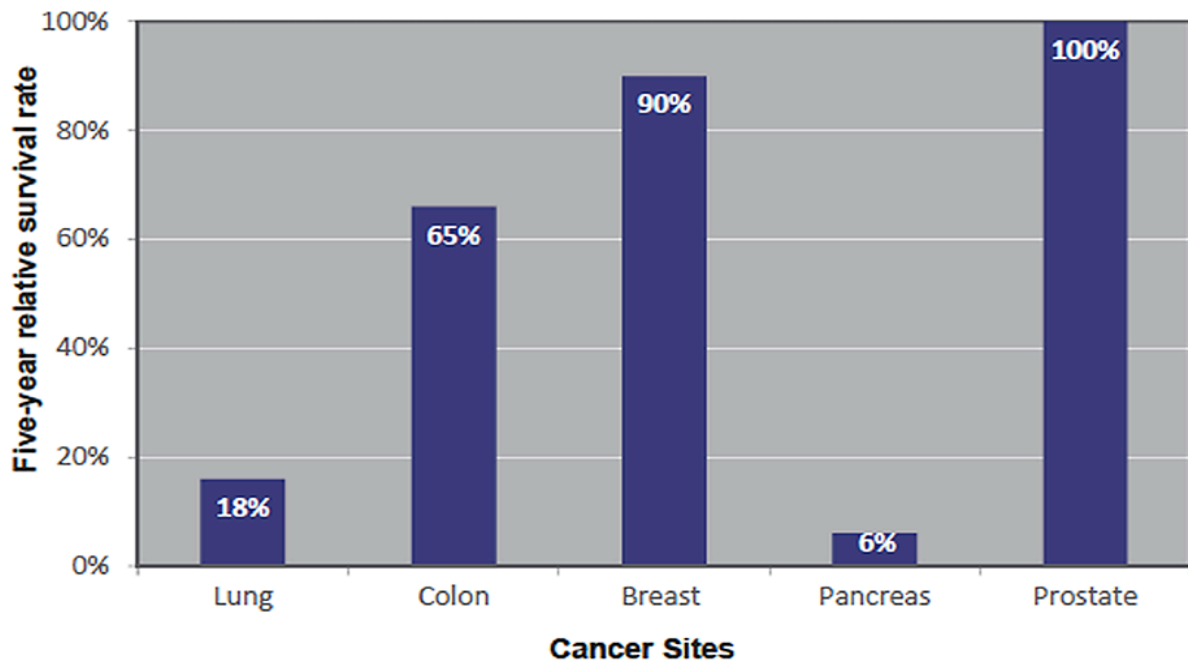
### INTRODUCTION

#### An Overview of Pancreatic Cancer

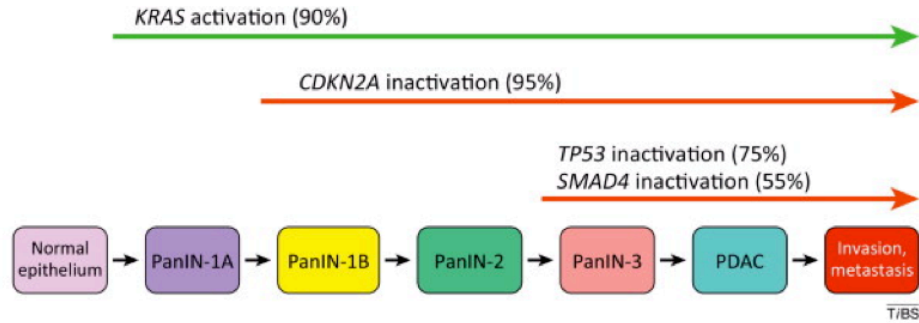
Pancreatic adenocarcinoma (PDAC) makes up approximately 85-95% of pancreatic cancer occurrences and is the fourth leading cause of cancer-related deaths. It is one of the most lethal cancers and is the only major cancer with a five-year relative survival rate in the single digits of approximately 6% (1-3) (**Figure 1.1**). Typically, patients are 60-80 years of age and PDAC is twice as prevalent in males than females (4, 5). Most PDAC is prevalent in the head of the pancreas, followed by the body and the tail (4, 6). In approximately 90% of cases, oncogenic mutations in *Kras* (G12D) drive PDAC initiation by causing the development of pancreatic intraepithelial neoplasia (PanIN) lesions (8, 9). However, the minimal spontaneous progression of precursor lesions to invasive PDAC has suggested that additional genetic mutations are necessary for PDAC disease progression (8). In fact, studies have shown that cooperative inactivating mutations in the tumor suppressor genes *Cdkn2a*, *Trp53*, or *Dpc4/Smad4* accelerate the development of PanINs and PDAC in *Kras* mouse models and in humans (8, 17, 18) (**Figure 1.2**). Currently, surgery is the only cure, and increases the survival rate to 20% (4, 5). Unfortunately, PDAC is unresectable in 75% of patients (20). Lack of early detection contributes to a high incidence of metastasis at

diagnosis in 80% of patients (21). Resistance to chemo- and radiation- therapies also contributes to the lethality of this disease (22, 23). The chemotherapeutic drug gemcitabine is the standard treatment for advanced pancreatic cancer which has minimal effects on survival rates (1). Immunotherapies, vaccines and other immunomodulatory drugs have shown limited success in treating PDAC (24).

### 2014 Five-Year Relative Survival Rates Top Five Causes of Cancer Death



**Figure 1.1. 2014 Five-Year Relative Survival Rates of the Top Five Causes of Cancer Death.** Pancreatic cancer is currently the only top-five cancer in terms of death, with a five-year survival rate in the single digits at 6 percent. *Note.* Reprinted from the Pancreatic Cancer Action Network; Source: American Cancer Society, Cancer Facts and Figures 2014. ©2014 Pancreatic Cancer Action Network. Reprinted with permission.

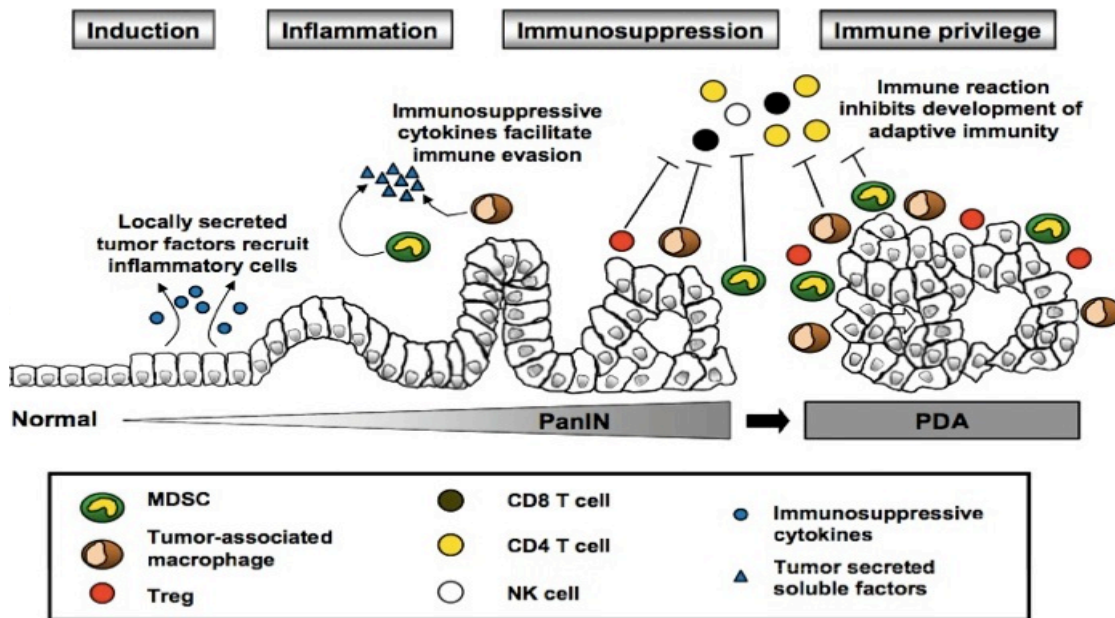


**Figure 1.2. Genetic and histological changes of pancreatic ductal adenocarcinoma (PDAC).** Kras activation leads to the development of pancreatic intraepithelial neoplasias (PanINs). Subsequent inactivation of cyclin-dependent kinase inhibitor 2A (Cdk2na), tumor protein 53 (TP53) and Smad4 further contribute to the progression of PanIN lesions and ultimately the development of PDAC and its invasion and metastasis. Green arrow – oncogene; red arrows- tumor suppressor genes. Percentages represent occurrence. *Note.* Reprinted from “KRAS: feeding pancreatic cancer proliferation” by Bryant et al., 2014, Trends Biochem Sci., 39, pg.91-100. Copyright © 2013 Elsevier Ltd. Reprinted with permission (7).

### Immune Responses in Pancreatic Cancer

Immune surveillance is the necessity of the immune system to detect and destroy tumors in a host (25). However, pancreatic cancer has employed a number of strategies to avoid and escape immune surveillance and is considered a ‘nonimmunogenic’ tumor (24). In particular, the absence of effective immune responses to pancreatic cancer is a major contributing factor to its poor prognosis. The immune cells in pancreatic cancer promote a local and systemic immunosuppressive microenvironment marred by the presence of inflammatory mediators, tumor-associated macrophages (TAMs), regulatory T cells (Tregs) and myeloid derived suppressor cells (MDSC) that inhibit innate and adaptive anti-tumor immune responses, especially that of T cells, leading to tumor progression (10, 26) (**Figure 1.3**).





**Figure 1.3. Immune Evasion in PDAC.** During the induction phase, mutations in oncogenes and tumor suppressor genes drive the development of PanIN lesions. In the inflammation stage, PanIN lesions secrete soluble factors that lead to the recruitment of inflammatory cells. This causes immunosuppression marked by the infiltration of tumor-associated macrophages (TAMs), regulatory T cells (Treg) and myeloid derived suppressor cells (MDSC) that eventually suppress adaptive immune responses –NK cells, CD4<sup>+</sup> T cells and CD8<sup>+</sup> T cells - through direct contact or the production of immunosuppressive cytokines. This ultimately leads to immune privilege and the progression to pancreatic ductal adenocarcinoma. *Note.* Reprinted from “Immunosurveillance of pancreatic adenocarcinoma: Insights from genetically engineered mouse models of cancer ” by Clark et al., 2009, Cancer Lett., 279, pg.1-7. © 2008 Elsevier Ireland Ltd. Reprinted with permission (10).

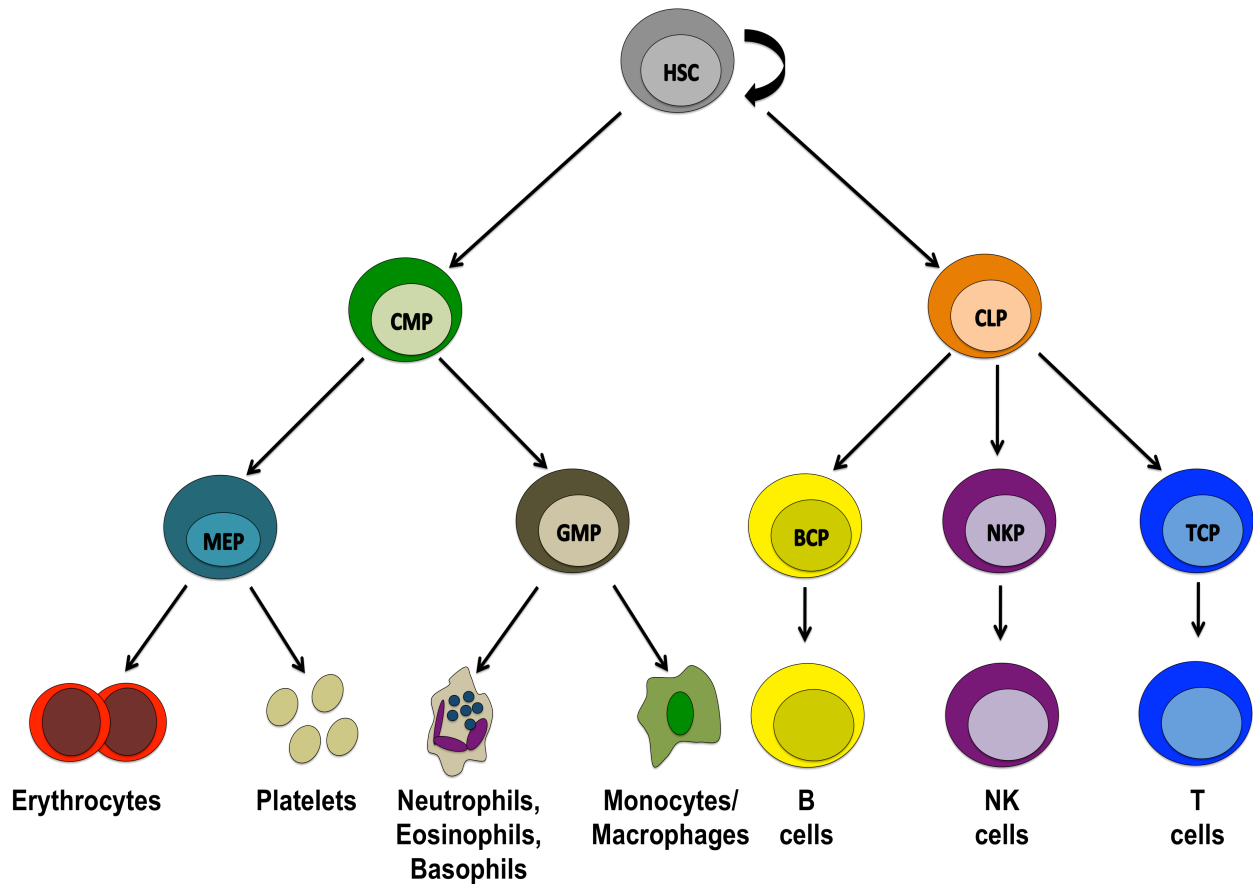
In pancreatic cancer patients, elevated Treg numbers are present both in the circulation and tumor site (27, 28) while effector T cells are scarce and have diminished functions (10). More so, increased Treg numbers correlate with worse survival (29) and a reduction in effector CD8<sup>+</sup> T cell numbers (28). Higher Treg:Teff ratios have been shown to indicate better prognosis in PDAC (30). Treg inhibitors, including cytotoxic T-

lymphocyte antigen-4 (CTLA-4) and programmed death-1 (PD-1) receptor, which are effective in other cancers, have shown no activity in PDAC (31-33). Therefore, identifying novel mechanisms that can potentially restore T cell homeostasis, thereby increasing Treg:Teff ratios, is essential in overcoming defects in the immune system that promote pancreatic cancer tumor progression and impede the success of treatment strategies.

### **Hematopoiesis and Lymphocyte Development**

Hematopoiesis, which occurs during embryonic development and adulthood, refers to the collective process involving the formation, development and differentiation of blood and immune cells (34) from pluripotent hematopoietic stem cells (HSCs) (35). Hematopoiesis functions to replenish short-lived blood and immune cells and occurs mainly in the bone marrow (36). HSCs can either self-renew or go on to produce daughter cells that become either common myeloid progenitor (CMP) or common lymphoid progenitor (CLP) cells. These cells further differentiate into erythroid, lymphoid and myeloid lineages. More specifically, CMPs generate granulocyte/macrophage precursors (GMPs) that produce granulocytes, macrophages/monocytes (includes dendritic cells (DCs)) and osteoclasts and megakaryocyte/erythroid precursors (MEP) that produce megakaryocytes, platelets and erythroid cells. These cells of the myeloid lineage mainly form components of innate immunity (36). CLPs give rise to lymphoid cells including natural killer (NK) cells, B cells and T cells (35), responsible for the innate and adaptive immune response (36) (**Figure 1.4**). Therefore, studying the mechanisms

that govern hematopoiesis and thus, immune cell development and differentiation, may provide insight into the development of blood disorders and cancers (37).



**Figure 1.4. Hematopoiesis.** Representation of the process of hematopoiesis through which blood and immune cells develop. Hematopoietic stem cells (HSCs) can self-renew or differentiate into common myeloid progenitors (CMP) or common lymphoid progenitors (CLP). CMPs then become either megakaryocyte/erythroid precursors (MEP) or granulocyte/macrophage precursors, which can develop into erythrocytes and platelets and neutrophils, eosinophils, basophils, monocytes/macrophages, respectively. CLPs differentiate into B cell precursors (BCP), NK cell precursors (NKP) and T cell precursors (TCP) giving rise to B cells, NK cells and T cells. Arrow – self renewal.

## **Antigen-Presenting Cells**

Antigen-Presenting Cells (APCs) are a population of immune cells that process and present antigens to stimulate the activation of mature T cells (38). Dendritic cells, macrophages and B cells are considered 'professional' APCs (39). Dendritic cells are the most potent APCs as they express high levels of major histocompatibility (MHC) and co-stimulatory molecules (40). APCs internalize and degrade proteins into peptides, load these peptides to MHC class I molecules for CD8<sup>+</sup> T cells and MHC II for CD4<sup>+</sup> T cells. These peptides are recognized by T cell receptors (TCR) on T cells (41). APCs also express co-stimulatory molecules, which provide a second signal for T cell activation. Such co-stimulatory molecules include B7 that binds to CD28 on T cells (42). Therefore, defects in APC development and function can negatively impact T cell immunity (38).

## **T cells**

T cells are the main cellular players responsible for eliciting robust adaptive immune responses to foreign pathogens and regulating immune tolerance to self-antigens (43). T cells are divided into a number of subsets with distinctive properties and roles. The two major subsets of peripheral T cells are characterized by their expression of CD4 and CD8 coreceptor molecules (44). CD4<sup>+</sup> and CD8<sup>+</sup> T cells develop in the thymus and originate from double-positive thymocytes expressing both CD4 and CD8 molecules (45). T cells express a T Cell Receptor (TCR) that recognizes antigens presented by antigen-presenting cells (APC). The TCR of CD4<sup>+</sup> T cells are MHC class II restricted while CD8<sup>+</sup> T cells are MHC class I restricted. CD4<sup>+</sup> and CD8<sup>+</sup> T cells become

activated through their TCR receptors and costimulatory molecules, specifically CD28, which leads to their proliferation, cytokine production, survival and function (41). CD4<sup>+</sup> and CD8<sup>+</sup> T cells are critical regulators of immunosurveillance (46) as they are capable of eliciting robust immune responses to eradicate various disease states (47). T cells are found in lymphoid tissues and circulate via the bloodstream to identify and destroy foreign, diseased cells. Therefore, T cells are important players in cellular immunity against cancers (48). However, the tumor microenvironment utilizes a number of strategies that inhibit T cells (47). Therefore, mechanisms that can facilitate effective activation, development and function of T cells are essential for effective anti-tumor immune responses (49)

**CD4<sup>+</sup> T Cells.** CD4<sup>+</sup> T cells play a critical role in antibody-mediated immunity and activating and expanding CD8<sup>+</sup> T cells and CD8<sup>+</sup> memory T cells, characteristic of its “helper” cell phenotype (48). Historically, CD4<sup>+</sup> T cells were shown to activate CD8<sup>+</sup> T cells by the production of IL-2 (48) and the activation of dendritic cells to stimulate CD8<sup>+</sup> T cells (50). More recently, studies also suggest that CD4<sup>+</sup> T cells use CD40/CD45 binding to directly interact with CD8<sup>+</sup> T cells (51). The number and cytotoxic function of CD8<sup>+</sup> T cells are significantly increased in the presence of CD4<sup>+</sup> T cells. CD4<sup>+</sup> T cell depletion favors tumor progression and reduces survival of tumor-bearing hosts, highlighting its significance in maintaining CD8<sup>+</sup> T cell function (48, 52, 53). As a result, diminished CD8<sup>+</sup> T cell antitumor responses may be due to insufficient CD4<sup>+</sup> T cell activation. Apart from this “helper” role, CD4<sup>+</sup> T cells have also been shown to possess tumor-reactive abilities. These CD4<sup>+</sup> T cells develop cytotoxic activity, which can cause

tumor rejection by recognizing MHC-II antigens on tumor cells (54, 55) facilitated by their production of IFN- $\gamma$  and TNF- $\alpha$  (56, 57). CD4<sup>+</sup> helper T cells are further characterized into subsets whose generation, cytokine secretion, function and differentiation are regulated by specific “master” transcription factors (45). The three main helper T cell subsets are Th1, Th2 and Th17 cells. Regulatory T cells (Tregs) are another unique subset of CD4<sup>+</sup> T cells that maintain peripheral tolerance and cause immune suppression (58). Th1 cells fight intracellular pathogens. These cells express the transcription factor T-bet and immediate immune responses by producing IFN- $\gamma$ , which can activate macrophages (45). Th2 cells fight extracellular parasites and express the transcription factor GATA-3 that regulates its production of IL-4, IL-5 and IL-13 (45). In addition, both of these cells have roles in antitumor immunity. Th1 cells prevent blood vessel formation and recruit CD8<sup>+</sup>T and NK cells, which both have the ability to kill tumors. Th2 cell production of IL-4 and IL-13 recruits eosinophils while IL-5 production promotes tumorigenesis (59). Th17 cells express the transcription factor ROR $\gamma$  and produce the cytokines IL-17 and IL-21 (59). Th17 have inflammatory properties and play a role in autoimmune diseases. Limited production of IL-17 has been reported to aid in cancer progression (60).

**CD8<sup>+</sup> T cells.** CD8<sup>+</sup> T cells are critical for controlling and eliminating viral infections and tumor cells (61). CD8<sup>+</sup> T cells recognize and respond to these foreign antigens via MHC-I molecules expressed on infected cells or antigen presenting cells (APCs) (62). Apart from TCR and CD28 engagement, the production of inflammatory cytokines such as IL-12 and type I interferon, by immune cells, further facilitates CD8<sup>+</sup> T

cell accumulation and differentiation (62). Memory phenotype of CD8<sup>+</sup>T cells after exposure to antigen stimulation results in host protection to future exposure (63).

In instances of autoimmunity, self-reactive lymphocytes cause destruction of normal tissues. These autoreactive T cells are usually deleted during negative selection in the thymus but sometimes evade this process (64-69), This is where peripheral tolerance is crucial in restricting these autoimmune responses. However, CD8<sup>+</sup> T cell self-tolerance can also lead to defective anti-tumor immune responses as many tumor cells express self-antigens (70).

The ability of CD8<sup>+</sup> T cells to destroy and eliminate infected or tumor cells is referred to as their cytotoxic ability. CD8<sup>+</sup> cytotoxic T cells (CTLs) kill target cells by inducing apoptosis or cell death mediated by calcium-dependent release of granules in response to antigen recognition of the target cell. These granules contain two types of cytotoxic proteins stored in an active form but do not become functional until their release. Perforin is one of these cytotoxic proteins that polymerizes, forming pores in the membranes of target cells, allowing salt and water to end and leading to cell death. The other cytotoxic protein, granzyme, consists of three serine proteases that activate apoptosis in the cytoplasm of the target cell by caspase cleavage (71). CTLs can also induce apoptosis using perforin-independent mechanisms that involve the expression of Fas ligand (also expressed on Th1 cells) that can bind Fas in the target cell membrane. CTLs also produce IFN- $\gamma$ , which can increase MHC-I expression on target cells and activate macrophages in conjunction with TNF- $\alpha$  and TNF- $\beta$ . Cells undergoing programmed cell death or apoptosis have exposed phosphatidylserine on their cell membranes which allow their recognition and ingestion by phagocytes ((71). Current

research is extensively focused on therapeutic techniques to eradicate tumor cells. These mainly involve using the host's immune system, especially targeting T cells to increase their cytotoxic abilities, survival and migration to tumor sites (47, 63).

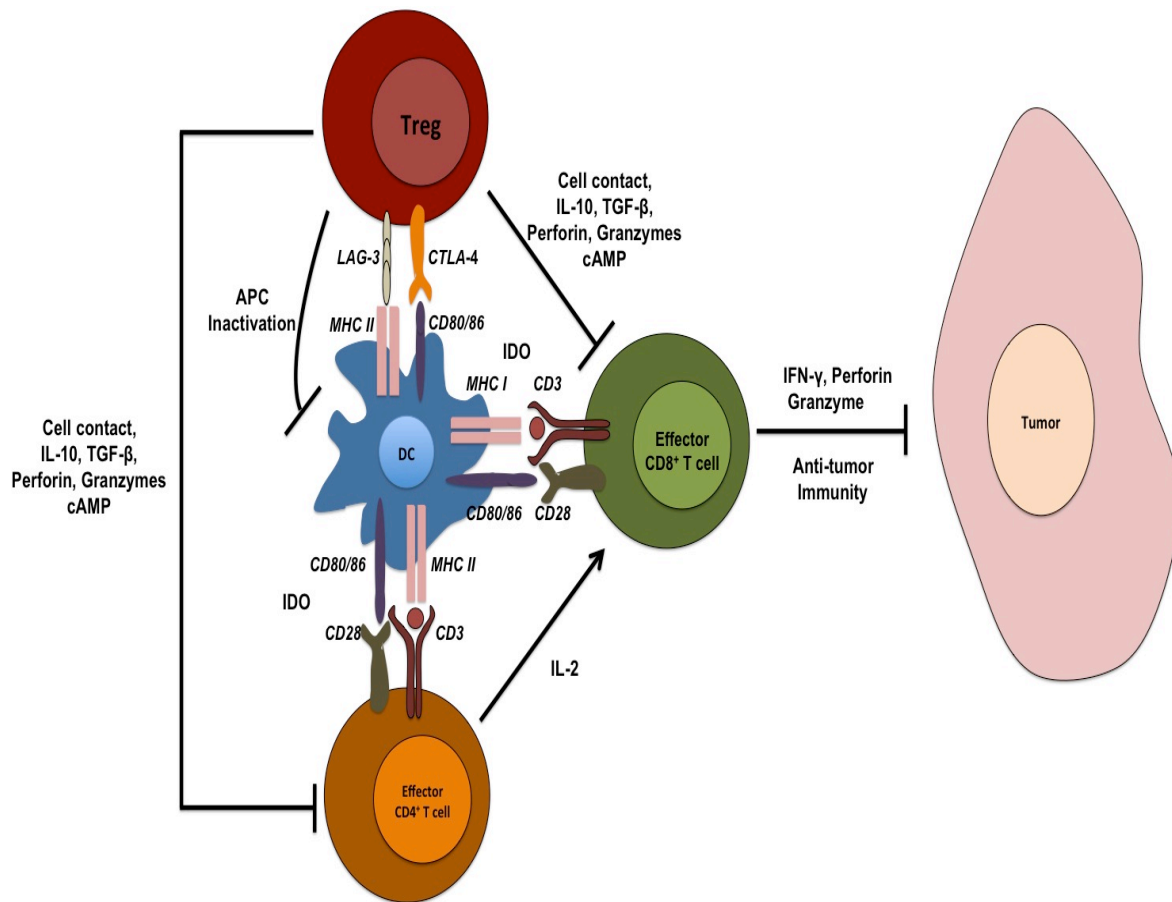
**Regulatory T Cells.** Regulatory T cells (Tregs) are characterized as a T cell population that can functionally suppress an immune response by influencing the activity of another cell type. Treg percentages are increased in the peripheral blood and tumor microenvironment of cancer patients including patients with pancreatic adenocarcinomas (72). Tregs are primarily involved in inducing and maintaining peripheral T cell tolerance to self-antigens (73). In tumors, activation of Tregs is considered one of the main tumor escape mechanisms (74). Tregs dampen T cell immunity to tumor-associated antigens and therefore, serve as the main obstacle to successful immunotherapy and active vaccination in tumor-bearing hosts (75, 76). Tregs can negatively affect CD4<sup>+</sup> and CD8<sup>+</sup> T cell numbers and function (63, 77, 78). Therefore, Treg-mediated suppression of immune responses leads to immune tolerance of tumor cells. Therefore, the ability to control Tregs is crucial to the development of effective immunotherapy strategies for treating cancer patients (79).

Classical Tregs are thymus-derived CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> T cells. However, there are several phenotypically distinct subsets of Tregs including natural (nTregs) and induced Tregs (iTregs). The classical Tregs are mainly nTregs that originate in the thymus and suppress via cell-cell contact mechanisms (90). Alternatively, iTregs consist of type I regulatory T cells (TR1) (CD4<sup>+</sup>IL-10<sup>+</sup>FoxP3<sup>-</sup>), which originate in the periphery and suppress through the production of IL-10 (91). T helper types 3 (TH3) (CD4<sup>+</sup>TGF-



$\beta^+$ ) Tregs are another subset of iTregs that suppress through TGF- $\beta$ -dependent mechanisms (92). TGF- $\beta$  induces FoxP3 expression in CD4<sup>+</sup>CD25<sup>-</sup> T cells, which leads to the accumulation of CD4<sup>+</sup>CD25<sup>+</sup> T cells with Treg suppressive functions (93).

FoxP3 is a transcription factor that serves not only as a key intracellular marker of Tregs but is also critical for their development and function (80, 81). The suppressive function of Tregs is mediated by FoxP3 transcriptional regulation of a number of important Treg genes including the activation of CTLA-4 and glucocorticoid-induced tumor necrosis factor receptor (GITR) and the repression of T effector cytokine genes, IL-2 and IFN- $\gamma$  (74, 82-84). Tregs suppress anti-tumor immune responses via a variety of mechanisms. Tregs may indirectly inhibit the activation of effector T cells by inhibiting the maturation and function of dendritic cells via co-stimulatory molecule CTLA-4 and lymphocyte-activation gene 3 (LAG-3) binding to CD80/86 and MHC II on DCs, respectively. This results in DC production of immunosuppressive indoleamine 2,3-dioxygenase (IDO) (85, 86). Tregs can also directly inhibit CD4<sup>+</sup> and CD8<sup>+</sup> T cells via cell-to-cell contact and the production of inhibitory cytokines including interleukin-10 (IL-10) and transforming growth factor beta (TGF- $\beta$ ) (87, 88). In addition, Tregs can also induce cytolysis of effector CD4<sup>+</sup> and CD8<sup>+</sup> T cells by producing perforin and granzymes that induce apoptosis (89). Tregs have also been shown to directly suppress effector T cells by transferring the inhibitory second messenger cyclic AMP (cAMP) into these cells using membrane gap junctions (90). In turn, effector T cell production of IL-2 can contribute to Treg suppressive function (91) and IL-10 and TGF- $\beta$  can induce Tregs (92, 93) (**Figure 1.5**).



**Figure 1.5 A representation of the mechanisms by which regulatory T cells (Treg) suppress anti-tumor immune responses.** Tregs cause the inactivation of antigen presenting cells (APC), especially dendritic cells (DC), causing them to produce indoleamine 2,3-dioxygenase (IDO). IDO inhibits the proliferation of effector CD4/CD8<sup>+</sup> T cells. Tregs can also directly inhibit CD4/CD8<sup>+</sup> T cells via cell-to-cell contact, the production of IL-10 and TGF-β, the cooperative activities of perforin and granzymes and cyclic AMP (cAMP) transfer. Overall, this results in the inability of CD4/CD8<sup>+</sup> T cells to generate effective immune responses against tumors via the production of IFN-γ by CD4<sup>+</sup> T cells to activate CD8<sup>+</sup> T cells as well as by perforin and granzyme production by CD8<sup>+</sup> T cells to cause apoptosis of tumor cells. *Note.* Modified from “Advances in Immune Regulation in Transplantation by Aldopgan, 2013, Discovery Medicine,15, pg.150-159. Copyright 2014 Discovery Medicine (16).

## Transcription Factors and Hematopoiesis

Transcription factors are regulators of gene expression and can include oncogenes and tumor suppressor genes (94). Transcription factors play a key role in

regulating hematopoietic cell fate decisions by regulating lineage-specific genes (95). In addition, master transcription factors also determine the fate of terminally differentiated or mature cells (96). These transcription factors work in concert with each other, epigenetic regulators and other regulatory elements within the genome to form “gene regulatory networks”. Dysregulation of these gene regulatory networks by transcription factor overexpression, mutations, deletion or oncogenic fusions may therefore lead to malignant phenotypes (95).

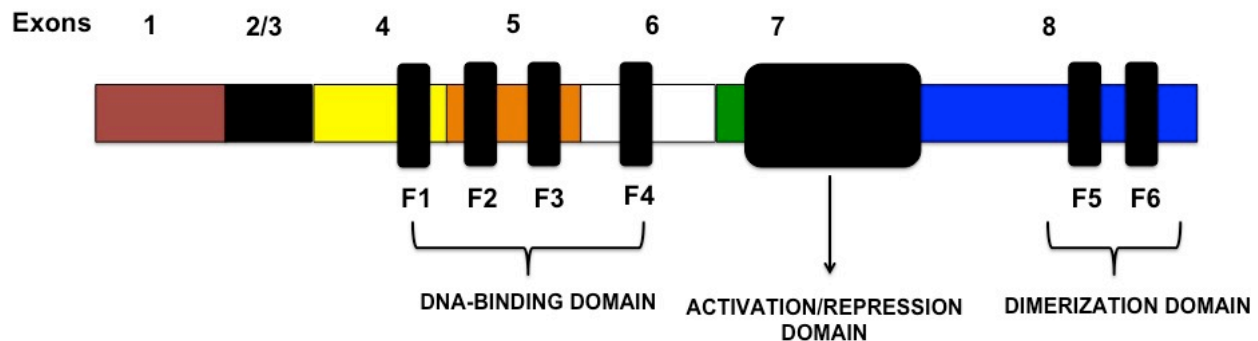
### **An Overview of the Ikaros Family**

The Ikaros family consists of five transcription factors – Ikaros, Helios, Aiolos, Pegasus and Eos – that are important regulators of hematopoiesis, especially lymphoid differentiation, proliferation and function (35, 97-99). These transcription factors interact with a complex network of gene-regulatory elements, other family members and a number of transcriptional regulators to control gene expression primarily via the recruitment of chromatin remodeling complexes (35). All family members share a similar Kruppel-type zinc-finger protein structure with four amino-terminal zinc fingers and two carboxy-terminal zinc finger motifs (35, 100-102). The carboxy-terminal zinc finger motifs encode the DNA binding domain (DBD) while those at the amino-terminal form the dimerization domain allowing interactions with self, family members and transcriptional regulators (35). Ikaros family proteins also have a bipartite activation domain adjacent to the carboxy-terminal zinc fingers, which stimulates basal transcriptional activation of target genes (103-105) (**Figure 1.6**). Ikaros, Aiolos, Helios and Eos contain four amino-terminal zinc finger motifs and all recognize the canonical

sequence “GGAAA” (35, 106), while Pegasus, which only contains three amino-terminal zinc finger motifs recognizes a “GNNNGNNG” sequence (107). Alternative splicing produces various isoforms of Ikaros family members with varying number of amino-terminal zinc finger motifs, which allow for functional differences in their DNA binding affinities (35). Isoforms containing less than three amino-terminal zinc finger domains in the DBD do not bind DNA and are considered dominant negative (DN) isoforms that are defective and can inhibit the activity of functional isoforms (101). Ikaros and Aiolos are the most highly conserved family members and are typically expressed in lymphoid tissues along with Helios. As a result, deregulated expression of these family members has been implicated in leukemias and lymphomas. Ikaros expression has also been detected in the brain and pituitary (108). Eos is more broadly expressed but its function has not been fully investigated. Pegasus, the most divergent family member, is also widely expressed with unknown functions (35).

### **An Overview of Ikaros**

Dr. Katia Georgopoulos’ group first isolated the Ikaros gene in 1992 during studies aimed at identifying transcription factors that control commitment to the T cell lineage (108). The Ikaros contains eight exons, an untranslated exon 1 seven translated exons (exon 2 to 8). Alternative splicing of the Ikaros gene gives rise to eight isoforms (Ik1-Ik8). Ikaros protein contains four zinc finger N-terminus DNA binding motifs and two C-terminus zinc fingers important for dimerization with an activation/repression domain **(Figure 1.6)**.



**Figure 1.6. A schematic of the Ikaros-1 (Ik-1) protein and its features.** Ikaros' 8 exons, including the untranslated exon 1 are depicted. Bars represent zinc fingers, F. Ik-1 has a DNA-binding domain (DBD) at its N-terminus characterized by the presence of four zinc finger motifs. Ik-1 also has a bipartite activation/repression domain in exon 7. At the C-terminus, Ik-1 contains two zinc finger motifs that form its dimerization domain. *Note.* Modified with permission from "Ikaros transcription factors: flying between stress and inflammation" by Chrousos et al, 2005, JCI,115, pg.844-848. Copyright © 2005, American Society for Clinical Investigation (14).

Ik1, Ik2 and Ik3 contain at least three N-terminal zinc fingers in the DBD and therefore bind DNA at the "GGAAA" motif. Ik-4 only contains two N-terminal zinc fingers binds tandem recognition sites that include this sequence. Ik5, Ik6, Ik7 and Ik8 contain one or no N-terminal zinc fingers and therefore do not bind DNA. Facilitation of homo- and heterodimer complexes through the conserved c-terminal zinc finger motifs allow for interactions of various Ikaros isoforms. The formation of such complexes between functional isoforms with N-terminal DNA-binding capabilities (i.e., Ik1, Ik2 and Ik3) increases their DNA affinities and transcriptional activities. Oppositely, complexes consisting of DN isoforms with these functional isoforms cannot bind DNA and are transcriptionally inactive (109). Functional Ik-1 and Ik-2 are the most highly expressed isoforms throughout development (106, 110). Subsequent mouse studies showed that

Ikaros is in fact required for lymphocyte development. Ikaros knockout mice (deletion of C-terminal resulting in functional inactive proteins that are rapidly degraded) lack B cells and their precursors as well as fetal T cells but their T cells develop normally after birth with increased CD4<sup>+</sup> T cells detected in the thymus (111). These Ikaros knockout mice also display defects in NK cells and dendritic cells (112-115). Mice expressing a DN form of Ikaros that is still able to dimerize with other Ikaros family members, exhibit more severe defects including the absence of T cells after birth which results in severe infections and death (105). Additionally, mice with one disrupted and one functional copy of Ikaros (+/-) display hyperproliferative T cells responses and develop T cell malignancies (116). The less marked phenotypes observed in the Ikaros knockout mutants are thought to reflect the functional redundancies of the Ikaros family members as the presence of other family members may compensate for the lack of Ikaros (35, 111). On the contrary, the DN Ikaros mutants produce functional proteins that may inactivate Ikaros itself and other family members (35, 105).

Studies by Schjerven et al aimed at identifying the role of individual zinc finger motifs of the Ikaros protein in regulating its transcriptional activity in lymphopoiesis and leukemogenesis (117). They found that Ikaros zinc fingers 2 and 3 mediate its DNA binding activity to its core consensus sequence while zinc fingers 1 and 4 bind to other specific sites (106, 118, 119). They generated mice lacking exons encoding Ikaros zinc fingers 1, which lacked full-length Ikaros protein, only expressing an alternatively spliced Ikaros isoform. They also generated mice lacking Ikaros zinc finger 4 which expressed mutant forms of both isoforms. These studies revealed that these zinc finger regions have distinct biological functions. Zinc finger 1 was found to be important for B cell

development while zinc finger 4 was mainly important for natural killer cell, dendritic and T cell development. Mice lacking zinc finger 4 developed thymic lymphomas, which, suggest that this motif is responsible for Ikaros' tumor suppressor activity. Overall, their studies show that zinc fingers 1 and 4 help Ikaros to recognize different DNA sequences but that the genes that are differentially targeted in the absence of either of these zinc finger motifs are few, suggesting that effects of Ikaros are mediated by its regulation of a small number of target genes (106, 118, 119).

**Ikaros Expression.** Ikaros expression was found to be primarily restricted to embryonic, fetal and adult hematopoietic cells. Ikaros mRNA is expressed in the developing embryo especially in the blood islands of the E8 yolk sac, mesodermal cells in the embryo proper and the fetal liver at E9.5 (97, 109). Ikaros is also expressed in the fetal thymus from E10.5, which coincides with the presence of fetal lymphoid precursors (97, 109). Within the bone marrow, Ikaros expression is first detected in pluripotent, self-renewing hematopoietic stem cells (HSC) and then in precursor myeloid and lymphoid cells (110, 120, 121). Subsequently, Ikaros expression is downregulated in mature monocytes, macrophages and erythrocytes but its expression is still detectable in granulocytes (121). On the contrary, Ikaros expression is upregulated in thymocyte precursors and is maintained at high levels in mature T cells in both fetuses and adults. Ikaros upregulation is also observed during B-cell differentiation (110). Overall, Ikaros expression is highest in double positive thymocytes and is highly expressed in mature T and B-lymphocytes as well as natural killer (NK) cells (108).

Therefore, it is apparent that Ikaros is especially critical for proper development, differentiation and homeostasis of lymphoid cells (122).

**Ikaros and Hematological Malignancies.** In humans, mutations in the IKZF1 gene encoding Ikaros and overexpression of DN isoforms often occur in many different types of leukemias such as acute lymphoblastic leukemias (ALLs), acute myeloid leukemia (AML) and chronic myeloid leukemia (CML) (123-126). These findings suggest that Ikaros may function as a tumor suppressor in human leukemias. Ikaros variants are also overexpressed in lymphomas such as histiocytic lymphoma, Burkitt lymphoma, anaplastic large cell lymphoma and non-Hodgkin lymphoma (127-129). Orozoco et al (2013), showed that in cases of overexpression of Ikaros DN isoforms, there is also reduced expression of functional isoforms, suggesting that the overexpression of DN isoforms is not directly responsible for malignant phenotypes but more so, the imbalance of the various isoforms, especially since DN isoforms are expressed in healthy individuals (130).

**Ikaros and Solid Cancers.** Little is known about the involvement of Ikaros in tumor development in solid cancers. Using microarray analyses, one study in particular has shown that Ikaros is widely expressed in solid tumors such as bladder, blood, breast, colorectal, gliomas, head and neck, lung, ovarian and skin cancers. Moreover, this study reported a correlation between Ikaros expression and the prognoses of 13 cancers including breast, lung, ovarian and skin cancers (131). In addition, Ikaros has been implicated in regulating the expression of genes that control the epithelial to



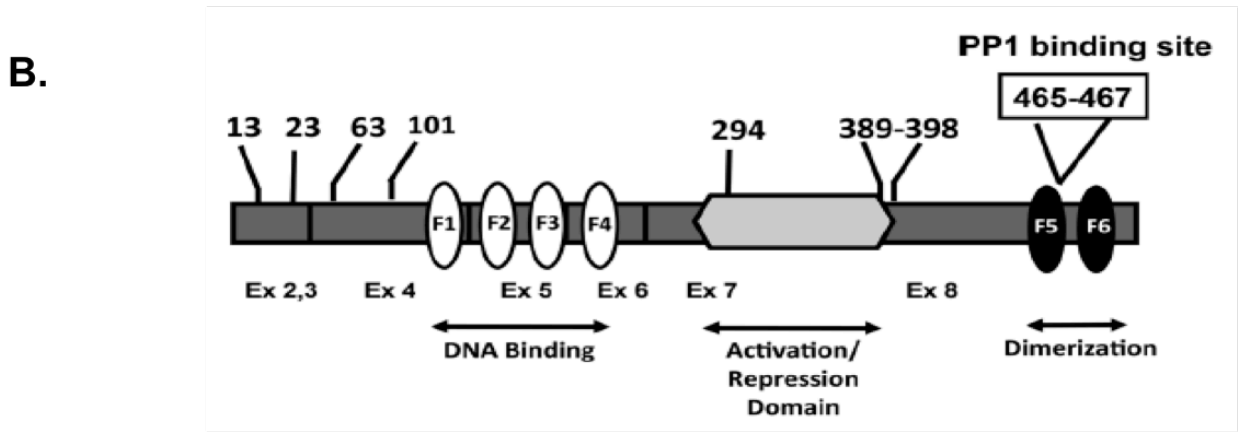
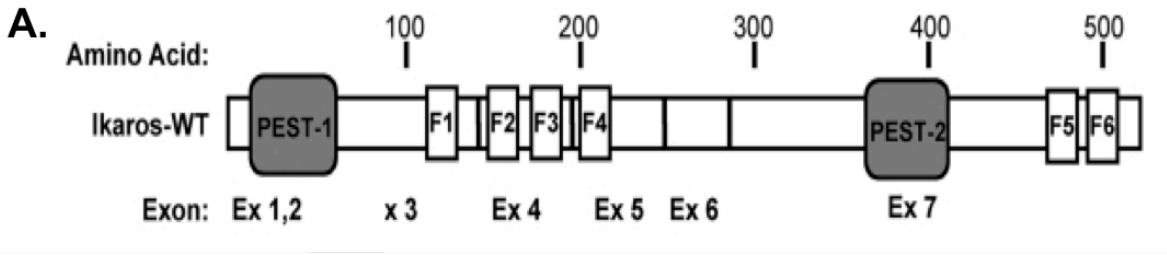
mesenchymal (EMT) transition process in ovarian cancer cells (132). Ikaros has also been shown to regulate the migration and invasion of lung cancer cells (133). Overall, a detailed understanding of the function of Ikaros in solid tumors is yet to be defined.

**Ikaros and Gene Regulation.** Ikaros can both activate and repress gene expression, primarily via chromatin remodeling mechanisms (100, 101, 134). In resting lymphocytes, Ikaros is localized to the nucleus in what is described as a diffuse, dot-like pattern. However, as it becomes activated, Ikaros takes on a ring-shaped tortoid structure near pericentromeric heterchromatin (135, 136). In lymphocytes, Ikaros forms a 2-MDa complex that contains 10–12 Ikaros molecules and other proteins (137). In T cells, Ikaros forms complexes with Mi-2b (an ATP-dependent chromatin remodeler) and histone deacetylases (HDACs) 1 and 2. These are all components of the nucleosome remodeling and deacetylation (NURD) complex. This Ikaros/NURD complex is involved in chromatin remodeling *in vitro* and histone deacetylation (137). Additionally, in mature lymphocytes, a small amount of Ikaros is also associated with the SWI-SNF remodeling complex as well as with the co-repressors Sin3, C-terminal binding protein (CtBP) and CtBP-interacting protein (CtIP) (138). Based on these findings, it has been proposed that Ikaros can regulate transcription via 1) the recruitment of chromatin remodeling complexes, which facilitate nucleosome remodeling and the binding of transcriptional activators or repressors 2) histone modifications mediated by the Ikaros/NuRd or Ikaros/Sin3 complexes that contain HDACs 1 and 2, therefore causing histone deacetylation and transcriptional silencing and 3) CtBP and CtIP-mediated suppression, independent of HDAC activity (139). Ikaros' regulation of gene expression via these

various mechanisms can therefore influence the development and function of immune cells.

**Ikaros and T Cells.** Defects in T cells and their resulting malignancies in Ikaros deficient mice pointed to its critical role in T cell development. In particular, Ikaros plays an important role in thymocyte differentiation and the development of mature T cells. Ikaros controls the differentiation of CD4<sup>+</sup>CD8<sup>+</sup> (double positive) thymocytes towards CD4 and CD8 T cell fates as seen in Ikaros null mice (140, 141). Terminaldeoxytransferase (TdT) is repressed by Ikaros at the early stages of thymocyte development (142). Ikaros also modules Cd4 gene expression using chromatin remodeling complexes (143) and activates the CD8a gene (144). Ikaros controls CD4<sup>+</sup> and CD8<sup>+</sup> proliferation in response to TCR signaling as T cells with reduced Ikaros activity require less engagement of their TCR for activation, proliferate abundantly in response to IL-2 and are less sensitive to inhibition of both TCR and IL-2 signaling (145). Ikaros also regulates key genes involved in T cell activation, anergy and tolerance such as CD3 delta activation (108), IL-2 in CD4<sup>+</sup> and CD8<sup>+</sup> T cells (146-148), IL-10 in CD4<sup>+</sup> T cells (149), IFN- $\gamma$  production in Th2 differentiation (150), IL-22 in CD4<sup>+</sup> T cells (151) and has recently been suggested to be involved in the differentiation of IL-17-producing T cells (152). Ikaros regulates the expression of several transcription factors involved in T cell differentiation including Signal Transducer and Activator of Transcription 4 (STAT4) (153) and T-bet (150, 154). Ikaros also regulates some Notch target genes that are critical for normal T cell development (155, 156).

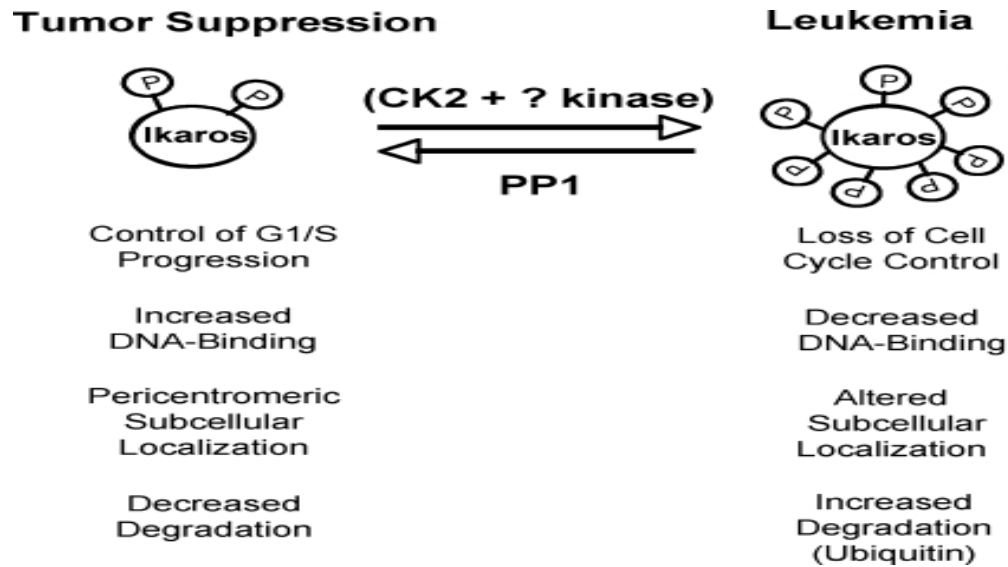
**Post-Translational Modifications of Ikaros.** Studies have revealed that Ikaros undergoes a number of post-translational modifications. The most well-studied post-translational modification of the Ikaros protein is phosphorylation. Phosphorylation of Ikaros was first identified in studies aimed at understanding how Ikaros is regulated cell cycle progression based on its role in lymphocyte development and proliferation (157). It was found that Ikaros was mainly phosphorylated at two major sites: a serine/threonine-rich region within amino acids 385 to 394 of exon 8 and S63 in the alternatively spliced exon 4, which affect its ability to negatively regulate the G1/s transition of the cell cycle (157) (**Figure 1.7**). Mutations that prevented phosphorylation in exon 8 led to increased ability of Ikaros to inhibit cell cycle progression and its DNA binding affinity (157). Protein kinase CK2 (formerly Casein Kinase II) was found to be mainly responsible for this observation (157). Subsequently, four novel CK2 phosphorylation sites at amino acids 13,23,101 and 294 in Ikaros were discovered (**Figure 1.7**). Mutations of amino acid 13 and 294 decreased Ikaros ability to bind DNA probes, altered its pericentromeric localization and prevented its regulation of its target gene, TdT, involved in thymocyte differentiation (158). These findings provided evidence that CK2 activity and/or hyperphosphorylation of Ikaros play a role in T cell differentiation (159). Dephosphorylation of Ikaros in its c-terminus is mediated by protein phosphatase 1 (PP1). PP1 dephosphorylation of Ikaros is necessary for its DNA binding activity, ability to localize to pericentromeric heterochromatic and protein stability. Increased CK2-mediated phosphorylation of Ikaros versus PP1 dephosphorylation at proline (P), glutamic acid (E), serine (S), and threonine (T) (PEST) regions leads to increased protein degradation of Ikaros via the ubiquitin/proteasome pathway (15) (**Figure 1.7**).



**Figure 1.7 Ikaros PEST and phosphorylation regions.** **A.** Schematic representation of two PEST regions in the Ikaros protein. **B.** Schematic representation of CK2 phosphorylation sites and PP1 binding site in Ikaros protein. **A. Note.** Reprinted from “Ikaros Stability and Pericentromeric Localization Are Regulated by Protein Phosphatase 1” by Popescu et al., 2009, J Biol Chem. 284, pg.13869-13880. Copyright © 2009, The American Society for Biochemistry and Molecular Biology, Inc. Reprinted with permission (15). **B. Note.** Reprinted from Protein Phosphatase 1 (PP1) and Casein Kinase II (CK2) regulate Ikaros-mediated repression of TdT in thymocytes and T-cell leukemia” by Wang et al., 2014, Pediatric Blood and Cancer. 61, pg. 2230-2235. © 2014 Wiley Periodicals, Inc. (19).

Overall, insight into the regulation of Ikaros showed that a balance between CK2 and PP1 is needed for normal Ikaros expression and function in regulating transcription, stability, cell cycle progression, T cell differentiation and the prevention of malignant transformation such as leukemias (15) (**Figure 1.8**). Both Spleen tyrosine kinase (syk) and bruton’s tyrosine kinase (btk) have also recently been found to phosphorylate

Ikaros leading to nuclear localization of Ikaros, DNA binding activity and transcription factor function (160, 161).



**Figure 1.8 A representation of Ikaros' regulation by CK2 and PP1.** PP1 dephosphorylates Ikaros maintaining its protein stability. However, CK2 hyperphosphorylates Ikaros, which facilitates its polyubiquitination and eventual protein degradation by the ubiquitin-proteasome system. *Note.* Reprinted from "Ikaros Stability and Pericentromeric Localization Are Regulated by Protein Phosphatase 1" by Popescu et al., 2009, J Biol Chem. 284, pg.13869-13880. Copyright © 2009, The American Society for Biochemistry and Molecular Biology, Inc. Reprinted with permission (15).

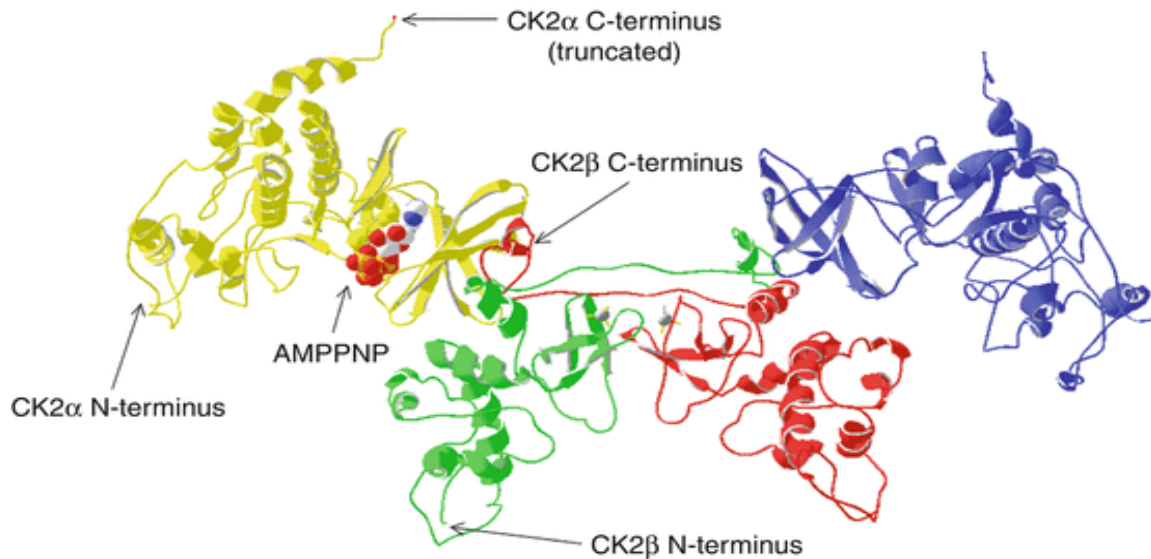
Recent studies have shown that pharmacological agents can regulate Ikaros protein stability. The immunomodulatory agents lenalidomide and pomalidomide are used to treat a number of blood cancers. These drugs cause proteasomal degradation of Ikaros and Aiolos in T cells, altering T cell function, via their effects on a ubiquitin ligase (162). Similar effects of these drugs on Ikaros protein degradation have also been discovered in multiple myeloma cells (163, 164).

Ikaros is also posttranslationally modified by SUMOylation (165). SUMOylation involves the conjugation of the small ubiquitin-related modifier (SUMO) protein via an enzymatic pathway similar to ubiquitination but instead being capable of maintaining protein stability. Ikaros SUMOylation occurs with the N-terminal repression domain at K58 and K240 negatively affecting its repressor functions by disrupting its interactions with HDAC-dependent and –independent chromatin remodeling complexes (165).

### **Protein Kinase CK2**

Since its discovery in 1954 by Burnett and Kennedy (166) , protein kinase CK2 has been a widely studied protein of interest in a number of pathological events (167). Protein kinase CK2 (~130 kDa) is a ubiquitously expressed, second messenger-independent, constitutively activated serine/threonine kinase (168, 169). CK2 forms a holoenzyme typically composed of two  $\alpha$  or  $\alpha'$  catalytic subunits (~42 and 28 kDa) linked by two molecules of the  $\beta$  regulatory subunit (~28 kDa) to form either a hetero- or homo-tetramer, depending on the cell type (168, 170-172) (**Figure 1.9**)

In most cases, the CK2 $\beta$  subunit has been shown to confer the enzyme's substrate specificity as well as its activity and stability (173, 174). However, studies have also revealed that the individual subunits also exist and function outside of the confines of the holoenzyme complex (168, 175). The requirement for protein kinase CK2 as a critical regulator of development and cellular proliferation/survival is evident based on studies, which showed embryonic lethality in CK2 $\beta$  knockout mice while CK2 $\alpha'$  knockout mice are viable but produce sterile offspring, indicating its role in spermatogenesis (176-178).



**Figure 1.9. Crystal structure of tetrameric CK2.** Ribbon representation of CK2 holoenzyme and subunits (PBD no. 1JWH) bound with non-hydrolysable ATP analogue adenylyl imidodiphosphate (AMPPNP). The catalytic  $\alpha$  subunits are presented in purple and yellow; the regulatory  $\beta$  subunits are in red and green. Gray circles represent  $Zn^{2+}$ . *Note.* Reprinted from “Casein Kinase II” by Turowec et al., 2012, Encyclopedia of Signaling Molecules, pg.234-242. © Springer, Part of Springer Science+Business Media. Reprinted with permission (11).

CK2 exhibits its effect through phosphorylation of its substrates at residues N-terminal to clusters of acidic residues with the minimal consensus sequence S/T-X-X-D/E (179), using either ATP or GTP as its phosphate donor (168, 180). The diversity of its numerous substrates is attributed to the fact that CK2 is ubiquitously expressed in mammalian cells and tissues (181, 182). CK2 shuttles between the cytoplasmic and nuclear compartments under various conditions, such as in response to growth stimuli (183, 184). However, its functionality, as it relates to cell growth and apoptosis, appears to primarily occur in the nucleus (185). As a result, the large repertoire of potential CK2 substrates found in various cellular compartments, are mainly transcription factors and genes, involved in cellular proliferation and play key roles in oncogenic signaling (170,

172, 186). CK2 substrates that contribute to cellular advantages in cancer cells have been extensively studied and described. It is well understood that CK2 regulates and interacts with a number of oncogenes and tumor suppressor and other important genes involved in transcription and chromatin remodeling, cell cycle arrest, cell proliferation and survival, apoptosis and metastasis and invasion, signal transduction pathways that are often dysregulated in cancers (171, 187).

CK2 is expressed in both normal and cancer cells and was therefore initially thought to be a marker of cellular proliferation (187, 188). However, studies subsequently proved that elevated levels of CK2 in the nucleus and its suppression of apoptosis and induction of dysplasia were specific to cancer cells (189-193). In fact, CK2 protein expression is upregulated in all cancers examined thus far (171, 172, 187), emphasizing its role in tumorigenesis. CK2 $\alpha$  in transgenic mice leads to the development of T cell leukemia and lymphoma ((194-197), similar to that observed in mice with impaired Ikaros function (145, 198-200).

### **CK2 Inhibitors**

With studies providing evidence that CK2 plays a critical role in cellular processes that lead to tumorigenesis(185), it is no surprise that emerging research has extensively focused on creating specific inhibitors of CK2 (13) (Summarized in **Table 1.1**).

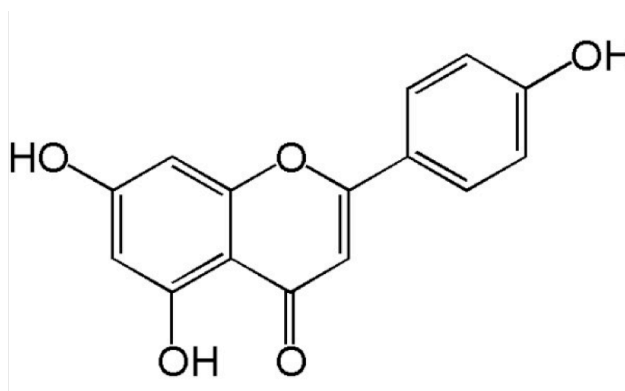


Inhibitor Family	Name	No. of PKs inhibited/No. of PKs tested	PKs inhibited
Benzoimidazole derivative	DRB	1/1	CK2
Benzoimidazole derivative	TBI	11/76	CK2, PKD1, CDK2, DYRK1a, DYRK2, DYRK3, PIM1, PIM2, PIM3, HIPK2, HIPK
Benzoimidazole derivative	DMAT	11/76	CK2, PKD1, CDK2, DYRK1a, DYRK2, DYRK3, PIM1, PIM2, PIM3, HIPK2, HIPK3
Benzoimidazole derivative	TBB	8/76	CK2, DYRK1a, DYRK2, DYRK3, PIM1, PIM2, PIM3, HIPK2
Carboxyl acid derivative	IQA	1/44	CK2
Anthraquinone	Emodin	4/33	CK2, SGK, GSK3 $\beta$ , DYRK1a
Natural derivative - flavonoid	Apigenin	12/33	CK2, MSK1, AMPK, GSK3b, S6K1, CHK1, PHK, SGK, CDK2, LCK, c-FCR, DYRK1a
ATP-Competitive Inhibitor	CX-4945	8/238	CK2, DAPK3, FLT3, HIPK3, TBK1, , CLK3, PIM1, CDK1

**Table 1.1 Common CK2 inhibitors.** CK2 inhibitors by inhibitor family, selectivity and protein kinases (PKs) inhibited. Selectivity is represented as the ratio between the total number of PKs inhibited by the molecule (with comparable or better efficacy than CK2; 500-fold greater than the IC<sub>50</sub> of CK2 for CX-4945) and the total number of PKs on which the activity has been tested. *Note.* Modified with permission from “How druggable is protein kinase CK2?” by Cozza et al., 2010, Med Res Rev, 30, pg.419-462. © 2009 Wiley Periodicals, Inc. (13).

CK2's ATP binding site contains unique bulky residues that favor the design of ATP-competitive inhibitors (201, 202). DRB (5,6-di-chloro-1-( $\beta$ -D-ribofuranosyl)-benzimidazole) is one of the first identified CK2 inhibitors (203). Natural compounds, specifically flavonoids a group of substances found in fruit, vegetables, wine and tea, have also been found to inhibit CK2 activity. These include, apigenin and quercetin, which are more potent inhibitors of CK2 activity than DRB but are also not very specific (202). Apigenin, (4',5,7-trihydroxyflavone,5,7-dihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one) (**Figure 1.10**), is present in plants and in high levels in fruits, vegetables, herbs and spices especially, chamomile and parsley (204). Apigenin is characterized as a selective CK2 inhibitor with a K<sub>i</sub> near 1 $\mu$ M in cell culture (205) and an

IC50 = 0.80  $\mu$ M (206). Apigenin is an ATP-competitive CK2 inhibitor (207). Apigenin displays pro-apoptotic, anti-inflammatory, anti-cancer and chemopreventative effects, which are thought to depend on its effects on CK2 (204).



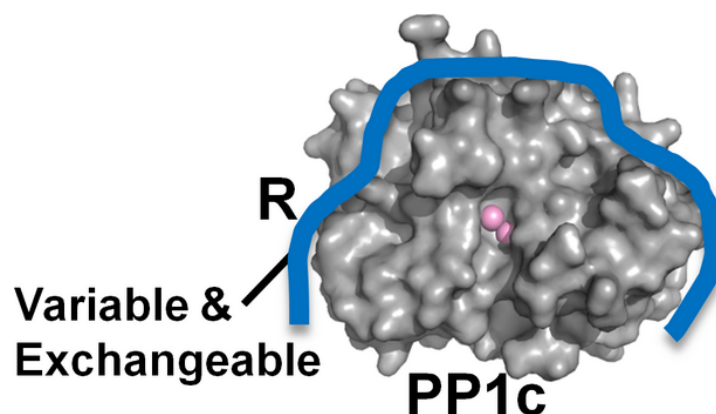
**Figure 1.10. The structure of apigenin (5,7-Dihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one).**

Another natural product shown to inhibit CK2 and other kinases is emodin (1,3,8-trihydroxy-6-methyl-antraquinone). However, its specificity is quite broad (208). Currently, the most commercially available CK2 inhibitors are TBB (4,5,6,7-tetrabromo-1H-benzotriazole) and DMAT (2-dimethylamino-4,5,6,7-tetrabromo-1H-benzimidazole). Analyses showed that these two inhibitors are fairly specific to CK2 (209, 210). TBB is a derivative of DRB (209) while DMAT is a derivative of TBB's analogue, TBI (tetrabromo-benzimidazole) (211). IQA (5-oxo-5,6-dihydro-indolo(1,2-a)quinazolin-7-yl]acetic acid) another CK2 inhibitor, shows enhanced selectivity and efficacy in vitro and in vivo compared to TBB, emodin and apigenin (202). More recently, Cylene Pharmaceuticals developed the first orally bioavailable CK2 inhibitor called CX-4945 (Silmitasertib). This potent and highly selective, small molecule inhibitor, inhibits both

CK2 $\alpha$  and CK2 $\alpha'$  and demonstrates causes cell cycle arrest, inhibition of cellular proliferation pathways and promotes apoptosis (212, 213). CX-4945 advanced to clinical trials in patients with solid tumors and was well-tolerated with minimal side effects, inhibited CK2 and AKT pathways and stabilized disease in some patients (214).

### Protein Phosphatase 1 (PP1)

PP1 (~38.5 kDa) is one of the major eukaryotic serine/threonine phosphatases belonging to the PP1 family, which includes PP1, PP2A, PP4, PP6, PP2B/calcineurin, PP5 and PP7 (12, 215). PP1 and PP2A are the most abundantly expressed with PP1 being expressed in all cells. The PP1 catalytic subunit (PP1c) can complex with over 200 regulatory subunits, converting PP1c into various holoenzymes (216) (**Figure 1.11**) with discrete substrates, subcellular localizations and regulatory mechanisms. On the contrary, PP1 inhibitory proteins bind to its active site containing metal ions that are necessary for its activity (216).



**Figure 1.11. Representation of the single catalytic domain of PP1. (PP1c – gray; pink - metal ions) from PDB 3EGG.** PP1c can bind to a plethora of regulatory (R) subunits (blue), which converts it to specific holoenzymes with different activities and substrate specificities. Note. Reprinted from “Structural basis for protein phosphatase 1 regulation and specificity” by Peti et al., 2012, FEBS Journal, 280, pg. 596-611 Copyright © 1999-2014 John Wiley & Sons, Inc. All Rights Reserved. Reprinted with permission (12).

As a result, PP1 plays roles in a number of cellular processes by regulating signaling cascades via independent mechanisms (217). In all eukaryotes, multiple genes encode PP1c, except in yeast (218). Four mammalian PP1c products were initially designated PP1 $\alpha$ , PP1 $\beta$  (also known as PP1 $\delta$ ), as well as PP1 $\gamma_1$  and PP1 $\gamma_2$ , which are alternative splice variants (217). PP1 $\alpha$ , PP1 $\beta/\delta$  and PP1 $\gamma_1$  are ubiquitously expressed, while PP1 $\gamma_2$  is testis-specific. Studies have identified over 200 PP1 regulatory subunits (12, 216) that bind PP1c and target it to particular subcellular compartments or substrates, enhancing its substrate specificity (217). On the contrary, inhibitory proteins can bind and block PP1c's active site that contains two metal ions (12, 219, 220). The primary PP1 binding motif, the RVxF motif, where x is any residue other than Phe, Ile, Met, Tyr, Asp or Pro, allows for binding of PP1 regulatory proteins and some PP1 substrates to PP1 but does not influence its activity (12, 221, 222). PP1 regulates cellular processes including cell cycle progression, protein synthesis, muscle contraction, carbohydrate metabolism, transcription and neuronal signaling (217).

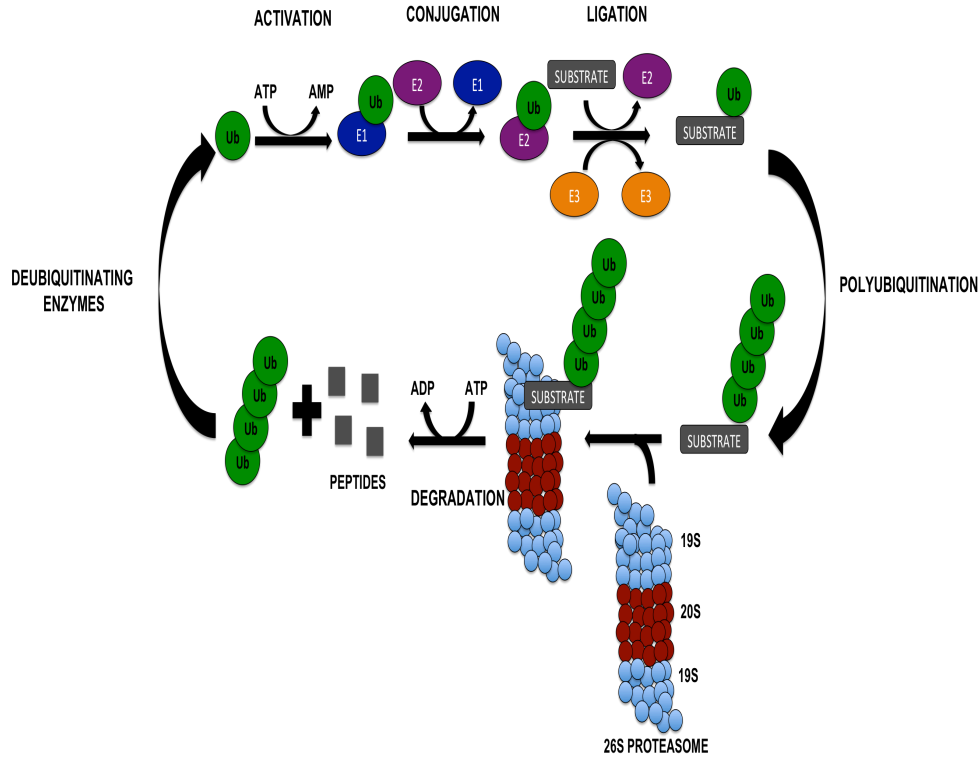
### **An Overview of Proteolysis**

Turn over of all intracellular and some extracellular proteins is a common occurrence resulting in protein hydrolysis to their amino acids components and new protein synthesis. Therefore, this process is important in maintaining protein homeostasis (223). Original studies pointed at the lysosome, where proteins and organelles are targeted by microautophagy and macroautophagy, respectively, and degraded by proteases, as the machinery primarily responsible for intracellular proteolysis. However, subsequent evidence suggested non-lysosomal mechanisms of

protein degradation of most intracellular proteins pointing to the ubiquitin-proteasome system (223).

### **Protein Degradation and the Ubiquitin-Proteasome System**

The ubiquitin-proteasome system plays an essential role in degradation of intracellular eukaryotic proteins. This irreversible process can lead to deregulation of pathways leading to cellular changes. This requires adaptation to new conditions that can cause various diseases (224). The ubiquitin-proteasome pathway consists of a series of enzymes that cooperatively add chains of ubiquitin (Ub; a small 76 residue, protein), a polypeptide co-factor, to proteins, to mark them for degradation (Glickman (225, 226). The polyubiquitination of a protein is an ATP-dependent, multi-step cascade carried out by three enzymes. E1 is the Ub-activating enzyme, E2 is the Ub-carrier or conjugating enzyme and E3 is the Ub-ligase, which is the key enzyme that recognizes proteins and catalyzes Ub transfer (224). E1 activates Ub at its C-terminus forming a linkage to itself. Next, the Ub is transferred to E2 enzymes, which are then conjugated with E3 enzymes that recognize proteins to be ubiquitinated (224). Ubiquitinated proteins are recognized by the 26S proteasome, a large multicatalytic protease that degrades them to small peptides (227). The 26S proteasome consists of two complexes, the 20S core particle, which contains the catalytic activity, and a 19S regulatory particle. The 20S unit is a barrel-shaped structure containing two outer  $\alpha$ -rings and two identical inner  $\beta$ -rings, which house the catalytic sites. The 20S subunit is also capped on one or both ends by the 19S regulatory particle, which recognizes polyubiquitinated (at least 5 Ubs) proteins, cleave the Ub chain and linearizes the



**Figure 1.12 Overview of the Ubiquitin-Proteasome System (UPS).** Ubiquitination of proteins is carried out in three steps: 1) Activation – Ubiquitin (Ub) binds to E1 activating enzyme by using one ATP to AMP 2) Conjugation – Ub is transferred to the E2 conjugation enzyme 3) E2 transfers Ub to a substrate bound to the E3 ligase. Polyubiquitylation occurs extending the ubiquitin chain on the substrate. The substrate then enters the 26S proteasome and is subject to degradation and the ubiquitin molecules are released by deubiquitination enzymes.

proteins facilitating its entry into the 20S subunit where they are digested into peptides and Ub is recycled (223, 224) (**Figure 1.12**).

The Ub-proteasome pathways also influences gene transcription. For example, many transcription factors are subject to proteasomal degradation (228). This often involves the overlap of transcriptional activation domains and ubiquitination signals (224). Degradation of regulators of transcription factors can also contribute to altered gene expression and localization (229, 230) and may thus contribute to oncogenesis.

## Content of the Dissertation and Central Hypothesis

Ikaros is defined as a tumor suppressor in T cells and has been extensively studied in hematological studies. However, its possible involvement in the regulation of T cells in solid cancers has not been fully investigated. This dissertation focuses on the regulation of Ikaros and its role in the maintenance of T cell homeostasis in murine pancreatic cancer. We hypothesize that in the pancreatic tumor microenvironment, there is an upregulation of protein kinase CK2 activity vs PP1 activity, which abrogates Ikaros' expression, thus leading to an imbalance in effector CD4/CD8<sup>+</sup> and regulatory T percentages and function. First, we show that Ikaros is downregulated at the protein but not mRNA level in splenocytes from our pancreatic tumor-bearing (TB) mice. Results suggest that downregulation may be attributed to its ubiquitin-mediated proteasomal degradation in response to factors produced by pancreatic cancer cells. Moreover, our results suggest that alternations in the balance of protein kinase CK2 and PP1, favoring increased CK2 activity may be the mechanisms involved. We also show that loss of Ikaros expression in T cells may lead to a disruption in the balance of effector CD4/CD8 T cells and regulatory T cells, needed for effective anti-tumor immunity. To further delineate the mechanism(s) by which Ikaros may be regulated and its involvement in T cell responses in our pancreatic cancer model, we evaluated the effects of apigenin, a natural plant flavonoid and selective CK2 inhibitor. We show that apigenin stabilizes Ikaros expression *in vitro* and *in vivo* while maintaining T cell homeostasis and immune function. Overall, these data suggest a previously undefined role for Ikaros in regulating effector and regulatory T cell development in murine pancreatic cancer thereby possibly making it a potential target for enhancing anti-tumor immune responses. Therefore,

pharmacological inhibition of CK2 may be a potential adjuvant therapy for treating murine pancreatic cancer.

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## CHAPTER TWO

### MATERIALS AND METHODS

#### Cell Culture

The murine pancreatic adenocarcinoma Panc02 cell line was established by Corbett et al. (1984) (1). This cell line was maintained in RPMI 1640 (Gibco) medium, supplemented with 10% fetal bovine serum (FBS), (HyClone), 2 mM L-glutamine, 100 U/ml penicillin and 100µg/ml streptomycin (Gibco) (known as Complete Media). Cells were harvested from frozen stock (provided by Dr. Nasreen Vohra), thawed in a water bath and suspended in complete growth media in a 15ml conical tube. The cells were centrifuged at 1200rpm for 5 minutes; the supernatant was discarded and the cells were resuspended in 15ml of complete growth media and transferred to a vented T-175 flask (BD Falcon). The flask was placed in an incubator at 37°C with 5%CO<sub>2</sub>. Cells were subcultured every 2 days using 0.025% trypsin-EDTA, at approximately 80% confluency. Media was removed from the flask and the cells were washed with 1X Dulbecco's Phosphate Buffered Saline (no calcium or magnesium) (Life Technologies) (1XDPBS herein referred to as PBS). The PBS was removed and 5ml of 0.025% trypsin-EDTA (Life Technologies) was added to the flask. The flask was placed in the incubator for 5 minutes. When the cells were dislodged, complete media was added to the flask, and the cells were split into a new T-175 flask and returned to the incubator

(2). Cells were similarly harvested with trypsin for all experiments. Panc02 cells at passage (P) <10 were used for all *in vitro* and *in vivo* experiments.

## Mice

Female C57BL/6 mice aged 6 to 8 weeks were purchased from Harlan Laboratories (Indianapolis). The Institutional Animal Care and Use Committee of the University of South Florida approved protocol R4152, in compliance with the Guide for the Care and Use of Laboratory Animals. The mice were maintained in a pathogen-free animal facility with 4 to 5 mice per cage. All mice were left undisturbed 1 week prior to commencing the experiments. Panc02 cells were harvested as previously described, resuspended in PBS and counted, using a 0.4% trypan blue (Thermo Scientific), a hemacytometer and an inverted microscope. The total number of cells was determined and the volume of PBS needed to resuspend the cells at a concentration of  $7.5 \times 10^5$  cells/ml was calculated. Cells were then centrifuged at 1200 rpm for 5 minutes, the supernatant was discarded and the pellet was resuspended in the calculated volume of PBS. Mice were randomly assigned into tumor-bearing (TB), control (ctrl) and apigenin (TB-API) groups, as needed. At week 7, all TB mice were subcutaneously (s.c.) injected with 100 $\mu$ l of the  $7.5 \times 10^5$  cells/ml ( $1.5 \times 10^5$ ) murine Panc02 cells while ctrl mice received 100 $\mu$ l PBS via subcutaneous (s.c.) injection, both administered on the lower, left, ventral abdomen using a 25 5/8-gauge needle. A cohort of TB mice was treated with 25mg/kg apigenin via intraperitoneal injection (i.p.) on the lower, right ventral abdomen, every three days, after the appearance of palpable tumors. Every three days, the mice were also weighed using a scale and tumors were measured using a digital caliper (2).



Approximately 28 days post Panc02 injection, mice were sacrificed by CO<sub>2</sub> asphyxiation and cervical dislocation. Animals were dissected using sterile instruments; the spleens were harvested and stored in PBS for processing. Spleens from spontaneous transgenic LSL-Kras<sup>G12D/+</sup>;LSL-Trp53<sup>R172H/+</sup>;Pdx-1-Cre mice, known as triple mutant (TrM) mice, were similarly harvested and used in this study (3). Spleens from 11 to 12-week-old female BALB/c mice were harvested and used for allogeneic mixed leukocyte reactions (MLR) (4). For *in vitro* experiments, spleens from female naïve (no injection) C57BL/6N aged 6 to 8 weeks were used.

### **Single Cell Suspension of Spleens**

Spleens from all mice were collected in PBS and made into single cell suspensions using a cell dissociation sieve and tissue grinder kit (Sigma). The single cell suspension was strained into a 50ml conical tube using a 70µm cell strainer (BD Biosciences). The single cell suspension was centrifuged at 1200 rpm for 5 minutes and the supernatant was discarded. The cell pellet was resuspended in 5ml of 1X red blood cell (RBC) lysis buffer (eBioscience), per spleen, and incubated for 5 minutes at room temperature, with pipetting. The reaction was stopped by adding 20ml of PBS. Furthermore, the cells were centrifuged at 1200 rpm for 5 minutes, the supernatant was discarded and the resulting splenocytes (leukocytes) were resuspended in PBS. Cells were diluted 1:10 in 0.4% trypan blue (Thermo Scientific), and counted for downstream experiments using a hemacytometer and inverted microscope.

## Western Blotting

Protein lysates were prepared from splenocytes of control, TB, TrM mice and *in vitro* treated naïve splenocytes. Splenocytes were resuspended in modified radioimmunoprecipitation (RIPA) Buffer (Millipore), supplemented with Na<sub>3</sub>VO<sub>4</sub> (Sigma-Aldrich) and protease inhibitor cocktail (Sigma-Aldrich) then rocked for 45 minutes at 4°C. Cells were centrifuged at 14000 rpm for 15 minutes and protein lysate supernatants were transferred to new tubes and stored at -20°C. Protein concentrations were determined using the BCA Protein Assay Kit (Thermo Fisher Scientific) and protein lysates diluted at a ratio of 1:10. A maximum of 40 µg cell protein lysates diluted in 4x NuPAGE® LDS Sample Buffer or 2x Laemmli Buffer were loaded and resolved using NuPAGE® 4–12% Bis-Tris pre-cast polyacrylamide Gels (Invitrogen) or 12% hand-cast gels, respectively, for 45 minutes at 200V by SDS-PAGE electrophoresis. Proteins were then transferred from the gels to nitrocellulose membranes (Whatman) at 25V for 2.5 hours. The membranes were blocked with 5% non-fat milk in 0.1% Tween-20/PBS for 1hr at room temperature and washed three times, using 0.1% Tween-20/PBS for 5 minutes each. The following antibodies were diluted in 3% bovine serum albumin (BSA) (Sigma-Aldrich) and 2% non-fat milk in 0.1% Tween-20 (Fisher Scientific)/PBS: anti-Ikaros (Cell Signaling), at a dilution of 1:1000, anti-p53 (Santa Cruz), anti-CK2 $\alpha$  (Santa Cruz Biotechnology), and anti-PP1 (Santa Cruz Biotechnology) at a dilution of 1:200. The blots were incubated with these antibodies overnight at 4°C and then washed three times using 0.1% Tween-20/PBS for 5 minutes each. Primary antibodies were detected using their respective secondary IgG, HRP-conjugated antibodies (Jackson Immunoresearch), at a dilution of 1:10000 in 3% BSA and 2% non-

fat milk in 0.1% Tween-20/PBS and incubated for 1hr at room temperature. The blots were washed three times using 0.1% Tween-20/PBS for 5 minutes each. Secondary antibodies were identified using Super Signal West Pico and Femto Chemiluminescent Substrates (Thermo Fisher Scientific). As an internal control for equal protein loading, all blots were stripped and re-probed with anti- $\beta$ -actin (Sigma-Aldrich) at a dilution of 1:20,000 or anti-GAPDH (Santa Cruz Biotechnology) diluted 1:200 in 3% BSA, 2% non-fat milk in 0.1% Tween-20/PBS, for 90 minutes at room temperature; the blots were later identified using Super Signal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific). Membranes were either exposed to x-ray films (Phoenix) and developed using a Kodak M35-X OMAT Processor or imaged using a ChemiDoc XRS Imaging System (Bio-Rad). Band intensities were quantified using Quantity One 1-D densitometry and Image Lab softwares (Bio-Rad) (2).

### **Quantitative Real-Time Polymerase Chain Reaction (qPCR)**

Total RNA was extracted from single-cell suspensions of control and TB whole splenocytes using TRI Reagent (Molecular Research Center).  $5-10 \times 10^6$  Splenocytes were resuspended and lysed in 1ml TRI reagent and stored at  $-80^{\circ}\text{C}$ , until needed. Phase lock gel heavy tubes (5 Prime) were pelleted at 1500g for 30 seconds. Lysates were thawed at room temperature and transferred to phase lock gel heavy tubes. Phase separation was carried out by adding chloroform to the samples and vigorously shaken for 15 seconds. Tubes were centrifuged at 1200g for 10 minutes at  $4^{\circ}\text{C}$ . The resulting aqueous phase was transferred to newly labeled tubes, precipitated using isopropanol, and incubated at room temperature for 10 minutes. Samples were centrifuged at

12000g for 10 minutes at 4°C. The supernatant was discarded and the pellets were washed twice with 75% ethanol, vortexed and centrifuged at 12000g for 5 minutes at 4°C. Supernatants were discarded, pellets were air-dried for 5 minutes at room temperature and dissolved in RNase free water (Life Technologies). In order to degrade DNA impurities from the obtained RNA sample, DNase treatment was performed using the Ambion TURBO DNA-free™ Kit (Life Technologies), according to the manufacturer's protocol. Resulting RNA was then quantitated using a Nanodrop 2000 Spectrophotometer (ThermoScientifi). If deemed necessary, RNA clean-up was performed using the NucleoSpin® RNA Clean-up (Macherey-Nagel), according to the manufacturer's protocol and requantitated using the Nanodrop 2000. cDNA was then synthesized with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) using up to 2µg of RNA diluted in RNase-free water, as stated per the manufacturer's instructions. As a negative control, reactions were prepared without the Multiscribe™ reverse transcriptase kit component. Reverse transcription was performed using a thermal cycler (Biometra) under the following conditions: Step 1- at 25°C for 10 minutes; Step 2 - at 37°C for 120 minutes; Step 3- at 85°C for 5 minutes and Step 4-, hold. cDNA was either stored at -20°C for long-term storage or 4°C with EDTA at a final concentration of 1mM up to 24 hours. qPCR was performed using 1µl of cDNA, the SYBR Green JumpStart Taq Ready Mix (Sigma-Aldrich) and an ABI StepOne Plus Real-Time PCR System (Applied Biosystems). No template controls (NTCs) reactions were prepared without cDNA templates. The following conditions were used: 95°C for 10 min followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min, and primers: forward, 5'-CAT AAA GAG CGA TGC CAC AA-3' , reverse, 5'-CAG GAC AAG GGA

CCT CTC TG-3' (5). Each sample was assayed in triplicate. GAPDH was amplified as the internal control and reference gene. Normalization to GAPDH was used to determine relative mRNA frequency using the Comparative CT method (2).

### **CD3<sup>+</sup> T Cell Enrichment**

Whole splenocytes from control, TB and naïve mice were processed into single-cell suspensions, as previously described. CD3<sup>+</sup> T cells were purified (~90% purity) from whole splenocytes by positive selection using either anti-CD3-1-PE (eBioscience) and anti-PE-magnetic microbeads (Miltenyi) on an automated magnetic activated cell sorting (AutoMACS) Pro Separator (Miltenyi) (6) or the EasySep Mouse T Cell Enrichment kit (Stem Cell Technologies), according to the manufacturer's protocol. For automated magnetic activated cell sorting, single-cell suspensions of splenocytes were resuspended at  $2 \times 10^7$  cells/ml in 0.5% BSA/PBS, centrifuged and the supernatant was discarded. Pellets were resuspended in 45 $\mu$ l of 0.5% BSA/PBS. 5 $\mu$ l of anti-CD3-PE (eBioscience) per for  $10^7$  cells was added; the cells were incubated for 10 minutes in the refrigerator. Cells were washed, centrifuged and supernatants were discarded. Pellets were then resuspended in 160 $\mu$ l of 0.5% BSA/PBS and 40 $\mu$ l of anti-PE microbeads per for  $10^7$  cells, and incubated for 10 minutes in the refrigerator. Cells were centrifuged and washed using 0.5% BSA/PBS and resuspended in 3ml of 0.5% BSA/PBS. The cells were then filtered into a 50ml conical tube using a 70 $\mu$ m strainer. On the AutoMACS Pro Separator, cells were separated using positive selection and the POSSEL program (6). Using the EasySep Mouse T Cell Isolation Kit, splenocytes were resuspended at  $1 \times 10^8$  cells/ml in 2% fetal bovine serum (FBS)/PBS in a 5ml round

bottom polystyrene tube (BD Falcon). A maximum of 2ml of cells ( $2 \times 10^8$  cells) were used. 50 $\mu$ l/ml of normal rat serum and 50 $\mu$ l/ml of antibody cocktail were added then incubated for 10 minutes at room temperature. The provided Rapidspheres™ were vortexed for 30 seconds; 75 $\mu$ l/ml of cells were added and incubated for 2.5 minutes at room temperature. The total volume was increased to 2.5 ml using the 2%FBS/PBS buffer. The tube was placed on the EasySep magnet for 5 minutes at room temperature and the enriched CD3<sup>+</sup> T cells (unlabeled) were poured into a new tube and counted for downstream experiments. In both techniques, the percent enrichment was determined by analyzing the CD3<sup>+</sup> T cell percentages, pre- and post-enrichment, using flow cytometry and CD3-PE antibodies (eBioscience). Protein lysates were then prepared as previously described.

### ***In vitro* Assays**

Single-cell suspensions of whole and CD3<sup>+</sup> enriched T cells from splenocytes and from naïve mice were prepared and counted as previously described.  $4 \times 10^5$  splenocytes were cultured in 6-well plates containing 3ml of complete RPMI growth media. For co-culture assays, Panc02 cells ( $P < 10$ ) were grown to approximately 70% confluency in 6-well plates, at which time the media was removed, and resuspended splenocytes were added to each well. The proteasome inhibitor Cbz-LLL (carbobenzoxyl-L-leucyl-L-leucyl-L-leucine) (MG132; Sigma-Aldrich) and apigenin (Sigma-Aldrich) were resuspended in dimethyl sulfoxide (DMSO) (Fisher Scientific) at stock solutions of 100mM, and the appropriate working dilutions prepared. Calculated volumes for the indicated concentrations were directly added to the cultures. Control

wells were treated with the vehicle. All treated cells were cultured in an incubator for four hours *in vitro*. Suspension splenocytes were harvested from adherent Panc02 cells. Protein lysates of these *in vitro* treated-splenocytes were prepared and analyzed for Ikaros protein expression using western blot analysis, as previously described.

### ***In vitro* CK2 Kinase Assay**

CK2 kinase activity was measured using the CK2 assay kit (Millipore), according to the manufacturer's instructions. 15µg of protein lysates for each sample were diluted in 10µl assay dilution buffer I (ADBI) in microcentrifuge tubes. Three control and TB samples were assayed in triplicates. A control for each sample was included which lacked the CK2 specific substrate to account for background and non-specific binding. Therefore, two sets of triplicates, labeled "with substrate" (+S) and "without substrate" (-S), were prepared for each control and TB sample. 10µl of CK2 specific substrate (RRRDDDSDDD), at a final concentration of 200µM, was added to the tubes with substrate (+S) while 10µl of ADBI was added to the tubes without substrates (-S). 10µl of a PKA inhibitor cocktail was also added to each tube to inhibit the activity of other serine/threonine kinases. 10µl of 100µCi of [ $\gamma$ -<sup>32</sup>P]ATP (3000 Ci/mmol) (Elmer Perkins), diluted 1:10, was added to each tube. Reactions were incubated for 10 minutes at 30 °C with agitation and then stopped by adding 40µl of trichloroacetic acid (TCA) to each tube. 25µl aliquots of each reaction were transferred to numbered P81 phosphocellulose paper. The papers were washed 6 times with 0.75% phosphoric acid, followed by an acetone wash, dried and transferred to scintillation vials containing scintillation fluid. Samples were quantitated using a scintillation counter. CK2 activity

was calculated by subtracting the mean counts per minute (CPM) of samples in the absence of substrate from the mean CPM of samples in the presence of the substrate.

### **Immunofluorescence Microscopy**

Single-cell suspensions of control and TB splenocytes were prepared, as previously described, and resuspended at  $3.5 \times 10^5$  cells/ml in complete RPMI media. Cytospins were prepared using 100 $\mu$ l of the cell suspension, Shandon™ Single Cytoslides™ (ThermoScientific), cytology funnels (VWR) and a Shandon CytoSpin™ 2 Centrifuge (Shandon) at 300rpm for 3 minutes. Cytospin slides were air-dried and fixed at -20°C in methanol:acetone (3:1) for a minimum of 20 minutes and a maximum of 2 months. Slides were washed in Tris plus Triton-X for 3 minutes with shaking. This was followed by a Tris buffer wash with shaking for 10 minutes with buffer changes every 2 minutes. Slides were then stained with a rabbit polyclonal against Ikaros (Santa Cruz Biotechnology), diluted 1:200 in 0.1% NP-40 (NP-40; Sigma-Aldrich) in 1% BSA (Sigma-Aldrich) in PBS for 1 hr at room temperature. Slides were washed with Tris buffer with shaking for 10 minutes, with buffer changes every 2 minutes. Slides were stained with a secondary goat anti-rabbit Alexa Fluora 594 antibody (Life Technologies), diluted 1:200 in 0.1%, NP-40 in 1% BSA in PBS, for 30 mins at room temperature. Appropriate isotype controls were used to check for non-specific binding which was not detected. Slides were washed with PBS 3 times for two minutes each, followed by dH<sub>2</sub>O twice for 2 minutes each. Slides were dried and cover slips were applied and mounted using ProLong® Gold Antifade Mountant with DAPI (Life Technologies).



Immunofluorescence was imaged using a Zeiss Olympic Microscope, and analyzed using Image J Software (7).

### **Flow Cytometry**

Splenocytes were harvested from control, TB and TrM mice and were processed into single-cell suspension, as previously described. Cells were then suspended at  $2 \times 10^7$  cells/ml in 3%FBS/PBS. 50 $\mu$ l of cells ( $1 \times 10^6$  cells) were transferred to their respective wells of a 96-well V-bottom plate for mixed staining with T-cell antibodies. Cells were also added for single-stain compensations. Cells were blocked with 50 $\mu$ l Mouse Fc block (Purified Rat Anti-Mouse CD16/CD32 - BD Biosciences) diluted in 3%FBS/PBS for 15 minutes at 4°C. The plate was centrifuged and supernatants were removed from each well. A master mix was prepared containing T cell surface markers including: anti-CD3-FITC (eBioscience), anti-CD4-Pe-Cy7 (BD Pharmingen), anti-CD8-APC-H7 (BD Pharmingen), anti-CD25 PE (eBioscience) diluted in 3%FBS/PBS. 50 $\mu$ l of the master mix was added to each well containing cells for mixed staining with T-cell antibodies. Single-stains for each fluorochrome was added to wells for single-stain compensations. The cells were incubated for 30 minutes at 4°C. The cells were then washed twice with 3%FBS/PBS and transferred to labeled 5ml round-bottom polystyrene tubes (BD Biosciences) containing 75ng/ml DAPI (except for compensations). Flow Cytometry was performed using a BD LSRII (BD Biosciences Immunocytometry Systems) and the data was analyzed with FlowJo software (Tree Star Inc.) (2).

## T Cell Activation and Intracellular Staining

Single-cell suspensions of splenocytes from control, TB and TB-Api mice were processed as previously described and resuspended at  $2 \times 10^6$  cells/ml in complete RPMI media. 1ml of cells ( $2 \times 10^6$  cells) from each mouse was plated into 6-well plates. The desired volume of mouse T-Activator CD3/CD28 Dynabeads® (Life Technologies) was prepared according to the manufacturer's protocol. Briefly, the dynabeads were resuspended by vortexing for 30 seconds and transferred to a 5ml round bottom polystyrene tube (BD Falcon). 1ml of 0.1%BSA/PBS buffer was added to the tube and the dynabeads were vortexed for 5 seconds. The tube was placed on a DynaMag™-5 Magnet for 1 minute and the supernatant discarded. The tube was removed from the magnet and the dynabeads were resuspended in the same volume of complete RPMI as the desired volume of beads originally used.  $2 \times 10^6$  beads were then added to each well of the 6-well plate containing splenocytes, for a bead-to-cell ratio of 1:1. Complete RPMI media was added to each well to bring the total volume up to 3ml. The cells were placed in an incubator for 4 days. After four days, the cells from each well of the 6-well plate were removed and transferred to labeled 5ml round bottom polystyrene tubes (BD Falcon). The tubes were placed on the DynaMag™-5 Magnet for 1 minute and the supernatants transferred to newly labeled tubes.  $1 \times 10^6$  cells surface stained with anti-CD8-PerCPCy5.5 (BD Biosciences) as previously described. The cells were then resuspended in 1ml of diluted Foxp3 Fixation/Permeabilization (eBioscience) buffer (1 part Fixation/Permeabilization Concentrate to 3 parts Foxp3 Fixation/Permeabilization Diluent) and vortexed. The tubes were incubated at  $4^\circ\text{C}$  for 30 minutes in the dark. The cells were then washed with 1x permeabilization buffer and centrifuged. The

supernatants were discarded and the cells were resuspended in 1x permeabilization buffer, then stained with anti-IFN- $\gamma$ -PE and incubated at room temperature, in the dark, for 30 minutes. The cells were washed, centrifuged again and resuspended in 3%FBS/PBS. Flow Cytometry was performed using a BD LSRII (BD Biosciences Immunocytometry Systems) and the data was analyzed with FlowJo software (Tree Star Inc.) (2).

### **Mixed Leukocyte Reaction (MLR)**

Splenocytes from BALB/c mice (responders) were prepared, as previously described, and resuspended at  $1 \times 10^7$  cells/ml in 0.1%BSA/PBS. 5mM 5-(and 6)-Carboxyfluorescein diacetate succinimidyl ester (CFSE) stock from the CellTrace™ CFSE Cell Proliferation Kit (Life Technologies) was prepared by adding 18 $\mu$ l DMSO to CFSE vial. 5mM stock CFSE was diluted to 2 $\mu$ M CFSE using 0.1%BSA/PBS. An equal volume of 2 $\mu$ M CFSE was added to resuspend BALB/c splenocytes, while stirring, for a final concentration of 1 $\mu$ M CFSE. The reaction was placed in a 37°C water bath for 10 minutes, with stirring every 2 minutes. The reaction was stopped by adding 3 times the volume of ice-cold complete RPMI media and quenched on ice for 5 minutes. The cells were centrifuged and washed three times with ice-cold complete RPMI media. CFSE labeling was confirmed by flow cytometry analysis of cells pre- and post- CFSE labeling. The cells were counted and resuspended at  $4 \times 10^6$  cells/ml in complete RPMI media. 50 $\mu$ l ( $2 \times 10^5$  cells) were added to their respective wells of a 96-well U bottom plate. Whole splenocytes from control, TB and TB-Api mice (stimulators) were irradiated at 2000 rads (one-way allogeneic MLR) and resuspended at  $8 \times 10^6$  cells/ml in complete

RPMI media. 50µl ( $4 \times 10^5$  cells) were added to their respective wells of a 96-well U bottom plate (4). 100µl volume of complete RPMI media was added to each well for a total volume of 200µl. The plate was placed in an incubator at 37°C, 5% CO<sub>2</sub>. On day four, all cells were harvested and the proliferation of CD8<sup>+</sup> responder BALB/c cells was evaluated by flow cytometry and stained with anti-CD3-PerCP and anti-CD8-APCH7. The CFSE dilution profile of CFSE<sup>+</sup>CD3<sup>+</sup>CD8<sup>+</sup> cells was analyzed by FlowJo software (Tree Star Inc.)

### Statistical Analysis

All *in vivo* and *in vitro* results described in this study are representative of the mean ± S.E.M. of at least three independent experiments analyzed with two-tailed Student's test using PRISM 5 software (GraphPad, San Diego, CA). Differences were considered significant at  $p < 0.05$ .

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## CHAPTER THREE

### MURINE PANCREATIC ADENOCARCINOMA REDUCES IKAROS EXPRESSION AND DISRUPTS T CELL HOMEOSTASIS

#### Note to Reader

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#### Keywords

Pancreatic Cancer, Ikaros, Transcription Factors, T Cells, Tumor Immunity

#### Abstract

Maintenance of T cell immune homeostasis is critical for adequate anti-tumor immunity. The transcription factor Ikaros is essential for lymphocyte development including T cells. Alterations in Ikaros expression occur in blood malignancies in humans and mice. In this study, we investigated the role of Ikaros in regulating T cell immune balance in pancreatic cancer mouse models. Using our Panc02 tumor-bearing (TB) mouse model, western blot analysis revealed a reduction in Ikaros proteins while qRT-PCR showed no differences in Ikaros mRNA levels in TB splenocytes compared to

control. Treatment of naïve splenocytes with the proteasomal inhibitor, MG132, stabilized Ikaros expression and prevented Ikaros downregulation by Panc02 cells, *in vitro*. Western blot analyses showed a reduction in protein phosphatase 1 (PP1) and protein kinase CK2 expression in TB splenocytes while CK2 activity was increased. Immunofluorescence microscopy revealed altered punctate staining of Ikaros in TB splenocytes. Flow cytometry revealed a significant decrease in effector CD4<sup>+</sup> and CD8<sup>+</sup> T cell percentages but increased CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells in TB splenocytes. Similar alterations in T cell percentages, as well as reduced Ikaros and CK2 but not PP1 expression, were observed in a transgenic, triple mutant (TrM) pancreatic cancer model. Ikaros expression was also reduced in enriched TB CD3<sup>+</sup> T cells. MG132 treatment of naïve CD3<sup>+</sup> T cells stabilized Ikaros expression in the presence of Panc02 cells. Western blots showed reduced PP1 and CK2 expression in TB CD3<sup>+</sup> T cells. The results of this study suggest that the pancreatic tumor microenvironment may cause proteasomal degradation of Ikaros, possibly via dysregulation of PP1 and CK2 expression and activity, respectively. This loss of Ikaros expression may contribute to an imbalance in T cell percentages. Ikaros may potentially be a therapeutic target to restore T cell homeostasis in pancreatic cancer hosts, which may be critical for effective anti-tumor immunity.

## **Introduction**

Pancreatic ductal adenocarcinoma is currently the fourth leading cause of cancer-related deaths in the United States. Despite recent advances, successful treatment options against pancreatic cancer have had limited success due in part, to

dampened anti-tumor immune responses that promote tumor progression (1, 2). Effector CD4<sup>+</sup> and CD8<sup>+</sup> T cells play important roles in the host's anti-tumor immune responses as they facilitate destruction of tumor cells (3). Regulatory T cells (Tregs) are a population of T cells that maintain peripheral immune tolerance against self-antigens and foreign antigens (1). However, the critical balance between effector T cells and Tregs is lost in pancreatic cancer TB hosts, which may negatively impact anti-tumor immunity (4). In particular, CD4<sup>+</sup>CD25<sup>+</sup> Treg percentages are elevated in the peripheral blood of pancreatic cancer patients (5) as well as lymphoid organs in mice (6). These elevated Treg numbers are associated with decreased CD8<sup>+</sup> T cell percentages and lower survival rates (4). Therefore, an imbalance in effector CD4<sup>+</sup> and CD8<sup>+</sup> T cells and regulatory T cells is a significant impediment to treating pancreatic cancer.

The Ikaros family of zinc finger transcription factors - Ikaros, Aiolos, Helios, Eos and Pegasus - play critical roles in hematopoiesis and lymphocyte development (7). Ikaros, the founding member, encoded by the gene *Izkl1*, can activate and repress gene transcription and acts as a tumor suppressor in T cell lineages (8, 9). Mice expressing a non-DNA binding dominant-negative (DN) isoform of Ikaros exhibit severe defects including the absence of T cells after birth (10). Additionally, mice with one disrupted and one functional copy of Ikaros display lymphocyte hyperproliferation and develop T-cell leukemias and lymphomas (8).

Ikaros is alternatively spliced, which produces functional and DN isoforms. The interaction of functional Ikaros isoforms with DN isoforms inhibit its activity (11). Ikaros is also regulated by posttranslational modifications, which include phosphorylation (12, 13). Protein Kinase CK2 (formerly casein kinase 2) phosphorylation of Ikaros impairs its



DNA binding ability, alters its subcellular localization and leads to its ubiquitin-mediated proteasomal degradation via phosphorylation in PEST regions (regions containing proline (P), glutamate (E), serine (S), and threonine (T) bordered by positively charged residues). In contrast, dephosphorylation of Ikaros by protein phosphatase 1 (PP1) maintains Ikaros stability and activity (14).

The necessity of Ikaros for normal lymphocyte development makes it a critical target to be examined in regulating immune responses in various diseases. Our study is one of the first to investigate Ikaros in pancreatic cancer, especially as it relates to effector and regulatory T cells. In this study, we provide evidence that loss of Ikaros expression occurs in pancreatic TB hosts. We show that this occurs, at least in part, by ubiquitin-mediated proteasomal degradation in response to pancreatic cancer factors. This protein degradation of Ikaros may be as a result of alterations in known regulators of its stability, PP1 and CK2. Loss of Ikaros expression may contribute to the observed imbalance in effector and regulatory T cell percentages, favoring an immunosuppressive microenvironment. Therefore, Ikaros may be a T-cell specific therapeutic target for maintaining T cell homeostasis in pancreatic cancer patients.

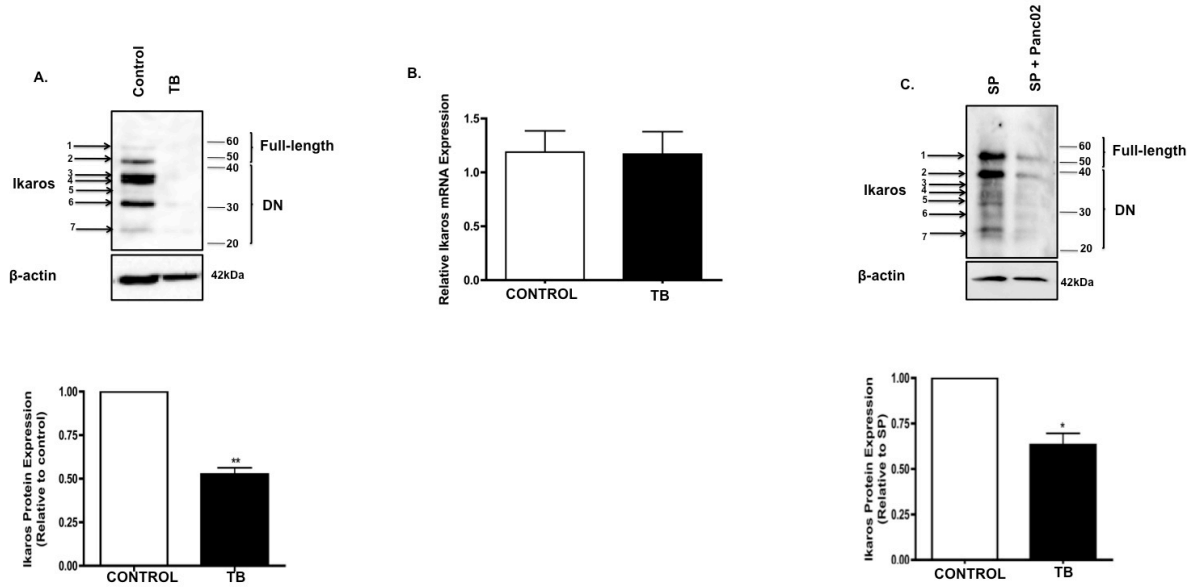
## **Results**

### **Reduced Ikaros expression in TB mice**

Ikaros is a critical regulator of lymphocyte development and is characterized as a tumor suppressor gene (15). More specifically, loss of Ikaros activity due to genetic or functional inactivation leads to the development of leukemias and lymphomas in mice and humans (8, 16, 17). However, investigations into the role of Ikaros in solid cancers,

especially as it relates to immune cell development, have been limited. We therefore wanted to determine whether defects in Ikaros might occur in a pancreatic tumor microenvironment. Ikaros protein expression was detected in the peripheral blood, bone marrow (data not shown) and spleen of our TB mice. However, its expression was most abundant in the spleen, which was used in this study. We first evaluated Ikaros protein expression in splenocytes from control and TB mice by using an antibody to the conserved C-terminus to detect all possible isoforms expressed. Western blot analyses revealed the expression of at least 7 Ikaros isoforms in control splenocytes which, based on their molecular weight (MW), appear to correspond to full-length isoforms Ik-1, and Ik-2/3 (arrows 1 and 2; **Figure 3.1A**) and five smaller (<46), DN isoforms (18, 19) (arrows 3-7; **Figure 3.1A**). Expression of these isoforms was downregulated in TB splenocytes and accounted for a significant (two-fold) decrease in total Ikaros protein expression in TB splenocytes compared to control (**Figure 3.1A**). Next, we evaluated mRNA expression of Ikaros in control and TB mice to determine whether differences in Ikaros protein expression were due to changes in its transcript. Using primers that detect Ikaros isoforms through conserved regions, we found no significant difference in total Ikaros mRNA expression between TB and control splenocytes (**Figure 3.1B**). Observing reduced Ikaros protein expression in TB mice, we then investigated whether this downregulation was in response to Panc02 factors (soluble and non-soluble). We recapitulated the *in vivo* tumor microenvironment by co-culturing splenocytes from naïve C57BL/6 mice with murine Panc02 cells *in vitro*. This co-culture resulted in reduced Ikaros protein expression in splenocytes as revealed by western blot analysis (**Figure**

**3.1C).** Thus far, these results suggest that pancreatic cancer factors may downregulate Ikaros expression in TB mice.



**Figure 3.1. Reduced Ikaros expression in TB mice.** **A.** Western blot analysis of Ikaros protein expression in control and TB splenocytes. To control for equal protein loading the blot was reprobbed with an antibody specific to  $\beta$ -actin. The arrows on the left indicate observed Ikaros isoforms. Representative quantification of normalized densitometric ratios of western blot data is shown. **B.** qRT-PCR analysis of Ikaros mRNA expression in control and TB mice. **C.** Western blot analysis of Ikaros protein expression in naive splenocytes co-cultured with Panc02 cells. To control for equal protein loading the blot was reprobbed with an antibody specific to  $\beta$ -actin. The arrows on the left indicate observed Ikaros isoforms. Representative quantification of normalized densitometric ratios of western blot data is shown. Represented is the mean  $\pm$  S.E.M. of control (n = 3) compared to TB (n = 3) mice. \*\*p<0.005 (by two-tailed Student's t test).

### Murine Panc02 cells cause ubiquitin-mediated proteasomal degradation of Ikaros *in vitro*

Our data proposes that downregulation of Ikaros protein expression in TB splenocytes may be due to a posttranslational modification affecting its protein stability.

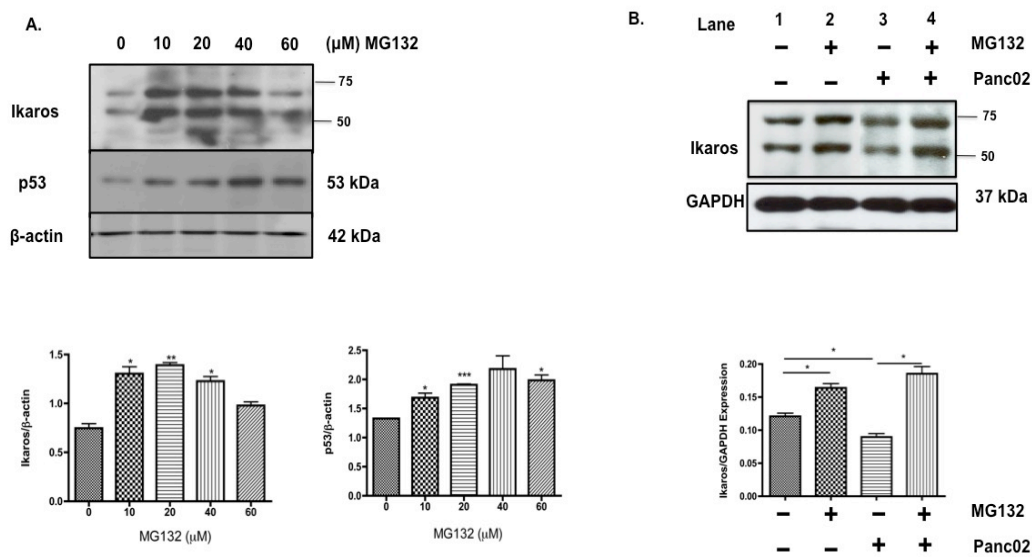
Studies have shown that Ikaros protein undergoes ubiquitin-proteasomal degradation

(14, 20-23). As Ikaros expression is significantly reduced in TB splenocytes, we treated naïve splenocytes with the proteasomal inhibitor, MG132, which was used as a molecular tool to test whether Ikaros protein undergoes proteasomal degradation. Results showed that in the presence of MG132, particularly at 10 $\mu$ M, 20 $\mu$ M and 40 $\mu$ M, there was a significant increase in Ikaros protein expression (**Figure 3.2A**). MG132 inhibition of the proteasome blocks apoptosis and stabilizes p53 expression (24). We therefore evaluated p53 expression to confirm MG132 activity in these experiments (**Figure 3.2A**). Furthermore, we wanted to determine whether the downregulation of Ikaros in TB mice was as a result of proteasomal degradation of Ikaros in response to Panc02 factors. Results of western blot analyses of splenocytes co-cultured with Panc02 cells showed that 10 $\mu$ M MG132 stabilized Ikaros expression (lane 2 vs. lane 1; **Figure 3.2B**). However, in the presence of Panc02 cells Ikaros protein expression was reduced in splenocytes (lane 3 vs. lane 1; **Figure 3.2B**). Interestingly, the addition of MG132 to the co-culture prevented Panc02-induced downregulation of Ikaros expression (lane 4 vs. lane 3; **Figure 3.2B**). These data suggest that pancreatic cancer factors may cause downregulation of Ikaros via protein degradation by the ubiquitin-proteasome pathway.

### **Altered PP1 expression, CK2 activity and Ikaros nuclear staining pattern in TB mice**

A balance between CK2 and PP1 is responsible for maintaining Ikaros' protein stability and function. In particular, lack of dephosphorylation by PP1 and hyperphosphorylation by CK2 leads to increased degradation of Ikaros (13, 14, 25).

Since our data suggests that Ikaros downregulation may be as a result of its protein degradation, we investigated the expression of CK2 and PP1 in splenocytes from our control and TB mice. We firstly evaluated PP1 expression by western blot analyses using an antibody that recognizes PP1 catalytic subunits. Western blot analyses detected two catalytic isoforms in control splenocytes.

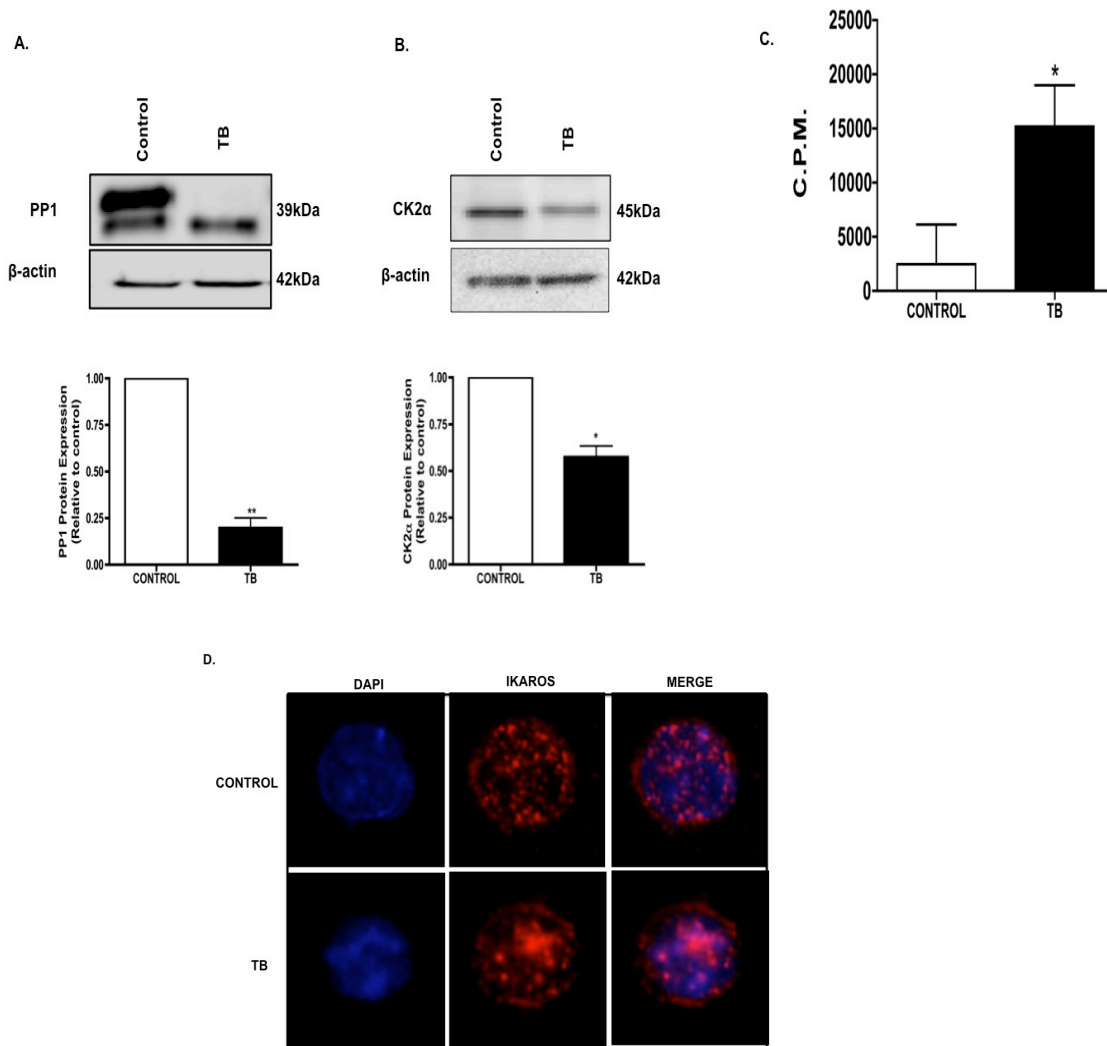


**Figure 3.2. Murine Panc02 cells cause ubiquitin-mediated proteasomal degradation of Ikaros *in vitro*.** **A.** Western blot analysis of Ikaros and p53 expression in naïve splenocytes treated with the proteasomal inhibitor, MG132 for four hours *in vitro*. To control for equal protein loading the blot was re-probed with an antibody specific to β-actin. Representative quantification of normalized densitometric ratios of western blot data is shown. **B.** Western blot analysis of Ikaros expression in naïve splenocytes co-cultured in the absence or presence of Panc02 cells and/or MG132. To control for equal protein loading the blot was re-probed with an antibody specific to GAPDH. Representative quantification of normalized densitometric ratios of western blot data is shown. Represented is the mean ± S.E.M. of three independent experiments. \* $p < 0.05$ , \*\* $p < 0.005$ ; \*\*\* $p < 0.0001$  (by two-tailed Student's t test).

However, the higher MW PP1 catalytic isoform was reduced in TB splenocytes (**Figure 3.3A**). Next, we evaluated CK2 by also evaluating the expression of its catalytic subunit. We found a reduction in CK2 $\alpha$  protein expression in TB splenocytes compared to control (**Figure 3.3B**). We also assayed CK2 activity which revealed a significant increase in CK2 activity in TB splenocytes compared to control (**Figure 3.3C**). Phosphorylation/dephosphorylation of Ikaros by CK2 and PP1 also affects its DNA binding ability and subcellular localization. The majority of Ikaros localizes at pericentromeric heterochromatin (PC-HC) where it functions in regulating gene expression (26). Therefore, having observed defects in PP1 and CK2 pathways, we evaluated Ikaros localization using immunofluorescence microscopy. In control splenocytes, we observed the characteristic nuclear, punctate staining pattern of Ikaros, indicative of its PC-HC localization (**Figure 3.3D**). However, in TB splenocytes, more diffuse staining of Ikaros was observed (**Figure 3.3D**). Overall, we observed reduced PP1 expression and increased CK2 activity as well as cytoplasmic subcellular localization of Ikaros in TB splenocytes. Therefore, differential expression of these two critical proteins may contribute to regulating Ikaros expression in our TB mice.

### **Altered T cell percentages in TB and TrM mice**

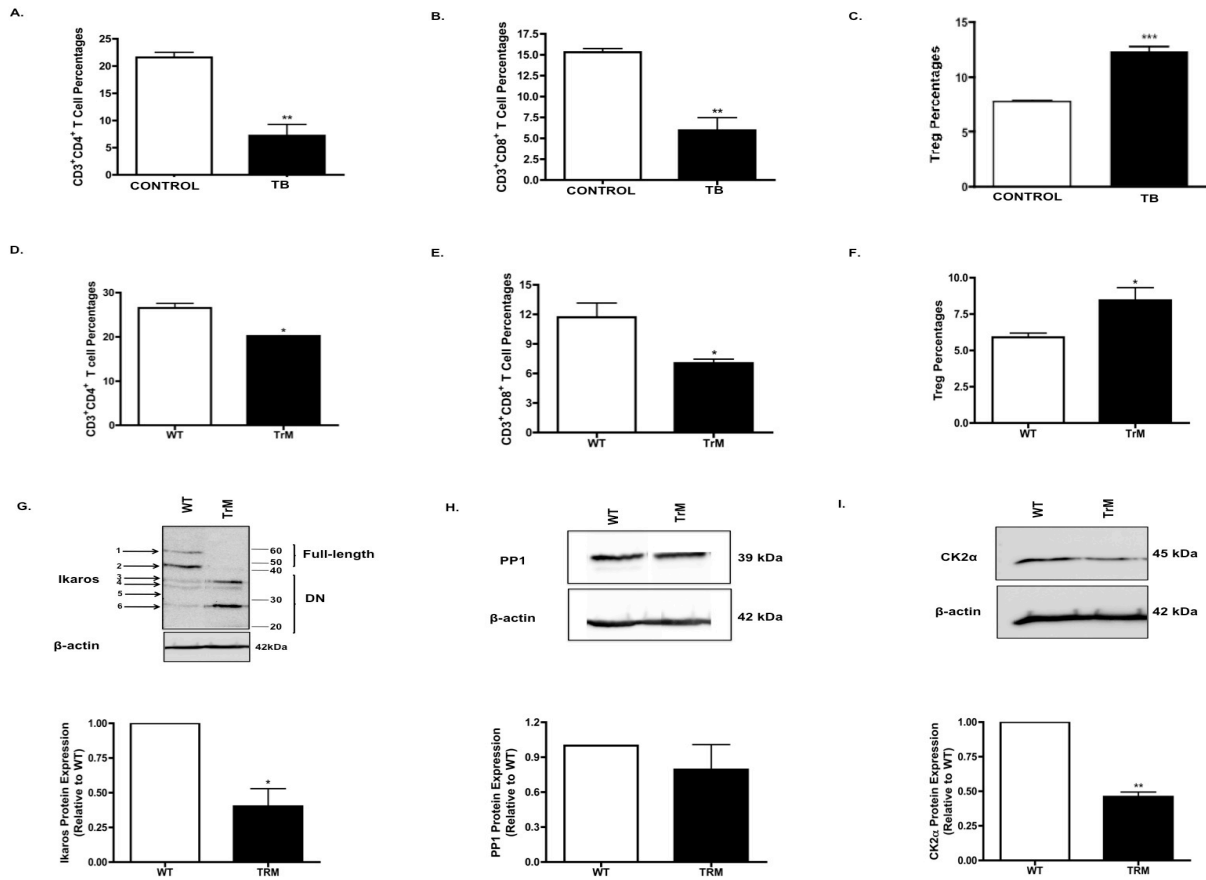
Ikaros has been identified as a regulator of T cell development (8, 27, 28). T cells, specifically CD4<sup>+</sup> and CD8<sup>+</sup> T cells, are key players in tumor protective immunity (29, 30). Having observed defects in Ikaros expression, we next evaluated whether T cell development is altered in response to murine pancreatic cancer. Flow cytometry results showed that there was a significant decrease in both CD4<sup>+</sup> (**Figure 3.4A**) and



**Figure 3.3. Altered PP1 expression, CK2 activity and Ikaros nuclear staining pattern in TB mice.** Western blot analysis of **A.** PP1 and **B.** CK2 $\alpha$  protein expression in control and TB splenocytes. To control for equal protein loading the blots were reprobed with an antibody specific to  $\beta$ -actin. Representative quantifications of normalized densitometric ratios of each western blot are shown. **C.** Counts per minute (C.P.M.) of CK2 activity in protein lysates from splenocytes from control and TB mice as assayed by an *in vitro* CK2 kinase assay. Represented is the mean  $\pm$  S.E.M. of control (n = 3 compared to TB (n = 3) mice). \* $p < 0.05$ , \*\* $p < 0.005$ ; (by two-tailed Student's t test). **D.** Immunofluorescence microscopy showing Ikaros expression and subcellular localization in control and TB splenocytes (n=25 cells). Nuclear DNA appears as blue (DAPI), Ikaros as red (Ikaros panel) and Ikaros and DAPI combined (Merged panel). (Magnification,  $\times 240$ ). Representative results from at least three independent immunofluorescence microscopy experiments.

CD8<sup>+</sup> (**Figure 3.4B**) effector T cell percentages in splenocytes from TB compared to control mice. Given the reduction in effector T cell percentages in splenocytes from TB mice, we investigated the percentages of immunosuppressive regulatory T cells. Flow cytometry results showed that there was a significant increase in CD4<sup>+</sup>CD25<sup>+</sup> Tregs in splenocytes from TB compared to control mice (**Figure 3.4C**). We previously published that CD4<sup>+</sup>CD25<sup>+</sup> Tregs from TB mice suppress antigen-specific CD8<sup>+</sup> T cell responses in a dose dependent manner, at a greater rate as compared to control Tregs (6). Thus far, our results suggest that defects in Ikaros expression may be associated with a loss of T cell equilibrium in our pancreatic TB mice. Our next step was to determine whether this disruption in effector and regulatory T cell balance occurred in another highly translatable, transgenic mouse model of pancreatic cancer. The LSL-Kras<sup>G12D/+</sup>;LSL-Trp53<sup>R172H/+</sup>;Pdx-1-Cre transgenic mouse model (TrM mice) has mutations in Kras and p53, leading to spontaneous development of pancreatic cancer (31), that recapitulates pancreatic cancer in humans (32). Flow cytometry analyses showed a reduction in effector CD4<sup>+</sup> (**Figure 3.4D**) and CD8<sup>+</sup> T (**Figure 3.4E**) cells but an increase in regulatory T cells (**Figure 3.4F**) in splenocytes from TrM mice compared to wild-type (WT) littermates. We then evaluated the expression of Ikaros, CK2 and PP1 in these TrM mice to delineate their possible involvement in regulating T cell immune homeostasis in this model. Western blot analyses revealed a significant reduction in overall Ikaros expression in splenocytes of triple mutant mice compared to wild-type (WT) mice (**Figure 3.4G**). However, in the TrM splenocytes, Ikaros DN isoforms were



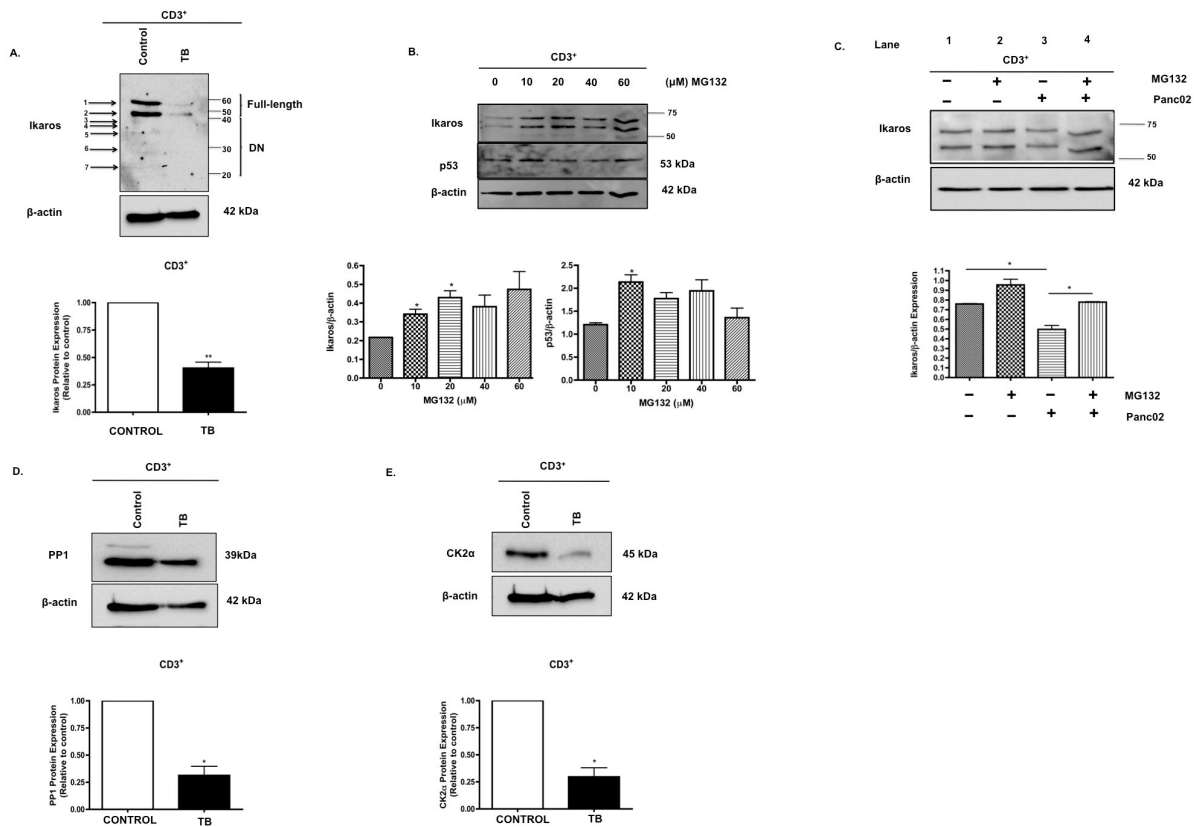


**Figure 3.4. Altered T Cell Percentages in TB and TrM mice.** Flow cytometry analysis of T cell percentages in TB and TrM mice. **A.** CD4<sup>+</sup> T cell, **B.** CD8<sup>+</sup> T cell and **C.** Treg percentages in splenocytes from control and TB mice. **D.** CD4<sup>+</sup> T cell, **E.** CD8<sup>+</sup> T cell and **F.** Treg percentages in splenocytes from wild-type (WT) and TrM mice. Western blot analysis of **G.** Ikaros **H.** PP1 and **I.** CK2α protein expression in WT and TrM splenocytes. To control for equal protein loading the blot was reprobed with an antibody specific to β-actin. The arrows on the left indicate observed Ikaros isoforms. Representative quantifications of normalized densitometric ratios of western blot data are shown. Represented is the mean ± S.E.M. of control (n = 3 compared to TB (n = 3) mice). \*p<0.05; \*\*p<0.005; \*\*\*p<0.0001 (by two-tailed Student's t test).

mainly expressed (**Figure 3.4G**). There was no significant difference in protein expression of PP1 catalytic subunits (**Figure 3.4H**) but CK2 $\alpha$  expression was reduced (**Figure 3.4I**) in TrM compared to WT mice. This implies that Ikaros dysregulation, the possible involvement of PP1 and/or CK2, and the resulting imbalance in T cell profiles, may have clinical relevance in pancreatic cancer.

### **Dysregulation of Ikaros, PP1 and CK2 in CD3<sup>+</sup> enriched T cells**

Thus far, we have observed a loss of Ikaros expression and T cell homeostasis in whole splenocytes from TB compared to control mice. Reports show that modulation of Ikaros expression in T cells affects their polarization, proliferation and differentiation (27, 33). Next, we investigated whether Ikaros expression was specifically altered at the T cell level in our animal model and could account for the loss of T cell homeostasis observed. Correlating with results in whole splenocytes, western blot analysis showed that Ikaros protein expression was also significantly reduced in enriched CD3<sup>+</sup> T cells from TB mice compared to control (**Figure 3.5A**). The isoforms detected appear to correlate with Ik-1 and Ik-2/3, which have previously been reported to be predominantly expressed in T lymphocytes (13). We then evaluated whether Ikaros expression in T cells is also regulated by ubiquitin-mediated proteasomal degradation. CD3<sup>+</sup> T cells enriched from naïve splenocytes were treated with increasing concentrations of MG132 *in vitro* as previously described. Western blot analyses of Ikaros expression revealed that MG132 did in fact significantly increase Ikaros expression in CD3<sup>+</sup> T cells at 10 and 20 $\mu$ M concentrations with MG132 activity evaluated by p53 expression (**Figure 3.5B**). Next, we co-cultured these enriched CD3<sup>+</sup> T cells with Panc02 cells in the absence or



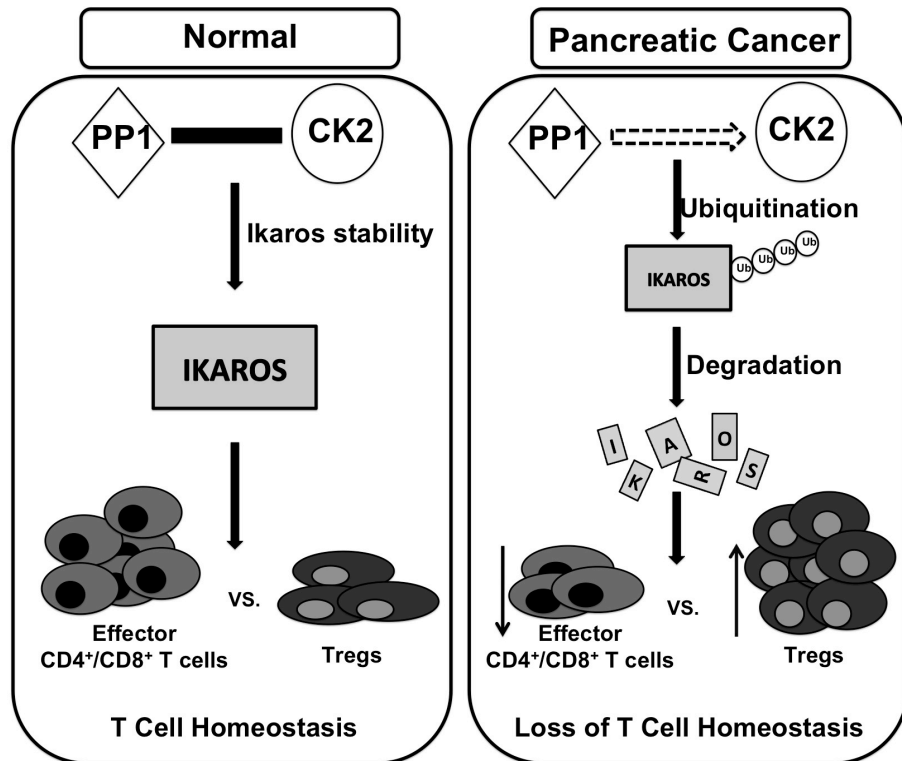
**Figure 3.5. Dysregulation of Ikaros, PP1 and CK2 in CD3<sup>+</sup> enriched T cells.** **A.** Western blot analysis of Ikaros protein expression in control and TB CD3<sup>+</sup> T cells. To control for equal protein loading the blot was reprobbed with an antibody specific to β-actin. The arrows on the left indicate observed Ikaros isoforms. Representative quantification of normalized densitometric ratios of western blot data is shown. **B.** Western blot analysis of Ikaros and p53 expression in naïve CD3<sup>+</sup> T cells treated with the proteasomal inhibitor, MG132 for four hours *in vitro*. To control for equal protein loading the blot was reprobbed with an antibody specific to β-actin. Representative quantification of normalized densitometric ratios of western blot data is shown. **C.** Western blot analysis of Ikaros expression in naïve CD3<sup>+</sup> T cells co-cultured in the absence or presence of Panc02 cells and/or MG132. To control for equal protein loading the blot was reprobbed with an antibody specific to GAPDH. Western blot analysis of **D.** PP1 and **E.** CK2α protein expression in control and TB CD3<sup>+</sup> T cells. To control for equal protein loading the blots were reprobbed with an antibody specific to β-actin. Representative quantifications of normalized densitometric ratios of western blot data are shown. Represented is the mean ± S.E.M. of control (n = 3 compared to TB (n = 3) mice). \*p<0.05, \*\*p<0.005; (by two-tailed Student's t test).

presence of MG132. MG132 stabilized Ikaros expression in T cells (lane 2 vs. lane 1;**Figure 3.5C**). Panc02 cells caused reduced Ikaros expression in T cells (lane 3 vs. lane 1;**Figure 3.5C**). However, this downregulation was prevented in the presence of 10 $\mu$ M MG132 (lane 4 vs. lane 3;**Figure 3.5C**), suggesting that Panc02 factors contribute to proteasomal degradation of Ikaros in CD3<sup>+</sup> T cells. Our next step was to determine the expression of PP1 and CK2, regulators of Ikaros, in these isolated CD3<sup>+</sup> T cells. Similar to our results in whole splenocytes, there was a reduction in PP1, especially of the higher MW catalytic isoform in TB CD3<sup>+</sup> T cells compared to control (**Figure 3.5D**). There was also a significant reduction in CK2 $\alpha$  expression in TB CD3<sup>+</sup> T cells compared to control (**Figure 3.5E**). These data indicate that dysregulation of Ikaros in CD3<sup>+</sup> T cells, possibly as a result of altered in PP1 and CK2 expression and activity, may contribute to loss of T cell homeostasis in pancreatic TB mice.

## Discussion

Ikaros is a critical regulator of lymphocyte development, especially T cells. In fact, Ikaros has been proposed to function as a tumor suppressor in hematological malignancies (34-36). However, the role of Ikaros in solid cancers has not been fully investigated. In this study, we identified the possible involvement of Ikaros in T cell homeostasis in pancreatic cancer mouse models. Our results suggest that pancreatic cancer (soluble and non-soluble) factors cause a reduction in Ikaros expression in splenocytes. We provide evidence that suggests these pancreatic cancer factors cause ubiquitin-mediated proteasomal degradation of Ikaros, which may be as a result of dysregulation in PP1 and CK2 pathways. Furthermore, we showed that this loss of

Ikaros coincides with an imbalance in T cell immune responses resulting in decreased percentages of effector CD4<sup>+</sup> and CD8<sup>+</sup> T cells and increased regulatory T cell percentages (**Figure 3.6**). Our study therefore proposes a putative and novel role for Ikaros in regulating T cell homeostasis in pancreatic cancer hosts.



**Figure 3.6. Proposed Model. Murine pancreatic cancer causes Ikaros degradation and alters T Cell Homeostasis.** We propose a potential molecular mechanism of Ikaros regulation by which under normal conditions, the balance (represented by the solid, black bar) in the concerted action of PP1 and CK2 stabilizes Ikaros protein expression. This results in maintenance of effector CD4/CD8<sup>+</sup> and regulatory T cell percentages. However, in a pancreatic cancer microenvironment, our findings suggest that there is a decrease in PP1 but an increase in CK2 activity (represented by broken arrow), which leads to ubiquitin-mediated protein degradation of Ikaros. This loss of Ikaros expression results in a loss of T cell homeostasis marked by a reduction of effector CD4/CD8<sup>+</sup> T cell percentages and an increase of regulatory T cells. Ikaros may therefore be important for regulating T cell immune responses in pancreatic cancer.

The Ikaros gene is alternatively spliced to generate multiple full-length DNA binding and DN isoforms (16). We observed at least 7 splice variants of Ikaros are expressed in control splenocytes, all of which are downregulated in TB mice. TrM LSL-Kras<sup>G12D/+</sup>;LSL-Trp53<sup>R172H/+</sup>;Pdx-1-Cre transgenic mice have mutations in p53 and Kras that model the genetic diversity of humans with pancreatic cancer, making it a highly translatable model. In WT littermates for TrM mice, at least 6 isoforms were observed. We are in the process of identifying these specific isoforms and comparing them to the currently identified isoforms and their variants (19). Ik-1 and Ik-2/3, are reduced in our TB and TrM models, which both have defects in T cell immune balance. These isoforms are amongst the predominant isoforms generally expressed in T cells (13). Interestingly, these isoforms are also reduced in our CD3<sup>+</sup> TB T cells and are the main isoforms undergoing proteasomal degradation. Therefore, these full-length Ikaros isoforms may be critical for maintaining T cell immune balance, while the overexpression of DN isoforms may cause an imbalance and needs to be further investigated. In addition, we are also investing the alternative splicing mechanisms that may govern Ikaros isoform expression in our pancreatic cancer models. Defects in Ikaros expression as well as the shift in T cell immune balance in the spontaneous pancreatic cancer mice, provide evidence that Ikaros may indeed have clinical relevance in regulating effector and regulatory T cell immune responses in pancreatic cancer hosts.

The reduction in protein but not mRNA expression of Ikaros in TB mice led us to believe that Ikaros protein may be regulated posttranslationally. Initial studies showed that Ikaros is subject to protein degradation via the ubiquitin-proteasome pathway (14) and eliminated the involvement of proteolysis by calpains (20). The ability of MG132 to

increase Ikaros expression in both whole and CD3<sup>+</sup> T cell enriched splenocytes provided evidence that the ubiquitin proteasome pathway may regulate Ikaros expression in immune cells in our pancreatic cancer mouse model. A number of recent studies support our findings as they have shown that Ikaros is indeed subject to proteasomal degradation (22, 23), especially as it relates to T cells (21). Our study further suggests that pancreatic cancer factors may trigger this proteasomal degradation of Ikaros. We published that Panc02 cells produce a number of inflammatory factors (37) and are investigating the molecular mechanism(s) by which inflammatory factors may modulate Ikaros' expression in our pancreatic cancer models.

We also aimed to determine the pathway(s) involved in regulating Ikaros degradation in our TB mice. The concerted action of CK2 and PP1, controls Ikaros' stability, DNA binding ability and subcellular localization (14). Hyperphosphorylation by CK2 induces Ikaros' degradation while dephosphorylation of Ikaros by PP1 increases its stability (14). Having observed that downregulation of Ikaros protein may be as a result of ubiquitin-mediated proteasomal degradation, we hypothesized that this mechanism may be due to increased CK2 vs. PP1 activity in TB mice. We observe a reduction in PP1, specifically of a particular catalytic isoform, which is currently being investigated for its possible role in regulating Ikaros expression in TB mice. We also detected a reduction in CK2 $\alpha$  expression but increased CK2 activity in TB splenocytes. In our TrM model, the lower MW catalytic subunit of PP1 was expressed and there was no difference in its expression compared to WT littermate controls. However, CK2 $\alpha$  expression was also downregulated in these TrM mice but its activity has not yet been assayed. We are investigating how CK2 activity is regulated in our models. We have

preliminary evidence that suggest that use of a selective CK2 inhibitor, increases Ikaros expression *in vitro* and *in vivo*, and restores effector and regulatory T cell balance in TB mice compared to control (unpublished data). This data further suggests that CK2 may be regulating Ikaros expression and function in our pancreatic cancer model. Currently, experiments using specific CK2 and PP1 inhibitors are being performed to confirm the roles of these two proteins in regulating Ikaros expression in pancreatic TB mice.

Defects in PP1 and CK2 can also affect Ikaros' function in binding DNA and its subcellular localization (14). In control splenocytes, Ikaros may be functional as its normal nuclear punctate staining was observed, which is characteristic of Ikaros localization to PC-HC which is essential for DNA binding and dimerization abilities (38). The diffuse, nuclear staining pattern suggests that Ikaros may not be localized to PC-HC and may therefore be functionally inactive in TB mice. In fact, this phenotype is similar to that observed in Ikaros mutants unable to interact with PP1 (14). It is also common in leukemic cells from infants with newly diagnosed ALL in which DN isoforms are prevalent (39), further supporting our findings.

We have shown that defects in Ikaros expression appear to limit the normal balance of T lymphocytes in our pancreatic cancer models. However, we have not specifically identified which T cell subsets mentioned ( $CD4^+$ ,  $CD8^+$ , regulatory T cells) have intrinsic defects in Ikaros. Other  $CD3^+$  T cell populations such as T helper (Th) cells (Th1, Th2, Th9, Th17), induced regulatory T cells (iTregs), natural regulatory T cells (nTregs), natural killer T (NK/T) cells, and  $CD8^+$  regulatory T cells, etc. may also be regulated by Ikaros and could potentially be included in our results. We have observed defects in some of these T cell populations in our TB mice and are attempting



to analyze these populations individually to identify possible defects in Ikaros expression, regulation and function. This will also allow us to identify other possible mechanisms by which Ikaros may be regulating T cell homeostasis in our model. These mechanisms may involve dysregulation of essential transcription factors (40, 41) and cytokines (42, 43) as well as alterations in cellular processes such as polarization, differentiation, proliferation (27, 33), anergy (44) and apoptosis (20) as reported in other studies. Helios, another Ikaros family member, has been reported to complex with Ikaros in T cells and may limit Ikaros function (45). Helios also plays a role in T cell activation and proliferation (46) and is involved in regulatory T cell development and function (47, 48). We have generated preliminary evidence that shows that Helios expression is downregulated in our TB mice (unpublished data) and are currently investigating its involvement along with Ikaros. We are also investigating Eos and Aiolos, other Ikaros family members, due to loss of homeostasis of other lymphocyte populations in this murine pancreatic cancer model (unpublished data).

This study is one of the firsts to investigate the possible involvement of Ikaros in regulating T cell immune homeostasis in pancreatic cancer. Our results show that pancreatic cancer factors cause reduced Ikaros expression in splenocytes, which may be as a result of Ikaros protein degradation by the ubiquitin/proteasome pathway. We also provide evidence that activation of this pathway may involve dysregulation of the balance between PP1 phosphatase and CK2 kinase. Furthermore, we show that this apparent functional inactivation of Ikaros potentially contributes to T cell imbalance and may have clinical relevance as a similar trend was observed in a translatable,

pancreatic cancer mouse model. In conclusion, this study highlights the importance of Ikaros in regulating T cell immune responses in pancreatic cancer hosts.

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## CHAPTER FOUR

### CK2 INHIBITION INCREASES IKAROS EXPRESSION AND RESTORES EFFECTOR AND REGULATORY T CELL IMMUNITY IN MURINE PANCREATIC CANCER

#### Keywords

Pancreatic Cancer, Ikaros, CK2, Apigenin, T Cells

#### Abstract

Pancreatic cancer evades immune destruction by favoring the development of regulatory T cells (Tregs) that inhibit effector T cells. The transcription factor Ikaros is critical for lymphocyte development, especially T cells. We have previously shown that downregulation of Ikaros occurs as a result of its protein degradation by the ubiquitin-proteasome system in our Panc02 tumor-bearing (TB) mouse model. Mechanistically, we observed a deregulation in the balance between CK2 and PP1, which suggested that increased CK2 activity is responsible for the regulating Ikaros' stability in our model. We also showed that this loss of Ikaros expression is associated with a significant decrease in CD4<sup>+</sup> and CD8<sup>+</sup> T cell percentages but increased CD4<sup>+</sup>CD25<sup>+</sup> Tregs in TB mice. In this study, we evaluated the effects of the dietary flavonoid apigenin, on Ikaros expression and T cell immune responses. Treatment of splenocytes from naïve mice with apigenin, stabilized Ikaros expression and prevented Ikaros downregulation in the

presence of Panc02 cells, *in vitro*, similar to the proteasome inhibitor, MG132. *In vivo*, treatment of TB mice with apigenin (TB-Api) reduced tumor weights and prevented splenomegaly. Apigenin treatment also restored protein expression of some Ikaros isoforms, which may be attributed to its moderate inhibition of CK2 activity. This partial restoration of Ikaros expression was accompanied by a significant increase in CD4<sup>+</sup> and CD8<sup>+</sup> T cell percentages and a reduction in Treg percentages. In addition, apigenin treated TB mice were better able to prime allogeneic BALB/c CD8<sup>+</sup> T cell responses, compared to TB mice. These results provide further evidence that Ikaros is regulated by CK2 in our pancreatic cancer model. More importantly, our findings suggest that apigenin may be a possible therapeutic agent for stabilizing Ikaros expression and function to maintain T cell homeostasis in murine pancreatic cancer.

## Introduction

Pancreatic adenocarcinoma is one of the most aggressive and most lethal solid malignancies (1). The pancreatic tumor microenvironment favors the recruitment of immunosuppressive cells that dampen anti-tumor immune responses, allowing tumor cells to evade immune surveillance and leading to tumor progression (2, 3). Understanding the mechanisms by which these anti-tumor immune responses, specifically those mediated by T cells, are regulated in pancreatic cancer is therefore critical to developing new, targeted treatment options.

Effector CD4<sup>+</sup> and CD8<sup>+</sup> T cells both play important roles in the host's immune response to cancer (4). Early studies showed a conventional "helper" role for CD4<sup>+</sup> T cells by primarily influencing immune responses by regulating CD8<sup>+</sup> cytotoxic T

lymphocytes (CTLs) (5). The percentages and function of CD8<sup>+</sup> T cells are significantly decreased in the peripheral blood of pancreatic cancer patients, compared to healthy controls (6). One contributing mechanism to this diminished anti-tumor response in pancreatic cancer patients is the induction and recruitment of suppressive cells by tumor-derived factors (2, 3). In particular, immunosuppressive regulatory T cells (Tregs) are a subpopulation of CD4<sup>+</sup> T cells that express the forkhead boxP3 (FoxP3) gene (7) and function by maintaining peripheral immune tolerance against self-antigens and foreign antigens by suppressing CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses (8). The percentages of Tregs are elevated in pancreatic cancer hosts (9-11). Delineating the mechanisms by which this balance in T cells is lost is critical for the generation of effective anti-tumor immune responses in pancreatic cancer hosts.

Alterations in transcription factors that play critical roles in the commitment and maintenance of lymphocyte development often promote malignant transformation (12). One such example is the Ikaros family of zinc finger transcription factors that includes Ikaros, Aiolos, Helios, Eos and Pegasus proteins. These transcription factors regulate cell-fate decisions during hematopoiesis and are thus important players in the development of immune cells (13). In particular, Ikaros, the founding member is particularly important for normal T cell development (14-16). Ikaros is regulated posttranscriptionally by alternative splicing, which produces functional and dominant-negative (DN) isoforms, which can inhibit its activity (17, 18). Ikaros is also regulated by posttranslational modifications, which primarily include phosphorylation (19). Phosphorylation by protein kinase CK2 and dephosphorylation by protein phosphatase 1 (PP1) can negatively affect Ikaros' stability, localization and function (20). More

specifically, CK2 phosphorylation of Ikaros impairs its DNA binding ability, regulation of cell cycle progression, and its function in T cells, alters its subcellular localization and leads to its ubiquitin-mediated proteasomal degradation via phosphorylation in PEST regions (20-22). On the contrary, dephosphorylation of Ikaros by PP1 maintains its stability and function (20, 21, 23). CK2 is a ubiquitously expressed and highly conserved serine/threonine kinase that regulates a number of critical cellular processes, including cell proliferation and apoptosis (24-26). CK2 is widely studied in blood and solid malignancies (27). Overexpression of its tetrameric subunits and deregulation of its activity have been linked to numerous cancers (24). Overexpression of CK2 in mice leads to T cell leukemias and lymphomas (28-30). However, limited studies have focused on CK2's involvement in regulating immune responses.

Apigenin (API) is a natural plant flavonoid and selective CK2 inhibitor that targets CK2-dependent signaling pathways. Apigenin has a number of reported biological effects including anti-proliferative, anti-oxidant anti-inflammatory and anti-carcinogenic properties, which are thought to be integral part of its anti-cancer property (31). Recently, there has been increased exploration of the use of apigenin as a chemopreventive agent in a number of cancer models (32). More specifically, apigenin has been shown to induce cell death and also enhance the anti-proliferative effects of chemotherapy agents in human pancreatic cancer cells (33-35).

We have previously shown that Ikaros undergoes proteasomal degradation, which may contribute to altered effector and regulatory T cell development in murine pancreatic cancer. Our studies suggested that a shift in the balance between CK2 and PP1, favoring CK2 activity may be responsible. Therefore, to further delineate CK2's

involvement in regulating Ikaros expression and thus, T cell responses, we investigated the effects of apigenin in our model. We found that apigenin is able to stabilize Ikaros' expression *in vitro* and *in vivo* while also restoring the balance between effector CD4/CD8<sup>+</sup> and regulatory T cells. This correlated with increased immune function as indicated by increased proliferation of allogeneic CD8<sup>+</sup> T cells in apigenin treated mice in a one-way allogeneic mixed leukocyte reaction (MLR). This study highlights the importance of CK2 in regulating Ikaros expression and its possible influence on T cell immune responses in murine pancreatic cancer.

## Results

### Apigenin prevents Ikaros downregulation *in vitro*

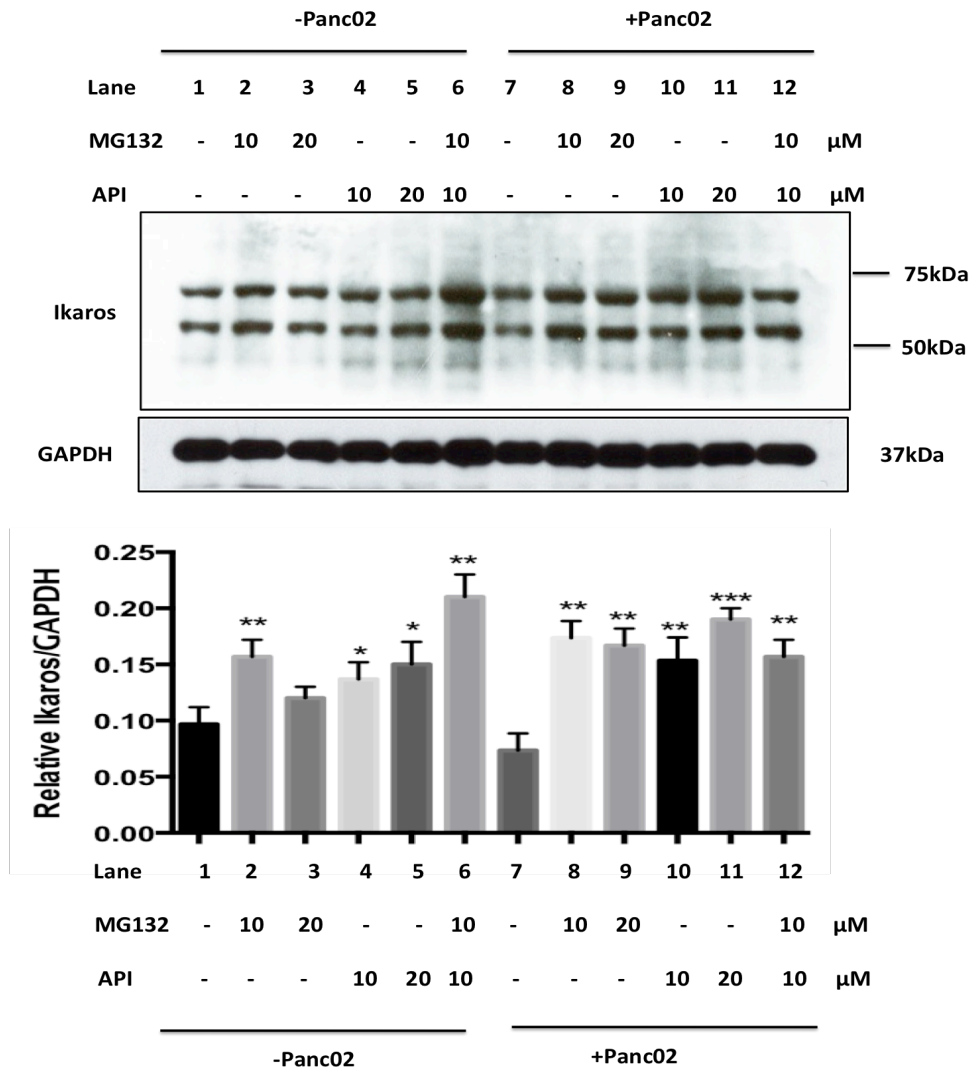
We previously showed that MG132, is able to stabilize Ikaros expression *in vitro*, providing evidence that Ikaros undergoes ubiquitin proteasomal degradation. A balance between CK2 and PP1 regulates Ikaros stability and function (20, 21, 36). In particular, increased CK2 activity is thought to cause Ikaros degradation (21). Therefore, inhibiting CK2 should therefore stabilize Ikaros expression and prevent its degradation, similar to MG132. We treated naïve splenocytes with the CK2 inhibitor, apigenin, as well as MG132, both at 10 $\mu$ M and 20 $\mu$ M, to compare their effects on Ikaros expression. Both apigenin and MG132, stabilized Ikaros expression (**Figure 4.1**; Lanes 2 and 3 vs. Lane 1; Lanes 4 and 5 vs. Lane 1) and also displayed a synergistic effect, which shows accumulation of ubiquitination ladders (**Figure 4.1**; Lane 6). In addition, both treatments appeared to induce ubiquitination ladders of Ikaros expression and stabilize p53 expression, a target of the ubiquitin-proteasome pathway (**Figure 4.1**; Lanes 2 and 3 vs.

Lane 1; Lanes 4 and 5 vs. Lane 1). Next, we recapitulated the *in vivo* pancreatic environment *in vitro* by adding murine Panc02 cells to the culture of naïve splenocytes. As previously reported, the addition of murine Panc02 cells causes a reduction, although not significant, in Ikaros protein expression (**Figure 4.1**; Lane 7) and MG132 treatment prevented this downregulation at both 10 $\mu$ M and 20 $\mu$ M (Figure 3.1; Lanes 8 and 9 vs. Lane 7) in naïve splenocytes. Interestingly, apigenin treatment also prevented Panc02 reduction of Ikaros protein expression in splenocytes at the same concentration as MG132 (**Figure 4.1**; Lanes 10 and 11 vs. Lane 7). However, the synergistic effect of the two drugs were not as apparent in the presence of Panc02 cells (**Figure 4.1**; Lane 12). Overall, these results suggest that apigenin is able to stabilize Ikaros and prevent its downregulation in a pancreatic tumor microenvironment. Moreover, the similarities to MG132 and their additive effect, also further suggests that apigenin may be preventing Ikaros' proteasomal degradation, possibly via its inhibition of CK2.

### **Apigenin reduces tumor burden *in vivo***

Due to the effects of apigenin *in vitro*, we next determined whether apigenin could have similar effects on Ikaros *in vivo*. Apigenin has been shown to have anti-tumor effects in a number of tumor models including breast cancer and melanoma and (37, 38). We therefore evaluated the effects of apigenin treatment on tumor burden using our pancreatic cancer model. Treatment of TB mice with 25 mg/kg apigenin (TB-Api) caused a significant decrease in tumor weight compared to vehicle-treated TB mice (**Figure 4.2A**).

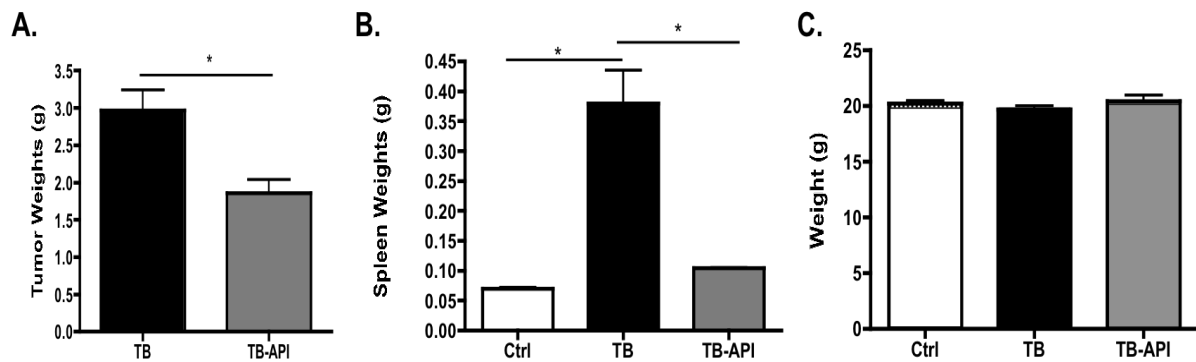




**Figure 4.1 Apigenin prevents Ikaros downregulation *in vitro*.** Western blot analysis of Ikaros in naïve splenocytes co-cultured in the absence or presence of Panc02 cells, treated with apigenin (API) and/or MG132 for four hours at 10 $\mu$ M and 20 $\mu$ M, *in vitro*. To control for equal protein loading the blot was reprobbed with an antibody specific to GAPDH. Representative quantification of normalized densitometric ratios of western blot data is shown. Represented is the mean  $\pm$  S.E.M. of three independent experiments. Lanes 1-6 vs. Lane 1; Lane 7 vs. Lane 1; Lanes 8-12 vs. Lane 7 \* $p$ <0.05, \*\* $p$ <0.005; \*\*\* $p$ <0.0001 (by two-tailed Student's t test).

Next, we evaluated whether apigenin treatment may have any toxicity effects *in vivo* by weighing all mice at the end of the study. Results showed that there was no

significant difference in the weights of TB-Apigenin mice compared to TB mice at the end of the study (**Figure 4.2B**). We previously published that our TB mice displayed splenomegaly, marked by a significant increase in spleen weights (39). We found that *in vivo* apigenin treatment reversed this pancreatic cancer induced splenomegaly and caused a significant reduction in spleen weights in TB-Apigenin compared to TB mice (**Figure 4.2C**).

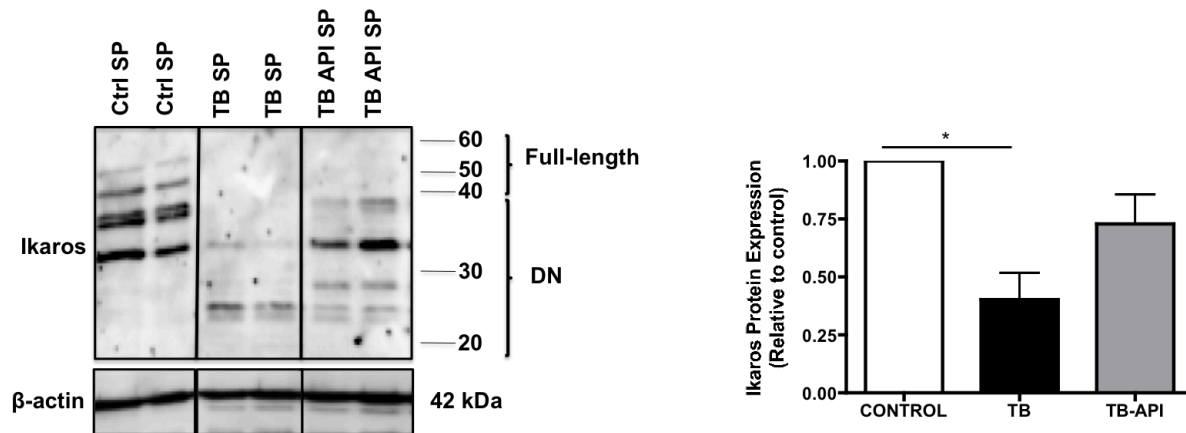


**Figure 4.2 Apigenin reduces tumor burden *in vivo*** **A.** Tumor weights of tumor-bearing (TB) and TB-apigenin treated (TB-Apigenin) mice on the last day of the study. **B.** Spleen weights of control (ctrl), TB and TB-Apigenin mice on the last day of the study. **C.** Body weights of control (ctrl), TB and TB-Apigenin mice on the last day of the study. . Represented is the mean  $\pm$  S.E.M. of three independent experiments. (n=3) \*p<0.05, (by two-tailed Student's t test).

### Apigenin partially stabilizes Ikaros expression *in vivo*

Our studies suggest that increased CK2 may be modulating Ikaros expression in our TB mice. Furthermore, since our *in vitro* data shows that apigenin can stabilize Ikaros expression, especially in the presence of murine Panc02 cells, we evaluated the effect of apigenin treatment on Ikaros protein expression in an *in vivo* pancreatic tumor microenvironment. Western blot analyses revealed that apigenin partially restored

Ikaros expression in TB-Api mice compared to TB mice (**Figure 4.3**). More specifically, DN Ikaros isoforms, described as less than 46 kDa) (40), were specifically increased in TB-Api compared to TB mice (**Figure 4.3**).



**Figure 4.3 Apigenin partially stabilizes Ikaros expression *in vivo*.** **A.** Western blot analysis of Ikaros protein expression in splenocytes from control, TB and TB-Api mice. To control for equal protein loading, the blot was reprobbed with an antibody specific to  $\beta$ -actin. Lines represent cropped images from the same western blot. Representative quantification of normalized densitometric ratios of western blot data is shown. Represented is the mean  $\pm$  S.E.M. of three independent experiments. (n=3) \*p<0.05, (by two-tailed Student's t test).

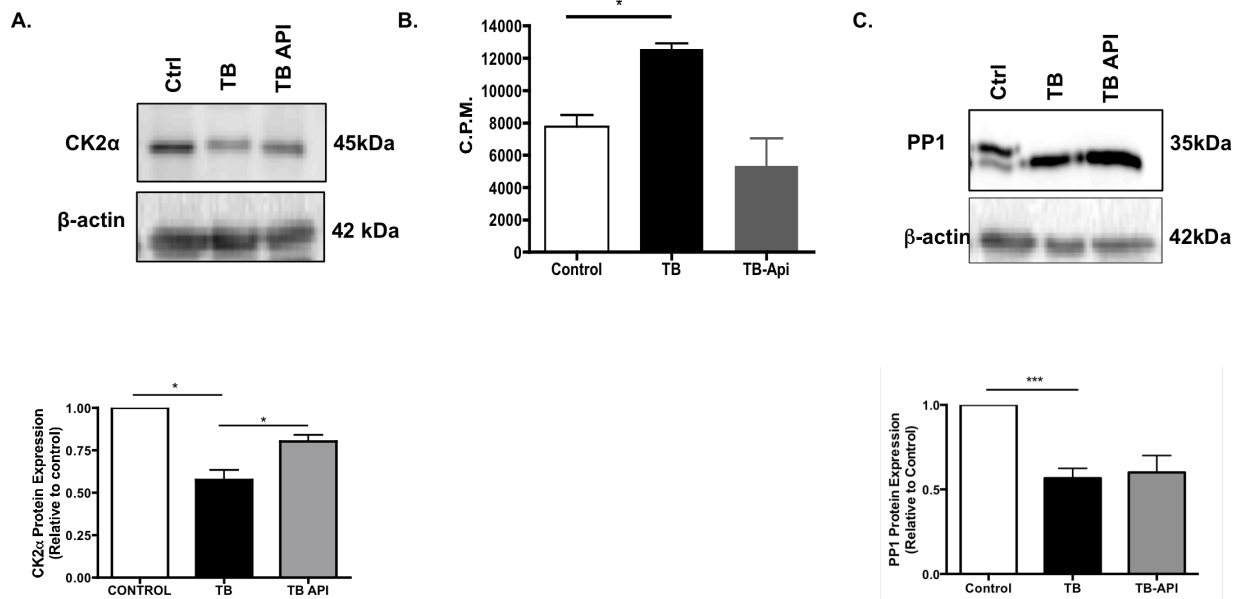
### Apigenin inhibits CK2 activity *in vivo*

We also evaluated apigenin's effect on CK2 expression using an antibody specific to its catalytic alpha subunit by western blot analysis. Splenocytes from TB-Api mice showed a slight decrease in the molecular weight of CK2 $\alpha$  than seen in TB mice, similar to that seen in control splenocytes (**Figure 4.4A**). To further delineate the effect of apigenin on CK2 in our pancreatic TB model, we evaluated CK2 activity and found that apigenin treatment caused a reduction in CK2 activity in TB-Api mice, compared to

TB. However, this inhibition was not significant (**Figure 4.4B**). We also evaluated PP1 expression and saw no difference in the expression of PP1 isoforms expressed in TB-Api mice compared to TB mice, while a higher MW isoform is also observed in control mice (**Figure 4.4C**). These data strongly suggest that apigenin is able to stabilize Ikaros expression *in vivo*, which may depend on its ability to inhibit CK2 activity.

### **Apigenin partially restores T cell homeostasis and immune responses *in vivo***

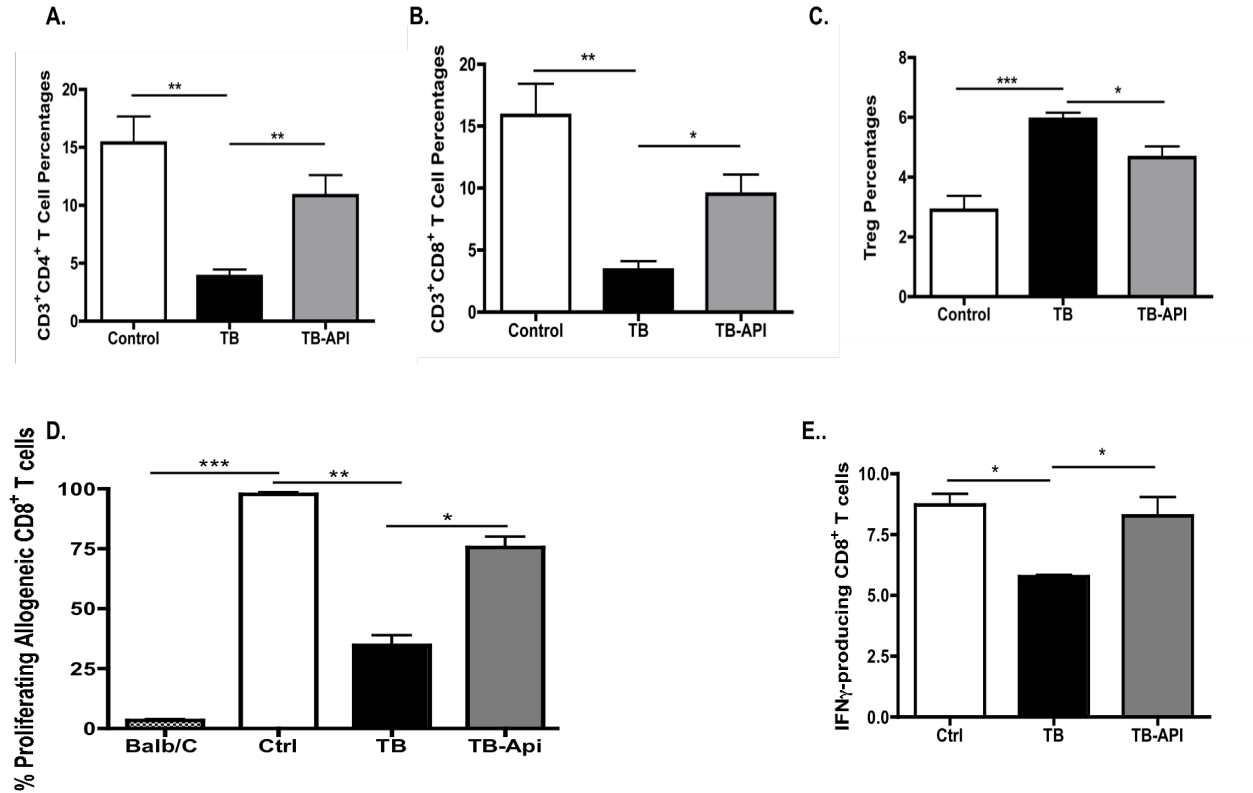
Next, we evaluated whether apigenin's restoration of Ikaros expression had any effect in the previously observed shift in T cell numbers in TB mice. TB-Api mice had significantly increased CD4<sup>+</sup> (**Figure 4.5A**) and CD8<sup>+</sup> T cell percentages (**Figure 4.5B**) but reduced Treg percentages compared to TB mice (**Figure 4.5C**). These results suggest that Ikaros expression may in fact influence T cell development in our pancreatic cancer model, in response to CK2 regulation. Next, we determined whether apigenin could influence immune responses in our TB mice. We performed a one-way allogeneic mixed leukocyte reaction (MLR) in which splenocytes from control, TB and TB-Api mice were used as stimulators to CFSE-labeled BALB/c responders. As expected, TB whole splenocytes were deficient in their ability to prime allogeneic CD8<sup>+</sup> T cell immune responses compared to control splenocytes (**Figure 4.5D**). In contrast, TB-Api whole splenocytes restored this ability to prime allogeneic responses compared to TB splenocytes (**Figure 4.5D**). Having observed that TB-Api mice may could elicit enhanced immune responses, we then evaluated IFN- $\gamma$  production of CD8<sup>+</sup> T cells from



**Figure 4.4 Apigenin inhibits CK2 activity *in vivo*.** **A.** Western blot analysis of CK2 $\alpha$  protein expression in control, TB and TB-API splenocytes. **B.** Counts per minute (C.P.M.) of CK2 activity in protein lysates from splenocytes from control, TB and TB-API mice as assayed by an *in vitro* CK2 kinase assay. Representative of three independent experiments. Represented is the mean  $\pm$  S.E.M. of three independent experiments. (n=3) \* $p$ <0.05 (by two-tailed Student's t test). **C.** Western blot analysis of PP1 catalytic protein expression in control, TB and TB-API splenocytes. **A. and C.** Representative quantification of normalized densitometric ratios of western blot data is shown. To control for equal protein loading, the blots were reprobbed with an antibody specific to  $\beta$ -actin. Representative of three independent experiments. Represented is the mean  $\pm$  S.E.M. of three independent experiments. (n=3) \* $p$ <0.05, \*\*\* $p$ <0.0001 (by two-tailed Student's t test).

these mice. Activated CD8<sup>+</sup> T cells produce IFN- $\gamma$ , which is critical to their effector function in eliminating tumor cells (41). Intracellular staining and flow cytometry analyses revealed that there were defects in CD8<sup>+</sup> T cell IFN- $\gamma$  production in TB mice, which was significantly enhanced with apigenin treatment (**Figure 4.5E**). These findings suggest a correlation between Ikaros expression, T cell development and immune

responses in a pancreatic tumor microenvironment and points to the involvement of CK2 is regulating this mechanism.



**Figure 4.5 Apigenin partially restores T cell homeostasis and immune responses *in vivo***. Flow cytometry analysis of **A.** CD4<sup>+</sup> T cell **B.** CD8<sup>+</sup> T cell and **C.** CD4<sup>+</sup>CD25<sup>+</sup> Treg percentages in splenocytes from control TB and TB-Api mice. **D.** Allogeneic CD8<sup>+</sup> T cell proliferation responses of CFSE-labeled BALB/c splenocytes (responders) in response to control, TB and TB-API splenocytes (stimulators) in a one-way mixed-leukocyte reaction (MLR), as analyzed by flow cytometry analysis. **E.** Flow cytometry analysis of IFN- $\gamma$  production of CD8<sup>+</sup> T cells in splenocytes from control, TB and TB-Api mice. Represented is the mean  $\pm$  S.E.M. of two independent experiments. (n=3) \*p<0.05, \*\*p<0.005; \*\*\*p<0.0001 (by two-tailed Student's t test).

## Discussion

Although widely studied in hematological malignancies (13), the role of Ikaros in solid cancers has not been fully investigated. We have previously identified the possible involvement of Ikaros in maintaining effector and regulatory T cell homeostasis in a preclinical pancreatic cancer model. Our data suggested that loss of Ikaros was as a result of its ubiquitin-mediated proteasomal degradation in response to increased CK2 activity versus PP1. In the current study, we make use of a selective CK2 inhibitor, apigenin, to further delineate the mechanism by which Ikaros is regulated and to provide functional evidence for its involvement in modulating T cell anti tumor immune responses. *In vitro*, apigenin stabilized Ikaros expression in naïve splenocytes and prevented its downregulation in the presence of murine Panc02 cells, similar to MG132 treatment. *In vivo*, apigenin treatment of TB mice reduced tumor burden, reduced CK2 activity and restored expression of some Ikaros isoforms. This coincided with increased effector CD4/CD8<sup>+</sup> T cell numbers while decreasing Treg numbers in TB-API compared to TB mice. Apigenin treatment of TB mice also increased CD8<sup>+</sup> T cell proliferation of allogeneic splenocytes in a one-way MLR. Our study sheds insight into Ikaros' regulation of T cell immunity in pancreatic cancer and defines one possible mechanism by which it is regulated. Overall, these results further suggest that pharmacological CK2 inhibition restores Ikaros expression and can influence T cell immune responses in murine pancreatic cancer.

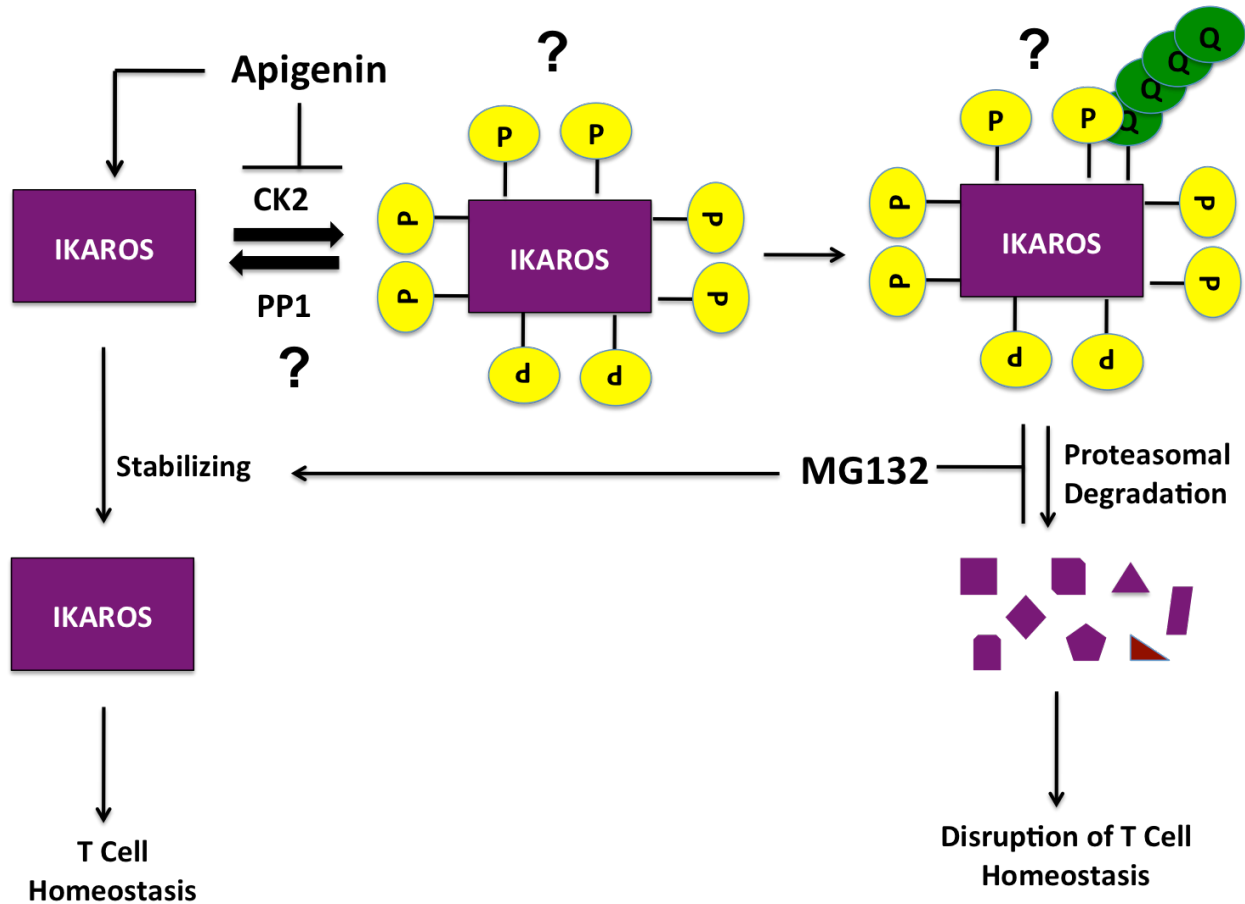
Phosphorylation of Ikaros by CK2 induces Ikaros degradation while dephosphorylation by PP1 maintains its stability (20, 21, 36). *In vitro*, we found that apigenin appeared to mimic the effects of MG132 by stabilizing Ikaros expression,

causing the accumulation of its ubiquitination ladders. These data suggests that apigenin may be similarly preventing ubiquitin-proteasomal degradation of Ikaros via its ability to inhibit CK2 activity. The combined effects of MG132 and apigenin further provide evidence for this mechanism. As a result, our current working hypothesis it that apigenin may be inhibiting the upstream effector of the pathway, CK2 and its ability to hyperphosphorylate Ikaros leading to its ubiquitination and degradation. On the contrary, we propose that MG132 works downstream of this pathway by inhibiting the proteasome. Ultimately, both inhibitors would lead to stability of Ikaros expression, and thus its function in regulating T cell homeostasis (**Figure 4.6**).

Alternatively, apigenin has also been reported to regulate proteasomal degradation. More specifically, apigenin has been shown to potentially inhibit the chymotrypsin-like activity of the proteasome (37), similar to MG132 (42, 43). It is therefore possible that apigenin may stabilize Ikaros expression by inhibiting both CK2 and/or proteasomal activity, which needs to be further investigated. Furthermore, clinically available proteasomal inhibitors exhibit some toxic effects (44), highlighting the need for safer alternatives such as natural, non-toxic compounds like apigenin.

*In vivo*, apigenin treatment significantly reduced tumor weights of TB-API compared to TB mice. These findings suggest that apigenin may have anti-tumor properties in murine pancreatic cancer. However, apigenin treatment could have been more effective in reducing tumor burden and tumor size. This may be attributed to the frequency and dosage of apigenin administered, as although CK2 activity was inhibited by at least half, it was not significant ( $p=0.053$ ). In depth pharmacokinetics and dose-dependent studies need to be done to determine a more effective dosage of apigenin





**Figure 4.6 Proposed Model. A representation of Ikaros' regulation by CK2 and PP1.** We have not delineated PP1's role but hypothesize that PP1 dephosphorylates Ikaros maintaining its protein stability. However, we propose that CK2 hyperphosphorylates Ikaros, which facilitates its polyubiquitination and eventual protein degradation by the ubiquitin-proteasome system. We propose that apigenin inhibits CK2 activity, stabilizing Ikaros expression and resulting in T cell (effector and Treg) homeostasis. We also propose that MG132 acts downstream by directly inhibiting the activity of the ubiquitin-proteasome system, preventing proteasomal degradation of Ikaros and thereby stabilizing its expression and maintaining T cell homeostasis.

treatment for our pancreatic TB mice. Western blot analyses of CK2 $\alpha$  expression showed an increase in expression in splenocytes from TB-API compared to TB mice. However, this was accompanied by a reduction in the MW of the observed band, similar to that of control mice. This suggests that apigenin treatment may be inhibiting a

posttranslational modification event of CK2. Phosphorylation of CK2 by kinases increases its activity (45, 46). Therefore, future experiments will be conducted to evaluate whether kinases such as ERK (45), and Cdk1/cycliB1 (47, 48) may be responsible for modulating CK2 activity in our pancreatic TB model.

Apigenin treatment appeared to primarily increase expression of DN Ikaros isoforms *in vivo*. Typically, the overexpression of DN isoforms is said to inhibit the activity of Ikaros and is associated with T cell malignancies (49). However, since the increase in expression of these isoforms conferred a return to T cell homeostasis and immune function, they may be of importance in regulating immune cells in our pancreatic cancer mice. Confirmation of these isoforms and further experiments to understand their role in immune responses and pancreatic tumor progression are therefore needed. It is possible that an increased dosage of apigenin and/or more frequent treatments, may lead to a significant decrease in tumor growth, CK2 activity and thus stability of more or all Ikaros isoforms as well as T cell percentages and function.

Apigenin treatment significantly increased CD4<sup>+</sup> and CD8<sup>+</sup> T cells but decreased Tregs percentages. Our results showed functional evidence that apigenin modulates immune responses in our TB mice since apigenin treatment increased IFN- $\gamma$  production of CD8<sup>+</sup> T cells. This is an indication of CD8<sup>+</sup> T cell activity and cytotoxic function (50, 51). Apigenin also significantly increased the ability of antigen-presenting cells (APCs) to prime allogeneic CD8<sup>+</sup> T cell immune responses. In our one-way MLR, allogeneic BALB/c CD8<sup>+</sup> T cell responses are stimulated by APCs, of control, TB and TB-API mice. Dendritic cells (DC) are the most potent APCs. Their function is often evaluated by their

ability to induce proliferation of allogeneic T cells in MLR assays (52). Therefore, the ability of TB-API splenocytes to effectively stimulate allogeneic CD8<sup>+</sup> T cell proliferation may be as a result of apigenin's effects on DC function, which has previously been reported (53). Apigenin's effects on DC function may be as a result of its reduction of Treg percentages, which can inhibit DC function and T cell immune responses (8). However, these Treg percentages were not fully restored to those of control mice, which may also explain why allogeneic CD8<sup>+</sup> T cell proliferation was fully not restored to control levels. In addition, we have previously published that other immunosuppressive cells such as myeloid derived suppressor cells (MDSC) are expanded in our TB mice (39). MDSC are immature macrophages, dendritic cells and granulocytes (54). Apigenin reduction in MDSC percentages may be as a result of maturation of these immature cells, producing mature dendritic cells and other APCs, which could also account for increased allogeneic immune responses. Our unpublished findings suggest that apigenin reduces MDSC percentages, which may also account for the increased proliferation of allogeneic T cell proliferation in TB-Api mice MLR assay. Overall, our results with apigenin provide evidence that Ikaros may be especially involved in regulating T cell immune responses in our TB model.

In conclusion, this study highlights the importance of CK2 in regulating Ikaros expression and T cell immune responses in a solid pancreatic microenvironment. Our results suggest that the natural flavonoid, apigenin, may be therapeutically beneficial in stabilizing Ikaros expression, thus restoring T cell homeostasis and enhancing anti-tumor immune responses, which may lead to better treatment options for pancreatic cancer.

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## CHAPTER FIVE

### DISCUSSION

#### Overview

Pancreatic cancer is one of the most lethal cancers, which is mainly attributed to its resistance to conventional treatment options and late stage diagnosis (1). Currently, research is focused on developing immunological approaches to treat pancreatic cancer. However, pancreatic cancer's induction of a highly immunosuppressive microenvironment is the main hurdle that is limiting treatment success in patients. It is therefore critical to understand the molecular mechanisms that govern suppression of anti-tumor immunity in pancreatic cancer hosts (1). In particular, the ratio and function of effector  $CD4^+$  and  $CD8^+$  T cells and suppressor Tregs is skewed to favor immunosuppression (2, 3). Therefore, it is especially important to understand how these T cells, which are central to the generation of effective anti-tumor immunity, are regulated in pancreatic cancer hosts. As a result, the outcome of this study may lead to the development of T-cell specific therapies to treat pancreatic cancer.

The Ikaros family of transcription factors is regulators of hematopoiesis and is essential for the development of a functional immune system (4). However, these transcription factors have mainly been extensively studied in hematological malignancies (5, 6). Ikaros, the founding and most characterized family member, acts a

tumor suppressor in T cells (7). Ikaros' role in regulating T cells in solid tumors has not been investigated. Ikaros regulates important genes for T cell differentiation and development (8-10), activation and proliferation (8, 11) and cell cycle progression (12). Thus, loss of Ikaros, due to genetic defects or functional inactivation, leads to T-cell leukemogenesis (13, 14). Ikaros' function is regulated by posttranslational modifications, primarily phosphorylation. CK2 is mainly responsible for phosphorylation of Ikaros thereby influencing a multitude of its functions, including its DNA binding ability and pericentromeric localization (15-18). These studies suggest that CK2-phosphorylation inactivates Ikaros function, especially in T cells (16, 18). Furthermore, CK2 phosphorylation of Ikaros in PEST regions, leads to its increased degradation by the ubiquitin-proteasome pathway, reducing Ikaros protein levels in cells (15). On the contrary, PP1 dephosphorylation maintains Ikaros function and protein stability (15). Therefore, tight regulation of these two pathways is critical for sustaining Ikaros activity and function in T cell differentiation and development (17). In this dissertation, we discuss the involvement of Ikaros in maintaining effector and regulatory T cell homeostasis and immune responses. We also identify a possible mechanism governing Ikaros' regulation by CK2 and PP1 in pancreatic cancer models.

### **Discussion of Data, Limitations and Future Studies**

Our *in vitro* results suggest that factors produced by Panc02 cells contribute to the observed downregulation of Ikaros protein expression in splenocytes. Our data also shows that this downregulation is at least due, in part, to Ikaros undergoing ubiquitin-proteasomal degradation. This was deduced by the use of the proteasomal

inhibitor MG132. Increased Ikaros expression, *in vitro* and *in vivo*, using the CK2 inhibitor apigenin and the observed increase in CK2 activity in TB splenocytes, suggest that CK2 may be responsible for this mechanism. Therefore, our results provide evidence that tumor-derived factors (TDF) produced by Panc02 cells may trigger CK2-dependent proteasomal degradation of Ikaros in our pancreatic TB model. We previously published that murine Panc02 cells produce a number of TDF including IL-6, IL-10 and MCP-1 (19). Some of these inflammatory factors, especially IL-6 and IL-10, have been reported to stimulate CK2 activity (20, 21). It is therefore possible that these and other TDF produced by Panc02 cells, may regulate CK2 activity in our pancreatic TB model. Future studies using cytokine and chemokine microarrays and qPCR will be used to further characterize the inflammatory factors produced in TDF. The effects of identified factors on CK2 activity will also be assessed using specific blocking peptides.

Litchfield et al (1991) identified that CK2 phosphorylation regulates its activity. Litchfield et al (1999) also found that changes in CK2 activity may be independent of changes in its amount (22). This may explain the shift in molecular weight observed in our TB splenocytes. This observation suggests that CK2 may be phosphorylated and its activity increased, despite a reduction in its expression. Mass spectrometry techniques will allow us to identify possible CK2 phosphorylation sites. Extracellular signal-regulated kinase (ERK) (23) and cyclin-dependent kinase (Cdk1/cyclin B1) (24) have both been shown to phosphorylate CK2 and will be investigated amongst other kinases.

As mentioned previously, a balance between CK2 and PP1 is needed to maintain Ikaros expression and function, and thus normal lymphocyte development (17). Our

results provide evidence for the involvement of CK2 in regulating Ikaros expression, based on our data with apigenin. Similar studies using apigenin in the TrM model, which also displayed defects in Ikaros and CK2, are needed to provide further clinical evidence for the involvement of CK2 in regulating Ikaros expression in pancreatic cancer. In addition, apigenin is a selective CK2 inhibitor that can inhibit several other kinases (**Table 1.1**). Therefore, our observed results could be as a result of apigenin's inhibition of CK2 and/or other potential kinases. Future experiments in both our TB and TrM models using DRB, a very specific and potent CK2 inhibitor (25) (**Table 1.1**), will allow us to further delineate CK2's involvement in regulating Ikaros expression, and function, in pancreatic cancer. The identification of other potential kinases that can phosphorylate Ikaros in sites located within its PEST regions could also help to highlight other kinases that may regulate Ikaros in our pancreatic cancer models.

On the contrary, analyses of PP1 suggested that its activity might be reduced in TB mice. However, we have not confirmed this by assaying PP1's activity. We did, however, detect two PP1 catalytic (PP1c) isoforms in control mice, one of which was absent in TB and TB-Api mice. Based on previous studies, these isoforms may correspond to PP1 $\alpha$  and PP1 $\beta/\delta$ , isoforms, which are highly expressed in mouse spleens (26). However, use of specific antibodies to each isoform is necessary to confirm their identities. The absence of the higher molecular weight PP1c isoform in whole splenocytes and CD3<sup>+</sup> T cells from TB mice, suggests that this isoform may be important in maintaining Ikaros expression and T cell homeostasis. However, our results in the TrM model did not reflect the differential expression of these PP1 isoforms observed in TB mice. Further studies using PP1 specific inhibitors, such as



PP1 inhibitor-2 (15), will help to determine if PP1 is essential in regulating Ikaros expression and function in our pancreatic cancer models. At this point, the mechanisms that regulate PP1 expression in our model are unknown to us. It is possible that alterations in alternative splicing machinery are responsible for the differential expression of PP1c isoforms observed. In addition, degradation of PP1 isoforms, specifically PP1 $\delta$  (27), has been reported and may account for differences in PP1 isoforms observed at the protein level.

The observed pattern of multiple Ikaros products in mouse splenocytes, representing DNA and non-DNA binding isoforms, is consistent with previous studies (28). Downregulation in Ikaros isoform expression was observed in splenocytes of both our heterotopic (TB) and mutation-induced (TrM) models of pancreatic cancer. Pancreatic cancer development in the TrM model is spontaneously induced by mutations in Kras and p53, which occur in humans with pancreatic cancer (29). Therefore, downregulation of Ikaros isoforms in this TrM model may provide clinical relevance for Ikaros' role in pancreatic cancer. We were unable to evaluate Ikaros expression in the bone marrow and peripheral blood of our mice as Ikaros was lowly expressed in these tissues. Methods to increase leukocyte yields from these organs and possibly immunoprecipitating Ikaros, may allow us to evaluate whether Ikaros expression is also altered in these systemic regions. Analyses of Ikaros expression in lymphoid tissues of pancreatic cancer patients will also provide translational evidence of its involvement in pancreatic cancer progression.

Furthermore, in both TB and TrM models, isoforms corresponding to full-length Ikaros were primarily reduced while DN isoforms were primarily expressed. Since full-

length Ikaros are generally functional and overexpression of DN isoforms inhibits the function of full-length (30), our data suggests that Ikaros may be functionally inactive in these pancreatic cancer models. Immunofluorescence microscopy data showing altered punctate staining of Ikaros in TB splenocytes also suggests that Ikaros function may be disrupted as functional Ikaros binds to DNA in pericentromeric heterochromatin (PC-HC), creating this distinct staining pattern. Mutations in Ikaros that prevent its dephosphorylation by PP1, inhibit Ikaros' targeting to PC-HC and its ability to bind to upstream regulatory elements in its target genes (15). This suggests that the observed disruption in punctate staining in TB mice may be due to altered subcellular localization to PC-HC as a result of lack of dephosphorylation by PP1. Testing the DNA binding ability of Ikaros between control and TB mice is necessary to confirm its activity. In addition, future experiments using protein sequencing are needed to confirm the identity of isoforms observed in TB and TrM models and their possible interactions. This may allow for the identification of any potential novel isoforms and may also provide further insight into specific isoforms that may be critical to T cell regulation.

Our results indicate that Ikaros is undergoing ubiquitin-mediated proteasomal degradation, which may be a result of a loss in the balance between CK2 and PP1 activity. We are currently performing *in vivo* time-course experiments to determine how Ikaros expression is regulated in response to pancreatic tumor progression using our TB mice. These experiments will also us to determine whether increased tumor burden can affect Ikaros' phosphorylation, ubiquitination and degradation, over time. We will also be able to determine how CK2 and PP1 activity correlate with these findings. Co-immunoprecipitation assays and immunofluorescence microscopy experiments will

provide insight into Ikaros localization and interactions with CK2/PP1 in these time-course experiments. Comparative experiments using apigenin or DRB will determine whether CK2 inhibition modulates Ikaros' phosphorylation, ubiquitination, localization and degradation in TB-API mice compared to TB mice. In addition, ongoing experiments are being conducted to evaluate the effects of MG132 on Ikaros expression, T cell responses and tumor regression, *in vivo*.

Expression of some Ikaros isoforms was increased by apigenin treatment of TB mice *in vivo*. This may be attributed to moderate inhibition of CK2 activity. Therefore, dose-dependent studies using apigenin, modifications in the route of administration and increased study time may provide more significant results. Apigenin treatment also showed immunological benefits as it significantly increased effector CD4<sup>+</sup> and CD8<sup>+</sup> T cells, while reducing Tregs percentages. Apigenin also increased allogeneic CD8<sup>+</sup> T cell immune responses and restored IFN- $\gamma$  production of CD8<sup>+</sup> T cells from TB-API mice. These results suggest that apigenin's regulation of CK2, and possibly Ikaros, may result in favorable anti-tumor immune responses in our TB mice. However, both the T cells percentages and allogeneic CD8<sup>+</sup> T cell proliferative responses were not restored to those of control mice, which may again be a reflection of the efficacy of the dose of apigenin administered in inhibiting CK2 activity.

We also observed T-cell specific defects in Ikaros expression in our pancreatic TB mice. Ikaros protein expression was significantly reduced in CD3<sup>+</sup> T cells isolated from TB mice. More interestingly, two isoforms that may correlate with full-length Ikaros 1 and 2 were predominantly expressed in isolated T cells and correlates with findings in other studies (28, 31, 32). Both of these isoforms were reduced in TB CD3<sup>+</sup> T cells

(all T cells), which indicates that Ikaros downregulation may be T-cell specific. More interestingly, the upper molecular weight band of PP1c was also absent in TB CD3<sup>+</sup> T cells. CK2 $\alpha$  expression was also downregulated in TB CD3<sup>+</sup> T but its activity has not been evaluated as yet. Confirmation of CK2 and PP1 activity as well as microscopy studies to determine whether Ikaros localization and interactions are altered in TB CD3<sup>+</sup> T cells, are needed to further delineate Ikaros' regulation in T cells.

The reduction in Ikaros protein expression in T cells and altered T cell numbers in both TB and TrM mice, suggests that Ikaros may be affecting the differentiation and/or development of T cells in these pancreatic cancer models. However, these mechanisms are currently unknown. It is possible that Ikaros may be modulating the development of these T cells via its regulation of critical T cell genes. Ikaros has been shown to positively regulate CD8 $\alpha$  gene expression (9) and Ikaros deficient mice display reduced CD8<sup>+</sup> T cell numbers (33). Ikaros has also been shown to upregulate the transcription of the Cd4 gene (34). Ikaros also regulates T cell production of cytokines, including IFN $\gamma$  (35), whose production was increased in CD8<sup>+</sup> T cells from TB-Api mice. Deregulation of these important genes, as a result of reduced Ikaros expression, may explain the reduction in effector CD4<sup>+</sup>T and CD8<sup>+</sup> T cells and their immune responses, observed in our pancreatic cancer models. As is relates to Tregs, Ikaros -/- CD4<sup>+</sup> T cells display increased numbers of CD25<sup>+</sup>FoxP3<sup>+</sup> T cells (36). These findings suggest that Ikaros deficiency results in increased Treg numbers, which coincide with our results. The FoxP3 transcription factor is essential for Treg development and function (37). We have recently identified potential Ikaros binding sites in the FoxP3 promoter using the transcription factor binding site prediction

program PROMO (ALGGEN) (38). It is therefore possible that Ikaros may negatively regulate the transcription of the FoxP3 gene, thereby influencing Treg development and function.

Ikaros also regulates Src homology-2 domain-containing inositol 5-phosphatase 1 (SHIP-1) (39). SHIP-1 is important for lymphocyte development (40, 41), especially T cells (42, 43). We previously published that defects in SHIP-1 expression and its signaling pathways occur in our TB model (19). The relationship between SHIP-1 and Ikaros, and their regulation and involvement in T cell development are currently being investigated in our pancreatic cancer models. Ikaros also regulates the expression of Notch target genes (44-46). Notch signaling is critical for T cell development (47). It is also proposed that Ikaros and Notch play a cooperative role in T-cell malignancies (44). As Notch signaling also plays a role in pancreatic cancer tumorigenesis (48), future studies will also investigate the possible interplay between Notch and Ikaros in regulating T cell responses in murine pancreatic cancer. Helios, another Ikaros family member, complexes with Ikaros and is considered important for regulating Ikaros function in T cells (49). However, there are conflicting reports on the importance of Helios in T cell development and function (50-52). It would be interesting to determine whether Helios is involved in Ikaros' regulation of T cells in our study. It would also be of interest to determine whether Helios or other Ikaros family members compensate for defects in Ikaros expression and function in T cells.

Ikaros has also been shown to regulate apoptosis, during which it undergoes proteasomal degradation in the early stages (53). More specifically, Ikaros regulates the expression of BCL-2 family proteins (54, 55). Our previous study showed that anti-

apoptotic pathways were active in our TB mice (19). Regulation of apoptosis induction may be another potential mechanism by which Ikaros is regulating T cell percentages in our pancreatic cancer mice. Ikaros also plays an essential role in cell cycle progression. More so, phosphorylation of Ikaros by CK2 influences its ability to regulate G1/S transition (32). It was also reported that Ikaros phosphorylation increased in response to T cell activation, but its expression decreased over time (32), which could be explained by the fact that hyperphosphorylation of Ikaros by CK2 leads to its ubiquitination and ultimately its degradation (15). This would also explain our results and further implies that the reduction of Ikaros in T cells is a result of CK2-mediated proteasomal degradation of Ikaros. This reduction in Ikaros expression could therefore influence effector and Treg proliferation via Ikaros' effect on the cell cycle.

We have yet to purify CD4<sup>+</sup>, CD8<sup>+</sup> and CD25 (Tregs) T cells from control, TB and TB-Api mice to evaluate Ikaros expression in these T cell populations. This would allow us to determine which of these T cells have intrinsic defects in Ikaros expression and may further allow us to identify Ikaros-specific target genes and/or cellular processes, that may contribute to their altered percentages and function in a pancreatic tumor microenvironment. We also need to test the function of these T cells to understand how Ikaros influences their immune responses. We are currently evaluating granzyme B and perforin production of CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells and Tregs (56, 57). We also plan to evaluate CD4<sup>+</sup> T activation of CD8<sup>+</sup> T cells, Treg suppression of antigen-specific immune responses and CD8<sup>+</sup> T cell activity from pancreatic cancer models.

## Considering CK2 Inhibitors as Therapeutic Agents for Pancreatic Cancer

This study highlights the possible therapeutic benefit of targeting upstream regulators of Ikaros, such as CK2, to contribute to the generation of effective anti-tumor immune responses in pancreatic cancer. The elucidation of the molecular mechanism(s) by which Ikaros is regulated highlights the involvement of CK2 in maintaining Ikaros expression, and possibly function, in T cell immunity, in our pancreatic TB mice. Compared to Ikaros, CK2 has been well-investigated as an attractive target for cancer drug discovery (58). CK2 regulates a vast number of cellular processes, playing a critical role in several physiological and pathological outcomes (59). More importantly, due to its tetrameric structure and ATP-binding site, CK2 is considerable a druggable target (60). More recently, CK2 inhibitors have been reported to enhance Ikaros' tumor suppressor activity in hematological malignancies (61). In addition, CK2 inhibitors, especially natural compounds such as apigenin, have therapeutic benefits in inducing apoptosis of pancreatic cancer cells (62, 63). CK2 inhibition also enhances chemosensitivity to gemcitabine in human pancreatic cancer cells (64). Our study focuses on the role of CK2 inhibition on Ikaros expression and function in murine immune cells, specifically T cells. Therefore, the combined effects of CK2 inhibition, possibly causing both tumor destruction and enhancing T cell function by targeting Ikaros, suggests that CK2 may indeed be an attractive target for treating pancreatic cancer.

## Significance of this Study

Pancreatic cancer is resistant to a number of therapeutic treatments and there is dire need for the development of new treatment options. Immunotherapy is a promising approach to treating pancreatic cancer (65). However, defects in generating effective anti-tumor immune responses to treatment options are a substantial hurdle in successfully combating pancreatic cancer (1, 66). This is attributed to decreased T cell immunity, mainly due to the persistent immunosuppressive environment. This immunosuppression is characterized by the presence of immunoregulatory cells such as Tregs (2), which inhibit effector CD4<sup>+</sup> and CD8<sup>+</sup> T cell proliferation and function (67). Therefore, understanding the mechanisms that can regulate T cell balance to favor the induction of anti-tumor immunity may prove beneficial in developing novel therapeutic targets to treat pancreatic cancer. This study is especially significant because it highlights the novel and potential role of Ikaros in regulating immune T cell balance in pancreatic cancer. It also proposes proteasomal degradation as one molecular mechanism by which Ikaros is regulated in murine pancreatic cancer. More importantly, this study provides evidence that CK2 inhibition may be therapeutically beneficial in maintaining Ikaros expression and function, and restoring T cell immune homeostasis in pancreatic cancer.

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## APPENDIX

### IACUC Protocol

### RESEARCH INTEGRITY AND COMPLIANCE INSTITUTIONAL ANIMAL CARE & USE COMMITTEE

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#### MEMORANDUM

---

TO: Tomar Ghansah, PhD

FROM: Farah Moulvi, MSPH, IACUC Coordinator  
Institutional Animal Care & Use Committee  
Division of Research Integrity & Compliance

DATE: 11/18/2014

PROJECT TITLE: Chemotherapy Treatment of Pancreatic Cancer Murine Model  
USF department, institute, center, etc.

FUNDING SOURCE:

IACUC PROTOCOL #: T IS00000447

PROTOCOL STATUS: **APPROVED**

---

Your request for continuation of this study was received and will be reported to the Institutional Animal Care and Use Committee (IACUC). The IACUC acknowledges that this study is currently on going as previously approved. Please be advised that **continuation of this study is in effect for a one-year period beginning 2/13/2015:**

Please take note of the following:

- **IACUC approval is granted for a one-year period at the end of which, an annual renewal form must be submitted for years two (2) and three (3) of the protocol through the eIACUC system.** After three years all continuing studies must be completely re-described in a new electronic application and submitted to IACUC for review.
- **All modifications to the IACUC-Approved Protocol must be approved by the IACUC prior to initiating the modification.** Modifications can be submitted to the IACUC for review and approval as an Amendment or Procedural Change through the eIACUC system. These changes must be within the scope of the original research hypothesis, involve the original species and justified in writing. Any change in the IACUC-approved protocol that does not meet the latter definition is considered a major protocol change and requires the submission of a new application.

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Anne Milo [amilo@pancan.org]

**To:** Schmidt, LeEtta M.

Wednesday, February 04, 2015 1:24 PM

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Thank you,

**ANNE MILO**

PUBLIC RELATIONS MANAGER  
1500 Rosecrans Ave. Suite 200  
Manhattan Beach, CA 90266  
310.706.3357 office  
562.810.3016 cell



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**From:** Schmidt, LeEtta M. [mailto:imschmidt@usf.edu]  
**Sent:** Wednesday, February 04, 2015 10:04 AM  
**To:** Anne Milo  
**Subject:** reuse of chart: 2014 Five-Year Relative Survival Rates...

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## ABOUT THE AUTHOR

Nadine Nelson was raised in Guyana, South America, until the age of seventeen, when she migrated to the United States to attend college. She attended the University of Maryland, College Park and graduated with a B.Sc. in Cell Biology and Molecular Genetics in 2007. She then matriculated into the Ph.D. Program in Biomedical Sciences at the University of South Florida in 2009.

In 2010, Nadine joined Dr. Ghansah's lab in the department of Molecular Medicine. Over the past five years, Nadine has been productive as a graduate student, having published a number of papers. During her time at USF, Nadine has served as secretary of the Association of Medical Science Graduate Students (AMSGS). Nadine has also been awarded the best poster presentation award (Cancer Biology) at the USF Health Research Day in both 2013 and 2014. Nadine has also received the National Science Foundation Bridge to the Doctorate and the Florida Education Fund McKnight Doctoral Completion Fellowships during her Ph.D. studies.