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Regulation of systemic autoimmunity and CD11c+ Tbet+ B cells by SWEF proteins

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Abstract

Recent studies have revealed the existence of a T-bet dependent subset of B cells, which expresses unique phenotypic and functional characteristics including high levels of CD11c and CD11b. In the murine system this B cell subset has been termed Age/autoimmune-associated B cells (ABCs) since it expands with age in non-autoimmune mice and it prematurely accumulates in autoimmune-prone strains. The molecular mechanisms that promote the expansion and function of ABCs are largely unknown. This review will focus on the SWEF proteins, a small family of Rho GEFs comprised of SWAP-70 and its homolog DEF6, a newly identified risk variant for human SLE. We will first provide an overview of the SWEF proteins and then discuss the complex array of biological processes that they control and the autoimmune phenotypes that spontaneously develop in their absence, highlighting the emerging involvement of these proteins in regulating ABCs. A better understanding of the pathways controlled by the SWEF proteins could help provide new insights into the mechanisms responsible for the expansion of ABCs in autoimmunity and potentially guide the design of novel therapeutic approaches.

Keywords

Def6; Swap70; Autoimmunity; Age-associated B cells

1. Introduction

Precise regulation of the expansion and function of T and B cell subsets is critical for the prevention of autoimmune disorders like Systemic Lupus Erythematosus (SLE), a

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prototypical systemic autoimmune disease characterized by autoantibody production and multi-organ involvement that preferentially affects females [1]. Indeed, generation of autoantibodies and lupus pathogenesis has been associated with the aberrant expansion and dysregulation of several T_H effector subsets including T_H-17 and follicular T helper (T_{FH}) cells [2–4]. Moreover, in addition to well-known abnormalities in germinal center B cells and plasma cells [5, 6], premature accumulation of $CD11c^+Tbet^+$ B cells has recently been shown to accompany the development of autoimmune disorders like SLE. The regulatory mechanisms that prevent the inappropriate accumulation and pathogenicity of $CD11c^+Tbet^+$ B cells are largely unknown. In this review, we will highlight recent findings from our laboratory implicating a small family of molecules, the SWEF proteins, in the regulation of $CD11c^+Tbet^+$ B cells. We will first provide a brief overview of the key features of the SWEF proteins, and then outline the present knowledge on their role in immune responses and SLE pathogenesis. Finally, we will discuss our initial insights into the mechanisms by which the SWEF proteins control $CD11c^+Tbet^+$ B cells and the potential role that these cells may play in the spontaneous autoimmune disease that develops in the absence of these molecules.

2 The SWEF proteins: General characteristics

Structure

The SWEF family of proteins is a small family comprised of only two members, SWAP-70 and DEF6 (also known as IRF4-Binding Protein or SLAT) [7–11]. The two proteins share a high degree of sequence and structural homology [10, 11]. They are comprised of an N-terminal domain, which contains a putative EF hand, a central Pleckstrin Homology (PH) domain, and a C-terminal domain termed DH-like domain because it bears weak homology to the Dbl homology (DH) domain, which is normally found in Rho-GEFs (Rho-Guanine Exchange Factors) and promotes the activation of Rho GTPases (Fig. 1) [10–13]. In contrast to conventional GEFs, the DHL domain in SWEF proteins is flanked by a PH domain at its N-terminus, rather than at its C-terminus [10–13]. This unusual arrangement coupled with the low degree of homology of the SWEF DHL to canonical DH domains suggests that the SWEF proteins represent a subfamily of GEFs with potentially unique characteristics. As will be described below in more detail, SWAP-70 and DEF6 control a broad-range of biological responses and their activity is regulated in a highly dynamic and complex manner. Their ability to mediate diverse biological effects may be partly due to their capacity to localize to distinct cellular compartments. Indeed, SWAP-70 and DEF6 can be found in cytoplasmic and nuclear compartments and can also be recruited to the plasma membrane in a stimulation-dependent manner [14–17]. Recent studies have furthermore shown that Def6 contains a glutamine-rich coiled-coil region within its C-terminal region that enables it to aggregate and localize to P-bodies upon cellular stress [18].

Expression

High levels of expression of SWAP-70 and DEF6 are primarily detected in the immune system where the two molecules exhibit a distinctive expression pattern. Particularly intriguing is their expression profile in $CD4^+$ T cells whereby naïve T helper (T_H) cells express only DEF6, while $CD4^+$ $Foxp3^+$ Tregs express both DEF6 and SWAP-70 [19]. Both molecules are also present in B cells where the expression of SWAP-70 has been shown to

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be upregulated upon activation with LPS or αCD40 and IL-4 [10, 20]. The SWEF proteins can also be detected in several other immune cell subsets, although DEF6 tends to be expressed at higher levels and more broadly than SWAP-70 (http://www.immgen.org). Their expression in hematopoietic precurors and non-immune cells like fibroblasts [7, 13, 21] has also been reported, suggesting a broad role for these molecules in biology, although most of the functional studies on this family have focused on their roles in the immune system.

3. Role of the SWEF proteins in T cells

Given that DEF6 is the predominant SWEF family member expressed in T_H cells [19, 20], studies investigating the role of SWEF proteins in this compartment have focused on DEF6. Extensive mutational analyses coupled with imaging studies have provided important insights into the regulation of DEF6 function and have lent support to the idea that, in resting T_H cells, DEF6 is present in an inactive form, which becomes activated upon antigenic stimulation. TCR engagement leads to the tyrosine phosphorylation of the N-terminal region of DEF6 by Lck, presumably resulting in a conformational change, which enables the relocalization of DEF6 to the immunological synapse where DEF6 can promote the activation of Rac and Cdc42 via its DHL domain [15, 16] (Fig. 2A). Several downstream effects have been associated with the DEF6-mediated activation of Rho GTPases including regulation of actin dynamics and immunological synapse formation as well as Ca^{2+}/NFAT signaling [16, 22]. Indeed the absence of DEF6 results in a number of abnormalities in T_H cells that include aberrancies in the ability of the TCR to properly gauge the strength of engagement, likely due to improper cytoskeletal reorganization and immunological synapse formation, and defective T_H1 and T_H2 responses that have been deemed secondary to impaired NFAT activation [22, 23]. Additional regulatory events, such as Itk-mediated phosphorylation, have also been recently shown to contribute to the regulation of DEF6 localization and activity [18, 24].

While the ability of DEF6 to control cytoskeletal dynamics has garnered the most attention, DEF6 can also mediate additional functions. Indeed, our laboratory originally cloned DEF6 during studies aimed at identifying proteins interacting with IRF4. IRF4 is a member of the Interferon Regulatory Factor (IRF) family of transcription factors [25, 26] and plays a fundamental and complex role in the activation of both T and B cells [27–29]. In particular, in T cells, IRF4 is essential for the differentiation of T_H17 cells and follicular T helper (T_{FH}) cells [28]. The interaction of DEF6 with IRF4 is primarily observed in the nuclear compartment and results in the modulation of a subset of IRF4-mediated biological activities (Fig. 2B) [17]. Notably DEF6 restrains the ability of IRF4 to drive the differentiation of T_H-17 cells and to promote the production of high-levels of IL-17 and IL-21, key cytokines produced by this subset. DEF6 regulates IRF4 function via a dual mechanism involving both its ability to physically interact with and “sequester” IRF4 [17] as well as its capacity to inhibit the phosphorylation of IRF4 by the serine-threonine kinase, ROCK2 [30]. This latter mechanism likely relates to the ability of DEF6 to activate Rac, since Rac activation is known to antagonize the activity of another small GTPase, RhoA, and decrease the activation of downstream RhoA effectors like the ROCKs [31, 32]. Whether these dual mechanisms are employed separately or are tightly coordinated remains to be determined.

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In addition to regulating cytoskeletal reorganization and gene expression, our laboratory has recently found that DEF6 can also control protein synthesis by regulating mTORC1, a multi-protein complex that is a key controller of protein translation and is composed of the mTOR serine/threonine kinase, raptor and other components [33–36]. Regulation of mTORC1 by DEF6 is mediated via effects on an alternative docking system that controls the recruitment of mTORC1 to the lysosomes in response to amino acid availability, a step necessary for the ability of mTORC1 to respond to additional signals like those mediated by growth factors [33–36]. This alternative docking system relies on the assembly of a trimolecular complex composed of raptor, p62, and TRAF6, an E3 ligase that activates mTOR via K63-linked polyubiquitination [37, 38]. DEF6 inhibits the assembly of this tri-molecular complex by directly interacting with p62 and TRAF6 (Yi W et al, submitted) (Fig. 2C). Interestingly, this pathway does not affect global protein synthesis but selectively controls the ability of mTORC1 to regulate the translation of a specific group of proteins that include Bcl6, the master regulator of T_{FH} differentiation (Yi W et al, submitted). In line with these findings, DEF6 regulates the expansion of T_{FH} cells *in vivo* in a manner dependent on mTORC1 in T cells (Yi W et al, submitted).

The capacity of DEF6 to control multiple aspects of T cell biology, which range from cytoskeletal reorganization to gene expression to the control of protein translation, supports the idea that DEF6 may serve as a multifunctional signaling hub that enables T cells to precisely coordinate these critical processes in a rapid manner when faced with quickly changing environmental conditions. Importantly, the presence of multiple phosphorylation sites and signaling motifs within DEF6 suggests that post-translational modification of DEF6 upon TCR engagement can help link TCR signaling to the proper acquisition of effector functions. Consistent with this idea, the lack of DEF6 is accompanied by alterations in the ability of T cells to acquire specific T_H cell differentiation fates and this occurs in a highly context-dependent manner [17, 22, 23, 39]. Particularly relevant to the field of lupus and systemic autoimmunity is the finding that, in the absence of DEF6, mice spontaneously develop aberrant expansion of both T_H-17 cells and T_{FH} cells [17, 40] (Yi W et al, submitted), although, as discussed below, the precise autoimmune manifestations that accompany these abnormalities are shaped by the presence/absence of additional genetic manipulations.

4. Role of the SWEF proteins in B cells

While work in the T cell compartment has primarily focused on DEF6, much of the current knowledge of the roles of the SWEF proteins in B cells derive from studies on SWAP-70. Consistent with its role as an activator of Rho GTPases [13] and with its ability to bind F-actin [41], SWAP-70 has been shown to regulate the cytoskeletal reorganization of B cells. Indeed, *in vitro* studies indicate that SWAP-70 inhibits integrin-mediated adhesion and is required for cell polarization and migration [42]. In line with these findings, B cells deficient in SWAP-70 aberrantly accumulate at integrin ligand-rich regions of the stroma and exhibit delayed entry into lymph nodes during an immune response [42]. The effects of SWAP-70 on integrin-mediated adhesion may contribute to the accumulation of transitional T1 B cells and the decreased development of marginal zone B cells that is observed in SWAP-70 deficient mice [43]. Similar to DEF6, the ability of SWAP-70 to control cytoskeletal

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processes is regulated by post-translational modifications, as in the case of its binding to F-actin, which can be inhibited by Syk-dependent phosphorylation [41]. Understanding the regulation of SWAP-70 in response to distinct B cell activating signals will thus be critical to fully appreciate the spectrum of the cytoskeletal effects mediated by SWAP-70 and their overall contribution to the development and activation of B cells.

In addition to the role of SWAP-70 in mediating cytoskeletal rearrangements, SWAP-70 contains three nuclear localization signals and translocates to the nucleus upon B cell activation [14, 20], where it regulates processes involved in B cell differentiation and activation. Nuclear SWAP-70 was originally postulated to be a central player in class switch recombination. As such, SWAP-70 was initially discovered in a screen aimed at identifying proteins involved in mediating class switch recombination [8] and was subsequently shown to directly bind to the Iε promoter in IL-4 stimulated B cells and to promote switching to IgE by regulating the STAT6-BCL6 antagonism [44]. Consistent with these observations, mice deficient in SWAP-70 exhibit selective impairments in IgE responses while producing relatively normal levels of other isotypes [45], suggesting that SWAP-70 plays a unique role in the control of IgE production.

In addition to selective effects on the production of IgE, SWAP-70 has also been shown to regulate plasma cell development. The role of SWAP-70 in this process appears to differ depending on the type of activating signal. Indeed, SWAP-70 was shown to play a positive role in inducing IRF4 expression and promoting plasma cell differentiation in response to T-independent signals, such as TLR ligands [46]. Subsequent studies instead illustrated that SWAP-70 negatively regulates plasma cell differentiation in response to T-dependent signals *in vitro* [40]. In further support of an inhibitory role for SWAP-70 in plasma cell generation, mice deficient in SWAP-70 exhibit enhanced formation of high-affinity plasma cells following immunization with T-dependent antigens [47]. Interestingly, these *in vivo* studies demonstrated that the lack of SWAP-70 also results in impaired germinal centers [47]. These effects were B-cell intrinsic and suggest that SWAP-70 regulates the decision of B cells to either form germinal centers or undergo extrafollicular responses.

The involvement of SWEF proteins in the regulation of B cell differentiation has been further reinforced by work comparing the role of the two family members in the B cell compartment. These studies have demonstrated that DEF6 and SWAP-70 function sequentially to regulate IRF4 activity and thus B cell activation and terminal differentiation [40]. Indeed, DEF6 was shown to control the ability of IRF4 to drive the early steps of B cell activation and its absence was associated with increased AID expression *in vitro* and enhanced GC formation *in vivo* [40]. SWAP-70, in contrast, was shown to regulate the later steps in B cell differentiation by inhibiting the ability of IRF4 to regulate the expression of Blimp1, the key regulator of plasma cell differentiation, thus providing a molecular explanation for the *in vivo* phenotype of the SWAP-70 deficient mice described above. Notably these effects were primarily observed in response to IL-21 and encompassed not only direct inhibition of IRF4 but also interference with the ability of IRF4 to cooperate with STAT3, which becomes activated in response to IL-21 [40]. These findings support the notion that one of the key biological functions of the SWEF proteins is to regulate the responsiveness of B cells to IL-21 in a manner that is most appropriate for the differentiation

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stage of a specific B cell. This multi-stage control system is likely to be one of the reasons why mice deficient in only one of the two SWEF proteins exhibit distinct and milder phenotypes as compared to mice lacking both members of this family, which will be described in the subsequent section.

5. The SWEF proteins and systemic autoimmunity

Given the multifaceted role played by the SWEF proteins in several cell compartments, it is not surprising that genetic manipulations targeting the expression of these molecules have resulted in interesting, albeit complex, *in vivo* phenotypes. While both lupus-like and RA-like phenotypes can develop in these mice depending on the strain and the presence of additional manipulations [17, 22, 40], here we will focus our discussion primarily on the spontaneous lupus-like disease that develops in the absence of both of these proteins given the insights that this model may shed onto the biology of $CD11c^+$ $Tbet^+$ B cells. The relevance of these murine studies to the pathogenesis of human SLE has recently been strengthened by genetic association studies that have identified DEF6 as a new SLE susceptibility locus [48].

The first hint that the SWEF proteins might play an *in vivo* immunoregulatory role emerged from studies of mice deficient in SWAP-70 alone or DEF6 alone, which were analyzed on either a 129 or a mixed 129/B6 background, respectively. On such backgrounds, SWAP-70-deficient mice developed mild autoimmune symptomatology characterized primarily by the development of autoantibodies [45], while DEF6-deficient female mice developed a much more severe systemic autoimmune disease marked by lymphadenopathy, splenomegaly, and autoantibody production with several of the mice succumbing to glomerulonephritis [22]. In line with the relative resistance of the C57BL/6 strain to the development of lupus, backcrossing of mice deficient in DEF6 or SWAP-70 alone onto such a background resulted in milder immunological abnormalities, which were insufficient to lead to the development of autoantibodies *in vivo* [40]. Generation of C57BL/6 mice lacking both SWEF proteins (Double-knockout=DKO mice), however, resulted in robust production of autoantibodies against DNA and other nuclear antigens and in the deposition of immune complexes leading to glomerulonephritis [40]. Remarkably, and akin to what is observed in human SLE, disease development in DKO mice exhibits a striking sex-bias with autoantibodies being detected preferentially in female, but not male, mice [40].

The insights into the cellular pathways regulated by the SWEF proteins described earlier have been invaluable in providing critical information into the mechanisms that underlie the development of autoimmunity in DKO mice. In particular, it has become evident that the spontaneous development of autoimmunity in these mice is marked by a number of abnormalities in T_H cells, which, as observed in other lupus strains as well as SLE patients, encompasses the accumulation of multiple T_H effector subsets including T_{FH} cells and T_H-17 cells [17, 40] (Yi W et al, submitted). These different subsets appear to mediate different aspects of disease pathogenesis with T_{FH} cells preferentially promoting humoral autoimmunity and T_H-17 cells contributing to end-organ damage. Similarly to other lupus models [49], the T_{FH} cells that accumulate in DKO mice express what has been deemed to be a “pathogenic” cytokine profile marked by the dual production of IFNγ and IL-21, a

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pattern that could be particularly conducive to the accumulation of $CD11c^+$ $Tbet^+$ B cells. Development of disease on a background like C57BL/6 that is highly amenable to genetic manipulation has furthermore enabled key molecular features uncovered by the *in vitro* studies to be directly tested *in vivo*. These studies have further confirmed the crucial roles of the mTORC1 and ROCK pathways in the aberrant expansion and function of DKO T_H cells (Yi W et al, submitted).

Analysis of DKO mice has demonstrated that the abnormalities in the T_H compartment are closely coupled with aberrancies in the activation and terminal differentiation of B cells [40]. These dual abnormalities may represent self-sustaining disease-amplifying loops as illustrated by the IL-21 system, whereby increased production of IL-21 by DKO T cells is coupled with enhanced responsiveness of DKO B cells to this cytokine, which could then augment the initial T_{FH} expansion. These findings may explain the requirements for the lack of both SWEF proteins in disease development and/or the need for additional genetic predisposing factors since they suggest that SWEF-deficient T_H cells can only promote disease in settings, such as the “B-cell permissive” milieu of DKO mice, where their potential for pathogenicity is reinforced and maintained by additional cellular interactions.

A similar scenario may also apply to the aberrant molecular networks that characterize SWEF-deficient T and B cells, as in the case of dysregulated IRF4 activity, which is observed in both DKO T and B cells [17, 40]. Interestingly, the shared dysregulation in IRF4-mediated molecular networks observed in DKO T_H and B cells also extends to $CD4^+Foxp3^+$ regulatory T cells where it exerts a beneficial rather than pathogenic role. Indeed DKO female mice exhibit an expansion of effector Tregs, which express a number of key activation markers such as CD44, ICOS and GITR as well as an increased capacity to produce IL-10 [19]. The accumulation of these cells was shown to depend on an IRF4-mediated gene expression program, which involves upregulation of a number of autophagy genes that enable Tregs to survive under the chronic inflammatory conditions encountered in DKO mice [19]. The expansion of these effector Tregs was required to limit pathology in DKO female mice suggesting that long-lived effector Tregs are critical in modulating disease severity in lupus. Increased IRF4 activity is, however, not a universal feature of all the abnormalities observed in DKO mice as illustrated by studies of DKO dendritic cells, which are hyper-responsive to various TLR ligands due to both IRF4-dependent and IRF4-independent mechanisms [50].

6. SWEF proteins and ABCs

While our dissection of the cellular abnormalities that characterize the systemic autoimmune disorder that develops in DKO female mice initially focused on the “classical” B cell compartment, the recent finding that ABCs represent a unique subset of B cells that prematurely expands in autoimmunity [51] have led us to examine the effects of the lack of SWEF proteins on the presence and function of ABCs. A detailed investigation has revealed that DKO female mice demonstrate a marked increase in ABCs by 24 wks of age, when these mice begin to produce autoantibodies (Manni M, unpublished observations). ABCs can be detected, albeit in smaller numbers, even in young DKO female mice before the development of any overt autoimmunity. Surprisingly, DKO male mice also exhibit an

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accumulation of ABCs, although to a slightly lesser extent than DKO female mice. The lack of either DEF6 or SWAP-70 alone was not sufficient to promote the accumulation of ABCs *in vivo* indicating that the premature expansion of ABCs in DKO mice is dependent on the concomitant absence of both DEF6 and SWAP-70.

As expected, ABCs in DKO mice express all the typical phenotypic features of previously described ABCs including high levels of T-bet, Class II MHC and costimulatory molecules (Manni M, unpublished observations). Furthermore, ABC cells sorted from female DKO mice secrete anti-dsDNA IgG2c antibodies upon TLR7 stimulation, supporting the idea that ABCs can directly contribute to the autoimmune syndrome in DKO mice by producing autoantibodies. Interestingly, no autoantibody production was observed when ABCs were sorted from male DKO mice and stimulated *in vitro* with TLR7 ligands, indicating that sex-dependent mechanisms may primarily control the function rather than the frequencies of ABC cells in this model.

In addition to TLRs, the generation of ABCs can also be promoted by cytokines like IL-21 [52]. Given our initial studies supporting a key role for IL-21 in the abnormalities that develop in DKO mice, we have directly investigated the pathogenic role of this cytokine by generating DKO mice that concomitantly lack IL-21. Removal of IL-21 had profound effects on several of the aberrancies that are observed in DKO female mice. DKO mice lacking IL-21 failed to accumulate T_{FH} cells, GC B cells, and plasma cells and lacked production of anti-dsDNA autoantibodies (Manni M, unpublished observations). Notably, accumulation of ABCs was completely abrogated in these mice as compared to age-matched female DKO mice. Preliminary results from *in vitro* experiments have demonstrated that addition of IL-21 leads to a significantly greater population of ABCs in cultures of DKO compared to wt B cells. Thus, the absence of SWEF proteins may alter the responsiveness of ABC cells to IL-21 supporting the *in vivo* findings that IL-21 is critical for the aberrant expansion of ABCs in DKO mice.

While IL-21 production is classically associated with T_{FH} cells, it can also be produced by innate sources [53]. To gain insights into the specific requirements for T_{FH} cells in the generation of DKO ABCs, we have assessed their presence in DKO mice that lack SAP (SLAM-associated protein). SAP is required to mediate sustained interactions between T_{FH} cells and B cells and its absence leads to impaired GC formation and humoral responses [54]. Interestingly, the concomitant absence of SAP in DKO mice resulted in a profoundly diminished accumulation of ABCs. This was again accompanied by marked reductions in T_{FH} cells, GC B cells, plasma cells and anti-dsDNA autoantibody titers (Manni M, unpublished observations). Thus, the aberrant accumulation of ABCs observed in DKO mice is dependent on cognate interactions with T_{FH} cells, suggesting that these cells are the critical source of the IL-21 driving the ABC expansion.

7. Conclusions

The SWEF proteins are multifunctional signaling hubs, which play a unique and critical role in immunoregulation as demonstrated by the spontaneous development of systemic autoimmunity in their absence. In addition to their ability to control the expansion and

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function of T helper cells and the “classical” B cell compartment, recent evidence shows that the SWEF proteins can also regulate the accumulation of ABC cells. The precise molecular mechanisms by which the SWEF proteins control ABCs are presently under investigation. Both *in vitro* and *in vivo* studies, however, support the notion that the ability of SWEF proteins to control the IL-21-IL-21R pathway is fundamental to restraining the premature expansion of ABCs. Importantly, mice deficient in SWEF proteins may provide a very informative platform to dissect the contribution of ABCs to the development and/or maintenance of systemic autoimmunity. These findings could be directly relevant to human autoimmune disorders. Indeed, in addition to the recent recognition of DEF6 as a genetic risk factor for SLE, several of the abnormalities in SWEF deficient mice such as the expansion of T_H17 cells and T_{FH} cells have been reported in SLE patients and studies in this model could facilitate an in-depth understanding of the potential cross-talk that may exist between ABCs and these T_H subsets. Even more importantly, the profound sex-bias that underlies disease development in SWEF deficient mice closely reflects the known female predisposition that accompanies human SLE and could enable the garnering of new insights into the sex-specific pathways that help shape ABCs.

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Figure 1. Diagram of DEF6 and SWAP-70

DEF6 and SWAP-70 are highly homologous and are comprised of an N-terminal domain, which contains a putative EF hand, a central Pleckstrin Homology (PH) domain, and a C-terminal domain termed DH-like. Y: Tyrosine residues.

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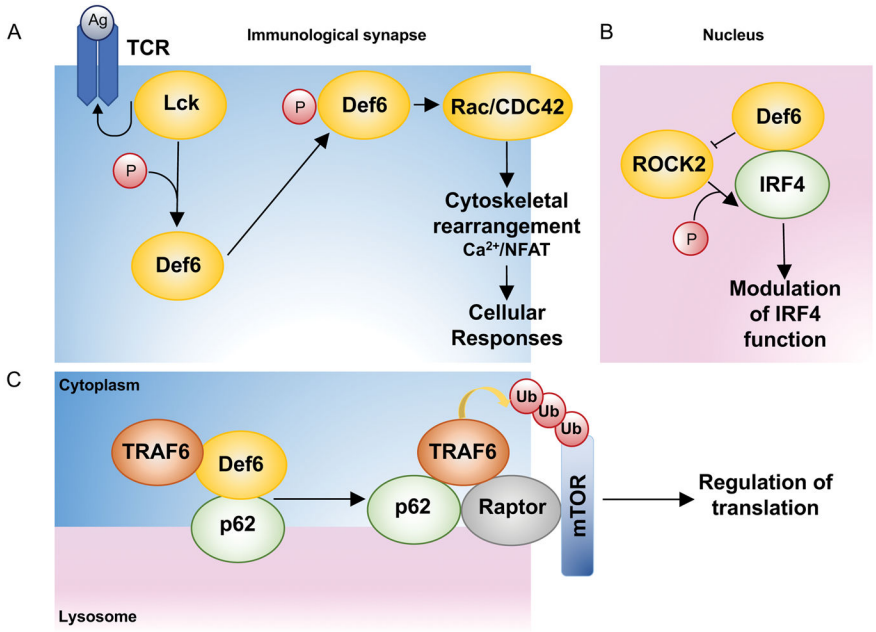

Figure 2. DEF6 localization and function

A) TCR engagement leads to the tyrosine phosphorylation of the N-terminal region of DEF6 by Lck, resulting in a conformational change, which enables the relocalization of DEF6 to the immunological synapse where DEF6 can promote the activation of Rac and Cdc42 via its DHL domain. B) In the nucleus DEF6 regulates IRF4 function by sequestering IRF4 and by inhibiting IRF4 phosphorylation by the serine-threonine kinase, ROCK2. C) DEF6 inhibits the assembly of the p62-TRAF6-Raptor tri-molecular complex by directly interacting with p62 and TRAF6 thus controlling the ability of mTORC1 to regulate the translation of a specific group of proteins.