Regulation of T-cell adhesion and megakaryopoiesis by immune adaptor ADAP

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ABSTRACT

The immune adaptor ADAP possesses versatile roles in a variety of immune cells, including T cells, dendritic cells, macrophages, and platelets, etc. The most extensively-studied role of ADAP is that it couples TCR activation to integrin activation and T-cell adhesion. However, the regulation of this adaptor during integrin activation and T-cell adhesion remains unclear. Meantime, the functions of ADAP linked to other immune cells are largely unknown. Work in this thesis have identified Ubc9, the sole SUMO E2 conjugase, as an essential regulator of ADAP in T-cell adhesion. We show that ADAP interacted directly with Ubc9 *in vitro* and *in vivo*, and the association was further strengthened in response to anti-CD3 stimulation. The Ubc9 binding domain on ADAP was mapped to a nuclear localisation sequence (aa 674-700) within ADAP. Knockdown of Ubc9 by shRNA or expression of the Ubc9-binding-deficient ADAP mutant significantly decreased TCR-induced integrin adhesion to ICAM-1 and fibronectin, as well as LFA-1 clustering, while having little effect on the TCR proximal signalling responses and TCR-induced IL-2 transcription. Furthermore, downregulation of Ubc9 impaired TCR-mediated Rac1 activation and attenuated the membrane targeting of Rap1 but not RIAM. Taken together, our data demonstrate for the first time that ADAP forms a functional interplay with Ubc9 and Ubc9 plays a selective role in integrin-mediated T-cell adhesion via modulation of Rap1 membrane recruitment and Rac1 activation. Another important finding of this thesis is the identification of a negative regulatory role for ADAP in the megakaryopoiesis. Here we show that in the bone marrow and spleen of $ADAP^{-/-}$ mice, a significant increase in the number of megakaryocytes were observed, and the ADAP-deficient megakaryocytes exhibited potentiated capacity in differentiation and development compared to the WT megakaryocytes. Mechanistically, ADAP directly interacted with STAT1, an indispensable modulator in megakaryopoiesis. Analysis on the activation of STAT1 showed that depletion of ADAP resulted in potentiated STAT1 phosphorylation and transcriptional activity, as well as upregulations of STAT1-regulatory genes. Collectively, these results suggest a novel role of ADAP in megakaryocytes, where ADAP attenuates megakaryopoiesis by direct interaction

with STAT1 and negatively modulates the STAT1 activities. In summary, the work in this thesis have illustrated the diverse roles of ADAP in TCR-mediated integrin activation and megakaryopoiesis, and altogether contributed to our current knowledge of the many facets of ADAP in immunity.

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LIST OF ABBREVIATIONS

AchE	acetylcholinesterase
ADAP	adhesion and degranulation-promoting adapter protein
AML	acute myeloid leukaemia
ANOVA	Analysis of variance
AP-1	activator protein 1
APC	antigen-presenting cell
BCL-10	B-cell lymphoma/leukemia 10
CAR	chimeric antigen receptor
CARMA1	caspase recruitment domain-containing membrane-associated guanylate kinase protein 1
CCD	coiled-coil domain
CCR7	C-C chemokine receptor type 7
CSF-1	colony-stimulating factor 1
CTL	cytotoxic T lymphocyte
CTLA-4	cytotoxic T-lymphocyte-associated protein 4
DAG	diacylglycerol
DBD	DNA binding domain
DC	Dendritic cell
ERK	extracellular regulated protein kinase
ET	essential thrombocythemia
EVH1	Ena/VASP homology 1
FACS	Fluorescence-activated cell sorting
FBS	foetal bovine serum
FYB	fyn-binding protein
G-CSF	granulocyte colony-stimulating factor
GADS	Grb2-related adaptor downstream of Shc
GAS	γ-activated sequence
GATA-1	GATA-binding factor 1
GEMs	glycospingolipid enriched microdomains
Grb2	growth factor receptor-bound protein 2

GST	Glutathione S-transferase
HA	haemagglutinin
HPK1	hematopoietic progenitor kinase 1
HPK1	Hematopoietic Progenitor Kinase 1
HSCs	haematopoietic stem cells
ICAM-1	intercellular adhesion molecule-1
IFN	interferon
IKKβ	IκB kinase β
IL-2	Interleukin-2
IP_3	inositol 1, 4, 5-trisphosphate
IPTG	isopropyl-beta-D- thiogalactopyranoside
ISRE	interferon-stimulated response element
ITAM	immunoreceptor tyrosine- based activation motifs
ITC	isothermal titration calorimetry
ITP	immune thrombocytopenia purpura
JAKs	Janus Kinases
JNK	c-Jun N-terminal kinases
LAD	leukocyte adhesion deficiency
LAT	linker for activation of T cells
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
Lck	lymphocyte-specific protein tyrosine kinase
LFA-1	Lymphocyte function-associated antigen 1
LPS	lipopolysaccharides
LTQ	Linear Trap Quadrupole
MALT1	Mucosa-associated lymphoid tissue lymphoma translocation protein 1
MAPK	Mitogen-activated protein kinases
MHC	major histocompatibility complex
MPNs	myeloproliferative neoplasms
MST1	macrophage-stimulating 1
Nck	non-catalytic region of tyrosine kinase adaptor protein 1
NF-AT	nuclear factor of activated T-cells
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NFE2	nuclear factor erythroid 2

NKT	Natural killer T
NLS	nuclear localisation sequence
NTD	N-terminal domain
PAG	phosphoprotein associated with GEMs
PAK	p21 activated kinase 1
PAK	p21 activated kinase
PBD	p21 binding domain
PBD	p21 Binding Domain
PD-1	programmed cell death protein 1
PDGF	platelet-derived growth factor
PI	propidium iodide
PIP_2	phosphatidylinositol-4, 5 bisphosphate
PKD	protein kinase D
PLCγ1	phospholipase Cγ1
PMA	phorbol 12-myristate 13-acetate
PMA	phorbol myristate ester
PV	polycythemia vera
Rap1	ras-related protein 1
RapL	regulator of cell adhesion and polarization enriched in lymphoid tissues
RIAM	Rap1 interacting adaptor molecule
RPMI	Roswell Park Memorial Institute medium
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SH2	Src Homology 2
SIT	SH2-interacting transmembrane adaptor protein
SKAP1	src kinase associated phosphoprotein 1
SLAP-130	SLP-76-associated protein
SLP-76	SH2 domain containing leukocyte protein of 76 kDa
SMAC	supramolecular activation cluster
SMAD3	Mothers against decapentaplegic homolog 3
STAT1	signal transducer and activator of transcription 1
SUMO	small ubiquitin-related modifier
TAD	transcriptional activation domain
TAK1	TGFβ-activated kinase 1

TCR	T cell receptor
TGFβ	Transforming growth factor beta
TNF	tumour necrosis factor
TPO	thrombopoietin
TRAF6	tumour necrosis factor receptor associated factor 6
TYK2	tyrosine kinase 2
VASP	vasodilator-stimulated phosphoprotein
VCAM-1	vascular cell adhesion molecule-1
VLA-4	very late antigen-4
Vsx-1	visual system homeobox 1
VWF	von Willebrand factor
WASP	Wiskott-Aldrich syndrome protein
WT	wild-type
ZAP-70	zeta associated protein-70

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Chapter 1 Introduction

The immune system and immune adaptor proteins

The immune system

The immune system is the body's defence system against invasion by nonself entities, including infectious microorganisms such as bacteria, viruses, fungi, and parasites. It distinguishes self from nonself and eliminates potentially harmful nonself pathogens from the body. The immune system is also capable to recognise and destroy abnormal cells that derive from host tissues, such as cancer cells. The normal functioning of the immune system relies on an amazingly complex network of proteins, cells, tissues, and organs that cooperates to defend the body. Harnessing the fundamental machinery of the immune system not only helps to generate appropriate immune responses against the invading entities, but also provides an opportunity to cure cancer. Very recently, a ground-breaking personalized therapy by Novartis called Chimeric Antigen Receptor (CAR)-T cell cancer therapy, has been recommended for FDA approval for the treatment of relapsed or refractory paediatric and young patients with B-cell acute lymphoblastic leukaemia. Different from typical small molecules or biologic therapies, the CAR-T cell therapy utilises the patient's immune system for the treatment of cancer. T cells are drawn from the blood of each individual patient, and subsequently reprogrammed and genetically coded to expressed a chimeric antigen receptor to recognise and fight cancer cells (Novartis, 2017). Not long ago, the modulation of signalling via coinhibitory or costimulatory receptors expressed on T cells, e.g. the "checkpoint-blocking" antibodies that block the CTLA-4 and PD-1 receptors, has been proven to be a potent way to amplify antitumour immune response in the treatment for melanoma and bladder cancer (FDA, 2015). Therefore, a careful elaboration on the fundamental machinery of immune system is important for us to interpret the immune functions, and ultimately develop promising treatments or therapies in fighting diseases.

The human body rests on two major pillars of immune response against invaders: the innate (natural) immune response and the adaptive (acquired) immune response. These two categories are very different with respect to the degree of specificity and the mechanisms underlying the recognition of the nonself entity, although these distinctions are not mutually exclusive. The innate immunity refers to nonspecific defence mechanisms that respond

immediately to an invader in the body by cells of both hematopoietic and non-hematopoietic origin. The hematopoietic cells that perform a task of innate protection involves macrophage, dendritic cells, mast cells, neutrophils, eosinophils, natural killer cells and natural killer T cells. It also requires the response from non-hematopoietic cells, such as epithelial cells lining the skin, and respiratory, gastrointestinal and genitourinary tracts. To augment the cellular response, the innate immune system also utilises humoral elements such as complement protein, lipopolysaccharides (LPS) binding protein, C-reactive protein and other acute phase reactants, and anti-microbial peptides (Turvey and Broide, 2010). If the broadly effective innate response is unsuccessful in eliminating the pathogens, a specific and more accurate adaptive immune response sets in. In contrast to the innate immunity, upon the engagement of foreign antigens derived from the invading entities, the adaptive immunity boasts an extremely diverse, and randomly generated repertoire of receptors expressed by T and B cells that are highly specific to the precise invading entity. Moreover, another defining characteristic of the acquired immune system is that it remembers past exposure to pathogens by producing memory cells. Following the initial encounter with pathogens, T cells and B cells that expressed antigen-specific receptors are subjected to clonal expansion. Upon a second encounter with this specific pathogen, a much stronger and faster immune response than the first one is triggered and initiates subsequent effectors functions to clear the pathogen. Similar with the innate immune system, to augment the cell-mediated immune response by T cells and B cells, the acquired immune system should utilise additional humoral components, such as soluble antigen-specific antibodies secreted by B cells. The innate and acquired immunity do not function in isolation, but instead cooperate with and complement the other to achieve a maximum defence against invaders.

T-cell signalling

T cell is one of the two main arms of acquired immune system. The engagement of T cells with antigen-presenting cells (APCs) evokes a plethora of signalling transduction events that are crucial to the T-cell functions including production of cytokines and chemokines, T-cell development and lineage commitment, generation of cytotoxic T cells, as well as the T-cell homeostasis (Koretzky and Myung, 2001, Wilkinson et al., 2004). Dysregulation of the signalling events can result in marked shifts in the T-cell receptor repertoire, and eventually lead to immunodeficiency or autoimmunity diseases (Rassenti et al., 2004). The most proximal T-cell signalling events are initiated by the Src kinase $p56^{lck}$ ligation with

coreceptors CD4/CD8. Following TCR engagement, the CD4/CD8-$p56^{lck}$ complexes are recruited to the proximity of TCRζ/CD3 chains, leading to the phosphorylation of intracellular portions of the TCR-associated CD3 complex called immunoreceptor tyrosine-based activation motifs (ITAMs). As a result, SYK-family protein tyrosine kinase zeta associated protein-70 (ZAP-70) is then recruited to and engaged by the doubly tyrosine phosphorylated ITAMs where $p56^{lck}$ activates ZAP-70 by specific phosphorylation at tyrosine 493 and 319. In turn, ZAP-70 phosphorylates several downstream effectors, one of which is the phospholipase Cγ1 (PLCγ1). Recruitment and activation of PLCγ1 hydrolyses phosphatidylinositol-4, 5 bisphosphate (PIP_2) to inositol 1, 4, 5-trisphosphate (IP_3) and diacylglycerol (DAG), second messengers responsible for inducing the release of intracellular Ca^{2+} and protein kinase C activation, respectively (Rao et al., 1997). These pathways are important for the translocation of transcription factors, such as nuclear factor of activated T-cells (NF-AT) and activator protein 1 (AP-1) to the nucleus, where they are required for Interleukin-2 (IL-2) transcription (Smith-Garvin et al., 2009, Raab et al., 1997, Zhang et al., 1998, Bubeck Wardenburg et al., 1996, Liu et al., 2010, Jordan et al., 2003). TCR engagement also signals to small GTP-binding proteins and integrins, leading to cytoskeleton reorganisation and integrin activation (Wilkinson et al., 2004).

Adaptor proteins in T-cell signalling

A coherent response of T cells to T-cell receptor (TCR) stimulation is contingent vitally on the adaptor proteins. Adaptor proteins are molecular scaffolds that recruit effectors and are central to signalling transductions in immune cells. They lack intrinsic enzymatic activities or direct effector functions, but contain modular domains through which multiple molecular signalling complexes are assembled via specific protein-protein and protein-lipid interactions (Jordan et al., 2003). Adaptor proteins are widely expressed in various hematopoietic cells, and function in diverse ways, including operating as ‘molecular bridging’ proteins that recruit positive or negative regulators into signalling pathways, regulating effector functions by inducing the intramolecular conformational changes, and by targeting other proteins for degradation (Koretzky and Myung, 2001).

In TCR signalling, adaptor proteins function as the link between proximal and distal signalling events. For instance, following TCR stimulation, the activation of ZAP-70 results in the formation of a signalosome complex nucleated by adaptor proteins linker for activation

of T cells (LAT) and SH2 domain containing leukocyte phosphoprotein of 76 kD (SLP-76) (Purbhoo et al., 2010, Liu et al., 2010). LAT is a transmembrane adaptor with tyrosine residues that bind to the SH2 domains of Grb2-related adaptor downstream of Shc (GADS), growth factor receptor-bound protein 2 (Grb2) and PLCγ1 (Asada et al., 1999). GADS binds to LAT and recruits SLP-76 by means of SH3 domain binding to a unique motif in SLP-76. The SH2 domain of SLP-76 in turn binds to another adaptor, adhesion and degranulation promoting adapter protein (ADAP) and the hematopoietic progenitor kinase 1 (HPK1) via YDDV motifs (Yablonski et al., 1998, Pivniouk et al., 1998, Clements et al., 1998, Sylvester et al., 2010). Adaptor proteins can function as positive or negative regulators of T-cell functions. The SLP-76, LAT, and GADS have positive effects in the TCR signalling, while the SH2-interacting transmembrane adaptor protein (SIT), and the phosphoprotein associated with glycospingolipid enriched microdomains (GEMs) (PAG) have a negative function. Meantime, Adaptors such as ADAP and the Src kinase-associated phosphoprotein 1 (SKAP1) positively regulate the T-cell adhesion and the formation of T cells conjugates with antigen-presenting cells (APC) (Wilkinson et al., 2004). To expand our knowledge of the functions of adaptor proteins in immune responses, the research interest of this thesis focuses on understanding the roles of immune adaptor ADAP in various immune cells.

Adhesion and degranulation promoting adapter protein (ADAP)

The immune adaptor ADAP is widely expressed in various hematopoietic cells including T cells, mast cells, platelets, myeloid cells, natural killer cells, dendritic cells, granulocytes, macrophage, microglia, and hippocampal neuronal cells (Wang and Rudd, 2008, Engelmann et al., 2013, Thiere et al., 2016). The functions of ADAP have been characterized in multiple signalling pathways targeting for cell motility, proliferation, activation, differentiation, and cytokine production (Peterson et al., 2001, Parzmair et al., 2017, Medeiros et al., 2007).

Structure and functional domains of ADAP

ADAP, formerly known as SLP-76-associated protein (SLAP-130) or fyn-binding protein (FYB), exists as two isoforms of 120 kDa and 130 kDa, which differ by an extra insertion of 46 amino acids at the C-terminus of the 130 kDa isoform (Musci et al., 1997). It was originally cloned as the binding partner of SLP-76 and Fyn (da Silva et al., 1997a, Musci et al., 1997). As an adaptor protein, ADAP lacks discernible enzymatic or transcriptional activity, but contains domains for scaffolding molecules of various classes to facilitate the

formation of signalling complexes. ADAP contains a proline-rich domain, an Ena/vasodilator-stimulated phosphoprotein (VASP) homology 1 (EVH1) domain-binding site, two putative nuclear localization sequences, multiple tyrosine motifs (Y^{595}, Y^{625}, and Y^{651} in the human; Y^{547}, Y^{584}, and Y^{687} in the mouse), and two SH3 domains (da Silva et al., 1997a, Geng et al., 1999, Wang and Rudd, 2008).

The N-terminus proline-rich domain constitutively associates with the SH3 domains of adaptor proteins SKAP1 and SKAP-Homologue (SKAP-HOM)(Liu et al., 1998, Marie-Cardine et al., 1998b). The EVH1-binding domain at the N-terminus connects ADAP to the cytoskeleton by association with the EVH1 domain in Ena/VASP family of actin regulatory proteins (Krause et al., 2000). The first SH3 domain at the N-terminus interacts with caspase recruitment domain-containing membrane-associated guanylate kinase protein 1 (CARMA1), while the second SH3 domain at the C-terminus is a second binding site for a tyrosine-based motif in SKAP1 (Medeiros et al., 2007, Duke-Cohan et al., 2006). ADAP also harbours multiple phosphotyrosine motifs which mediate well-characterised bindings with the SH2 domains of binding partners, e.g. FYN (at Y^{625}DGI), SLP-76 (at Y^{595}DDV and Y^{651}DDV), NCK (at Y^{595}DDV and Y^{651}DDV) (Musci et al., 1997, Raab et al., 1999, Sylvester et al., 2010). A very C-terminus of ADAP (aa 691-708) can bind to the TGFβ-activated kinase 1 (TAK1) as well (Sylvester et al., 2010).

Taking advantage of the gene-deficient cell lines and gene-knockout mice, significant advances have been made in understanding the functions of adaptor protein in immune cells, where the functions of ADAP have been most-extensively investigated in T cells.

ADAP positively regulates T-cell activation and T-cell adhesion

T-cell activation

The study on the role of ADAP as a positive or negative regulator of T-cell activation using ADAP overexpression in various cell lines was initially controversial (Veale et al., 1999, Raab et al., 1999, Boerth et al., 2000). A major milestone in the field was achieved with the generation of chimeric and knockout ADAP mice by parallel studies from two independent groups (Griffiths et al., 2001, Peterson et al., 2001). The characterization of ADAP knockout mice show that T cells are defective in early cell activation. Upon TCR stimulation, ADAP-deficient T cells exhibit defective proliferation and cytokines production e.g. IL-2 and IFN-γ, as well as diminished CD69/CD25 upregulation. The defect of ADAP-

deficient T cells in response to CD3/CD28 stimulation *in vitro* subsequently translates into a defective immune response to antigen *in vivo.* Interestingly, T cells from ADAP-deficient mice can respond if provided sufficient costimulation. Proliferation of ADAP-deficient T cells is partially rescued by CD3 stimulation plus phorbol ester, PMA, while simultaneous stimulation with PMA and ionomycin completely rescue proliferation and cytokine production (Peterson et al., 2001). TCR/CD28-mediated activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and the Mitogen-activated protein kinases (MAPK) c-Jun N-terminal kinases (JNK signalling) in ADAP-deficient T cells are impaired, whereas the activation of MAPK extracellular regulated protein kinase 1/2 (ERK1/2) are not affected (Srivastava et al., 2012, Peterson et al., 2001, Griffiths et al., 2001). These findings suggest that ADAP may function in the downstream of many proximal signalling events that culminate in T-cell activation.

Mechanistically, ADAP regulates T-cell activation via modulation of the transcription factor NF-κB (Medeiros et al., 2007). ADAP interacts with CARMA1 and is required for the assembly as well as the membrane relocalisation of the CARMA1-BCL10-MALT1-complex, thereby regulates the NF-κB translocation to the nucleus. Additionally, ADAP drives the activation of NF-κB by association with TAK1 and subsequently recruiting TAK1 to the CARMA1-BCL10-MALT1-complex to mediate the phosphorylation of IκB kinase β (IKKβ) and further NF-κB translocation into the nucleus(Srivastava et al., 2010).

Interestingly, while ADAP is dispensable for the TCR proximal signalling events, including calcium flux and tyrosine phosphorylation of PLC-γ, SLP-76, Lck, LAT, and Vav, ADAP is rather a critical regulator of TCR-induced integrin clustering and adhesion (Griffiths et al., 2001, Peterson et al., 2001). ADAP is the first protein identified that couples TCR and chemokine receptor stimulation to the activation of integrins via the “inside-out” signalling pathway.

T-cell adhesion

Integrins are heterodimeric transmembrane proteins that can undergo conformation changes to increase their affinity and avidity upon stimulation through the TCR-CD3 complex or chemokine receptors. Integrins induce bidirectional signalling, whereby signal from the binding of integrin extracellular domain to ligand is passed “outside-in” to regulate cellular functions while cellular activation is transmitted “inside-out” to the adhesive domain to activate integrin (Mor et al., 2007, Zhang and Wang, 2012). The molecular events leading

to integrin activation have been collectively termed the "inside-out" signalling (Hogg et al., 2004). The activation state of integrins is of particular importance to the adhesiveness of T cells. On resting T cells, integrins are expressed in inactive states that adopt a low-affinity conformation for their ligands (Hogg et al., 2004, Hogg et al., 2011, Kinashi, 2005). The initial contact of T cells with APCs is made by random encounters and by chemokines that can partially activate integrins on the surface of T cells (Wang and Rudd, 2008). The engagement of TCR on T cells with peptide-major histocompatibility complex (MHC) on APCs enables transmitting an "inside-out" signals to the adhesive surface thereby leading to a firm conjugation between T cells and APCs mediated by high-avidity integrins. Conjugation is accompanied by a rearrangement of receptors that form a supramolecular activation cluster (SMAC) at the interface (termed as "immunological synapse"). The central SMAC is composed of the engaged TCRs in microclusters, while a ring enriched with the integrin LFA-1 and talin forms a peripheral SMAC (Monks et al., 1998, Dustin and Shaw, 1999). A stable and strong T-cell adhesion enables the TCR-pMHC interaction to be sustained long enough to complete a full T-cell activation (Davis et al., 2003). T-APC conjugation and cell adhesion are crucial prerequisites for the execution of effector functions of T cells, including T-cell migration, transmigration, proliferation, cytokine production, and T-cell cytotoxicity.

Given the importance of integrin-mediated adhesion to T-cell immunity, unravelling the nature of the intracellular signalling pathways responsible for TCR-mediated "inside-out" signalling is of major interest. Disruption on the earliest signalling events of TCR ligation or upstream TCR signalling components such as $p56^{lck}$, ZAP-70, SLP-76, or GADS-SLP-76 interaction results in a generic impairment on LFA-mediated T-cell adhesion (Bunnell et al., 2001, Goda et al., 2004, Jordan et al., 2007). Despite this, major advances have been made over the past two decades in identify the key downstream components/complexes of the "inside-out" signalling pathway nucleated by the adaptor protein ADAP (Wang et al., 2003, Griffiths et al., 2001, Burbach et al., 2007, Kliche et al., 2006, Horn et al., 2009).

ADAP-deficient T cells exhibited altered β1- and β2- mediated cell adhesion to purified integrin ligands ICAM-1, VCAM- 1 and fibronection, attenuated LFA-1 activation, and impaired conjugate formation with APCs upon TCR stimulation (Peterson et al., 2001, Griffiths et al., 2001, Wang et al., 2004). In addition to its role in TCR-mediated adhesion and T cell-APC conjugation, ADAP is also involved in chemokine receptor CCR7-mediated "inside-out" signalling, that affects both affinity/avidity regulation of LFA-1, adhesion, homing, as well as intranodal T-cell motility *in vivo* (Kliche et al., 2012).

Emerging evidences have pointed to functional partnerships between ADAP and SKAP1, as well as ADAP and SLP-76 for the regulation of integrin activation and adhesion. ADAP constitutively interacts with SKAP1, for that approx. 70% of endogenous ADAP binds SKAP1 while all SKAP1 molecules in T cells are associated with ADAP. Meantime, ADAP regulates the stability of SKAP1 by intervening with SKAP1 proteolysis such that ADAP-deficient T cells lack expression of SKAP1 (da Silva et al., 1997b, Marie-Cardine et al., 1998a, Huang et al., 2005). It was not clear initially whether the defects in integrin activation and T-cell adhesion displayed in ADAP-deficient mice was indeed due to ADAP alone or a combinative effect of ADAP and SKAP1 until the SKAP1-deficient mice was generated. In SKAP1-deficient mice, ADAP expression is normal. Interestingly, a comparable degree of impaired integrin activation and adhesion was observed in SKAP1-deficient T cells, suggesting that SKAP1 functions independently of ADAP and is the effector within the ADAP/SKAP1 module in the regulation of integrin activation and cell adhesion (Wang et al., 2007, Wang and Rudd, 2008). Disruption of the interactions impairs TCR-mediated adhesion and LFA-1 activation, T cell-APC conjugation and assembly of the peripheral SMAC (Kliche et al., 2012, Kliche et al., 2006, Raab et al., 2010, Peterson et al., 2001). In addition to the functional module of ADAP/SKAP1, ADAP also associates with SLP-76 in the regulation of integrin activation and T cell-APC conjugation. Overexpressing ADAP with mutation in the SLP-76 binding motif fails to enhanced TCR-induced T cell-APC conjugation and displayed defects in integrin clustering and assembly of the peripheral supramolecular activation complex (Wang et al., 2004). Conversely, an SLP-76 mutant unable to bind ADAP fails to support T-cell adhesion to integrin ligands, but retains the ability to form clusters following TCR stimulation(Baker et al., 2009). Interesting, while SLP-76 is indispensable for TCR-mediated integrin activation and adhesion, the chemokine-mediated “inside-out” is not affected by the loss of SLP-76 (Horn et al., 2009). These findings suggest a differential requirement for SLP-76 in the regulation of TCR- or chemokine-mediated “inside-out” signalling, and that SLP-76 in the ADAP/SLP-76 module may have a non-redundant role as to ADAP.

Aside from adaptor proteins, various signalling molecules are required for maintaining integrin-dependent T-cell adhesion by transmitting the “inside-out” signals from TCR to integrins via functional cooperation with adaptor proteins. These intracellular signalling proteins acting as downstream targets of ADAP/SKAP include the GTPase Rap1 and its binding partners regulator of cell adhesion and polarization enriched in lymphoid tissues (RapL) and Rap1-GTP-interacting adaptor molecule (RIAM). Constitutively active

Rap1 promotes TCR-induced LFA-1 activation and adhesion, by means of associating with its effector RapL. RapL interacts with the cytoplasmic domain of αL subunits on LFA-1 upon TCR stimulation, thereafter inducing and stabilizing a high affinity conformation of LFA-1. RIAM suppression inhibits Rap1 membrane targeting and directly binds adhesion molecule talin to facilitate its membrane targeting and access β integrin tail to enhance integrin affinity (Lafuente et al., 2004, Katagiri et al., 2003, Katagiri et al., 2004). Two complexes are assembled in T cells for integrin activation, one of which is the ADAP/SKAP1/RIAM/Mst1/Rap1/talin/kindlin-3 complex interacts with the αL subunit of LFA-1 while the other is ADAP/SKAP1/RapL/Mst1 interacts with β subunit of LFA-1. Upon TCR and chemokine receptor triggering, these two distinct complexes were recruited to the plasma membrane in close proximity to the integrin LFA-1, and induce the activation of LFA-1, thus eventually promote T cell-APC conjugation (Kliche et al., 2006, Kliche et al., 2012, Raab et al., 2010, Menasche et al., 2007).

The other component of the bidirectional integrin signalling is "outside-in" signalling, where ligand bound integrins transmit an "outside-in" signal to the T cell and thereby promote activation, proliferation, and migration of T cells (Hogg et al., 2011). ADAP may also play a unique role in outside-in signalling from integrins in T cells. Following very late antigen-4 (VLA-4) and LFA-1 stimulation, ADAP is phosphorylated and induces formation of a ring-shaped actin reorganisation called actin cloud in a SLP-76-dependent manner (Suzuki et al., 2007, Hunter et al., 2000). It was further discovered that ADAP and SLP-76-ADAP binding are coupled to LFA-1–mediated costimulation of IL-2 production, F-actin clustering, T cell polarization, and T-cell motility (Wang et al., 2009).

Diverse regulations by ADAP in immune cells and functions

ADAP is a versatile protein involved in many aspects of biological functions. Besides the well-established regulations on T-cell activation, proliferation, and integrin-mediated cell adhesion in T cells, the role for ADAP is further supported by work in other cell functions, e.g. thymic selection, $CD8^+$ homeostasis, $CD8^+$ cytotoxicity, as well as by those in macrophage, dendritic cells, natural killer cells, mast cells, and platelet.

Thymocyte development and selection

ADAP is required for efficient thymocyte development and selection. An inspection into the T-cell compartment in ADAP-deficient mice has identified modest reduction in splenic T cells and mildly decreased thymocyte number compared to the wild-type littermates. Nevertheless, the percentage of $CD4^+$ and $CD8^+$ T cells revealed no differences (Peterson et al., 2001). Moreover, while dispensable for the development of nonconventional thymocytes including NKT, CD88aa, and gamma/delta-TCR T cells, ADAP is required for optimal development of conventional alpha/beta-TCR T cells in the thymus (Dluzniewska et al., 2007). Whereas gross changes in thymic selection have not been observed, loss of ADAP affects both positive and negative selection of thymocytes in MHC class I- as well as class II-restricted TCR transgenic mice (Wu et al., 2006, Peterson et al., 2001).

$CD8^+$ T-cell functions

It has been well-acknowledged that ADAP plays a critical role in $CD4^+$ T cell functions, as T-cell activation, proliferation, and adhesion are severely compromised in CD4+ T cells from mice lacking ADAP (Parzmair et al., 2017, Mueller et al., 2007). However, only a few studies have addressed the roles of ADAP in $CD8^+$ T cells. $CD8^+$ T cells express a comparable level of ADAP compared to $CD4^+$ T cells (Parzmair et al., 2017). In naïve T cells, ADAP-deficient $CD8^+$ T cells displayed a decreased response to stimulatory antigen, but an enhanced response to weak agonist peptide ligands. In addition, uninfected ADAP-deficient mice exhibit more $CD8^+$ T cells with a memory phenotype due to an enhancement of the homeostatic cytokine IL-15 signalling (Fiege et al., 2015). A similar increase in the frequency of memory $CD8^+$ T cell precursors were observed in the secondary lymphoid organs from ADAP-deficient mice after a systemic infection with *Listeria monocytogenes* and stomatitis virus during the contraction phase (Fiege et al., 2016). Although loss of ADAP modestly affects activation and clonal expansion, whether it impinges on $CD8^+$ effector functions remained controversial. There are studies reported that the pathogen clearance and cytotoxic capacity of $CD8^+$ CTL cells was comparable in ADAP-deficient mice with that observed in WT animals in response to *Listeria monocytogenes* and viral infections, as well as to an allogeneic graft (Parzmair et al., 2017, Tian et al., 2010, Fiege et al., 2016). However, there is another study suggests that tumour-specific CTLs from ADAP-deficient mice exhibited enhanced cytolytic functions and anti-tumour immunity by suppressing the inhibitory receptor PD-1 expression in a DC vaccine-based tumour prevention and therapeutic mouse model (Li et al., 2015b). Of note, a study from the same

group discovered that infection of ADAP-deficient mice with a highly pathogenic avian influenza virus H5N1 caused enhanced mortality that was associated with increased numbers of $CD8^+$ T cells in the lung but reduced levels of TGF-β1, CD102 and VLA-1 expressions by these $CD8^+$ T cells. ADAP was found forming a complex with TRAF6 and TAK1 in $CD8^+$ T cells, and functions to increase autocrine TGF-β1 production by activated SMAD3 (Li et al., 2015a). Taken together, the seemingly divergent roles of ADAP in $CD8^+$ T-cell immunity may due to the tissue-specific differences in access to signals from different pathogens. Future work will be needed to further characterize these processes and define the mechanism by which ADAP regulates these tissue-specific $CD8^+$ T cells.

Macrophage and dendritic cell

Implications of ADAP in macrophage and dendritic cell are similarly strong as in T cells, e.g. cytoskeleton modulation, although the cell-specific signalling events that connect ADAP to the attributed functions are diverse and, sometimes controversial.

ADAP forms a large signalling complex with VASP, SLP-76, Nck and Wiskott-Aldrich syndrome protein (WASP) at actin rich sites upon triggering of the Fcgamma receptor during phagocytosis in macrophage. These complex converge to regulate actin polymerization and cytoskeleton remodeling that are essential for macrophage phagocytosis (Coppolino et al., 2001). In addition, ADAP N-terminal interacts with a C-terminally located SH3 domain of monomeric actin-binding protein 1 (mAbp1, also known as HPK1-interacting protein) in the leading edge of macrophage (Yuan et al., 2005b). These pieces of evidence that suggest a role of ADAP in actin polymerization are in contrast to those in T cells, where actin polymerization in T cells from ADAP-deficient mice was normal upon TCR stimulation (Griffiths et al., 2001, Peterson et al., 2001). Moreover, ADAP is found involved in the macrophage-mediated Yersinia infections. The Yersinia species is a pathogenic bacterial family that has evolved a virulence mechanism by which the tyrosine phosphatase YopH translocated into macrophage and cause disruption of focal complex structures and blockage of the phagocytic process via its dephosphorylating activity. Interestingly, ADAP is a substrate of YopH in macrophage. Mechanistically, a central part of YopH binds to ADAP C-terminus and dephosphorylates ADAP as well as SKAP-HOM, leading to reduced phagocytosis by affecting signalling for cytoskeletal rearrangement (Yuan et al., 2005a, Black et al., 2000, Deleuil et al., 2003, Hamid et al., 1999).

Dendritic cells are potent APCs that have specialized roles in initiating adaptive MHC-restricted, antigen-specific T-cell responses. While the extensive *in vivo* investigations have pointed a crucial role of ADAP in T cells forming conjugation with APCs, e.g. dendritic cells (Raab et al., 2010), ADAP is also required for optimal integrin-mediated dendritic cellular responses. Upon stimulation with CD11c integrin, ADAP-deficient dendritic cells showed significantly diminished production of proinflammatory cytokines IL-6, TNF-α, and IL-10, and enhanced actin polymerization, whereas the antigen uptake, adhesion, maturation, migration into the draining lymph nodes, antigen-specific T-cell activation, and proliferation remained normal in ADAP-deficient dendritic cells (Togni et al., 2012).

Platelet

Platelets are one of the major cellular components in blood that contribute to maintaining of the integrity of the cardiovascular system via haemostasis. Platelet dysfunction has linked to a wide range of pathological conditions, such as bleeding or thrombosis, and cancer. Emerging experimental and clinical evidences have identified a link between cancer spreading and platelet activation, which is pivotal to understand the hypercoagulable state found in most cancer patients (Jain et al., 2010, Nash et al., 2002). Platelet targeting therapy may be advantageous as adjuncts to current cancer immunotherapy (Wang et al., 2017).

Binding of von Willebrand factor (VWF) to platelet adhesion receptor GP Ib-IX-V is important for the initial capture of platelets to the extracellular matrix of injured blood vessel, and initiates activation of integrin αIIbβ3, which is crucial for platelet adhesion, aggregation and spreading (Kasirer-Friede et al., 2007, Kasirer-Friede et al., 2004). ADAP functions downstream of the VWF/GP Ib-IX-V signalling. Upon GP Ib IX-V stimulation, ADAP is subjected to src kinase-dependent tyrosine phosphorylation and facilitates the αIIbβ3 activation by presenting the cytoplasmic protein talin and kindling-3 to the β3 cytoplasmic tail. ADAP deficient platelets exhibited attenuated platelet aggregation, adhesion and spreading (Kasirer-Friede et al., 2014, Kasirer-Friede et al., 2004, Kasirer-Friede et al., 2007). In contrast to those in T cells, ADAP functions in a way that is independent of SKAP1 and RIAM, given that SKAP1 is not expressed in platelets, and that interactions of ADAP/talin/kindlin-3 do not involve RIAM in platelet (Kasirer-Friede et al., 2014). The *in vivo* consequences of ADAP deficiency were evidenced by that ADAP-deficient mice displayed increased rebleeding from tails wounds and abnormal thrombus formation after

carotid artery injury by chemicals (Kasirer-Friede et al., 2010). Moreover, ADAP is required for platelet responses to collagen-mediated inside-out signalling to α2β1 as well (Jarvis et al., 2012).

Implication of ADAP in animal disease models

Recent studies have described the implication of ADAP in animal disease models. ADAP-deficient mice developed milder clinical course of autoimmune encephalomyelitis and showed a strong reduction of all inflammatory leukocyte populations invading the central nervous system (Engelmann et al., 2013). It was further shown that loss of ADAP attenuated neutrophil recruitment in an ischemia–reperfusion-induced acute kidney injury model (Block et al., 2012). In those transplantation models, ADAP-deficient mice showed prolonged heart graft survival and a decrease in the infiltration, proliferation and activation of T cells in the allograft (Tian et al., 2007). Nevertheless, ADAP-deficient TCR transgenic mice also display lymphopenia that leads to enhanced autoimmune diabetes incidence (Zou et al., 2008).

Clinical Relevance

The integrin-mediated adhesion participates in the pathogenesis of various diseases, one of which is the disease leukocyte adhesion deficiency (LAD-1). Patients with LAD-1 present with recurrent bacterial infections, impaired pus formation, poor wound healing, and increased bleeding disorder tendency, which is caused by the dysfunction of the β2 and β3 integrin (van de Vijver et al., 2012, Hogg et al., 1999, Hogg et al., 2002). The importance of β2 integrin LFA-1 expression and activation is highlighted by this disease. The β2-subunit conserved domain harbours two mutations S138P and G273R in LAD-1 patients. The mutated S138P subunit was expressed but did not support function, whereas the G273R mutant was not expressed in these patients(Hogg et al., 1999).

The first clinical case of ADAP dysfunction was found in a familial thrombocytopenia with small size platelets in a highly consanguineous family from Northern Iraq. A homozygous pathogenic variant (loss of function) was presented in the FYB gene encoding ADAP, and this frameshift mutation was defined as the underlying causes of autosomal recessive thrombocytopenia in these patients (Hamamy et al., 2014). Coincidentally, an independent group from Israel also reported FYB gene dysfunction in congenital autosomal recessive small-platelet thrombocytopenia from a family of

consanguineous sibships of Arab Christian descent (Levin et al., 2015). A homozygous deleterious nonsense mutation c.393G>A was found in the FYB gene, which resulted in a compromised function of ADAP with all the known interaction and phosphorylation site located downstream from the mutation site.

Taken together, the links between integrin signalling and ADAP dysfunctions with human diseases have suggested critical roles played by ADAP and integrin in the disease development. A comprehensive understanding towards the mechanism of integrin-mediated signalling, as well as the many facets of ADAP regulations would give pivotal insight into the biological processes and the pathogenesis of diseases.

Conclusion

Studies of ADAP have suggested a versatile role of ADAP in the regulation of a wide variety of important signalling events and cell types, including the T-cell activation signalling/TCR signalling, and the integrin activation signalling in T cells, platelets and macrophages. Of note, substantial variations exist in each individual cell type and signalling pathway, e.g. triggering receptors, composition of signalling molecules, dynamic interactions with binding partners, etc. It is therefore not feasible to pinpoint one single and unified molecular machinery that is able to link ADAP with various signalling pathways in the context of diverse functions. Furthermore, the participations of ADAP in multiple, distinct signalling pathways are achieved by virtue of the multiple intermolecular binding domains within ADAP that enable its interactions with a changing repertoire of binding partners.

As named for its ability to promote adhesion, ADAP has been most-extensively studied in integrin-mediated adhesion. This work starts by addressing the potential interplay between ADAP and SUMOylation, a posttranslational modification by small ubiquitin-like modifiers (SUMO) proteins, in TCR-mediated integrin activation and T-cell adhesion (Chapter 2). SUMOylation is a key regulatory modification that has a particular effect in tuning the protein-protein interaction, protein subcellular localization, stability and activity in a vast range of proteins (Geiss-Friedlander and Melchior, 2007). The hypothesis is that the SUMO pathway participates in the regulation of integrin-mediated T-cell adhesion via a direct functional interplay with ADAP. The identification of SUMO pathway regulating integrin-mediated adhesion would provide a novel modulatory layer on this process. The other

research interest of the thesis is to unravel the implication of ADAP in other immune cell functions than those in T cells. Given that a mild thrombocytopenia as evidenced by the low platelet counts is seen in ADAP-deficient mice (Griffiths et al., 2001, Peterson et al., 2001) and megakaryocyte is the sole source of platelet in the body, the potential function of ADAP in megakaryopoiesis, a complex process involves the differentiation of megakaryocytes from haematopoietic stem cells, is explored and comprises Chapter 3. Finally, Chapter 4 offers a summary of this work's findings, as well as a discussion on its contribution to our current understanding towards ADAP regulations in the field.

Chapter 2 Regulation of ADAP on integrin activation and T-cell adhesion via SUMO E2 conjugase Ubc9[1]

ABSTRACT

While the immune adaptor ADAP acts as a key mediator of integrin inside-out signalling leading to T-cell adhesion, the regulation of this adaptor during integrin activation and T-cell adhesion remains unclear. We now identify Ubc9, the sole SUMO E2 conjugase, as an essential regulator of ADAP where it is required for Rap1 membrane recruitment and activation of the small GTPase Rac1 in T-cell adhesion. We show that Ubc9 interacted directly with ADAP *in vitro* and *in vivo*, and the association was increased in response to anti-CD3 stimulation. The Ubc9 binding domain on ADAP was mapped to a nuclear localisation sequence NLS (aa 674-700) within ADAP. Knockdown of Ubc9 by shRNA or expression of the Ubc9-binding-deficient ADAP mutant significantly decreased TCR-induced integrin adhesion to ICAM-1 and fibronectin, as well as LFA-1 clustering, while having little effect on the TCR proximal signalling responses and TCR-induced IL-2 transcription. Furthermore, downregulation of Ubc9 impaired TCR-mediated Rac1 activation and attenuated the membrane targeting of Rap1 but not RIAM. Taken together, our data demonstrate for the first time that Ubc9 acts as a functional binding partner of ADAP and plays a selective role in integrin-mediated T-cell adhesion via modulation of Rap1 membrane recruitment and Rac1 activation.

[1] Most of the data presented in Chapter 2 has been published in the following form: XIONG, Y., YE, C., YANG, N., LI, M. & LIU, H. 2017. Ubc9 Binds to ADAP and Is Required for Rap1 Membrane Recruitment, Rac1 Activation, and Integrin-Mediated T Cell Adhesion. *J Immunol,* Volume 199, Number 12.

INTRODUCTION

T-cell adhesion is mediated by integrins on the surface of T cells binding to integrin ligands on APCs or the endothelium. T-cell adhesion is essential for T-cell functions; it permits T cells to transmigrate across endothelium and enter into inflamed tissue to form immunological synapses with APCs and carry out immune functions including cytokine production and T-cell cytotoxicity. T-cell adhesion also enables the T cell to remain bound to the APC long enough for the T cell to become activated, increase TCR sensitivity to the MHC, and lower the threshold for T-cell activation (Billadeau et al., 2007, Wang and Rudd, 2008, Hogg et al., 2011). Regulation of T-cell adhesion is achieved by controlling the activity of integrins on the cell surface.

The major integrin receptors on T cells that mediate T-cell adhesion are β2-integrins of LFA-1. LFA-1 is a heterodimeric transmembrane receptor consisting of a unique α subunit (αL; CD11a) and a β2 subunit (CD18), which are receptors for ICAMs on APCs (Billadeau et al., 2007). In addition, VLA-4 of the β1-integrin family is another group of integrin receptors for T-cell adhesion. VLA-4 is composed of an α4 subunit (CD49d) and a β1 subunit (CD29) and its ligands are fibronectin or VCAM-1 on APCs (Chen and Zhu, 2013). Integrins need to be converted to an active state to bind their ligands. A conformational change is induced that augments affinity for integrin ligand followed by clustering of these receptors on the surface of T cells, which enhances avidity for ligand binding (Kinashi, 2005). The molecular events leading to integrin activation have collectively been termed “inside-out” signalling (Hogg et al., 2004), which involves an array of signalling molecules including the adaptor proteins SLP-76, ADAP, and SKAP1 (Wang et al., 2003, Griffiths et al., 2001, Burbach et al., 2007, Kliche et al., 2006, Horn et al., 2009). These adaptor proteins lack enzymatic activity, but instead, carry binding modules or sites for the assembly of supra-molecular complexes that are critical for the “inside-out” adhesion pathway (Wang and Rudd, 2008).

ADAP, a key mediator in the “inside-out” signalling of T cells, contains domains for scaffolding molecules of various classes including a proline-rich region for binding to SKAP1, tyrosines (Y) in the two YDDV sites that can be phosphorylated and mediate SLP-76 and Fyn binding, an E/K-rich region for binding to CARMA1, two putative nuclear localization sequences (NLSs), and an FPPPP sequence for binding the EVH1 domain of Ena/VASP (Wang and Rudd, 2008, Medeiros et al., 2007, da Silva et al., 1997a, Marie-Cardine et al., 1998a). The scaffolding function of ADAP for effectors SKAP1 and SLP-76 is necessary to support TCR-

dependent integrin-mediated adhesion (Wang et al., 2004, Menasche et al., 2007). Abrogating the interaction of ADAP with SKAP1 or SLP-76 reduces TCR-mediated integrin activation and adhesion, as well as impairs immunological synapse formation and T cell-APC conjugation (Wang et al., 2004, Baker et al., 2009, Menasche et al., 2007, Peterson et al., 2001).

ADAP constitutively associates with and stabilizes SKAP1 (Huang et al., 2005). The downstream effector molecules of the ADAP-SKAP1 module include the small GTPase Rap1 and its effector proteins RapL and RIAM in the TCR-mediated regulation of integrin function and T-cell adhesion (Kinashi, 2005). T-cell activation induces the formation of a complex of activated Rap1 and RIAM, which is brought to the plasma membrane in close proximity to the β-chain of LFA-1, and is required to mediate binding of LFA-1 to talin, thereafter facilitating the activation of LFA-1(Kliche et al., 2012). The ADAP-SKAP1 module is recruited to the plasma membrane following TCR activation (Menasche et al., 2007), which is required for the plasma membrane recruitment of Rap1 and RIAM in TCR-mediated T-cell adhesion. T cells from $ADAP^{-/-}$ mice exhibited deficient TCR-mediated upregulation of adhesion (Peterson et al., 2001, Griffiths et al., 2001). In addition to its central role in TCR-mediated adhesion, ADAP also participates in the signalling pathways downstream of the TCR that result in T-cell activation (Griffiths et al., 2001, Peterson et al., 2001). ADAP-deficient T cells displayed reduced IL-2 production and cell proliferation (Griffiths et al., 2001, Peterson et al., 2001).

Ubc9, the sole E2 conjugating enzyme of the SUMOylation cycle, mediates the transfer of SUMO to the target proteins (Johnson and Blobel, 1997). Ubc9-mediated SUMOylation has been implicated in many processes, such as DNA replication/repair, cell division, movement, nuclear transport, and transcription (Flotho and Melchior, 2013, Gareau and Lima, 2010). In addition, Ubc9 functions as a cellular chaperone and transcriptional co-regulator, which are SUMOylation-independent (Kurtzman and Schechter, 2001, Liu et al., 2007, Kurihara et al., 2005). To date, a link between SUMOylation and T-cell adhesion has remained unclear. In the present study, we determined that Ubc9 has a role in TCR-mediated integrin activation and T-cell adhesion. We show that Ubc9 downregulation impaired the TCR-mediated integrin adhesion and LFA-1 clustering, whilst the CD3-induced IL-2 transcription was not affected. Mechanistically, Ubc9 interacted directly with ADAP and the Ubc9-ADAP interaction is essential for plasma membrane recruitment of Rap1 and Rac1 activation upon TCR stimulation. Our data have for the first time identified that the SUMO E2 conjugase Ubc9 regulates integrin-mediated T-cell adhesion through an interaction with the immune adaptor ADAP.

RESULTS

Knockdown of Ubc9 impairs integrin-mediated T-cell adhesion and LFA-1 clustering

While the post-transcriptional modification with SUMO, namely SUMOylation, is one of the important mechanisms that regulate a series of key cellular processes, a role for SUMOylation in T-cell adhesion has remained unclear. Ubc9 is the sole SUMO E2 conjugase, and thus depletion of Ubc9 leads to the switching off of SUMO pathway activity (He et al., 2015, Lin et al., 2003, Nacerddine et al., 2005). The binding of integrin molecules on the surface of T cells to ICAM-1 (e.g. β2 integrin LFA-1) and fibronectin (e.g. β1 integrin VLA-4) facilitates T-cell adhesion and enables T cells to form firm conjugates with APCs (Hogg et al., 2004).To examine the functional role of the SUMO pathway in TCR-mediated T-cell adhesion, we initially assessed the effect of Ubc9 knockdown on β1- and β2-integrin mediated T-cell adhesion and LFA-1 clustering. Stable Ubc9-knockdown Jurkat T cells and control shEGFP Jurkat T cells were generated using lentiviral delivery of shRNA expression vectors for the expressions of two individual sequence-specific shRNAs against Ubc9 and shRNA against EGFP, respectively. Western blotting analysis confirmed both shRNAs targeted against Ubc9 showed efficient knockdown (80%) of Ubc9 in stable cells (**right panels in Fig. 2.1, A and B**). The *in vitro* ICAM-1 binding assays were performed by plating Ubc9-knockdown cells or control shEGFP cells on ICAM-1- or fibronectin-coated plates. Adhesion was analysed by counting the bound T cells on the plate as previously described (Wang et al., 2007). Stimulation with anti-CD3 increased the number of control shEGFP Jurkat T cells bound to ICAM-1-coated plates and fibronectin-coated plates by 5.7- and 1.8-fold, respectively (**Fig. 2.1, A and B, left panels, shEGFP, anti-CD3 vs. unstimulated**). By contrast, Ubc9-knockdown cells displayed a substantially attenuated binding to ICAM-1-coated plates upon anti-CD3 stimulation (**Fig. 2.1A, left panel, shUbc9#1 and shUbc9#2 vs. shEGFP**). Similar effects were observed for stable expressions of shRNAs targeted to different sites of Ubc9. Meantime, a reduction in the number of cells binding to fibronectin-coated plate was shown in the Ubc9-knockdown cells as well (**Fig. 2.1B, left panel)**. Further, similar results were also found using mouse primary T cells. Ubc9 was knocked-down in primary T cells by lentiviral-shRNA infections. While the primary T cells infected with shEGFP showed a 2 - 3-fold increase in adhesion to ICAM-1- or fibronectin-coated plates after anti-CD3 stimulation (**Fig. 2.1, C and D, shEGFP, anti-CD3 vs. unstimulated**), a marked decrease in adhesion to both ICAM-1- and fibronectin-coated plates was apparent in shUbc9#2-infected cells upon anti-CD3 stimulation (**Fig. 2.1, C and D, shUbc9#2 vs. shEGFP**).

Integrin, especially LFA-1, forms clusters or accumulates at one side of the cell to increase its avidity for ICAM-1 binding (van Kooyk and Figdor, 2000). Thus, we examined whether the impaired anti-CD3-induced adhesion of Ubc9-knockdown T cells to ICAM-1 and fibronectin was also accompanied by a reduction in LFA-1 clustering on the surface of T cells. In the control shEGFP Jurkat T cells, anti-CD3 stimulation resulted in a 4-fold increase in the number of cells with LFA-1 clusters (**Fig. 2.1E, shEGFP, anti-CD3 vs. unstimulated, from 5% to 20%**), while fewer Ubc9-knockdown cells displayed clustering after parallel stimulation with anti-CD3 (**Fig. 2.1E, shUbc9#2, anti-CD3 vs. unstimulated, from 4% to 10%**). Examples of cells with LFA-1 clustering were shown in the left panel of **Fig. 2.1E**. Therefore, these data indicated that Ubc9 is needed for TCR-induced integrin mediated T-cell adhesion and LFA-1 clustering.

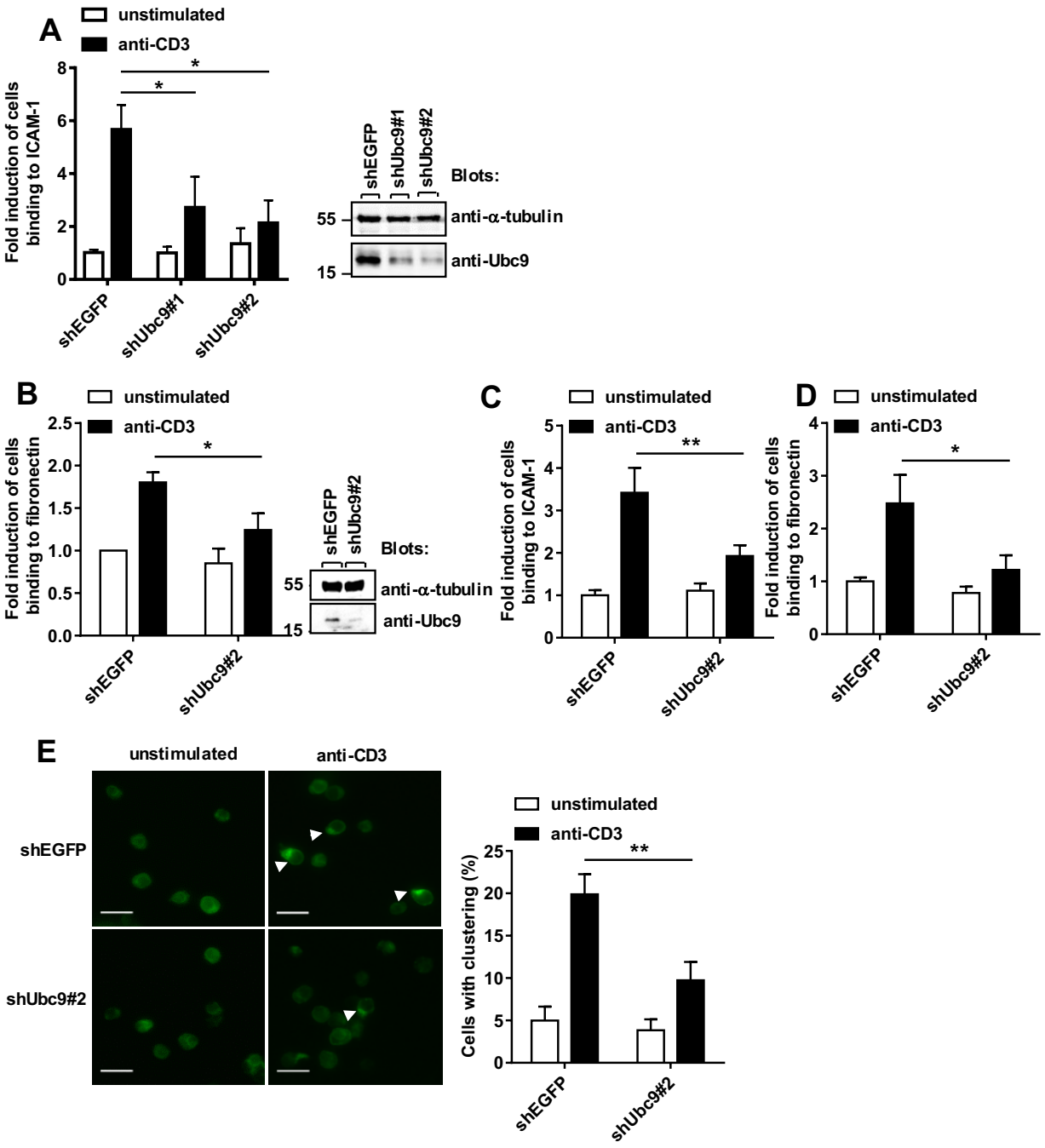


FIGURE 2.1. Knockdown of Ubc9 impairs integrin-mediated T-cell adhesion and LFA-1 clustering.

(A-B) Stable Ubc9-knockdown Jurkat T cells (shUbc9#1 and shUbc9#2) or control shEGFP Jurkat T cells (shEGFP) were either left untreated or stimulated with anti-CD3, followed by the measurement of cell adhesion to ICAM-1-coated plates (A) and fibronectin-coated plates (B) as described in Materials and Methods. Error bars indicate the s.e. from three individual experiments for the ICAM-1 binding assay and four individual experiments for the fibronectin binding assay. Immunoblots on the right panels show anti-Ubc9 western blot of whole-cell lysates of cells used in the ICAM-1/fibronectin binding assays. (C-D) Murine primary T cells infected with lentivirally-encoded shEGFP or shUbc9#2 were stimulated with

anti-CD3 and assessed for adhesion to ICAM-1-coated plates (C) and fibronectin-coated plates (D). Error bars indicate the s.e. from three individual experiments. (E) Left panel: Stable Ubc9-knockdown and control shEGFP Jurkat T cells were stimulated with anti-CD3 for 30 min and imaged for LFA-1 clustering as described in Materials and Methods. Arrow designates LFA-1 cluster, and the scale bars represent 20 μm. Right panel: Histogram showing the percentage of T cells with LFA-1 clustering. Error bars indicate the s.e. from four individual experiments.

Ubc9 binds directly to the immune adaptor ADAP but not SKAP1 *in vivo* and *in vitro*

Cytosolic adaptor proteins ADAP and SKAP1 have been implicated in integrin-mediated T-cell adhesion and identified as crucial mediators for TCR-mediated inside-out signalling (Wang et al., 2003, Wang et al., 2004, Wang et al., 2007). Since we showed here that Ubc9 is required for integrin-mediated T-cell adhesion, we hypothesized that there may be a functional interaction between Ubc9 and the adhesion-related adaptors ADAP and SKAP1. To test this, we performed co-immunoprecipitation experiments in 2B4 mouse T-cell hybridoma cells with or without anti-CD3 stimulation. Western blot analysis of immunoprecipitates with anti-Ubc9 antibodies revealed that Ubc9, at approximately 18-kDa, was co-immunoprecipitated with ADAP (**Fig. 2.2A, lanes 4 and 5**) and with SKAP1 (**Fig. 2.2A, lanes 6 and 7**). However, in comparison, the amount of Ubc9 was more abundant in the anti-ADAP precipitates than in the anti-SKAP1 precipitates (**lanes 4 and 5 vs. lanes 6 and 7 in Fig. 2.2A**). As a positive control (Swaminathan et al., 2004), Ubc9 was readily detected in the anti-RanGAP1 immunoprecipitates (**Fig. 2.2A, lane 8**). Importantly, anti-CD3 stimulation significantly increased the level of Ubc9 in the precipitations (**lanes 5 vs. 4 and lanes 7 vs. 6**).

The effect of anti-CD3 stimulation on the binding of Ubc9 to ADAP was also assessed using 2B4 cells stimulated with anti-CD3 over a time course of 30 mins. Endogenous ADAP was immunoprecipitated with anti-ADAP antibody and assayed for interaction with Ubc9 by immunoblotting with antibodies against ADAP and Ubc9. Anti-CD3 stimulation induced the co-precipitation of Ubc9 with ADAP, which peaked at 10 min post anti-CD3 stimulation (**Fig. 2.2B**). Interestingly, the binding of Ubc9 to ADAP declined at 15 min post anti-CD3 stimulation (**Fig. 2.2B, lane 4 vs. lane 3**). This data indicate that Ubc9 binds to the immune adaptors ADAP and SKAP1, and anti-CD3 stimulation promotes their interaction in T cells.

To determine whether the binding of Ubc9 to ADAP and/or SKAP1 is direct or indirect, we carried out immunoprecipitation assays in HEK 293T cells in which endogenous ADAP and SKAP1 are both absent. Ubc9 was co-expressed with either HA-tagged ADAP or SKAP1

proteins in HEK 293T cells, followed by anti-HA precipitation and anti-Ubc9 blotting. Ubc9 was readily co-precipitated with HA-tagged ADAP (**Fig. 2.2C, lanes 3**). The presence of Ubc9 in anti-ADAP immunoprecipitates was further confirmed by LC-MS/MS analysis. Coomassie brilliant blue staining identified proteins that were coprecipitated by anti-ADAP. Six peptides corresponding to Ubc9 were detected (**Fig. 2.2D**). However, Ubc9 failed to co-immunoprecipitate with HA-tagged SKAP1 in HEK 293T cells (**Fig. 2.2C, lane 4**), suggesting that the co-immunoprecipitation of Ubc9 with SKAP1 in Jurkat T cells was indirect (**Fig. 2.2A, lane 6-7**) and likely mediated via ADAP. Thus, we concluded that Ubc9 interacts directly with ADAP but not SKAP1 in the ADAP-SKAP1 module.

Next, we asked which site/region in ADAP contributes to its interaction with Ubc9. ADAP consists of a proline-rich domain, multiple tyrosine sites, two SH3 domains, an EVHI domain binding site, and two putative NLSs (Wang and Rudd, 2008). We first constructed different HA-tagged truncated deletions of ADAP (**Fig. 2.2E**). Ubc9 was then co-expressed with either HA-tagged ADAP WT or its deletion mutants in HEK 293T cells, followed by anti-HA immunoprecipitation and immunoblotting with anti-Ubc9. WT ADAP co-precipitated with Ubc9 (**Fig. 2.2F, lane 2**). Deletion of NLS1 (aa 469 to 505) resulted in an approximate 50% decrease in binding of Ubc9 compared to WT ADAP (**lane 3 vs. lane 2**). By contract, deletion of NLS2 (aa 674 to 700) abolished the binding of Ubc9 with ADAP (**lane 4 vs. lane 2-3**). Similar amounts of HA-tagged ADAP WT and mutants expressions were confirmed by anti-ADAP blotting (**lower panel**). These data indicated that the NLS2 of ADAP was essential for the binding of ADAP with Ubc9.

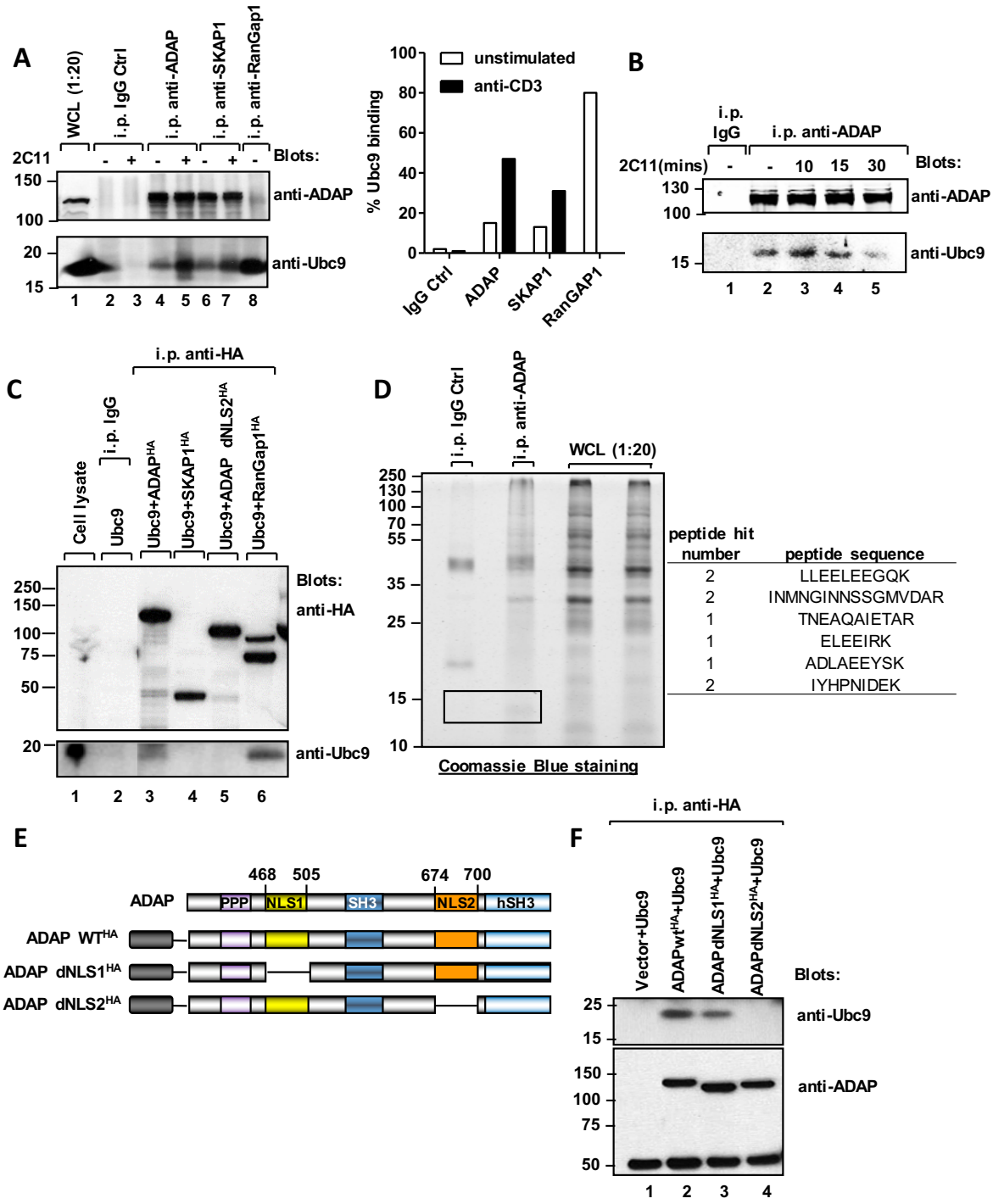


FIGURE 2.2. Ubc9 binds directly to the immune adaptor ADAP but not SKAP1 *in vivo* and *in vitro*.

(A) Left panel: Cell lysates of resting and anti-CD3 (2C11) stimulated 2B4 cells were prepared for immunoprecipitation with either IgG control, anti-ADAP, anti-SKAP1, or anti-RanGAP1 followed by blotting with anti-ADAP (top) and anti-Ubc9 antibody (bottom). Right panel: Histogram of relative Ubc9 binding based on densitometric readings. (B) Time course of Ubc9 binding to ADAP following anti-CD3 stimulation. 2B4 cells were stimulated with anti-CD3 for the indicated time periods. Endogenous ADAP was immunoprecipitated with anti-ADAP antibody and assayed for interaction with Ubc9 by immunoblotting with

antibodies against ADAP or Ubc9. (C) HEK293 T cells were transfected with Ubc9 alone, or together with either HA-ADAP, HA-SKAP1, HA-ADAP dNLS2, or HA-RanGAP1 constructs. Immunoprecipitation was performed using anti-HA antibody, followed by blotting with anti-HA (top) or anti-Ubc9 antibody (bottom). (D) Detection of Ubc9 in anti-ADAP immunoprecipitates using mass spectrometry analysis. Left panel: Coomassie Brilliant Blue staining analysis of anti-CD3 stimulated Jurkat T cell lysate prepared for immunoprecipitation with IgG control and anti-ADAP. Bands indicated with rectangle were excised and subjected to LC-MS/MS analysis as described in Materials and Methods. Right panel: List of detected peptides assigned to Ubc9 and their abundance. (E) Schematic drawings of ADAP WT and NLS1deletion mutant (ADAP dNLS1), and NLS2 deletion mutant (ADAP dNLS2) constructs. (F) HEK 293T cells were co-transfected with Ubc9 and vector control, ADAP WT, ADAP dNLS1, or ADAP dNLS2, followed by precipitation with anti-HA antibody and immunoblotting with anti-HA (upper) and anti-ADAP antibody (lower).

ADAP-Ubc9 binding fails to affect TCR proximal signalling events and TCR-induced IL-2 transcription

TCR engagement with APCs is rapidly followed by tyrosine phosphorylation of downstream substrates including receptors, kinases, and adaptor proteins, which is critical for signal initiation and integrin inside-out signalling (Balagopalan et al., 2009, Raab et al., 2010). To assess whether the attenuation of TCR-induced T-cell adhesion in Ubc9 knockdown cells was a result of impaired TCR signalling, we compared the level of CD3-mediated tyrosine phosphorylation between control shEGFP T cells and Ubc9-knockdown Jurkat T cells (**Fig. 2.3A**). The total tyrosine phosphorylation level of Ubc9-knockdown cells and control shEGFP cells was examined by blotting with the anti-phosphotyrosine antibody 4G10. Both control shEGFP and Ubc9-knockdown T cells responded effectively to anti-CD3 stimulation as indicated by a significant increase in the overall level of tyrosine phosphorylation upon anti-CD3 stimulation (**Fig. 2.3A, lane 2 vs. 1 for control shEGFP T cells and lane 4 vs. 3 for Ubc9-knockdown cells**). In addition, stimulation of Ubc9-knockdown T cells with anti-CD3 revealed no significant difference in total tyrosine phosphorylation as compared to control shEGFP T cells (**Fig. 2.3A, lane 3-4 vs. lane 1-2**). As a control, the expression level of Ubc9 in Ubc9-knockdown cells was confirmed to be significantly reduced as shown by the anti-Ubc9 blot. We next examined the effect of Ubc9 knockdown on IL-2 transcription, a long term parameter of T-cell activation. Jurkat T cells were co-transfected with either shEGFP or shUbc9#2 constructs in combination with IL-2 promoter-driven luciferase reporter. The luciferase assay demonstrated that stimulation with anti-CD3 induced significant enhancement in IL-2 promoter activity. However, shEGFP-transfected cells and shUBC9#2-transfected cells

showed a similar level of increase in IL-2 promoter activity (**Fig. 2.3B, shEGFP vs. shUBC9#2, 9-fold vs. 7-fold)**.

In addition to its central role in TCR-mediated adhesion, ADAP participates in the signalling pathways downstream of the TCR that result in T-cell activation (Griffiths et al., 2001, Peterson et al., 2001). Next, it was important to assess whether Ubc9-ADAP binding is required for the proper functions of ADAP in TCR proximal responses including the formation of signalling complex of ADAP with its binding partners such as SKAP1 and SLP-76, as well as TCR induced IL-2 transcription in T cells. SKAP1 or SLP-76 were co-expressed with either ADAP, ADAP mutant dNLS1, or the Ubc9 binding-deficient ADAP mutant dNLS2 in Jurkat T cells or COS-7 cells followed by co-immunoprecipitation with anti-SKAP1 or anti-SLP-76 and blotting with anti-ADAP. ADAP dNLS2 bound similar amounts of SKAP1 (**Fig. 2.3C, left panel, lane 3 vs. lane 1-2**) and SLP-76 (**Fig. 2.3C, right panel, lane 3 vs. lane 1-2**) compared to WT ADAP and the ADAP dNLS1 mutant.

ADAP positively regulates the signalling downstream of the TCR, and its phosphorylation at both the Tyr595 and Tyr651 sites is essential for IL-2 production related to T-cell activation (Geng et al., 1999, Wang et al., 2004). To determine whether Ubc9-ADAP binding is required for ADAP-mediated TCR signalling events, we next examined if the Ubc9-ADAP interaction is required for TCR-induced IL-2 transcription. Jurkat T cells were co-transfected with an IL-2 promoter-driven luciferase reporter together with either WT ADAP or ADAP mutants, including the Ubc9 binding deficient ADAP mutant dNLS2. The luciferase assay revealed that in the absence of CD3 stimulation, the luciferase activity was minimal. In the presence of CD3 stimulation, a significant 7-fold increase in luciferase activity was observed (**Fig. 2.3D, vector, anti-CD3 vs. unstimulated**). Transfection with the ADAP-WT expression construct caused a further 2-fold increase in the luciferase activity when anti-CD3 stimulation was present (**Fig. 2.3D, ADAP WT vs. vector**). Surprisingly, luciferase activity in cells transfected with the Ubc9 binding-deficient mutant dNLS2, or the other mutant dNLS1, showed levels of TCR-mediated IL-2 transcription comparable to those of cells transfected with WT ADAP (**Fig. 2.3D, ADAP dNLS2 and dNLS1 vs. ADAP WT**). To assess whether deletion of Ubc9-ADAP binding site affects the subcellular distribution of ADAP following T cell activation, we next transfected Jurkat T cells with HA-tagged ADAP WT or truncation mutants, stimulated the cells with ionomycin plus phorbol 12-myristate 13-acetate (PMA) followed by visualisation with immunofluorescence microscopy using anti-HA antibodies. The staining pattern revealed a distinct localisation of ADAP proteins throughout the cytoplasm,

whereas cells expressed ADAP dNLS1 and ADAP dNLS2 displayed no difference in the subcellular distribution **(Fig. 2.3E)**.

Together, these data suggest that the Ubc9-ADAP interaction is not required for TCR proximal signalling responses as well as for the positive regulatory effects of ADAP on TCR induced IL-2 transcription.

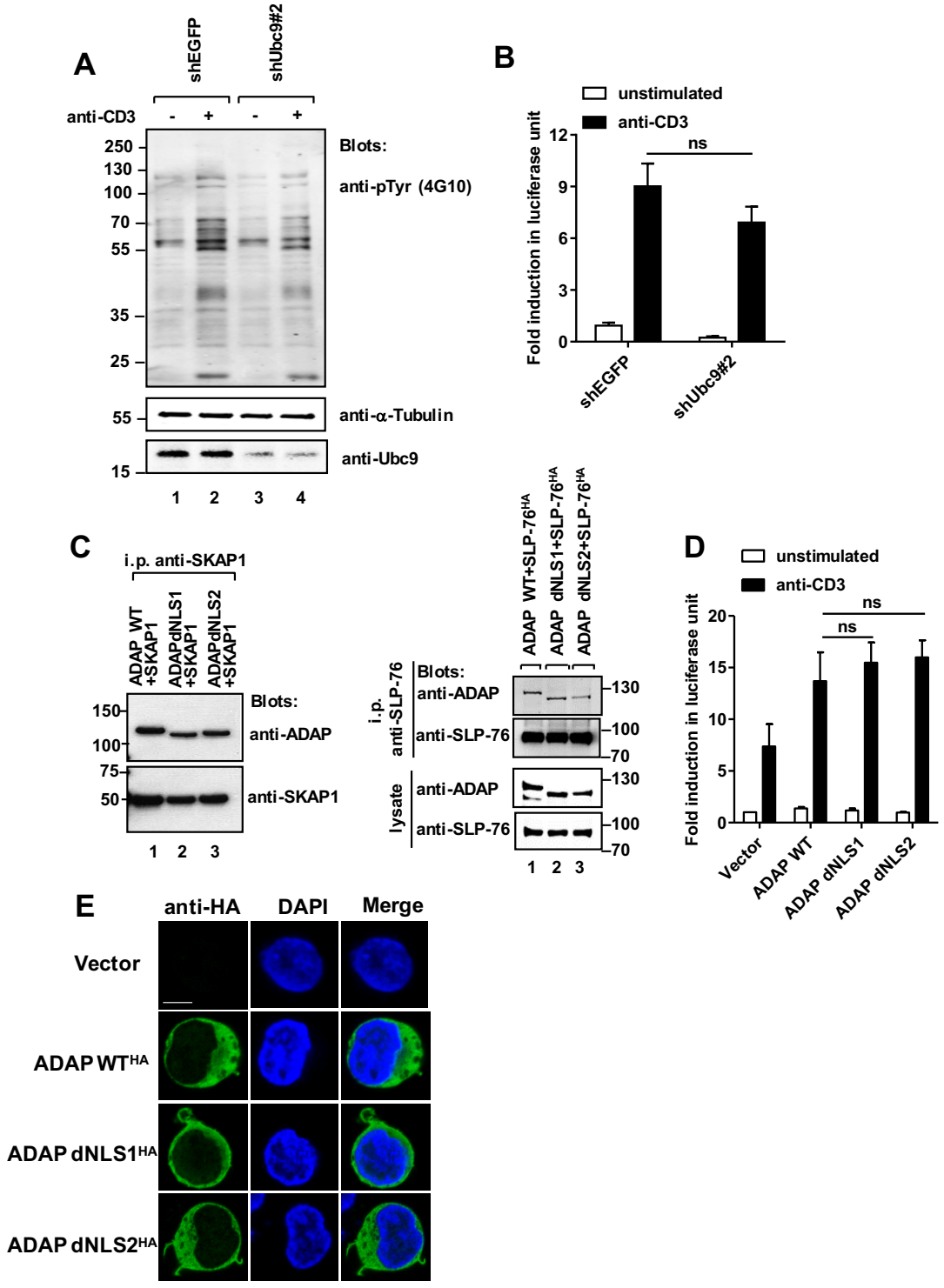


FIGURE 2.3. ADAP-Ubc9 binding fails to affect TCR proximal signalling events and TCR-induced IL-2 transcription.

(A) Stable Ubc9-knockdown and control shEGFP Jurkat T cells were either left untreated or stimulated with anti-CD3 (OKT3) for 3 min. The cell lysates were extracted and subjected to western blotting with antibodies against phosphotyrosine, α-tubulin, and Ubc9. (B) Jurkat T cells were co-transfected with IL-2 promoter-driven luciferase reporter with either shEGFP or shUbc9#2 constructs and were stimulated with anti-CD3 for 6h, followed by a measurement of luciferase activity as indicated in Materials and Methods. Error bars indicate the s.e. from three individual experiments. (C) Left panel: Jurkat T cells were co-transfected with SKAP1 and either ADAP WT, ADAP dNLS1, or ADAP dNLS2 constructs, followed by immunoprecipitation with anti-SKAP1 antibody and blotting with anti-ADAP and anti-SKAP1. Right panel: COS-7 cells were co-transfected with SLP-76 and either ADAP WT, ADAP dNLS1, or ADAP dNLS2 constructs, followed by immunoprecipitation with anti-SLP-76 antibody and immunoblotting with anti-ADAP and anti-SLP-76. (D) Jurkat T cells were co-transfected with IL-2 promoter driven luciferase reporter and either vector, ADAP WT, ADAP dNLS1, or ADAP dNLS2 constructs, stimulated with anti-CD3 for 6 h followed by luciferase activity analysis as described in Materials and Methods. Error bars indicate the s.e. from three individual experiments. (E) HA-tagged ADAP WT and deletion mutants were expressed in Jurkat T cells and stimulated with ionomycin plus PMA for 30 mins. Confocal images of the cells showed subcellular localisations of ADAP WT and deletion mutants (green) by immunofluorescence microscopy using anti-HA antibody. Scale bar represents 5 μm.

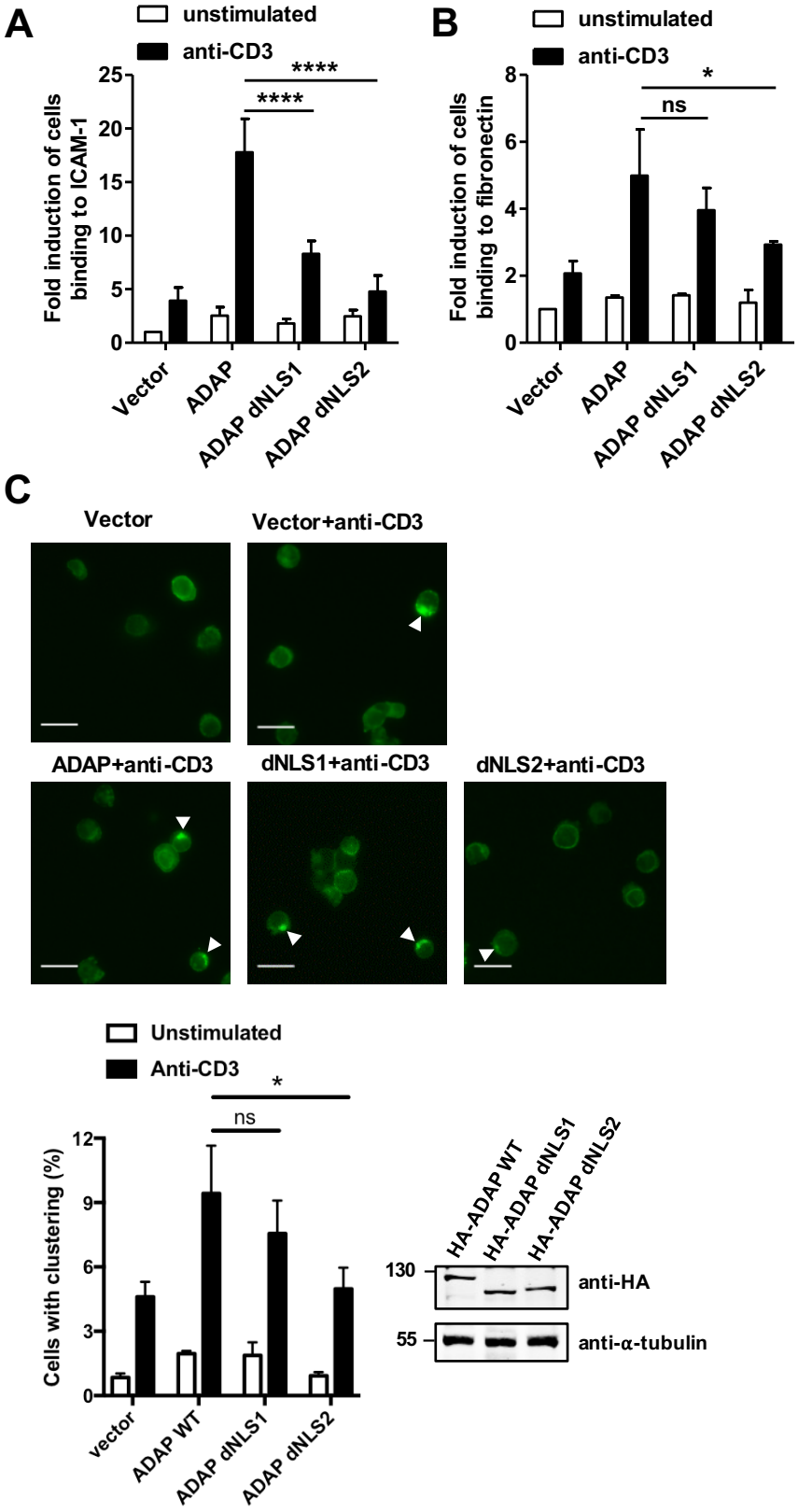
The Ubc9-binding domain of ADAP is required for integrin adhesion and clustering

ADAP plays an important role in the adhesion of integrin to ICAM-1 and fibronectin in T cells (Wang et al., 2004, Griffiths et al., 2001). Given that TCR stimulation increased the association between ADAP and Ubc9 (**Fig. 2.2A, lanes 5 vs. 4**), we next assessed whether the Ubc9-ADAP module is essential for TCR-mediated T-cell adhesion by an *in vitro* ICAM-1 binding assay in Jurkat T cells. Consistent with previous reports, over-expression of ADAP enhanced T-cell adhesion to ICAM-1-coated plates, which was further increased approximately 5-fold upon TCR activation (**Fig. 2.4A, ADAP vs. Vector**). Interestingly, this profound potentiation of T-cell adhesion induced by anti-CD3 stimulation was diminished sharply by 70% in cells expressing the Ubc9 binding deficient mutant ADAP dNLS2 (**Fig. 2.4A, ADAP dNLS2 vs. ADAP**). In addition, similarly impaired anti-CD3-induced adhesion to fibronectin, another ligand of β1 integrins, was observed in ADAP dNLS2-transfected Jurkat T cells compared to ADAP WT-transfected Jurkat T cells (**Fig. 2.4B, ADAP dNLS2 vs. ADAP**).

Since knockdown of Ubc9 caused impaired LFA-1 clustering in response to anti-CD3 stimulation, we next assessed if expression of ADAP dNLS2 has a similar effect on this event. Cells were considered to have clustered LFA-1 if the staining pattern showed LFA-1 polarized to one side of the cell. In vector-transfected Jurkat T cells, clustered LFA-1 (arrows point) was

observed and anti-CD3 stimulation increased the percentage of cells with clustering from a basal 1% to 4% (**Fig. 2.4C, upper panel**). Transfection of ADAP or ADAP dNLS1 further increased the percentages of cells with clustering by two-fold to about 9%. In contrast, expression of the Ubc9 binding-deficient mutant ADAP dNLS2 failed to further augment LFA-1 clustering, with percentages comparable to that in the vector control cells (**Fig. 2.4C, lower left panel**). Expressions of the transfected ADAP WT and mutant proteins were confirmed by immunoblotting with anti-ADAP (**Fig. 2.4C, lower right panel**). These data indicated that dissociation of Ubc9 from ADAP impairs TCR-induced integrin adhesion and clustering of T cells.

Thus, either knockdown of Ubc9 or expression of the Ubc9 binding-deficient ADAP mutant led to impaired integrin-mediated T-cell adhesion and LFA-1 clustering, indicating that ADAP-Ubc9 interaction is required for T-cell adhesion.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ADAP dNLS1
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dNLS2+anti-CD3
Unstimulated
Anti-CD3
Cells with clustering (%)
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9
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3
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*
ns
vector
ADAP WT
ADAP dNLS1
ADAP dNLS2
HA-ADAP WT
HA-ADAP dNLS1
HA-ADAP dNLS2
130
anti-HA
55
anti-α-tubulin

FIGURE 2.4. The Ubc9-binding domain of ADAP is required for integrin adhesion and clustering.

(A-B) Jurkat T cells transfected with either the empty vector, ADAP WT, ADAP dNLS1, or ADAP dNLS2 construct were either left unstimulated or stimulated with anti-CD3 for 30 min, followed by an *in vitro* assay for adhesion to either ICAM-1-coated plates (A) or fibronectin-coated plates (B). Error bars indicate the s.e. from four individual experiments for the ICAM-1 binding assay, and s.e. from three individual experiments for fibronectin-binding assay. (C) Upper left panel: Representative microscopy images of Jurkat T cells transfected with either empty vector, ADAP WT, ADAP dNLS1, or ADAP dNLS2 construct. Cells were stimulated with anti-CD3 for 30 min, fixed and stained with anti-CD11a and Alexa Fluor 488-conjugated anti-mouse antibodies. Arrow designates LFA-1 clustering, and the scale bars represent 20 μm. Lower left panel: Histogram showing the percentage of T cells with LFA-1 clustering. Error bars indicate the s.e. from four individual experiments. Right panel: Anti-ADAP western blot analysis of cells transfected with either ADAP WT, ADAP dNLS1, or ADAP dNLS2 construct.

Effects of Ubc9 knockdown on the membrane localisation of Rap1-RIAM and Rac1 activation in response to anti-CD3 stimulation

Rap1 and its binding protein RIAM are critical components of β2 integrin activation downstream of the TCR-ADAP-SKAP1 axis, and ADAP-mediated plasma membrane recruitment of Rap1 and RIAM following TCR activation is required for LFA-1 activation (Menasche et al., 2007, Kinashi, 2005). Thus, we investigated whether Ubc9 is required for the TCR-induced plasma membrane translocation or recruitment of Rap1 and RIAM. The membrane fractions extracted from control shEGFP Jurkat T cells or Ubc9-knockdown T cells with or without anti-CD3 stimulation were subjected to western blot analysis for the presence of Rap1 and RIAM. As shown in **Fig. 2.5A**, TCR stimulation substantially increased the plasma membrane recruitments of Rap1 in control shEGFP Jurkat T cells (**Fig. 2.5A, shEGFP, lane 6 vs. lane 5**). In contrast, the anti-CD3 induced membrane targeting of Rap1 was significantly reduced in the Ubc9-knockdown T cells. However, the membrane recruitment of RIAM is unaffected (**Fig. 2.5A, lane 8 vs. lane 6**).

Additionally, double immunofluorescence with Rap1 and LFA-1 was also performed to assess the effect of downregulation of Ubc9 on the plasma membrane translocation of Rap1 and LFA-1 clustering in response to anti-CD3 stimulation. The level of Rap1 localised on the plasma membrane was analysed by quantifying the Rap1 signal intensity of the plasma membrane area compared to the whole cell. As shown in **Fig. 2.5B**, in the absence of anti-CD3 stimulation, Rap1 distributed diffusely over the cells in both control shEGFP (**panel a**) and

Ubc9-knockdown T cells (**panel c**). In the control shEGFP cells, anti-CD3 stimulation led to an increase in the level of Rap1 at the plasma membrane, and the Rap1 at the plasma membrane was co-localised with clustered LFA-1 (**indicated by the white arrows in panel b**). In contrast, the level of anti-CD3-induced increases in the translocation of Rap1 on plasma membrane was significantly decreased in the Ubc9-knockdown cells, accompanied by a reduction in LFA-1 cluster formation (**panel d; shUbc9#2, anti-CD3 vs. unstimulated in panel e**).

Rac1 belongs to the Rho subgroup of a family of small GTPases, and the active GTP-bound Rac1 is critical for α4β1 (VLA4)-mediated T-cell adhesion to fibronectin (Dios-Esponera et al., 2015, Faroudi et al., 2010, Price et al., 1998, D'Souza-Schorey et al., 1998). ADAP facilitates Rac1 activation and α4β1-mediated adhesion (Dios-Esponera et al., 2015). To address whether the contribution of Ubc9 to T-cell adhesion is involved in Rac1 activation signalling, we performed a Rac1 activity assay by detecting the level of active Rac1 (GTP-bound Rac1) in the lysate of control shEGFP Jurkat T cells or Ubc9-knockdown T cells with or without anti-CD3 stimulation. Consistent with a previous observation (Kaminuma et al., 2001), anti-CD3 treatment led to an increase in the amount of GTP-Rac1 detected in control shEGFP Jurkat T cells (**Fig. 2.5C, shEGFP, anti-CD3 vs. unstimulated**), indicating that Rac1 was activated upon TCR activation. In contrast, the anti-CD3 stimulation-induced Rac1 activation was significantly decreased in Ubc9-knockdown T cells in both unstimulated and anti-CD3-stimulated cells (**Fig. 2.5C, anti-CD3, shUbc9#2 vs. shEGFP**). These results suggested that the effect of Ubc9 on T-cell adhesion relies on its modulation of TCR-mediated Rac1 activation and plasma membrane recruitment of Rap1 but not RIAM.

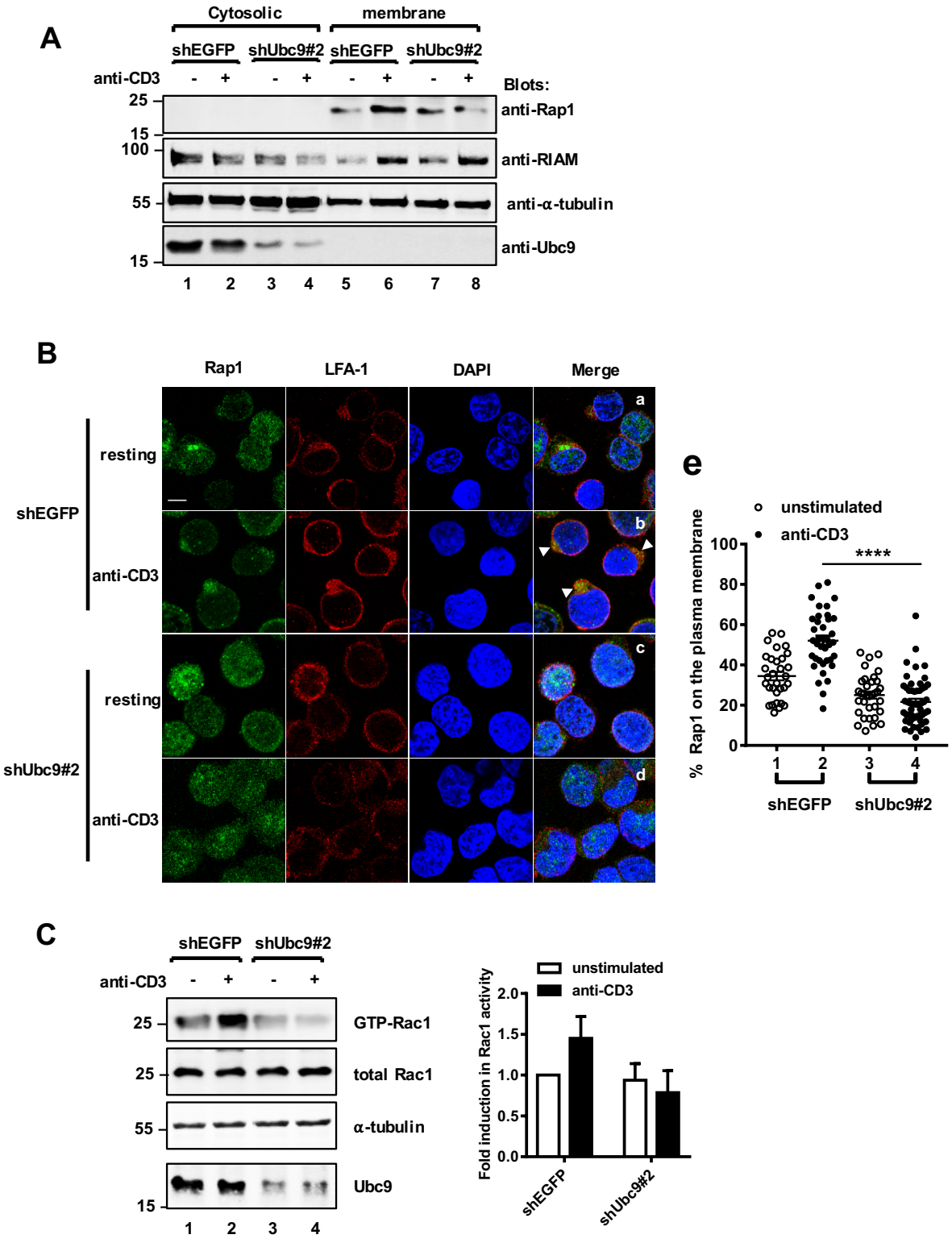


FIGURE 2.5. Effects of Ubc9 knockdown on LFA-1-mediated membrane localisation of Rap1-RIAM and fibronectin-mediated Rac1 activation in response to anti-CD3 stimulation.

Stable Ubc9-knockdown and control shEGFP Jurkat T cells were either left unstimulated or stimulated with 4 µg/ml OKT3 for 10 min at 37 °C. (A) Cytosolic and plasma membrane fractions were isolated as described in the Materials and Methods and subjected to western

blotting using anti-Rap1, anti-RIAM, anti-α-tubulin, and anti-Ubc9. (B) Double immunofluorescence staining of Rap1 (green) and LFA-1 (red) in stable Ubc9-knockdown or control shEGFP Jurkat T cells with or without anti-CD3 stimulation. Cells were fixed, permeabilised, stained, and visualized for LFA-1(red) and Rap1 (green) as described in the Materials and Methods. Images are representative of three independent experiments and the scale bar represents 10 μm. The graph indicates the percentage of Rap1 localised at the plasma membrane from a total of 30 - 50 cells in each condition. (C) Rac1 activity was assessed by pulldown assays using GST-PBD-PAK. GTP-bound Rac1 and total Rac1 were determined by immunoblot with anti-Rac1 antibody. Right panel: the bar graph represented the relative intensity of the bands (Rac-GTP/Total Rac1) measured by ImageJ software. The fold induction of each sample was normalized to that of unstimulated shEGFP cells. Error bars indicate the s.e. from three individual experiments.

DISCUSSION

While the immune adaptor ADAP acts as a key mediator of integrin inside-out signalling leading to T-cell adhesion, the regulation of this adaptor during integrin activation and T-cell adhesion remains unclear. We investigated the role of Ubc9, the sole SUMO E2 conjugase of the SUMO pathway, in the regulation of T-cell signalling and adhesion. The present study demonstrates that Ubc9 regulates T-cell adhesion via a mechanism involving a direct interaction with the immune adaptor ADAP: (1) While proximal T-cell signalling responses are not affected, T-cell adhesion is impaired in Ubc9-knockdown T cells; (2) Ubc9 directly binds to ADAP within a segment of NLS spanning residues 674-700, and the binding is enhanced by anti-CD3 stimulation; (3) Ubc9-ADAP interaction is required for integrin-mediated T-cell adhesion and LFA-1 clustering but not for TCR-mediated IL-2 transcription; (4) The mechanism of Ubc9 regulation of T-cell adhesion involves the plasma membrane translocation of Rap1, but not RIAM, and Rac1 activation upon TCR stimulation. Our study revealed the importance of Ubc9 in ADAP-mediated T-cell adhesion, and further presented the underlying mechanisms of the regulation via a direct interaction between Ubc9 and immune adaptor ADAP that is required for the TCR-induced plasma membrane translocation of Rap1, but not RIAM, as well as for Rac1 activation.

The *in vivo* co-immunoprecipitation in T cells showed that Ubc9 was pulled-down not only by anti-ADAP but also by anti-SKAP1 (**Fig. 2.2A**), suggesting that there is a formation of a signalling complex containing Ubc9, ADAP, and SKAP1 at least at some stage of the event. However, in an overexpression system in HEK 293T cells where both ADAP and SKAP1 are absent, Ubc9 only associated with overexpressed ADAP but not with SKAP1 (**Fig. 2.2C**), suggesting that Ubc9 interacts directly with ADAP rather than SKAP1 within the ADAP-SKAP1 module. Given the fact that ADAP constitutively associates with SKAP1 and expression of ADAP is required for stable expression of SKAP1 in T cells (Kliche et al., 2006), the precipitation of Ubc9 by SKAP1 may be bridged via ADAP. In addition, dynamic association of Ubc9 and ADAP was shown in a time course of TCR stimulation. The Ubc9-ADAP association peaked at 10 min and declined at 15 min post anti-CD3 stimulation (**Fig. 2.2B**). These data suggest that the functional role of Ubc9 in T-cell adhesion relies on its interaction with ADAP in the ADAP-SKAP1 module.

There are two NLS sites within ADAP with unknown functions. The Ubc9 binding site in ADAP was mapped to the second NLS site, NLS2, which was in line with previous reports that Ubc9 was found to bind to the NLS sites of its other binding partners. Ubc9 binds to the

NLS at the NH2 terminus of the homeodomain of visual system homeobox 1 (Vsx-1) and mediates the nuclear localisation of Vsx-1 independent of SUMO conjugation (Kurtzman and Schechter, 2001). Another example is the poly (A) polymerase interaction with Ubc9 for SUMOylation via its NLS site, which facilitates the nuclear localisation of poly (A) polymerase and contributes to its stability (Vethantham et al., 2008). The ADAP NLS2 deletion mutant displayed impaired TCR-mediated integrin adhesion and clustering. Our data reveals a new function for the NLS2 of ADAP as the binding site for Ubc9, which is required for TCR-mediated integrin activation and adhesion. Identification of Ubc9 as a new ADAP interaction partner has expanded the repertoire of ADAP binding partners, and increase the understanding of its regulation by virtue of the multiple and diverse domains within the molecule.

Apart from its enzymatic activity as to conjugate SUMO moieties to protein substrates, Ubc9 also functions independently of SUMOylation in regulating cellular physiology (Kaul et al., 2002, Kurtzman and Schechter, 2001, Chakrabarti et al., 1999). Proteins that interact with Ubc9 without being SUMOylated include high mobility group A1, Vsx-1, and chicken ovalbumin upstream promoter transcriptional factors (Li et al., 2007, Kurtzman and Schechter, 2001, Kobayashi et al., 2004). Our co-immunoprecipitation data suggest that ADAP was not SUMOylated by Ubc9 with or without anti-CD3 stimulation, indicating that Ubc9 modulation of integrin-mediated T-cell adhesion is independent of Ubc9-mediated SUMO modification of ADAP. However, it remains possible that Ubc9 promotes the SUMOylation of ADAP binding partners.

Downregulation of Ubc9 or interruption of the Ubc9-ADAP interaction by overexpression of the ADAP mutant deficient in Ubc9 binding led to impaired TCR-induced integrin adhesion. Previous studies identified two layers of modulation on the inside-out T-cell adhesion pathway, the adaptor proteins SLP-76-ADAP-SKAP1 module (Wang and Rudd, 2008, Raab et al., 2010) and the Rap1-RapL-RIAM module (Kinashi, 2005). Distinct pools of the ADAP-SKAP1 module in T cells selectively associate with RapL and RIAM at the plasma membrane but collectively facilitate TCR-mediated adhesion (Kliche et al., 2012). Rap1 is an important coordinator between integrins and TCR signals. T-cell activation results in the enrichment of active Rap1 at the plasma membrane destined for LFA-1 activation (Katagiri et al., 2006). Active Rap1 binds with RapL in response to TCR ligation, and their interaction is required for the spatial distribution of LFA-1 in T cells. Rap1 binding with a second effector RIAM is also necessary for the association of talin with the β-chain of LFA-1 (Kliche et al., 2012, Katagiri et al., 2003). Our data showed that Ubc9 downregulation caused a significant decrease in the TCR-mediated membrane targeting of Rap1, concomitant with the impaired

formation of LFA-1 clusters (**Fig. 2.5B**). Interestingly, in cells where Ubc9 was downregulated, while the TCR-induced RIAM membrane translocation was not affected, the TCR-induced Rap1 membrane targeting was abolished (**Fig. 2.5A**). These results suggest that Ubc9-ADAP interaction plays a selective role in LFA-1 activation via facilitating the TCR-induced translocation of Rap1, but not RIAM, to the membrane. Given that RIAM is only one of Rap1 effector molecules for LFA-1 activation, it is likely that Ubc9 utilizes Rap1 and other effector molecules downstream of Rap1 such as RapL and PKD for LFA-1 activation.

In addition to LFA-1, the other integrin molecules on the surface of T cells mediating T-cell adhesion are VLA-4 (α4β1 integrin), whose ligands are fibronectin and VCAM-1 on APCs (Chen and Zhu, 2013). ADAP facilitates the Rac1 activation and regulates the Rac1-mediated α4β1 integrin activation and T-cell adhesion (Dios-Esponera et al., 2015, Faroudi et al., 2010, Price et al., 1998, D'Souza-Schorey et al., 1998). Our data also showed that Ubc9 downregulation resulted in a significant reduction of T-cell adhesion to fibronectin (**Fig. 2.1, B and D**) as well as the abolishment of Rac1 activation (**Fig. 2.5C**). Thus, the involvement of Ubc9-ADAP in the regulation of T-cell adhesion is a combinative effect via a mechanism of facilitating the activation of Rac1 and the membrane targeting of Rap1 but not RIAM.

In addition to its central role in TCR-mediated adhesion, ADAP participates in the signalling pathways downstream of the TCR by an interaction with SLP-76, thereby causing T-cell activation (Griffiths et al., 2001, Peterson et al., 2001). ADAP also plays a key role in IL-2 production and cell proliferation (Griffiths et al., 2001, Peterson et al., 2001). While the binding of Ubc9 to ADAP was required for integrin-mediated T-cell adhesion, downregulation of Ubc9 did not affect the overall anti-CD3-induced tyrosine phosphorylation in T cells (**Fig. 2.3A**). Further, the Ubc9 binding site deletion mutant retained the ability to form a signalling complex with SKAP1 and SLP-76 (**Fig. 2.3C**). Moreover, cells transfected with the Ubc9 binding-deficient mutant dNLS2 showed comparable levels of TCR-mediated IL-2 transcription to cells transfected with WT ADAP (**Fig. 2.3D**). These data demonstrate that Ubc9-ADAP is not required for TCR proximal signalling responses or TCR-mediated IL-2 transcription. This suggests that the ADAP that associates with Ubc9 for adhesion regulation is segregated from the pool of ADAP that interacts with SLP-76 for T-cell activation as indicated by IL-2 production. Similar findings were observed in the Grb2-related adaptor downstream of Shc (Gads)-deficient T cells, where Gads is required for TCR-mediated IL-2 release but not TCR-induced adhesion (Bilal et al., 2015). As the region of ADAP involved in Ubc9-dependent regulation of T-cell adhesion is distinct from that involved in the regulation of TCR-mediated IL-2 transcription for its binding partner SLP-76, it is not surprising that the

Ubc9-ADAP module regulates TCR-mediated T-cell adhesion and IL-2 transcription differently. Thus, these results provide evidence for a novel interaction involving ADAP and Ubc9 that has profound and specific consequences for TCR-dependent adhesion but not for T-cell signalling.

The TCR activates integrin-mediated T-cell adhesion, which is mediated by either the LFA-1 or VLA-4 binding to the ICAMs or fibronectin, respectively. Our findings support a model where Ubc9 serves as a molecular switch for integrin-mediated T-cell adhesion via direct association with the immune adaptor ADAP (**Fig. 2.6**). In this model, TCR engagement stimulates the binding of Ubc9 to ADAP. The Ubc9-ADAP module participates in the regulation of T-cell adhesion via two distinct pathways. One is via the TCR-Rap1-LFA-1/ICAMs signalling axis whereby Ubc9 is required for TCR-induced translocation of Rap1 to plasma membrane and LFA-1 clustering. The other is via the TCR-Rac1-VLA-4/fibronectin axis whereby Ubc9 is required for TCR-induced Rac1 activation (**Fig. 2.6**). Given that the Ubc9-ADAP interaction is dynamic in response to anti-CD3 stimulation (**Fig. 2.2B**), an increase or decrease in the Ubc9-ADAP interaction triggered through the TCR represents an important component of adhesion-inducing mechanisms. In conclusion, our findings reconfigure the existing model of inside-out signalling and provide a new regulatory layer of the Ubc9-ADAP module on the controls of integrin-mediated T-cell adhesion.

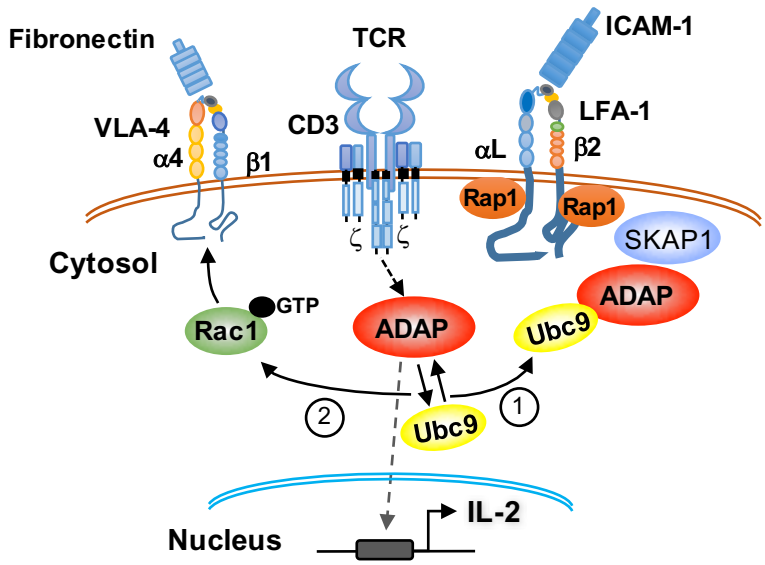


FIGURE 2.6. Proposed model for the role of Ubc9-ADAP in the regulation of TCR-mediated T-cell adhesion and signalling.

The immune adaptor ADAP possesses a dual role in mediating both the inside-out signalling for T-cell adhesion and the transduction of proximal and distal TCR signalling events. TCR activates integrin-mediated T-cell adhesion, which is mediated by either the LFA-1 or VLA binding to the ICAMs or fibronectin, respectively. In this model, we have identified Ubc9 as

a molecular switch for integrin-mediated T-cell adhesion via direct association with the immune adaptor ADAP. TCR engagement stimulates Ubc9 binding to ADAP. The Ubc9-ADAP module participates in the regulation of T-cell adhesion via two distinct pathways. One pathway is via the TCR-Rap1-LFA-1/ICAMs signalling axis whereby Ubc9 is required for TCR-induced translocation of Rap1 to plasma membrane and LFA-1 clustering. The other pathway is via the TCR-Rac1-VLA-4/fibronectin axis whereby Ubc9 is required for TCR-induced Rac1 activation. Thus, an increase or a decrease in the Ubc9-ADAP interaction triggered through the TCR represents an important component of adhesion-inducing mechanisms. However, Ubc9 binding to ADAP is not indispensable for ADAP-dependent TCR-induced IL-2 production. Thus, our findings reconfigure the existing model of inside-out signalling and introduce the Ubc9-ADAP module as a new regulatory layer on the controls of integrin-mediated T-cell adhesion.

MATERIALS AND METHODS

Cell culture and reagents

Jurkat T cells and murine T cell hybridoma cells were maintained in RPMI 1640 medium supplemented with 5% (v/v) FBS (Sigma) and 100 U/ml penicillin/streptomycin. Primary T cells from 6 - 8-week-old female C57BL/6J mice (SLAC laboratory Animal Co., Ltd, Shanghai, China) were cultured in RPMI 1640 medium with 10% (v/v) FBS, 100 U/ml penicillin/streptomycin, and 50 μM 2-mercaptoethanol. Primary cells were activated for 2 days by plate-bound 2 μg/ml anti-CD3 (2C11, eBioscience #16-0031-81) and 1 μg/ml anti-CD28 (eBioscience #16-0281-82) before experimental use. HEK 293T cells and COS-7 cells were maintained in DMEM supplemented with 5% (v/v) FBS and 100 U/ml penicillin/streptomycin. Reagents and antibodies were obtained from the following sources: mouse anti-human CD3 OKT3 (Biolegend #317315); mouse anti-human CD28 (Sigma #C7831); rabbit polyclonal anti-ADAP (1:2000, Millipore #07-546) and mouse monoclonal anti-phosphotyrosine (4G10, 1:1000, Millipore #05-1050); rabbit polyclonal anti-Ubc9 (1:1000, Santa Cruz Biotechnology #610748); mouse monoclonal anti-HA (1:10000, Sigma #H9658); mouse monoclonal anti-SKAP1 (1:2000, BD Transduction Laboratories #611236); rabbit polyclonal anti-SLP-76 (1:2000, Cell Signalling Technology #4958); mouse monoclonal anti-Rap1 (1:2000, BD Bioscience #610195); rabbit monoclonal anti-α-tubulin (1:10000, Abcam, #ab108629), rabbit monoclonal anti-RIAM (1:2000, Abcam #ab92537); rabbit polyclonal anti-CD18 (1: 500, Proteintech #10554-1-AP); recombinant human ICAM-1 Fc Chimera (R&D systems and Sino Biological Inc.); fibronectin (Sigma).

Transfection and lentivirus infection

Full-length human ADAP and SKAP55 cDNAs were cloned into pSRα expression vector containing an influenza haemagglutinin (HA) epitope tag at the N terminus. The HA-tagged ADAP dNLS1 mutant was generated by Quikchange-mediated deletion of a sequence coding for amino acids 469 to 505 using pSRα-HA-ADAP wild-type (WT) plasmid as a template. The HA-tagged ADAP dNLS2 mutant was generated by a Quikchange-mediated deletion of a sequence coding for amino acids 674 to 700. Ubc9 was expressed in a pcDNA3.1 expression vector. For transient transfection, Jurkat T cells were transfected by electroporation (Biorad Gene Pulser Xcell), using 250 V, 800 microfarads. For lentiviral infection, sequences targeted

against either Ubc9 or EGFP as a negative control were cloned into pLVX-shRNA1 expression vector using BamHI and EcoRI sites. Target sequences for shRNAs against Ubc9 are as follows: shUbc9#1 (5′-GAAGUUUGCGCCCUCAUAA-3′) and shUbc9#2 (5′-GGAACUUCUAAAUGAACCA-3′) (Kim et al., 2006, Kurihara et al., 2005). Jurkat T cells and primary T cells were transduced by spinoculation using lentiviral supernatants. Stable cell lines were selected with puromycin.

Integrin adhesion assay

Prior to the adhesion assay, an equal number of stable cells or freshly transfected cells were stimulated with anti-CD3 or left unstimulated for 30 min. The wells of a flat-bottomed 96-well plate were coated with either 10 μg/mL ICAM-1 human/mouse Fc chimera or 30 μg/mL fibronectin and the cells were incubated for 1h at 37 °C. Nonadherent cells were gently washed off, and the bound cells were counted as previously described (Wang et al., 2004).

Immunofluorescence and LFA-1 clustering assay

Immunofluorescence microscopy was conducted as described previously (Liu et al., 2015). Cells were seeded on poly-L-lysine (Sigma)-coated slides, fixed in 4% paraformaldehyde for 15 min, permeabilised with 90% methanol for 30 min, and blocked with 5% BSA for 1 h. Cells were subsequently stained with the indicated primary antibodies in blocking buffer overnight and visualised with Alexa Fluor 488-conjugated goat anti-mouse or Alexa Fluor 555-conjugated donkey anti-rabbit antibodies (Cell Signalling Technology) correspondingly. For LFA-1 clustering, cells were incubated with anti-CD3 (2 μg/10^6 cells) at 37 °C for 30 min and surface-bound antibody was removed by incubation on ice for 3 min in a PBS solution acidified to pH 2.0 with HCl and supplemented with 0.03 M sucrose and 10% FBS (Cefai et al., 1992, Cefai et al., 1998). Cells were then fixed and blocked in 5% BSA followed by incubation with anti-CD11a (1 μg/ml, BD Bioscience) and Alexa Fluor 488-conjugated goat anti-mouse antibody (Cell Signalling Technology). Cells were counterstained with DAPI for 10 min and mounted on microscopy slides. Samples were visualised on a Nikon Eclipse Ni-U microscope. For each experiment, a minimum of 200 cells from each condition were imaged and analysed for LFA-1 clustering. Anti-LFA-1 clustering was defined by the presence of a discrete polarized cap at one end of the cell (Wang et al., 2004). For the imaging of Rap1 and LFA-1 in cells, sequential immunofluorescence staining was performed using anti-Rap1 (BD

Bioscience) and anti-LFA-1 (Proteintech) followed by incubation with fluorescence-labelled secondary antibodies as described above. Cells were imaged with a Nikon Eclipse Ti laser scanning confocal system. The mean intensity of green fluorescence of Rap1 at the plasma membrane or the total amount per cell was determined using ImageJ software. For the imaging of HA-tagged proteins, cells were imaged with a ZEISS LSM 880 Confocal Laser Scanning Microscope.

Immunoprecipitation and Immunoblotting

Cell lysis, immunoprecipitation, and detection were performed as described previously with minor modifications (Wang et al., 2007). Briefly, 5×10^6 cells were lysed with 200 µl of lysis buffer (1% Triton X-100 (v/v) in 20 mM Tris-HCl (pH 8.3), 150 mM NaCl, 1 mM Na_4VO_3, and 0.1% protease inhibitor cocktail solution (Roche)). For immunoprecipitation, cell lysate was incubated with the indicated antibodies at 4 °C overnight before conjugating with 20 µl protein G sepharose beads (Amersham) at 4 °C for 1 h. Bound beads were washed three times with lysis buffer and precipitates were dissociated from beads by boiling in sample buffer for 10 min. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked with 5% BSA and incubated with indicated antibodies at 4 °C overnight. Bound antibodies were visualised using IRDye secondary antibodies (LI-COR) followed by detection with Odyssey Imaging Systems (LI-COR).

Isolation of cytosolic and plasma membrane fractions

Isolation of cytosolic and plasma membrane fractions has been previously described (Meller et al., 1996, Menasche et al., 2007). Briefly, freshly harvested lymphocytes from C57BL/6 mice were enriched with $CD4^+$ enrichment column and infected with lentiviruses expressing shRNAs against Ubc9 or EGFP, and cultured *ex vivo* for 2 days. Cells were either left unstimulated or stimulated with 4 µg/ml anti-CD3 (2C11) for 10 min, and washed in ice-cold PBS and resuspended on ice in a hypotonic buffer. Cells were sheared and lysates were centrifuged at low-speed to precipitate nuclei. The remaining supernatant was recentrifuged, and the supernatant (cytosolic fraction) was collected. The pellet (membrane fraction) was washed twice with hypotonic buffer and resuspended on ice in lysis buffer containing 1% NP-40. The protein concentrations of the cytosolic and membrane fractions were determined by

the Bradford assay (Sigma), and an equal amount of protein from each fraction was analysed by western blotting.

Rac1 activity assay

The Rac1 pull-down activation assay kit (Cytoskeleton, Inc.) was used to measure endogenous Rac1 GTPase activity. The assay uses the Cdc42/Rac Interactive Binding region (also called the p21 Binding Domain, PBD) of the Cdc42/Rac effector protein, p21 activated kinase 1 (PAK). The PAK-PBD is in the form of a GST fusion protein, and this allows Rac-GTP be precipitated from the cell lysate by binding to the PAK-PBD domain fused to glutathione affinity beads. Either resting or stimulated (4 μg/ml OKT3 for 60 min) control shEGFP cells or stable Ubc9-knockdown T cells (3×10^7) was washed with ice-cold PBS and lysed, and equal amounts of cell lysates were processed according to the manufacturer's protocol. The GTP-bound Rac1 protein and total Rac1 protein (10 μg) were detected by western blotting using a mouse monoclonal anti-Rac1 antibody (1:2000, Cytoskeleton, Inc #ARC03).

In-gel digestion and liquid chromatography tandem mass spectrometry (LC-MS/MS)

Cell lysates and precipitates were prepared as described in immunoprecipitation and immunoblotting and resolved in a 12% SDS polyacrylamide gel. The gel was subjected to GelCode Blue staining (Thermo Scientific) according to the manufacturer's instructions. Proteins of interest were excised and subjected to reduction, alkylation, in-gel digestion and extraction according to standard protocols (Shevchenko et al., 2006). Peptides were desalted with ZipTip C18 pipette tips (Millipore) before analysis by mass spectrometry. Samples were loaded and eluted into an EASY-nLC 1000 system coupled online to a Linear Trap Quadrupole (LTQ) Orbitrap Elite (Thermo Fisher Scientific) through an Acclaim PepMap 100 Column (75 μm × 15 cm, C18, 3 μm, 100 Å, Thermo Fisher Scientific) with a reversed-phase binary gradient (Solvent A: 0.1% formic acid; Solvent B: 0.01% formic acid, 100% acetonitrile in 2 h). Results were evaluated with Mascot software (Matrix Science). MS spectra were searched against the human UNIPROT non-redundant protein database using Mascot (Matrix Science). The Mascot database search was performed using the following parameters: trypsin enzyme specificity, one possible missed cleavage, 10 ppm mass tolerance for peptide ions, and 0.5 Da mass tolerance for fragment ions. Search parameters specified a differential modification of methionine oxidation, (+15.9949 Da) as well as a static modification of carbamidomethylation

(+57.0215 Da) on cysteine. To provide high confidence sequence assignments, Mascot results were filtered by a cut-off value of < 0.05.

Luciferase assay

Jurkat T cells (5×10^6) were transfected with 5 - 10 µg of plasmid constructs and in combination with 4 µg of luciferase driven IL-2 promoter as described previously (Raab et al., 1999). Cells were stimulated at 37 °C with 2 µg/mL anti-CD3 mAb (OKT3) or left unstimulated for 6 h after transfection. After stimulation, cells were lysed in 50 µl of lysis buffer (Promega kit). Luciferase activity was subsequently determined using a microplate reader (PHERAstar FS, BMG LABTECH GmbH). Luciferase units of the experimental vector were normalised to the level of control vectors in each sample.

Statistics

All data were analysed with Prism 7 (GraphPad Software). Differences between two group means were analysed using an unpaired Student's *t* test or the Mann Whitney test for nonparametric data. A one-way or two-way ANOVA followed by correction for Bonferroni's multiple comparison test was used to compare more than two groups. Wherever indicated, *P* values are as follows: *, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.0001$; ns, non-significant. Data are shown as mean ± s.e.

Chapter 3 ADAP negatively regulates megakaryopoiesis

ABSTRACT

ADAP is essential for the regulation on integrin-mediated platelet activation, and has been linked to thrombocytopenia in gene-knockout mice and FYB-defective human patients. However, the role of ADAP in platelet formation remains largely unknown. Here, we show that ADAP inhibited megakaryopoiesis, a process entails the development of megakaryocytes from haematopoietic stem cells. An increased number of megakaryocytes in bone marrow, as well as in spleen was found in ADAP$^{-/-}$ mice compared to the WT littermates. The number of bone marrow-derived ADAP-deficient megakaryocytes were further increased after *ex vivo* culturing with thrombopoietin (TPO), concomitant with a potentiated polyploidization. A similar inhibition of ADAP on megakaryocytic development is observed in megakaryocytic cell lines MEG-01, where knockdown of ADAP resulted in an increase in the number of differentiated MEG-01 with higher DNA ploidy class. Mechanistically, depletion of ADAP potentiated STAT1 phosphorylation and transcriptional activity through a direct protein-protein interaction between ADAP and STAT1. The binding sites were further mapped to the SH2 domain of STAT1 as well as a segment of far N-terminus of ADAP. Taken together, our data illuminate for the first time the implication and significance of ADAP in megakaryopoiesis, and further present a mechanism by which ADAP directly interacts with STAT1 and constrains the STAT1 activation during megakaryocytic development.

INTRODUCTION

Circulating blood platelets are indispensable for haemostasis, would healing, angiogenesis, inflammation, atherosclerosis, lymphatic development and tumour growth (Semple et al., 2011). Platelet formation is a constant continuum in development entails the differentiation of self-renewal HSCs into mature megakaryocytes (known as megakaryopoiesis), and release of platelets by megakaryocytes into the bloodstream (known

as thrombopoiesis) (Eto and Kunishima, 2016). Megakaryopoiesis and thrombopoiesis are the cumulative result of intricate mechanisms that integrate proliferation, endomitosis, differentiation, and cell death. Aberrations in these processes lead to the pathogenesis of diseases such as the myeloproliferative neoplasms (MPNs) (Nangalia et al., 2016) and immune thrombocytopenia (Khodadi et al., 2016). Megakaryocytes reside primarily in the bone marrow and are the largest (50-100 μm) and one of the rarest cells in the bone marrow (~0.05%-0.1% of the nucleated bone marrow cells)(Patel et al., 2005, Harker and Finch, 1969, Nakeff and Maat, 1974). Megakaryocytes are also present in the yolk sac, fetal liver, spleen, and lung (Machlus and Italiano, 2013, Lefrancais et al., 2017).

Megakaryopoiesis is governed by transcription factors (e.g. GATA-1, NF-E2, FOG1, C-myb), adhesion molecules (e.g. VWF, fibronectin, fibrinogen), cytokines (e.g. thrombopoietin (TPO), interferons, interleukins), and chemokines (e.g. SDF-1, CXCR4, CXCL4/PF4) (Deutsch and Tomer, 2013, Khodadi et al., 2016). Cytokine TPO has been identified as the primary growth factor and major regulator of megakaryopoiesis (Hitchcock and Kaushansky, 2014). Like most cytokine receptors, c-Mpl–the receptor for TPO, lacks intrinsic kinase activity and instead, employs the Janus Kinases (JAKs) as signalling intermediates to convey the downstream signals (Ward et al., 2000, Chen et al., 2012). JAKs are a family of four cytoplasmic tyrosine kinases (JAK1, JAK2, JAK3, and tyrosine kinase 2 (TYK2)) associated with the cytokine receptors (Ward et al., 2000). Upon TPO binding to c-Mpl, JAK2 and TYK2 become activated and resulted in the phosphorylation of a family of latent transcription factors signal transducer and activator of transcription (STAT) (Hitchcock and Kaushansky, 2014, Drachman et al., 1997).

STATs family are comprised of seven members (STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, and STAT6). Activated STATs form homodimers and heterodimers via the intrinsic STAT-SH2 domains and translocate into the nucleus, bind specific DNA elements, and initiate transcription. The JAK-STAT signalling stands as a paradigm of how signals from protein-protein contacts at the cell surface can be conveyed directly to the genes in the nucleus (Shuai et al., 1994, Darnell et al., 1994, Darnell, 1997, Stark and Darnell, 2012). This pathway is widely used by a variety of cytokines and growth factors, including interferons (IFNs), interleukins, erythropoietin, and granulocyte colony-stimulating factor (G-CSF). Therefore the JAK-STAT signalling pathway is particularly prominent to hematopoietic cell biology and haematological malignancies (Ward et al., 2000). Two key signalling molecules, JAK2 and STAT1, mediate TPO- and IFN-γ-associated signal transduction, respectively. IFN-γ has been

widely known to inhibit the growth of hematopoietic progenitor cells in general (Kato et al., 2003), however IFN-γ alone can promote megakaryopoiesis, with respect to the facilitation of proliferation, polyploidization and differentiation of megakaryocytes both *in vitro* and *in vivo* (Huang et al., 2007, Muraoka et al., 1997, Tsuji-Takayama et al., 1996).

STAT1 exists as two isoforms, a 94 kD STAT1α and an 84 kD splice variant STAT1β (Rane and Reddy, 2002). Structural analysis of STAT1 recognized six tandem domains, including a N-terminal domain (NTD), a coiled-coil domain (CCD), a DNA binding domain (DBD), a linker domain, a SH2 domain and a transcriptional activation domain (TAD) (Chen et al., 1998). STAT1 is essential for the biological effects of both type-I and type-II IFNs. First and foremost, it governs the transcriptions of more than two hundred IFNs-stimulated genes (Ramana et al., 2000). In addition to IFNs, STAT1 is activated in response to a wide range of growth factors, cytokines and signalling molecules in cell culture system, e.g. prolactin, IL-10, IL-6, platelet-derived growth factor (PDGF), and colony-stimulating factor 1 (CSF-1) (Meraz et al., 1996). Of note, many studies have demonstrated that in addition to the canonical stimulus as IFNs and IL-6, the megakaryopoiesis-associated TPO can induce the STAT1 tyrosine phosphorylation and potentiate its DNA-binding activity (Gurney et al., 1995, Brizzi et al., 1997).

It has been extensively studies that STAT1 plays an indispensable role in the immunity against virus and bacteria. STAT1-deficient mice display defective immune responses to either viral or bacterial infections because of the lack of responsiveness to IFNs (Meraz et al., 1996, Durbin et al., 1996), with exception on normal developmental and reproducing capability in pathogen-free environment (Meraz et al., 1996). Recent studies have recognized a unique role of STAT1 in megakaryocyte development (Huang et al., 2007, Chen et al., 2010, Duek et al., 2014). Aberrant function of STAT1 has linked to myeloid disorders and that increased STAT1 activity is implicated in certain JAK mutation-associated diseases and subtypes, such as myeloproliferative neoplasms (MPN) and acute myeloid leukaemia (AML). A canonical JAK mutation leading to increased STAT1 activity has been well studied in the context of JAK2-V617F. STAT1 and its downstream signalling pathway are hyper-activated in MPN patients harbouring a JAK2-V617F mutation. More importantly, the phenotype is selectively found in erythroblasts from patients with essential thrombocythemia (ET, promotes megakaryocytic differentiation) subtype, but not in those with polycythemia vera (PV, promotes erythromcytosis) (Chen et al., 2010). Further, AML patients bearing a somatic mutation of JAK1 (T478S or V623A) showed activated IFN signalling pathway via increased

phosphorylation of STAT1 (Xiang et al., 2008). Last but not least, activation of STAT1 upregulates megakaryocyte protein production by crosstalk with mTOR signalling pathway (Haas et al., 2015), and promotes megakaryopoiesis via a function link with GATA-1 (Huang et al., 2007).

ADAP has been implicated in the regulation of integrin activation and adhesion. The role of ADAP in the signal transduction of integrin activation has been largely explored in T cells. ADAP forms a functional module with SKAP1 and facilitates the activation of β1 and β2 integrins through direct linkages with small GTPase Rap1 and its effector proteins following TCR activation (Griffiths et al., 2001, Peterson et al., 2001, Kinashi, 2005). Meantime, ADAP also promotes the VWF-mediated β3 integrin and collagen-induced β1 integrin activation in platelets, using a SKAP1-indedpendent mechanism distinctly from those in T cells. ADAP is essential for the haemostatic platelet responses to vascular injury, with respect to platelet activation, adhesion, aggregation, spreading and thrombus stabilization (Kasirer-Friede et al., 2007, Kasirer-Friede et al., 2014, Jarvis et al., 2012, Kasirer-Friede et al., 2010, Jarvis et al., 2004). ADAP-knockout mice demonstrate deficient TCR-mediated T-cell adhesion and proliferation, as well as a defect in haemostasis and mild thrombocytopenia (Jarvis et al., 2012, Peterson et al., 2001, Kasirer-Friede et al., 2007). Interestingly, to date there are only two case reports that have linked pathogenic variants or mutations of the ADAP-coding gene FYB with human diseases, and both of them concern the recessive thrombocytopenia in humans (Hamamy et al., 2014, Levin et al., 2015). However, the involvement of ADAP in a function of megakaryocyte remains largely unknown. In an attempt to clarify the potential implication and provide insight into aetiology of thrombocytopenia, we characterized the effects of ADAP on the regulation of megakaryopoiesis using ADAP-deficient mice and stable ADAP-knockdown MEG-01 megakaryoblastic cells. We show that ADAP deficiency potentiated megakaryocytic differentiation and maturation. Importantly, ADAP bound to STAT1 *in vitro* and *in vivo*. The depletion of ADAP from cells significantly potentiated the STAT1 activity. Together, our data identify a new regulatory hierarchy through which ADAP inhibits megakaryopoiesis via direct interaction with STAT1.

RESULTS

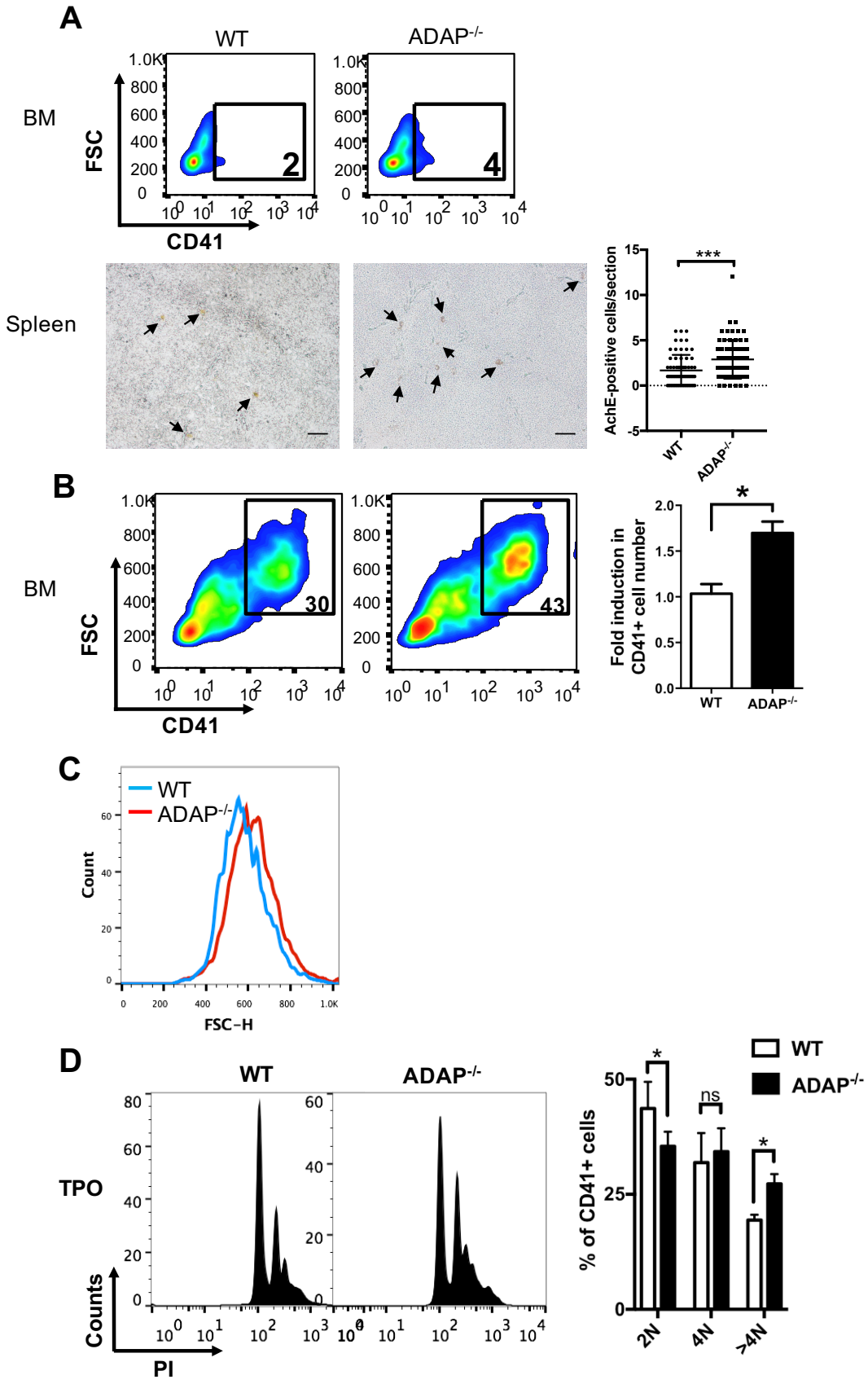
ADAP-deficient mice have enhanced megakaryocytic development *in vivo*

During the process of megakaryocyte maturation, megakaryocyte progenitors undergo proliferation, endomitosis, and maturation to generate platelets. Mature megakaryocyte is

morphologically characterized by polyploidization and the expansion of cytoplasmic mass before forming proplatelet to release platelet followed by eventual apoptosis (Italiano et al., 1999).

To dissect the role of ADAP in megakaryopoiesis, we characterized the megakaryocyte development and differentiation in ADAP-gene knockout mice. Primary bone marrow cells were isolated and examined for the percentage of megakaryocytes as indicated by the expression of early maturation marker CD41. In ADAP$^{-/-}$ mice, the percentage of megakaryocyte was 2-fold higher than those of WT littermates (**Fig. 3.1A, upper panel**). Similarly, ADAP$^{-/-}$ mice have a 1.7-fold increase in the average number of megakaryocytes in the spleen sections (**Fig. 3.1A, lower panel, 2.9 ± 0.2/section vs. 1.7 ± 0.2/section**), recognised by acetylcholinesterase (AchE) staining showing all mature and immature megakaryocytes (Ichikawa et al., 2004).

We next cultured the isolated bone marrow cells for 6 days in the presence of TPO to induce differentiation of bone marrow cells to megakaryocytes. The differentiation of megakaryocytes from bone marrow cells was successfully induced by TPO, resulted in an approx. 10-fold increase in the number of CD41-positive megakaryocytes compared to those in freshly isolated bone marrow cells in WT mice. This percentage was further enhanced in ADAP$^{-/-}$ mice resulted in a 1.5-fold increase compared to that of WT mice (**Fig. 3.1B, ADAP$^{-/-}$ vs WT**). Further, the increased number of bone marrow-derived megakaryocyte from ADAP$^{-/-}$ mice was accompanied by an enlarged cell size, as indicated by the forward side scatter of CD41-positive megakaryocytes determined by flow cytometric analysis (**Fig. 3.1C**). The increased cell size suggested a higher maturity of megakaryocyte. We next examine the cellular DNA content using propidium iodide staining. As shown in **Fig. 3.1D**, the ADAP-deficient CD41-positive megakaryocytes showed a higher level of polyploidy class (>4n) than the WT megakaryocytes. Taken together, these findings indicate that ADAP-deficiency in mice potentiates megakaryocyte development and differentiation in the bone marrow.


A
WT
ADAP-/-
BM
FSC
CD41
2
4
Spleen
AchE-positive cells/section
***
WT
ADAP-/-
B
BM
30
43
Fold induction in CD41+ cell number
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C
WT
ADAP-/-
Count
FSC-H
D
WT
ADAP-/-
TPO
Counts
PI
% of CD41+ cells
ns
2N
4N
>4N

FIGURE 3.1. ADAP deficiency in mice enhances megakaryocytic differentiation

(A) Upper panel: flow cytometric analysis of $CD41^+$ megakaryocytes in the bone marrow cells, determined by FITC-labelled anti-CD41 staining. Representative FACS profile from three independent experiments was showed. Lower panel: Cryostat sections of spleen containing megakaryocytes were stained for AchE. Scale bar represents 50 μm. Black arrows denote $AchE^+$ megakaryocytes. The average number of $AchE^+$ megakaryocytes were enumerated and shown as mean ± se in the right graph (n = 50 fields). (B) Bone marrow cells harvested from WT and $ADAP^{-/-}$ mice were cultured *in vitro* in the presence of TPO (50 ng/mL) for 6 days. Representative FACS profile showed $CD41^+$ megakaryocyte (left panel). Data are shown as mean ± se from three individual experiments (right panel). (C) The cell size of bone marrow-derived $CD41^+$ megakaryocyte was measured with forward side scatter using flow cytometry. (D) DNA polyploidy was analysed by staining the $CD41^+$ megakaryocytes with PI and measured using flow cytometry (left panel). Percentage of DNA ploidy were shown as mean ± se from 3 individual experiments (right panel).

ADAP inhibits megakaryocytic differentiation

To further characterize the implication of ADAP in megakaryopoiesis, we studied the effects of ADAP depletion on megakaryocytic development in a human megakaryoblastic leukemia cell line MEG-01 cells.

Lentiviral-mediated shRNA against ADAP was used to stably knock down the expression levels of ADAP in MEG-01 cells. Gene silencing efficiency were >90% for MEG-01 cells as determined by Western Blotting (**Fig. 3.2A, right panel**). Both the shEGFP MEG-01 cells or ADAP-knockdown MEG-01 cells were induced to undergo megakaryocytic differentiation by phorbol 12-myristate 13-acetate (PMA). PMA promoted several features of megakaryocytic differentiation of MEG-01 cells, as shown by increased expression of CD41, and high DNA ploidy class reflecting polyploidization (**Fig. 3.2, A and B, shEGFP, PMA vs. Resting**). Intriguingly, in stable ADAP-knockdown MEG-01 cells, the multiple aspects of megakaryocytic differentiation were further potentiated compared to shEGFP MEG-01 cells. As shown in **Fig. 3.2A**, in shEGFP MEG-01 cells, PMA significantly increased the number of CD41-positive cells from 7% to 18%, albeit to a much lesser extent than in stable ADAP-knockdown MEG-01 cells, where a marked increase from 5% to 25% was observed. In addition, stable ADAP-knockdown MEG-01 cells developed increased DNA ploidy compared to shEGFP MEG-01 cells, as evidenced by the percentage of cells with a high ploidy class (>4N) was significant increased (**Fig. 3.2B, PMA, shADAP vs. shEGFP, 19.4% vs. 11.7%)**. To further characterize the ADAP-mediated megakaryocytic differentiation, we measured the mRNA level of ITGA2B (CD41) and GP1BA (CD42) using quantitative RT-PCR (**Fig. 3.2C**),

which are early and late marker of megakaryocyte maturation respectively. In consistent with surface expression level determined by flow cytometry analysis shown in **Fig. 3.2A**, the mRNA level of ITGA2B in stable ADAP-knockdown MEG-01 cells was significantly upregulated compared to that in control shEGFP MEG-01 cells. In addition, ADAP-knockdown MEG-01 cells also displayed a modest increase in the GP1BA level (**Fig. 3.2C**), albeit no apparent difference was observed in the surface expression of CD61 compared to the control shEGFP cells (data not shown).

These data collectively suggested that depletion of ADAP from mice and from megakaryoblastic cell line augmented the many aspects of megakaryopoiesis and that ADAP may possess a detrimental role in megakaryopoiesis.

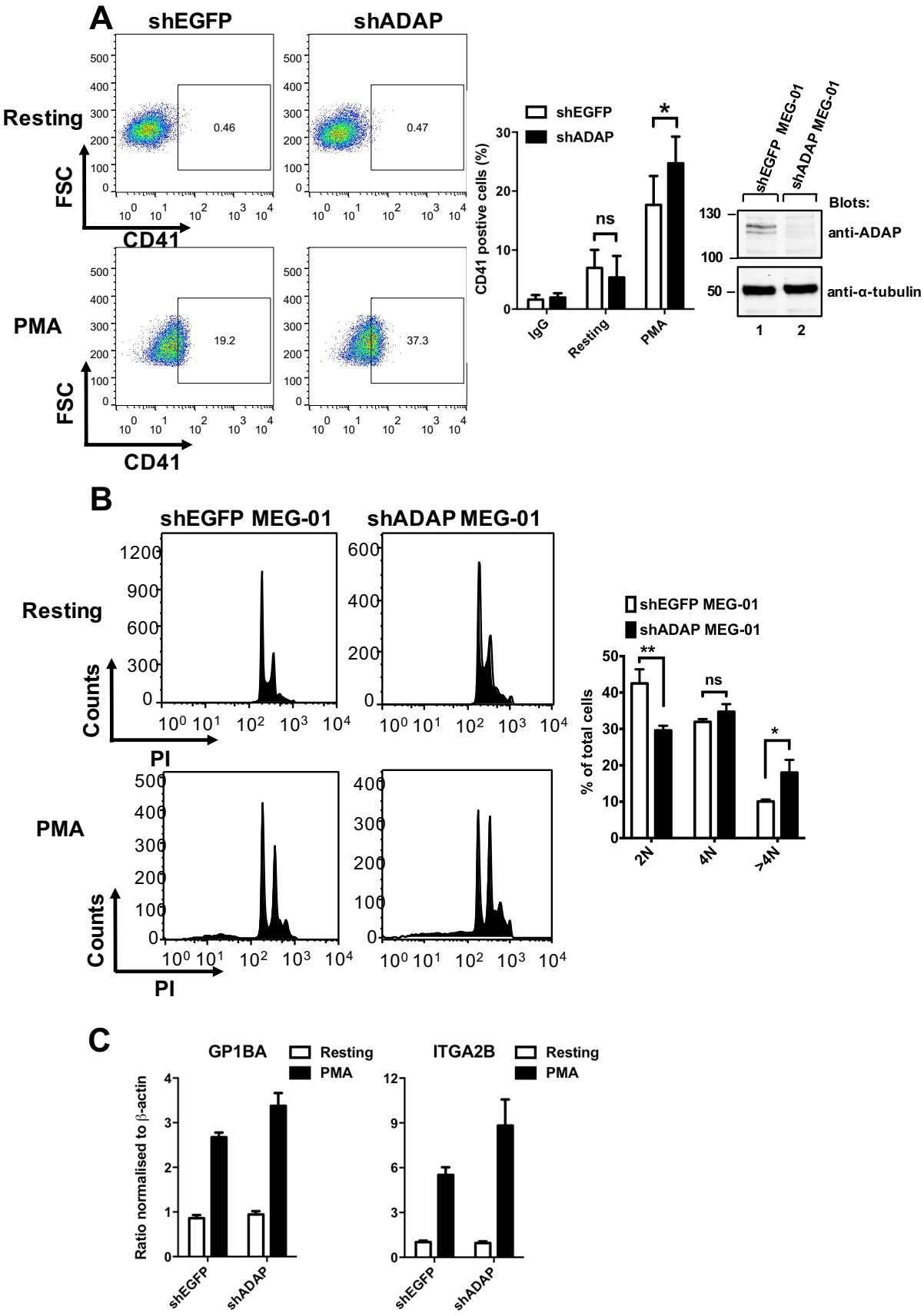

A
shEGFP
shADAP
Resting
PMA
FSC
CD41
0.46
0.47
19.2
37.3
shEGFP
shADAP
CD41 postive cells (%)
ns
*
IgG
Resting
PMA
shEGFP MEG-01
shADAP MEG-01
Blots:
anti-ADAP
anti-α-tubulin
130
100
50
1
2
B
shEGFP MEG-01
shADAP MEG-01
Resting
PMA
Counts
PI
% of total cells
**
ns
*
2N
4N
>4N
C
GP1BA
ITGA2B
Resting
PMA
Ratio normalised to β-actin
shEGFP
shADAP

FIGURE 3.2. ADAP inhibits megakaryocytic differentiation

Control shEGFP MEG-01 cells or stable ADAP-knockdown MEG-01 cells were left untreated or treated with PMA for 3 days to induce differentiation. (A) Representative FACS profile showed cell surface expression of CD41, determined by staining with FITC-labelled anti-CD41 antibody (left panel). Data are shown as mean ± se from 5 individual experiments (middle panel). Right panel shows immunoblot of whole-cell lysates of stable cells using anti-ADAP. (B) Representative FACS profile showed DNA polyploidy of the cells, analysed by propidium iodide staining using flow cytometry (left panel). Percentage of DNA ploidy were shown as mean ± se from three individual experiments (right panel). (C) Expressions of GP1BA (left panel), and ITGA2B (right panel) were determined by quantitative RT-PCR. Data are shown as mean ± se from three individual experiments performed in triplicates.

ADAP interacts with STAT1

ADAP harbours multiple intermolecular domains for binding with its binding partners or molecules to facilitates immune functions. We ask if ADAP could interact with one of the crucial modulators in megakaryopoiesis. The JAK-STAT pathway is central to the megakaryopoiesis (Hitchcock and Kaushansky, 2014, Ward et al., 2000), of which STAT1 plays an indispensable role in the megakaryocytic development (Huang et al., 2007, Chen et al., 2010, Duek et al., 2014). It remained unclear whether ADAP could interact with STAT1 in mammalian cells.

To address this question, we first performed coimmunoprecipitation experiments in MEG-01 cells with or without PMA stimulation. Western Blotting analysis of immunoprecipitates by anti-ADAP antibodies showed that STAT1 was coimmunprecipitated with ADAP after PMA stimulation (**Fig. 3.3A, upper panel, lane 4**), albeit STAT1 was not found in the precipitates prepared from resting MEG-01 cells by anti-ADAP antibodies. Further, the interaction between endogenous ADAP and STAT1 was discovered in Jurkat T cells as well. Anti-ADAP antibodies coprecipitated STAT1 from resting Jurkat T cells (**Fig. 3.3A, lower panel, lane 5**). Importantly, anti-CD3 ligation increased the level of coprecipitated STAT1 (**lane 6 vs. lane 5**). As a control, antibodies against SLP-76, another crucial immune adaptor in T cells, failed to coprecipitate STAT1 in either resting Jurkat T cells or anti-CD3 stimulated T cells (**lane 3 and 4**), while STAT1 was readily detected in the anti-STAT1 immunoprecipitates (**lane 7 and 8**). This results indicate that STAT1 binds to ADAP in both MEG-01 cells and T cells.

In line with this finding is the result that we found in HEK 293T cells where ADAP is not constitutively present. HA-tagged ADAP was coexpressed with FLAG-tagged STAT1 in

HEK 293 T cells, followed by anti-FLAG immunoprecipitation and anti-HA blotting. As shown in **Fig. 3.3B**, HA-ADAP was detected in the anti-FLAG immunoprecipitates (**lower panel, lane 2**), which indicates that ADAP binds directly to STAT1. We next constructed a series of truncation mutants of STAT1 to further determine which domain attributes to the interaction of STAT1 with ADAP (**Fig. 3.3B, upper panel**). STAT1 truncation mutants were prepared with an amino-terminal FLAG tag, coexpressed with HA-tagged ADAP, and immunoprecipitated with anti-FLAG antibodies. The presence of HA-ADAP in the precipitates was analysed with anti-HA blotting. Deletion of the SH2 and linker domain of STAT1 resulted in a steep decrease in coprecipitated ADAP by anti-FLAG antibodies, although they were well expressed as shown in the anti-FLAG immunoblot analysis (**Fig. 3.3B, lower panel, lane 5 and 4 vs. lane 2**). However, the amount of coprecipitated ADAP was much fewer when STAT1 lacked in SH2 domain than the linker domain (**lane 5 vs. lane 4**). In contrast, truncations on NTD or TAD domain retained similar amounts of coprecipitated ADAP as to those of full-length WT construct (**lane 3 and 6**). These data suggest that the association with ADAP minimally requires NTD and TAD domains of STAT1, but was predominantly mediated by the SH2 domain.

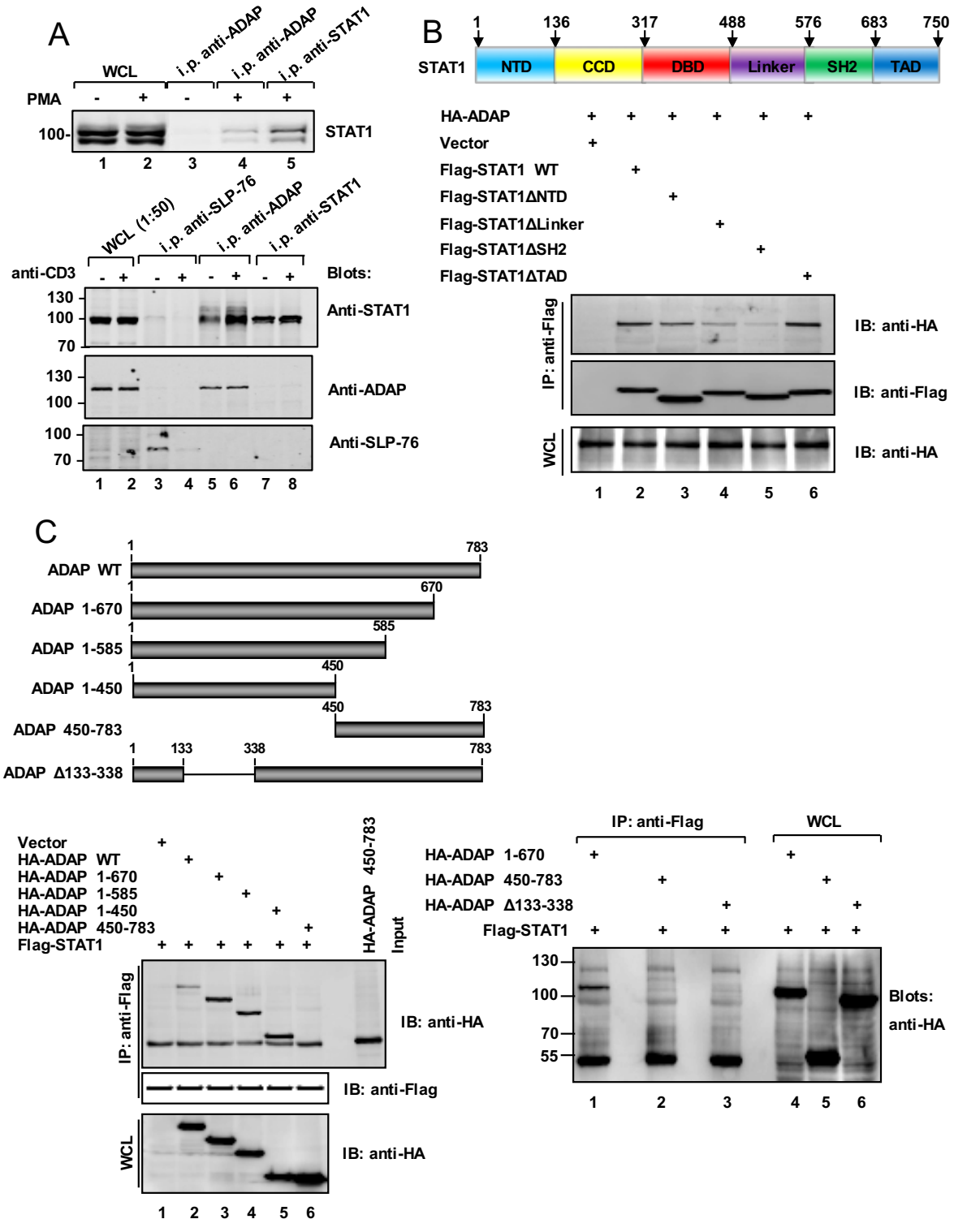
Conversely, we mapped the STAT1 binding site to the N-terminus of ADAP (**Fig. 3.3C**). A series of HA-tagged carboxyl-terminal deletion mutants and an amino-terminal deletion mutant (amino acids 450-783) were constructed (**upper panel**) and coexpressed with FLAG-tagged STAT1 constructs in HEK 293T cells. Cell lysates were subjected to immunoprecipitation with anti-FLAG antibodies, followed by immunoblot analysis using anti-HA antibodies. Analysis of the ADAP truncation mutants reveals that the carboxyl-terminal deletions of ADAP (i.e. ADAP/1-670, ADAP/1-585 and ADAP/1-450) retained the binding to STAT1 while the amino-terminal deletion mutant ADAP/450–783 failed to bind (**lower left, lane 3-5 vs. lane 6**). This data suggests that amino acids 1-450 on ADAP accommodates the STAT1-binding site. We further determined the binding region on ADAP as the amino acids 133 to 338. When coexpressed the amino acids 133 to 338-truncated mutant of ADAP (denoted as ADAP Δ133-338) along with STAT1, the truncation mutant showed no binding to FLAG-tagged STAT1 (**lower right panel, lane 3 vs. lane 1**). Note that the observed difference in binding was not due to differences in the expression level of each ADAP mutants as shown in the anti-HA immunoblot analysis.

To therefore directly assess the interaction between STAT1 SH2 domain and amino acids 133-338 of ADAP, we purified individual STAT1 SH2 domain as GST-fusion protein

and a His-tagged peptide encoding the amino acids 133-338 of ADAP. The GST protein was used as a negative control. These peptides were then subjected to Isothermal titration calorimetry (ITC) for the measurement on stoichiometry, binding constants, and thermodynamic parameters in a single interaction. ITC is routinely used in the characterization of thermodynamics of a biomolecular interaction by directly and quantitatively measuring the heat that is either released or absorbed upon binding of the two reactants (Wiseman et al., 1989). The ITC result determined at 25 ºC showed that the His-ADAP 133-338 peptide directly bound to GST-STAT1 SH2 domain with a high binding constant of K_D = 0.19 μM, while no apparent thermodynamic shift was observed between the His-ADAP 133-338 peptide and GST control protein (**Fig 3-3D, left vs. right panel**).

We next questioned if the localization of STAT1 could overlap with those of ADAP in intact cells. MEG-01 cells were differentiated with PMA for 3 days and imaged by confocal fluorescence microscopy. Endogenous STAT1 and ADAP were stained with anti-STAT1 and anti-ADAP antibodies, and visualized with an Alexa Fluo 488 and Alexa Fluo 555 secondary antibody respectively. ADAP predominantly localized at the cytoplasm, while a substantial portion of STAT1 protein was found in the nucleus or nuclear periphery area of differentiated MEG-01 cells (**Fig. 3.3E**). The nuclear periphery-localized STAT1 was found overlapped with endogenous ADAP in the cytoplasm.

Taken together, these data indicate that ADAP directly interacts with STAT1, and the interaction is mediated by SH2 domain of STAT1 and amino acids 133-338 of ADAP.


A
WCL
i.p. anti-ADAP
i.p. anti-ADAP
i.p. anti-STAT1
PMA
STAT1
WCL (1:50)
i.p. anti-SLP-76
i.p. anti-ADAP
i.p. anti-STAT1
anti-CD3
Blots:
Anti-STAT1
Anti-ADAP
Anti-SLP-76
B
STAT1
NTD
CCD
DBD
Linker
SH2
TAD
HA-ADAP
Vector
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Flag-STAT1ΔSH2
Flag-STAT1ΔTAD
IP: anti-Flag
IB: anti-HA
IB: anti-Flag
WCL
IB: anti-HA
C
ADAP WT
ADAP 1-670
ADAP 1-585
ADAP 1-450
ADAP 450-783
ADAP Δ133-338
Vector
HA-ADAP WT
HA-ADAP 1-670
HA-ADAP 1-585
HA-ADAP 1-450
HA-ADAP 450-783
Flag-STAT1
HA-ADAP 450-783
Input
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IB: anti-HA
IB: anti-Flag
WCL
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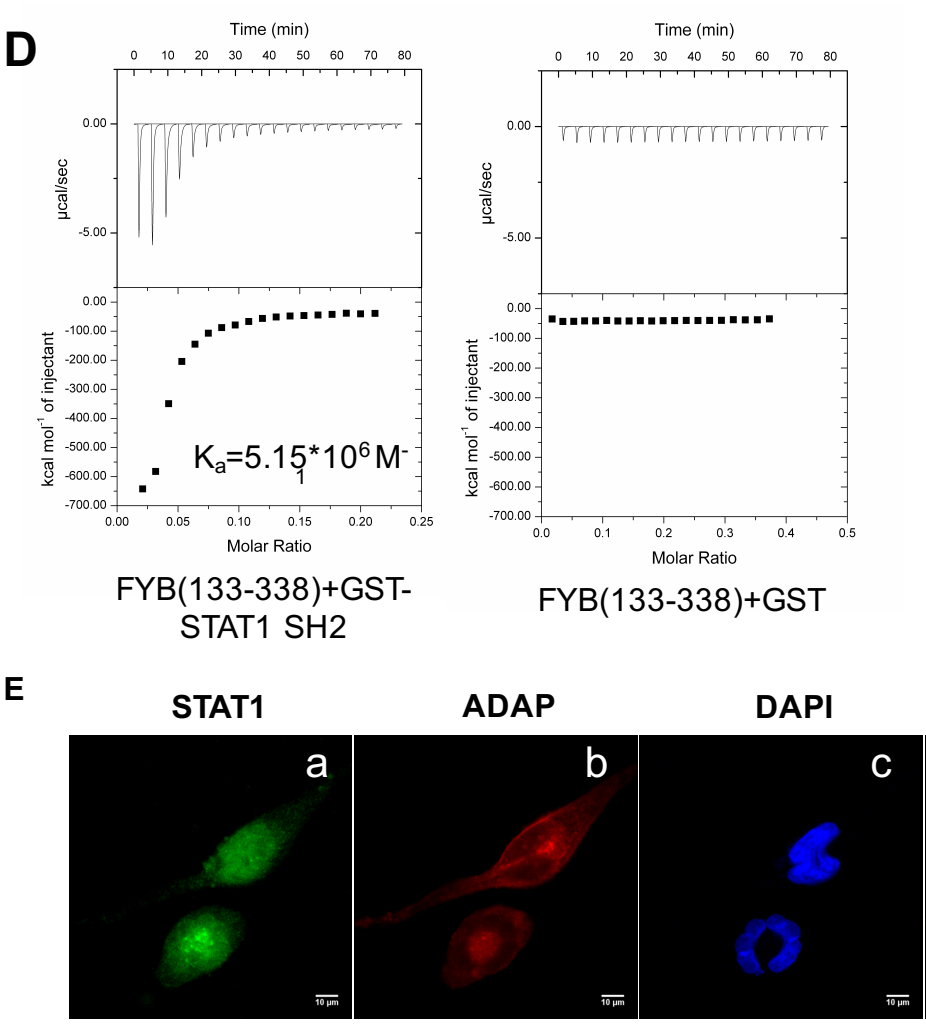


FIGURE 3.3. ADAP interacts with STAT1

(A) Cell lysate of resting and PMA-stimulated MEG-01 cells (upper panel), or anti-CD3 stimulated Jurakt T cells (lower panel) were prepared for immunoprecipitation immunoprecipitation with either anti-ADAP, anti-STAT1, or anti-SLP-76, followed by blotting with anti-STAT1, anti-ADAP, and anti-SLP-76. (B) Upper panel: schematic diagram of STAT1 protein structure. Lower panel: HEK 293T cells were transfected with HA-ADAP together with empty vector, FLAG-STAT1 WT, or FLAG-STAT1 truncation mutants as indicated. Immunoprecipitation was performed using anti-FLAG antibody, followed by blotting with anti-HA (top), anti-FLAG (middle), or anti-HA (bottom). (C) Upper panel: schematic representations of ADAP WT and deletion mutants. FLAG-STAT1 was coexpressed with either vector, HA-ADAP WT, or HA-ADAP deletion mutants (middle panel: HA-ADAP 1-670, HA-ADAP 1-585, HA-ADAP 1-450, HA-ADAP 450-783; lower panel: HA-ADAP 1-670, HA-ADAP 450-783, HA-ADAP Δ133-338) in HEK 293T cells. Cell lysates were subjected to immunoprecipitation using anti-FLAG antibody, followed by blotting with anti-HA and anti-FLAG. (D) Isothermal titration calorimetry of binding between aa.133-338 of ADAP and GST-STAT1 SH2 domain (left panel) or GST protein as a negative control (right panel). (E) Immunofluorescence staining of STAT1 (green) and ADAP (red) in MEG-01 differentiated with PMA for 3 days. Nuclei were counterstained with DAPI. Scale bar represents 10 µm.

ADAP negatively modulates STAT1 phosphorylation and transcriptional activities

STAT1 protein forms homodimers via a highly conserved SH2 domain to bind to DNA. The interaction between the two SH2 domains is regulated exclusively by the phosphorylated segment (residues 701-708) (Chen et al., 1998). STAT1 phosphorylation on tyrosine 701 and serine 727 is a prerequisite step for STAT1 to be converted to its active form and reach maximal activation (Wen et al., 1995). We have showed that the ADAP deficiency in mice and human MEG-01 cell lines resulted in an enhanced megakaryopoiesis, and that ADAP directly interacted with STAT1 via the SH2 domain. It raised the possibility that the binding of ADAP to STAT1 may negatively regulate the STAT1 activation and transcription activity, thus contribute to its inhibition on megakaryopoiesis.

Stable ADAP-knockdown MEG-01 cells and Jurkat T cells were induced to STAT1 activation by exposure to IFN-γ. As shown in **Fig. 3.4A**, control shEGFP and stable ADAP knockdown MEG-01 cells were treated with IFN-γ, and phosphorylation of STAT1 at Tyr701 or Ser727 was analysed by Western Blotting with phosphoTyr701- or phosphoSer727-specific antibodies. IFN-γ induced substantial STAT1 phosphorylation on Tyr701 as well as Ser727 in shEGFP MEG-01 cells (**Fig. 3.4A, lane 2 vs. lane 1**), while intriguingly, induced further increases in the levels of phosphorylation on both residues in shADAP MEG-01 cells (**Fig. 3.4A, lane 4 vs. lane 2**). In consistent with these data, we found similar results using stable ADAP knockdown Jurkat T cells. IFN-γ treatment induced enhancement in tyrosine phosphorylation (**Fig. 3.4B, left panel**) and serine phosphorylation (**right panel**) of STAT1 in control shEGFP Jurkat T cells, whereas these inductions were further potentiated in ADAP-knockdown Jurkat T cells. Note that the differences in the degree of phosphorylation induction were not due to the different expression levels of STAT1 proteins or loading amounts as showed in the anti-STAT1 and anti-α-tubulin immunoblotting analysis. These data suggest that depletion of ADAP from the cells increases the STAT1 phosphorylation on Tyr-701 and Ser-727 and that ADAP may function as a negative regulator of STAT1 activity.

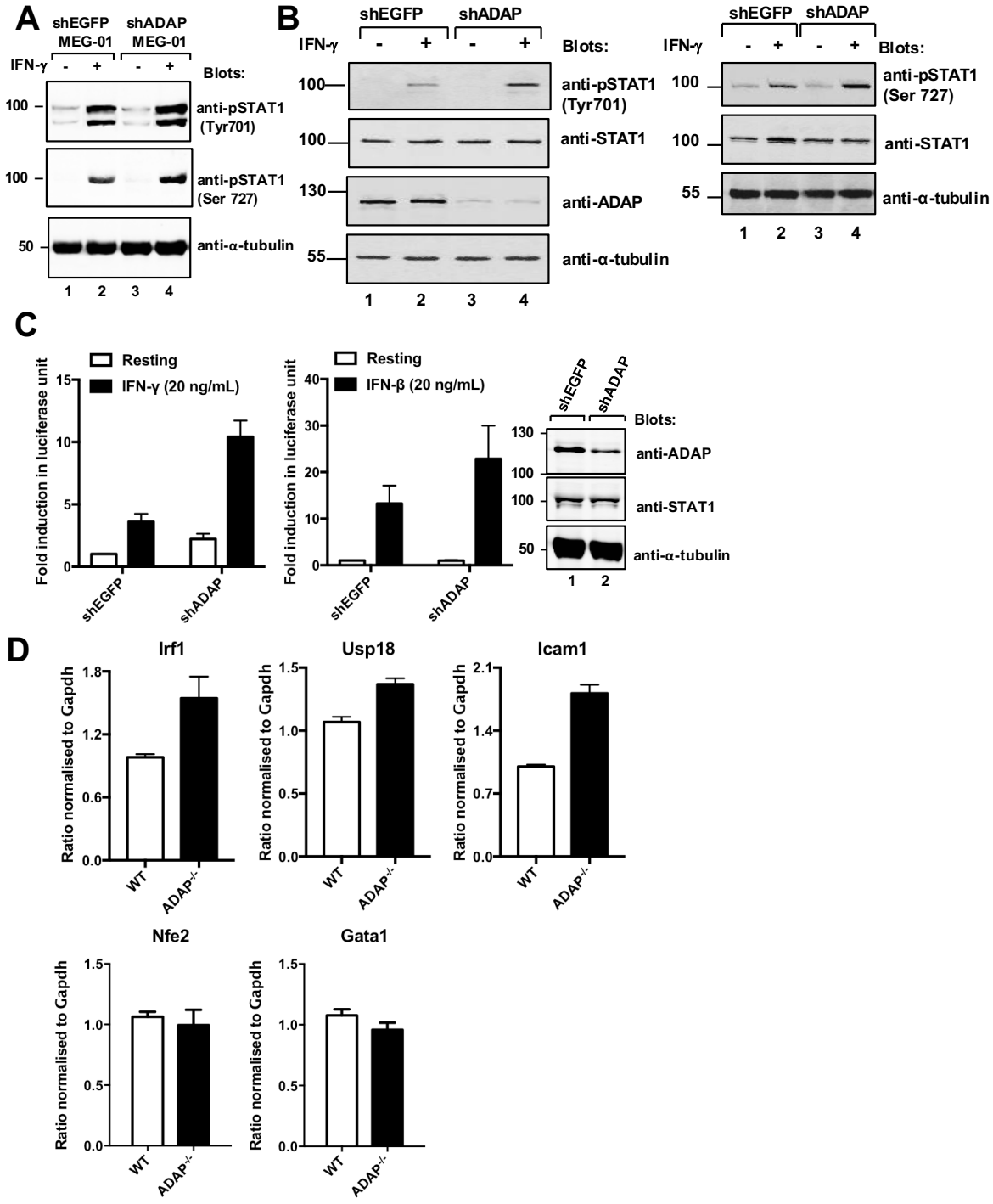
The activated STAT1 dimerizes, translocates to the cell nucleus, and binds specific DNA elements. In response to IFN-γ, STAT1 is phosphorylated and binds to specific elements termed γ -activated sequence (GAS) on IFN-γ-responsive promoters. While in response to IFN-α and -β, STAT1 binds to interferon-stimulated response element (ISRE) within the promoters of type I IFN-regulated genes (Darnell et al., 1994). Having demonstrated that both Tyr701 and Ser727 phosphorylation in STAT1 were potentiated in ADAP-knockdown cells in

response to IFN-γ stimulation, we tested for the effects of ADAP knockdown on IFN-γ and -β-induced STAT1 transcription. Equal number of control shEGFP Jurkat T cells and stable shADAP Jurkat T cells were transfected with GAS-luciferase reporter constructs or ISRE-luciferase reporter constructs. After 18 hours of transfection, the cells were treated with IFN-γ or left unstimulated for a further 6 hrs, and cell lysates were assayed for luciferase activity. As shown in **Fig. 3.4C**, in the control shEGFP cells, IFN-γ induced an approx. 4-fold increase in the GAS-driven luciferase signal compared to resting cells (**left panel, shEGFP, IFN-γ vs. resting**), whereas in the stable ADAP-knockdown cells, a steep 10-fold increase was observed after IFN-γ treatment (**left panel, shADAP, IFN-γ vs. resting**). In parallel, expression of the ISRE-driven luciferase reporter was strongly enhanced by IFN-β stimulation in stable shADAP knockdown Jurkat T cells compared to control shEGFP cells (**right panel, IFN-β, shADAP vs. shEGFP, 22-fold vs. 13-fold**). These data indicate that depletion of ADAP from the cells enhances the IFN-γ and -β induced STAT1-mediated transcription.

Next, to examine the role of ADAP in modulating STAT1 activity during the process of megakaryopoiesis, we measured the mRNA expressions of STAT1-regulatory genes in TPO-induced bone marrow-derived megakaryocytes and PMA-stimulated MEG-01 cells. The bone marrow-derived megakaryocytes were isolated from ADAP$^{-/-}$ mice and WT mice, and cultured in the presence of TPO for 6 days to induced megakaryocytic development. Expressions of three STAT1 target genes *Irf1(Kovarik et al., 2001)*, *Usp18(Grisouard et al., 2015)*, and *Icam1(Jahnke and Johnson, 1994)* were analysed by qPCR. As shown in **Fig. 3.4D**, the mRNA levels of *Irf1*, *Usp18* and *Icam1* is significantly increased in ADAP-deficient bone marrow-derived megakaryocytes compared to those of WT mice. Interestingly, the expression of *Gata1*, an upstream key regulator of STAT1 for megakaryopoiesis (Huang et al., 2007), was not affected in ADAP-deficient megakaryocytes, as well as those of Nfe2 (**Fig. 3.4D**), a late megakaryopoiesis gene regulating the terminal differentiation before platelet release (Vyas et al., 1999). This results demonstrate that ADAP specifically modulates the activities of STAT1, and might have minor effects on Gata1 and Nfe2. A similar trend towards the expressions of STAT1-regulatory genes was observed in MEG-01 cells as well. MEG-01 cells were incubated with PMA for 3 days to induced megakaryocytic development. PMA stimulation significantly enhanced the mRNA level of IRF1 gene by approx. 6-fold in shEGFP MEG-01 cells, however, in ADAP-knockdown MEG-01 this steep enhancement was further potentiated to approx. 9-fold after exposure to PMA. In line with those of IRF1 gene, upregulated mRNA levels in ADAP-knockdown MEG-01 against WT MEG-01 cells were also noted in those of USP18 and

ICAM-1. Consistent with the data obtained from mice, the expressions of GATA1 and NF-E2 was similar between stable ADAP-knockdown MEG-01 cells and shEGFP cells.

Taken together, these findings suggest that ADAP negatively regulates STAT1 with respect to its phosphorylation, transcription activity, and STAT1-mediated gene transcription.



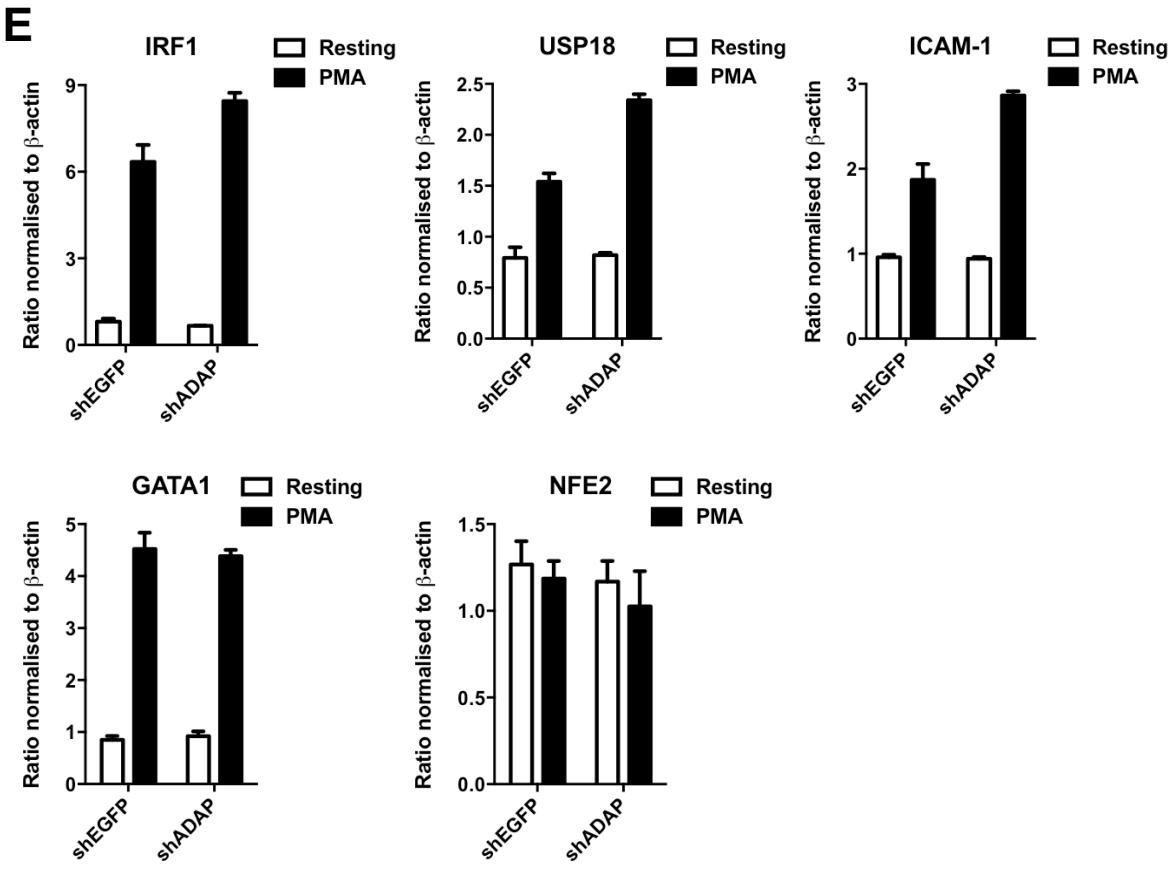


FIGURE 3.4. ADAP negatively modulates STAT1 phosphorylation and transcriptional activities

(A) shEGFP MEG-01 cells or stable ADAP-knockdown MEG-01 cells were left untreated or treated with IFN-γ (10 ng/mL) overnight. Phosphorylation of STAT1 were analysed by western blotting using antibodies against tyrosine 701- or serine 727-phosphorylated STAT1. (B) Control shEGFP and stable ADAP-knockdown Jurkat T cells were either left unstimulated or stimulated with IFN-γ (10 ng/mL) for 1 h or overnight. Tyrosine (left panel) and serine (right panel) phosphorylation of STAT1 were analysed by western blotting using antibodies as indicated. (C) Control shEGFP and stable ADAP-knockdown Jurkat T cells were transfected with GAS promoter-driven (left panel) or ISRE promoter-driven luciferase reporter (right panel). Cells were treated with or without IFN-γ or IFN-β for 6 h respectively, after which the luciferase activity was measured as described in Materials and Methods. Data are shown as mean ± se from three individual experiments. Expressions of ADAP and STAT1 in cells used in luciferase assay were analysed by western blotting, and the α-tubulin levels were shown as loading control.

DISCUSSION

ADAP is one of the major components of the signal transduction for integrin-mediated T-cell adhesion and platelet activation (Griffiths et al., 2001, Kasirer-Friede et al., 2007). ADAP-knockout mice show normal viability, growth, fertility and haematopoietic cellularity, with an exception of moderate thrombocytopenia, enlarged spleens, and reduced splenic T cells and thymocytes (Peterson et al., 2001). More importantly, defects in the ADAP/FYB gene have been linked to recessive thrombocytopenia in humans (Levin et al., 2015, Hamamy et al., 2014). However, there has been a major lacuna in understanding whether ADAP has a role in the biological processes leading to platelet production. In this study, we addressed the role of ADAP in the regulation of megakaryopoiesis, a process entails the differentiation and maturation of megakaryocytes from HSCs. We showed here that ADAP deficiency leads to an enhanced megakaryopoiesis *in vivo*. Mechanistically, ADAP inhibits STAT1 activity through the direct protein-protein interaction, and the binding sites were mapped to the SH2 domain of STAT1 as well as a segment of far N-terminus of ADAP. Taken together, our data illuminate the importance of ADAP in megakaryopoiesis, and further present a mechanism by which ADAP directly interacts with STAT1 and constrains the STAT1 activation during megakaryocytic development. Our findings thus shed light on the current understanding towards the pathogenesis of thrombocytopenia in ADAP-knockout mice and in FYB gene-defective humans.

ADAP-knockout mice have mild thrombocytopenia characterized by low platelet count (60-70% of the platelet levels in WT mice) in the whole blood (Jarvis et al., 2012, Peterson et al., 2001). Problems in any stage of the megakaryopoiesis or thrombopoiesis can affect the eventual platelet counts. We showed here that the ADAP deficiency in mice led to a more progressive megakaryopoiesis, as indicated by increased number of megakaryocytes with higher ploidy class. The increased numbers of megakaryocyte found in the bone marrow and spleen of ADAP-knockout mice are in concordance with a previous study showing a similar increment in the ADAP-deficient spleens (Jarvis et al., 2012). Moreover, we found that *ex vivo* culture of the ADAP-deficient bone marrow cells in the presence of TPO yield higher number of megakaryocytes, suggesting that the megakaryopoiesis process in bone marrow was enhanced. However, future work is required as to investigate the role of ADAP in thrombopoiesis and platelet haemostasis.

Beside the intrinsic insufficiency in megakaryopoiesis, thrombocytopenia may be due to a secondary (acquired) mechanism which concerns the peripheral destruction (Drachman,

2004, Audia et al., 2017), especially in immune thrombocytopenia purpura (ITP). Given the complexity of the aetiology of thrombocytopenia, it is not surprising to find normal or increased number of megakaryocytes in bone marrow and unchanged TPO levels in ITP patients (von Gunten et al., 2013). Thrombocytopenia can be trigger by innate immune responses in ITP patients. The antiplatelet autoantibodies produced by autoreactive B cells facilitate platelet and megakaryocyte phagocytosis by macrophages, especially in the spleen. In addition, platelets also exhibit increased apoptosis, probably due to the cytotoxicity of $CD8^+$ T cells (Audia et al., 2017, McMillan et al., 1974, Olsson et al., 2003, Houwerzijl et al., 2004). Therefore, the mild thrombocytopenia in $ADAP^{-/-}$ mice might be a consequence of aberrant immune response, where a potential mechanism concerning the platelet destruction might take action. A careful dissection on the immune system, e.g. detection of autoantibodies against platelet and megakaryocytes, intercellular communications between platelet/megakaryocyte and macrophage, would provide further insight into pathogenesis of thrombocytopenia in $ADAP^{-/-}$ mice. Interestingly, it has been well-documented that $ADAP^{-/-}$ mice have enlarged spleen (Peterson et al., 2001), and we observed a similar phenotype in our own experiment as well (data not shown). Spleen can retain a pool of platelets which increased with splenomegaly (Penny et al., 1966), and macrophage is a major effector for platelet destruction in spleen (Tavassoli and McMillan, 1975). The unexpected but not conflicted discrepancy between increased number of megakaryocytes and low platelet counts in ADAP-knockout mice may be collectively resulted from imbalance between enhanced megakaryopoiesis and peripheral platelet destruction, probably an increased clearance. It is therefore tempting to suspect the splenic macrophage may play a role in the course of thrombocytopenia in ADAP-knockout mice, which is an important aspect to be clarified in our future study.

Another clue on the action of ADAP on megakaryopoiesis may come from studying its binding partner. STAT1 can be activated by cytokines e.g. TPO and IFN-γ (Gurney et al., 1995, Brizzi et al., 1997). IFN-γ mainly signals through STAT1, and the levels of IFN-γ were increased in serum of mice with thrombocytosis and in patients with essential thrombocythemia (Duek et al., 2014). Importantly, the IFN-γ/STAT1 signalling promotes megakaryopoiesis. IFN-γ was found to augment the development and polyploidization of megakaryocyte colonies *in vivo* and support the TPO-independent proliferation and differentiation of human megakaryocytic cell line (Tsuji-Takayama et al., 1996, Griffin and Grant, 1990, Li et al., 1998). Moreover, activated STAT1 enhances megakaryocytic differentiation and maturation, while deletion of STAT1 favours erythropoiesis and reduces megakaryopoiesis (Chen et al., 2010,

Huang et al., 2007, Duek et al., 2014). Although the molecular machinery of STAT1 regulation on megakaryopoiesis remained elusive, one proposed mechanism is that STAT1 promotes megakaryopoiesis downstream of GATA1, key regulator for the erythoid and megakaryocytic lineage. The positive regulation of STAT1/GATA1 exerts by facilitating expression of multiple endomitotic regulators e.g. cyclins D1 and D2, as well as the expression of NF-E2 (Huang et al., 2007, Duek et al., 2014). In this study, we demonstrate that ADAP suppressed the megakaryocytic development, and that ADAP deficiency led to a hyper-activated STAT1 signalling, which may at least in part contribute to the underlying mechanism of ADAP regulation on megakaryopoiesis.

ADAP binds to the SH2 domain of STAT1, which is in line with previous reports that ADAP binds to the SH2 domain of its other binding partners. Interestingly, in most cases, they are Src-type SH2 domains. ADAP binds to the SH2 domain of SLP-76 and controls the assembly and stabilization of SLP-76 microcluster (Coussens et al., 2013). In addition, ADAP binds to c-Src via SH2 domain and their association is required for retaining the kinase activity of c-Src (Koga et al., 2005). Similar observation on ADAP binding to a SH2 domain can also be found in those of Src kinase Fyn (da Silva et al., 1993) and adaptor protein Nck (Lettau et al., 2014). The STAT family harbours a distinct type of SH2 domain, which primarily differs in the βE or βE-βF motif from those of Src-type SH2 domains (Gao et al., 2004). The SH2 domain of STAT1 mediates the tyrosine phosphorylation of STAT1, as well as subsequent dimerization and nuclear translocation of STAT1 (Shuai et al., 1994, Improta et al., 1994, Mowen and David, 1998). We showed here that ADAP binds to STAT1. To the best of our knowledge, it is the first report discovered that ADAP could bind to a member of the STAT family via its SH2 domain. And most importantly, unlike previously identified binding partners, ADAP negatively modulates the function of STAT1 by inhibiting the tyrosine and serine phosphorylation of STAT1, and eventually impairing the DNA binding to ISRE and GAS elements. A plausible explanation may proceed as that the binding of ADAP to STAT1 results in a blockade of SH2 domain, thus inhibits the recruitment of STAT1 to JAK kinases and dampens the JAK-induced phosphorylation of STAT1, and eventually attenuates the DNA binding activity of STAT1. Similar findings were observed in Hepatitis C virus (HCV) core protein, where HCV core protein specifically interacts with STAT1 at SH2 domain and blocks the STAT1 phosphorylation and dimerization (Lin et al., 2006). On the other hand, the STAT1 interaction domain resides in an N-terminal segment of ADAP (amino acid 133-338). Unfortunately, the function of N-terminus of ADAP has been underexplored so far. One clear

example is that the SKAP1 primarily binds to a proline-rich motif (amino acid 338-358) of ADAP. Expression of the ADAPΔ338-358 mutant fails to rescue T cell-APC conjugation formation, as well as impairs efficient LFA-1 function (Burbach et al., 2011). Our data thus expand the current knowledge on the function of N-terminus of ADAP by showing a novel interaction between amino acid 133-338 of ADAP with the SH2 domain of STAT1.

In conclusion, our data confer a novel role of ADAP in the regulation of megakaryopoiesis, and further provide a mechanism by which it directly interacts with STAT1 and negatively modulates the STAT1 activity during megakaryocytic development.

METHODS AND MATERIALS

Reagents and antibodies

Recombinant human (rh) and recombinant mouse (rm) TPO and IFN-γ, rh IFN-β, rm IL-3, rm SCF were purchase from Sino Biological. Inc. Antibodies were obtained from the following rabbit polyclonal anti-STAT1 (1:2000, Cell Signalling Technology #9172), rabbit monoclonal anti-pSTAT1 (Y701, 1:2000, Cell Signalling Technology #9167), rabbit polyclonal anti-pSTAT1 (S727, 1:2000, Cell Signalling Technology #9177); rabbit polyclonal anti-ADAP (1:2000, Millipore #07-546); mouse monoclonal anti-HA (1:10000 Sigma #H9658), mouse monoclonal anti-FLAG (1:1000, Sigma #F1804); rabbit monoclonal anti-α-tubulin (1:10000, Abcam #ab108629).

Cell culture

Jurkat T cells and MEG-01 cells (kind gift from Dr Yun Zhao from Soochow University) were maintained in RPMI 1640 medium supplemented with 5% (vol/vol) fetal bovine serum (FBS) and 100 U/ml penicillin/streptomycin. HEK293T cells were maintained in DMEM supplemented with 5% (vol/vol) (FBS) and 100 U/ml penicillin/streptomycin. To culture primary megakaryocytes, progenitor cells were harvested by flushing bone marrow of femurs and tibiae from 10-12-week-old C57BL/6J and ADAP$^{-/-}$ mice and cultured with 50 ng/ml TPO, 10 ng/ml IL-3 and 20 ng/ml SCF for 6 days as previously described (Kozuma et al., 2009). Following differentiation, megakaryocytes were enriched on a 1.5%/3.0% discontinuous BSA gradient (Geddis and Kaushansky, 2004).

Plasmids and lentiviral transduction

Human ADAP was cloned into pSRalpha vector and human STAT1 was cloned into p3xFlag-CMV expression vector. ADAP and STAT1 mutants were generated by site-directed mutagenesis method of DpnI digestion. Sequences targeting again ADAP (5'-GCAAAGGCCAGACGGCTTA-3') or against EGFP as negative control were cloned into pLVX-shRNA1 expression vector using BamHI and EcoRI sites. GAS element (AGGTTTCCGGGAAAGCAG) were constructed into promoter-firefly luciferase plasmid TA LUC-vector. ISRE-LUC construct was a kind gift from Prof. Xiaofeng Qin from Suzhou Institute of Systems Medicine, Chinese Academy of Medical Sciences. Jurkat T cells and MEG-01 cells were transduced by spinoculation using lentiviral supernatants. Stable cell line was selected with puromycine.

Protein Induction and purification

The SH2 domain of human STAT1 was expressed with a GST tag and residues 133-338 of human ADAP were expressed with a His tag in BL21(DE3) cells. Cells were grown at 37 ^{o}C in LB medium and protein expression was induced with 1 mM isopropyl-beta-D-thiogalactopyranoside (IPTG) for 3 hrs. The cells were harvested, lysed with sonication in PBS buffer and centrifuged at 10000 *g* for 30 min at 4 ^{o}C. The supernatant was collected and incubated with Glutathione-agarose (Sigma) and Ni-NTA agarose (Qiagen) respectively for 3 hr. The protein was eluted with a linear gradient of 250 mM imidazole. Peptides were then dialyzed and kept at -80^{o}C until being processed for ITC experiment.

Isothermal titration calorimetry (ITC) experiment

For ITC experiment, proteins were resuspended in PBS. Concentrations of the peptide solutions were determined from absorbance at 280 nm. ITC titrations were performed using a MicroCal VP-ITC calorimeter. For each titration experiment, the concentration of peptide was 40 ng/ul in the sample cell. Raw thermograms were integrated with automated shape analysis, and then imported in the ORIGIN 7.0 software for individual analysis or global analysis of multiple titrations, using models for 1:1 association schemes and non-linear least squares fitting.

Megakaryocyte ploidy analysis

BSA gradient-enriched megakaryocytes were stained with FITC-labelled anti-CD41 (BD bioscience) for 30 mins at 4^{o}C and fixed in 70% methanol at 4^{o}C overnight. The cells were

then washed with PBS and resuspended in 50 µg/ml of propidium iodide (Sigma) and 20 µg/ml RNase (Sigma) for 30 min at room temperature. Ploidy of CD41+ cells were determined by flow cytometry using a FACS Calibur (Becton Dickinson). Data analysis was performed using FlowJo software (Tree Star).

Acetylcholinesterase (AchE) staining

Spleen sections at 6 µm were cut on a cryostat (Leica) and mounted on slides. The specimens were fixed in 1% PFA for 10 min, and stained for AchE using the modified Karnovsky and Roots method (Karnovsky and Roots, 1964) with a commercially available kit (Leagene, Beijing).

Immunofluorescence microscopy

Immunofluorescence microscopy was conducted as described previously (Liu et al., 2015). For the visualisation of ADAP and STAT, cells were fixed with 4% paraformaldehyde, permeabilized with 90% methanol, and blocked with 5% BSA. Endogenous ADAP and STAT1 were detected using anti-ADAP and anti-STAT1 (BD Bioscience), and visualized with Alexa Fluor 488-conjugated goat anti-mouse antibody and Alexa Fluor 555-conjugated donkey anti-rabbit antibody (Cell Signalling Technology). Cells were counterstained with DAPI (Sigma). Cell were imaged with a Nikon Eclipse Ti laser scanning confocal system. Images were processed in ImageJ software.

Immunoprecipitation and Immunoblotting

Cell lysis, immunoprecipitation, and immunoblotting were performed as described previously (Wang et al., 2007). The protein concentration was determined by the Bradford assay (Sigma) and equal amount of cell lysate was analysed by Western Blotting or subjected to immunoprecipitation. Protein samples were resolved with SDS-PAGE, transferred to nitrocellulose membrane and immunoblotted with primary antibodies. Bound antibodies were visualized using IRDye secondary antibodies (LI-COR) followed by detection with Odyssey Imaging System.

Quantitative RT-PCR

Total RNA was isolated from the cells using TRI-reagent (Sigma). cDNA was prepared from RNA using RevertAid reverse transcriptase (Thermo Fisher) following the manufacturer's manual. Quantitative PCR analysis and data collection were performed on a QuantStudio 5 Real-time PCR system (Thermo Fisher) using the primer pairs listed below. Gp1ba: forward,

5’-AGGTGATGGAACTGGGTGGT-3’, reverse, 5’-TAGTGCTCCCATCCTGGCTAA-3’; Nfe2: forward, 5’-TTATCACAGCTGCCTGTTGGG-3’, reverse,5’-GCTGGGAGCTCATAAGATGGTG-3’;Itga2b: forward, 5’-AGTGGACACCACTGTTCTTGG-3’, reverse, 5’-AATACGGCTCCAGTCTCCTCT-3’; Gata1: forward, 5’-CTTGGGATCACCCTGAACTCG-3’, reverse, 5’-CCAGGGCAGAATCCACAAACT-3’; Irf1: forward, 5’-TAGGACGTGCTTTCACAGTCTAA-3’, reverse, 5’-GCATTCGAGTGATTGGCATGG-3’; Usp18: forward, 5’-GGTCTTCGCTCTTTGGCTTG-3’, reverse, 5’-CTGACTGGCAGGGTTTCCTC-3’; Icam1: forward, 5’-TCCGCTACCATCACCGTGTA-3’, reverse, 5’-TAGCCAGCACCGTGAATGTG-3’; Gapdh: forward, 5’-GGGTCCCAGCTTAGGTTCATC-3’, reverse, 5’-ACTGTGCCGTTGAATTTGCC-3’; GP1BA: forward, 5’-GCCGAAAGACACAACCATCC-3’, reverse, 5’-TGAGGCGAGTGTAAGGCATC-3’; NFE2: forward, 5’-AAGATCTGACTCTGCCTTTAGCC-3’, reverse,5’-TGTATCACCCTGTTCCTGCTC-3’; ITGA2B: forward, 5’-TTCCTGTGGAGGAATCTGAAGG-3’, reverse, 5’-CACTGGGTCCAGGTTCAAGG-3’; GATA1: forward, 5’-GTGAACCGGCCACTGACCAT-3’, reverse, 5’-CCACCACCATAAAGCCACCA-3’; USP18: forward, 5’-GGCTCCTGAGGCAAATCTGT-3’, reverse, 5’-CAACCAGGCCATGAGGGTAG-3’; IRF1: forward, 5’-TGAGGCATGTGCCATAGGTG-3’, reverse, 5’-CAGCCGTGAGGACCTTTCTT-3’; ICAM-1: forward, 5’-GGTAGCAGCCGCAGTCATAA-3’, reverse, 5’-GTGGCTTGTGTGTTCGGTTT-3’; ACTB: forward, 5’-AATCTGGCACCACACCTTCTAC-3’, reverse, 5’-ATAGCACAGCCTGGATAGCAAC-3’. Quantification was normalised to Gapdh and ACTB gene.

Luciferase Assay

Luciferase assay was performed as described previously (Liu et al., 2013). Equal number of shEGFP Jurkat T cells or stable shADAP Jurkat T cells were transfected with 5-10 μg of GAS/ISRE-luciferase reporter plasmid. Cells were either left unstimulated or stimulated with at 37 ^{o}C with 20 ng/mL IFN-γ or IFN-β for 6 h after transfection. Cell were harvested and lysed in 50 μl of lysis buffer (Promega kit). The luciferase activity was determined subsequently using a microplate reader (PHERAstar FS, BMG LABTECH GmbH). Luciferase units of the experimental vector were normalized to the level of control vectors in each experiment.

Statistical analysis

All data were analysed with Prism 7 (GraphPad Software). Differences between two group means were analysed using an unpaired Student's *t* test or the Mann Whitney test for nonparametric data. A one-way or two-way ANOVA followed by correction for Bonferroni's multiple comparison test was used to compare more than two groups. Wherever indicated, *P* values are as follows: *, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.0001$; ns, non-significant. Data are shown as mean ± s.e.

Chapter 4 Summary and conclusion

ADAP is a haematopoietic-specific immune adaptor. It acquired versatile roles in the regulations on various types of cells, e.g. T cell, dendritic cells, macrophages, and platelets, etc. By virtue of the multiple intermolecular binding domains, it binds and forms functional links with a wide range of proteins to facilitate its regulations. The most extensively-studied role of ADAP is that it couples TCR activation to integrin activation and T-cell adhesion. However, it remained elusive how the signals from TCR are conveyed to integrin via the signalling axis of ADAP and how the ADAP is regulated in T cells. Meantime, the functions of ADAP linked to other immune cells were largely unknown. Work in this thesis have characterized the implication of ADAP in TCR-mediated integrin activation and megakaryopoiesis via its interactions with two novel binding partners, Ubc9 and STAT1, respectively. Although the functions of ADAP in integrin activation have been extensively studied for many years, this work provides novel evidence suggesting that ADAP forma s functional link with Ubc9 in the regulation of TCR-mediated integrin activation and adhesion. While the interaction of ADAP-Ubc9 is not necessary for maintaining the integrity of TCR proximal signalling cascades, it is mandatory for supporting the TCR-induced integrin clustering and T-cell adhesion, with a selective role in facilitating the membrane targeting of Rap1. It has been documented that ADAP-deficient mice showed modest thrombocytopenia and defects in platelet aggregation as well. Although the discoveries that integrin-mediated inside-out signalling is positively governed by ADAP in platelet answer for the defects in platelet aggregation and thrombus formation, the mechanism underlying thrombocytopenia in ADAP-deficient mice remained elusive. The analysis of mice lacking ADAP expression in this work revealed a novel role of this adaptor protein in megakaryopoiesis. Significant increases in the number of megakaryocytes were observed in the bone marrow and spleen of $ADAP^{-/-}$ mice, and the ADAP-deficient megakaryocytes displayed potentiated capacity in differentiation and development compared to WT megakaryocytes. Mechanistically, ADAP interacts with STAT1, a crucial signalling molecules/transcriptional factor in megakaryopoiesis. Analysis on the activities of STAT1 suggested that the interaction between ADAP and STAT1 posed an inhibitory effect on STAT1, which might be responsible for the negative role of ADAP in megakaryopoiesis. In summary, by characterizing the functional interplays of ADAP with Ubc9 and STAT1 in T-

cell adhesion and megakaryopoiesis, the work in this thesis have contributed towards a better understanding of the versatile roles of ADAP in immunity.

TCR-mediated integrin activation and T-cell adhesion

The initial studies on ADAP have proposed a positive and pivotal role in modulating the proximal TCR signalling in T cells, as well as integrin signalling destined for integrin activation and adhesion in T cells, dendritic cells, and platelets (reviewed in Chapter 1). However, these signalling pathways are not supported by ADAP solely, but rather contingent upon the interactions between ADAP and its interacting partners. The past two decades have witnessed an expanding repertoire of ADAP binding partners and identified the indispensable roles of these interactions in the signal transductions. In the context of TCR signalling and integrin signalling in T cells, ADAP was reported to form signalling complexes with 1) adaptor proteins SLP-76, SKAP1, SKAP-HOM, and Nck; 2) tyrosine kinases Fyn, Tak1, and phosphatase SHP-1; 3) signalling molecules VASP/Ena and CARMA1; 4) small GTPase family Rap1/RapL/RIAM via the effector protein SKAP1; 5) adhesion molecules talin and kindlin via the ADAP/SKAP1 complex (da Silva et al., 1993, Liu et al., 1998, Geng et al., 1999, Marie-Cardine et al., 1998b, Coppolino et al., 2001, Lettau et al., 2014, Medeiros et al., 2007, Burbach et al., 2011, Sauer et al., 2016, Menasche et al., 2007, Raab et al., 2011). Work from Chapter 2 has identified a novel interaction partner to ADAP–Ubc9, the sole SUMO E2 ligase. The association between ADAP and Ubc9 was facilitated by the NLS2 domain of ADAP, and was further strengthened upon TCR stimulation. Thus, this piece of evidence has expanded the current repertoires of ADAP binding partners by pinpointing the interactions with the sole SUMO E2 ligase in T cells.

Given that SUMOylation is a key posttranscriptional modification that has a particular effect in tuning the protein-protein interaction (Geiss-Friedlander and Melchior, 2007), the potential impinge of SUMO pathway on the T-cell adhesion as well as on ADAP was examined by knockdown of the sole E2 ligase in T cells at first. Ubc9 is conserved from yeast to human and is expressed ubiquitously, and gene disruption of Ubc9 in mice is lethal (Seufert et al., 1995, Hayashi et al., 2002). Despite that many reports have addressed the regulations of Ubc9 on cell movement (Flotho and Melchior, 2013, Gareau and Lima, 2010), its regulation is exerted in a manner that is dependent on the specific cell types. For instance, Ubc9 promotes migration and invasion of human small cell lung cancer cells (Li et al., 2013), and those of fibroblast-like synoviocytes in Rheumatoid Arthritis mouse (Li et al., 2014). However, the underlying mechanism of Ubc9 on cell migration or motility remained poorly

addressed. The findings in this thesis showed for the first time Ubc9 is required for TCR-mediated T-cell adhesion. Knockdown of Ubc9 in T cells resulted in impaired integrin activation and T-cell adhesion upon TCR stimulation. The underlying mechanism was further unravelled by the identification of a functional cooperation with ADAP. While not affecting the ADAP-mediated TCR proximal signalling events, the association of Ubc9 with ADAP is mandatory for TCR-mediated integrin activation and adhesion, with a particular impact on the membrane targeting of small GTPase Rap1. These findings have therefore conferred a novel role of Ubc9 in T-cell adhesion, and for the first time described a functional interplay between the SUMO pathway with T-cell adhesion.

TCR-mediated integrin activation and T-cell adhesion is tightly controlled by two major regulatory layer as mentioned in Chapter 2, the adaptor proteins SLP-76-ADAP-SKAP1 module (Wang and Rudd, 2008, Raab et al., 2010) and the Rap1-RapL-RIAM module (Kinashi, 2005). The ADAP/SKAP1 module in T cells selectively associate with the Rap1-RapL-RIAM module at the plasma membrane but collectively facilitate TCR-mediated adhesion (Kliche et al., 2012). The findings in this thesis suggested that Ubc9-ADAP interaction plays a selective role in integrin activation via facilitating the TCR-induced translocation of Rap1, but not RIAM, to the membrane. It is, therefore, a further evidence supporting the interrelated partnership between these two modules. And more importantly, the Ubc9-ADAP cooperation represents a new layer of the temporal and spatial regulation on TCR-mediated integrin activation and T-cell adhesion.

Megakaryopoiesis

In addition to T cells, ADAP is widely expressed in other hematopoietic lineages including mast cells, platelets, and macrophage (Wang and Rudd, 2008, Engelmann et al., 2013, Thiere et al., 2016). Characterization of the ADAP$^{-/-}$ mice in the early studies showed that ADAP$^{-/-}$ mice are viable but exhibit modest thrombocytopenia, and ADAP-deficient platelet failed to retain the platelet aggregation, adhesion and spreading (Kasirer-Friede et al., 2014, Kasirer-Friede et al., 2004, Kasirer-Friede et al., 2007). Meantime, SLP-76$^{-/-}$ mice displayed impaired viability and haemorrhaging, due to defects in platelet activation (Clements et al., 1999). These findings indicate a role for the adaptor proteins in platelets, and are further supported by evidences from human patients. In two case report on the familial thrombocytopenia patients, defects in ADAP-coding gene FYB were reported (Hamamy et al., 2014, Levin et al., 2015). Interestingly, the ADAP$^{-/-}$ mice have enlarged spleens as compared to the wild-type animals (Peterson et al., 2001), which may be

responsible for the increased removal of platelets from circulation system and eventually leads to thrombocytopenia. However, the detailed mechanism underlying the reduced number of platelet in the ADAP$^{-/-}$ mice and FYB-defective human patients was poorly addressed so far.

The work in this thesis have described a negative role of ADAP in megakaryopoiesis *in vivo*. Increased number of megakaryocytes were found in the bone marrow and spleen from ADAP-deficient mice. The ADAP-deficient megakaryocytes are further showed an accelerated differentiation compared to the WT-mice derived megakaryocytes, suggesting that ADAP impinges on megakaryocytic development. These results have therefore conferred a new role of ADAP as important regulator for megakaryopoiesis. Given that the platelet formation is a constant continuum in platelet development, the low platelet counts might be a collective consequence from imbalance between megakaryocytic development and platelet destruction. For instance, IL-21 increases the development and differentiation of megakaryocytes generated from HSC, as well as the platelet generation, but also increases platelet clearance, leading to a consequent reduction in blood cell counts (Benbarche et al., 2017). The results in this thesis, together with the observation as low platelet counts in ADAP-deficient mice and FYB-defective human patients, have highlighted ADAP as important for platelet formation. However, further studies are needed to assess the functionality of platelet destruction in this mice. A thorough characterization on the balance between megakaryopoiesis and platelet homeostasis in the ADAP-deficient mice will surely provide critical insight into the current understanding of thrombocytopenia in ADAP-deficient mice and FYB-defective human patients.

Additional clues to the mechanisms of ADAP functions may come from studying its binding partners. We have for the first time described a novel interaction between ADAP and STAT1, a transcriptional factor that is implicated in the control of megakaryopoiesis. A segment of far N-terminus of ADAP facilitate the binding with the SH2 domain of STAT1. Although the discovery that ADAP interacts with the SH2 domain of its binding partner have been well-documented, e.g. Fyn, SLP-76, and NCK, their associations are formed via phosphotyrosine motifs on ADAP. To our knowledge this is the first evidence linking the far N-terminus of ADAP with a function of cells. Therefore, these findings have provided another evidence on the functional complexes formed by ADAP, and thus expanded the repertoire of ADAP binding partners and our knowledge on the functions of each intermolecular domains.

While quite distinct from its classically positive roles in integrin signalling in T cells, platelets, and macrophage, ADAP poses a negative impact on the megakaryopoiesis. This inhibitory role is exerted via impingement on the activity of STAT1. In ADAP-deficient cells, a potentiated STAT1 tyrosine and serine phosphorylation, along with enhanced transcriptional activity and upregulations of STAT-1-targeted genes were observed compared to the WT cells. Further work is needed as to rescue the ADAP-deficient phenotype by re-expression of ADAP, thus allowed the examination of ADAP binding-deficient mutants for their ability to restore the negative regulations on STAT1 activities and megakaryopoiesis. Interestingly, in line with these findings, it's a previous study discovered that ADAP-deficient CD8 T cells exhibit enhanced activation of STAT5 during the memory phenotype CD8 T cell generation (Fiege et al., 2015). Given that STAT1 is one of the many crucial factors that regulates megakaryopoiesis, other members of STAT family may also play a role in the regulation, e.g. STAT3 and STAT5 (Chen et al., 2010). Future work on clarifying the potential associations between ADAP and STAT3 and STAT5, as well as the effects of ADAP on STAT3 and STAT5 activities during megakaryopoiesis are required. Furthermore, it is of equal importance to characterize the impacts of STAT1 poses on ADAP *vice versa*.

Conclusion

Work in this thesis together with many precedents have implicated ADAP in a plethora of cellular functions including TCR activation for cytokine production, integrin activation for adhesion and cytoskeleton rearrangement, thymocyte development and selection, cytotoxicity of CD8 T cells, hippocampal neuron development, and megakaryopoiesis. Appreciation of the significance of ADAP in regulating intracellular signalling shall also be attributed to the identifications of a growing number of binding partners. The ADAP molecular complex has now served as a paradigmatic example of how the diverse cellular functions can be regulated within a core of signalling molecules, but engages a changing repertoire of binding partners. How ADAP fulfils the versatile roles in multiple cell types, and those yet to be uncovered *in vivo* will surely reveal new insights on to our knowledge towards immune functions, and ultimately, towards the development of therapies targeting specific signalling pathways for the treatment of diseases.

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