Published in final edited form as:

Oncogene. 2021 January ; 40(2): 345–354. doi:10.1038/s41388-020-01533-3.

Deficiency of Wiskott–Aldrich syndrome protein has opposing effect on the pro-oncogenic pathway activation in nonmalignant versus malignant lymphocytes

Seong-Su Han¹, Kuo-Kuang Wen¹, Yatin M. Vyas¹

¹Division of Pediatric Hematology-Oncology, Carver College of Medicine and the Stead Family University of Iowa Children's Hospital, Iowa City, IA 52242, USA

Abstract

Immunodeficiency is associated with cancer risk. Accordingly, hematolymphoid cancers develop in Wiskott–Aldrich syndrome (WAS), an X-linked primary immunodeficiency disorder (PID) resulting from the deficiency of WAS-protein (WASp) expressed predominantly in the hematolymphoid cell lineages. Despite the correlation between WASp deficiency and hematolymphoid cancers, the molecular mechanism underlying the oncogenic role of WASp is incompletely understood. Employing the WASp-sufficient and WASp-deficient cell-pair model of human T and B lymphocytes, we show that WASp deficiency differentially influences hyperactivation versus inhibition of both CDC42:ERK1/2 and NF- κ B:AP-1 pro-oncogenic signaling pathways in nonmalignant versus malignant T and B lymphocytes. Furthermore, WASp deficiency induces a cell-type specific up/down-modulation of the DNA-binding activities of NF- κ B, AP-1, and multiple other transcription factors with known roles in oncogenesis. We propose that WASp functions as a putative “tumor-suppressor” protein in normal T and B cells, and “oncoprotein” in a subset of established T and B cell malignancies that are not associated with the NPM-ALK fusion.

Introduction

In Wiskott–Aldrich syndrome, life-threatening complications such as autoimmunity and hematolymphoid malignancies, especially lymphomas, develop in a subset of patients [1, 2]. Although these complications are typically reported in patients lacking WASp-expression and manifesting higher clinical-grades of the disease, they also occur in patients expressing mutant-WASp and manifesting milder clinical-grades (e.g., X-linked thrombocytopenia). How WASp insufficiency triggers oncogenic transformation of normal T and B cells, and conversely, how it modulates oncogenicity in the established T and B cell malignancies is ill-understood. Although, impaired immunosurveillance from deficient NK cell and/or T cell

Yatin M. Vyas, yatin-vyas@uiowa.edu.

Author contributions SSH generated all CRISPR/Cas9-mediated *WAS* gene KO cellular models and performed the majority of molecular and cellular assays. KKW performed all Western blots, flow studies, and cell-survival assays. YMV conceived the study, designed the experiments, analyzed and interpreted the data, and wrote the paper.

Conflict of interest The authors declare that they have no conflict of interest.

Supplementary information The online version of this article (<https://doi.org/10.1038/s41388-020-01533-3>) contains supplementary material, which is available to authorized users.

function contributes to oncogenesis (cell-extrinsic model), the finding that revertant T cells (spontaneous re-expression of WASp in WAS T cell lineage only) are insufficient to prevent B cell lymphoma/B cell lymphoproliferation in WAS [3], proposes also a cell-autonomous defect contributing to B cell oncogenesis in WAS (cell-intrinsic model). In support of the cell-intrinsic model, both human- and murine-WASp were recently shown to function as a tumor-suppressor protein [4, 5]. WASp deficiency triggered early-onset malignancies in $p53^{+/-}$ mice [4], and enabled oncogenicity in human NPM-ALK⁺ anaplastic large T cell lymphoma (ALCL) by increasing CDC42-GTP and MAPK/ERK activation [5]. The findings in the ALCL model demonstrate that WASp deficiency renders an “already” malignant cell more aggressive, yet whether WASp deficiency can incite similar pro-oncogenic signal-activation in normal (nonmalignant) B cells or T cells, and in B cell or T cell leukemias/lymphomas that are not driven by NPM-ALK fusion or p53 mutation remains unknown. Furthermore, whereas WASp-expression is downregulated in NPM-ALK⁺ ALCL [5], in other NPM-ALK⁻ lymphomas [diffuse large B cell lymphomas (DLBCLs), Burkitt] and T cell leukemias, WASp is not spontaneously downregulated, which points to the fundamental difference in how WASp interfaces with the biology of different hematolymphoid cancers. Adding yet another layer of complexity to WASp role in lymphomagenesis are the findings that WASp promotes oncogenesis and invasiveness of NPM-ALK⁺ ALCL, and conversely, knock-down of WASp decreases in vivo ALCL tumor growth [6]. These findings implicate WASp as an “oncoprotein”. Such paradoxical roles of WASp in oncogenesis point to a gap in knowledge about the contextual subversion of molecular mechanisms/pathways by WASp deficiency, and raise the possibility for unique differences in how WASp deficiency manifests in T versus B lymphocytes, and in nonmalignant versus malignant lymphocytes. Our studies have revealed that WASp role in promoting or repressing oncogenic signal-activation is context-dependent, by discovering the opposing effects of WASp deficiency on the activation of CDC42:ERK and NF- κ B:AP-1 signaling pathways in a subset of non-malignant versus malignant T and B cells.

Results

Differential expression patterns of N-WASp and WIP in WASp-deficient T cells and B cells

In order to directly test the role of WASp in oncogenesis, we generated wild-type/*WAS* gene knock-out (WT/WKO) isogenic cell line pairs, nonmalignant and malignant, as a way to also normalize any salutary/deleterious effects of disparate genotypes, cancer subtype, or viral (HTLV-1/EBV) infection on pro-oncogenic signaling and cellular behavior. First, WASp expression was undetected in all T and B cell-types, malignant or nonmalignant, subjected to *WAS* gene knock-out (WKO) by CRISPR/Cas9, and in nonmalignant B cells carrying pathogenic *WAS* mutations (Fig. 1a, b). Second, because neural-WASp (N-WASp) and WASp-interacting protein (WIP) have been shown to promote oncogenicity in different cancer models [7–10], we tested how WASp-loss influences the expression of these WASp-family proteins. In WASp-deficient nonmalignant T and B cells, the expression of N-WASp was largely unaffected, whereas that of WIP was increased 2- to 3-fold relative to WT, the latter however observed only in non-malignant B cells (WAS03, WAS68) but not in T cells (Fig. 1a, b). By contrast, in WASp-deficient malignant T and B cells, WIP expression was largely unaffected, whereas that of N-WASp was modestly decreased in Farage, OCI-Ly19,

and Raji (Fig. 1a, b). Notably, unlike NPM-ALK⁺ T cell lymphoma (ALCL), where WASp and WIP were both downregulated [5], neither protein was spontaneously downregulated in any wild-type (WASp-sufficient) malignant T or B cell leukemia/lymphomas tested (Fig. 1a). Accordingly, the secondary effect of WASp deficiency on N-WASp and WIP protein expression is cell-type and cancer specific, which could further modulate the effect of WASp deficiency on oncogenicity.

WASp deficiency has opposite effects on CDC42-GTP activation in non-malignant versus malignant T and B lymphocytes

Because CDC42 hyperactivation promotes tumorigenesis [11], and “active” CDC42-GTP is ectopically increased in WASp-deficient ALCL relative to WT ALCL [5], we quantitated the relative abundance of CDC42-GTP fraction in our WASp-sufficient and WASp-deficient T cell and B cell pairs (Fig. 1c). In WASp-deficient, nonmalignant T cells (ND1-WKO) and B cells (WAS03, WAS68), the fraction of activated CDC42-GTP level (quantified by G-LISA assay) normalized to their respective total CDC42 protein expression level (quantified by gel densitometry of Western blot) was increased by 20–60% relative to their WASp-sufficient (WT) controls (Fig. 1c, bottom panel). Thus, the effect of WASp deficiency on the ectopic hyperactivation of CDC42-GTP in nonmalignant T cells and B cells is similar to that in a WASp-deficient malignant T cell lymphoma (NPM-ALK⁺ ALCL) [5]. By contrast, in 5 WASp-deficient (WKO), malignant T and B cell lines (Jurkat-WKO, Farage-WKO, HBL-1-WKO, OCI-Ly19-WKO, Raji-WKO), the fraction of activated CDC42-GTP level, when normalized to total CDC42, was decreased by 5–60% relative to their corresponding WT pair (Fig. 1c, bottom panel). (Note, although the absolute CDC42-GTP level in OCI-Ly19-WKO cells was increased relative to WT, the relative fraction of CDC42-GTP was decreased when normalized to an ectopically increased total CDC42 expression in OCI-Ly19-WKO cells). Thus, the effect of WASp deficiency on CDC42 hyperactivation in a subset of malignant B cell lymphomas and a T cell leukemia (Jurkat) is opposite to that in WASp-deficient NPM-ALK⁺ ALCL. Hence, WASp deficiency can both decrease or increase active CDC42-GTP levels in human T/B cells, an outcome that is influenced in part by the malignant versus nonmalignant cellular context.

WASp deficiency has opposite effects on MAPK/ERK activation in non-malignant versus malignant T and B lymphocytes

“Active” CDC42-GTP coordinates multiple signal transduction events including those that increase pro-oncogenic signaling via the PI3K/AKT/mTOR or MAPK/ERK pathways, which collectively contributes to malignant transformation (Fig. 2a) [5, 11–15]. We therefore tested how these pro-oncogenic signaling pathways downstream of CDC42 are modulated by WASp deficiency, and if any outcome is further modulated by malignant versus nonmalignant (normal) cellular state. We show that the level of phosphorylated(p) pERK1/2 (Thr202, Tyr204) (MEK1/2-effector) was increased in WASp-deficient, nonmalignant B cells and T cells (Fig. 2b), whereas that of pP38 (MKK3/6-effector) was unchanged, which suggests specificity of WASp effect on the MAPK sub-pathways. By contrast, in malignant WASp-deficient B cells, pERK1/2 level was decreased in DLBCLs (Farage, HBL-1, OCI-Ly19) and Burkitt-lymphoma (Raji) (Fig. 2c). In malignant T cells (Jurkat), however, WASp-deficiency caused an increase in pERK1/2 level relative to the WT control. Notably,

the expression of total MEK1 protein, which phosphorylates ERK1, was comparable in all WT and WASp-deficient, malignant or nonmalignant, B and T cell pairs (Fig. 2c), thereby discounting MEK1-deficiency as an explanation for pERK1/2 deficiency in WASp-deficient lymphomas. Like pERK1/2, the expression of pAKT (Ser473, Thr308) was also increased by WASp-deficiency in nonmalignant B cells and in a subset of malignant B cells (OCI-Ly19-WKO, Farage-WKO). pAKT level remained unchanged in WASp-deficient nonmalignant T cells (T cell-WKO), malignant T cells (Jurkat-WKO) and in a subset of malignant B cells (HBL-1-WKO, Raji-WKO). Finally, the expression level of pP70-S6 kinase (mTOR-effector) was unaffected by WASp-deficiency in nonmalignant T or B cells (Fig. 2b), and hence was not further evaluated in malignant T or B cells. We conclude that WASp-deficiency induces ectopic hyperactivation of pERK1/2 (MAPK:MEK1/2 pathway) in nonmalignant lymphocytes, but their paradoxical hypoactivation in malignant lymphomas, a finding that tracks with the relative abundance/paucity of the CDC42-GTP fraction (Fig. 1c).

WASp deficiency causes differential modulation of the constitutive DNA-binding activity of NF- κ B and AP-1 in nonmalignant versus malignant T and B lymphocytes

Because hyperactivation of pERK1/2 (MAPK) and pAKT (PI3K) signaling pathways promotes oncogenesis, in part by increasing activation of NF- κ B and AP-1 transcription factors [14–17], we next asked if the above-observed effects of WASp deficiency on the activation of CDC42, ERK, and AKT in malignant and nonmalignant cells is mirrored in the constitutive DNA-binding activities of NF- κ B/AP-1 (Supplementary Fig. 1B), and of multiple other transcription factors known to influence oncogenicity. In WASp-deficient, nonmalignant lymphocytes, the DNA-binding activity of NF- κ B, AP-1, ATF2 (AP-1 family-member) and CREB (seen only in WAS B cells), all four transcription factors known to promote oncogenesis [14, 17–19], was markedly increased (Fig. 3a, b, Supplementary Fig. 2). However, the constitutive DNA-binding activities of multiple other transcription factors (OCT1, STAT1, SP1, YY1, MYC, NFATc) were unaffected by WASp deficiency (Supplementary Fig. 2), a finding that underscores the specificity of WASp effect on modulating certain oncogenic signaling pathways in nonmalignant lymphocytes. Conversely, in malignant lymphocytes (Jurkat T cell leukemia, multiple B cell lymphomas), WASp deficiency paradoxically decreased the DNA-binding activity of both NF- κ B and AP-1 (Fig. 3a, b), whereas, its effect on other transcription factors was mixed (Supplementary Fig. 2). Accordingly, WASp deficiency causes an increase or decrease in the DNA-binding activity of NF- κ B and AP-1 in nonmalignant or malignant lymphocytes, respectively.

Inhibition of the hyperactivated CDC42-GTP suppresses NF- κ B:AP-1 activity and cell-growth in WASp-deficient, nonmalignant T and B lymphocytes

To identify which upstream signaling aberration is inducing NF- κ B:AP-1 hyperactivation in WASp-deficient, nonmalignant T and B cells, we chemically inhibited each of three hyperactivated signaling effectors (CDC42-GTP, pERK1/2, pAKT) (Fig. 4a). First, using ML141 (CDC42-GTPase inhibitor), we show a substantive reduction in CDC42-GTP levels in the ML141-treated WASp-deficient lymphocytes relative to their DMSO-treated controls (Supplementary Fig. 3A), this without affecting the expression of total-CDC42 (Supplementary Fig. 3B). Significantly, the ML141-treated WASp-deficient, non-malignant

T and B cells showed a dramatic decrease in the DNA-binding activities of NF- κ B, AP-1, and CREB, monitored by electrophoretic mobility shift assay (EMSA) (Fig. 4a). Notably, these changes occur contemporaneously with a parallel decrease in the accumulation of pERK1/2 and pAKT in the ML141-treated WASp-deficient lymphocytes (Fig. 4b), suggesting that the hyperactivation of CDC42:ERK:AKT pathway is causally-linked to the hyperactivation NF- κ B:AP-1: CREB pathways, and that CDC42-GTP hyperactivation is a driver-defect in WASp-deficient, nonmalignant T and B cells.

To further substantiate this conclusion, we tested if chemically-inhibiting pERK1/2 by SCH772984 or pAKT by LY294002 phenocopy the effect obtained by chemically-inhibiting CDC42-GTP by ML141. First, we show a dramatic reduction in pAKT and pERK1/2 signals, without affecting their total protein expression by Western blot, in WASp-deficient T and B cells treated with their respective inhibitors (Supplementary Fig. 3C). Second, the DNA-binding activities of NF- κ B and AP-1 in these inhibitor-treated cells was reduced, but that of CREB was unaffected (Fig. 4a). Third, inhibiting any component of the hyperactivated CDC42: ERK:AKT signaling pathway by ML141, LY294002, or SCH772984 resulted in a reproducible reduction in cell growth of WASp-deficient, nonmalignant T and B cells, measured by the MTS colorimetric assay (Fig. 4c). However, only CDC42-GTP inhibition by ML141, which we show inhibits the DNA-binding activities of NF- κ B, AP-1, and CREB (Fig. 4a), resulted in the most pronounced, and statistically significant ($p < 0.01$ in B cells; $p:0.02$ in T cells), cell growth suppression in WASp-deficient, nonmalignant T and B cells, in a dose-dependent fashion (Fig. 4c, Supplementary Fig. 4C). As such, without any chemical inhibitors, WASp deficiency *per se* does not cause a statistically-significant change in either cell growth or cell death parameters in nonmalignant T and B cells (Supplementary Fig. 4A, B).

Finally, we selectively inhibited NF- κ B with LC-1 and AP-1 with SR-11302 to test how the magnitude of cell growth suppression caused by directly inhibiting these two transcription factors compares with CDC42-GTP, pERK, or pAKT inhibition. After ascertaining that LC-1 and SR-11302 successfully decreases the DNA-binding activity of NF- κ B and AP-1 respectively (Fig. 4a), we show that inhibiting NF- κ B or AP-1 alone also suppresses cell growth of WASp-deficient, nonmalignant T and B cells, the magnitude (%) of which is higher than in the WT control (Fig. 4c). Moreover, the % of cell growth suppression in WASp-deficient relative to WT cells caused by NF- κ B or AP-1 inhibition alone is comparable to that caused by CDC42-GTP inhibition alone, but is much higher to that caused by pERK or pAKT inhibition alone (Fig. 4c). These findings suggest that the aberrant hyperactivation of CDC42-GTP funnels through NF- κ B and AP-1 to influence cell growth behavior in WASp-deficient T and B cells.

Discussion

Dysregulation of cell-signaling pathways contributes to the development and progression of cancers. Our study has uncovered that WASp-deficiency triggers dysregulation of CDC42:MAPK and NF- κ B:AP-1 cell-signaling pathways that have well-established, interconnected roles in oncogenesis [13, 14, 16, 17, 19]. Similar to the coregulators of transcriptional gene activation, WASp functions to either facilitate (“coactivator”) or impede

(“corepressor”) oncogenic pathway activation in malignant versus nonmalignant lymphocytes, respectively. Accordingly, our model proposes that WASp is part of both “tumor-suppressor” and “oncoprotein” complexes, an outcome modulated by the cellular context (Fig. 3c).

In nonmalignant T and B cells, loss of WASp induces heightened activation of cytoplasmic signaling effectors CDC42-GTP, MAPK/ERK1/2, and/or PI3K/AKT (the latter only in B cells), contemporaneously with the hyperactivation of nuclear transcription factors, NF- κ B, AP-1, CREB and ATF2 (the latter 2 only in B cells). Although the precise mechanism by which WASp deficiency induces these signal-activation events in human T and B cells was not addressed here, a previously-established phenomenon in which WASp (or N-WASp)-deficiency increases murine B cell activation by promoting CD19: B cell receptor (BCR) coactivation may contribute in part to the development of “signal-activation” phenotype in WASp-deficient lymphocytes [20, 21]. Regardless, given the role of hyperactivated CDC42-GTP: ERK and NF- κ B:AP-1 signaling pathways in promoting malignant transformation [5, 11, 16, 17], and of ATF2 and CREB proto-“oncoprotein” activation in cancer [18, 19], these results suggest that WASp functions within the “*tumor-suppressor*” apparatus in nonmalignant (normal) T and B cells, and that WASp deficiency de-represses pro-oncogenic programs, at least those driven by the hyperactivated NF- κ B:AP-1 and/or CREB:ATF2 transcriptional circuits. Indeed, chemically-inhibiting any component of the hyperactivated CDC42-GTP:NF- κ B:AP-1 signaling pathway impedes cell proliferation in vitro, the magnitude of which is higher in WASp-deficient relative to WASp-sufficient T and B cells. As such, only CDC42-GTP inhibition caused cell-growth inhibition that was statistically-significant over control, and one that also was comparable to the cell-growth inhibition caused by directly inhibiting NF- κ B or AP-1 alone.

Together, the finding supports the model that hyperactivated CDC42-GTP and NF- κ B:AP-1 signaling circuits contribute in part to the initiation of oncogenesis in WAS lymphocytes. Because WASp loss causes ectopic hyperactivation also of pERK1/2 and pAKT (the latter only in B cells), these aberrations may also contribute to the oncogenic-signal activation in WAS lymphocytes. Accordingly, similar to NPM-ALK⁺ ALCL (T cell lymphoma), WASp functions as a putative “tumor-suppressor” protein in nonmalignant T and B lymphocytes.

Because B cell malignancies occur more frequently than any other malignancies in WAS patients [3], our identification of a linkage between WASp deficiency and hyperactivation of CDC42:ERK:AKT:NF- κ B:AP1 prooncogenic signaling pathway, specifically in WAS B cells (Fig. 3c), provides a potential mechanism for malignant transformation in WAS B cells (i.e., B cell lymphomagenesis). Moreover, because we show that the ectopic increase in the expression of other WASp-family proteins (N-WASp, WIP) is not sufficient to rescue the abnormal hyperactivation of prooncogenic signaling pathways in WASp-deficient B cells, further emphasizes the tumor-suppressor function of WASp in normal B cells, and its loss in the genesis of B cell malignancies. Intriguingly, WAS mutations are described in patients who develop juvenile myelomonocytic leukemia (JMML) in which Ras/MAPK hyperactivation drives oncogenesis [22], raising the possibility that WASp might function as a “tumor-suppressor” protein also in normal myelomonocytes, and that WASp deficiency

may enable oncogenicity by hyperactivating the CDC42: ERK:NF- κ B:AP-1 signaling network in the myeloid cell-lineage as well.

By contrast, in the “established” malignant T and B cells (Jurkat T cell leukemia; multiple B cell lymphomas), WASp likely functions within the “*tumor-promoting*” apparatus (“oncoprotein” role), such that WASp deficiency represses the ongoing activation of pro-oncogenic signaling pathways linked to CDC42:ERK and NF- κ B:AP-1 [16, 17]. Because YY1 and SP1 transcription factors also promote oncogenesis [23–26], the reduced DNA-binding activity of both YY1 and SP1 induced by WASp deficiency, specifically in Burkitt lymphoma (Raji) and T cell leukemia (Jurkat) cells, further support our model that WASp functions as an “oncoprotein” by modulating additional transcriptional circuits in a subset of cancers. Notably, we show that decreased NF- κ B:AP-1 activity tracks with decreased CDC42-GTP levels in all malignant cell-types lacking WASp. As such, for all lymphomas tested (3 DLBCLs and 1 Burkitt), loss of WASp expression correlates with decreased activation of CDC42-GTP, pERK1/2, NF- κ B, and AP-1. In a T cell leukemia cell-line (Jurkat), however, WASp deficiency correlates with decreased activation of CDC42-GTP, NF- κ B, and AP-1, but with an increased pERK1/2 activation. Accordingly, CDC42-GTP levels differentially modulate pERK1/2 activation in malignant B cells versus malignant T cells, in the context of WASp-deficiency. As such, the effect of Rho-protein GTPases (Rho, Rac, CDC42) on NF- κ B activation is also not always linear, i.e., Rho GTP-ases can either activate or repress NF- κ B, depending on the context. [27]

Regardless, at the minimum, our findings consolidate the contribution of WASp in enabling NF- κ B:AP-1-mediated signal-activation in malignant cells. In support of this argument, a previous study showed that knock-down of *WAS* gene in established NPM-ALK⁺ ALCL decreases colony-formation, invasiveness, and in vivo tumor-growth [6]. Likewise, WAVE3, a WASp-family protein, also functions as an “oncoprotein” in human breast adenocarcinoma, such that WAVE3 deficiency decreases survival and invasiveness of adenocarcinoma cells by repressing NF- κ B: KT-activity [28]. In murine lymphoma and melanoma models, suppressing Wave2 also reduced invasiveness/metastasis [29, 30]. WIP deficiency also impedes growth of the established glioblastoma and breast cancer tumor models [7]. N-WASp deficiency induced in pancreatic adenocarcinoma or cervical cancer represses oncogenicity [8, 9], and in a chemical-induced skin cancer murine model, loss of N-WASp similarly represses tumor growth [10]. The collective evidence therefore suggests that WASp-family proteins (N-WASp, WIP, WAVE) function as putative “oncoproteins” (tumor-promoters) in these cancers. Accordingly, a secondary reduction of N-WASp expression caused by a primary deficiency of WASp, as seen in Farage-WKO, OCI-Ly19-WKO, and Raji-WKO cells (Fig. 1a), may further aid in repressing oncogenicity. This result fits well with our model (Fig. 3c), in which WASp functions as a putative “oncoprotein” in “established” malignant cells, such that WASp deficiency impedes activation of prooncogenic signaling pathways in a subset of B lymphoid malignancies.

Although, this dual-role of WASp in modulating oncogenic signaling pathways mirrors multiple other proteins, including N-WASp, which also acts to either promote or suppress oncogenicity [31–33], how these signaling changes in WASp-deficient cells translate into cellular behavior at the organismal level remains to be elucidated. Our finding that the short-

term, net cell-growth is not increased in WASp-deficient, nonmalignant T and B cells, although unexpected, aligns with the effect of genotoxic carcinogens that also impede, not promote, cell-growth [34]. However, because chemical-inhibition of CDC42-GTP significantly suppresses cell-growth suggests that the deregulated CDC42-mediated signaling contributes in part to ectopic cell-growth in WASp-deficient, nonmalignant T and B cells. Indeed, in the WASp-deficient NPM-ALK⁺ ALCL cells, decreased cell-growth was observed only after inducing a concomitant deficiency of Cdc42 [5].

Certain limitations of our study should be acknowledged. To define WASp role in oncogenesis, we have investigated a limited panel of molecular and cellular readouts in a small subset of cancer subtypes, especially for T cell leukemia that was studied only in Jurkat cell line. Indeed, oncogenic transformation of WASp-deficient lymphocytes will likely involve multiple events that are both cell-intrinsic and cell-extrinsic. Because WASp aids in multiple aspects of the RNA Pol II-mediated gene-transcription [35–38], WASp deficiency could potentially disrupt the expression patterns of three broad classes of genes (oncogenes, tumor-suppressor genes, stability genes), whose imbalanced activation could positively/or negatively influence oncogenicity (cell-intrinsic event) [39]. Other cell-intrinsic events already reported in WASp-deficient lymphocytes that could enable oncogenicity are: (1) increased genomic-instability caused by an aberrant accumulation of transcriptional byproducts called R-loops (mRNA:DNA hybrids) [40], (2) irradiation-induced double-strand breaks (DSBs) [41], and (3) deficient repair of DSBs due to impaired homology-directed repair (HDR) pathway [42]. Our present work and those of others [5] propose ectopic hyperactivation of pro-oncogenic CDC42:ERK:AKT and NF- κ B:AP-1:ATF2:CREB cell-signaling pathways as yet another cell-intrinsic event promoting the benign-to-malignant transformation of WASp-deficient B lymphocytes. These cell-intrinsic aberrations could be further aided by the cell-extrinsic defect of impaired tumor-immunosurveillance from systemic-immunodeficiency in WAS, and the development of an inflammatory microenvironment caused by chronic activation of the DNA damage response (DDR) in the genome-unstable WAS cells [43]. Finally, because WASp localizes to the cytoplasm and nucleus [35, 36], its mis-localization could also influence oncogenicity by causing compartment-delimited functional deficiency as shown for p53, BRCA1, etc [32]. Therefore, testing how pathogenic WAS mutations, including those affecting nucleocytoplasmic transport domains of WASp [36], modulate oncogenic-signaling and biological parameters proposed by Hanahan and Weinberg [44], namely clonal-outgrowth, inhibition of differentiation, senescence, genome-instability, cell-metabolism, cancer-promoting inflammation in the nonmalignant, malignant, and cancer stem-cells, will further refine WASp role in oncogenesis.

Materials and methods

Cells

To generate WASp-sufficient (WT) and WASp-deficient cell-pairs, we used human Th (T helper)- and B cells, normal or malignant. These are: (1) normal Th-cells (HTLV1-immortalized, ND1) described previously [40], (2) leukemic Th-cells (Jurkat), (3) normal-donor (ND03179) and WAS patient-derived B cells (EBV-immortalized), harboring E133K

(WAS03; ID00003) and V75M (WAS68; GM21868) missense mutations were purchased (Coriell Institute, Camden, NJ). (4) B cell lymphomas: Farage, HBL-1, OCI-Ly19, (Diffuse large B cell lymphomas, DLBCLs), Raji (Burkitt lymphoma), were either purchased from American Type Culture Collection (Manassas, VA) or a gift from Dr. S. Janz (Univ. of Iowa). To generate an isogenic WASp-deficient cell-line pair, wild-type (WT) cells were transfected with 2 µg of WASP-CRISPR/CAS9 GFP-tagged plasmid (sc-400712-KO-2, Santa Cruz biotechnology, Santa Cruz, CA) using Amaxa-nucleofaction. GFP⁺ cells were FACS-sorted, plated into 96-well plates by serial dilution, and samples screened by PCR, DNA-sequencing, and Western blot to verify complete *WAS* knock-out (WKO) and WASp-depletion, as we previously described [40]. Mycoplasma contamination was ruled out for all cell lines used, prior to conducting downstream assays (Supplementary Fig. 1A)

Measurement of GTP-bound CDC42-activity

G-LISA kit (Cytoskeleton, Denver, CO) was employed to quantify CDC42-GTP levels using 50µg protein/reaction, as previously described [5].

Pharmacological inhibitors

We employed following chemical inhibitors: ML141 for CDC42-GTP-inhibition (EMD-Millipore, Danvers, MA), SCH772984 for MAPK/ERK-inhibition (Enzo-Biomol, Plymouth, PA), LY294002 for PI3K/AKT-inhibition (Promega, Madison, WI), Lactacystin (LC-1) for NF-κB-inhibition (Enzo), and SR11302 for AP-1-inhibition (Tocris, Ellisville, MO). Other reagents/antibodies used are shown in Supplementary file 1.

Electrophoretic mobility shift assay (EMSA) and Western blot

EMSA, as we described previously [38], was performed in 5 µg nuclear-extract co-incubated with double-stranded oligonucleotide probes that were 5' end radio-labeled with [³²P] ATP. Oligonucleotides for AP-1 (E3201), NF-κB (E3292), OCT1 (E3241), SP1 (E3231), CREB (E3281), MYC (SC-2509), STAT1 (SC-2573), YY1 (SC-2533), and NFATc (SC-2577) were also purchased (Promega or Santa Cruz Biotech). ATF2-oligonucleotide (5'-GATAGATGACCTCA TTTTAATCTTGC-3') was synthesized by the Integrated DNA technologies, IDT (Coralville, IA). Antibodies for the super-shift assay NF-κB p50 (SC-114X), NF-κBp65 (SC-109X) JUN-B (SC-210X), WASp (W0890f), IgG (H-270) purchased from Millipore-Sigma (Burlington, MA). Western Blot assays were performed as we described previously [37].

Cell-proliferation and cell-survival assays

MTS-assay using CellTiter96® Aqueous Non-Radioactive Cell-Proliferation Assay (MTS) (Promega) was employed to assess cell-proliferation. Briefly, 3×10^4 cells (100 µl medium/well) were plated into 96-well plates and incubated for 24 h, 48 h, and 72 h. 20 µl MTS/PMS solution (Promega) was added to each well 4 h before measurement at the 490 nm absorbance using Multiskan-Spectrum Spectrophotometer (Thermo-Scientific, Hudson, NH). Cell survival was assayed by flow cytometry after dual-labeling cells with Propidium iodide/Triton X-100 staining solution and Annexin V. Mycoplasma contamination was tested by LookOut® Mycoplasma PCR detection kit (Sigma-Aldrich).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This work was supported in part by the NIH, National Institute of Allergy and Infectious Diseases (NIAID) grants R21AI138051 and R01AI146380 (to YMV), the University of Iowa Dance Marathon (UIDM) research award (to SSH), Research Bridge Award from the Carver College of Medicine (to YMV), and the Endowment from the Mary Joy and Jerre Stead Foundation (to YMV). A subset of data was obtained at the Flow Cytometry Facility, which is a Carver College of Medicine/Holden Comprehensive Cancer Center core research facility at the University of Iowa. We thank the UIDM for supporting the research laboratory space where this work was carried out.

Funding This work was supported in part by the NIH grant R21AI138051 and R01AI146380 (to YMV).

References

1. Ochs HD, Thrasher AJ. The Wiskott-Aldrich syndrome. *J Allergy Clin Immunol*. 2006;117:725–38. [PubMed: 16630926]
2. Bosticardo M, Marangoni F, Aiuti A, Villa A, Grazia, Roncarolo M. Recent advances in understanding the pathophysiology of Wiskott-Aldrich syndrome. *Blood* 2009;113:6288–95. [PubMed: 19351959]
3. Cheminant M, Mahlaoui N, Desconclois C, Canioni D, Ysebaert L, Dupré L, et al. Lymphoproliferative disease in patients with Wiskott-Aldrich syndrome: analysis of the French Registry of Primary Immunodeficiencies. *J Allergy Clin Immunol*. 2019;143:2311–5. [PubMed: 30796981]
4. Keszei M, Kritikou JS, Sandfort D, He M, Oliveira MMS, Wurzer H, et al. Wiskott-Aldrich syndrome gene mutations modulate cancer susceptibility in the p53[±] murine model. *Oncoimmunology* 2018;7:e1468954. [PubMed: 30393584]
5. Menotti M, Ambrogio C, Cheong TC, Pighi C, Mota I, Cassel SH, et al. Wiskott-Aldrich syndrome protein (WASP) is a tumor suppressor in T cell lymphoma. *Nat Med*. 2019;25:130–40. [PubMed: 30510251]
6. Murga-Zamalloa CA, Mendoza-Reinoso V, Sahasrabudhe AA, Rolland D, Hwang SR, McDonnell SR, et al. NPM-ALK phosphorylates WASp Y102 and contributes to oncogenesis of anaplastic large cell lymphoma. *Oncogene* 2017;36:2085–94. [PubMed: 27694894]
7. Escoll M, Gargini R, Cuadrado A, Anton IM, Wandosell F. Mutant p53 oncogenic functions in cancer stem cells are regulated by WIP through YAP/TAZ. *Oncogene* 2017;36:3515–27. [PubMed: 28166194]
8. Hou J, Yang H, Huang X, Leng X, Zhou F, Xie C, et al. N-WASP promotes invasion and migration of cervical cancer cells through regulating p38 MAPKs signaling pathway. *Am J Transl Res*. 2017;9:403–15. [PubMed: 28337270]
9. Hidalgo-Sastre A, Desztics J, Dantes Z, Schulte K, Ensarioglu HK, Bassey-Archibong B, et al. Loss of Wasl improves pancreatic cancer outcome. *JCI Insight*. 2020;5:e127275.
10. Li H, Petersen S, Garcia Mariscal A, Brakebusch C. Negative Regulation of p53-Induced Senescence by N-WASP Is Crucial for DMBA/TPA-Induced Skin Tumor Formation. *Cancer Res*. 2019;79:2167–81. [PubMed: 30894371]
11. Stengel K, Zheng Y. Cdc42 in oncogenic transformation, invasion, and tumorigenesis. *Cell Signal*. 2011;23:1415–23. [PubMed: 21515363]
12. Etienne-Manneville S. Cdc42-the centre of polarity. *J Cell Sci*. 2004;117:1291–300. [PubMed: 15020669]
13. Maldonado MDM, Dharmawardhane S. Targeting Rac and Cdc42 GTPases in Cancer. *Cancer Res*. 2018;78:3101–11. [PubMed: 29858187]
14. Wagner EF, Nebreda AR. Signal integration by JNK and p38 MAPK pathways in cancer development. *Nat Rev Cancer*. 2009;9:537–49. [PubMed: 19629069]

15. Fruman DA, Rommel C. PI3K and cancer: lessons, challenges and opportunities. *Nat Rev Drug Disco.* 2014;13:140–56.
16. Karin M. Nuclear factor-kappa B in cancer development and progression. *Nature* 2006;441:431–6. [PubMed: 16724054]
17. Chaturvedi MM, Sung B, Yadav VR, Kannappan R, Aggarwal BB. NF- κ B addiction and its role in cancer: 'one size does not fit all'. *Oncogene* 2011;30:1615–30. [PubMed: 21170083]
18. Shankar DB, Cheng JC, Kinjo K, Federman N, Moore TB, Gill A, et al. The role of CREB as a proto-oncogene in hematopoiesis and in acute myeloid leukemia. *Cancer Cell.* 2005;7:351–62. [PubMed: 15837624]
19. Lopez-Bergami P, Lau E, Ronai Z. Emerging roles of ATF2 and the dynamic AP1 network in cancer. *Nat Rev Cancer.* 2010;10:65–76. [PubMed: 20029425]
20. Rey-Suarez I, Wheatley BA, Koo P, Bhanja A, Shu Z, Mochrie S, et al. WASP family proteins regulate the mobility of the B cell receptor during signaling activation. *Nat Commun.* 2020;11:439. [PubMed: 31974357]
21. Liu C, Bai X, Wu J, Sharma S, Upadhyaya A, Dahlberg CI, et al. N-wasp is essential for the negative regulation of B cell receptor signaling. *PLoS Biol.* 2013;11:e1001704. [PubMed: 24223520]
22. Stieglitz E, Taylor-Weiner AN, Chang TY, Gelston LC, Wang YD, Mazor T, et al. The genomic landscape of juvenile myelomonocytic leukemia. *Nat Genet.* 2015;47:1326–33. [PubMed: 26457647]
23. Castellano G, Torrisi E, Ligresti G, Nicoletti F, Malaponte G, Traval S, et al. Yin Yang 1 overexpression in diffuse large B-cell lymphoma is associated with B-cell transformation and tumor progression. *Cell Cycle.* 2010;9:557–63. [PubMed: 20081364]
24. Ramkumar C, Cui H, Kong Y, Jones SN, Gerstein RM, Zhang H. Smurf2 suppresses B-cell proliferation and lymphomagenesis by mediating ubiquitination and degradation of YY1. *Nat Commun.* 2013;4:2598. [PubMed: 24121673]
25. Wang W, Li D, Sui G. YY1 Is an Inducer of Cancer Metastasis. *Crit Rev Oncog.* 2017;22:1–11. [PubMed: 29604932]
26. Beishline K, Azizkhan-Clifford J. Sp1 and the 'hallmarks of cancer'. *FEBS J.* 2015;282:224–58. [PubMed: 25393971]
27. Tong L, Tergaonkar V. Rho protein GTPases and their interactions with NF κ B: crossroads of inflammation and matrix biology. *Biosci Rep.* 2014;34:e00115. [PubMed: 24877606]
28. Davuluri G, Augoff K, Schiemann WP, Plow EF, Sossey-Alaoui K. WAVE3-NF κ B interplay is essential for the survival and invasion of cancer cells. *PLoS ONE.* 2014;9:e110627. [PubMed: 25329315]
29. Meacham CE, Ho EE, Dubrovsky E, Gertler FB, Hemann MT. In vivo RNAi screening identifies regulators of actin dynamics as key determinants of lymphoma progression. *Nat Genet.* 2009;41:1133–7. [PubMed: 19783987]
30. Kurisu S, Suetsugu S, Yamazaki D, Yamaguchi H, Takenawa T. Rac-WAVE2 signaling is involved in the invasive and metastatic phenotypes of murine melanoma cells. *Oncogene* 2005;24:1309–19. [PubMed: 15608687]
31. Lee EY, Muller WJ. Oncogenes and tumor suppressor genes. *Cold Spring Harb Perspect Biol.* 2010;2:a003236. [PubMed: 20719876]
32. Wang LH, Wu CF, Rajasekaran N, Shin YK. Loss of tumor suppressor gene function in human cancer: an overview. *Cell Physiol Biochem.* 2018;51:2647–93. [PubMed: 30562755]
33. Morris HT, Fort L, Spence HJ, Patel R, Vincent DF, Park JH, et al. Loss of N-WASP drives early progression in an Apc model of intestinal tumorigenesis. *J Pathol.* 2018;245:337–48. [PubMed: 29672847]
34. Farber E. Cell proliferation as a major risk factor for cancer: a concept of doubtful validity. *Cancer Res.* 1995;55:3759–62. [PubMed: 7641190]
35. Taylor MD, Sadhukhan S, Kottangada P, Ramgopal A, Sarkar K, D'Silva S, et al. Nuclear role of WASp in the pathogenesis of dysregulated TH1 immunity in human Wiskott-Aldrich syndrome. *Sci Transl Med.* 2010;2:37ra44.

36. Sadhukhan S, Sarkar K, Taylor M, Candotti F, Vyas YM. Nuclear role of WASp in gene transcription is uncoupled from its ARP2/3-dependent cytoplasmic role in actin polymerization. *J Immunol.* 2014;193:150–60. [PubMed: 24872192]
37. Sarkar K, Sadhukhan S, Han SS, Vyas YM. Disruption of hSWI/SNF complexes in T cells by WAS mutations distinguishes X-linked thrombocytopenia from Wiskott-Aldrich syndrome. *Blood* 2014;124:3409–19. [PubMed: 25253772]
38. Sarkar K, Sadhukhan S, Han SS, Vyas YM. SUMOylation-disrupting WAS mutation converts WASp from a transcriptional activator to a repressor of NF- κ B response genes in T cells. *Blood* 2015;126:1670–82. [PubMed: 26261240]
39. Vogelstein B, Kinzler KW. Cancer genes and the pathways they control. *Nat Med.* 2004;10:789–99. [PubMed: 15286780]
40. Sarkar K, Han SS, Wen KK, Ochs HD, Dupré L, Seidman MM, et al. R-loops cause genomic instability in T helper lymphocytes from patients with Wiskott-Aldrich syndrome. *J Allergy Clin Immunol.* 2018;142:219–34. [PubMed: 29248492]
41. Wen KK, Han SS, Vyas YM. Wiskott-Aldrich syndrome protein senses irradiation-induced DNA damage to coordinate the cell-protective Golgi dispersal response in human T and B lymphocytes. *J Allergy Clin Immunol.* 2020;145:324–34. [PubMed: 31604087]
42. Schrank BR, Aparicio T, Li Y, Chang W, Chait BT, Gundersen GG, et al. Nuclear ARP2/3 drives DNA break clustering for homology-directed repair. *Nature* 2018;559:61–66. [PubMed: 29925947]
43. Kawanishi S, Ohnishi S, Ma N, Hiraku Y, Murata M. Crosstalk between DNA Damage and Inflammation in the Multiple Steps of Carcinogenesis. *Int J Mol Sci.* 2017;18:1808.
44. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell* 2011;144:646–74. [PubMed: 21376230]

Fig. 1. The up/or down modulatory effect of WASp deficiency on CDC42-GTP levels is context-dependent.

a Western blot analyses of the indicated isogenic T cell and B cell pairs, nonmalignant (normal) or malignant (B cell lymphomas; T cell leukemia), expressing endogenous WASp (WT control) or lacking WASp (*WAS* gene knock-out by CRISPR/Cas9, WKO) sequentially re-probed with the indicated antibodies. The data are representative of at least three independent experiments performed at different intervals. **b** Gel densitometric quantitation of the Western blot bands from (a) shown as mean + SD for the WASp-deficient sample relative to their corresponding WT sample, after normalizing the values to their respective loading control (β -actin, shown as 1.0). Western blot data are representative of 3–4 independent assays. **c** Quantitation of active CDC42-GTP levels in the indicated cell pairs, WT and WASp-deficient, using the ELISA assay kit (G-LISA). Top panel, shows the actual experimental values as mean + SD, $n = 3–4$ experiments. (*) denotes p values < 0.001 determined by unpaired, two-tailed Student's t test with Welch's correction. Positive (CDC42 protein) and negative (no protein, background signal) controls are also shown. Bottom panel, shows the relative abundance or paucity of active CDC42-GTP fraction normalized to total CDC42 expression (shown as % increase or decrease) in WASp-deficient sample compared to WT control (shown as 1.0).

Fig. 2. The up/or down modulatory effect of WASp deficiency on MAPK/ERK and PI3K/AKT pathways is also context-dependent.

a Diagrammatic depiction of the key downstream effectors of the CDC42, MAPK, PI3K, and mTOR signaling pathways funneling into NF- κ B/AP-1 activation that influences oncogenicity. **b** and **c** Western blots of the whole cell lysates from nonmalignant (**b**) or malignant (**c**) T and B cells, WT and WASp-deficient, sequentially re-probed with the indicated antibodies. Isogenic T cell pair (WT and WKO), heterologous B cells (WT is from a normal donor; WAS03, WAS68 are from 2 WAS patients). Small “p” denotes phosphorylation at the residues shown in parenthesis. The data are representative of at least three experiments conducted at different intervals. Gel densitometric quantitation of the Western blot bands from **b** and **c** are shown under the corresponding images as mean + SD for the WASp-deficient sample relative to their corresponding WT sample (WT = 1.0), after normalizing the values to their respective loading control (β -actin).

Fig. 3. WASp deficiency alters the constitutive DNA-binding activities of NF-κB, AP-1, and multiple other transcription factors in a context-dependent manner.

a The DNA-binding activity of the indicated transcription factors to their respective DNA-sequences (radioactive DNA-probes) monitored by electrophoretic mobility shift assay (EMSA) is shown for the indicated cell types, malignant and nonmalignant. Verification of the NF-κB and AP-1 EMSA-bands was performed by super-shift and competition assays, shown in Supplementary Fig. 1B. The data are representative of at least three independent EMSA assays for each factor. **b** Shows the tabulated summary of the ELISA (CDC42-GTP) and Western blot results (pERK, pAKT) (from Figs. 1 and 2) along with the DNA-binding activities of NF-κB and AP-1 (from Fig. 3a). Increased activation is denoted by up arrow in green, decreased activation by down arrow in red, and unchanged by “unch”. A similar set of data showing modulation of other transcription factors by WASp-deficiency is summarized in Supplementary Fig. 2. **c** Cartoon summarizing the key findings of the above table, which forms the basis to propose a model for WASp’s double-role in oncogenesis in nonmalignant versus malignant lymphocytes. Symbols ↑ or ↓ denotes increased or decreased activation/activity of the indicated factor in the indicated cell type.

Fig. 4. Pharmacological inhibition of hyperactivated CDC42-GTP and ERK pathways in nonmalignant WASp-deficient T and B cells decreases the DNA-binding activity of transcription factors and cell-growth.

a EMSA assays show changes in the DNA-binding activity of three transcription factors (NF-κB, AP-1, CREB) induced by chemically inhibiting (+) or not inhibiting (–) different effectors of the indicated signaling pathways in WASp-deficient cells. Data are representative of 2–3 independent experiments. **b** Western blot showing the effect of suppressing CDC42-GTP by ML141 on pERK1/2 and pAKT activation in the indicated WASp-deficient T and B cells. Gel densitometric quantitation of the Western blot bands are shown as mean + SD for the WASp-deficient sample relative to their corresponding WT sample (WT = 1.0), after normalizing the values to their respective loading control (β-actin). **c** MTS cell proliferation assay to monitor percentage of cell-growth inhibition induced by different signaling factor inhibitors (inh), individually or in combination. The data are shown as mean ± SD, $n = 3$ independent experiments. p value, two-way ANOVA comparing WT against WASp-deficient; ns nonsignificant.