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WASH1 AS AN INTERACTION HUB

WASH1 jako interakční centrum

Bachelor´s thesis

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Declaration

I declare that I wrote this thesis on my own and that I stated all used literature and other information sources. I did not use this work or its significant part to previously acquire any other academic title.

In Prague, 29.4.2025

Abstract

Proteins that have many interacting partners also subsequently perform a variety of functions. WASH1 protein enables branching of the actin network by activating the Arp2/3 complex on the endosomal membrane. In addition to its cytoskeletal function, WASH1 plays a significant role in regulating gene expression, autophagy, and maintaining genome integrity. This thesis provides a summary of the major WASH1 interaction partners not only within the WASH complex but also in its non-canonical functions.

Keywords

WASH1, actin polymerization, cytoskeleton, Arp2/3, NPF, interactions, gene expression

Abstrakt

Proteiny, které mají mnoho interakčních partnerů, také následně plní celou řadu funkcí. Protein WASH1 umožňuje větvení aktinové sítě prostřednictvím aktivace Arp2/3 komplexu na endozomální membráně. Kromě cytoskeletální funkce hraje WASH1 významnou roli při regulaci genové exprese, autofagie a udržování integrity genomu. Tato práce poskytuje přehled hlavních interakčních partnerů WASH1 nejen v rámci WASH komplexu, ale také v jeho nekanonických funkcích.

Klíčová slova

WASH1, polymerace aktinu, cytoskelet, Arp2/3, NPF, interakce, genová exprese

List of Abbreviations

AHR = Aryl Hydrocarbon Receptor

AMBRA1 = Activating molecule in BECN1-regulated autophagy protein 1

Arid1a = AT-rich interactive domain-containing protein 1A

Arp2 = Actin-Related Protein subunit 2

Arp3 = Actin-Related Protein subunit 3

Arp2/3 = Actin-Related Protein 2/3

BAF = BRG1-Associated Factor

BLOC-1 = Biogenesis of lysosome-related organelles complex 1

BLOS2 = Biogenesis of lysosome-related organelles complex 1 subunit 2

BPTF = Bromodomain and PHD finger-containing transcription factor

Btk29A = Burton tyrosine kinase

CCDC53 = Coiled-coil domain-containing protein 53

cDNA = complementary DNA

ChIP = Chromatin Immunoprecipitation

c-Myc = cellular Myelocytomatosis oncogene

DNA-PKcs = DNA-dependent protein kinase catalytic subunit

dNTPs = deoxynucleotide triphosphates

DSB = double-strand break

FAM21 = family with sequence similarity 21

GTP = Guanosine-5'-triphosphate

GST = Glutathione S-Transferase (pokud vyhodim ten fialový odstavec na konci, tak tam nebude)

HeLa = Henrietta Lacks

HSBP1 = heat shock factor binding protein 1

H2AX = H2A histone family member X

ILC3s = group 3 innate lymphoid cells

ISWI = Imitation SWItch

KO = knockout

MAGE-L2 = Melanoma-associated antigen family member L2

MCM = minichromosome maintenance protein complex

NHEJ = non-homologous end joining

NK = natural killer

NKp46 = Natural killer cell p46-related protein

NLS = Nuclear Localization Signal

NPF = Nucleation Promoting Factor

NURF = nucleosome remodelling factor

PCR = polymerase chain reaction

PIP = Phosphatidylinositol phosphate

PI3P = Phosphatidylinositol (3,4,5)-trisphosphate

PRR = Proline Rich Region

PTMs = post-translational modifications

p16-Arc = Actin-related protein 2/3 complex subunit 5

Rab = Ras-related protein

Rbbp4, RBAP46/48 = Retinoblastoma-binding protein 4

Rho = Ras-like GTP-binding protein

RNF2 = RING finger protein 2

SCAR = Suppressor of cAMP receptor

SNF2L = Nucleosome-remodeling factor subunit SNF2L

SWIP = Strumpellin and WASH-interacting protein

TRIM27 = Tripartite motif-containing protein 27

USP7 = ubiquitin-specific protease 7

VCA = verprolin, cofilin, acidic

Vps35 = Vacuolar protein sorting-associated protein 35

WAS = Wiskott-Aldrich Syndrome

WASH1 = Wiskott-Aldrich Syndrome Protein and SCAR Homolog 1

WASP = Wiskott-Aldrich Syndrome Protein

WHD1,2 = WASH Homology Domain 1,2

WT = wild type

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1 Introduction

WASH1 (Wiskott-Aldrich Syndrome Protein and SCAR [Suppressor of cAMP Receptor] Homolog 1) is primarily recognized as a core component of the pentameric WASH complex, which comprises WASH1, FAM21 (Family with Sequence Similarity 21), SWIP (Strumpellin and WASH-Interacting Protein), CCDC53 (Coiled-Coil Domain-Containing Protein 53), and Strumpellin. This complex plays a critical role in actin nucleation on endosomal vesicles, thereby facilitating the proper sorting and trafficking of transmembrane proteins and their ligands, often referred to as “cargo”. WASH1 is evolutionarily conserved from *Entamoeba* to humans and is ubiquitously transcribed across most tissues (Linardopoulou et al., 2007; Su et al., 2004).

WASH1 was identified in 2007 as a novel member of the WASP (Wiskott-Aldrich Syndrome Protein) family of nucleation-promoting factors (NPFs). These NPFs play a critical role in the activation of Arp2/3, the complex responsible for the nucleation of branched actin filaments (Fokin and Gautreau, 2021; Linardopoulou et al., 2007). WASH1 name derives from the Wiskott-Aldrich syndrome, a genetic disorder caused by mutations in the WAS (Wiskott-Aldrich Syndrome) gene located on the X chromosome (Marchand et al., 2001). This gene encodes WASP, another NPF family member with key roles in actin cytoskeleton regulation.

Despite its moderate molecular size (~50 kDa), WASH1 interacts with a broad array of cellular partners. While several of these partners are subunits of the WASH complex, emerging evidence suggests that WASH1 also engages with additional, non-canonical binding partners. Through these interactions, WASH1 contributes to diverse cellular processes, including the regulation of autophagy (Xia et al., 2014a, 2013), transcriptional control, and the maintenance of genome integrity (Hong et al., 2022; Wang et al., 2022; Xia et al., 2017, 2014b).

This thesis aims to summarize the currently known direct interaction partners of WASH1 and to evaluate the functional relevance of these interactions in sustaining cellular homeostasis and viability. The thesis begins with a brief overview of the structural features and subcellular localization of WASH1, followed by a comprehensive examination of both its canonical and non-canonical interactions.

2 Structure and important domains of WASH1

WASH1 protein contains three essential segments: an N-terminal segment with two WASH homology domains (WHD1 and WHD2), a proline-rich region, and a C-terminal VCA (verprolin, cofilin, acidic) domain (see Fig. 1).

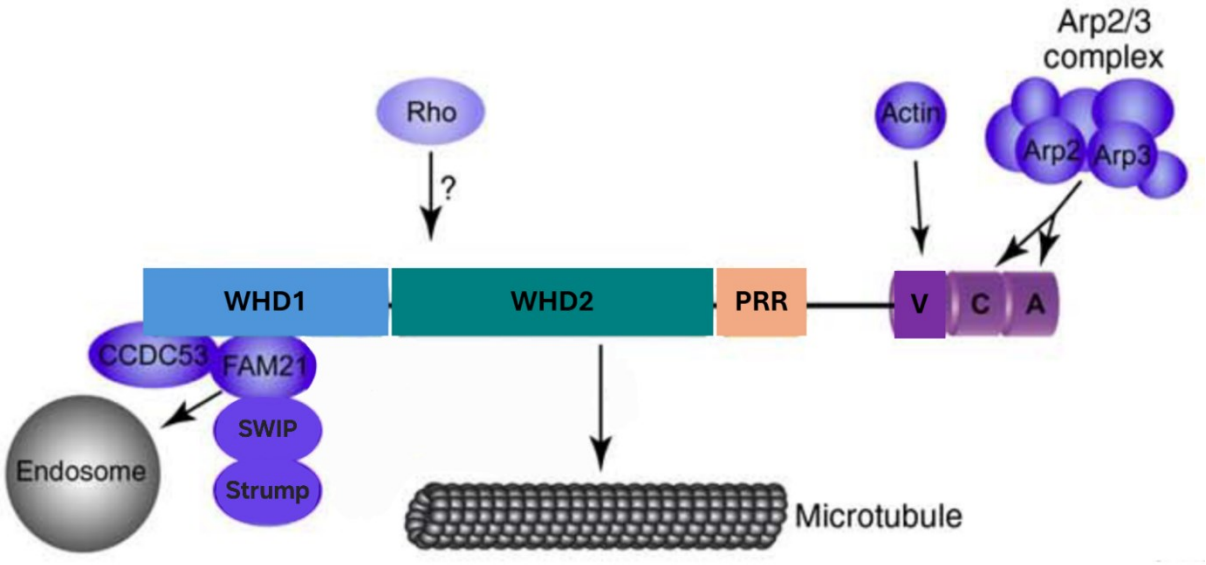

Figure 1 – Important domains of WASH1 protein and their interaction partners. The WHD1 domain interacts with FAM21, holding the WASH1 protein in the WASH complex and targeting it to the endosomal membrane. WHD2 interacts with α-tubulin, indicating an association with microtubules. Interaction of WASH1 with the Rho GTPase has been shown in *Drosophila*, but there is no evidence of this interaction in mammalian cells (represented in the figure with a query). The VCA domain interacts with the Arp2/3 complex and G-actin monomers. (adapted from Rottner et al., 2010)

2.1 WHD1 and WHD2 domains

The WASH homology domains, also referred to as WAHD domains, mediate specific interactions with various WASH1-binding partners and contain conserved residues that serve as sites for post-translational modifications (PTMs) (Linardopoulou et al., 2007).

WHD1 is critical for the interaction between WASH1 and FAM21, a key component of the WASH complex. FAM21 plays an essential role in anchoring the complex to the endosomal membrane through its interaction with retromer, another complex involved in endosomal

protein sorting. The WASH1 WHD1 domain is therefore important for the localization of WASH1 to the endosomal membrane (Gomez and Billadeau, 2009). Moreover, this domain undergoes phosphorylation on conserved tyrosine residues, particularly Tyr141, which has been identified as a crucial step in the activation of the WASH complex (Huang et al., 2016).

WHD2, also known as the tubulin-binding region (TBR), has been shown to directly interact with α-tubulin, which enables WASH1 association with microtubules (Gomez and Billadeau, 2009). Similar to WHD1, WHD2 contains conserved tyrosine residues, the phosphorylation of which contributes to WASH complex activation. In *Drosophila*, this phosphorylation occurs at Tyr273 (Tsarouhas et al., 2019). However, this specific tyrosine is absent in the human WASH1 protein sequence, and the precise residues in human WHD2 responsible for this regulatory modification have not yet been identified.

2.2 Proline-rich region (PRR)

The proline-rich region (PRR), also referred to as the polyproline region (PPR), is situated between WHD2 and the VCA domain. The roles of WASH1 PRR are not fully understood, but there is some evidence of its functions in other WASP family members. For example, in WASP and SCAR, the PRR has been shown to interact with proteins containing Src homology 3 (SH3) domains (Bompard and Caron, 2004; Machesky and Insall, 1998). Given that proline-rich sequences are known to mediate binding to SH3 domains (Li, 2005), it is plausible that the PRR in WASH1 similarly facilitates such interactions.

2.3 VCA domain

The VCA domain, also referred to as the WCA domain, is a highly conserved motif present in all members of the WASP family of NPFs. It enables WASH1 to exert its function in activating the Arp2/3 complex.

As an NPF, WASH1 facilitates actin polymerization by simultaneously binding to monomeric G-actin and the Arp2/3 complex. This dual binding promotes the spatial proximity of these two components, thereby inducing a conformational change in the Arp2 (Actin-

Related Protein subunit 2) and Arp3 (Actin-Related Protein subunit 3) subunits from globular-like to filamentous-like conformation. In the filamentous-like conformation, Arp2 and Arp3 mimic the minus end of a nascent daughter actin filament, anchored to the pre-existing mother filament (see Fig. 2), and allow the attachment of additional G-actin subunits from the cytoplasm to the nascent filament (Rotty et al., 2013).

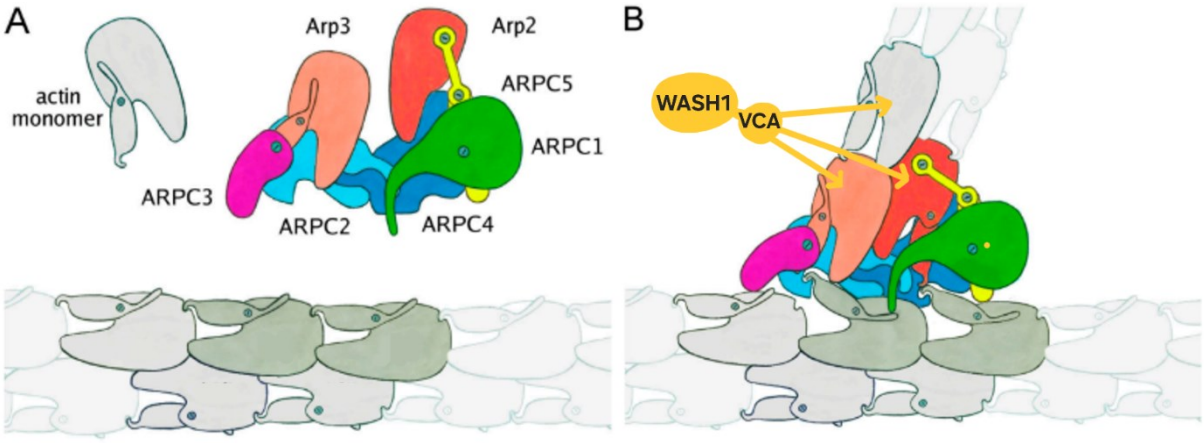

Figure 2 – Arp2/3 complex undergoes conformational changes and binds to the mother actin filament after activation by WASH1. A) Inactive Arp2/3 complex, Arp2 and Arp3 subunits in globular conformation. B) Approximation of the Arp2 and Arp3 subunits and their conformational change from globular to filamentous, mimicking the first two subunits of the daughter filament. Binding of Arp2/3 and the G-actin monomer by the WASH1 VCA domain (represented in the figure with yellow arrows). (adapted from Rouiller et al., 2008)

The VCA domain consists of three motifs: the V motif (verprolin-homology, also known as WH2 - WASP homology), the C motif (cofilin-homology, also known as central), and the A motif (acidic domain).

The V motif has been found in numerous actin cytoskeleton regulators. It is required for binding of the monomeric G-actin, which is essential for the initiation of actin branching. Structural studies of the V motif have identified several critical residues involved in actin binding, four of which (Met7, Ile10, Leu18, and Lys19) are conserved across all WASP family members (Paunola et al., 2002; Van Troys et al., 1996).

The CA region of the VCA domain, encompassing both the C and A motifs, mediates binding to the Arp2/3 complex, specifically interacting with the Arp2 and Arp3 subunits. This function is

evolutionarily conserved among WASH1 homologs across species (Linardopoulou et al., 2007). The A motif is required for binding to the Arp3 subunit of the Arp2/3 complex (Machesky and Insall, 1998), while the interaction of the C motif with Arp2/3 triggers the conformational changes of Arp2/3 that are necessary to stimulate actin nucleation. The C motif contribution to Arp2/3 conformational changes, however, has not been described in detail for the WASH1 protein. Panchal et al. estimate that the C motif role within the NPFs WASP and N-WASP consists of the creation of an amphipathic helix, which binds to an extended hydrophobic surface on Arp2/3. The interaction energy, according to Panchal et al., „could be used to drive an activating conformational change“ (Panchal et al., 2003).

3 *wash* gene

The *wash* gene is located in subtelomeric chromosomal areas. These regions are poorly conserved, showing great variability even within a single species. This variability can contribute to intraspecific phenotypic differences (Gomez and Billadeau, 2009; Linardopoulou et al., 2007). Subtelomeric regions are known to be hotspots for DNA breaks and repair, a feature that underlies their genomic instability and dynamic nature (Rudd et al., 2007). Frequent duplication events in these regions have played a significant role in the primate evolution of the *wash* gene (Cerdán-Vélez and Tress, 2024; Linardopoulou et al., 2007).

The *wash* gene was first identified in 2002 as part of the Mammalian Gene Collection project and was assigned the name MGC52000. Due to its subtelomeric location and resulting sequence variability, the gene has been studied in various species and reading frames, and given different names (Ciccodicola, 2000; Gianfrancesco et al., 2001; Hansen et al., 2005). However, its function remained unclear until 2007, when Linardopoulou et al. proposed that the MGC52000 gene encodes a new member of the WASP family, and they named it *wash*.

The number of *wash* gene copies among species is not conserved. While *Drosophila* and mouse, the two common model organisms, possess just one active copy, 13 paralogs of the *wash* gene have been identified in humans so far (Fig. 3) (Cerdán-Vélez and Tress, 2024). However, only three of them are situated so that the entire gene is expressed and a functional WASH1 protein is produced. The products of the other copies are truncated, either due to frame-shifts or because they contain in-frame stop codons. Alternatively, other *wash* gene copies are missing terminal stop codons (Cerdán-Vélez and Tress, 2024; Kollmar et al., 2012; Linardopoulou et al., 2007).

Given the multiple duplications and resulting paralogs of the *wash* gene, the identification of the ancestral or original gene copy has been a subject of investigation. Recent studies by Cerdán-Vélez et al. suggest that the gene *WASH8P*, located on chromosome 12p (see Fig. 3), represents the most likely original copy of *wash* (Cerdán-Vélez and Tress, 2024; Linardopoulou et al., 2007).

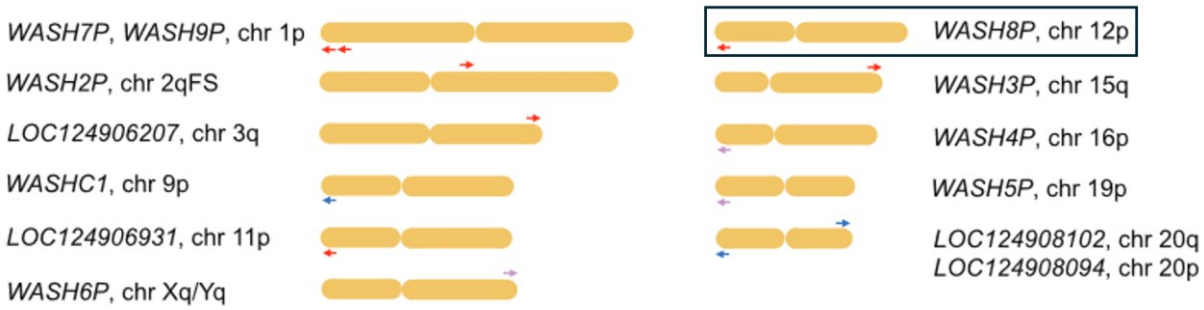

Figure 3 – 13 human paralogs of the *wash* gene. Individual paralogs are depicted by red, purple, and blue arrows. Blue arrows represent active genes. The box represents the original *wash* gene. chr = chromosome (adapted from Cerdán-Vélez and Tress, 2024)

4 Subcellular localization of WASH1 protein

In contrast to other WASP family NPFs, which predominantly localize to the cell cortex, WASH1 is primarily associated with the endosomal membrane, facilitating its tubulation and formation of cargo-containing vesicles.

WASH1 is distributed along most of the endolysosomal pathway. It exhibits partial colocalization with the early/sorting endosome marker Rab5 (Ras-related protein), as well as with the recycling endosome markers Rab4 and Rab11 (Derivery et al., 2009). In contrast, its colocalization with the late endosomal and lysosomal marker Rab7 is weak, indicating that WASH1 localization to lysosomes is rather occasional (Derivery et al., 2009). It was shown that WASH1 plays a functional role in both the recycling and degradative pathways, with an emphasis on the recycling pathway (see Fig. 4) (Seaman et al., 2013). Additionally, WASH1 is not uniformly distributed across the entire limiting membrane. It localizes to discrete, dynamic subdomains that vary in position and composition over time. This dynamic distribution is likely influenced by interactions of the WASH complex with specific membrane lipids (Derivery et al., 2009).

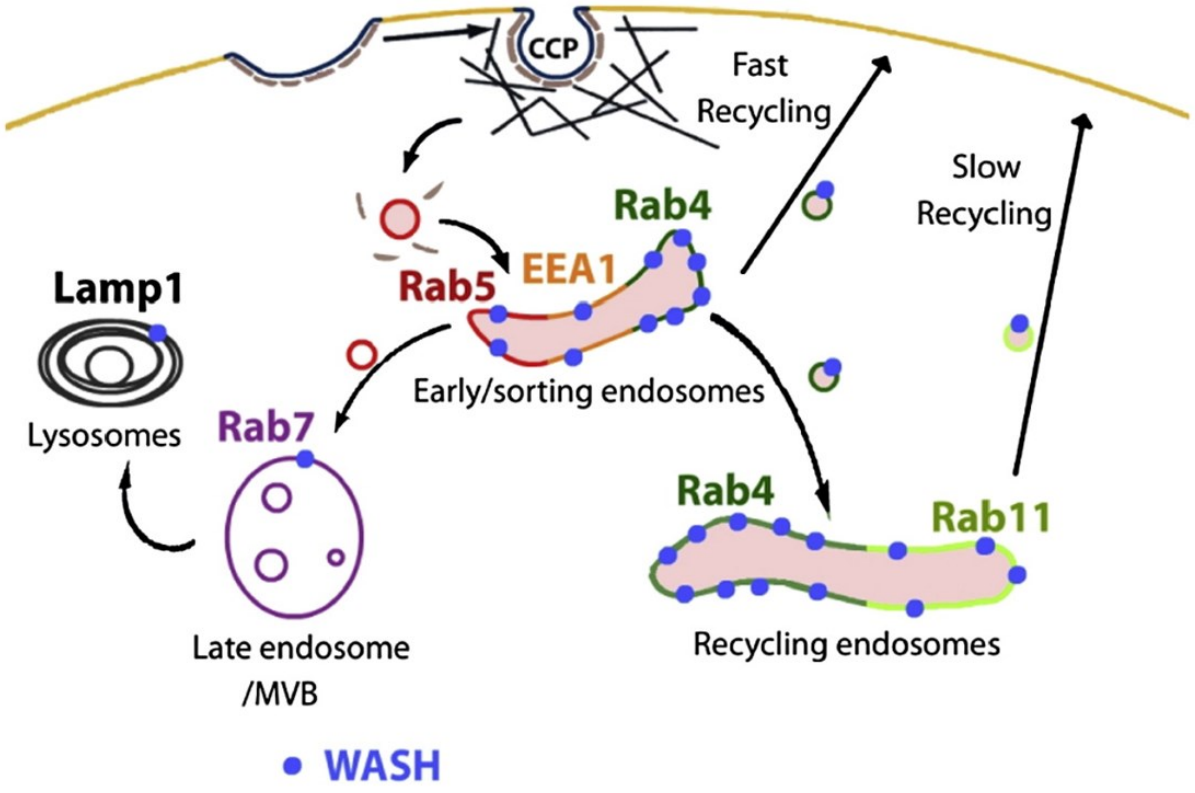

Figure 4 – WASH1 localization in the endolysosomal pathway. Different colours represent different membrane compositions. (adapted from Derivery et al., 2009)

WASH1 localization to the endosomal membrane is dependent on its interaction partners. The association can be mediated by FAM21, which directly interacts with the Vps35 (Vacuolar protein sorting-associated protein 35), a core component of the retromer complex. This interaction facilitates the recruitment of the entire WASH complex to the endosomal membrane (Harbour et al., 2012). The WASH complex recruitment is further supported by binding of the SWIP subunit to membrane phospholipids (Dostál et al., 2023) (see Fig. 5).

In addition to endosomal membranes, WASH1 localizes to centrosomes, where it interacts with γ-tubulin (to be specified in the chapter *Tubulins*) (Monfregola et al., 2010). The pericentriolar matrix is the place of WASH complex assembly (Visweshwaran et al., 2018), and WASH1 also performs its function in initiating actin branching there, possibly influencing centrosome architecture and function (Farina et al., 2016; Obino et al., 2016).

WASH1 is also present in the nucleus (Hochheimer et al., 2002; Linardopoulou et al., 2007). The presence of WASH1 in the nucleus is supported by the fact that it contains an NLS (nuclear localization signal) (Linardopoulou et al., 2007). Moreover, the NLS motifs found in other WASH complex components—except CCDC53, which lacks an NLS—may also facilitate the nuclear import of WASH1 (Verboon et al., 2015). WASH1 nuclear functions are not well understood, but several studies suggest its roles in gene expression (Xia et al., 2017, 2014b), replication initiation (Hong et al., 2022), and maintenance of chromosomal stability (Hong et al., 2022).

5 WASH1 in the context of the WASH complex – canonical interactions of WASH1

In cells, WASH1 canonically exists as part of the pentameric WASH complex, which comprises FAM21, CCDC53, SWIP, and Strumpellin (Hong et al., 2022) (see Fig. 5). These subunits perform essential regulatory and structural functions that significantly enhance WASH1's efficiency as an NPF.

To avoid overactivation of Arp2/3 and excessive actin network branching, WASH1 activity must be regulated within the complex. The results of experiments investigating the role of the WASH complex in regulating the actin-polymerizing function of WASH1 have been inconsistent. Derivery et al. reported that the WASH complex purified from 3T3 cells is constitutively active in promoting Arp2/3-dependent actin nucleation (Derivery et al., 2009). In contrast, *in vitro* experiments by Jia et al. using a human WASH complex purified from insect cells suggest that the complex maintains WASH1 in an inactive state by sequestering its VCA domain (Jia et al., 2010). This discrepancy may stem from methodological differences. Importantly, WASH1 itself does not possess intrinsic autoinhibitory activity. Therefore, the findings by Jia et al., indicating that the WASH complex is necessary to keep WASH1 inactive and prevent excessive actin filament branching, appear more plausible.

5.1 WASH1 interacting partners within the WASH complex

As previously noted, the WASH complex comprises five subunits. However, to date, only two of these subunits—FAM21 and CCDC53—have been demonstrated to directly interact with WASH1 (Fokin and Gautreau, 2021). Both interactions are mediated through the WHD1 domain of the WASH1 protein (Rottner et al., 2010).

5.1.1 FAM21

FAM21 is one of the subunits responsible for the recruitment of WASH1 to the endosomal membrane (Jia et al., 2010). This recruitment is mediated through the interaction between FAM21 and the Vps35 subunit of the retromer complex (Harbour et al., 2012) (see Fig. 5).

Gomez et al. demonstrated a direct interaction between FAM21 and WASH1. Using co-immunoprecipitation assays, they showed that WASH1 could co-precipitate FAM21, suggesting a physical association between the two proteins. This was further supported by immunostaining, which revealed their colocalization. However, this method did not confirm direct binding. To identify the interaction domains, the authors generated a series of WASH1 mutants, each lacking specific regions of the protein. They identified a 51-amino-acid N-terminal segment within the WHD1 domain of WASH1 as essential for binding FAM21. A WASH1 mutant lacking this segment failed to co-precipitate FAM21 (Gomez and Billadeau, 2009). Conversely, a 356-amino-acid N-terminal region of FAM21 was found to be necessary for interaction with WASH1. Deletion of this region abolished WASH1 binding (Gomez and Billadeau, 2009; Jia et al., 2010).

In addition to its role in recruitment, FAM21 may contribute to the stabilization of WASH1. Depletion of FAM21 led to a marked decrease in WASH1 protein levels (Gomez and Billadeau, 2009). This stabilizing effect appears to extend beyond FAM21, as the abundance of individual WASH complex components is influenced by the presence or absence of other subunits (Dostál et al., 2023; Fokin and Gautreau, 2021; Visweshwaran et al., 2018).

5.1.2 CCDC53

CCDC53 plays a crucial role in the early stages of WASH complex assembly, which takes place in the pericentriolar matrix—a region enriched in the assembly factor HSBP1 (Heat Shock Factor Binding Protein 1). CCDC53 interacts with HSBP1 to form a heterotrimer composed of two HSBP1 molecules and one CCDC53 molecule. This heterotrimer is stabilized by two salt bridges, formed between the CCDC53 and the two strands of HSBP1 (Visweshwaran et al., 2018).

In the absence of HSBP1, CCDC53 exists as a homotrimer within the pericentriolar matrix. The assembly factor HSBP1 is thus required for the dissociation of CCDC53 from the homotrimeric complex, so it can interact with other WASH complex subunits.

Within the HSBP1-CCDC53 complex, CCDC53 interacts with WASH1 and FAM21 via its coiled-coil domain, and together these proteins form a so-called ternary complex (CCDC53, WASH1, and FAM21) (Visweshwaran et al., 2018). This ternary complex subsequently associates with

the remaining WASH complex subunits—SWIP and Strumpellin—to assemble the functional pentameric WASH complex (Visweshwaran et al., 2018). The precise molecular mechanisms driving the transition from the ternary complex to the complete pentamer remain unclear and are currently the subject of ongoing research. However, this lies beyond the scope of this work.

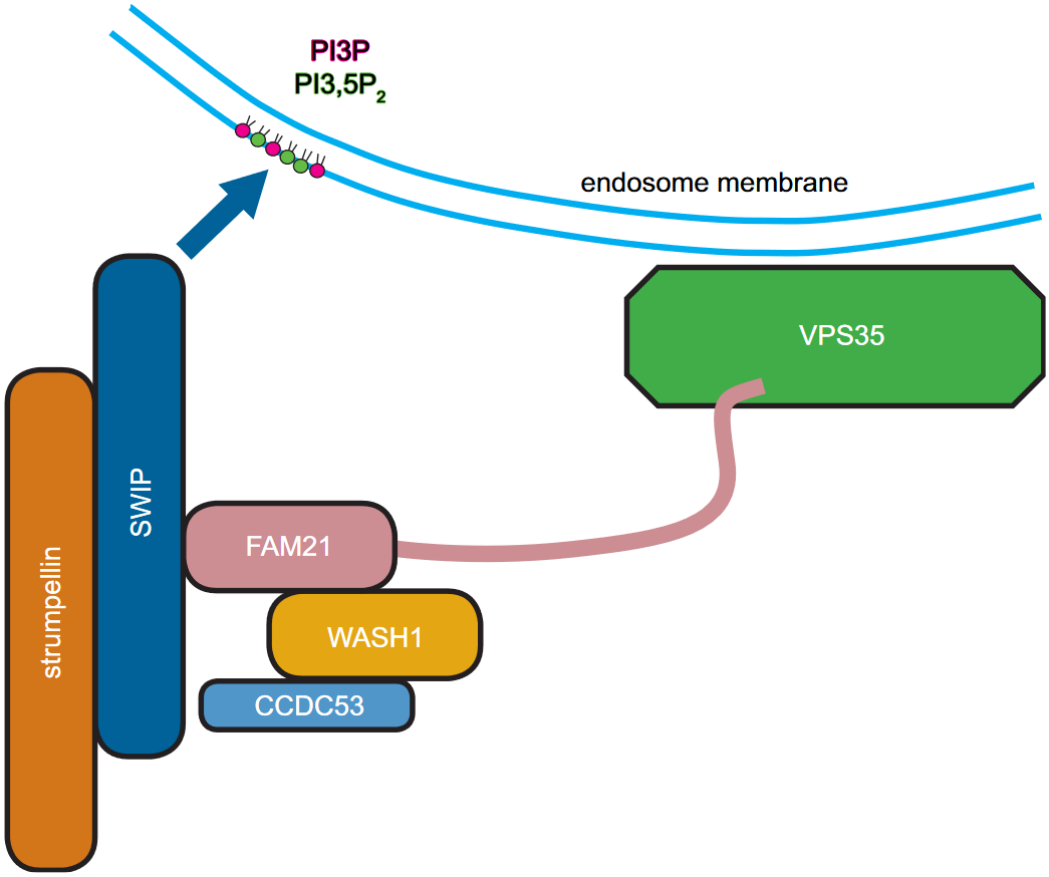

Figure 5 – Schematic model of the WASH complex and its interaction with the endosomal membrane. WASH1 directly associates with CCDC53 and FAM21. The WASH complex-endosomal membrane association is mediated by FAM21, which interacts with a retromer subunit Vps35, and further supported by binding of SWIP to membrane phospholipids. (adapted from Dostál et al., 2023)

5.2 Activation of the pentameric WASH complex

The trimeric ternary complex, composed of WASH1, CCDC53, and FAM21, is functionally active immediately upon its assembly. In contrast, activation of the fully assembled pentameric WASH complex requires a series of post-translational modifications (PTMs) (Fokin and Gautreau, 2021). These modifications include phosphorylation events on conserved tyrosine residues located within the WHD1 and WHD2 domains of the WASH1 protein, as well as

K63-linked ubiquitination at lysine 220 (Lys220). These PTMs are essential for enabling the WASH complex to carry out its cellular functions effectively.

5.2.1 Post-translational modifications

Phosphorylation of WASH1 is mediated by Src family protein kinases. One critical phosphorylation site is tyrosine 141 (Tyr141), located within the WHD1 domain of the WASH1 protein. In human natural killer (NK) cells, the lymphocyte-specific Src family kinase Lck was shown to phosphorylate this residue. This kinase directly interacts with WASH1, as demonstrated by yeast two-hybrid assays, and the phosphorylation event was found to regulate NK cell cytotoxicity. Additionally, other members of the Src family kinases were also shown to phosphorylate WASH1 (Huang et al., 2016).

Another kinase contributing to WASH1 activation is Btk29A (Bruton's tyrosine kinase 29A). In *Drosophila*, Btk29A phosphorylates tyrosine 273 (Tyr273), which is located in the WHD2 domain of the WASH1 protein (Tsarouhas et al., 2019).

In addition to phosphorylation, K63-linked ubiquitination also contributes to the activation of the WASH complex. This modification occurs at Lys220, the only lysine residue in WASH1 known to be ubiquitinated (Kim et al., 2011). The ubiquitination is performed by the MAGE-L2-TRIM27 complex, where TRIM27 (Tripartite motif-containing protein 27) is the E3 RING ubiquitin ligase and MAGE-L2 (Melanoma-associated antigen family member L2) acts as its enhancer (Hao et al., 2013). This ubiquitination induces a conformational change that releases the WASH complex from its autoinhibited state (Hao et al., 2013; Jia et al., 2010).

USP7 (ubiquitin-specific protease 7) plays a dual role in regulating WASH1 activity. It prevents TRIM27 from proteasomal degradation, thereby promoting WASH1 ubiquitination, while also preventing WASH1 overactivation by its direct deubiquitination. In USP7 knockdown cells, a significantly increased pool of ubiquitinated WASH1 was observed compared to wild-type cells, along with elevated levels of Arp2/3 and F-actin on endosomes (Hao et al., 2015). These findings demonstrate that USP7 fine-tunes WASH1 activity and thereby regulates the extent of branched actin formation on endosomes.

5.2.2 Rho GTPase

Many cellular processes and signaling pathways are regulated by the activity of small GTPases. Among them, Rho (Ras-like GTP-binding protein) family GTPases are well-known regulators of actin dynamics and are reported to interact with several WASP family NPFs (Antón et al., 2020; Lane et al., 2014).

Until 2009, Rho GTPase was only known in connection with nucleators of linear actin filaments, such as formins (Kühn and Geyer, 2014). However, Liu et al. (2009) demonstrated that Rho GTPases can also influence branched actin networks through direct interaction with WASH1. Specifically, in Drosophila, the Rho1 GTPase was shown to bind directly to WASH1, predominantly in its GTP (Guanosine-5'-triphosphate)-bound (active) state. Despite this interaction, Rho1 does not enhance WASH1-mediated activation of the Arp2/3 complex or branched actin nucleation, leaving the functional implications of this interaction unclear (Liu et al., 2009).

In contrast to the *Drosophila* findings (Liu et al., 2009), a regulatory role of Rho GTPases in WASH1 activation has not been observed in mammalian cells. Studies by Hao et al. and Jia et al. reported that Rho GTPases do not directly activate human WASH1 *in vitro* (Hao et al., 2013; Jia et al., 2010). Despite ongoing research efforts, no evidence has yet confirmed a role for Rho GTPases in regulating WASH1 activity in mammalian cells. (Fig. 1)

6 WASH1 non-canonical interactions

In addition to its canonical role in activating the Arp2/3 complex and initiating actin filament branching on endosomal membranes, WASH1 also performs several non-canonical functions that are essential for diverse cellular processes. WASH1 is localized to the nucleus, where it contributes to transcriptional regulation and the maintenance of genome integrity. Furthermore, it plays a role in the regulation of autophagy, cell survival, and centrosome function. While many of these non-canonical activities involve WASH1-mediated, Arp2/3-dependent actin polymerization, there are also WASH1 functions that are independent of actin dynamics.

6.1 Tubulins

WASH1 has been reported to directly interact with α-tubulin, a functional unit of microtubules, via its WHD2 domain (Gomez and Billadeau, 2009). This interaction was demonstrated by a GST (Glutathione S-Transferase) pull-down assay, where α-tubulin could coprecipitate WASH1 and *vice versa*, suggesting a potential direct binding relationship. However, while the authors interpreted these findings as evidence of a direct interaction, it is important to note that GST pull-down assays do not unambiguously demonstrate direct domain-specific binding. Given that the WHD domains of WASH1 are known to engage with multiple binding partners, it is plausible that the observed association with α-tubulin may be mediated indirectly through other interacting proteins. Additionally, the potential involvement of other WASH complex subunits in facilitating this interaction cannot be excluded. To rigorously confirm a direct interaction between the WHD2 domain of WASH1 and α-tubulin, alternative methods such as the yeast two-hybrid system could be employed, which allow for more precise mapping of binary protein–protein interactions.

The functional significance of WASH1-α-tubulin interaction may relate to microtubule-dependent endosomal tubulation. In cells depleted of WASH1, elongated tubules are frequently observed protruding from endosomes. These structures likely represent nascent vesicles that cannot undergo proper membrane scission. Such phenotypes are absent in WT cells, suggesting a role for WASH1 in the regulation of endosomal tubulation and vesicle

scission. This hypothesis is further supported by the reported interaction between WASH1 and dynamin, a GTPase essential for vesicle scission, as demonstrated by co-immunoprecipitation experiments (Derivery et al., 2009).

Moreover, WASH1 interacts with γ-tubulin in centrosomes (Monfregola et al., 2010), indicating the role of WASH1 in microtubule organisation centres.

6.2 Rbbp4

WASH1 is involved in transcriptional regulation, where it plays a pivotal role in recruiting chromatin-remodeling complexes to chromatin. Among these, its interaction with the nucleosome remodeling factor (NURF) complex has been the most thoroughly investigated. The NURF complex, a member of the ISWI (Imitation SWItch) subfamily of chromatin-remodeling complexes, comprises three subunits in mammals: BPTF (bromodomain and PHD finger-containing transcription factor), Rbbp4 (Retinoblastoma-binding protein 4, also known as RBAP46/48), and SNF2L (Nucleosome-remodeling factor subunit SNF2L) (Alkhatib and Landry, 2011). Of these, Rbbp4 directly associates with WASH1 (Xia et al., 2014b).

WASH1 knockout (KO) in adult mice led to an accumulation of long-term hematopoietic stem cells, herein referred to as hematopoietic stem cells. This increase was not due to increased proliferation, but rather to the inability of these cells to differentiate (Xia et al., 2014b). After the addition of differentiation factors to WASH1 KO and control WT hematopoietic stem cells, subsequent differentiation was observed only in control cells, which demonstrates an essential role of WASH1 in hematopoietic stem cells differentiation.

The phenotype of WASH1-deficient hematopoietic stem cells resembled that of *c-Myc* (cellular Myelocytomatosis oncogene)-deficient mice (Kalkat et al., 2017; Wilson et al., 2004). Quantitative real-time PCR analysis confirmed that *c-Myc* and its downstream target genes were significantly downregulated in WASH1 KO hematopoietic stem cells (Xia et al., 2014b).

Chromatin immunoprecipitation (ChIP) assays revealed that both WASH1 and the NURF complex associate with the *c-Myc* promoter. Notably, the recruitment of NURF to the promoter was dependent on WASH1. Furthermore, Xia et al. identified the exact region on the promoter that WASH1 binds to. It is a section 750-550 bp upstream from the transcription start site.

Moreover, the interaction between WASH1, NURF, and the c-Myc promoter increased significantly during differentiation in WT hematopoietic stem cells. These results indicate that WASH1 mediates the interaction between NURF and the c-Myc promoter, and this interaction is involved in cell differentiation (Xia et al., 2014b).

At the molecular level, immunoprecipitation experiments showed that anti-WASH1 antibodies co-immunoprecipitate all three subunits of the NURF complex. However, only the Rbbp4 subunit was confirmed by yeast two-hybrid assay to directly interact with WASH1, indicating that Rbbp4 mediates the WASH1–NURF complex association with the c-Myc promoter.

They further focused on whether the ability of WASH1 to recruit the NURF complex to the c-Myc promoter is related to the WASH1 actin-polymerizing function. They showed that WASH1 can initiate actin polymerization in the nuclear extract as well as in the cytosol. This nuclear actin polymerization was shown to be VCA-domain dependent, as only WASH1 variants with an intact VCA domain retained polymerizing activity in the nuclear extract. Furthermore, immunoprecipitation assays indicated that Rbbp4 associates with actin in WT but not in WASH1 KO cells, suggesting that WASH1 facilitates the connection between actin filaments and the NURF complex (Xia et al., 2014b).

The actin polymerizing nature of WASH1's function in this process was also demonstrated by changes in by the changes in DNA accessibility. As a chromatin remodelling complex, NURF can change the structure of chromatin so it is more or less accessible for enzymes to perform gene expression (Hamiche et al., 1999). DNA accessibility of the *c-Myc* promoter was significantly reduced after application of cytochalasin D, an inhibitor of actin polymerization. Moreover, the DNA accessibility of the c-Myc promoter was reduced in WASH1 KO cells compared to WT cells (Xia et al., 2014b). Together, these observations suggest that actin polymerization initiated by WASH1 is necessary for the NURF complex recruitment on the chromatin.

All together, these findings suggest that WASH1 induces actin polymerization on Rbbp4, thereby allowing the NURF complex to remodel chromatin, so the c-Myc transcription factor could be expressed and allow cell differentiation. The interaction of WASH1 with NURF has also been previously confirmed in *Drosophila* (Hochheimer et al., 2002), indicating that the interaction of the WASH1 protein with chromatin remodelling complexes is evolutionarily conserved.

Although many aspects of the nuclear NURF-associated functions of WASH1 have already been described, much still remain elusive and need to be further investigated. For example, cytoplasmic WASH1 is known to be intrinsically inactive and needs several PTMs, including phosphorylation and ubiquitination, to become activated (Hao et al., 2013). Whether nuclear WASH1 also requires some PTMs to be able to perform its actin-nucleating function has not been investigated so far. The function of WASH1 in actin polymerization in the nucleus had not been previously demonstrated and has not been confirmed by any other study to date. Furthermore, while Xia et al. suggest that WASH1 links actin filaments to Rbbp4, the precise molecular mechanism remains unclear, and no similar function has been reported for WASH1 previously. Previous studies have only shown the relationship of WASH1 to actin polymerization as the initiation of branching in the Arp2/3 dependent manner. There is also no evidence of WASH1 working alone in mediating the NURF complex attachment to the c-Myc promoter. The interaction surely is mediated by WASH1 protein, but there are no experiments proving that it has no other interaction partners (e.g., from the WASH complex), that are required for this interaction.

6.3 Arid1a

In addition to c-Myc, WASH1 affects the expression of other genes as well. These include, for example, transcription factor *AHR* (Aryl Hydrocarbon Receptor), whose expression is required for the maintenance of NKp46 (Natural killer cell p46-related protein)-positive ILC3s (group 3 innate lymphoid cells). In these cells, WASH1 facilitates *AHR* transcription by recruiting Arid1a (AT-rich interactive domain-containing protein 1A), a key subunit of the BAF (BRG1-associated factor) chromatin remodeling complex (Wilson and Roberts, 2011), to the *AHR* promoter (Xia et al., 2017).

The direct interaction between WASH1 and Arid1a was demonstrated using a yeast two-hybrid assay. Furthermore, both proteins were found to bind to the same region of the *AHR* promoter, and two specific regions within the Arid1a protein (amino acids 968–1,484 and 1,935–2,283) were identified as responsible for binding WASH1. Additional components of the BAF complex were also observed at the *AHR* promoter, and in WASH1 knockout cells, the BAF complex failed to associate with this promoter. These findings strongly suggest that WASH1 is required for BAF complex recruitment to the *AHR* promoter (Xia et al., 2017).

Importantly, WASH1's role in promoting *AHR* expression is dependent on its ability to activate the Arp2/3 complex via its VCA domain, thereby initiating actin polymerization. WASH1 variants lacking the VCA domain were unable to induce *AHR* transcription, in contrast to the full-length protein. Similarly, treatment with cytochalasin D, an inhibitor of actin nucleation, resulted in the suppression of *AHR* expression (Xia et al., 2017). These data confirm the importance of actin polymerization in the process of activating AHR expression.

6.4 MCM2-7

One of the non-canonical functions of WASH1 is maintaining chromosomal stability and playing a role in replication initiation through interaction with MCM2-7. MCM (minichromosome maintenance protein complex) is a DNA helicase that is important for unwinding DNA strands during replication. It consists of six subunits (MCM2-MCM7) forming a heterohexamer that encircles the DNA strand. It is part of the pre-replication and pre-initiation complexes, required for licensing (in late M phase and early G1 phase) and firing (in S phase) of replication origins (Tye, 1999).

Hong et al. discovered that WASH1 interacts with the subunits of MCM2-7 helicase in the HeLa (Henrietta Lacks) cell line. Their experiments demonstrated that WASH1 and FAM21 co-precipitated with MCM helicase components, suggesting their interaction. However, WASH1 silencing did not affect the interaction of MCM with FAM21, suggesting that the interaction between WASH1 and MCM is mediated by FAM21. This is consistent with the fact that WASH1 does not affect the G1/S cell cycle progression in HeLa cells under standard conditions (Hong et al., 2022).

While WASH1 is not required for S-phase progression under unstressed conditions, its role becomes critical during replication stress. Upon hydroxyurea treatment—a replication inhibitor that depletes dNTP pools—WASH1 knockdown cells exhibited reduced proliferation and colony formation, suggesting impaired replication (Hong et al., 2022).

Although WASH1 is not essential for S phase progression and initiation of DNA replication in cells under unperturbed conditions, its importance becomes apparent after application of a replication inhibitor. In this assay, hydroxyurea, an inhibitor of ribonucleotide reductase, was used, which led to a decrease in the pool of dNTPs (deoxynucleotide triphosphates) and thus

the inability to form a new DNA strand. WASH1 knockdown cells showed reduced proliferation and colony formation after application of hydroxyurea (Hong et al., 2022), which may have been caused by impaired replication.

WASH1 depletion also leads to increased chromosomal instability. Hong et al. observed an increased percentage of micronuclei in WASH1 knockdown HeLa and 3T3 cell lines. The formation of micronuclei can be caused by several factors. The main one is chromosomal instability caused by impaired replication. Moreover, when a rescue experiment was performed and WASH1 was re-expressed in WASH1 KO cells, it led to the regained chromosomal stability. Hong et al., therefore, conclude that loss of WASH1 induces chromosomal instability in response to replication stress (Hong et al., 2022).

It is generally known that only a fraction of licensed replication origins will undergo firing and become full-fledged origins in which replication is initiated. On the contrary, most of them, up to 90% (McIntosh and Blow, 2012), are dormant and become active only when the originally active origins are inhibited. The most common reason for the inhibition of these origins is replication stress. When cells are under replication stress (here achieved by hydroxyurea application), origin licensing is ensured by an excess of MCM2-7 (Ibarra et al., 2008; Woodward et al., 2006).

WASH1 has been shown to co-localize with MCM subunits at replication origins. Furthermore, WASH1 knockdown caused a reduction in the amount of MCM7 subunit associated with replication origins, suggesting that WASH1 is important for MCM recruitment to replication origins (Hong et al., 2022).

In conclusion, WASH1 is suggested to be important for loading excess MCM to replication origins in cells under replication stress, providing chromosomal stability and survival of these cells. WASH1 is thought to constitutively interact with MCM components, which was proved using the PLA (proximity ligation assay) for WASH1, FAM21, and MCM7. However, the exact nature of interaction remains unclear.

As WASH1 has been shown to interact with chromatin remodeling complexes, Hong et al. hypothesized that interactions with these complexes could be involved in WASH1-dependent loading of excess MCM on origins. However, there is no direct evidence for this hypothesis, and much remains to be investigated in this field. For example, the question of whether

WASH1-dependent MCM loading on replication origins is dependent on the VCA domain and WASH1-dependent actin polymerization may be the subject of further research.

6.5 Ku

The WASH1-dependent loading of excess MCM on replication origins is not the only WASH1 interaction that contributes to maintaining genome integrity and cell survival. WASH1 is also involved in DNA DSB (double-strand break) repair via NHEJ (non-homologous end joining). It is recruited to the sites of DSBs by the Ku protein, its nuclear-specific interaction partner. The Ku protein is involved in the initial phases of the NHEJ. It is needed for the DSB recognition and recruitment of other NHEJ factors to the DNA damage site (Downs and Jackson, 2004). Once recruited to the DSB, WASH1 contributes to the phosphorylation of histone H2AX (H2A histone family member X) via activation of DNA-PKcs (DNA-dependent protein kinase catalytic subunit), which consequently promotes chromatin relaxation (Wang et al., 2022).

WASH1 and Ku both localize to DNA damage sites. Immunoblotting experiments demonstrated that Ku is required for WASH1 recruitment to double-strand breaks (DSBs), whereas WASH1 is not necessary for Ku localization. This suggests that Ku mediates the recruitment of WASH1 to the sites of DSBs. Furthermore, WASH1 KO cells exhibit increased sensitivity to etoposide (topoisomerase II inhibitor that induces DSBs) compared to WT cells, supporting a role for WASH1 in DSB repair (Wang et al., 2022).

WASH1 KO cells showed more phosphorylated histone H2AX foci, which are considered a biomarker of DSBs. H2AX phosphorylation is required for chromatin relaxation (Lu et al., 2019; Nakamura et al., 2010; Ziv et al., 2006), which enables other proteins of the NHEJ machinery to access the DNA damage site. It is provided by DNA-PKcs, which was less activated in WASH1 KO cells compared to WT. This suggests that WASH regulates the phosphorylation of H2AX through DNA-PKcs activation (Wang et al., 2022).

Notably, the VCA domain of WASH1 is essential for its role in DSB repair. Cells expressing WASH1 lacking the VCA domain exhibited fewer phosphorylated H2AX foci, leading to reduced chromatin relaxation at damage sites. Furthermore, treatment with CK666, an Arp2/3 complex inhibitor, confirmed the importance of Arp2/3-mediated actin polymerization in DSB repair, as CK666-treated cells displayed a higher number of phosphorylated H2AX foci that persisted

even in the presence of fully functional WASH1. Together, these findings indicate that the role of WASH1 in DSB repair lies in the VCA-dependent activation of the Arp2/3 complex (Wang et al., 2022).

While the basic properties of WASH1 in DSB repair have been identified, the details of its protein interactions remain poorly understood and require further investigation. The interaction between WASH1 and Ku was detected through co-immunoprecipitation assays. However, a direct interaction has not yet been confirmed. Notably, this interaction is not mediated by DNA, as the addition of ethidium bromide, which disrupts nearly all DNA-protein interactions (Lai and Herr, 1992), did not affect the co-immunoprecipitation results. This finding suggests that WASH1 and Ku either interact directly or are connected via an unidentified intermediary protein, the presence of which cannot be distinguished by co-immunoprecipitation.

Moreover, the precise mechanism by which DNA-PKcs is activated during DSB repair remains unclear, and WASH1's involvement in this process requires further investigation. Another open question concerns the role of WASH1's VCA domain. While VCA-dependent activation of the Arp2/3 complex has been shown to be necessary for efficient DSB repair, it remains uncertain whether this function is related specifically to actin nucleation or if alternative mechanisms are involved.

6.6 BLOS2 and pallidin

Another interaction partner of WASH1 is BLOS2 (Biogenesis of Lysosome-related Organelles Complex 1 Subunit 2), a centrosomal protein that acts as a scaffold during the biogenesis of lysosome-related endosomal compartments and forms part of the BLOC-1 (Biogenesis of Lysosome-related Organelles Complex 1) complex (Lee et al., 2012).

The direct interaction between WASH1 and BLOS2 was confirmed by yeast two-hybrid assay, and further analysis showed that WASH1 binds BLOS2 through its N-terminal region (Monfregola et al., 2010). In addition to their cytoplasmic distribution, WASH1 and BLOS2 also colocalized in centrosomes, suggesting that, in addition to WASH complex assembly, WASH1 may have other functions in centrosomes.

Besides BLOS2, WASH1 also partially colocalizes with another BLOC-1 subunit, pallidin, which is responsible for linking the BLOC-1 complex to actin filaments (Starcevic and Dell'Angelica, 2004). Based on these findings, Monfregola et al. proposed that WASH1 could represent a novel subunit of the BLOC-1 complex, potentially contributing to its actin filament binding (Monfregola et al., 2010). However, a direct interaction between WASH1 and pallidin has not yet been confirmed.

Additionally, Monfregola et al. demonstrated that the interaction of WASH1 with BLOS2 leads to WASH1 activation in neuronal cells, suggesting that BLOS2 overexpression can trigger WASH1-mediated Arp2/3 activation and branched actin polymerization (Monfregola et al., 2010).

6.7 Autophagy

Another non-canonical function of WASH1 is the regulation of autophagy. Autophagy is a cellular process in which intracellular proteins and organelles are degraded within an autophagosome—a double-membrane structure that encapsulates material destined for degradation and subsequently fuses with a lysosome. This fusion forms an autolysosome, where the material is degraded (Klionsky, 2005; Lee et al., 2010). WASH1 has been found to inhibit autophagy induction through direct interaction with Beclin1 and RNF2 (RING finger protein 2) (Xia et al., 2014a, 2013).

In WASH1 KO mouse embryos, an increased presence of autophagic structures was observed compared to WT embryos, as detected using electron microscopy. Similarly, numerous autophagosome-like structures were observed in WASH1 KO mouse fibroblasts under starvation conditions (Fig. 6), indicating that WASH1 deficiency enhances autophagy induction. Furthermore, Xia et al. demonstrated that WASH1 localizes to closing and recently closed autophagosomes, but not to autolysosomes, suggesting that WASH1 is involved in the regulation of autophagosome formation (Xia et al., 2013).

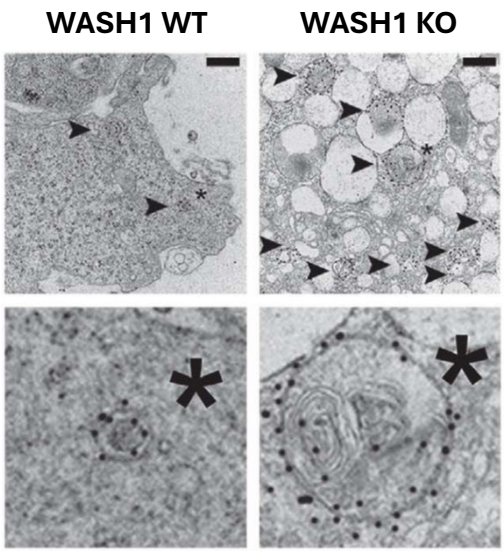

Figure 6 – WT and WASH1 KO mouse fibroblasts after 1h starvation, containing autophagosome-like structures (represented in the picture by black arrowheads). Visualisation by immuno-electron microscopy (antibody against LC3). Scale bar, 500 nm. (adapted from Xia et al., 2013)

Xia et al. screened a human spleen cDNA (complementary DNA) library using WASH1 as bait and identified Beclin1 and RNF2 as new WASH1-interacting partners. The direct nature of these interactions was confirmed using a yeast two-hybrid assay, and further validated by pull-down assays for RNF2 and co-immunoprecipitation experiments for Beclin1 (Xia et al., 2014a, 2013).

Beclin1 significantly influences the autophagy induction. In cells exhibiting autophagy, Beclin1 associates with Vps34, a member of PI3K, to form the Beclin1-Vps34 complex (Kihara et al., 2001). Associated with Beclin1, Vps34 becomes activated and phosphorylates PIP (Phosphatidylinositol phosphate) to form PI3P (Phosphatidylinositol (3,4,5)-trisphosphate), which leads to autophagosome formation (Pattingre et al., 2005; Weidberg et al., 2011). WASH1 inhibits Vps34 activation by suppressing Lys437 ubiquitination of Beclin1, thus preventing Beclin1 from binding to Vps34. This is evidenced by a significantly higher level of Beclin1–Vps34 coprecipitation observed in WASH1 KO cells compared to WT (Xia et al., 2013).

To further confirm the direct interaction between WASH1 and Beclin1, Xia et al. identified the specific region within the WASH1 protein responsible for binding Beclin1. This region spans amino acids 121 to 221 of the WASH protein. A mutant cell line lacking this region exhibited the same phenotype as WASH1 KO cells. Additionally, a corresponding region in the Beclin1

structure, between amino acids 200 and 238, was found to be critical for the interaction with WASH1 (Xia et al., 2013).

The role of WASH1 in autophagy inhibition is independent of its function in Arp2/3-dependent actin polymerization. Silencing of p16-Arc (Actin-related protein 2/3 complex subunit 5), a component of the Arp2/3 complex, did not affect autophagy induction, nor did silencing of FAM21, a WASH complex subunit essential for its attachment to the endosomal membrane. Moreover, WASH1 lacking the VCA domain was able to restore the inhibition of excessive autophagy in WASH1 KO cells, and since the VCA domain is responsible for Arp2/3 dependent actin polymerization, this is direct evidence that the canonical function of WASH1 is not related to the inhibition of autophagy (Xia et al., 2013).

In addition to preventing Beclin1 ubiquitination, WASH1 also directly interacts with RNF2, as mentioned earlier. RNF2 is indirectly linked to Beclin1 ubiquitination through the E3 ligase AMBRA1 (Activating Molecule in BECN1-Regulated Autophagy Protein 1). In the absence of RNF2, AMBRA1 ubiquitinates Beclin1, thereby promoting autophagy. WASH1 interacts with RNF2 and mediates its association with AMBRA1 (Xia et al., 2014a, 2013). This interaction was confirmed by immunoprecipitation experiments, where anti-RNF2 antibody failed to immunoprecipitate AMBRA1 in WASH1-depleted cells, while in the WT cells the precipitation was well pronounced (Xia et al., 2014a). Moreover, even greater AMBRA1 precipitation by RNF2 was detected under autophagy, which stands for the fact that WASH1 enhances the association of RNF2 and AMBRA1. Finally, Xia et al. showed that RNF2 ubiquitinates AMBRA1 via K48-linked ubiquitination, targeting it for proteasomal degradation, which consequently leads to the suppression of autophagy (Xia et al., 2014a).

As the association between AMBRA1 and Beclin1 was dramatically disrupted in WASH1 overexpressed cells, Xia et al. concluded that WASH1 and AMBRA1 compete for the binding site on Beclin1, while AMBRA1 ubiquitinates it, and the competitive binding of WASH1, on the other hand, prevents ubiquitination (Xia et al., 2013).

In conclusion, during autophagy induction, AMBRA1 ubiquitinates Beclin1 via K63-linkage, enabling Beclin1 to bind and activate the Vps34 kinase, which subsequently drives autophagosome formation. WASH1 functions as a negative regulator of autophagy by recruiting the E3 ligase RNF2 to AMBRA1. RNF2 ubiquitinates AMBRA1 via K48-linkage,

targeting it to proteasomal degradation. As a result, Beclin1 is not ubiquitinated and therefore cannot activate Vps34, leading to autophagy suppression. The balance between WASH1 and AMBRA1, which may compete for binding to Beclin1, appears to regulate the extent of Beclin1 ubiquitination and thus the induction of autophagy. Through these mechanisms, WASH1 suppresses autophagy via both RNF2- and Beclin1-dependent pathways.

7 Discussion and conclusion

WASH1 is an interaction hub involved in numerous cellular processes. Its canonical function is to activate the Arp2/3 complex on endosomes, which is essential for initiating branched actin network formation on vesicular membranes. Such activation is exerted by the WASH1 VCA domain. WASH1 performs its canonical function as a part of the pentameric WASH complex. The interactions of the complex members with retromer and membrane phospholipids enable WASH1 to localize to the membrane of endosomal vesicles, the main site of WASH1-dependent actin network formation.

WASH1 also exhibits non-canonical functions beyond its established role in the WASH complex. Specifically, WASH1 is implicated in gene transcription regulation, maintenance of genome integrity, and suppression of excessive autophagy. Current studies describing such non-canonical functions do not mention WASH1 interactions with other WASH complex subunits, suggesting that WASH1 may perform these roles independently of the complex partners. However, direct evidence for the absence of other WASH complex subunits during these non-canonical interactions is lacking.

Most non-canonical WASH1 interactions have been identified using yeast two-hybrid assays, which demonstrate direct binding between WASH1 and its partners. Nevertheless, these studies have not rigorously excluded the possibility that WASH1 remains associated with other WASH complex subunits while playing non-canonical roles. This is particularly relevant given that the stability of WASH1 is compromised when other WASH complex components are depleted, indicating strong interdependence among subunits. Thus, it remains unresolved whether WASH1 exists as two distinct subpopulations: one integrated within the WASH complex and another functioning independently to mediate non-canonical activities. While current data support the hypothesis that WASH1 can fulfill non-canonical functions without other WASH complex subunits, definitive proof is still lacking.

Further research is also needed in the field of WASH1 nuclear roles and its dependency on actin polymerization. It was proved that actin polymerization is needed for WASH1-dependent loading of the NURF chromatin remodelling complex on chromatin. Authors of the same study suggested that WASH1 attaches an actin filament to the NURF complex subunit. However, this

function of WASH1 has not been proven so far, and it remains largely elusive how this attachment could be performed.

In conclusion, in recent years, many WASH1 interactions (summarized in Table 1) have been identified that have contributed to the overall understanding of the role of WASH1 in organisms. However, these are probably only a small fraction of what WASH1 performs in cells, and much remains to be elucidated.

Protein	Cellular function of the interaction	References
G-actin	Branched actin nucleation	(Rotty et al., 2013)
Arp2	Branched actin nucleation	(Rotty et al., 2013)
Arp3	Branched actin nucleation	(Rotty et al., 2013)
FAM21	WASH1 localization on the endosomal membrane	(Jia et al., 2010)
CCDC53	WASH complex assembly	(Visweshwaran et al., 2018)
Lck	WASH complex activation (WASH1 phosphorylation)	(Huang et al., 2016)
TRIM27	WASH complex activation (WASH1 ubiquitination)	(Hao et al., 2013)
USP7	Preventing WASH1 overactivation (WASH1 deubiquitination)	(Hao et al., 2015)
α-tubulin	WASH1 association with microtubules	(Gomez and Billadeau, 2009)
γ-tubulin	WASH1 association with microtubules	(Monfregola et al., 2010)
Rbbp4	Transcriptional regulation	(Xia et al., 2014b)
Arid1a	Transcriptional regulation	(Xia et al., 2017)
MCM2-7	Replicational regulation and maintenance of chromosomal stability	(Hong et al., 2022)
Ku	NHEJ DSB repair	(Wang et al., 2022)
BLOS2	Centrosomal function	(Monfregola et al., 2010)
Pallidin	Centrosomal function	(Monfregola et al., 2010)
Beclin1	Autophagy regulation	(Xia et al., 2013)
RNF2	Autophagy regulation	(Xia et al., 2014a)

Table 1 – Summary of WASH1 direct interaction partners and the cellular functions of these interactions

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