Characterization of nuclear import and export signals determining the subcellular localization of WD repeat-containing protein 42A (WDR42A)

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ABSTRACT
WD repeat-containing protein 42A (WDR42A) is a member of the WD40-repeat proteins. Here, we investigated the localization pattern of WDR42A in living cells. By mutational analysis, a nuclear localization signal, 114PRRRVQRKR122, was for the first time determined. The dominant negative, co-immunoprecipitation and GST pull-down results further demonstrated that the nuclear import of WDR42A was mediated by karyopherin-α1/β1 in conjunction with the GTPase Ran. Additionally, a nuclear export signal, 39IEVEASDLSLSL50, was verified to be a functional NES, which mediated the nuclear export through Chromosome Region Maintenance 1 dependent pathway. All these data suggest WDR42A is a nucleocytoplasmic shuttling protein.

1. Introduction
WD repeat-containing protein 42A (WDR42A) is a member of the WD40-repeat proteins, also named DDB1- and CUL4-associated factor 8 (DCAF8) [1,2]. The WD (also called Trp-Asp or WD-40 motif) is a ~40-amino acids stretch typically ending with a Trp-Asp dipeptide, but exhibiting only limited amino acid sequence conservation [3,4]. Almost all structurally characterized WD-repeat proteins are composed of seven such repeats and fold into seven-bladed propellers [5]. The WD40 domain was ranked as the top interacting domain in Saccharomyces cerevisae and eukaryotic genomes [4,6]. Members of the WD40-repeat protein family don’t have an obvious common function but are involved in diverse cellular pathways including signal transduction, RNA-processing, chromatin dynamics, transcriptional regulation, apoptosis, chemotaxis, cytoskeleton assembly and mitotic-spindle formation, regulation of vesicle formation as well as vesicular trafficking and controlling various aspects of cell division [3,4,7]. Some results have established WD40 β-propellers as a class of ubiquitin-binding domains and that Ub binding may contribute critical yet mechanistically distinct functions to the proteins that contain them [8]. However, no WD40 domain has been found with intrinsic enzymatic activity despite often being part of large molecular machines [6]. Recently, WD40 proteins are proposed to function as an interchangeable substrate receptor to target different substrates selectively and serve as scaffolds to facilitate the function and activity of other proteins, and thus propel cellular networks [6,7].

As an important step toward understanding the detailed functions of WDR42A in vivo is to determine its precise subcellular localization, a study has been undertaken to characterize the subcellular localization and nucleocytoplasmic trafficking mechanism of WDR42A. Maintenance of cellular functions requires transport of many bio-macromolecules into and out of the nucleus. A typical signal-mediated nuclear protein contains a transferable basic nuclear localization signal (NLS), which is recognized by a karyopherin (importin) receptor [9,10]. The karyopherin superfamily also includes plentiful exportins responsible for nuclear export, which recognize a hydrophobic nuclear export signal (NES) [9]. Chromosomal Region Maintenance 1 (CRM1) has been identified as an export receptor recognizing NES directly and is responsible for the export of NES containing proteins [11].

In this study, we employed fluorescence microscopy in live cells and mutational analysis, co-immunoprecipitation and GST pull-
down techniques to characterize its subcellular localization and transport mechanism of WDR42A. Significantly, we identified a functional NLS and NES in WDR42A and characterized the molecular determinants responsible for its subcellular localization and nucleocytoplasmic trafficking, which will shed light on its function in vivo.

2. Materials and methods

2.1. Plasmids construction

The WDR42A gene amplified from the human cDNA library and its deletion mutants were cloned into pEYFP-N1 (Clontech). For expression of HA/Flag-WDR42A, we subcloned the WDR42A from pWDR42A-EYFP to the vector pCMV-N-HA/Flag. The plasmid pGEX6p-1Q69L Ran was a generous gift from Dr. Yoshinari Yasuda and Q69L Ran was then sub-cloned into pEFP-N1 (Clontech) to yield a dominant negative mutant pRan-Q69L-ECFP [12]. In addition, the plasmid pRev-NES-EGFP was a generous gift from Dr. Gillian Elliott, and Rev-NES was sub-cloned into pEFP-N1 to yield pRev-NES-EYFP.

Karyopherin-α1 dominant negative mutant (DN-κα1) and karyopherin-β1 dominant negative mutant (DN-κβ1) were also generous gifts from Dr. Christopher F. Basler [13] and Dr. Haitao Guo [14], respectively, and plasmids pDN-κα1-ECFP and pDN-κβ1-ECFP were constructed in a similar way. In addition, plasmids pcDNA-Flag-importin α3, pcDNA-Flag-κα1, pGEX-HA-κβ1 and pcDNA-Flag-κβ1 were generous gifts from Dr. Yoshihiro Yoneda [15], and the plasmid importin α3 was then sub-cloned into pEFP-N1 to yield importin α3-EYFP. The plasmid GST-WDR42A was a gift from Dr. Johannes C. Walter [1]. All constructs were verified by sequencing and all primers used in this study are available by request.

2.2. Cell culture, treatment and transfection

COS-7 and 293T cells were maintained at 37°C in a humidified atmosphere of 5% CO2 in DMEM (Gibco-BRL) supplemented with 2% fetal bovine serum (Gibco-BRL). COS-7 cells were transfected with 1.0 μg plasmid DNA with Lipofectamine 2000 plus reagent (Invitrogen), according to the instructions of the manufacturer. 293T cells were transfected by standard calcium phosphate precipitation. When indicated, LMB (Sigma) was added to the culture medium at a final concentration of 20 ng/ml for 4 h. At 24 h post-transfection, cells were washed with fresh growth medium and processed for fluorescence microscopy (Zeiss, Germany). All the photomicrographs were taken under a magnification of 40×.

2.3. Indirect immunofluorescent microscopy

COS-7 cells were transfected with the indicated plasmids and immunofluorescent assays were performed as described in our previous study [16].

2.4. Co-immunoprecipitation (Co-IP)

The Co-IP assay was performed as described in our previous study [17]. In brief, 293T cells were transfected with the indicated plasmids for 24 h. For each immunoprecipitation, a 0.4 mL aliquot of lysate was incubated with 0.5% NP40-TB buffer and then centrifuged at 12,000g for 30 min. Glutathione Sepharose-4B beads (GE Healthcare) immobilized with GST-WDR42A were incubated with cell lysates for 4 h at 4°C. The beads were washed with 0.5% NP40-TB buffer four times and the proteins bound to beads were eluted by sample buffer and analyzed by western blot.

3. Results

3.1. Subcellular localization of WDR42A-EYFP fusion protein in transfected cells

To determine the exact subcellular localization of WDR42A, COS-7 cells were transfected with the plasmid pCMV-HA-WDR42A and visualized by indirect immunofluorescence assay. WDR42A exhibited predominant nuclear localization (Fig. 1A), which is consistent with the previous report [18]. Additionally, fluorescence microscopy of live cells technique and enhanced yellow fluorescent protein (EYFP) tagged variants were applied to investigate the intracellular localization of WDR42A (Fig. 1C), and the proteins bound to beads were eluted by sample buffer and analyzed by western blot.

![Fig. 1. Analysis of the subcellular location and sequence of WDR42A. (A) Indirect immunofluorescent assay of the WDR42A protein in COS-7 cells. COS-7 cells were transiently transfected with pCMV-HA-WDR42A for 24 h before fixing with paraformaldehyde, and immunostained with anti-HA mAb. The nuclei were stained with Hoechst. (B) Co-immunoprecipitation of the WDR42A protein in COS-7 cells. COS-7 cells were transiently transfected with pCMV-HA-WDR42A-EYFP or pEFP-N1, and live cells were examined by fluorescence microscopy 24 h after transfection. Light-translucent pictures are introduced to show cellular morphology. The percentages of cells with nuclear (N), predominantly nuclear (N > C), or predominantly cytoplasmic (N < C), cytoplasmic (C) and nuclear and cytoplasmic (N = C) localization were depicted. At least 100 cells were evaluated for each sample. Data were averages of three independent experiments, shown as mean ± S.D.](image-url)
distribution of WDR42A. The plasmid pWDR42A-EYFP encoding WDR42A and EYFP fusion protein was constructed. Representative micrographs showed that the fluorescence of WDR42A was principally restricted in the nucleus, with a faint staining in the cytoplasm. On the contrary, the fluorescence of EYFP monomer was evenly distributed throughout the cell (Fig. 1B). To address whether the nuclear accumulation of the WDR42A was an artifact of over expression, a time course of expression showed that WDR42A accumulated in the nucleus as early as 3 h after transfection when expression levels were very low (data not shown). Taken together, these results indicated that WDR42A localized mainly to the nucleus and this prompted us to characterize the molecular mechanism responsible for its subcellular localization.

### 3.2. Mapping and identification of the nuclear localization signal in the WDR42A

The subcellular localization of WDR42A in fixed cells transfected with pCMV-HA-WDR42A was similar to that observed in live cells transfected with pWDR42A-EYFP, so WDR42A-EYFP was used to map the NLS and NES of WDR42A. The well characterized nuclear targeting sequence contains a contiguous stretch of basic amino acids [19,20]. Program prediction (PSORT II, http://psort.hgc.jp/form2.html) and amino acid (aa) sequence analysis demonstrated that WDR42A contained two potential NLSs in the arginine-rich regions, namely, PRRRVQRKR at aa 114 to 122, and RQRRHHRRWR at aa 553–562, which were designated as NLS1 and NLS2, respectively (Fig. 2A).

To determine whether any or both of the nuclear localization motifs were functional, a series of deletion mutants of WDR42A-EYFP encompassing aa 1–113, 1–122, 51–122, 123–597 and 51–597 fused with EYFP, were constructed as depicted in Fig. 2B. COS-7 cells were transfected with these constructs, and the subcellular distributions of the WDR42A-EYFP mutants were analyzed by fluorescence microscopy at 24 h and 48 h post-transfection. The subcellular localization patterns of live cells at 24 h and 48 h were found to be identical (data not shown). Fluorescence images showed that aa 1–113-EYFP was found mainly in the cytoplasm, while...
aa1–122–EYFP, aa51–122–EYFP and aa51–597–EYFP localized primarily in the nucleus, with faint fluorescence in the cytoplasm, but excluded from the nucleolus. In contrast, aa123–597–EYFP distributed throughout the cell evenly (Fig. 2C). Taken these observations together, we concluded that NLS1, but not NLS2, is responsible for targeting WDR42A to the nucleus.

The function of a putative NLS can be tested by fusion with a cytoplasmic reporter protein and by mutation. To confirm the functional NLS in WDR42A nuclear localization, specific mutations of NLS1 and NLS2 were generated as shown in Fig. 2B. The results showed that mutation of lysine and arginine residues to alanine in NLS1 114PAAAVQAKA122 (aa51–597–mNLS1–EYFP) abrogated the...
nuclear localization of WDR42A51–597 (Fig. 2D). However, mutation in NLS2 553AQAAHHAWAY562 (aa51–597-mNLS2-EYFP) did not alter the subcellular localization pattern of aa51–597-EYFP. Similar results were obtained with mutation of these residues in the context of full-length WDR42A. Furthermore, NLS1 was fused to the N-terminus of EYFP, yielding pNLS1-EYFP. The fluorescent result showed that the NLS1 drove EYFP efficiently to the nucleus, indicating that the NLS1 of WDR42A were sufficient to mediate the nuclear import of a heterologous protein (Fig. 2D). Collectively, these results demonstrated that the nuclear translocation of WDR42A was Ran dependent and required GTP hydrolysis.

3.3. Characterization of the nuclear import mechanism of WDR42A

The Ran protein has been verified to be required for classic NLS-dependent nuclear transport [12]. To further explore the nuclear import mechanism of WDR42A, a dominant negative Ran protein, Ran-GTP Q69L, which is deficient in GTP hydrolysis was introduced to determine whether Ran is indispensable for the nuclear transport of WDR42A. At first, the localization of Ran-GTP Q69L was investigated, as shown in Fig. 3A, Ran-Q69L-ECFP mainly localized to nucleus, which is similar to the wild-type Ran and is in accordance with previous reports [21]. COS-7 cells were co-transfected with pWDR42A-EYFP and pRan-Q69L-ECFP and the subcellular localization was monitored. As shown in Fig. 3B, co-transfection of Ran-GTP Q69L significantly blocked the nuclear import of WDR42A (51–597) whereas WDR42A alone targeted to the nucleus. These results demonstrated that the nuclear translocation of WDR42A was Ran dependent and required GTP hydrolysis.

To further test whether nuclear transport of WDR42A protein is karyopherin-mediated, dominant negative proteins karyopherin-b1 (DN-kb1) and karyopherin-b1 (DN-kb1), which lack the ability to bind karyopherin-b1 and karyopherin-α1, respectively [14,22], were employed to determine whether they are required for the nuclear transport of WDR42A. The DN-kb1-ECFP or DN-kb1-ECFP expressed alone in COS-7 cells accumulated mainly in the nucleus with a little cytoplasmic staining (Fig. 3A), which is consistent with the wild type karyopherin-α1 and karyopherin-b1 [23,24]. Subsequently, COS-7 cells were co-transfected with paa1–597-EYFP and pDN-kb1-ECFP or pDN-kb1-ECFP. As shown in Fig. 3C, co-transfection of dominant negative karyopherin-α1 or karyopherin-b1 blocked the nuclear import of WDR42A, and the former functioned less effectively which meant that there might be some other karyopherin-α receptors mediating the process. As negative controls, the nuclear transport of HSV US11 and importin α3 were not influenced by the dominant-negative karyopherin-α1 and karyopherin-β1, respectively (Fig. 3C), which is consistent with previous reports [25]. To investigate whether WDR42A could interact with karyopherin-α1/b1, 293T cells were co-transfected with pcDNA-Flag-karyopherin-α1 (Flag-kα1) and pCMV-Flag-WDR42A, pGEX-HA-karyopherin-β1 (HA-kβ1) and pCMV-Flag-WDR42A, respectively. At 24 h post transfection, cell lysates were prepared and immunoprecipitation was carried out using anti-Flag or anti-HA monoclonal antibody (mAb) or control mouse IgG. The results showed that WDR42A was co-immunoprecipitated with karyopherin-α1 as well as karyopherin-β1, whereas no such protein was immunoprecipitated by control IgG (Fig. 3D–E). To further confirm these results, we performed the GST pull-down assay using GST-WDR42A protein in the lysates of the Flag-kα1/b1-expressing 293T cells, and found that GST-WDR42A could specifically pull-down Flag-kα1 but not Flag-kβ1. It is known that karyopherin-α1 functions as a heterodimer with karyopherin-β1 [26,27]. Therefore, we added karyopherin-α1 and found that Flag-κ1 co-precipitated with GST-WDR42A when κ1 was present (Fig. 3F). Taken together, these results suggested that the nuclear transport of WDR42A was mediated by karyopherin-α1/b1 under the regulation of Ran GTPase.

3.4. Identification and characterization of the nuclear export signal of WDR42A

NES is characterized by its consensus hydrophobic residue rich sequence, φX2–3φX2–3φXp, where φ is a hydrophobic residue and X is any amino acid residue [20]. Our results above showed that aa51–597-EYFP has stronger nuclear localization than aa1–113-EYFP. Furthermore, the cytoplasmic localization of aa11–113-EYFP suggested that there might be a nuclear export domain in the N terminus of WDR42A (Fig. 2B). An examination of the N-terminal region of WDR42A and NetNES 1.1 Server (http://www.cbs.dtu.dk/services/NetNES/) prediction revealed that it contained a leucine-rich motif, 39IEVEASDLSLSL50, resembling the most prevalent NES in which the leucine residues are critical for its function [28]. To validate this hypothesis, the putative NES was mutated by replacing the leucine and isoleucine residues with alanine residues to create 39AEVEASDASASA (WDR42A-mNES-EYFP; Fig. 4A). COS-7 cells were transfected with WDR42A-mNES-EYFP and examined by live cell fluorescence microscopy. As anticipated, WDR42A-mNES-EYFP was exclusively localized in the nucleus. To determine whether...
the NES alone can direct nuclear export, NES was fused to the N-terminus of EYFP, yielding pNES-EYFP. Representative micrographs showed that the NES drove EYFP efficiently to the cytoplasm, indicating that the NES of WDR42A was sufficient to mediate the nuclear export of a heterologous protein (Fig. 4B).

3.5. Characterization of the nuclear export mechanism of WDR42A

Leucine-rich NESs have been identified in an increasing number of cellular proteins executing quite diverse biological functions. Most studies pertaining to nuclear export have implicated CRM1, which is a nuclear export receptor for proteins containing leucine-rich NES [11,20]. However, an antibiotic compound, LMB, a potent and specific nuclear export inhibitor, can alkylate and inhibit CRM1. To investigate the nuclear export mechanism of WDR42A, COS-7 cells transfected with pNES-EYFP, paa1–113-EYFP, and positive control pRev-NES-EYFP as well as negative control pEYFP-N1 were treated with LMB at a final concentration of 20 ng/ml for 4 h. Similar to the fact that LMB treatment could almost completely abolish the nuclear export of pRev-NES-EYFP [29], the nuclear export activities of aa1–113-EYFP and the NES were almost completely abolished by LMB, whereas the subcellular distribution of EYFP was not affected (Fig. 5A). To further confirm the effects of CRM1 on export of WDR42A, we probed the interaction between WDR42A and CRM1 via Co-IP. The result showed that CRM1 was co-immunoprecipitated with WDR42A (Fig. 5B), indicating that WDR42A was able to bind CRM1 in vitro. All of these data further demonstrated the WDR42A NES itself was the target of export receptor, CRM1. Collectively, these results indicated that the ∆IEVEASDL50 sequence of WDR42A constituted a functional NES and the export of WDR42A from the nucleus to the cytoplasm was CRM1 dependent.

4. Discussion

Chemical fixation may lead to the artifact of the protein localization, so EYFP was introduced in this study. EYFP was used as a marker to visualize the subcellular localization of WDR42A in live cells and to identify its NLS and NES. Amino acid sequence analysis and R/A mutant analysis revealed that the NLS1 possessed nuclear targeting activity. However, although the NLS was functional, both aa51–122-EYFP and aa51–597-EYFP showed faint fluorescence in the cytoplasm, which could be explained that the common function between the majorities of WD40-repeat proteins involved in various protein–protein interactions [4].

There are at least six isoforms of karyopherins-α in mammals that can be divided into three distinct subfamilies: the Rch1, the Qip1, and the NPI-1 subfamily based on their sequence similarity [26]. In Fig. 3B–C, partial fluorescence of WDR42A was observed in the nucleus under the cotransfection of these dominant-negative mutants, which indicated that there may be some other karyopherins-α and karyopherins-β for mediating its nuclear localization. Furthermore, the exact configuration of how these transport receptors recognize WDR42A awaits further investigation. In eukaryotic cells, proteins containing a classical NLS are recognized and imported by a heterodimeric receptor consisting of karyopherin-α/β [26]. Since karyopherin-β1 could not be directly pulled down by GST-WDR42A (Fig. 3F), it is probably that karyopherins-α1 binds to WDR42A and links them to karyopherins-β1, which ferries the ternary complex through nuclear pore complex.

The N terminal portion of WDR42A (aa1–113) localized into the cytoplasm and prompted us to reexamine its amino acid sequence. A leucine-rich NES mediating WDR42A nuclear export was found in the N terminus. However, partial nuclear fluorescence was also observed in the cells transfected with paa1–113-EYFP and
pNES-EYFP (Fig. 2C, Fig. 4B, and Fig. 5A). These results deepened our understanding of the protein and two explanations are illustrated as follows: Firstly, phosphorylation events (adjacent to NES or at any site within the protein) could cause conformational changes in the protein, which may weaken the relative NES strength ratio and finally affect its nuclear export [30]. Other modifications couldn't be excluded as well. Secondly, we couldn't preclude the influence of the orientation of the fused protein.

Previous data indicated that the DDB1–CUL4 ubiquitin ligase machinery regulated diverse cellular functions including DNA replication, DNA repair, transcription and chromatin modifications in the nuclei of eukaryotes [31]. Consequently, the nuclear localization of WDR42A is very important to elucidate its biological functions. By bridging a cellular target to the DDB1–CUL4 ubiquitin ligase machinery, the substrate receptor WDR42A ensured the specificity of E3 ubiquitin ligase and its physiological function in the nucleus. Recently, in Arabidopsis thaliana, DDB1–CUL4 associated factor 1 (DCAF1) was found colocalized with DDB1 and CUL4 in the nucleus and the DDB1–CUL4–DCAF1 complex; as a nuclear E3 ubiquitin ligase, it was involved in multiple plant developmental processes [32]. Furthermore, based on the data DDB1 translocates to the nucleus following UV irradiation [33], we also predict that WDR42A may also be involved in the complicated nucleocytoplasmic transporting process.

In summary, we identified an arginine-rich NLS mediating nuclear import of WDR42A by karyopherin-α1/β1 heterodimer in conjunction with the small GTPase Ran and an N-terminal leucine-rich NES functioning through a CRM1-dependent nuclear export pathway. Interestingly, the NLS appears to be stronger than the NES, as most of WDR42A was found in the nucleus in transfected cells. It is noteworthy that the substrate, and therefore function, of WDR42A is unknown. Our studies uncovered the subcellular localization of WDR42A in living cells and opened up new avenues toward delineating its function and physiological significance in vivo.

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