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# Proteomic investigation of the interactome of FMNL1 in hematopoietic cells unveils a role in calcium-dependent membrane plasticity

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ABSTRACT

Formin-like 1 (FMNL1) is a formin-related protein highly expressed in hematopoietic cells and overexpressed in leukemias as well as diverse transformed cell lines. It has been described to play a role in diverse functions of hematopoietic cells such as phagocytosis of macrophage as well as polarization and cytotoxicity of T cells. However, the specific role of FMNL1 in these processes has not been clarified yet and regulation by interaction partners in primary hematopoietic cells has never been investigated. We performed a proteomic screen for investigation of the interactome of FMNL1 in primary hematopoietic cells resulting in the identification of a number of interaction partners. Bioinformatic analysis considering semantic similarity suggested the giant protein AHNAK1 to be an essential interaction partner of FMNL1. We confirmed AHNAK1 as a general binding partner for FMNL1 in diverse hematopoietic cells and demonstrate that the N-terminal part of FMNL1 binds to the C-terminus of AHNAK1. Moreover, we show that the constitutively activated form of FMNL1 (FMNL1γ) induces localization of AHNAK1 to the cell membrane. Finally, we provide evidence that overexpression or knock down of FMNL1 has an impact on the capacitative calcium influx after ionomycin-mediated activation of diverse cell lines and primary cells.





*Abbreviations:* FMNL1, formin-like 1; PBMC, peripheral blood mononuclear cell; CLL, chronic lymphatic leukemia; GO, gene ontology; MF, molecular function; CC, cellular component.

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## 1. Introduction

Formins belong to a protein family represented by 15 different members in humans which are involved in actin-dependent key cellular processes as polarization, migration, vesicle trafficking and cytokinesis [1]. Formin-like 1 (FMNL1) is particularly expressed in hematopoietic cells as well as overexpressed in malignant cell lines. It is functionally involved in a number of diverse cell-type specific functions such as phagocytosis in macrophages, centrosome orientation and cytotoxicity as well as maintenance of the Golgi complex in T cells [2–5]. It is so far not well understood how these different processes are regulated and which interaction partners are involved. Previously, diverse Rho GTPases have been described to interact with and regulate FMNL1 as shown by direct interaction in pull down assays [4,6,7]. Direct pull down assays further identified FMNL1-specific interaction partners as Profilin 1 and Profilin 2 in FMNL1-transfected cell lines [4]. Large proteomic screens revealed FMNL1 as an interaction partner for several proteins, such as growth arrest-specific protein 7 (GAS7), PRPF40A, transcription elongation regulator 1 (TCERGI) [8] as well as X-ray repair cross-complementing protein 6 (XRCC6) [9]. However, interaction partners of human FMNL1 in primary hematopoietic cells have never been investigated so far. This, however, might be essential for a deeper understanding of the multifaceted role of this protein in hematopoietic cells.

We here applied a systematic proteomic interaction screen in order to identify direct interaction partners of FMNL1 within different human primary hematopoietic cell populations. We thereby identified a number of interaction partners and confirmed several by direct immunoprecipitation. The highest number of unique peptides, as found by mass spectrometry, was derived from the scaffold protein AHNAK1. Bioinformatics analysis additionally suggests that AHNAK1 is a crucial interaction partner of FMNL1. We confirm AHNAK1 as the interaction partner of FMNL1 in diverse hematopoietic cells and demonstrate specific binding of the N-terminal part of FMNL1 to the C-terminal part of AHNAK1. Moreover, we here demonstrate that membrane localization of AHNAK1 can be directly modulated by a splice variant of FMNL1 (FMNL1γ). Finally, membrane localization of AHNAK1 has been previously demonstrated to be calcium-dependent [10–14] and we present evidence that FMNL1 is involved in the modulation of the capacitative calcium influx induced by ionomycin.

These data provide insights into the regulation of FMNL1 in primary hematopoietic cells and demonstrate that AHNAK1 is a common interaction partner of FMNL1 potentially conjointly involved in calcium-dependent membrane processes during excitative responses of hematopoietic cells.

## 2. Materials and methods

### 2.1. Cells and cell lines

Peripheral blood mononuclear cells (PBMC) from healthy donors and patients with chronic lymphatic leukemia (CLL) were collected with donors' informed consent following the requirements of the local ethical board and the principles expressed in the Helsinki Declaration. Patients had diagnosis of CLL by morphology, flow cytometric analysis and cytogenetics. PBMC were obtained by density gradient centrifugation on Ficoll/Hypaque (Biochrom). PBMC subpopulations from healthy donors were isolated by negative or positive magnetic bead depletion (Invitrogen). K562 (ATCC CCL-243) and breast carcinoma cell line MDA-MB 231 (CLS) were used for adenoviral transduction of FMNL1 splice variants. HEK293T embryonal kidney cells (ATCC CRL-1573) were utilized for FMNL1 protein expression after transfection whereas 293A (Invitrogen) cells were used for production of adenoviral supernatant as previously described [15]. Peptide-pulsed T2 cells were used for T cell polarization [16,17]. Autologous EBV— B cell lines cultured with IL-4 on CD40L expressing feeder cells [17] were used for colocalization studies of FMNL1 and AHNAK1.

### 2.2. Antibodies

The following antibodies were used: N-terminal-specific rat anti-human FMNL1 antibody 8A8 and C-terminal-specific rat anti-human FMNL1 antibodies 6F2, 5A1, 5B12 and 5C9 [15,18], rat anti-mumps-hemagglutinin-specific monoclonal IgG antibody TQL, mouse anti-human GAPDH monoclonal IgG (Sigma-Aldrich), rabbit anti-human AHNAK1 polyclonal antibody [19] and mouse anti-human AHNAK1 monoclonal antibody (Abnova, clone 3G7), mouse anti-HIS antibody (Anaspec, 61250), polyclonal rabbit anti-human ARHGAP4 (ATLAS) and polyclonal rabbit anti-human anti-ARHGAP17 (Abcam), anti-CD8–FITC (V5T-HIT8a), goat anti-mouse immunoglobulin antibody (Jackson), goat anti-rabbit immunoglobulin antibody (Jackson), goat anti-rat immunoglobulin antibody (Jackson), Cy3-labeled goat anti-rat immunoglobulin antibody (Jackson) and Cy5-labeled goat anti-rabbit and anti-mouse immunoglobulin antibody (Jackson).

### 2.3. Immunoprecipitation

Activated T cells, B cells and CLL cells were pelleted and lysed in CHAPS buffer for 30 min on ice followed by incubation with Sepharose G beads for preclearing. Freshly prepared Sepharose G beads were washed and the FMNL1 N-terminal-specific antibody 8A8 using the IgG2c control TQL antibody or the FMNL1 C-terminal-specific antibodies 6F2, 5A1, 5B12 and 5C9 using IgG2b control anti-flag antibody were added followed by rotating for 1 h at 4 °C. Sepharose G beads were then washed and incubated with precleared protein lysate on the rotator for 3 h at 4 °C. Elution was performed and probes were forwarded for SDS-PAGE on two separate gels. One gel was used for immunoblotting to analyze the efficiency of immunoprecipitation of FMNL1 using the FMNL1-specific antibody 6F2. Silver staining was performed with the other gel to visually detect specific immunoprecipitated bands. Samples from different IPs were subjected to in gel digestion by trypsin before MS analysis. The different IP samples have been analyzed reduced and alkylated as well as non-reduced.

### 2.4. LC–MS/MS analysis

Digested peptides were analyzed by nano-HPLC (Ultimate 3000, Dionex) coupled to a linear quadrupole ion trap-Orbitrap (LTQ



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Orbitrap XL) mass spectrometer (Thermo Fisher) equipped with a nano-ESI source. A nonlinear gradient using 2% ACN in 0.1% formic acid in water and 0.1% formic acid in 98% acrylonitrile was used with a flow rate of 250 nl/min. The mass spectrometer was operated in the data-dependent mode to automatically switch between Orbitrap-MS and LTQ-MS/MS. The method used allowed sequential isolation of the ten most intense ions depending on signal intensity for fragmentation on the linear ion trap. High resolution MS scans in the orbitrap and MS/MS scans in the linear ion trap were performed in parallel.

All MS/MS data were analyzed using MASCOT (Matrix Science) version: 2.2.06 searched the human uniref 100 (version from 19.04.2011 (126,233 protein entries for *Homo sapiens*)) and NCBI databases (version from 15.05.2011 (237,237 protein entries for *Homo sapiens*)) assuming the digestion enzyme trypsin, including a fragment ion mass tolerance of 0.8 Da and a parent ion tolerance of 10 PPM. Iodoacetamide derivative of cysteine as stable and oxidation of methionine, deamidation of arginine and glutamine was specified in MASCOT as variable modifications. Scaffold (version Scaffold_2_02_03, Proteome Software) was used to validate MS/MS-based peptide and protein identifications. Protein identifications were accepted if they could be established at greater than 95.0% probability and contained at least 2 unique peptides.

### 2.5. *Bioinformatics analysis*

Gene Ontology (GO) enrichment analysis was adopted to uncover the common biological processes played by the interaction partners of FMNL1 in different cell types, with $p<0.05$. A hypergeometric model was implemented to assess whether the number of selected genes associated with the GO term was at a frequency greater than expected by chance. Q-values [20] were estimated to control the false discovery rate (FDR) for multiple hypotheses testing, with $q$-value$<0.05$. These analyses were calculated by Bioconductor package clusterProfiler [21]. The analysis result contained some redundant biological processes, since the GO categories to which genes are assigned are not independent. To solve this issue, semantic similarity [22] among GO categories was used to aggregate closely related categories. The final result was visualized by clusterProfiler [21].

Based on the semantic similarities of GO terms used for gene annotation, we rank the protein inside the interactome by the average functional similarities between the protein and its interaction partners. GO semantic similarity, which has been verified in terms of the correlation with gene expression profiles [23], provides the basic for functional comparison of gene product, and thus has been widely applied in bioinformatics, such as protein–protein interaction analysis [24], pathway analysis [25] and gene function prediction [26]. Here, we measure the functional similarity among proteins. Functional similarity, which is defined as the geometric mean of their semantic similarities in molecular function (MF) and cellular component (CC) aspect of GO, is designed for measuring the strength of the relationship between each protein and its partners by considering function and location of proteins. Semantic similarities among interactome proteins in MF and CC were measured through the GOSemSim package [22] using the Wang method which performs in a more accurate and unbiased manner by taking the GO topological structure into account [27]. Functional similarities were further estimated by the geometric mean of semantic similarities in MF and CC. The distributions of functional similarities were demonstrated in Fig. 1. Proteins, which have strong relationship in function and location among the proteins within the interactome, were essential for the interactome to exert their functions. Here, we used the average of functional similarities to rank protein in the FMNL1 interactome. A cutoff value of 0.75 was chosen.

### 2.6. *T cell polarization assay*

T2 cells were pulsed with the tyrosinase-derived peptide YMNGTMSQV as previously described [16,18]. T cells that retrovirally transduced with the tyrosinase-specific T cell clone IVSB [28] were co-incubated with T2 cells pulsed with the relevant peptide for 15 min and then processed for immunofluorescence staining as previously described [15].

### 2.7. *Constructs and protein expression*

DNA of different FMNL1 splice variants, FMNL1ΔDAD and the G2TA4T mutant variant for adenoviral transduction were produced as previously described [15]. Adenovirally transduced

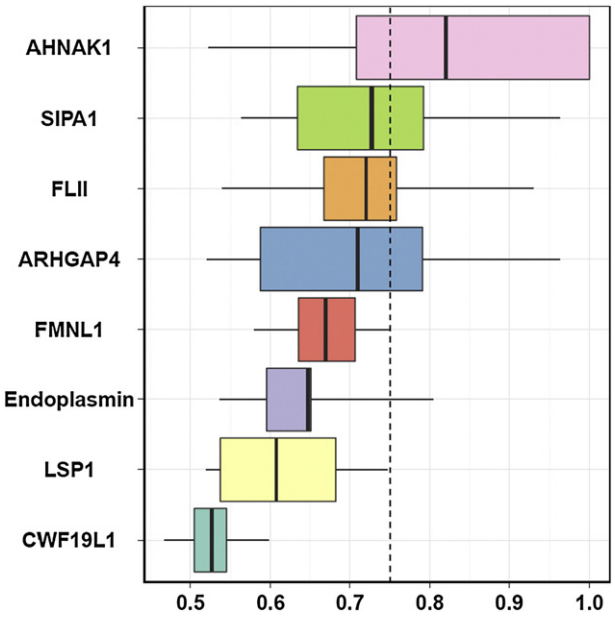


**Fig. 1 – Summary of functional similarities of the FMNL1 interactome in T cells. The distributions of functional similarities were summarized as boxplots. The boxes represent the middle 50% of the similarities; the upper and lower boundaries show the 75th and 25th percentile. The lines in the boxes indicate the mean of the functional similarities. Proteins with a higher average functional similarity (cutoff>0.75) were defined as party proteins, which are considered as the central proteins within the FMNL1 interactome in T cells. The dashed line represents the cutoff value.**



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cells were then used for confocal microscopy and calcium signaling experiments.

Constructs with truncated and complete FMNL1 (Fig. 2B) were cloned into a eukaryotic pCMV-His-tagged expression vector (Invitrogen). T293 cells were transfected with the plasmid of interests by calcium phosphate transfection. T293 cells were lysed with CHAPS lysis buffer and supernatant of lysed cells was used for pull down assay.

C-terminal GST-tagged AHNAK1 constructs cloned into pGEX-4T1 (C1, C2 and R4) have been previously described [29]. BL-21-Codon Plus (DE3)-RIL bacteria (Stratagene) were transformed with GST-tagged AHNAK1 constructs. Protein expression was induced by IPTG (Sigma-Aldrich) incubation for 2 h. Bacteria were collected by centrifugation and lysed with NETN lysis buffer followed by sonification and centrifugation. Supernatant was removed and used for pull down assay.

### 2.8. *Immunofluorescence*

Non-adherent cells were dropped on poly-L-lysine-coated coverslips. Cells were fixed in 3% paraformaldehyde and then washed, blocked with 10% FCS and stained with specific primary and secondary antibodies as indicated. DAPI (Invitrogen) was used for nuclear staining. Glass coverslips were mounted on the cells in Mounting Medium (Vector Laboratories) and investigated by Confocal microscopy (Leica).

### 2.9. *Pull-down assay*

Bacterial supernatant containing GST-tagged AHNAK1 constructs was incubated with GST-Sepharose 4B beads (GE Healthcare) rotating for 60 min at 4 °C. Beads were washed intensively and proteins were verified by Coomassie simple blue staining as well as immunoblot. Supernatant derived from lysed 293T cells transfected with different FMNL1 constructs was equilibrated with GST-Sepharose beads containing GST-tagged AHNAK1 constructs rotating for 2 h at 4 °C. After washing, elution was performed with 1×LDS+DTT and probes were further analyzed by SDS-PAGE and western blot analysis.

### 2.10. *siRNA knock down*

Several FMNL1-specific siRNA were produced using the Silencer siRNA Construction Kit (Applied Biosystems). The best efficacy in down regulation of FMNL1 was reached with the following sequences (MWG): 5′FMNL1 siRNA-AAAGGCG TACCTGGACAATATCCTGTC and 3′FMNL1-AAATATTGTCCAG GTAGCCCTCCTGTCTC. T cells stimulated with OKT3 and IL-2 were transfected by nucleofection using program T-20 (Amaxa Biosystems, Lonza). 4 h after transfection, cells were collected, washed and cultured for 3 days prior to calcium measurement and immunoblot.

### 2.11. *Intracellular calcium measurement*

Cells were transfected or transduced with different FMNL1 constructs. 2 days later, cells were harvested and adjusted to $10^6$/ml in pre-warmed medium in round bottom tubes. Indo-1AM stain (Sigma-Aldrich) was added and cells were then incubated for 30 min at 37 °C mixing every 10 min during incubation. Stained cells were diluted to 0.1 Mio/ml and kept in the dark until analysis. The free intracellular calcium was analyzed by flow cytometry MoFlow (Dako) recording the ratio of violet fluorescence (405 nm) and green fluorescence (530 nm).

## 3. Results

### 3.1. *Identification of interaction partners of FMNL1 in primary hematopoietic cells by proteomic analysis*

Since FMNL1 has been described to be predominantly expressed in hematopoietic cells and involved in diverse cellular functions, we wanted to identify novel key interaction partners of FMNL1 in primary hematopoietic cells including malignant cells derived from patients with chronic lymphocytic leukemia (CLL). We first focused on activated T cells and established IP procedures for activated T cells using 5 FMNL1-specific antibodies (N- and C-terminal). We then applied a proteomic approach by immunoprecipitation of FMNL1 followed by Nano-HPLC and LC–MS/MS analysis. As the N-terminal antibody turned out to reveal the best results, this antibody has been further applied for B cells and CLL cells. We thereby identified a number of proteins, which were immunoprecipitated together with FMNL1 but not with the control antibodies. In Table 1 and Table S1–S4 best protein scores for a protein in all LC–MS/MS approaches are shown. Table 1 shows the most abundant hits also indicating the number of biological repeats for T cells and all analyzed samples. The different IP samples have been additionally analyzed reduced and alkylated and partially also non-reduced further confirming actual results (data not shown). We have identified a large number of novel interaction partners which can be grouped into clusters involved in diverse subcellular functions as proteins associated to the GTPase signaling pathway (e.g. ARHGAP4, ARHGAP17, SIPA1, BTB/POZ domain-containing adapter for CUL3-mediated RhoA degradation protein 3), scaffold proteins (e.g. AHNAK1, Plectin-1), chaperons (e.g. Endoplasmin) and others. The biological processes regulated by these interaction partners of FMNL1 were further analyzed by clusterProfiler [21], which implemented a hypergeometric model for identifying predominant biological themes (Supplement, Fig. S1). The function of cytoskeleton organization is over-represented across all the hematopoietic cell types consistent with the fundamental role of the formin defining FH2 domain in actin filament elongation [30]. Moreover, the clusterProfiler analysis indicated a cell-type specific interaction pattern of the FMNL1 interactome suggesting that FMNL1 may display different functions in different cell types (Supplement, Fig. S1).

In order to validate our data by other experimental approaches we confirmed three interaction partners with high numbers of identified unique peptides by direct immunoprecipitation. These experiments identified two of them, AHNAK1 and ARHGAP4, as interaction partners of FMNL1 in different probes derived from primary hematopoietic cells (Fig. 2A, Supplement, Fig. S2A). In contrast, ARHGAP17 was only identified as interaction partner of FMNL1 in B and CLL cells (Supplement, Fig. S2B). We have also observed a number of interaction candidates of FMNL1 identified only in CLL cells



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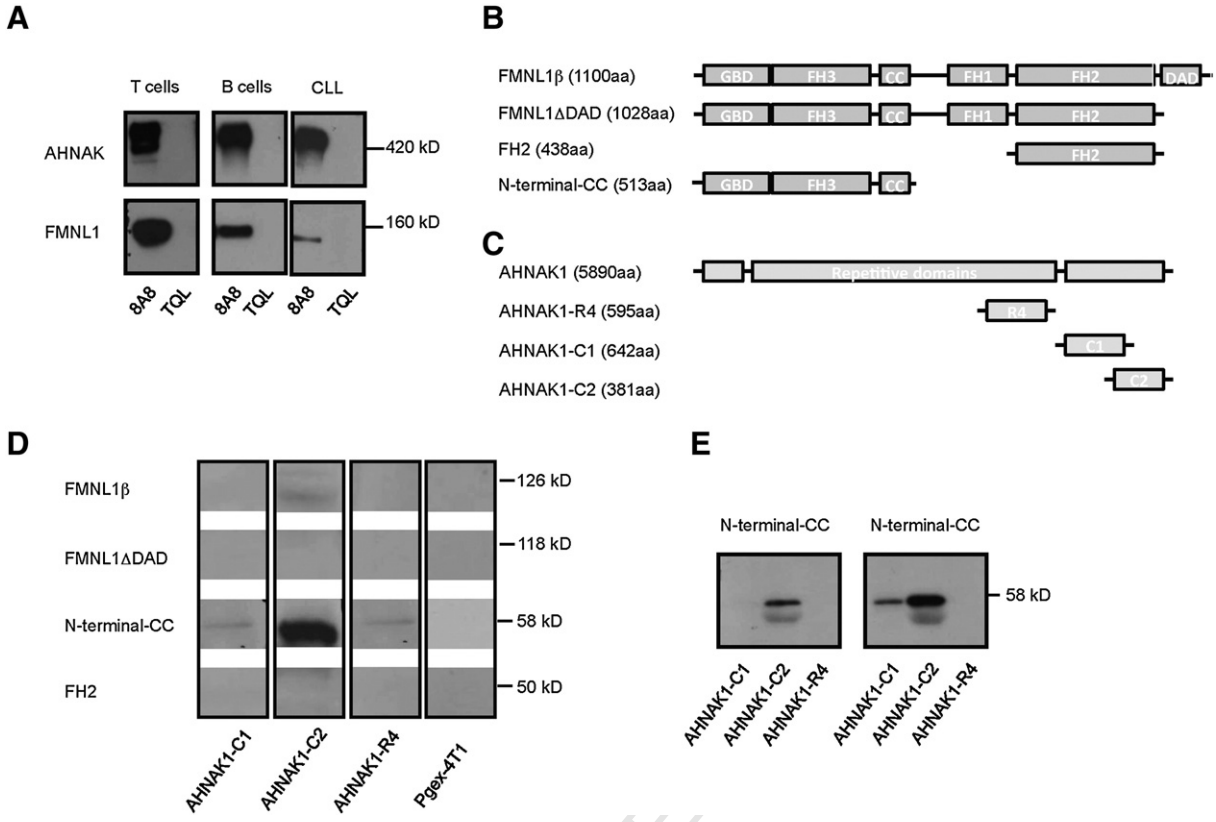


**Fig. 2 – Co-immunoprecipitation of AHNAK1 and FMNL1 in T cells and identification of the interaction site. (A) FMNL1 was immunoprecipitated using the FMNL1-specific antibody 8A8. The TQL antibody served as isotype control. Co-immunoprecipitation of AHNAK1 in T cells non-specifically stimulated with IL-2 and OKT3, B cells and CLL cells was confirmed by immunoblot. (B) His-tagged constructs of FMNL1 cloned for pull down assays. (C) Scheme of AHNAK1 and cloned GST-tagged C-terminal AHNAK1 constructs R4, C1 and C2 used for pull-down assay. (D and E) Pull-down assays using GST-tagged AHNAK1 constructs and control GST. GST-tagged proteins were coupled to GST-Sepharose 4B and used in pull-down assays with His-tagged FMNL1 constructs. Proteins retained by defined AHNAK1 constructs were separated by SDS-PAGE and analyzed by immunoblotting with antibodies against HIS. Pull-down of the N-terminal-CC construct was demonstrated especially by AHNAK1 C2 (D and E) and in a single experiment also by AHNAK1-C1 (E).**

(Table 1, Supplement, Table S3 and S4). One of them, Gemin5, was identified in both CLL samples. However, further analyses are necessary to segregate the interactome of FMNL1 in diverse hematopoietic and leukemic cells.

### 3.2. *Evaluation of the interactome of FMNL1 I in T cells*

In order to identify essential proteins of the FMNL1 interactome in T cells, we ranked proteins by their average functional similarity relationships among proteins within the interactome [31]. AHNAK1, SIPA1 and FLII were the three top-ranked proteins potentially playing central roles in the FMNL1 interactome in T cells. AHNAK1 was the only protein with a cutoff value >0.75 (Fig. 1) which is widely used to separate significant and non-significant correlations [32,33]. However, FLII demonstrating the average functional similarity score of 0.72 has been previously demonstrated to enhance actin assembly activity by directly binding to the formins DAAMI and mDIA1 [34]. Thus our data suggest a similar role of FLII in its interaction with FMNL1. AHNAK1, which has not yet been previously identified as an interaction partner of FMNL1 or formins, has been previously reported to play an important role in T cell activation [35]. As AHNAK1 has the highest average functional similarity in our analyses, we chose AHNAK1 for further functional investigation.

### 3.3. *AHNAK1 interacts at its C-terminus with the N-terminal region of FMNL1*

AHNAK1 was consistently identified as an interaction partner of FMNL1 by mass spectrometry with a high number of unique peptides in T cells and other hematopoietic cells (Table 1, Supplement, Table S1 and data not shown). We further confirmed the interaction by co-immunoprecipitation experiments with FMNL1 in T cells and other hematopoietic cells (Fig. 2A). In order to localize the interaction site of FMNL1 with AHNAK1, we cloned different truncated His-tagged constructs of FMNL1 (Fig. 2B) and used previously described GST-tagged constructs of AHNAK1 (Fig. 2C and [29]) for pull down experiments. We were able to detect specific binding of the FMNL1-derived construct N-terminal-CC to the AHNAK1-derived C-terminal construct C2 (Fig. 2D) There was no specific interaction observed for the other constructs of FMNL1. However, one out of three experiments also demonstrated weak interaction with the AHNAK1-derived C-terminal construct C1



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(Fig. 2E). These data demonstrate that AHNAK1 interacts at its C-terminus with the N-terminal region of FMNL1.

### 3.4. Distinct patterns of FMNL1 and AHNAK1 localization in primary T cells and K562 cells overexpressing diverse FMNL1 splice variants

We investigated localizations of FMNL1 and AHNAK1 in primary T cells as well as K562 cells transduced with different splice variants of FMNL1. In non-specifically stimulated T cells, we observed localization of FMNL1 in the cytoplasm (Fig. 3A and B) as previously described [15]. In contrast, AHNAK1 showed different patterns of localization, either in the cytoplasm or at the cell membrane (Fig. 3A and B). Quantification by counting demonstrated that AHNAK1 was located intracellularly in most cases (Fig. 3C). Regarding specifically stimulated T cells, AHNAK 1 was polarized to the immunological synapse (Fig. 3D and E). AHNAK1 partially colocalized with FMNL1 at the immunological synapse (Fig. 3D). We additionally overexpressed different splice variants of FMNL1 in K562 cells. Wildtype K562 cells or K562 cells adenovirally transduced with GFP showed a mainly intracellular distribution of FMNL1 and variable AHNAK1 localization (Fig. 4A, B). K562 cells transduced with diverse FMNL1 splice variants showed a splice-variant dependent localization of both FMNL1 and AHNAK1. Whereas K562 cells transduced with FMNL1α showed almost completely divergent localization of FMNL1 and AHNAK1 (Fig. 4C), and transduction with FMNL1β resulted in colocalization of both proteins mainly in the cytoplasm (Fig. 4D). In contrast, K562 cells adenovirally transduced with FMNL1γ showed a clear membranous localization of both, FMNL1γ and AHNAK1 (Fig. 4E). Moreover, we could also detect co-localization of FMNL1γ and AHNAK1 in blebbing cells (Fig. 4F) [15].

### 3.5. FMNL1 enhances ionophore-mediated calcium influx

AHNAK1 is a scaffold protein, which plays an important role in plasma membrane enlargement, exocytosis and repair [11,12,36]. It has been previously reported that localization of AHNAK1 is calcium-dependent and that ionophore-mediated calcium influx induces AHNAK1 localization to the plasma membrane [10–13]. Moreover, AHNAK1 has been shown to be involved in calcium signaling and effector functions of T cells [35,37]. Since transduction of K562 cells with FMNL1γ induces translocation of AHNAK1 to the cell membrane (Fig. 4E, F), we hypothesized that FMNL1 may be also involved in calcium-dependent membrane processes. Therefore, we aimed to investigate if overexpression or down regulation of FMNL1 may affect ionophore-mediated calcium influx. We therefore transduced MDA-MB-231 cells not expressing FMNL1 with different splice variants and constructs of FMNL1 as previously described [15]. Overexpression of FMNL1 in MDA-MB-231 cells induced enhanced calcium influx after stimulation with ionomycin as measured by Indo-1-AM-staining (Fig. 5A). Of interest, transduction of FMNL1α showed only a minor effect

**Table 1 – Identification of potential interaction partners of FMNL1 by LC–MS/MS analysis in different hematopoietic cell populations.**

| Eluted protein identified by mass spectrometry | Description | UniProt accession number | T cells [a] | Number of different biological samples in T cells | B cells [a] | CLL1 [a] | CLL2 [a] | Total number of different biological samples including T, B and CLL cells |
|---|---|---|---|---|---|---|---|---|
| FMNL1 | Formin-like 1 | O95466 | 25 | 5 | 47 | 56 | 29 | 8 |
| AHNAK1 | AHNAK nucleoprotein | Q09666 | 40 | 4 | 273 | 311 | 286 | 7 |
| ARHGAP4 | Rho GTPase activating protein 4 | P98171 | 16 | 2 | 63 | 44 | 28 | 5 |
| CWF19L1 | CWF19-like 1 | Q69YN2 | 10 | 1 | 23 | 34 | 19 | 4 |
| PARP | Poly [ADP-ribose] polymerase 1 | P09874 | 8 | 4 | 46 | 21 | 3 | 7 |
| Endoplasmin | Heat shock protein 90 kDa beta | P14625 | 2 | 3 | 19 | 3 | 3 | 6 |
| FLI1 | Flightless-1 homolog | Q13045 | 3 | 3 | – | – | – | 3 |
| LSP-1 | Lymphocyte-specific protein 1 | P33241 | 2 | 4 | – | – | – | 4 |
| SIPA1 | Signal-induced proliferation associated protein 1 | Q96FS4 | 2 | 3 | – | – | – | 3 |
| ARHGAP17 | Rho GTPase activating protein 17 | Q68EM7 | – | – | 17 | 4 | – | 2 |
| PLTN | Plectin-1 | Q15149 | – | – | 15 | 36 | – | 2 |
| TIA-1 | Nucleolysin TIA-1 isoform p40 | P31483 | – | – | 2 | 4 | 2 | 3 |
| hBACURD3 | BTB/POZ domain-containing adapter for CUL3-mediated RhoA degradation protein 3 | Q9H3F6 | – | – | 2 | 7 | – | 2 |
| Gemin 5 | Gem-associated protein 5 | Q8TEQ6 | – | – | – | 22 | 2 | 2 |

[a] Number of unique peptides identified.

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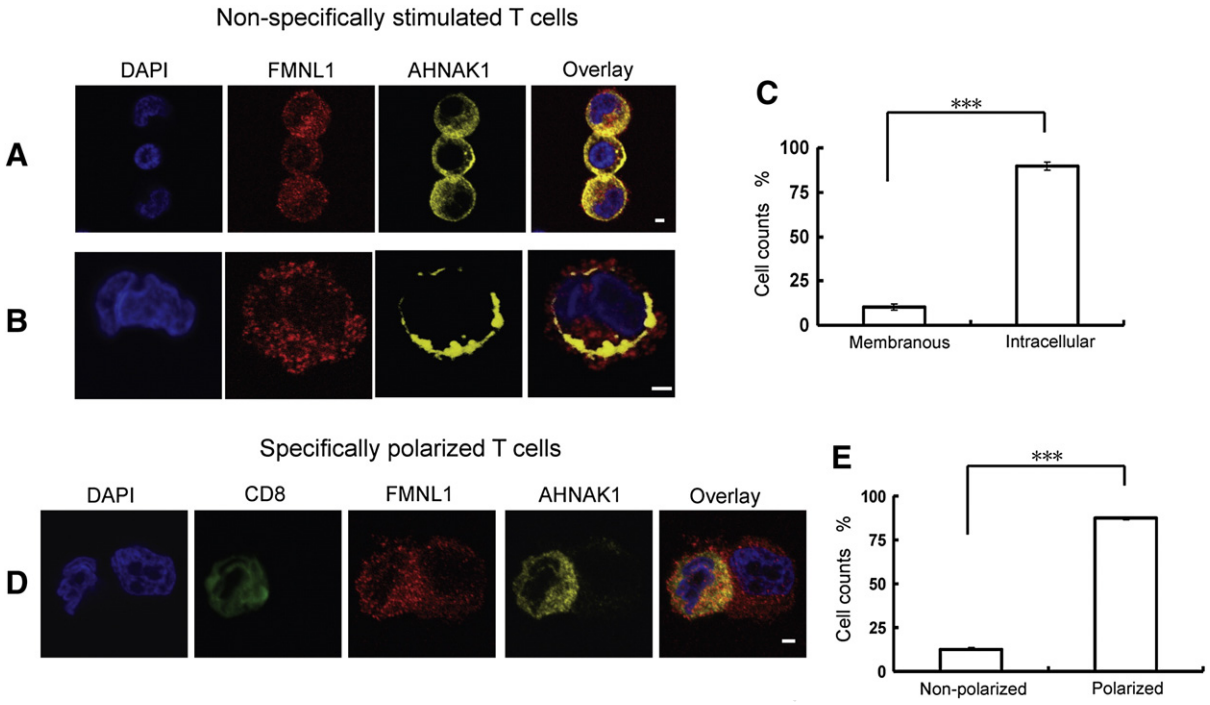


**Fig. 3 – Immunofluorescence staining of AHNAK1 and FMNL1 shows diverse patterns in non-specifically and specifically stimulated T cells. (A, B) T cells non-specifically stimulated with IL-2 and OKT3 were stained with DAPI for nuclear staining (blue), rat anti-human FMNL1 6F2 followed by Cy3-labeled goat anti-rat antibody (red) and mouse anti-human AHNAK1 followed by Cy5-labeled goat anti-mouse antibody (yellow). Cells were analyzed at the confocal microscope, scale bar: 2 μm. (C) Quantification of localization of AHNAK1 in non-specifically stimulated T cells. *** indicates $p<0.0001$ (D) The tyrosinase-specific T cell clone IVSB [28] was used for polarization assay and stimulated with T2 cells pulsed with the HLA-A2-binding tyrosinase-derived peptide 369 (YMNGTMSQV) for 15 min. Mixed cell populations were then fixed and stained with DAPI for nuclear staining (blue), mouse anti-human CD8 FITC (green), rat anti-human FMNL1 6F2 followed by Cy3-labeled goat anti-rat antibody (red) and mouse anti-human AHNAK1 followed by Cy5-labeled goat anti-mouse antibody (yellow). Cells were analyzed at the confocal microscope, scale bar: 2 μm. (E) Quantification of polarization of AHNAK1 in specifically stimulated T cells. *** indicates $p<0.0001$. Two sample t-tests were applied to calculate $p$ value. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)**

compared to the other splice variants including constitutively activated FMNL1γ and FMNL1ΔDAD. However, transduction of the G2TA4T construct containing a mutation of the N-terminal myristoylation site of FMNL1 showed no impact on calcium signaling (Fig. 5A), suggesting that modulation of calcium influx by FMNL1 is not necessarily associated with its membrane localization mediated by N-terminal myristoylation. A similar effect on calcium signaling of FMNL1 has also been observed in other cell lines as HEK293T cells and HT1080 cells transfected or adenovirally transduced with FMNL1 (Supplement, Fig. S3). We additionally investigated calcium influx of stimulated T cells by ionomycin after knock down of FMNL1 (Fig. 5B). In fact, calcium influx was reduced after knock down of FMNL1 when compared to the control siRNA (Fig. 5B).

## 4. Discussion

FMNL1 has been previously demonstrated to be involved in a number of different functional processes in diverse hematopoietic cell types as polarization and cytotoxicity of T cells [3] and phagocytosis as well as podosomal dynamics in macrophages [4,6,38]. More recently, a role of FMNL1 in maintenance of structural integrity of the Golgi complex and lysosomes has been reported [5]. We performed a proteomic interaction screen in primary hematopoietic cells as activated T cells, B cells and CLL cells to identify novel interaction partners of FMNL1 potentially providing further insights into the regulation and function of this multi-faceted protein.

We thereby repeatedly identified a number of interaction proteins in diverse biological samples and technical repeats. Among these were several Rho GTPase modulating proteins as ARHGAP4 and ARHGAP17 which have been repeatedly identified with a significant number of unique peptides. Both proteins were verified by co-immunoprecipitation and direct immunoblot. ARHGAP4 belongs to the srGAP family and may bind to the proline-rich FH1 domain of FMNL1 by its SH3 domain as previously reported for srGAP2 [7]. ARGHAP4 is highly expressed in embryonic neuronal tissue as well as hematopoietic cells [39,40]. It has been demonstrated to localize to the Golgi and microtubules and to mediate inhibition of axon outgrowth and cell motility [39,41]. However, ARHGAP4 activity may be dispensable for FMNL1 regulation as deletion of ARHGAP4 in patients with additional

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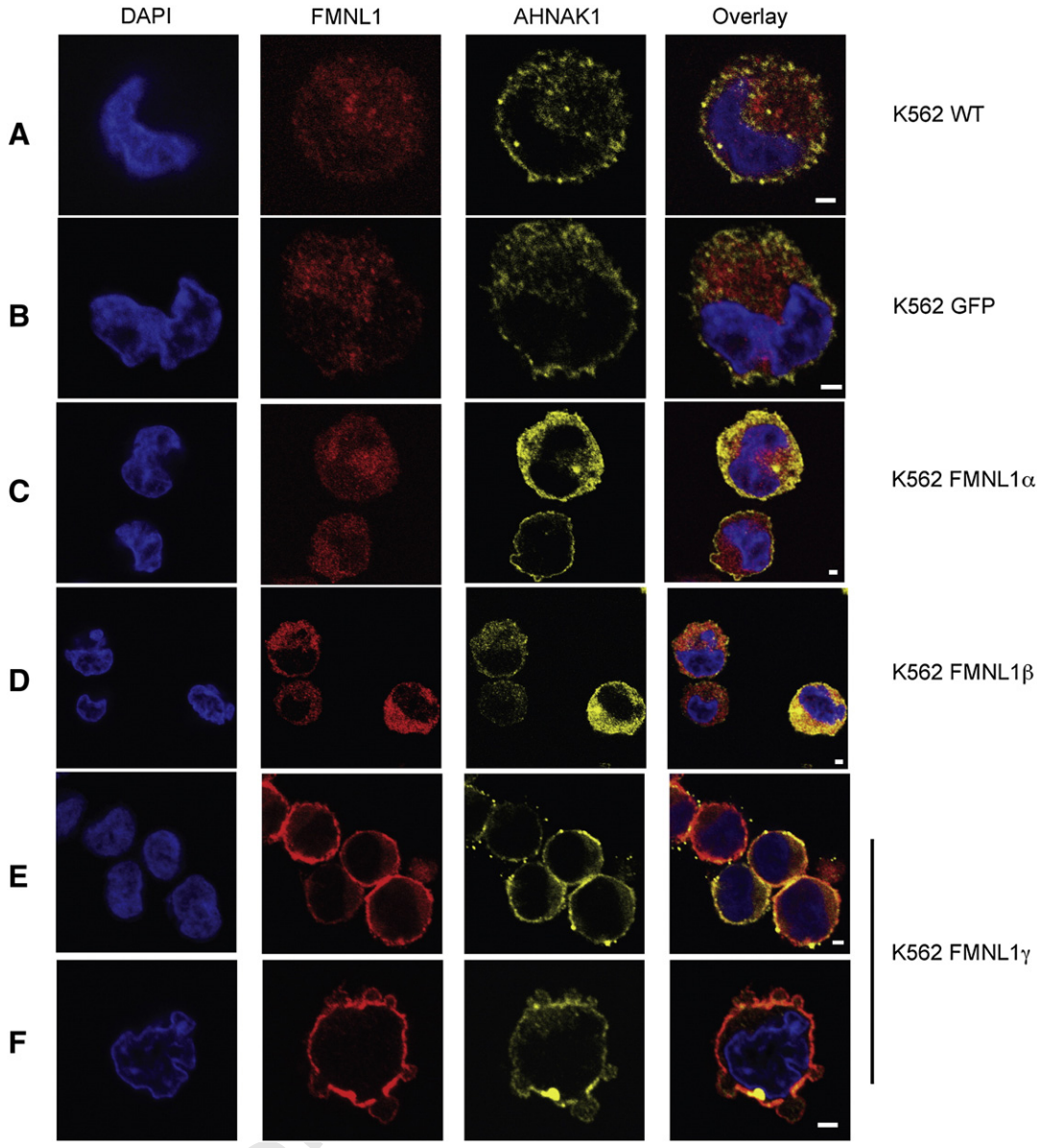


**Fig. 4 – Colocalization of AHNAK1 and FMNL1 in K562 cells overexpressing different splice variants of FMNL1. K562 cells were adenovirally transduced with different FMNL1 splice variants as well as GFP control. Transduced cells and wildtype (WT) control were stained with DAPI for nuclear staining (blue), rat anti-human FMNL1 6F2 followed by Cy3-labeled goat anti-rat antibody (red) and mouse anti-human AHNAK1 followed by Cy5-labeled goat anti-mouse antibody (yellow). Cells were analyzed at the confocal microscope, scale bar: 2 μm. (A) wildtype, (B) GFP, (C) FMNL1α, (D) FMNL1β, (E, F) FMNL1γ. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)**

AVPR2 deletion and nephrogenic diabetes insipidus does not result in major perturbations of the immune system [40]. In contrast to ARHGAP4, ARHGAP17 (Rich1) has been identified as interaction partner of FMNL1 in B cells and one CLL cell sample but not in T cells suggesting a preferential cell-type specific regulation of FMNL1 by ARHGAP17 which was confirmed by western blot. The protein also belongs to the srGAP family containing a conserved N-terminal F-BAR domain, a central Rho-family GAP domain and a C-terminal SH3 domain. ARHGAP17 has been previously described to regulate Cdc42 and to be involved in organization of apical polarity in epithelial cells [42]. Moreover, ARHGAP17 is obviously involved in Rac1 regulation [43–45] and has been suggested to play a role in inhibition of calcium-dependent exocytosis [43] as well as inhibition of Rac1 and the Ras-MAPK signaling pathways under growth suppressive conditions [45].

Bioinformatic analysis was performed and visualized by clusterProfiler in order to identify significant biological processes regulated by FMNL1 concentrating on proteins with enhanced statistical probability [46]. The analysis result suggested a cell-type specific interaction pattern. In order to further select proteins potentially significant in the FMNL1 interactome in T cells we applied further bioinformatics analyses by ranking through integration of protein semantic similarity [31,46]. AHNAK1, SIPA1 and FLII were the three top-ranked proteins potentially playing central roles in the FMNL1 interactome in T

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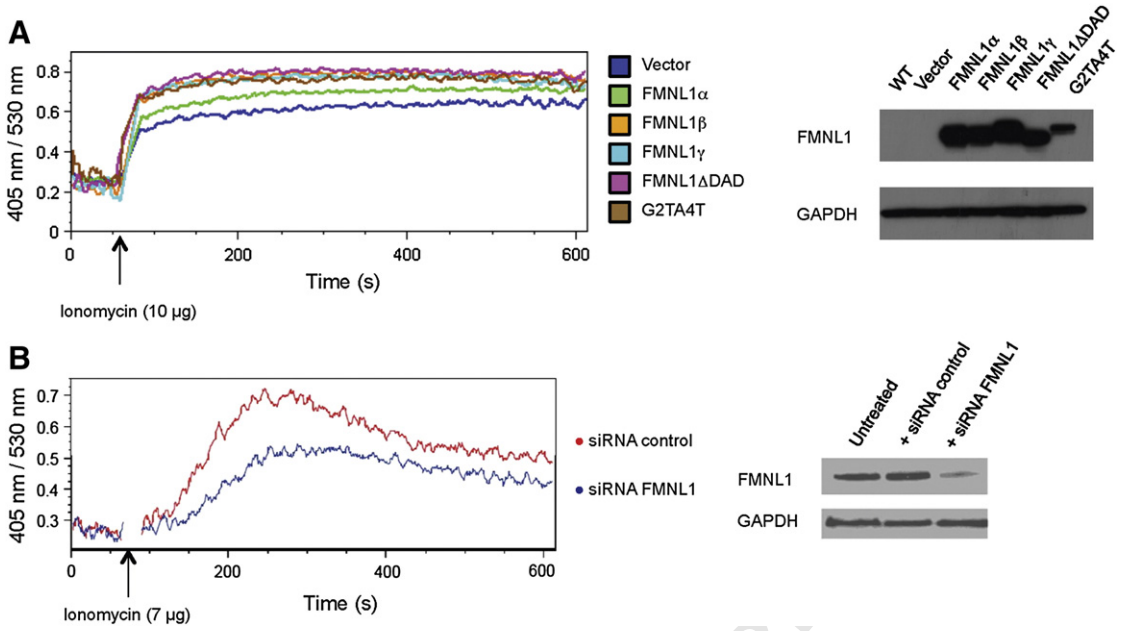


**Fig. 5 – FMNL1 modulates ionophore-mediated calcium influx. (A) MDA-MB-231 cells were adenovirally transduced with different FMNL1 splice variants as well as the FMNL1ΔDAD and G2TA4T mutant constructs. Cells were harvested and stained with Indo-1AM stain. Intracellular calcium was measured after stimulation of cells with ionomycin (10 µg) and analyzed by flow cytometry recording the ratio of violet fluorescence (405 nm) and green fluorescence (530 nm) (left). Expression of FMNL1 constructs was verified by SDS-PAGE and immuoblot of cell lysates using the rat anti-human FMNL1-specific antibody 6F2 (right). One out of at least three independent experiments for MDA-MB-231 cells is shown. Two other experiments using different cell lines showed similar results (Fig. S3). (B) T cells non-specifically stimulated with IL-2 and OKT3 were transfected with an FMNL1-specific siRNA as well as a control siRNA by nucleofection. 3 days later, harvested cells were stimulated with ionomycin (7 µg) and analyzed by flow cytometry recording the ratio of violet fluorescence (405 nm) and green fluorescence (530 nm) (left). Knock-down of FMNL1 was confirmed by SDS-PAGE and immuoblot of cell lysates using the rat anti-human FMNL1-specific antibody 6 F2 (right). One out of two independent experiments is shown. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)**

cells. FLII has been previously demonstrated to be a positive regulator of Rho-induced linear actin assembly in Daam1 and mDia1 [34]. Thus, our results suggest that FLII may be also a positive regulator for actin polymerization for other formins as FMNL1. AHNAK1 showed the highest functional similarity in our analyses. This protein represents a scaffold protein involved in different cellular processes such as calcium signaling in T cells and cardiomyocytes [35,47,48] as well as membrane plasticity and repair [11,12,36]. It has not been previously identified as an interaction partner of FMNL1 or other formins. We confirmed interaction of FMNL1 with AHNAK1 by co-immunoprecipitation and subsequent immunoblot. We were further able to define interaction sites of both proteins, as we observed specific binding of the N-terminal construct of FMNL1 harboring also the coiled-coil region to the C-terminal end of AHNAK1. The binding localization of FMNL1 to the C-terminus of AHNAK1 is similar to the previously reported interaction of AHNAK1 with diverse proteins as Cav1.2 channel, actin, and the S100A10-annexin A2 complex [29,49]. It has been previously shown that the C-terminal end of AHNAK1 is critically involved in subcellular localization of AHNAK1 [13]. Moreover, structural analysis of the S100A10-annexin A2-AHNAK1 complex recently suggested both proteins to be involved in plasma membrane translocation of AHNAK1 [49]. Apart from the similar interaction site we here demonstrate that in K562 cells plasma membrane localization of AHNAK1 to the cell membrane can be induced by overexpression of FMNL1γ but not the other FMNL1 splice variants. These data indicate that especially FMNL1γ may also be involved in subcellular localization of AHNAK1 at the plasma membrane and confirm that diverse splice variants of FMNL1 may harbor distinct functions [5,15].

AHNAK has been previously repeatedly shown to be involved in calcium-dependent membrane repair [11,36,50,51]. Subcellular localization of AHNAK1 has been demonstrated to be calcium-dependent and AHNAK1 is located to the cell membrane after capacitative calcium influx induced by ionomycin [10–13]. In addition, AHNAK1 has been demonstrated to be involved in Cav1.1 channel regulation in T cells [35,37]. Thus, AHNAK1 seems to be regulated by calcium modulation but also to represent a calcium modulator itself. By investigation of the impact of FMNL1 expression on the ionophore-mediated calcium influx we have observed that overexpression of FMNL1 in diverse cell lines results in enhanced capacitative calcium influx after ionomycin treatment whereas FMNL1 knock down reduces calcium influx in primary T cells. AHNAK1 itself might not be involved in ionomycin-mediated calcium modulation as ionomycin-mediated calcium influx has not been altered in AHNAK1 knock-out mice [35]. However, calcium modulation of FMNL1 may further modulate the activity of AHNAK1 and both proteins may interact in

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calcium-dependent membrane processes [3,4,12,15,36]. The exact nature of calcium modulation by FMNL1 needs to be further investigated. Other identified interaction partners of FMNL1 as the voltage-dependent anion-selective channel protein 1 as well as chaperones as endoplasmin may be involved in calcium modulation induced by FMNL1 [52,53]. Of note, similar to FMNL1, AHNAK1 has been also previously shown to be up-regulated in CLL cells [54]. Thus it will be important to further segregate the role of FMNL1, AHNAK1 and interaction partners selectively identified in CLL samples as Gemin5 in healthy and malignant cells.

## 5. Conclusion

In conclusion, the proteomic screen of the interactome of FMNL1 provided novel information about general and cell-type specific interaction partners of FMNL1, demonstrated interaction of FMNL1 and AHNAK1 as well as a splice-variant dependent close cooperation between AHNAK1 and FMNL1 in modulation of membrane plasticity. Data point to a novel role of FMNL1 as modulator of capacitative calcium influx. Although these data need to be further confirmed they suggest that FMNL1 is involved in modulation of stimulation-associated calcium-dependent cytoskeletal membrane processes.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jprot.2012.11.015.

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