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Full Length Research Paper

Identification of FANCA interacting proteins in mammalian cells using tandem affinity purification and mass spectrometry

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Tandem affinity purification (TAP) allows for the isolation of protein complexes under close-to-physiological conditions for subsequent protein identification by mass spectrometry. Although TAP has been successfully applied to yeast system, there are only a few reports in mammalian cells. In this study, the gene *fanca* was cloned into the commercially available TAP construct from Stratagene and transiently transfected into human embryonic kidney cells (Hek 293). The FANCA interacting proteins were TAP purified and subsequently identified by tandem mass spectrometry under both mitomycin C (MMC) treated and non-treated conditions. Several novel protein-protein interactions were identified by liquid chromatography (LC) tandem mass spectrometry (MS) under both conditions. The interaction between FANCA and Huntingtin (HTT), which was induced by MMC treatment, was also confirmed by western blot analysis. Although more studies need to be done to determine the biological implications of these interactions, this study provides a useful method for understanding protein functions through identification of protein-protein interactions.

Key words: Tandem affinity purification, fanconi anemia, proteomics, mass spectrometry.

INTRODUCTION

Fanconi anemia (FA) is a rare autosomal recessive disease with an occurrence of one in 360,000 live births (Reuter et al., 2003). Patients with FA have a reduced average life expectancy of ~20 years (Reuter et al., 2003). FA is characterized by aplastic anemia, congenital abnormalities, birth defects and increased susceptibility to cancer (Reuter et al.. 2003; Folias et al., 2002). Cells from FA patients exhibit increased sensitivity to DNA crosslinking agents such as mitomycin C (MMC) and diepoxybutane (Reuter et al., 2003). FA cells have also been shown to exhibit abnormal cell cycle progression and reduced cell survival (Naf et al., 1998) . To date, thirteen complementation groups have been identified, including FA-A, -B, -C, -D1, -D2, -E, -F, -G, -I, -J, -L, -M, and -N (Kennedy and D'Andrea, 2005). FA patients are assigned to one of the complementation groups, with the majority falling into group A (65%), followed by group C (15%), and Group G (10%) for most populations (D'Andrea and Grompe, 2003). All of the FA genes have been cloned with the exception of FA-I (Kennedy and D'Andrea, 2005), but the function of each gene product remains largely unknown. FA proteins lack significant sequence homology to each other and to any proteins of known function. Although the general phenotypic feature of bone marrow failure in FA patients is the same across all complementation groups, each FA protein is likely to have specific cellular functions. FA proteins likely share a

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Abbreviations: CBP, calmodulin binding peptide; HPRD, human protein reference database; FA, fanconi anemia; HEK, human embryonic kidney; HTT, Huntingtin disease protein; IPI, international protein index; LC, liquid chromatography; MMC, mitomycin C; MS, mass spectrometry; SBP, streptavidin binding peptide; TAP, tandem affinity purification.

Table 1. FANCA interacting proteins that have been previously published using methods including yeast two-hybrid, immunoprecipitation, co-immunoprecipitation, and affinity purification.

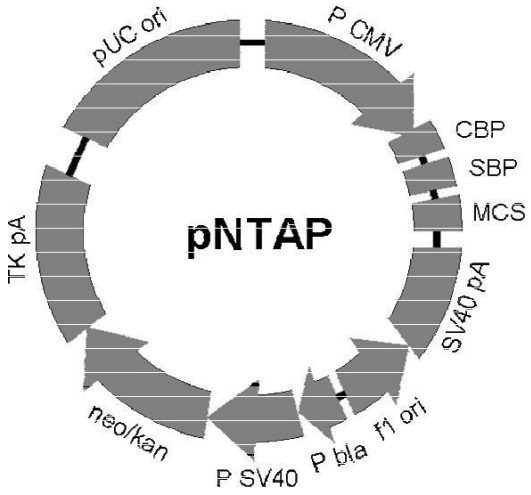
Biological process	Protein	Condition	Reference
Cell communication; Signal transduction	AKT	Yeast two-hybrid, untreated and co-immunoprecipitation, untreated	(Reuter et al., 2003; Otsuki, et al., 2002)
	DAXX	Yeast two-hybrid, untreated	(Reuter et al., 2003)
	IKK2	Yeast two-hybrid, untreated, coimmunoprecipitation, untreated and TNF-α treated	(Otsuki, et al., 2002)
	PKR	Immunoprecipitation, untreated	(Zhang et al.,2004)
	Ran	Yeast two-hybrid, untreated	(Reuter et al., 2003)
Cell growth and/or maintenance	Alpha spectrin (SPTA2)	Yeast two-hybrid, untreated	(Reuter et al., 2003)
DNA repair	FANCC	Immunoprecipitated, untreated	(Naf et al., 1998)
	FANCG	Co-immunoprecipitation, untreated	(Waisfisz et al., 1999)
Metabolism; Energy pathways	FANCL	Immunoprecipitation, untreated	(Medhurst et al., 2006)
Regulation of gene expression, epigenetic	BRCA1	Yeast two-hybrid, untreated and co-immunoprecipitation, untreated and IR-treated	(Folias et al., 2002)
Regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism	BLM	Immunoprecipitation, untreated and ethidium bromide treated	(Meetei et al., 2003)
	BRG1 (SMARCA4)	Yeast two-hybrid, untreated and immunoprecipitation, untreated	(Reuter et al., 2003; Otsuki et al., 2001)
	FANCE	Affinity purification, untreated and MMC treated	(Thomashevski et al., 2004)
	FANCF	Coimmunoprecipitation, untreated	(de Winter et al., 2000)
	XPF (ERCC4)	Yeast two-hybrid, untreated	(Reuter et al., 2003)
Transport	SNX5	Yeast two-hybrid, untreated	(Reuter et al., 2003; Otsuki et al., 1999)
Unknown	FANCB	Immunoprecipitated, untreated	(Meetei et al., 2004)

Biological process was determined from the Human Protein Reference Database (http://www.hprd.org/).

role in maintaining normal hematopoesis within the cell by either acting together in a multi-protein complex and/or by playing individual roles in other signaling pathways. It is suggested that assembly of a nuclear complex consisting of eight of the FA proteins (FANCA, FANCB, FANCC, FANCE, FANCF, FANCG, FANCL and FANCM) is required for FANCD2 to be monoubiquitinated on Lys 561 during S phase of the normal cell cycle (D'Andrea and Grompe, 2003). The monoubiquitination of FANCD2 at Lys561 is required for targeting of FANCD2 to the cell nuclei. FANCD2 mono- ubiquitination can be induced by DNA-damaging agents such as ionizing radiation and mitomycin C (D'Andrea and Grompe, 2003) . Although patients with FA clearly have a defect in DNA repair, the precise mechanism of how the FA proteins function within the cell remains largely unknown. Identification of FA protein interacting partners can facilitate the understanding of how each FA protein functions in the cell. Previous studies using several different methods, including yeast two-hybrid, affinity purification, immunoprecipitation and co-immunoprecipitation have identified a line of proteins that interact with members of the FA family. These previously identified FANCA interacting partners are shown in Table 1.

While some of these methods have been successful, the TAP method is advantageous for its ability to identify all members of a protein complex, not only for direct interactions, but for its ability to identify protein interactions that are based on post-translational modifycations, especially in mammalian systems. In addition, the two-step purification employed by TAP minimizes non-specific protein-protein interactions.

TAP is a relatively new method for discovery of proteins involved in a protein complex. TAP was first developed in yeast (Rigaut et al., 1999). Several TAP constructs have been developed by for use in mammalian systems (Burckstummer et al., 2006). In TAP, all the proteins that are in the same protein complex with the protein of interest will be pulled down after a two-step affinity purifycation. For example, the pNTAP vector contains Streptavidin Binding Peptide (SBP) and Calmodulin Binding Peptide (CBP) affinity tags adjacent to the 5' end of the



pNTAP Multiple Cloning Site Region (sequence shown 889-1051)

SBP tag (3' end)

P S G G C K L G

Srf I *BamH I* *Pst IEcoR I*

EcoR V *Hind III* *Acc 1 / Sal I* *Xho I* *Apa I*

Multiple stop codons

T7 promoter

* In pNTAP-A, no bases inserted; in pNTAP-B, T inserted; in pNTAP-C, TT inserted

Figure 1. Map of the pNTAP expression vectors.

multiple cloning sites (MCS) where a gene of interest can be inserted (Figure 1). The SBP tag allows for the protein of interest and its associated proteins to be captured through the tight binding of SBP to streptavidin resin and purified by eluting with excess biotin. The CBP tag allows for a second purification step in which the CBP tag binds to calmodulin resin and is released by EGTA through Ca^{2+} chelation. The final eluate contains all the proteins in the same complex with the protein of interest, and can be subsequently identified by tandem MS (MS/MS).

In this study, one of the FA proteins, FANCA, was TAP tagged using the pNTAP vector from Stratagene. Protein complexes under normal and DNA damaging conditions were TAP purified and identified by LC-MS/MS. Several novel FA protein interacting partners were identified and confirmed by western blot.

MATERIALS AND METHODS

Materials

Dulbecco's Modification of Eagles Medium (DMEM) with 4.5 g/L glucose, L-glutamine and sodium pyruvate was purchased from Cellgro (Herndon, VA, USA) . Fetal Bovine Serum (FBS) Premium was purchased from Atlanta Biologicals (Lawrenceville, GA, USA). 0.25% Trypsin-EDTA and Lipofectamine 2000 were obtained from Invitrogen (Carlsbad, CA, USA). pNTAP vectors (Figure 1) and Interplay Mammalian TAP Purification Kit were from Stratagene (La Jolla, CA, USA). The pFlag- CMV2- FANCA expression vector was obtained from Dr. D. Wade Clapp (Indiana University School of Medicine). Restriction endonucleases, *Xba I, Hind III,* and *EcoRI* .

, and modified trypsin were purchased from Promega (Madison, WI, USA). Shrimp alkaline phosphatase and antarctic phosphatase were from New England Biolabs (Ipswich, MA, USA). The rapid DNA ligation kit was from Roche (Indianapolis, IN, USA). Qiagen Miniprep kit, Qiagen plasmid Maxi kit, QIAexII Gel Extraction kit, QIAquick PCR purification kits were all from Qiagen (Valencia, CA, USA). 7.5% Tris-HCl Ready gels and Immun-Blot PVDF Membrane were from Bio-Rad (Hercules, CA, USA). Huntingtin antibody ab7667 was purchased from Abcam (Cambridge, MA, USA). Supersignal West Dura Extended Duration substrate and Immobilized Protein G were from Pierce (Rockford, IL, USA).

Mitomycin C (MMC) was from Sigma-Aldrich (St. Louis, MO, USA) DTT, Triethylphosphine (TEP) and 2-iodoethanol were purchased from Sigma Aldrich (St. Louis, MO, USA). Modified trypsin was purchased from Promega (Madison, WI, USA). Acetonitrile and MS grade water were purchased from Honeywell Burdick and Jackson (Morristown, NJ, USA).

Cloning

The gene, *fanca*, was cloned into the pNTAP vector using conventional molecular biology techniques. A new restriction site *Xba I* was created downstream of the *Xho I* site in the pNTAP-B vector (Figure 1) to generate a new vector, pNTAP-B-Xba I. The *fanca* gene was cut out of the pFlag-CMV2-FANCA vector using

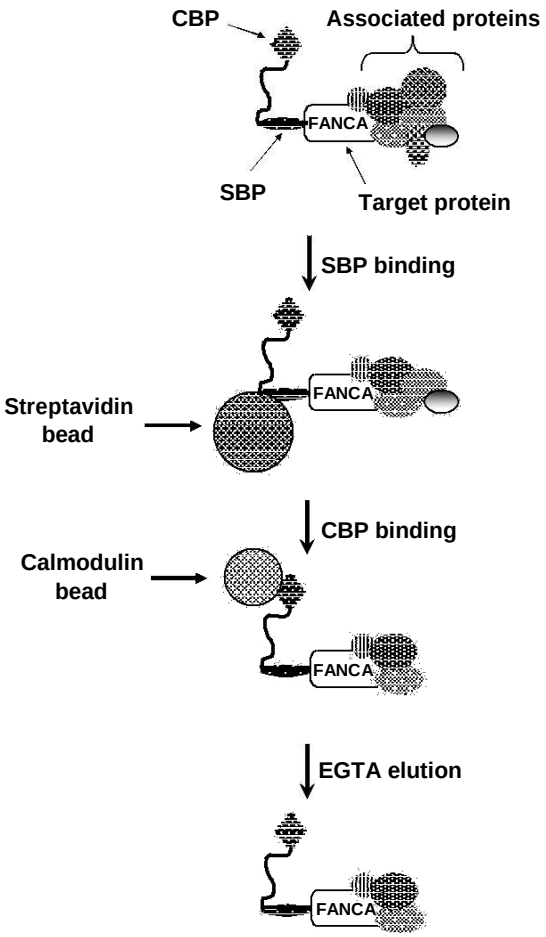


Figure 2. Overview of the TAP procedure. The high-affinity streptavidin-binding peptide (SBP) and calmodulin-binding peptide (CBP) tags allow for a two-step specific selection of the tagged protein and its associated proteins under mild conditions. EGTA eluted proteins are subsequently identified by tandem mass spectrometry.

both *Hind III* and *Xba I,* and then inserted into the pNTAP-B-Xba I vector. The *fancg* was cut out of the pFlag-CMV2-FANCG vector using *EcoRI* and then inserted into the pNTAP-C vector. The resulting vectors, pNTAP-B-Xba I, pNTAP-B- FANCA, and pNTAP-C-FANCG were verified by DNA sequencing.

Cell culture and transfection

The human embryonic kidney cells (HEK293) were maintained in DMEM with 10% FBS. The HEK293 cells were transiently transfected with pNTAP-B-FANCA or pNTAP-B-Xba I (empty vector, served as a negative control) vectors, respectively, using Lipofectamine 2000 transfection kit. For the co-expression experiment, HEK293 cells were transiently transfected with both the pNTAP-C-FANCG and pFlag-CMV2- FANCA vectors using Lipofectamine 2000. For each sample, ten 150 mm 90 - 95% confluent tissue culture plates were transfected with each vector in order to obtain a sufficient amount of proteins for mass spectrometric analysis following TAP purification. After 24 h of transfection, one group of pNTAP-B-FANCA transfected plates was treated with MMC to a final concentration of 40 ng/mL. All plates of HEK293 cells (treated and untreated) were collected 44 h post transfection. The efficiencies of transfection under different conditions (untreated and treated) were tested using a GFP-tagged TAP vector as control, and they showed very similar transfection efficiencies.

Tandem affinity purification (TAP)

A general TAP strategy is shown in Figure 2. The cells were purified using the Interplay Mammalian TAP Kit. Briefly, the cells were collected into 50 mL tubes and washed three times with cold PBS and lysed following the manufacture's protocol. Whole cell lysate from the cells was first bound to streptavidin beads. Unbound proteins were washed twice with the streptavidin binding buffer (SBB) and bound proteins were eluted with the streptavidin elution buffer which contains 2 mM biotin. The eluate was subsequently bound to Ca^{2+} -activated calmodulin beads. Unbound proteins were washed twice with calmodulin binding buffer (CBB) and bound proteins were eluted with the calmodulin elution buffer (CEB) which contains the chelating agent EGTA at 5 mM concentration. The final eluate containing the protein of interest (FANCA) and the proteins that associate with it were subsequently analyzed by tandem mass spectrometry.

Tandem mass spectrometric (LC-MS/MS) analysis

The TAP purified proteins were first precipitated using trichloro-acetic acid (TCA) to remove salts and other mass spec unfriendly materials. The precipitated proteins were denatured with 8 M Urea. Then 100 mM ammonium carbonate (pH 10.8) was added to each sample to increase the pH. The samples were reduced and alkylated by a solution containing 0.5% triethylphosphine (TEP), 2% 2- iodoethanol, and 97.5% acetonitrile at 37 ºC for 1.5 h and placed in a speed-vacuum overnight (Hale, et al. 2004). The dried samples were resuspended in a 100 mM ammonium bicarbonate solution and digested with modified trypsin (6 ng/mL) overnight at 37ºC. The tryptic peptides were then analyzed by nano-flow electrospray ionization (ESI) LC-MS/MS using a Thermo-Finnigan LTQ linear ion-trap mass spectrometer. Both the trapping column (i.d. = 200 µM, length = 2 cm) and analytical column (i.d. = 75 µM, length = 10 cm) were self-packed with Neucleosil 100 - 5 C_{18} resin (Macherey–Nagel). The peptides were eluted using the a nano-flow pump (Agilent 1100) with a linear gradient from 4 to 50% acetonitrile developed over 120 min at a flow rate of 250 nL/min, and effluent was electro-sprayed into the LTQ mass spectrometer. The data were collected in "Triple-Play" mode (MS scan, Zoom scan, and MS/MS scan). The acquired data were filtered and analyzed by a proprietary algorithm that has been described by Higgs et al. (2005). Database searching for the identities of the interacting proteins were determined using both the SEQUEST® and X!Tandem algorithms along with a proprietary algorithm that was recently developed by Higgs, et al. (2005) . Following data analysis, a list of proteins was generated based on their protein identification confidence levels (Reiner et al., 2003). Proteins with a q-value (estimated false discovery rate) less than 0.05 are declared significant, which means it is expected that 5% of the identified proteins will be false positives (Reiner et al., 2003).

Data interpretation

Each protein identified by TAP and LC-MS/MS was searched using the Human Protein Reference Database (HPRD, http://www.hprd.org/) to determine its function in a biological pro-

Table 2. FANCA interacting proteins under normal condition.

Biological function	Protein name	IPI number	Protein identification confidence (q-value)
Cell communication and Signal transduction	COUP transcription factor 1	IPI00021431.1	0.0011
	DNA-PK catalytic subunit*	IPI00296337.2	2.54e-06
Cell growth and/or Maintenance	TUBB1 human beta tubulin 1 class VI*	IPI00006510.1	2.76e-06
	Alpha-Actin, skeletal muscle	IPI00021428.1	0.0002
Chaperone activity	Heat shock 90 kDa protein 1 beta*	IPI00414676.2	2.06e-05
DNA repair	FANCA*	IPI00006170.1	2.12 e-06
Metabolism and energy pathways	SLC25A5 protein*	IPI00007188.2	3.19e-06
	Steroid dehydrogenase homolog*	IPI00007676.1	2.58e-05
Nuclear organization and biogenesis	Lamin A associated polypeptide 2 isoform beta/gamma	IPI00030131.2	0.0002
Protein metabolism	Heat shock cognate 71kDa protein	IPI00003865.1	9.11e-06
	Heat shock protein 86	IPI00031523.1	0.0040
	HSP89 alpha delta N	IPI00382470.1	0.0393
	KIAA0219 protein*	IPI00001159.6	1.17e-05
	RPLP1	47496625	7.21e-05
Regulation of cell shape	RHO interacting protein 3	IPI00166518.3	0.0002
Regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism	60kDa SS-A/Ro ribonucleoprotein	IPI00019450.2	0.0003
	Transcription intermediary factor 1 beta	IPI00438229.1	8.47e-06
Transport	Co-atomer beta subunit	IPI00295851.3	1.61e-05
	Hypothetical DKFZp686D22141*	IPI00464999.1	6.08e-06
	Importin alpha re-exporter	IPI00022744.4	1.96e-05
Unknown	Hypothetical FLJ14658*	IPI00477345.1	7.05e-06
	hypothetical protein DKFZp434G0222*	IPI00100160.1	0.0070
	Retinoblastoma associated factor 600*	IPI00180305.3	2.28e-05

All proteins listed have a q-value < 0.05 (>95% confidence). Biological process was determined from the Human Protein Reference Database. *Proteins also found in the FANCA MMC sample.

process. A query was done by manually inputting each protein name and its accession number from the TAP results (Tables 2 and 3). Pathway Studio® 4.0 from Ariadne Genomics was used to analyze known biological pathways in which these proteins are involved. This software allows the user to input a list of proteins that can be searched for direct interactions as well as for the shortest known pathway(s) between two proteins. This software also has the ability to expand the search to look for all proteins connected to the proteins in the list.

Immunoprecipitation

For each sample, three 150 mm 90 - 95% confluent tissue culture plates were transiently transfected with Flag-FANCA using Lipofectamine 2000. After 24 h of transfection, one group of Flag-FANCA transfected plates was treated with MMC to a final concentration of 40 ng/mL. All plates of HEK293 cells (treated and untreated) were collected 44 h post transfection into 50 mL tubes and washed three times with cold PBS. The cell pellets were resuspended in IP lysis buffer (150 mM NaCl, 10 mM HEPES, 5 mM EDTA, 1% Triton X-100, 1 mM PMSF, and 1% protease inhibitor cocktail) and incubated on ice for 20 min. Then they were centrifuged at 16,000 x g for 20 min at 4ºC. The lysates (supernatants) were saved and directly used for immunoprecipitation. Briefly, 5 µg of anti-Huntingtin (HTT) antibody (ab7667) was added to Flag-FANCA and Flag-FANCA MMC-treated lysates, respectively. The lysates were rotated at 4ºC overnight. Then 100 µL of

Figure 3. Western blot analysis of Huntingtin. TAP purified samples along with HEK293 whole cell lysate were run on a 7.5% acrylamide gel and transferred to PVDF membrane. Huntingtin antibody [HDB4E10] (ab7667 from Abcam) was used for western blot analysis. Lane 1, Precision Blue protein ladder; Lane 2, NTAP-MMC (negative control); Lane 3, FANCA-MMC; Lane 4, FANCA (no treatment); Lane 5, HEK293 whole cell lysate.

Figure 4. Immuoprecipitation of FANCA with Huntingtin antibody. Flag-FANCA was transiently transfected into HEK293 using Lipofectamine 2000. Huntingtin antibody was used to pull down Huntingtin and its associated proteins. The protein complex containing both Huntingtin and Flag-FANCA was analysis by western blot using Anti-Flag M2 antibody (for detection of Flag-FANCA). Lane 1, Precision Blue protein ladder; Lane 2, Flag-FANCA MMC; Lane 3, Flag-FANCA untreated.

Protein G beads were added to each sample and incubated with rotation for additional 2 h at room temperature. Then the lysates were centrifuged at 2,500 x g for 5 min at room temperature. The beads were washed with 1 mL each of the buffer containing 150 mM NaCl, 25 mM Tris-HCl, pH 6.8, 1 mM PMSF, and 1% protease inhibitor cocktail six times, and once with 0.5 mL of deionized water. The resins containing Huntingtin complex were resuspended in 90 µL of 2X Laemmli buffer (125 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 4% β-mercaptoethanol, 0.01% bromophenol blue) and incubated at 95ºC for 5 min. The resins were centrifuged at 2,500 x g for 5 min. The supernatants were saved and examined by western blot using anti-Flag antibody (M2 from Sigma-Aldrich).

Western blot analysis

Samples for pNTAP-MMC (empty vector control), FANCA, and FANCA-MMC were TAP purified from four tissue culture plates of HEK293 cells. Each of the samples, along with HEK293 whole cell lysate, were run on a 7.5% acrylamide gel and transferred to PVDF membrane. The blot was then blocked in 1xTBST (25 mM Tris -HCl, pH 7.4, 2.7 mM KCl, 137 mM NaCl, 0.1% Tween-20) plus 5% non-fat milk for 1 h. The blot was incubated in anti-HTT antibody (ab7667) at a 1:2,500 dilution in 1xTBST plus 5% non-fat milk for 1 h. The blot was then washed in 1xTBST three times for 10 min. The blot was incubated in Rabbit polyclonal to Mouse IgG H and L (ab6728) at a 1:15,000 dilution in 1xTBST plus 5% non-fat milk for 50 min and then washed four times with 1xTBST for 10 min. Supersignal West Dura Extended Duration Substrate was used to develop the blot.

Samples for Flag-FANCA and Flag-FANCA-MMC were immunoprecipitated with anti-HTT antibody [HDB4E10] (ab7667), then run on a 7.5% acrylamide gel and transferred to PVDF membrane. The same procedure as described above was applied except a monoclonal anti-Flag M2 antibody in 1:500 dilution was used for detection of Flag-FANCA.

RESULTS

Identification of FANCA interacting proteins

All of the proteins that were identified from the FANCA and FANCA MMC-treated TAP experiments were compared with the proteins that were pulled-down with the empty vector negative control (pNTAP-B-Xba I) . The redundant proteins (due to non- specific interactions) were excluded from the final protein lists. The lists of proteins identified with the bait FANCA with and without DNA damaging agent (MMC) are shown in Tables 2 and 3, respectively. The proteins identified with >95% confidence level under each condition were sorted based on biological function as determined from the protein database HPRD. Twenty-two FANCA interacting proteins were identified in the untreated sample (Table 2), while twenty- five proteins were identified in the FANCA-MMC treated sample (Table 3). Of all the proteins identified under both conditions, ten were found in both conditions (as indicated by a "*" in Tables 2 and 3) . The ten proteins that were found in both samples may contribute to the "core" of proteins that FANCA interacts with at all times regardless of DNA damage. The proteins that were found in only the FANCA untreated sample may be replaced by those proteins found in only the FANCA MMC-treated sample as a result of DNA damage. The proteins that were unique to the FANCA MMC-treated sample may be important in DNA damage recognition or repair.

Validation by Western blotting and immunoprecipitation

A novel interaction between FANCA and HTT was identified by LC -MS/MS. This interaction was only identified in the FANCA MMC-treated sample, and confirmed by repeating TAP followed by a western blot for HTT as well as by an alternative affinity purification approach, immunoprecipitation (IP). The TAP experiment was carried out using anti-HTT antibody (Figure 3) to confirm the tandem mass spectrometry results. For the IP, two groups of HEK293 cells were transiently transfected with Flag-FANCA in which one group was treated with MMC. After transfection, the whole cell lysates, which contain both endogenous HTT and over-expressed Flag-FANCA, were co-purified with the anti- HTT antibody. The purified complex was then run on an SDS-PAGE and a western blot analysis was carried out was repeated in HEK293 cells for FANCA with and without MMC treatment. After TAP, western blot analysis using an anti-Flag M2 antibody (Figure 4). The IP results were consistent with the TAP results. As shown in Figures 3

Table 3. FANCA interacting proteins in the presence of DNA damaging agent MMC.

Biological function	Protein name	IPI number	Protein identification confidence (q-value)
Cell communication and Signal transduction	CRM1 protein	IPI00298961.3	0.0002
	DNA-PK catalytic subunit*	IPI00296337.2	9.14e-07
	Ras-GTPase activating protein binding protein 2	IPI00009057.1	1.76e-05
Cell growth and/or Maintenance	TUBB1 human beta tubulin 1 class VI*	IPI00006510.1	8.70e-07
Chaperone activity	Heat shock 90 kDa protein 1 beta*	IPI00414676.2	0.0008
DNA repair	FANCA*	IPI00006170.1	8.70e-07
Metabolism and energy pathways	ATP5A1 protein	IPI00440493.1	9.29e-07
	Dynein cytoplasmic heavy chain	IPI00456969.1	2.23e-06
	Lactate dehydrogenase A	IPI00217966.1	0.0003
	Lactate dehydrogenase A like 6B	IPI00016768.2	0.0360
	SLC25A5 protein*	IPI00007188.2	1.92e-06
	Steroid dehydrogenase homolog*	IPI00007676.1	2.24e-06
Protein metabolism	Heat shock 70kDa protein 8 isoform 2	51095054	0.0004
	KIAA0010 protein	IPI00329519.4	0.0047
	KIAA0219 protein*	IPI00001159.6	5.34e-06
Regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism	E3 ubiquitin protein ligase URE-B1	IPI00445401.1	8.10e-06
	HNRPF protein	IPI00003881.2	0.0182
	Huntingtin	IPI00002335.1	0.0002
	Hypothetical protein FLJ20425	IPI00015838.1	2.46e-06
	Interleukin enhancer binding factor 3		
	THO complex subunit 2	IPI00158615.4	0.0027
Transport	Hypothetical protein DKFZp686D22141*	IPI00464999.1	1.27e-06
	Solute carrier family 25 member A6	IPI00291467.2	1.04e-06
Unknown	Hypothetical protein DKFZp434G0222*	IPI00100160.1	1.06e-06
	Hypothetical protein FLJ14658*	IPI00477345.1	4.41e-06
	Regulatory associated protein mTOR	IPI00166044.1	1.95e-06
	Retinoblastoma associated factor 600*	IPI00180305.3	3.78e-06

All proteins listed have a q- value < 0.05 (>95% confidence). Biological process was determined from the Human Protein Reference Database. *Proteins also found in the FANCA untreated sample.

and 4, HTT or Flag-FANCA was only detected under MMC treated conditions. Protein interaction studies have been previously done with both FANCA (Table 1) and HTT, as well as with mutant HTT. But none of those

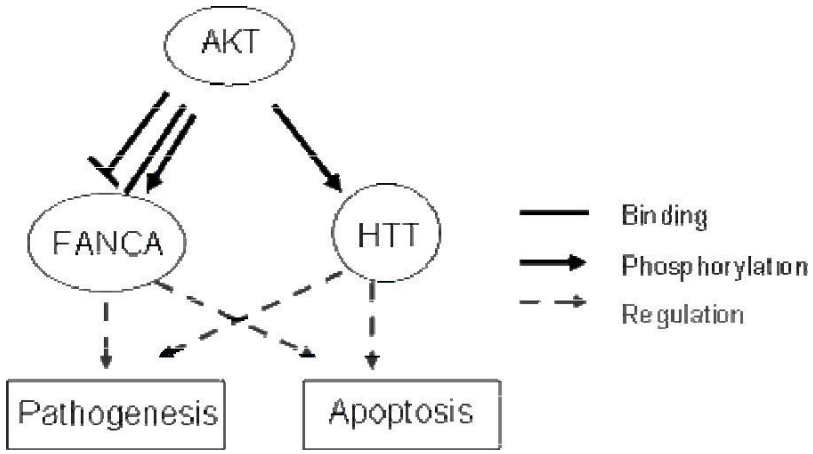


Figure 5. Pathway Analysis. Pathway Studio® 4.0 was used to search for the shortest pathway(s) between FANCA and HTT. Akt was the only protein found to interact with both proteins.

showed the interaction between FA and HTT. Huntington disease, an autosomally dominant degenerative disorder, results from an expansion of polyglutamine repeats (> 37) in the N-terminal of huntingtin protein (Li et al., 2002). The function of HTT has been linked to cellular trafficking through interaction with both Huntingtin- associated protein-1 (HAP1) and Huntingtin-interacting protein 1 (HIP1) which are both involved in cellular trafficking (Hyun and Ross, 2004; Rong et al., 2007). Since both FANCA and HTT are found in multiple cellular compartments, the significance of their interaction may be that HTT can assist FANCA moving between cellular compartments.

DISCUSSION

Understanding protein- protein interactions in a cell is a crucial step toward the understanding of the function of a protein. There are many methods that have been employed to identify protein-protein interactions (Bauer and Kuster, 2003) that exhibit various pros and cons. One of the more widely used ones is the yeast two-hybrid method (Serebriiskii et al., 2002). Yeast two-hybrids have the highest false negative rate when compared with affinity purification and TAP (Dziembowski and Seraphin, 2004). Reuter et al. (2003) successfully used yeast two-hybrid screens for FANCA, FANCC and FANCG, and several proteins were identified as having a direct involvement with the FA pathway. However, in their yeast two-hybrid screens, none of the screens identified a second FA protein (Reuter et al., 2003). One possible conclusion to the yeast two-hybrid screens failure to detect the interaction between FANCA and FANCG could be because the interaction is dependent on protein post-translational modifications which might differ or not occur in yeast relative to mammalian cells (Reuter et al., 2003).

Two of the FA proteins, FANCA and FANCG, are known phosphoproteins (Mi, et al. 2004); therefore it is reasonnable to speculate that other protein interactions with FANCA or FANCG may likely be dependent on their phosphorylation status. Another common method used to identify protein-protein interactions is affinity purification, which can be achieved with a variety of tags including Myc, HA, and Flag tags (Bauer and Kuster, 2003). The affinity purification method can detect protein interactions that are dependent on post-translational modifications if the proteins are expressed in mammalian cells. Unfortunately, the two problems associated with the affinity purification method are: 1) the possibility that an added tag on a protein might alter the structure of a protein or interfere with its activity (Puig et al., 2001); and 2) the non-specific interactions of the protein tag with other proteins in the sample, which can lead to a high false-positive rate (Dziembowski and Seraphin, 2004). Immunoprecipitation is another method to detect protein-protein interactions. The problem with Immunoprecipitation, however, is that it requires the use of an antibody which may have cross-reactivity within the sample (Bauer and Kuster, 2003). While these methods are still widely used by many investigators, the use of the TAP method minimizes the non-specific interactions through a two-step purification procedure, which appears to be a more feasible and efficient method for protein complex determination in mammalian systems (Dziembowski and Seraphin, 2004). An advantage of the TAP method over the yeast two-hybrid method is that TAP allows for identification of all components in the complex whether they are direct or indirect interactions (Riguat et al., 1999). Proteins in nature can have a broad specificity for other proteins and may bind tightly to one protein and still bind loosely to another.

In this study, TAP was used to identify proteins that

were in the same protein complex with FANCA under normal and DNA damaging conditions. A total of thirty-seven proteins were identified from both the FANCA and FANCA MMC-treated samples. Previous research had identified several proteins including; FANCB, FANCC, FANCE, FANCF, FANCG, FANCL, BLM, BRCA1, SPTA2, PKR, IKK2, DAXX, BRG1, XPF, AKT, RAN, and SNX5, which interact with FANCA as shown in Table 1. Unexpectedly, none of the previously identified FANCA interacting proteins were pulled down in either the FANCA or FANCA MMC-treated samples. One reason for this could be due to the extremely low endogenous expression levels of other known FANCA interacting proteins. Interestingly, the previously identified FANCA interacting proteins are involved in the same biological processes as those identified by TAP with the addition of DNA repair and regulation of gene expression, epigenetic (Tables 1 - 3). The TAP method begins with whole cell lysate that is obtained using mild buffer conditions, which are maintained throughout the purification. Separation of a nuclear fraction where FANCA is known to reside requires much higher salt concentrations in the lysate. This condition would be too harsh for proteins that are not tightly bound to the FANCA complex to be recovered after TAP. The goal of our study is to identify all the components of the FANCA protein complex, whether they are tightly or loosely associated under native conditions. TAP is different from the previously used methods for identification of FANCA interacting proteins, which include: yeast two-hybrid, co-immunoprecipitation, in vitro translated protein immunoprecipitation and immunoprecipitation (Burckstummer et al., 2006; Otsuki et al., 2001; Otsuki et al., 1999). Thus, it is not a surprising that we observed a different set of FANCA interacting proteins identified by TAP method. As all of our TAP experiments displayed, “bait” protein FANCA was always the top “hit” from database searching results, suggesting a high level of confidence in protein purification and identification.

Many of the methods used previously to confirm FANCA protein interactions involved over-expression of both FANCA and the target protein. In addition, some of these methods started with a nuclear extract compared with the whole cell lysate used in TAP. To confirm the results obtained from the TAP experiments, and the reason that we did not pull down other members of the FA complex is likely due to their low endogenous protein expression levels, we performed another TAP experiment in which pNTAP-C-FANCG and Flag-CMV2- FANCA were co-transfected in HEK293 cells. Following TAP purify-cation and tandem mass spectrometry, FANCG with 94 peptides and a q- value of 3.86e-07 (>99.99% confidence) and FANCA with 37 peptides and a q-value of 3.86e-07 (>99.99% confidence) were top two proteins identified along with other FANCA interacting proteins. This result not only confirmed our reasoning that other members of FA complex are at low endogenous levels, but also validated our TAP method.

Functional classification based on biological processes for all thirty-seven proteins identified includes: 1) cell communication and signal transduction, 2) cell growth and/or maintenance, 3) chaperone activity, 4) DNA repair, 5) metabolism and energy pathways, 6) nuclear organization and biogenesis, 7) protein folding, apoptosis, and regulation of immune response, 8) protein metabolism, 9) regulation of cell shape, 10) regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism, and 11) transport, which suggests that FANCA plays multiple roles in the cell. Of the thirty-seven proteins identified, only ten were common in both the FANCA untreated and FANCA MMC-treated samples. One explanation for the small number of overlapping proteins could be due to the method of transfection. Each TAP experiment was performed by transient transfection of each construct. It is possible that the number of proteins found between the untreated and MMC-treated FANCA samples might increase if stable cell lines expressing the TAP vectors (pNTAP-B-Xba I and pNTAP-B-FANCA) could be established. Another possibility is that the ten proteins common in both conditions are part of a “core” complex of proteins that bind tighter to FANCA than the proteins that are unique to either the untreated or treated FANCA samples. Proteins that are not part of the “core” complex are likely to be more loosely bound to the “core” complex, and a unique set of these proteins may be inter-changeable depending on the cellular condition. In our case, we found six proteins that are involved in the regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism in the MMC-treated sample versus two in the untreated sample (Tables 2 and 3). The proteins that were found only in the MMC- treated sample could have important implications about the function of FANCA in DNA damage recognition or repair because MMC is a known DNA cross-linking agent. In our case, we found six proteins that are involved in the regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism in the MMC-treated sample versus two in the untreated sample (Tables 2 and 3). Interestingly, there were five proteins that were previously identified to interact with FANCA which are also involved in the regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism (Table 1). FANCA clearly binds to other proteins that function in the regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism. Many of the proteins that function in the regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism also have been shown to bind to DNA, which could again implicate the role FANCA plays in DNA repair.

Using both western blot and immunoprecipitation methods, we successfully confirmed a novel HTT/FANCA interaction that is induced upon MMC treatment. This observation is important and may help us to better understand the function of FANCA in DNA repair. To establish the link between FANCA and HTT, we then used a path

way analysis tool to elucidate whether there was a known pathway that links these two proteins. A search using Pathway Studio® 4.0 was performed to look for the shortest pathway(s) between FANCA and HTT. A serine/-threonine kinase Akt (also called protein kinase B or PKB) was the only protein found (Figure 5). Akt has been shown to phosphorylate both proteins (Humbert et al., 2002) . Otsuki et al. (2002) showed that FANCA is a substrate of Akt and is phosphorylated by Akt at S1149 (Otsuki et al., 2002).They also found that Akt can regulate the phosphorylation status of FANCA (Otsuki et al., 2002). When WT-FANCA was expressed in HEK293 cells along with constitutively active (CA)-Akt, the phosphorylation of WT-FANCA was blocked, but when WT-FANCA was expressed with dominant-negative (DN)-Akt, the phosphorylation of FANCA was increased (Otsuki et al., 2002).

Otsuki et al. (2002) also hypothesized that Akt might participate with another kinase to modify the phosphorylation status of FANCA which in turn could lead the cell to become pro- or anti-apoptotic (Otsuki et al., 2002). For HTT, Humbert et al. (2002) showed that the active form of Akt can phosphorylate HTT at S421 in human HTT with 23 glutamines, whereas the inactive form of Akt cannot (Humbert et al., 2002).

They also showed that IGF-1 treatment of human neuroblastoma SH-SY5Y cells induced phosphorylation at S421, and that this phosphorylation plays a protective role against neuronal cell death (Humbert et al., 2002).

The HTT/FANCA interaction is likely based on their phosphorylation status given the role of Akt in both proteins. Determining whether or not the phosphorylation of FANCA and/or HTT increased or decreased upon MMC treatment would be interesting to explore. It would also be interesting to determine whether the HTT/FANCA interaction remained intact with the co-expression of CA-Akt or DN -Akt. Since it has been shown that phosphorrylation of HTT plays a protective effect on neuronal cells, it would be reasonable to expect that HTT is phosphorrylated upon MMC treatment to protect the cells from DNA damage.

The biological significance of the phosphorylation of FANCA is unknown, but could account for the variation in binding partners. Perhaps, phosphorylation at certain residue allows one protein to bind while blocking the binding of the second protein.

Although the HTT/FANCA interaction appears to be very interesting, more studies need to be carried out to deter-mine whether this interaction plays a role in cell survival and/or DNA repair. Under DNA damage conditions, both HTT and FANCA are probably phosphorylated by AKT, which then might allow HTT to interact with FANCA so that it can assist FANCA moving between cellular com- partments, and possibly bring FANCA to sites of DNA damage. Results obtained from these TAP experiments suggest that several FANCA interacting proteins need to be fur her studied before their roles in *Fanconi anemia* can be better understood.

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