

RESEARCH ARTICLE

Proteomic analysis reveals a FANCA-modulated neddylation pathway involved in CXCR5 membrane targeting and cell mobility

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ABSTRACT

The aim of this study was to identify novel substrates of the FANCore complex, the inactivation of which leads to the genetic disorder Fanconi anemia, which is associated with bone marrow failure, developmental abnormalities and a predisposition to cancer. Eight FANCore proteins participate in the nuclear FANCore complex, which functions as an E3 ubiquitin-ligase that monoubiquitylates FANCD2 and FANCI in response to replicative stress. Here, we use mass spectrometry to compare proteins from FANCore-complex-deficient cells to those of rescued control cells after treatment with hydroxyurea, an inducer of FANCD2 monoubiquitylation. FANCD2 and FANCI appear to be the only targets of the FANCore complex. We identify other proteins that are post-translationally modified in a FANCA- or FANCC-dependent manner. The majority of these potential targets localize to the cell membrane. Finally, we demonstrate that (a) the chemokine receptor CXCR5 is neddylation; (b) FANCA but not FANCC appears to modulate CXCR5 neddylation through an unknown mechanism; (c) CXCR5 neddylation is involved in targeting the receptor to the cell membrane; and (d) CXCR5 neddylation stimulates cell migration and motility. Our work has uncovered a pathway involving FANCA in neddylation and cell motility.

KEY WORDS: Fanconi anemia, CXCR5, NEDD8, Cell motility, Leukemia

INTRODUCTION

Protein activity, stability and/or subcellular localization are precisely regulated through post-translational modifications (PTMs) (Cai et al., 2012; Han and Martinage, 1992). Among several known PTMs, ubiquitylation is based on the addition of ubiquitin chains to target proteins in a three-step process. The ubiquitin protein is a 76-amino-acid polypeptide with a molecular mass of 8.5 kDa. It is first attached to the E1 ubiquitin-activating enzyme. It is then transferred to a ubiquitin-conjugating enzyme (Ubc or E2), which will then interact with an ubiquitin ligase (E3) that will complete the ubiquitin transfer to a specific lysine (K) on the target protein (Komander and Rape, 2012). Ubiquitylation marks protein for proteasomal degradation or acts to regulate their function or subcellular localization (Chen

and Sun, 2009). The hypothesis that ubiquitylation plays a regulatory role is supported by the identification of a family of deubiquitylating enzymes (DUBs) that are involved in the rapid and specific deubiquitylation of ubiquitylated proteins (Clague et al., 2012). Furthermore, other ubiquitin-like proteins (UBLs), such as small ubiquitin-like modifier (SUMO), neural precursor cell expressed developmentally downregulated 8 (NEDD8) and interferon stimulated gene 15 product (ISG15) have been identified; these proteins undergo processes analogous to the ubiquitylation pathway and play roles similar to that of ubiquitin. UBLs often target the same proteins at the same sites as ubiquitin. Among the several identified UBLs, NEDD8 is the closest relative to ubiquitin and might regulate the ubiquitylation process (Xirodimas, 2008). For example, NEDD8 targets the epidermal growth factor receptor (EGFR) and stimulates its ubiquitylation, which is required for receptor internalization and degradation or recycling (Oved et al., 2006). In addition, NEDD8 also targets the Cullin family of proteins, in which neddylation induces the conformational changes that are necessary for their activity (Pan et al., 2004). Whereas few E1, E2, E3 and deconjugating NEDD8 enzymes have been identified to date, the dynamic and reversible circuitry of the ubiquitylation and deubiquitylation processes involves two E1s and ~40 E2s, 600 E3s and 90 DUBs. The E3 family comprises two groups. The members of one group contain the ‘homologous to the E6-AP carboxyl terminus’ (HECT) domain, and the members of the other group contain a ‘really interesting new gene’ (RING) domain. The RING-E3 subfamily consists both of E3 proteins that directly link the E2 and the target protein, and E3 proteins that bind to only the E2 while the target protein interacts with another component of a multi-protein complex that includes the E3 (Acconcia et al., 2009; Chen and Sun, 2009; Deshaies and Joazeiro, 2009).

The FANCore complex is an E3 multiprotein complex that contains the E3 RING ligase FANCL, which, in collaboration with the E2 UBE2T, catalyzes the transfer of a monoubiquitin to the downstream targets FANCD2 and FANCI (Alpi et al., 2007; Alpi et al., 2008; Machida et al., 2006). FANCore complex partners, including FANCA, FANCB, FANCC, FANCE, FANCF, FANCG, FANCL and FANCM, and its two identified targets constitute the upper portion of a signaling cascade referred to as the FANCore pathway. This pathway also includes six downstream proteins, FANCD1, FANCI, FANCN, FANCO, FANCP and FANQ (also known as BRCA2, BRIP1, PALB2, RAD51C, SXL4 and XPF or ERCC4, respectively), which are more directly involved in homologous recombination (Bogliolo et al., 2013; Kashiwama et al., 2013; Kim and D’Andrea, 2012). A biallelic mutation in any of the 16 identified *FANCore* genes causes the rare DNA damage response (DDR) syndrome Fanconi anemia. The symptoms of this syndrome include bone marrow failure, genetic instability, a predisposition to acute myeloid

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leukemia and head and neck cancers (Soulier, 2011), altered free radical generation and detoxification (Pagano et al., 2012), and abnormalities in cytokine production and responses (Briot et al., 2008; Pang et al., 2001; Rosselli et al., 1992). This clinical and cellular heterogeneity could result from a general disturbance of the cell and tissue metabolism as a consequence of a DDR defect, but it might also be caused by alternative activities of a single FANCA protein or the existence of other currently unidentified targets of the FANCCore complex.

To further elucidate the cellular and clinical consequences of inactivation of the FANCA pathway, in this study, we aimed to identify and characterize novel targets of the E3 ubiquitin-ligase activity of the FANCCore complex. FANCD2 and FANCI appear to be the only true direct targets of the nuclear FANCCore complex. Surprisingly, the majority of the other proteins that were identified as potential FANCA or FANCC targets localize to the cell membrane. Finally, we demonstrate that the chemokine receptor CXCR5 is neddylated in a FANCA-dependent manner through a currently unknown mechanism and that CXCR5 neddylation is involved in the targeting of this receptor to the cell membrane and in optimizing cell migration and motility.

RESULTS

Identifying proteins that are post-translationally modified by the FANCCore complex

To identify novel substrates of the ubiquitin ligase activity of the FANCCore complex that might be involved in its heterogeneous phenotype, we used a recently developed approach based on the immunoaffinity enrichment of peptides that are modified by ubiquitin or ubiquitin-like (NEDD8 and ISG-15) chains using antibodies that are specifically directed against the K-GG moiety that remains on the modified lysine residues after the tryptic digestion of a cellular extract (Fig. 1A). We compared the proteins extracted from exponentially growing HSC-72 (FANCA-deficient) and HSC-536 (FANCC-deficient) FANCCore complex-deficient lymphoblasts with those of their corrected counterparts (HSC-72corr and HSC-536corr, respectively) after treatment with the replication inhibitor hydroxyurea, which is a strong inducer of FANCCore complex activity and FANCD2 monoubiquitylation.

The peptides containing the lysine targeted by the FANCCore complex in FANCD2 and FANCI were unambiguously identified as over-represented in the corrected cells and had a calculated FANCCore-proficient:FANCCore-deficient fold change of at least 20 (supplementary material Tables S1 and S2). We expected to

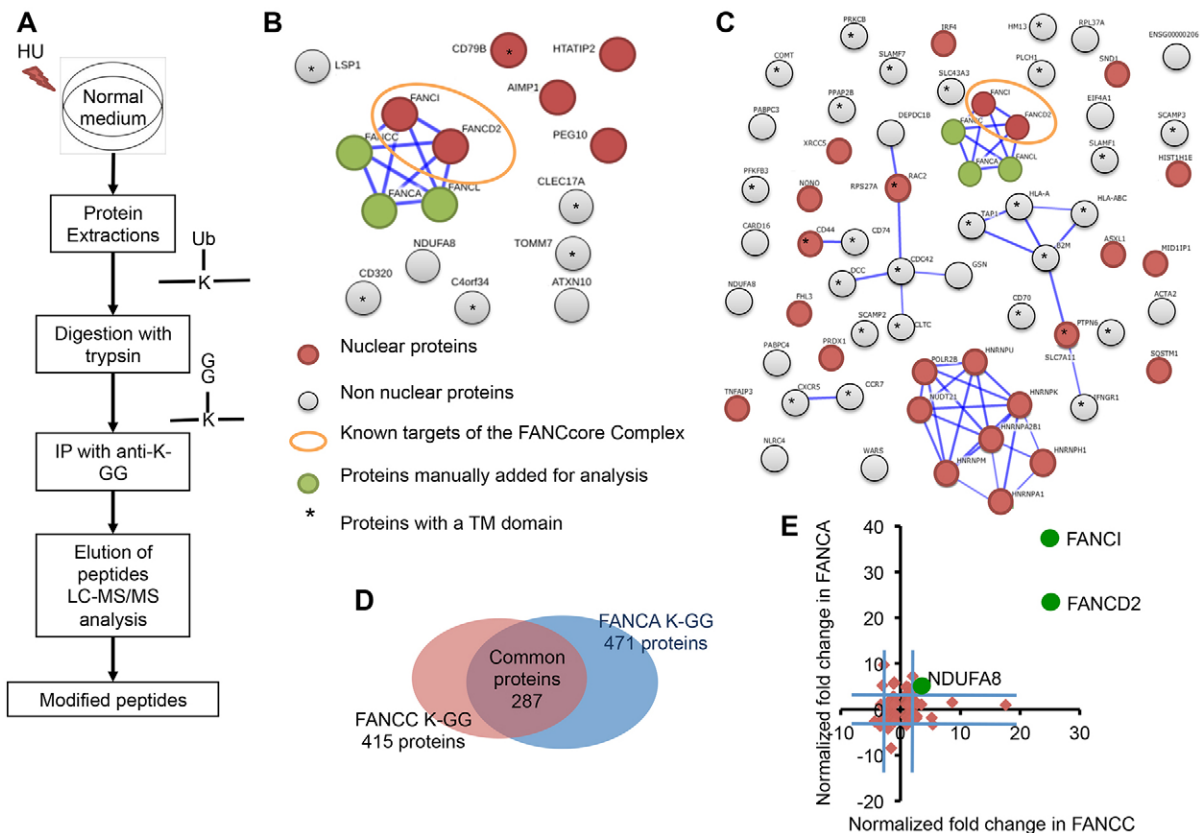


Fig. 1. Ubiquitylome analysis of FANCA- and FANCC-deficient cells and their corrected counterparts identifies FANCD2 and FANCI as the main targets of the FANCCore complex. (A) The enrichment strategy for ubiquitylated peptides is shown. Cells were treated with 5 mM hydroxyurea (HU) and collected after 8 h. IP, immunoprecipitation. (B,C) The interacting network of significant proteins (ratio of proficient:deficient of >2.5), as determined by the Stringdatabase Software, is shown for FANCC (B) and FANCA (C) cells. FANCD2 and FANCI are circled in orange. Green circles indicate the manually added proteins. Red circles indicate the nuclear-localized proteins. Gray circles indicate the non-nuclear proteins that eventually (*) associate with the membrane. TM, transmembrane. (D) A Venn diagram showing the overlap of potentially ubiquitylated proteins identified in the FANCA- and FANCC-deficient cell lines after K-GG immunoprecipitation. (E) A comparison of the quantified ratios obtained for the 287 proteins that were common to the screens of FANCA-deficient and FANCC-deficient cells. Blue lines define the region of exclusion. Green spots indicate proteins whose post-translationally modified peptide was found to be significantly different in both FANCA- and FANCC-deficient cells compared with the respective corrected controls.

identify peptides from other proteins at high concentrations in the corrected cells and with fold changes between the corrected and the Fanconi anemia cells that were similar to those observed for FANCD2 and FANCI in both the FANCA and FANCC analysis. Surprisingly, we did not identify other proteins with a ratio similar to that calculated for FANCD2 and FANCI, which suggests that FANCD2 and FANCI might be the only targets of the FANCCore complex.

The peptides that were identified when comparing the FANCC-deficient to the FANCC-corrected cell line revealed 415 individual proteins that were modified by the addition of ubiquitin or a ubiquitin-like moiety (supplementary material Table S1). In addition to FANCD2 and FANCI, we found relatively few proteins that had a significant Z-score and/or a robust (>2.5) FANCC-proficient:FANCC-deficient cell ratio (supplementary material Table S1; Fig. 1B). Only one polypeptide belonging to the uncharacterized transmembrane glycan-binding receptor on dividing B cells – C-type lectin domain family 17, member A isoform 1 (CLEC17A or prolectin) protein (Koh et al., 2011) – exhibited a fold change that was similar to the values obtained for FANCD2 and FANCI.

When we compared the FANCA-deficient cells with the FANCA-corrected cells, we identified 471 unique proteins, including FANCD2 and FANCI (supplementary material Table S2; Fig. 1C). Of the proteins identified in our FANCC analysis, 287 were also identified in the FANCA analysis (Fig. 1D,E; supplementary material Table S3). Of the 76 proteins identified in the FANCA analysis that had a high FANCCore-complex-proficient: deficient ratio (>2.5) and/or a significant Z-score, only three were also identified in the FANCC analysis (Fig. 1E; supplementary material Table S3). These three proteins were FANCD2, FANCI and the nuclear-encoded NDUFA8 subunit of NADH:ubiquinone oxidoreductase, which is part of the first mitochondrial respiratory chain complex (Triepels et al., 1998). Only FANCD2 and FANCI, however, had a highly significant FANCCore-corrected:FANCCore-deficient ratio, which was consistent with their already known direct ubiquitylation by the FANCCore complex.

Surprisingly, for both the FANCA and FANCC analyses, the majority of the identified proteins appeared to be localized outside of the nucleus and specifically appeared to be localized at the membranes (Fig. 1B,C; supplementary material Fig. S1). Because we failed to identify novel targets of the FANCCore complex, we next examined the sensitivity of our approach. Among the identified post-translationally modified proteins, we identified the well-known replication-stress-induced monoubiquitylated peptide of PCNA and a peptide from the DNA-replication-licensing factor MCM7. There was no significant difference in the level of these peptides between the Fanconi anemia and corrected cells. The monoubiquitylation of FANCD2 and PCNA was easily detected by immunoblot (supplementary material Fig. S2A,B), but we needed to perform an immunoprecipitation with anti-MCM7 or anti-ubiquitin (FK2) antibodies prior to western blot analysis in order to enrich and detect the ubiquitylated MCM7 form. Using this technique, we demonstrate that MCM7 is monoubiquitylated (supplementary material Fig. S2C). According to the mass spectrometry results, however, there was no significant difference in the MCM7 or PCNA monoubiquitylation levels that correlated with the proficiency of the FANCC pathway.

Because of these findings, we are confident that the approach used in this study was sufficiently sensitive and specific to achieve our goal. Therefore, the proteomics results from this

study suggest that the FANCCore complex is only involved in FANCD2 and FANCI monoubiquitylation and that some of its components might act independently of one another in other pathways and cellular locations.

CXCR5 is a neddylated protein, and FANCA is involved in its neddylation

Among the proteins identified in the FANCA analysis, CXCR5, B2M and SLAMF7 might be potential targets of a FANCA-regulated activity through currently unknown mechanisms. To confirm a role for FANCA in the differences observed in our study, we continued our characterization of the observed PTM of the transmembrane chemokine receptor CXCR5, which plays a role in B cell behavior, cancer progression, cancer invasion and auto-immune and pro-inflammatory diseases (Müller et al., 2003; Pevzner et al., 1999). To determine whether the difference observed in the mass spectrometry analysis was the result of a modified expression of CXCR5 in the Fanconi-anemia-derived cells or of a FANCA-induced CXCR5 overexpression, we used western blotting to determine the CXCR5 expression level at steady state and following treatment with hydroxyurea. The Fanconi anemia cells and their corrected counterparts have similar CXCR5 expression levels, excluding bias in the mass spectrometry data (Fig. 2A). Next, the proteins extracted from the FANCA- and FANCC-deficient cells and their corrected counterparts were immunoprecipitated using the anti-ubiquitin antibody FK2, and an immunoblot analysis was conducted using an anti-CXCR5 antibody and an anti-PCNA antibody as a positive control for the immunoprecipitation. Unexpectedly, we failed to identify a ubiquitylated form of CXCR5 (supplementary material Fig. S3A). The K-GG moiety that is created by the tryptic digestion of the protein extract before the mass spectrometry analysis and that is recognized by the specific antibody used to enrich ubiquitin-modified peptides is also a signature of NEDD8 and ISG15 PTMs. Consequently, we immunoprecipitated the protein extract from FANCC-proficient Epstein-Barr virus (EBV)-transformed HSC93 lymphoblasts using either an anti-NEDD8 or an anti-CXCR5 antibody and conducted an immunoblot analysis using an anti-CXCR5 antibody, which revealed the presence of a specific, albeit minor, neddylated form of CXCR5 (Fig. 2B). To confirm the observed CXCR5 neddylation, we followed two complementary approaches. First, we demonstrated that the presumed neddylated-CXCR5 band is no longer present in samples from cells treated with the NEDD8-specific inhibitor ML4924 (Wei et al., 2012) (Fig. 2C), which is able to eliminate the known neddylated form of Cul4A (supplementary material Fig. S2B) without modification of the CXCR5 level (supplementary material Fig. S2A). Second, we used proximity ligation assay (PLA) technology on fixed cells with anti-NEDD8 and anti-CXCR5 antibodies (Fig. 2D). Exposure to the NEDD8 inhibitor significantly reduced the number of CXCR5–NEDD8 spots per cell (Fig. 2E). Finally, we performed a NEDD8 immunoprecipitation using cellular extracts from the Fanconi anemia cells and corrected cells (Fig. 2F). The immunoblot demonstrates that the expression level of the neddylated CXCR5 form is elevated in the FANCA-corrected but not the FANCC-corrected lymphoblasts when compared with the respective Fanconi anemia cells (Fig. 2G).

In conclusion, by using a combination of different approaches, we have identified CXCR5 as a new member of the small group of neddylated proteins, validating the results from our proteomics analysis, and we have shown that FANCA,

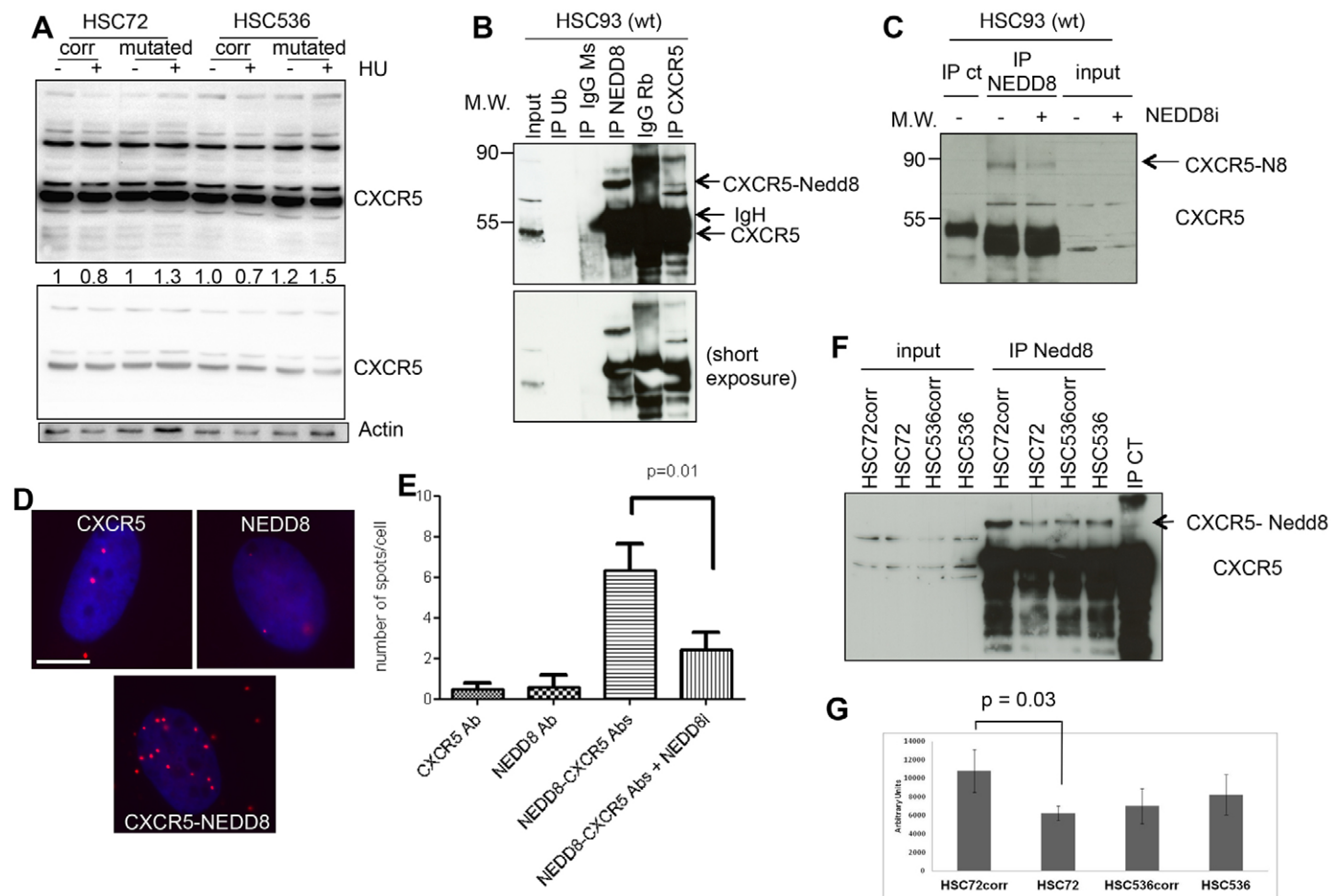


Fig. 2. CXCR5 is neddylated in a FANCA-dependent manner. (A) Western blot results showing the relative expression level of CXCR5 in FANCA-deficient (HSC72) cells, FANCC-deficient (HSC536) cells and their corrected counterparts (corr), with or without treatment with hydroxyurea (HU) (5 mM for 8 h). (B) Western blot showing the CXCR5 expression level after immunoprecipitation (IP) with antibody against NEDD8, CXCR5 or ubiquitin (Ub) in HSC-93 cells (FANCC-pathway-proficient; wild-type, wt). IgG Rb, rabbit IgG. (C) Western blot showing the CXCR5 neddylated band after immunoprecipitation with a NEDD8 antibody in untreated cells or cells exposed to the NEDD8 inhibitor MLN4924. Ct, control. (D) PLA (red dots) results with antibodies against CXCR5, Nedd8 or both are shown. The nucleus is stained with DAPI (blue). Scale bar: 10 μ m. (E) Quantification of the PLA dots in cells analyzed by the addition of antibodies (Abs) against CXCR5, NEDD8 or both, in absence or presence of the NEDD8 inhibitor MLN4924. (F) Western blot results showing the CXCR5 expression level after immunoprecipitation with the antibody against Nedd8 in FANCA-deficient cells, FANCC-deficient cells and their corrected counterparts. (G) Relative quantification of the immunoprecipitated band from three independent experiments is also shown. For E and G, data show the mean \pm s.d.; significance was calculated by using Student's *t*-test.

but not the FANCCore complex, plays a role in CXCR5 neddylated.

Neddylated is responsible for targeting CXCR5 to the cell membrane and for cell motility

Because neddylated has been shown to be involved in EGFR trafficking (Oved et al., 2006), we wanted to determine whether this was also the case for the CXCR5 neddylated identified here. Therefore, by flow cytometry analysis, we quantified the amount of CXCR5 localized to the plasma membrane in several cell lines. Consistent with the hypothesis that the addition of NEDD8 to CXCR5 is involved in the targeting of the latter to the cell membrane, the FANCA-corrected cells showed the highest level of membrane-associated receptor among the analyzed EBV-transformed lymphoblastoid cell lines (Fig. 3A,B).

To confirm and expand on our previous results showing that CXCR5 is neddylated in a FANCA-dependent manner and that neddylated is required for the optimal targeting of CXCR5 to the

membrane, we used different approaches. First, we treated the FANCA-corrected cells with the NEDD8 inhibitor MLN4924. As expected, the MLN4924 treatment reduced the level of membrane-associated CXCR5 in the FANCA-corrected cells in a dose-dependent manner (Fig. 3C). This finding supports the hypothesis that neddylated is important for the optimal targeting of CXCR5 to the cell surface. Next, we transfected U2OS cells with either a full-length wild-type form of CXCR5 or a mutant CXCR5-K339R form that cannot be neddylated (supplementary material Fig. S3C). Compared with the untransfected cells, there was an increase in the CXCR5 protein level on the membranes of the cells overexpressing wild-type CXCR5. Furthermore, the CXCR5-K339R mutant, which was expressed at the same level as the wild-type CXCR5, was less targeted to the membrane (Fig. 3D,E).

Finally, it has been reported that the overexpression of the oncogenic transcription factor MiTF in the metastatic melanoma cell line MEL501, which was isolated from a lymphatic node, is

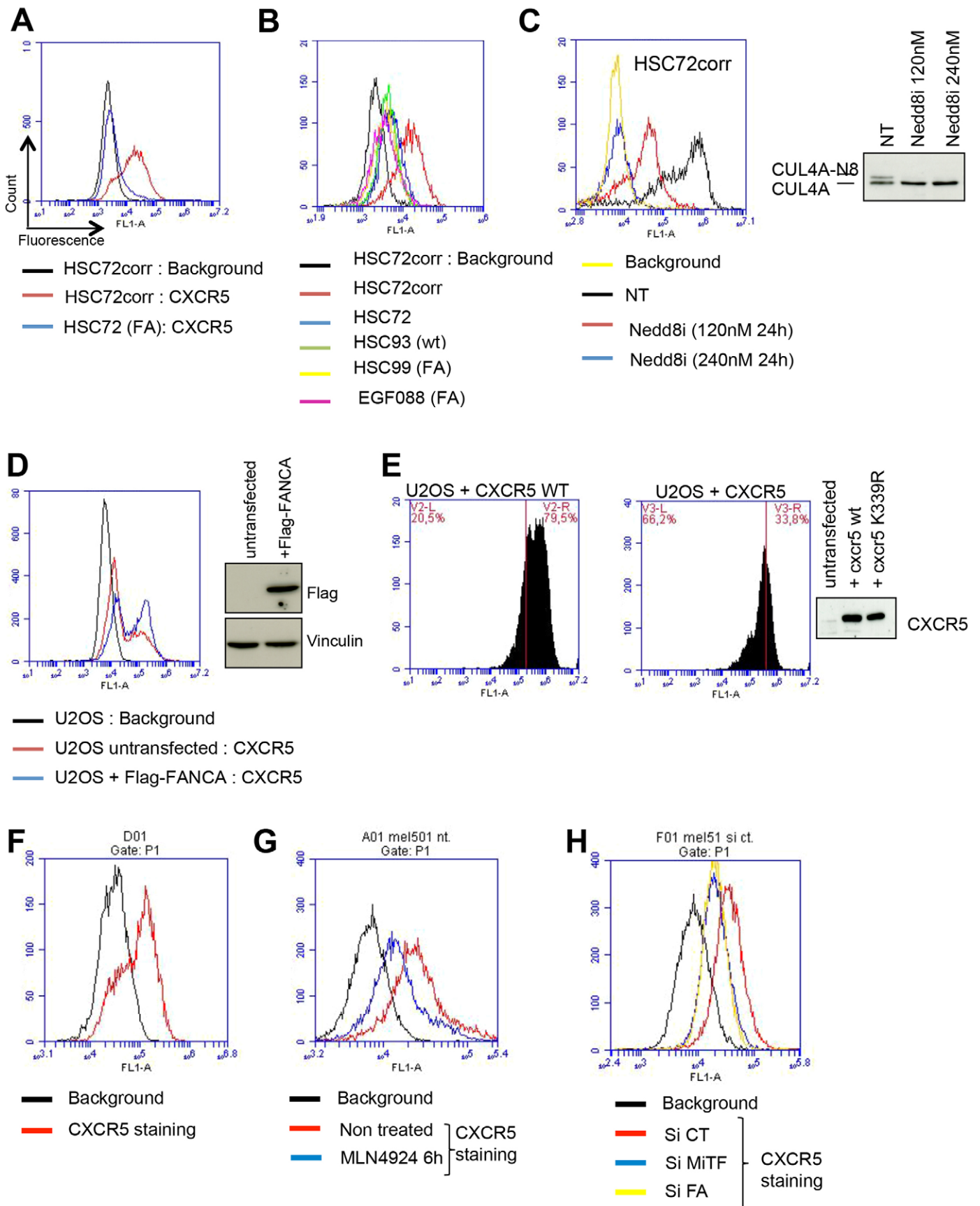


Fig. 3. See next page for legend.

Fig. 3. Neddylated CXCR5 on lysine 339 regulates its membrane association in a FANCA-dependent manner. (A) The results of flow cytometry analysis for the amount of membrane-associated CXCR5 in the FANCA-deficient and corrected (corr) cell lines are shown. (B) The results of flow cytometry analysis for the amount of membrane-associated CXCR5 in three FANCA-deficient cell lines (FA; HSC72, EGF088 and HSC99), one corrected cell line (HSC72corr) and one wild-type (wt; HSC93) cell line are shown. (C) Flow cytometry analysis results (left) for the amount of membrane-associated CXCR5 after treatment of the cells with two different doses of a Nedd8 inhibitor (Nedd8i). The western blot results for Cullin4A (right) are shown as a control for the efficiency of the treatment with the Nedd8 inhibitor. NT, non-treated. (D) Flow cytometry analysis results showing the CXCR5 membrane association in U2OS cells. U2OS were transiently transfected with either a plasmid coding for FLAG-FANCA or a control plasmid for 24 h and were stained as described elsewhere with an antibody against CXCR5 (left). The western blot results showing the efficiency of the transfection are also shown (right). (E) The results of flow cytometry analysis for the amount of membrane-associated CXCR5 in U2OS cells that were transfected with a plasmid coding for wild-type CXCR5 or mutated K339R CXCR5 (left). The red line indicates the limit of the isotype staining in each transfection. The percentage of positive and negative cells is indicated in red. The western blot results showing the efficiency of the transfection are also shown (right). (F,G) The results of flow cytometry analysis to show the amount of membrane-associated CXCR5 in Mel501 cells (F) and after treatment with Nedd8 inhibitor (G). (H) The results of flow cytometry analysis for the amount of membrane-associated CXCR5 in Mel501 cells after the MiTF or FANCA expression levels were reduced by the use of specific siRNAs (Si MiTF or Si FA, respectively). Mel501 cells were transfected with the indicated siRNA for 48 h and stained with the CXCR5-specific antibody as described elsewhere. Si CT, control siRNA.

associated with overexpression of FANCA mRNA (Strub et al., 2011). Taking advantage of this non-genetically manipulated FANCA-overexpressing model, we determined the CXCR5 expression level on the MEL501 cell membrane (Fig. 3F) and demonstrated that this level can be significantly decreased by

treating MEL501 cells with the neddylation inhibitor MLN4924 (Fig. 3G), as well as by the siRNA-mediated downregulation of the MiTF or FANCA (Fig. 3H).

Thus, using several different approaches and cell models, we have demonstrated that CXCR5 is post-translationally modified by the addition of NEDD8 through a mechanism regulated by FANCA and that this PTM is important for targeting CXCR5 to the plasma membrane. CXCR5 is involved in B cell motility and migration to the germinal centers in the spleen and in Peyer's patches in response to its ligand, CXCL13 (Förster et al., 1996). To determine whether CXCR5 neddylation plays a role in cell mobility, we used a wound-healing assay to compare the motility of U2OS cells expressing either a wild-type CXCR5 protein or the CXCR5-K339R mutant that cannot be neddylated. After creating a scratch wound in the U2OS monolayer culture, wound closure was significantly reduced when the K339R CXCR5 mutant was overexpressed (Fig. 4A,B). This finding indicates that a defect in CXCR5 neddylation is associated with a motility defect.

DISCUSSION

To further elucidate the molecular pathways that are altered in Fanconi anemia and involved in its heterogeneous phenotype, we wanted to identify novel substrates of the ubiquitin ligase activity of the FANCCore complex in response to replication stress. By using mass spectrometry analysis of immunoprecipitated peptides containing a K-GG moiety after tryptic digestion, we identified FANCD2 and FANCI peptides, which are specific targets of the FANCCore complex, as being overrepresented in the extracts from the FANCA- and FANCC-corrected cells. Surprisingly, FANCD2 and FANCI were the only proteins that were identified with high confidence as being under-represented in the FANCA- and FANCC-deficient cells compared with their respective corrected counterparts.

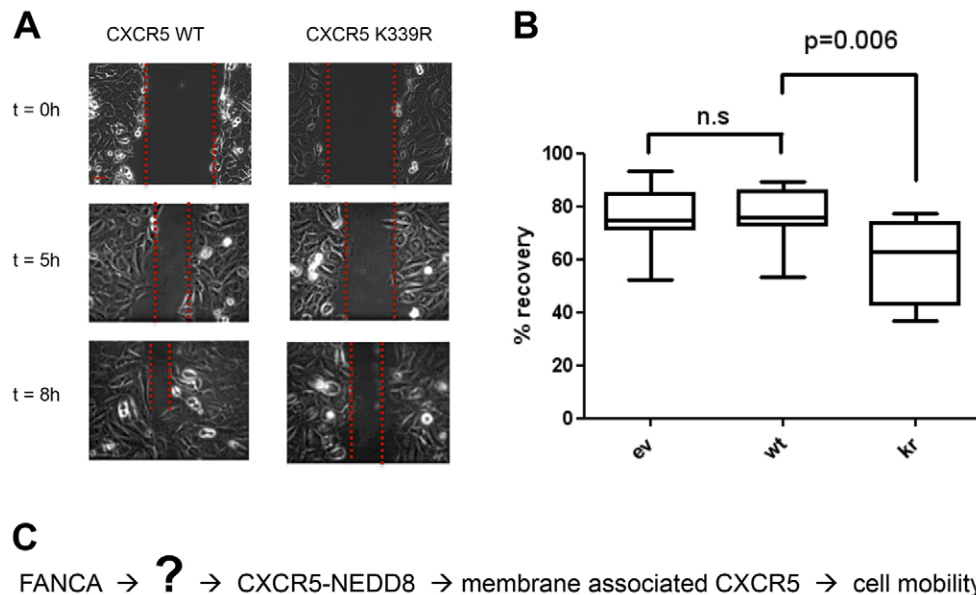


Fig. 4. CXCR5 neddylation on lysine 339 is required for normal cell mobility. (A) A representative image of cells after the cell layer was scratched at the start of the wound assay is shown. U2OS cells were transfected with wild-type (WT) or K339R CXCR5; after 48 h, the cells were scratched with a fine pipette tip and incubated in fresh medium for 8 h for recovery. Scale bar: 40 μ m. (B) Graph showing the percentage of recovery for untransfected (ev) cells and for cells transfected with the wild-type CXCR5 or the K339R mutant (kr) (both conditions from one experiment). Similar results were obtained three times, with at least 12 measurements taken each time. The boxes show the median, 25th and 75th percentiles; the whiskers show the minimum and maximum; significance was calculated using Student's *t*-test; n.s., non-significant. (C) A simple linear model highlighting our results is shown. FANCA overexpression induces CXCR5 neddylation through a currently unknown mechanism and results in the membrane association of CXCR5 that allows for high cell motility.

Among the proteins with a K-GG PTM that were significantly reduced in the Fanconi anemia cells, the receptor, channel, transporter and cell surface protein class, i.e. membrane-associated proteins, was surprisingly the only class that was significantly enriched in the FANCA- and FANCC-deficient cells (Fig. 1). This observation supports the hypothesis that FANCA and FANCC independently regulate the PTMs of transmembrane and membrane-associated proteins, and this function could play an important role in the Fanconi anemia phenotype. Even though the FANCCore complex is assembled in the nucleus, several of the FANCCore complex proteins can be found in cytoplasmic subcomplexes. For example, FANCA has been isolated in association with FANCG, FANCB and FANCL, and FANCC interacts with a FANCE–FANCF subcomplex (Medhurst et al., 2006). Whether these subcomplexes directly or indirectly regulate independent pathways involved in protein PTM remains to be determined in future studies.

In this study, we demonstrate that (a) the chemokine receptor CXCR5 is a neddylation protein, (b) FANCA modulates CXCR5 neddylation through a currently unknown mechanism, (c) CXCR5 neddylation is involved in the targeting of the receptor to the cell membrane and (d) cell migration and motility is stimulated or optimized by CXCR5 neddylation (Fig. 4C). Further in-depth studies are needed to elucidate the precise role that neddylation plays in CXCR5 function, identify the enzymes that are directly involved in this process, determine how FANCA regulates CXCR5 neddylation and determine the role that CXCR5 plays in the Fanconi anemia cellular and clinical phenotypes. CXCR5-dependent signaling is involved in B cell terminal differentiation, immune responses, cancer progression, cancer invasion and pro-inflammatory diseases (Hardtke et al., 2005; Yu et al., 2012). The existence of a mechanism that regulates the precise interplay between the FANCCore and neddylation pathways is supported not only by the findings from our present study but also by several published observations. NEDD8 target proteins are involved in chromatin remodeling and DNA repair and replication (Jones et al., 2002; Xirodimas, 2008), in which the FANCCore pathway is also involved. Inhibition of neddylation increases both CHK1 stability and the activation of the G2/M checkpoint (Yang et al., 2012), which are two important cellular features associated with Fanconi anemia (Guervilly et al., 2011). Additionally, neddylation negatively regulates the transcriptional activity of NF- κ B and p53 (Abida et al., 2007; Gao et al., 2006; Xirodimas et al., 2004), and the activity of these transcription factors is increased in FANCCore-pathway-deficient cells. These findings further support the hypothesis that there is a potential neddylation defect in Fanconi anemia. Finally, NEDD8 and neddylation are involved in FANCD2 monoubiquitylation and relocalization to chromatin foci (Kee et al., 2012). Consequently, inhibition of the neddylation pathway results in a Fanconi-anemia-like cellular phenotype.

Barroca and colleagues recently reported that a CXCR4–CXCL12 signaling defect results in changes in the migration of FANCG-knockout mouse embryonic fibroblasts (MEFs) and LSK cells (Barroca et al., 2012), which supports the hypothesis that defects in the signaling pathway occur downstream of the C-X-C receptors and that these defects could contribute to the bone marrow failure and developmental abnormalities associated with Fanconi anemia syndrome. Previous studies have shown that there is a functional crosstalk between CXCR4 and CXCR5 that is important in B cell and secondary lymphoid organ behavior

(Schmidt et al., 2013). Because FANCA and FANCG should form a cytoplasmic subcomplex (Medhurst et al., 2006), and Barroca and colleagues failed to identify the cause of the CXCL12–CXCR4 signaling defects in the FANCG-knockout cells (Barroca et al., 2012), it is tempting to speculate that the cause of this defect is dependent on the changes in CXCR5 neddylation that we observed in this study.

In conclusion, our findings reveal a connection between FANCCore proteins, the neddylation pathway and membrane receptor signaling and allude to new potential mechanisms that provide a more integrated view of the Fanconi anemia syndrome. Moreover, our findings might serve as a starting point to discover how crosstalk between the signaling pathways involved in ubiquitylation, neddylation and other PTMs regulates cell physiology and developmental processes.

MATERIALS AND METHODS

Cell lines, culture conditions and treatments

We used EBV-immortalized lymphoblastoid cell lines HSC-72 (FANCA deficient) and HSC-536 (FANCC deficient), their respective corrected counterparts HSC-72corr and HSC-536corr, HSC-93 (wild type), EGF088 (FANCA deficient) and HSC-99 (FANCA deficient) as well as U2OS and Mel501 cell lines. Cells were grown in RPMI or DMEM supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 μ g/ml streptomycin and 0.5 g/ml puromycin (all from Invitrogen) at 37°C and under 5% CO₂.

Cells were collected by centrifugation at 8 h after exposure to 5 mM hydroxyurea, flash frozen and stored at –80°C. Protein extraction, mass spectrometry and proteomic analysis were performed by the Cell Signaling Ubiquitin core facility. Treatments with the NEDD8 inhibitor (MLN4924, Millennium Pharmaceutical), prepared as a 10 mM stock solution in DMSO, were performed at the doses and times indicated.

Proteomics analysis

For the K-GG peptide immunoprecipitation and liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis, cell pellets were sent on dry ice to Cell Signaling Technology for UbiScan analysis using the ubiquitin branch antibody. The *in silico* protein interactions were determined using the StringDatabase with a confidence score >0.7.

Transfection

The control siRNA (5'-CGUCGACGGAAUACUUCGA-3') was purchased from Eurogentec. siRNA against MiTF (SMARTpool), FANCC (SMARTpool) and FANCA (SMARTpool) were purchased from Dharmacon. siRNA (20 nM) was transfected into cells using INTERFERin (Polyplus), and the experiments were conducted 48 h later. CXCR5 K339 was mutated (K339R) using the QuikChange II Site-Directed Mutagenesis Kit (Agilent) and the following primers: 5'-GCGGCTCCTGACGAGGCTGGGCTGTAC-3' and 5'-GTACAGCCCAGCCTCGTCAGGAGCCGC-3'. For the overexpression experiment, 2 μ g of the FANCA–FLAG-expressing vector (a gift from Angelos Constantinou; Institut de Génétique Humaine - CNRS, Montpellier, France), wild-type CXCR5 (from DNASU) and K339R were transfected using Turbofect (Fermentas) according to the manufacturer's protocol, and the experiments were conducted 24 h later.

Antibodies

We used the following antibodies: rabbit anti-MCM7 (Abcam), mouse anti-FANCD2 (Santa Cruz), rabbit anti-FANCD2 (Abcam), mouse anti-ubiquitin (FK2; Biomol), rabbit anti-CXCR5 (Genetex and BD Pharmingen for the Alexa-Fluor-488-conjugated antibody), mouse anti-CXCR5 (Abcam), mouse anti-vinculin (Abcam), goat anti-actin (Abcam), mouse anti-PCNA (Santa Cruz), rabbit anti-NEDD8 (Cell Signaling Technology), goat anti-mouse (Tebu), donkey anti-rabbit

(Tebu) and donkey anti-goat (Tebu). The isotype antibodies mouse IgG1 and IgG2a,b and rabbit IgG were obtained from Dako.

Western blot analysis

The collected cells were disrupted in lysis buffer [50 mM Tris-HCl pH 7.9, 40 mM NaCl, 1 mM MgCl₂, 0.1% SDS and 1% Benzodase (Novus), supplemented with protease and phosphatase inhibitors (Roche)]. After 20 min incubation at room temperature, the protein concentration was determined using the Bradford assay (BioRad), and samples were combined with 4× Laemmli buffer containing β-mercaptoethanol and denatured by boiling. The proteins (25 μg) were separated by SDS-PAGE. All western blot quantifications were performed using densitometry measures and the ImageJ software.

Protein immunoprecipitation

The proteins were extracted using NETN buffer (150 mM NaCl, 50 mM Tris-HCl pH 8.0, 0.5–1% NP-40 and 1 mM EDTA) and sonicated to fragment the DNA. The extracts were precleared using a non-specific antibody for 1 h at 4°C. The cleared extracts were added to magnetic Protein G beads (Millipore) that were coated with a specific antibody, and the extracts and beads were incubated overnight at 4°C. Control immunoprecipitations were conducted using a non-specific isotype control antibody under the same conditions. After five washes in NETN buffer, the immune complexes were eluted by boiling for 5 min in 35 μl of 2× Laemmli buffer and were denatured by boiling for another 5 min in the presence of 1 μl of 1 M β-mercaptoethanol.

Flow cytometry analysis

Cells were harvested and washed once in cold PBS before centrifugation at 150 g for 5 min. The pellets were resuspended in PBS containing 3% bovine serum albumin (BSA) with an Alexa-Fluor-488-conjugated CXCR5 antibody diluted 1:100 and incubated for 1 h at room temperature under dark conditions. After two washes with cold PBS, the cells were incubated in PBS containing 3% BSA and analyzed using a C6 cytometer with CFlow software (Accuri).

Proximity ligation assay

Briefly, cells were fixed with 4% paraformaldehyde (Sigma) for 10 min at room temperature and then permeabilized in PBS containing 0.05% Tween-20 and 0.5% Triton X-100 (both from Sigma) for 5 min. After being blocked in PBS containing 2% BSA, the cells were incubated in a solution of primary antibody diluted in PBS with 2% BSA for 2 h and then incubated in PLA probe for 30 min at 37°C. Next, ligation and amplification were performed following the manufacturer's protocol. Finally, the slides were mounted in fluorescent medium (Dako) supplemented with DAPI (Sigma) and analyzed at a magnification of 63× using fluorescence microscopy (Zeiss Axio Observer Z1). Images were captured using an Orca-ER Camera (Hamamatsu).

Wound-healing assay

U2OS cells were seeded at a density of 200,000 cells per well in six-well plates and transfected with CXCR5 plasmid for 48 h. The cells were incubated in a serum-free medium overnight. Next, the cell monolayer was scratched using a fine tip, and pictures were taken at different time-points (0, 5 and 8 h) with a Zeiss inverted microscope and an AxioCam MRm camera. The images were analyzed using AxioVision software.

Statistical analysis

Significance was determined by using a two-tailed *t*-test. *P*-values <0.05 were considered as significant.

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Competing interests

The authors declare no competing interests.

Author contributions

X.R. performed research, analyzed data and wrote the paper. J.-H.G. and S.A. designed the research. F.R. designed the study, analyzed data and wrote the paper.

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Supplementary material

Supplementary material available online at <http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.150706/-DC1>

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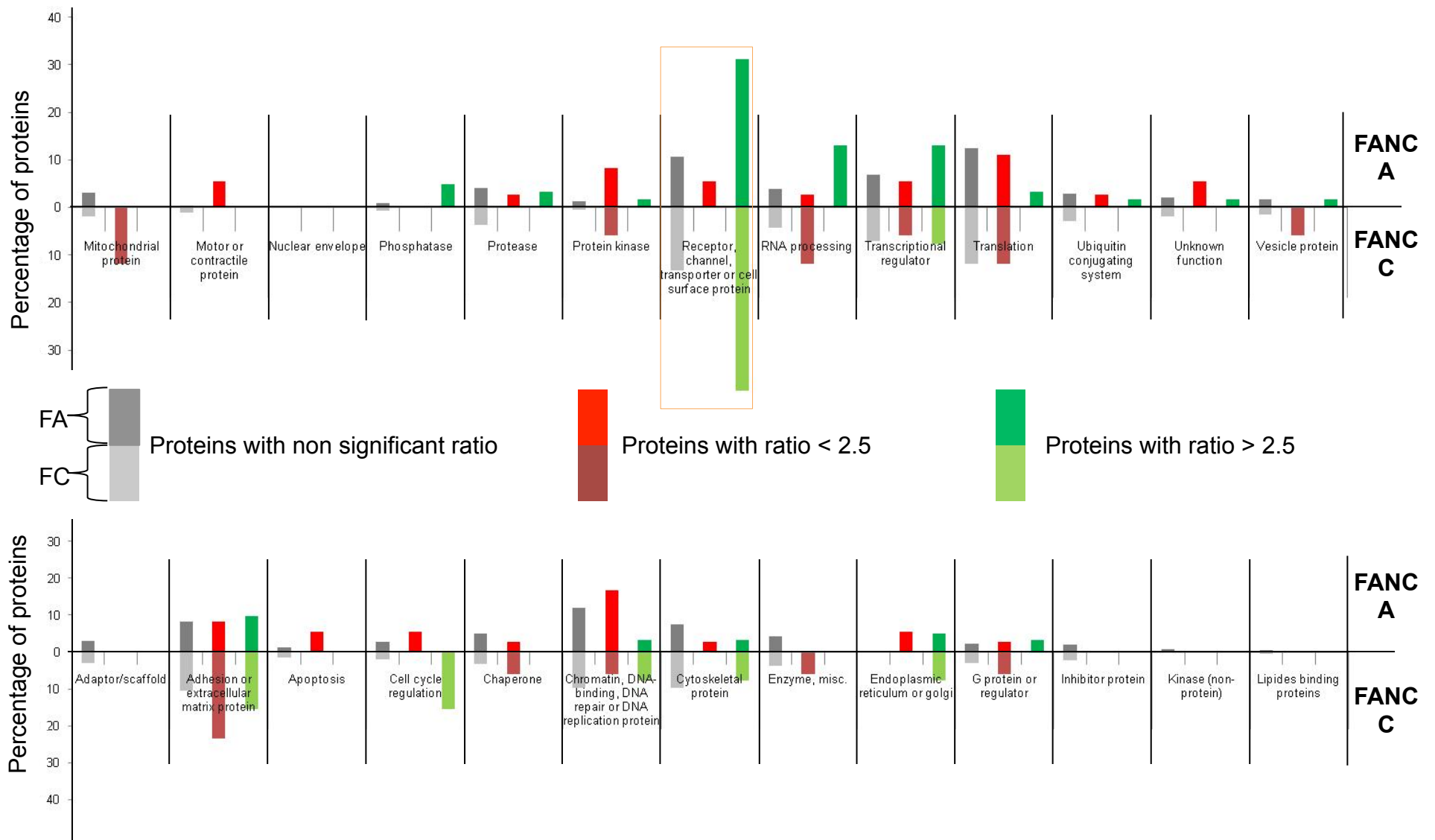


Fig. S1. Distribution of protein function/localization based on the K-GG analysis of the FANCA and FANCC cells and their corrected counterparts. Proteins with no significant differences are indicated in gray; significant negative ratios are indicated in red, and significant positive ratios are indicated in green.

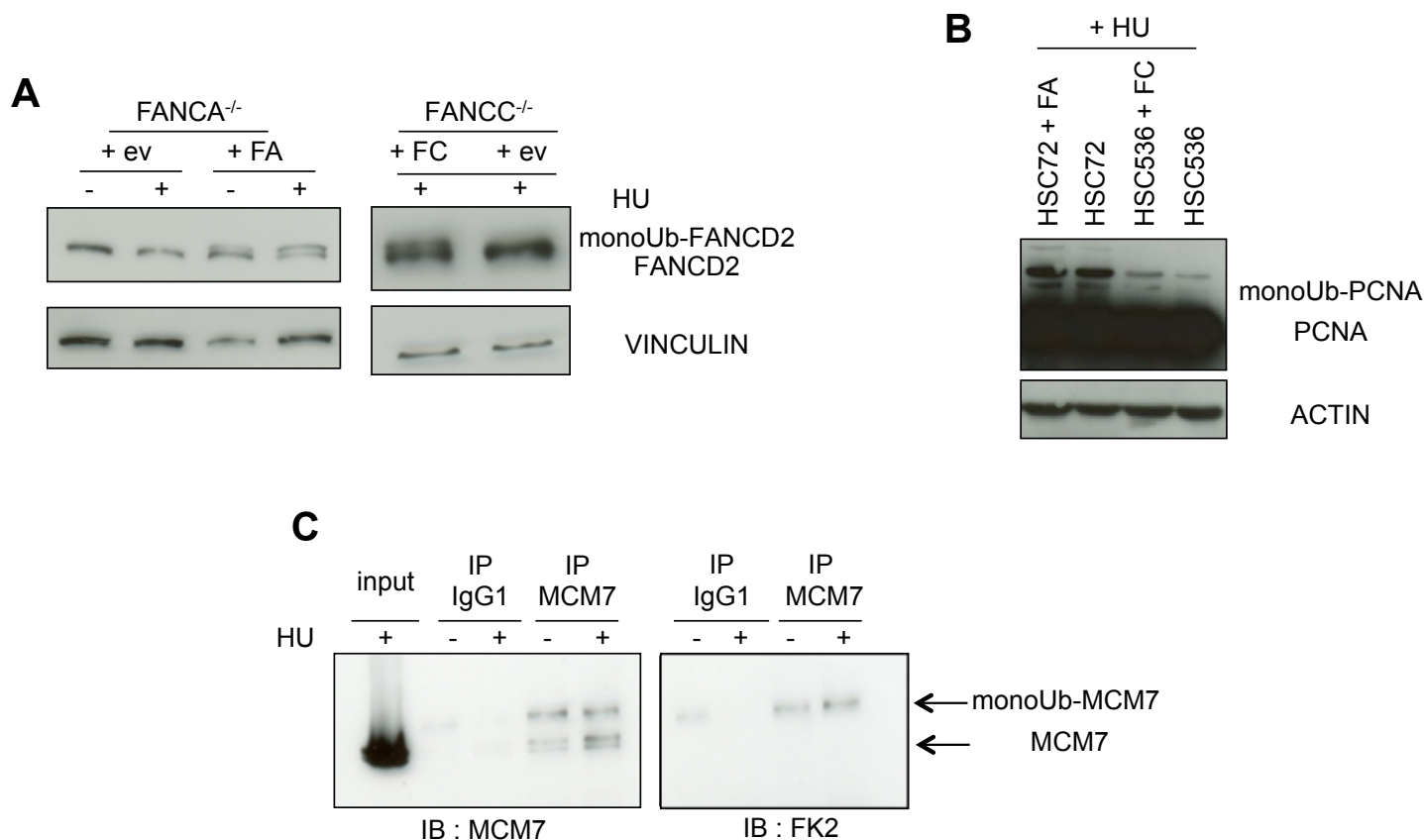


Fig. S2A. FA deficient cells are fully complemented for FANCD2 monoubiquitination. Western-blot showing FANCD2 monoubiquitinylation in untreated conditions or after 8h of exposure to 5mM. Whole cell extracts from FANCA-deficient and -proficient cells were analyzed by immunoblot using anti-FANCD2 or anti-Vinculin antibodies.

Fig. S2B. FANCA-deficient and -proficient cells monoubiquitinated normally PCNA.

Fig. S2C. MCM7 is a protein monoubiquitinated protein. Western-Blot analysis of immunoprecipitation with anti-MCM7 or anti-FK2 antibodies. Protein extracts from HeLa cells were incubated with appropriate antibody or isotypic controls (IgG1) over-night, and immunoprecipitated proteins were analyzed with the indicated antibody. The same IP was loaded twice for the different staining to avoid cross reaction.

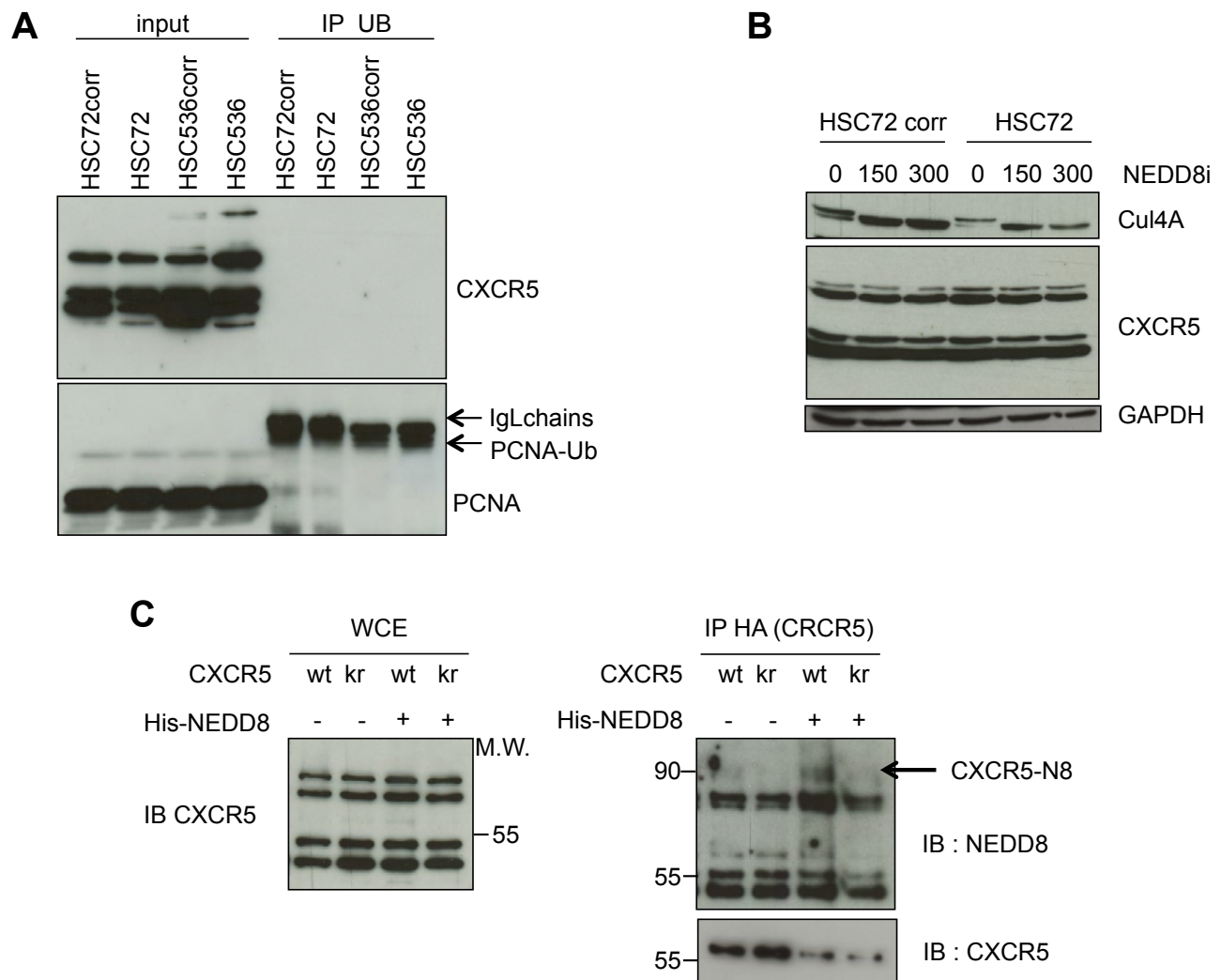


Fig. S3A. CXCR5 is not a ubiquitinated protein. Western blot results showing the expression level of CXCR5 after immunoprecipitation (IP) with a ubiquitin antibody (Ub) in FANCA cells, FANCC cells and their corrected counterparts after HU treatment (5 mM for 8 h). A PCNA blot is shown as a positive control for the IP.

Fig. S3B. The NEDD8 inhibitor does not modify endogenous levels of CXCR5. Effect of the NEDD8 inhibitor MLN4924 on expression level of CXCR5. Cul4A was analyzed as control of the efficiency of the NEDD8 inhibitor. The upper, neddylated band, disappears in response to MLN4924.

Fig. S3C. The CXCR5-KR mutant is not neddylated. Cells transfected with the WT of KR mutant of CXCR5 co-transfected or not with a His-NEDD8 expression vector. Cellular extracts were immunoprecipitated with anti HA antibodies and Western blot was revealed by a NEDD8 antibody. A neddylated band appears specifically in cells co-transfected with wt-CXCR5 and His-NEDD8 but not in cells co-transfected with the KR-CXCR5 mutant plus the His-NEDD8 fusion. Please note that the expression of His-NEDD8 induces a strong decrease of HA-CXCR5, likely inducing its degradation.

Table S1. K-GG peptides and proteins identified in FA-C and corrected cell lines.

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Table S2. K-GG peptides and proteins identified in FA-A and corrected cell lines.

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Table S3. Common proteins identified in both the FA-A and FA-C screening.

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