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SUMOylation negatively regulates the stability of CHFR tumor suppressor

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ABSTRACT

CHFR ubiquitin ligase acts as a checkpoint upon DNA damage and its functional inactivation is one of key characteristics of tumor development and metastasis. Despite the crucial role in maintaining genome integrity and cell cycle progression, little is known how CHFR stability is regulated. Here, we showed that CHFR is covalently modified by SUMO-1 at lysine 663 and subsequently destabilized by ubiquitin–proteasome system. While $CHFR^{K663R}$ substitution mutation does not alter its subcellular localization, SUMOylation-defective $CHFR^{K663R}$-stable cells exhibit substantial growth suppression due to the increased stability of $CHFR^{K663R}$. Moreover, protein level of CHFR, not $CHFR^{K663R}$, is rapidly declined under SUMOylation-promoting conditions, and SENP2 deSUMOylating enzyme reverses its SUMO-modification. Collectively, we demonstrated that CHFR stability is regulated by SUMOylation-dependent proteasomal degradation. Therefore, our study underscores the importance of CHFR SUMOylation as a new regulatory mechanism of CHFR and highlights the emerging role of SUMOylation in modulating protein stability.

1. Introduction

CHFR (checkpoint with FHA and RING finger domains), originally identified as a mitotic checkpoint [1], is an E3 ubiquitin (Ub) ligase for mitotic kinases, Aurora A and PLK1 to control their protein levels, thereby regulating cell cycle progression and chromosomal stability [2,3]. CHFR also destabilizes HDAC1, resulting in the upregulation of *p21*, *E-cadherin*, and *KAI1*, which in turn leads to G1 arrest and metastasis inhibition [4]. *CHFR* is mostly quiescent in human cancer cell lines and tumors either by epigenetic inactivation or by genetic mutation [1,5]. *Chfr*-deficient mouse embryonic fibroblast (MEF) cells incur mitotic defects and aneuploidy, and *Chfr* knockout mice have a high incidence of tumors [3]. Reduced *CHFR* expression by RNAi in normal-like human mammary epithelial cell line, MCF10A also exhibits cancer-like characteristics including a higher mitotic index and proliferation, increased cellular invasion, and aneuploidy. Many of these tumorigenic phenotypes are reversed when reconstituted with wild-type CHFR in Hs578T breast cancer cells [6]. Therefore, it has been suggested that CHFR expression status is highly correlated with cancer development and furthermore CHFR functions as a tumor suppressor.

Although the importance of CHFR in tumorigenesis has been implied by several studies, the exact regulatory mechanism how CHFR protein levels are maintained is still elusive. It has been known that protein stability and activity are regulated by post-translational modifications such as ubiquitination or SUMOylation [7]. CHFR is a dynamic component of nuclear foci where it colocalizes with promyelocytic leukemia (PML) proteins [8]. Since many SUMOylated proteins as well as components of SUMOylation machinery are also localized in PML nuclear bodies [9], it raises the possibility that CHFR can be SUMO-modified.

SUMOylation is the process of conjugating a small ubiquitin-like modifier (SUMO) to a target protein by an enzymatic cascade, consisting of an E1 SUMO-activating enzyme (SAE1 and SAE2), an E2 SUMO-conjugating enzyme (UBC9), and/or an E3 SUMO-ligase [10,11]. SUMOs are covalently attached to lysine residues within the SUMOylation consensus sequence, ΨKxE/D (where Ψ is a large hydrophobic residue, K is a lysine residue, x is any amino acid, and E/D is an acidic residue) in target proteins [12]. This SUMO-modification is a reversible cycle, intertwined by conjugation and deconjugation. SUMO-deconjugation from target proteins is carried out by SUMO-specific proteases (SENPs), and SENP1 and SENP2 have a broad specificity for SUMO paralogues [13]. SUMOylation is highly dynamic and plays a crucial role in controlling cellular localization, activity, and stability of target proteins [13–15]. Based on which target proteins get SUMOylated, many important biological processes including transcriptional regulation, DNA repair, cell cycle progression, cell proliferation, and apoptosis are modulated [11,13–16].

In the present study, we report that CHFR is conjugated by SUMO-1 at lysine 663 and this SUMO-modification is reversed by SENP2. SUMOylation of CHFR is responsible for its degradation by ubiquitin–proteasome system (UPS). Therefore, SUMOylation acts as a destabilization code for maintaining the cellular levels of CHFR protein.

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2. Materials and methods

2.1. Plasmids and cell culture

CHFR cDNA was subcloned into p3xFLAG-CMV10 (Sigma) and pFastBac (invitrogen) vectors [4], and lysine mutants were generated using the QuikChange site-directed mutagenesis Kit (Stratagene). HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% FBS (Gibco) at 37 °C in a humidified 5% CO_2 condition. Transient transfections were carried out using either polyethylenimine (Sigma) or PolyFect (Qiagen) according to the manufacturer's instructions.

2.2. Immunoblotting and antibodies

Cells were lysed in buffer A (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1 mM EDTA, and 0.2% Triton X-100) containing 1 × complete protease inhibitor cocktail (Roche Applied Science). Cell lysates were resolved by SDS–PAGE, transferred to a nitrocellulose membrane, and probed with appropriate antibodies. The following antibodies were used: anti-FLAG and anti-β-actin (Sigma), anti-MYC and anti-HA (Santa Cruz Biotechnology), anti-Xpress (Invitrogen), peroxidase-conjugated AffiniPure goat anti-rabbit and anti-mouse IgGs (Bio-Rad), and rabbit polyclonal anti-CHFR antiserum (raised against recombinant His-CHFR).

2.3. In vitro SUMOylation assay

Purified His-CHFR protein from Sf9 insect cells was incubated with GST-SAE1/SAE2 (0.5 μg), His-UBC9 (0.5 μg), GST-SUMO-1 (2 μg), and ATP-regenerating system (50 mM Tris–HCl, 5 mM $MgCl_2$, 10 mM creatine phosphate, 5 units/ml phosphocreatine kinase, and 5 mM ATP) at 37 °C for 30 min. Reactions were stopped by adding SDS-sampling buffer. Each sample was subjected to immunoblotting with an appropriate antibody.

2.4. Immunofluorescence staining

Cells on coverslips were fixed with 2% formaldehyde in PBS for 30 min at room temperature and permeabilized with 0.5% Triton X-100 in PBS. Nonspecific binding sites were masked by incubation for 30 min with blocking solution consisting of 10% goat serum and 1% gelatin in PBS. All subsequent dilutions and washes were performed with PBS containing 0.1% Triton X-100 (PBS-T). Cells were incubated with primary antibody for 1 h and washed 4 times with PBS-T every 10 min, and then incubated with FITC- or TRITC-conjugated secondary antibody for 1 h. Coverslips were washed 4 times with PBS-T and mounted using Vectashield (Vector Labs). DAPI was used to label the nuclei. Cells were visualized under a Zeiss Axioplan II microscope.

2.5. Cell proliferation assay

HeLa-CHFR stable cells (1×10^4) were plated into 35 mm culture dishes. The number of cells was counted everyday for a period of 6 days after plating. Values are expressed as mean ± S.E.M. for 3 independent experiments.

2.6. Colony formation assay

HeLa-CHFR stable cells (1×10^4) were plated in 60 mm culture dishes and grown in G418 (250 μg/ml)-containing DMEM for 8 days. G418-resistant colonies were subsequently fixed with 10% formaldehyde and stained with 0.5% crystal violet in 20% methanol.

3. Results and discussion

3.1. CHFR is SUMOylated in vivo and in vitro

Given that CHFR is a dynamic component of nuclear foci where SUMOylated proteins are recruited to PML nuclear bodies (PML-NB) [8], we tested the possibility whether CHFR is SUMO-modified. When CHFR was co-expressed with UBC9 and SUMO-1 in HeLa cells, which lack endogenous CHFR [4], a single discrete band corresponding to SUMO-1 conjugated form was detected from whole cell lysates as well as CHFR immunoprecipitates (Fig. 1A). To further validate SUMOylation of CHFR, we performed *in vitro* SUMOylation assay with purified CHFR protein. As shown in Fig. 1B, CHFR was readily conjugated with SUMO-1. Taken together, these data suggest that CHFR is indeed SUMO-modified both *in vivo* and *in vitro* at a single lysine residue.

3.2. Lysine 663 on CHFR is a major SUMO-modification site

In order to find a SUMOylation site within CHFR, we first used SUMOplot™ SUMO acceptor site search program (http://www.abgent.com/tools/sumoplot). However, none of such predicted candidates were found to be SUMOylated. We then constructed CHFR truncated mutants to narrow down which regions of CHFR would hold a SUMOylated lysine residue. Wild-type CHFR ($CHFR^{WT}$) and $CHFR^{\Delta N142}$ were SUMOylated when co-expressed with UBC9 and

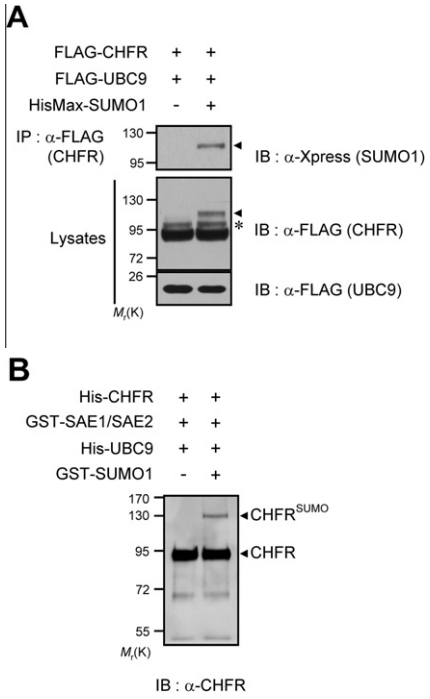

Fig. 1. CHFR is covalently modified by SUMO. (A) CHFR is SUMO-modified *in vivo*. HeLa cells were transfected with FLAG-CHFR, FLAG-UBC9, and HisMax-SUMO-1 and harvested at 36 h after transfection. Cell lysates were immunoprecipitated with anti-FLAG M2 resin and precipitates were analyzed by immunoblotting with anti-Xpress antibody for SUMOylated CHFR. Whole cell lysates were subjected to SDS–PAGE followed by immunoblotting with anti-FLAG antibody for CHFR and UBC9. Black arrowhead depicts SUMOylated CHFR ($CHFR^{SUMO}$). Asterisk depicts nonspecific band. (B) CHFR is SUMOylated *in vitro*. Recombinant His-CHFR purified from Sf9 insect cells was incubated with GST-SAE1/SAE2, His-UBC9, and GST-SUMO-1 at 37 °C for 30 min. Each sample was subjected to immunoblotting with anti-CHFR antibody.

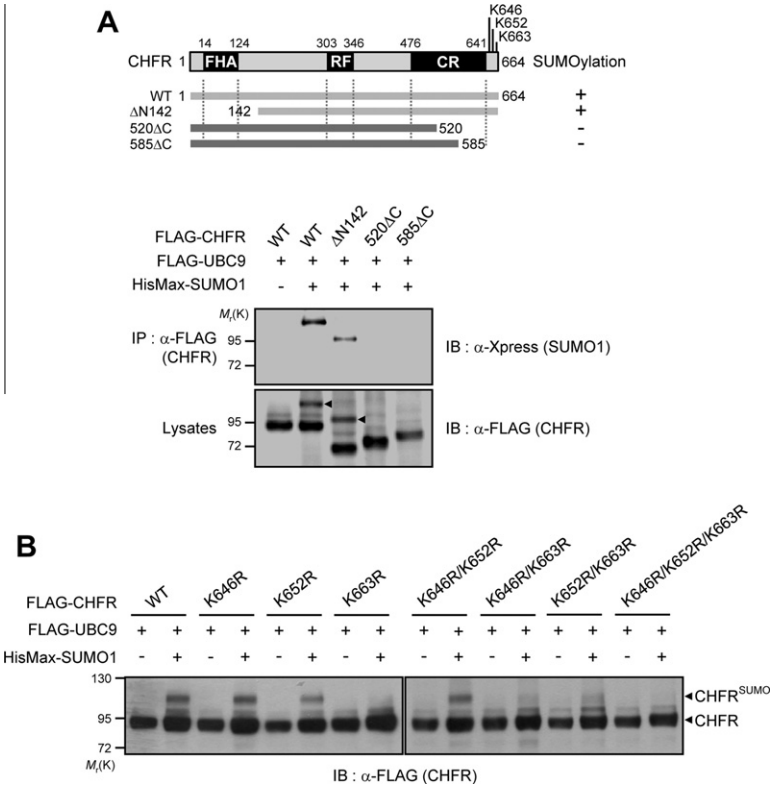

Fig. 2. CHFR is SUMOylated on lysine 663 at the C-terminal end. (A) C-terminal region of CHFR is SUMOylated. Schematic representation of CHFR deletion mutants is shown. FHA, forkhead associated domain; RF, RING finger domain; CR, cysteine rich domain. All constructs contained a N-terminal FLAG epitope tag. FLAG-CHFR^{WT} or deletion mutants were transfected with FLAG-UBC9 and HisMax-SUMO-1 in HeLa cells. At 36 h post-transfection, cell lysates were subjected to immunoblotting with anti-FLAG antibody. Black arrowhead depicts CHFR^{SUMO}. (B) K663 at the C-terminal end of CHFR is responsible for SUMOylation. FLAG-CHFR^{WT} or K to R substitution mutants (single, double, and/or triple) was transfected with FLAG-UBC9 and HisMax-SUMO-1 in HeLa cells. At 36 h post-transfection, cell lysates were analyzed by immunoblotting with anti-FLAG antibody.

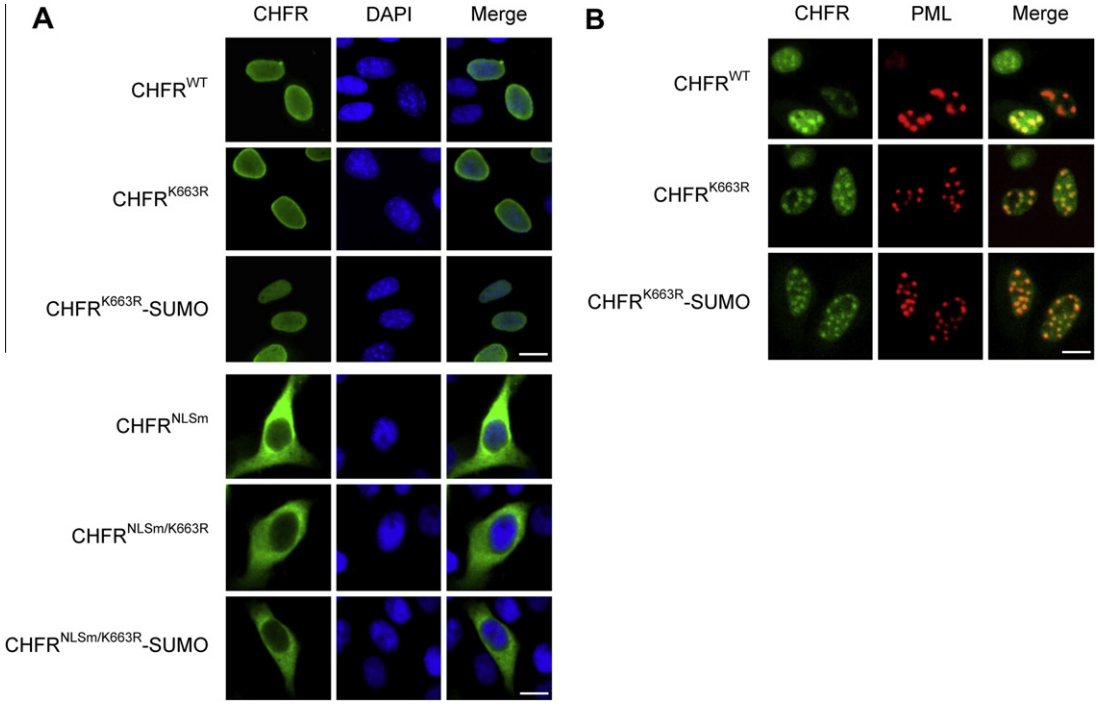

Fig. 3. Subcellular localization of CHFR is not dependent on its SUMOylation status. CHFR localization was examined by immunofluorescence microscopic analyses. Scale bars, 10 μm. (A) HeLa cells were transfected with FLAG-CHFR wild-type and mutants (K663R, K663R-SUMO, NLSm, NLSm/K663R, or NLSm/K663R-SUMO). Cells were stained with anti-FLAG antibody (green) and DAPI (blue). NLSm: K257A, K258A, K259A. (B) FLAG-CHFR^{WT}, FLAG-CHFR^{K663R}, or FLAG-CHFR^{K663R}-SUMO was transfected with HA-PML into HeLa cells. Cells were stained with anti-FLAG (green) and anti-HA (red) antibodies. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

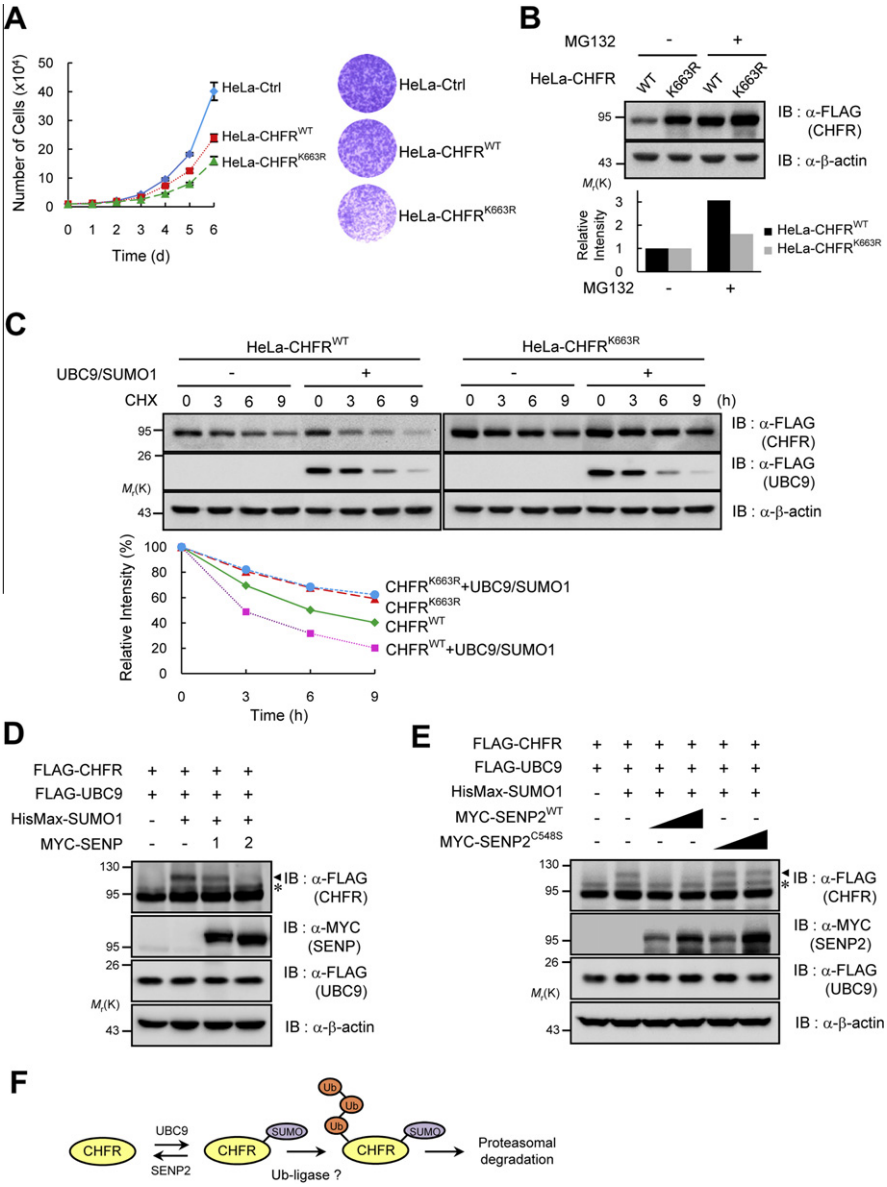

Fig. 4. SUMOylation of CHFR negatively influences its protein stability. HeLa cells stably expressing CHFR^{WT} or CHFR^{K663R} are used for analyses. (A) CHFR^{K663R} cells confer a substantial anti-proliferative activity. *Left panel*, HeLa-CHFR stable cells (1×10^4) were plated and counted every day for a period of 6 days. Values are expressed as mean ± S.E.M. from 3 independent experiments. *Right panel*, representative images of colony formation assay after 8 days of G418 selection using HeLa-CHFR stable cells. G418^R colonies were fixed and stained with crystal violet. (B) CHFR^{K663R} protein levels are higher than CHFR^{WT} and CHFR SUMOylation is associated with its proteasomal degradation. HeLa-CHFR stable cells were lysed and immunoblotted with anti-FLAG or anti-β-actin antibodies. Relative band intensity was quantified by densitometry using the ImageJ software (ImageJ, US National Institutes of Health). (C) CHFR^{WT} protein levels are rapidly declined under SUMOylation-promoting conditions. HeLa-CHFR stable cells were transfected with UBC9 and SUMO-1. At 24 h post-transfection, cells were treated with 200 μg/ml cycloheximide (CHX) and harvested at indicated times. Cell lysates were subjected to immunoblotting with anti-FLAG and anti-β-actin antibodies. (D, E) SENP2 reverses the SUMO modification of CHFR. FLAG-CHFR, FLAG-UBC9, HisMax-SUMO-1, and MYC-SENPs (-1, -2 wild-type, or -2 C548S catalytic mutant) were transfected into HeLa cells. At 36 h post-transfection, cells were lysed and immunoblotted with anti-FLAG, anti-MYC, or anti-β-actin antibodies. (F) Proposed model for the SUMOylation-dependent regulation of CHFR stability.

SUMO-1, on the other hand, neither CHFR$^{520\Delta C}$ nor CHFR$^{585\Delta C}$ was SUMOylated (Fig. 2A). Since C-terminal region of CHFR (aa 586–664) contains three lysine residues (K646, K652, and K663), we have replaced lysine with arginine to identify which lysine residue is responsible for SUMOylation. As shown in Fig. 2B, CHFR^{K663R} completely abolished SUMO-conjugation in any context. Therefore, we have concluded that lysine 663 at the C-terminal end of CHFR is a major SUMO-modification site.

3.3. SUMOylation of CHFR does not affect its subcellular localization

As the SUMOylation status of certain proteins may reflect changes in subcellular localization [16,17], we tested the possibility whether SUMOylation of CHFR influences its subcellular localization by immunofluorescence staining. To test the direct effect of SUMOylation, we generated CHFR^{K663R}-SUMO fusion proteins to faithfully simulate endogenous SUMOylated CHFR to its maximum extent. As the SUMOylation site (K663) is located at the C-terminal end of CHFR, we fused SUMO-1 directly to the C-terminus of CHFR^{K663R} mutant. When the subcellular localization of CHFR was examined, all of three tested CHFR, CHFR^{WT}, CHFR^{K663R}, and CHFR^{K663R}-SUMO mutants, were located in the nucleus (Fig. 3A), indicating that SUMOylation of CHFR does not affect nuclear localization. We have previously shown that CHFR is a nuclear protein and mutation in the nuclear localization signal (NLS) sequence shifts the localization of CHFR exclusively in the cytoplasm [18].

This NLS mutation (NLSm) was then introduced into CHFR^{K663R} or CHFR^{K663R}-SUMO to further test the effect of SUMOylation on CHFR localization. All three mutants, CHFR^{NLSm}, CHFR$^{NLSm/K663R}$, and CHFR$^{NLSm/K663R}$-SUMO, were localized in the cytoplasm (Fig. 3A), suggesting that SUMOylation of CHFR is not able to override the defective NLS. Since it has been reported that CHFR is recruited to the PML-NB through SUMO-modified PML [8], we tested whether SUMOylation influences specific subnuclear localization of CHFR. As shown in Fig. 3B, CHFR^{K663R}, CHFR^{K663R}-SUMO mutants, and CHFR^{WT} were colocalized with PML. Therefore, these results indicate that SUMO-modification of CHFR does not affect its subcellular/subnuclear localization.

3.4. SUMOylation of CHFR exerts a negative influence on its stability

SUMOylation can modulate the activity and/or the stability of target proteins. To explore the effect of SUMOylation on CHFR function, we established HeLa cells stably expressing CHFR wild-type or K663R mutant (HeLa-CHFR^{WT} or HeLa-CHFR^{K663R}, respectively). Given the fact that CHFR confers a growth suppressive activity [5], we performed a cell proliferation assay to investigate whether SUMOylation of CHFR influences cell growth. Interestingly, HeLa-CHFR^{K663R} cells grew slower and formed less G418-resistant colonies compared to HeLa-CHFR^{WT} cells (Fig. 4A), suggesting that SUMOylation resistance of CHFR^{K663R} contributes to substantial growth suppression. This stronger anti-proliferative activity of CHFR^{K663R} would be a mere reflection of the increased stability of CHFR^{K663R} protein compared to that of CHFR^{WT}. Accordingly, cellular protein levels of CHFR^{K663R} in a steady state without external stimuli were much higher than CHFR^{WT} in these HeLa-CHFR stable cells (Fig. 4B, lanes 1 and 2). CHFR^{WT} became highly stable in the presence of proteasome inhibitor MG132 (Fig. 4B, lanes 1 and 3), while CHFR^{K663R} remained relatively similar (Fig. 4B, lanes 2 and 4). These results indicate that SUMOylation of CHFR is linked to its UPS-dependent destabilization. In agreement with this, the half-life of CHFR^{K663R} was longer than CHFR^{WT}, as measured in a cycloheximide-chase assay. Notably, CHFR^{WT} protein levels were rapidly declined when co-expressed with UBC9 and SUMO-1, however, CHFR^{K663R} protein levels were not changed even in the presence of SUMOylating enzymes (Fig. 4C). Taken together, these results demonstrate that SUMOylation of CHFR negatively affects its stability and cellular growth potential.

3.5. CHFR is deSUMOylated by SENP2

It has been shown thus far that SUMOylated CHFR is quickly destabilized. Conversely, the deSUMOylation process may be required to sustain CHFR proteins. We therefore aimed to find which deSUMOylating enzyme is involved in the SUMO-modification cycle of CHFR. As SUMOylation is reversed by SUMO-specific proteases (SENPs), and SENP1 and SENP2 have a broad specificity for SUMO paralogues [13], we tested both SENPs for CHFR deSUMOylation. SENP2^{WT} was able to hydrolyze SUMO-1 moiety completely from SUMOylated CHFR, whereas SENP1 was partially responsible (Fig. 4D) and SENP2^{C548S} catalytic mutant was ineffective for CHFR deSUMOylation (Fig. 4E). These results demonstrate that SENP2 is a major deSUMOylating enzyme for CHFR and might be involved in modulating cellular levels of CHFR.

In the present study, we provide compelling evidence that CHFR is SUMO-modified at lysine 663 and this SUMOylation promotes the degradation of CHFR in a UPS-dependent manner. Therefore, Fig. 4F illustrates our current working model that CHFR stability is regulated by SUMOylation and subsequent proteolysis.

Known regulatory mechanisms of CHFR stability thus far are autoubiquitination followed by proteasomal degradation [19] and deubiquitination by ubiquitin-specific protease USP7/HAUSP [20]. Our current study adds another layer to the CHFR regulation by characterizing SUMOylation as its destabilization code. It would be of particular interest to determine the underlying mechanism of CHFR SUMOylation-dependent degradation and the possible interplay between SUMOylation and (auto-) ubiquitination.

Collectively, our data highlight that CHFR protein levels are regulated by SUMOylation-coupled proteolysis. As CHFR inactivation or destabilization has been implicated in cell-cycle regulation and cancer progression, our study will be useful for future investigations towards understanding how CHFR destabilization by SUMOylation influences many important biological processes mediated by CHFR.

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