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Regulation of fragile X mental retardation 1 protein by C-terminus of Hsc70-interacting protein depends on its phosphorylation status

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ABSTRACT

The fragile X mental retardation 1 (FMR1) protein binds mRNA and acts as a negative regulator of translation. Lack of FMR1 causes the most common neurological disorder, fragile X syndrome, while its overexpression is associated with metastasis of breast cancer. Its activity has been well-studied in nervous tissue, but recent evidence as well as its role in cancer indicates that it also acts in other tissues. We have investigated the expression of FMR1 in brain and other tissues of mouse and examined its regulation. We detected expression of FMR1 in liver and heart tissues of mice as well as in brain tissue, supporting other contentions that it acts in non-nervous tissue. Expression of FMR1 inversely correlated with expression of the C-terminus of Hsc70-interacting protein (CHIP) and, based on the known activity of CHIP in protein homeostasis, we suggest that CHIP regulates expression of FMR1. CHIP ubiquitinated FMR1 for proteasomal degradation in a molecular chaperone-independent manner. FMR1 expression was reduced following treatment with okadaic acid, a phosphatase inhibitor, but not in CHIP-depleted cells. Also, a non-phospho FMR1 mutant was much less efficiently ubiquitinated by CHIP and had a longer half-life compared to either wild-type FMR or a phospho-mimic mutant. Taken together, our results demonstrate that CHIP regulates the levels of FMR1 as an E3 ubiquitin ligase in phosphorylation-dependent manner, suggesting that CHIP regulates FMR1-mediated translational repression by regulating the levels of FMR1.

1. Introduction

FMR1 is a polysome-associated RNA-binding protein that inhibits translation. The function of FMR1 in the brain has been intensively investigated [1,2]. Inadequate levels of FMR1 result in defects in synaptic plasticity [3] and cause the most common inherited neurological disorder, fragile X syndrome [2,4]. Recent studies indicate that FMR1 also functions in non-brain tissues. These studies reveal that FMR1 is ubiquitously expressed [5,6] and binds to 5% of the mRNA molecules, only 20% of which are specific to neurological tissue [7]. Also supportive of a non-neural role is evidence linking FMR1 to human cancers. Specifically, fragile X syndrome patients are at decreased risk for cancer [8]; FMR1 mRNA is highly expressed in hepatoma [9,10], breast, colon, and bladder cancers [11]; and high levels of FMR1 are associated with lung metastasis of murine breast cancer [11]. We sought to reveal more information about the tissue specificity and regulation of FMR1.

Regulation of FMR1 protein is essential in long-term depression of metabotropic glutamate receptors (mGluR-LTD), an important mechanism for neural plasticity. Activation of mGluR-LTD is accompanied by a transient increase in the levels of FMR1. Proteasome inhibitors abolished this LTD, and the FMR1 proteins were rapidly degraded by the ubiquitin proteasome system [12,13]. Thus, FMR1 is regulated in an ubiquitin/proteasome-dependent manner; although, the mechanism of this regulation is uncertain.

C-terminus of Hsc70-interacting protein (CHIP) is a co-chaperone E3 ligase and regulates misfolded proteins presented by molecular chaperones such as Hsp70 or Hsp90. CHIP is ubiquitously expressed, and its function in the brain, where it acts as a protein quality control E3 ligase, is well defined [14]. Recent studies have shown that CHIP is also involved in tumorigenesis of various tissues via regulating specific target proteins [15,16]. Furthermore, CHIP levels are down-regulated in various cancers and negatively correlated with malignancy of breast cancer

Abbreviations: FMR1, fragile X mental retardation 1 protein; CHIP, C-terminus of Hsc70-interacting protein; OA, okadaic acid; 17-AAG, 17-*N*-allylamino-17-demethoxygeldanamycin; Hsp, heat shock protein; TPR, tetratricopeptide.

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[16–18]. Collectively, these findings suggest that CHIP, like FMR1, plays a role in tumorigenesis in non-brain tissues as well as regulation of protein expression in the brain.

In the present study, the levels of FMR1 were inversely correlated to CHIP expression in brain and other tissues, as well as in vivo and in vitro. We examined whether CHIP regulates the levels of FMR1 as an E3 ligase and whether phosphorylation is necessary for CHIP-mediated regulation of FMR1.

2. Materials and methods

2.1. Cell culture and transfection

HeLa cells were cultured in minimum essential medium. Human embryonic kidney 293T (HEK293T) cells and MDA-MB231 cells were cultured in Dulbecco's modified Eagle's medium. U2OS cells were cultured in McCoys'5A medium. Transfection was performed using polyethyleneimine (PEI) (Sigma) and Lipofectamine 2000 (Invitrogen) according to the manufacturers' instructions.

2.2. Antibodies and immunoprecipitation

The following antibodies were acquired and used for western blotting (WB) or co-immunoprecipitation (co-IP): anti-FMR1 (Millipore), anti-human influenza hemagglutinin (HA; Covance), anti-CHIP (Youngin Frontier), anti-Hsp70 (Santa Cruz Biotechnology), anti-EndoG (Enzo), anti-β-tubulin (Millipore), and anti-actin (Bethyl) were used for western blotting or co-immunoprecipitation. For co-IP, lysates were incubated with antibody overnight at 4 °C. Immunocomplexes were recovered with protein-A sepharose (Sigma), washed three times with buffer (20 mM Tris pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.1% Triton X100), and analyzed with WB.

2.3. Plasmids and site-directed mutagenesis

The mammalian expression vector for FMR1 was generated by inserting FMR1 PCR products from pINCY4-FMR1 (obtained from 21C Frontier Human Gene Bank, Daejeon, Republic of Korea) into pcDNA3-HA (Invitrogen). FMR1S500A and FMR1S500D were generated by site-directed mutagenesis according to manufacturer's instruction (Invitrogen).

2.4. In vivo ubiquitination assay and measurement of half-life

HEK293T cells were transfected as indicated, and 18 h after transfection, cells were treated with 20 μM MG132 for 6 h and lysed in lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% TritonX100, 10% glycerol, protease inhibitor cocktail). Ubiquitination was detected by co-IP with anti-FMR1 antibody and WB analysis with anti-HA. HEK293T cells were transfected with empty vector or HA-CHIP. Twenty-four hours after transfection, cells were treated with 100 μg/mL cycloheximide and harvested. Cells were lysed with RIPA buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 0.5% sodium-deoxycholate, protease inhibitor cocktail), and samples were subjected to WB.

2.5. RNA interference and okadaic acid treatment

U2OS cells were transfected with CHIP-specific siRNA (5′CGCUGGUGGCCGUGUAUUA3′), or scrambled RNA (Genolution) using Lipofectamine 2000 (Invitrogen). After 48 h, cells were washed with phosphate-buffered saline (PBS) and treated with 10 nM okadaic acid (OA) for 24 h. Cells were harvested and lysed for WB.

2.6. Animals and tissue homogenates

All experiments were approved by and carried out in accordance with the regulations of the Kyung Hee University Animal Care and Use Committee (KUACUC). C57BL/6 J mice were used for these experiments. Tissues were obtained from 10- or 40-week-old mice. Approximately 280 mg of tissue was homogenized in RIPA buffer, sonicated for 180 s, and centrifuged for 10 min at 12,000 rpm, 4 °C. The supernatants were collected and used for WB.

3. Results and discussion

3.1. The levels of FMR1 protein inversely correlated with CHIP expression

Levels of CHIP are greater in old mouse brain tissue than in young mouse brain tissues [16,17], and this is accompanied by greater expression of its target protein, Endonuclease G (EndoG), in old mouse brain tissue [17]. This negative correlation is presumably due to the degradation of EndoG by CHIP. We hypothesized that there were other targets of CHIP protein that would be similarly upregulated in old mouse brain compared to young. To test this hypothesis, we prepared whole-brain extracts from 10- or 40-week-old mice and compared the levels of several proteins. Of these proteins, FMR1 levels were greater in brains of the old mice than those of the young mice (Fig. 1A) and FMR1 therefore showed the same inverse correlation with CHIP as did its other target, Endo G. EndoG levels were greater, while CHIP levels were lower, in old mice than in the young (Fig. 1A), confirming the previous report [17].

Both FMR1 and CHIP are ubiquitously expressed, and CHIP has been shown to function in liver and heart [18,19]. We confirmed the expression of FMR1 in liver, heart, and brain extracts by WB. The size of the protein varied from 65 to 75 kDa (Fig. 1B), indicating that FMR1 is expressed in multiple forms in various tissues, probably due to alternative splicing. Twelve isoforms of FMR1 have been reported [20]; however, their functions have not been determined. The inverse correlation that we observed between CHIP and FMR1 in tissues of different ages was also detected in these tissues. Low levels of FMR1 and high levels of CHIP proteins were observed in the brain, and high levels of FMR1 and low levels of CHIP were found in the liver and heart (Fig. 1B).

Next, we expressed CHIP in HEK293T cells to examine the correlation between the CHIP expression and FMR1 levels. FMR1 levels were decreased as a result of CHIP expression and the decrease was proportional to the increased concentration of CHIP (Fig. 1C). Alternately, when we depleted CHIP expression with siCHIP in HeLa cells, endogenous FMR1 was substantially upregulated (Fig. 1D). The levels of FMR1 protein in this experiment were inversely correlated to CHIP expression in murine tissues and human cell lines and suggest that FMR1 levels are regulated by a CHIP-mediated post-transcriptional mechanism.

3.2. CHIP interacts with FMR1 via a non-tetratricopeptide repeat domain

Next, we asked if CHIP interacts with FMR1. WB following co-IPs with anti-FMR1 showed a strong interaction between CHIP and FMR1 (Fig. 2A). The N-terminus of CHIP has the tetratricopeptide repeat (TPR) domain, which confers the ability to bind molecular chaperones such as Hsp70 (or Hsp90). Its C-terminus has a U

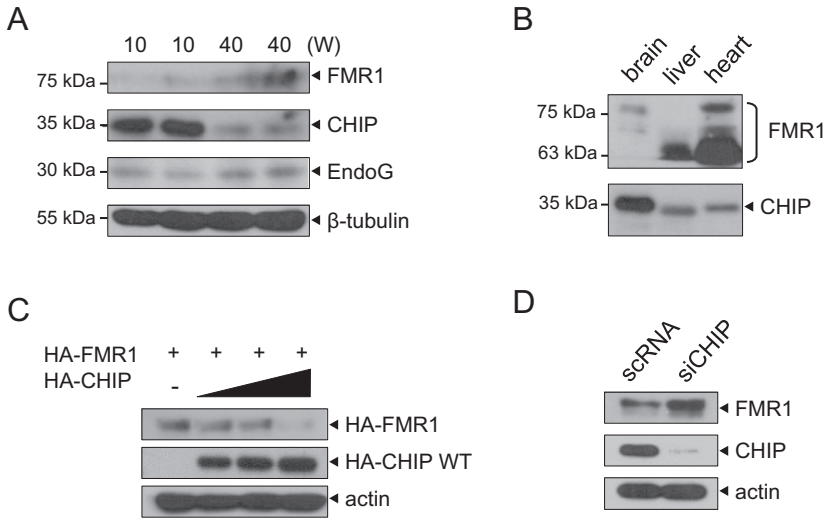

Fig. 1. The levels of FMR1 protein inversely correlated with CHIP expression. (A) Levels of FMR1 and CHIP proteins in mouse brain. Different amounts of brain lysates were loaded for WB with different antibodies for proper visualization: 70 μg with anti-FMR1, 50 μg with anti-EndoG, and 20 μg with anti-CHIP and anti-β-tubulin (10, 10-week-old mice; 40, 40-week-old mice). (B) Levels of FMR1 and CHIP proteins in 50 μg of lysates from different tissues of 30-week-old mice. (C) Levels of FMR1 and CHIP proteins in HEK293T cells co-transfected with HA-FMR1 and HA-CHIP and lysed for WB using anti-HA and anti-actin. (D) Levels of FMR1 and CHIP proteins in HeLa cells transfected with scRNA or siCHIP. The steady-state levels of FMR1 were elevated in CHIP-depleted cells.

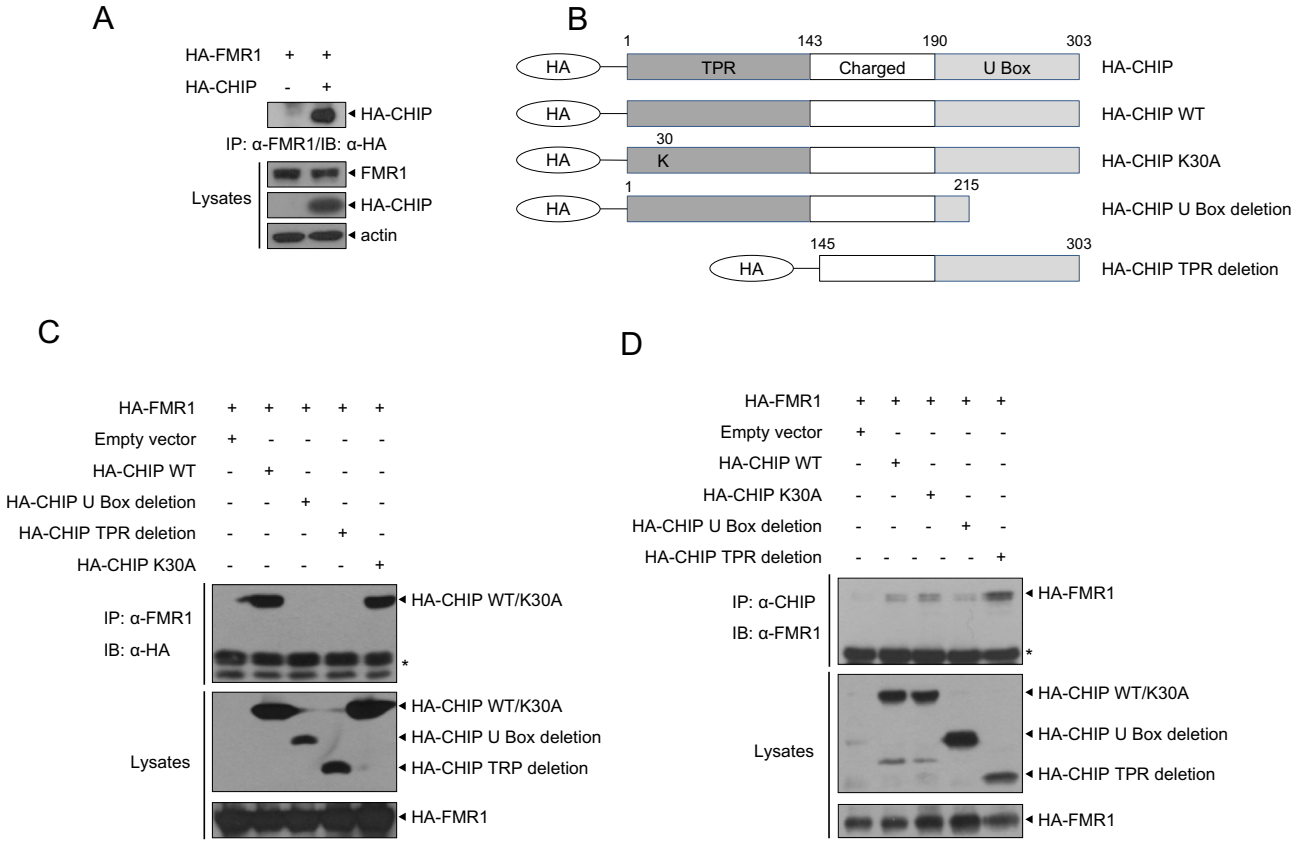

Fig. 2. CHIP interacts with FMR1 via a non-TPR domain. (A) Interaction of FMR1 with CHIP in HEK293T cells. (B) Schematic diagram of CHIP WT and various CHIP deletion mutants. (C) HEK293T cells were co-transfected with HA-FMR1 and various HA-CHIP constructs (as shown in B). After transfection, co-IPs were performed with anti-FMR1 and WB by anti-HA antibody. (* IgG light chain). (D) The cell culture and preparation of lysates were the same as in (C) but co-IP was performed with anti-CHIP and WB with anti-FMR1. (* IgG heavy chain).

box capable of interacting with E2 enzyme [21]. To investigate the region of CHIP required for interaction with FMR1, we generated various CHIP deletion mutants (Fig. 2B; [22]) and performed co-IPs with anti-FMR1 antibody. WB showed that FMR1 bound to the CHIP K30A mutant, which does not interact with Hsp70 (Fig. 2C; [21]), and also bound to CHIP wild type (WT). If FMR1 binds somewhere other than the TPR domain, either the CHIP U box deletion mutant or CHIP TPR deletion mutant should have

been detected with WB. Since it is possible that the protein bands of the mutants might be hidden in the strong immunoglobulin light chain band on WB, we performed co-IPs with anti-CHIP antibody. WB revealed a strong interaction between the FMR1 and CHIP TPR deletion mutant and little or no interaction between FMR1 and the CHIP U box deletion mutant (Fig. 2D). We conclude that CHIP interacts with FMR1 via non-TPR domains, likely via U box domain or a charged domain, in a manner that is independent of Hsp70. Interactions between CHIP and its target proteins are mostly mediated by Hsp70 (or Hsp90) via a TPR domain, although several proteins interact with CHIP independently of Hsp70 (or Hsp90) [23–25] and charged domains partially participate in the interaction with Runx1 [24]. We conclude that CHIP interacts with FMR1 in human cells lines via a non-TPR domain in an Hsp70-independent manner. CHIP might physically interact with FMR1 or the interaction between CHIP and FMR1 may be mediated by an unknown factor that replaces the role of Hsp70.

3.3. FMR1 is regulated by CHIP in an ubiquitin proteasome-dependent manner

We next examined if the CHIP expression-induced decrease of FMR1 (Fig. 1C and D) was due to proteasome activity. We expressed control vector or HA-CHIP in the presence or absence of the proteasome inhibitor MG132 in HEK293T cells. Decreased FMR1 protein expression following CHIP expression was stabilized upon MG132 treatment (Fig. 3A), indicating that CHIP is involved in the regulation of FMR1 protein in a proteasome-dependent manner.

Next, we examined whether CHIP regulates the levels of FMR1 via ubiquitination. An in vivo ubiquitination assay was performed with HEK293T cells. The results showed that FMR1 was ubiquitinated by CHIP WT or CHIP K30A. Also, ubiquitinated FMR1 accumulated in the presence of MG132, indicating that CHIP ubiquitinates FMR1 for proteasomal degradation (Fig. 3B). Interestingly, FMR1 ubiquitination by CHIP K30A appeared to be similar to, or even more efficient than, ubiquitination by CHIP WT (Fig. 3B). This observation is consistent with the interaction between FMR1 and CHIP K30A (Fig. 2C and D), suggesting that CHIP-mediated FMR1 ubiquitination does not depend on a molecular chaperone.

Thus, we next investigated whether regulation of FMR1 depends on Hsp70 (or Hsp90). 17-*N*-allylamino-17-demethoxygeldanamycin (17-AAG), a potent antitumor drug that inhibits Hsp90, causes destabilization of its client proteins via Hsp70 and CHIP [26]. The interaction of CHIP and its target proteins is mediated by Hsp70 or Hsp90; these target proteins are downregulated by 17-AAG [27,28]. To determine if CHIP-mediated FMR1 regulation requires a molecular chaperone, we treated the breast cancer cell line MDA-MB231 with 17-AAG. In these cells, functional relevance of FMR1 to metastasis has been shown. WB showed that FMR1 was not decreased but actually increased in these cells following treatment with 17-AAG. Expression of EndoG, the target protein regulated by CHIP in an Hsp70-dependent manner [20], was inhibited by 17-AAG treatment in a dose-dependent manner (Fig. 3C). The inhibition of EndoG expression is likely due to increased interaction with Hsp70 and ubiquitination via its E3 ligase CHIP (Fig. 3C). These results indicate that CHIP-mediated regulation of FMR1 is not dependent on Hsp70 or Hsp90, suggesting that another factor might be required to present FMR1 to CHIP or that CHIP regulates FMR1 via direct binding. CHIP has been shown to regulate BER [23], Runx1 [24], CtBP2 [25], and Profilin1 [22] in an Hsp70-independent manner. Together, these results demonstrate that CHIP ubiquitinated FMR1 as an E3 ligase and the FMR1 proteins were regulated by CHIP-driven ubiquitin proteasome system in Hsp70-independent manner.

3.4. CHIP-mediated FMR1 regulation depends on phosphorylation status of FMR1

Ribosomal protein S6 kinase 1 (S6K1) and protein phosphatase 2A (PP2A) determine the phosphorylation status of FMR1 in mGluR-LTD. Phosphorylation of FMR1 facilitates its degradation via proteasomes [29]. For instance, the E3 ligase SCF recognizes phosphorylated substrate proteins in their PEST sequences for proteasomal degradation [30]. Of note, a putative PEST site in FMR1 overlaps a phosphorylation site at Ser residue 499 (S499) of murine

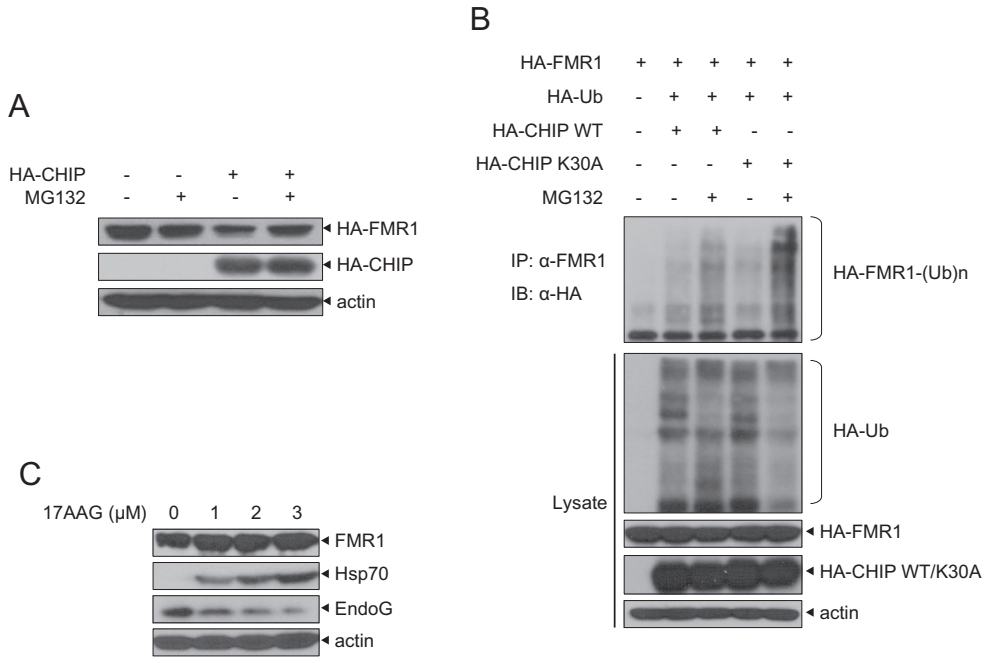

Fig. 3. FMR1 is regulated by CHIP in an ubiquitin proteasome-dependent manner. (A) CHIP is involved in the regulation of FMR1 protein via proteasome. (B) In vivo ubiquitination. (C) MDA-MB231 cells were treated with the indicated concentration of 17-AAG.

FMR1 [31]. We speculated that the regulation of FMR1 by CHIP is influenced by its phosphorylation status and employed regulators of phosphorylation enzymes to study this question. We treated U2OS cells with okadaic acid (OA), an inhibitor of PP2A, and measured the levels of FMR1. WB showed that the FMR1 bands were markedly reduced in the OA treatment compared to the untreated cells (Fig. 4A), suggesting that phosphorylation reduced the stability of FMR1. To test whether the reduced stability was due to CHIP, we depleted CHIP with siCHIP in U2OS cells in the presence of OA, with the result FMR1 levels were stabilized (Fig. 4B). We suggest that CHIP activity is involved in destabilization of FMR1 following OA treatment, presumably through degradation.

There is other evidence that CHIP-mediated degradation requires phosphorylation of target proteins. The kinase activity of HIPK2 is essential for proteasomal degradation of CtBPs [32] and for CHIP-mediated degradation of CtBP2 [25]. Akt is regulated by CHIP in a phosphorylation-dependent manner [33]. S6K1 phosphorylates S499 of murine FMR1 (S500 in humans) [29]. Therefore, we generated two FMR1 mutants: the FMR1 non-phospho mutant, in which S500 was mutated to Ala (HA-FMR1 S500A) and the FMR1 phospho-mimic mutant, in which S500 was mutated to Asp (HA-FMR1 S500D). The former mutant protein should behave as FMR1 in the dephosphorylated conformation and the latter as the phosphorylated conformation. The mutants were expressed in HEK293T cells and CHIP ubiquitination efficiency was determined. HA-FMR1 S500A, the non-phospho mutant, was not efficiently ubiquitinated by CHIP, while HA-FMR1 S500D, the phospho-mimic mutant, was ubiquitinated to a similar extent as FMR1 WT (Fig. 4C). Furthermore, the non-phospho mutant had a much longer half-life than FMR1 WT or the phospho-mimic mutant, even when CHIP was overexpressed (Fig. 4D). These data indicate that CHIP-mediated FMR1 regulation depends on the phosphorylation status of FMR1 in human cell lines.

Here, we report the first evidence that CHIP functions as an E3 ubiquitin ligase to regulate FMR1. The levels of FMR1 were inversely correlated to CHIP expression in both brain and several non-brain tissues. Considering that FMR1 and CHIP are ubiquitously expressed, these results suggest that CHIP regulates FMR1 protein in any tissue. However, the regulatory mechanism of FMR1 via phosphorylation may be tissue dependent. CHIP preferentially regulated phosphorylated FMR1 in non-neuronal human cell lines, but results in neurons are opposite to ours: mGluR-induced rapid degradation of FMR1 requires prior dephosphorylation of S499 in rat cortical neuron tissue and FMR1 S499A mutant is more efficiently ubiquitinated than FMR1 WT or FMR1 S499D in Neuro2a cell lines [34]. This discrepancy might be due to different signaling between neuron and non-neuronal cells. In fact, proteomic analyses have shown that several residues other than S500 of FMR1, such as S370, S511, T581 residues in HeLa cells and Y461 residue in lung cancer, are phosphorylated [35,36]. Furthermore, other kinases besides S6K1 could be involved in phosphorylation of FMR1 in non-neuronal cells. Casein kinase II [37] and p21-activated kinase 1 (PAK1) phosphorylate FMR1 [38] and polo-like kinase (plk) phosphorylates FMR1 in mitotic cells [39]. However, the relevance of

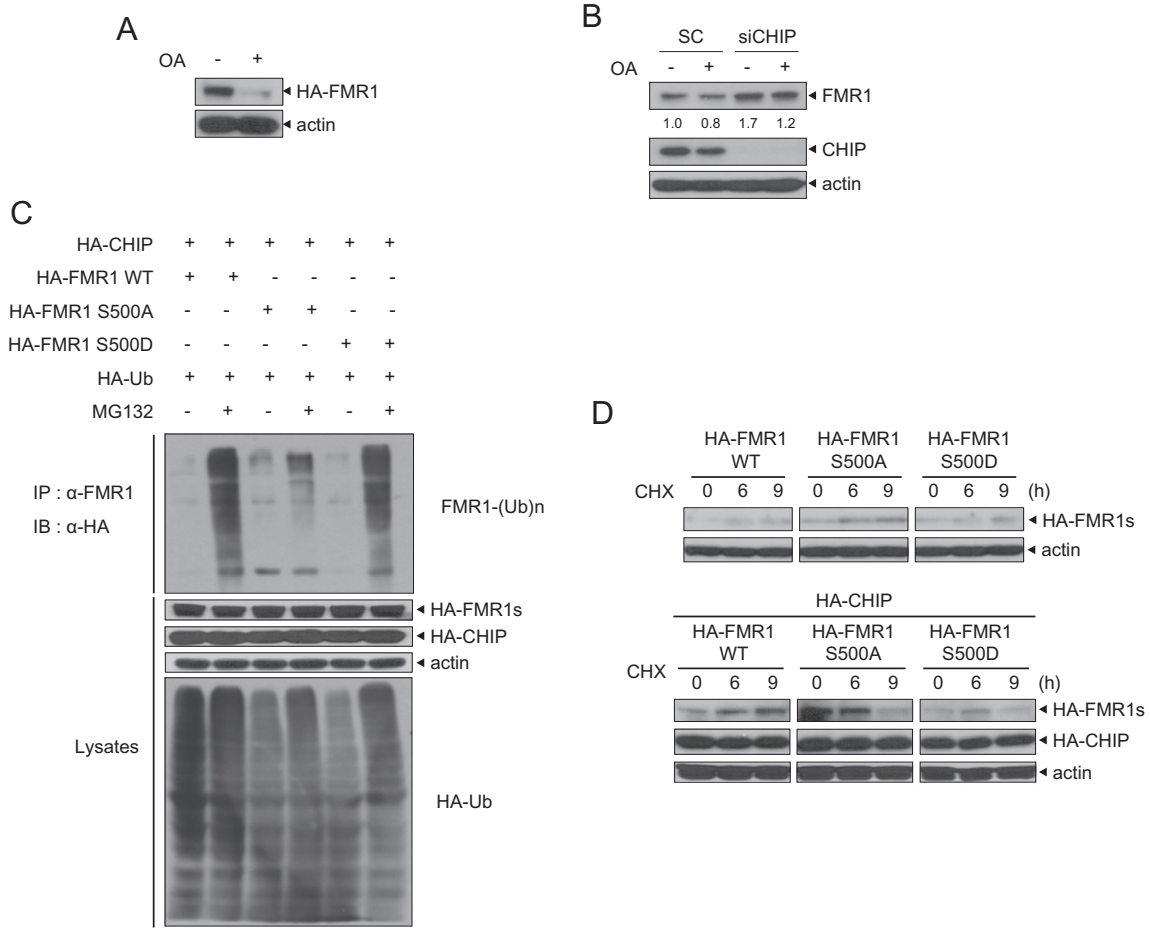

Fig. 4. CHIP-mediated FMR1 regulation depends on phosphorylation status of FMR1. (A) U2OS cells were treated with OA and WB was performed with anti-HA and anti-actin antibodies. (B) U2OS cells were transfected with scRNA or siCHIP, then treated with OA. (C) In vivo ubiquitination. (D) The half-life FMR1 WT and phosphorylation mutants as affected by HA-CHIP overexpression.

these phosphorylations or kinases to FMR1 regulation is unknown. The signaling pathway underlying the regulation of FMR1 protein in non-neuronal cells needs to be investigated to understand different regulation of FMR1 via phosphorylation in neuron and non-neuronal cells.

Recent evidence shows that FMR1 or CHIP is involved in the pathogenesis of breast cancer. Decreased CHIP accompanied increased malignancy of breast cancer [13] and high levels of FMR1 protein were associated with lung metastasis of murine breast cancer [9]. The possible interaction of CHIP and FMR1in the pathogenesis of human breast cancer has not been addressed. Our results imply that high levels of FMR1 were attributable to low levels of CHIP protein in breast cancer. Better understanding of FMR1 regulatory mechanism by CHIP may provide an unexpected strategy for treating breast cancers.

Acknowledgments

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