The artificial WSTF PHD_EL5 RING finger was designed via “α-helical region substitution”, and its structural model for the attachment of activated ubiquitin has been demonstrated. Chemical modifications of Cys residues, the circular dichroism spectra, and substrate-independent ubiquitination assays illustrated that the WSTF PHD_EL5 RING finger has E3 activity, and it is ubiquitinated via Lys14. Homology modeling calculations revealed that the WSTF PHD_EL5 RING finger possesses a classical RING fold for specific E2–E3 binding. The docking poses of the WSTF PHD_EL5 RING finger with the UbcH5b–ubiquitin conjugate provided insight into its functional E2 interaction and development of ubiquitination at the atomic level. The structural model of the artificial WSTF PHD_EL5 RING finger proposed by the present work is useful and may help to extend the strategy of α-helical region substitution.

The RING finger domain contains some chelating residues, namely Cys and/or His, and it binds to two zinc atoms1–3. Protein ubiquitination is an enzymatic cascade consisting of ubiquitin-activating (E1), ubiquitin-conjugating (E2), and ubiquitin-ligating (E3) enzymes4,5. Most RING fingers function as E3s and transfer activated ubiquitin from E2s to the ε-amino groups of the substrate Lys6. The enzymatic activities of E2 and E3 are associated with various diseases such as cancer and Parkinson’s disease7–9. In fact, overexpression of E2s is associated with tumor development in human cancers10. The detection of E2 activity in the ubiquitination system can facilitate the monitoring of tumor progression for diagnostic and prognostic purposes. E2s are attractive targets as tumor markers of various cancers. To detect E2 activity, both an E3 and a substrate are indispensable in the ubiquitination system.

Recently, the “α-helical region substitution” method was reported for designing artificial RING fingers11,12. E3 RING fingers always possess the essential α-helical region for E2–E3 binding13. If the α-helical region of the EL5 RING finger is inserted into the amino acid sequence between the sixth and seventh zinc ligands of the Williams–Beuren syndrome transcription factor (WSTF) PHD finger, then the artificial WSTF PHD_EL5 RING finger is created on the basis of the EL5 RING finger (Figure 1). The WSTF PHD_EL5 RING finger functions as E3 and cooperates with the same E2 UbcH5 as the EL5 RING finger12. In the absence of a substrate, the EL5 RING finger has no E3 activity, however the WSTF PHD_EL5 RING finger is poly-ubiquitinated via Lys63 of ubiquitin, but not Lys6 and Lys4811.

The WSTF PHD_EL5 RING finger is useful for detecting the E2 activity of UbcH5 because its substrate is inessential for ubiquitin transfer. There is growing interest in characterizing the ubiquitination mechanism of artificial RING fingers. However, detailed structural information about artificial RING fingers and their ubiquitination sites at the atomic level is not available. This knowledge is crucial for the molecular design of various artificial RING fingers as E3.

In this study, we investigated the structural features of the artificial WSTF PHD_EL5 RING finger using homology modeling and circular dichroism (CD) spectroscopy. In addition, the ubiquitination site of the WSTF PHD_EL5 RING finger was assessed via mutational analysis of five Lys residues. The present report provides important information about the ubiquitination mechanism of this artificial RING finger.

Results
Secondary structure of the artificial WSTF PHD_EL5 RING finger. To obtain information on the secondary structure of the WSTF PHD_EL5 RING finger and its five mutants (K4R, K8R, K9R, K14R, and K23R) shown in Figure 1, their CD spectra were recorded at room temperature in Solution A consisting of 20 mM Tris-HCl (pH 6.9), 50 mM NaCl, 1 mM dithiothreitol, and 50 μM ZnCl2. As the WSTF PHD_EL5 RING finger has E3 activity, the synthesized five mutations would be expected to alter the function of the protein and decrease its E3 ligase activity. As shown in Figure 2, all of the CD spectra were suggestive of the typical helical conformation with
double minima occurring at approximately 205 (π-π* transitions) and 225 nm (π-π* transitions). The molar ellipticity occurring at approximately 208 nm shows the existence of α-helical and β-sheet conformations. However, the CD spectrum of K23R displayed slight decreases at the negative ellipticities, although the shapes of these spectra were extremely similar. Furthermore, the zinc:protein stoichiometry was estimated by the chemical modification of the Cys residues. The protein-binding zinc ions were released by p-hydroxymercuribenzoic acid and quantified using the metallochromic indicator 4-(2-pyridylazo) resorcinol. The concentration of the WSTF PHD_EL5 RING finger were 2.0 μM, and that of the zinc atoms released by K4R, K8R, K9R, K14R, and K23R mutants was 4.5, 4.4, 4.0, 3.7, and 3.5 μM, respectively. The molar calculated ratios ([Zn]/[protein]) were 2.25, 2.20, 2.00, 1.85, and 1.75, respectively. This finding indicates that the artificial RING fingers bind to two zinc atoms for proper folding with C4HC3-type zinc coordination.

Identification of the ubiquitination site of the artificial RING finger. To identify the ubiquitination site of the WSTF PHD_EL5 RING finger, the E3 activities of the artificial RING finger and its five mutants were assessed via in vitro substrate-independent ubiquitination. The ubiquitin reaction, even in the absence of an E3 ligase, promotes the formation of ubiquitin thioester-linked conjugates of the E2 UbcH5b. The addition of the wild-type artificial RING finger or its mutants led to the accumulation of ubiquitin-E3 conjugates, and mono- and poly-ubiquitination were clearly observed (Figure 3). The products corresponding to the higher molecular weight around 148 kDa were observed, and thus the poly-ubiquitination was preferentially on the WSTF PHD_EL5 RING finger. K4R, K8R, and K9R possessed similar E3 activities as the wild-type artificial RING finger. Ubiquitination of the K23R mutant was somewhat weaker than that of the wild-type artificial RING finger. Excluding the K14R mutant, the mutants functioned as

![Figure 1](https://example.com/figure1.png)

**Figure 1** | Strategy for the creation of the artificial RING finger as E3 ligase. (A) Amino acid sequence of the EL5 RING finger. (B) Creation of an artificial RING finger by α-helical region substitution of the helical region (underlined). Zinc ligands are shown in red. L1 and L2 display the short loop between zinc ligands. Lys residues that were replaced with Arg are shown in blue.

![Figure 2](https://example.com/figure2.png)

**Figure 2** | CD spectra of the artificial WSTF PHD_EL5 RING finger and its five mutants. Spectra of 25 μM samples were collected in 20 mM Tris-HCl (pH 6.9), 50 mM NaCl, 1 mM dithiothreitol, and 50 μM ZnCl2 at room temperature. (1) K4R, (2) K8R, (3) K9R, (4) K14R, and (5) K23R are denoted by solid lines, and the dotted line displays the wild-type.
Figure 3 | Ubiquitination of the artificial WSTF PHD_EL5 RING finger and its five mutants. The wild-type (WT) WSTF PHD_EL5 RING finger and its mutants were used in the ubiquitination reaction together with randomly biotinylated ubiquitin, E1, and E2 (UbcH5b). The biotinylated ubiquitin on the PVDF membrane was reacted with streptavidin-horseradish peroxidase solution and a chemiluminescence reagent. The emitted signals were detected using a Luminescent Image Analyzer LAS-3000. The arrow shows the product of the mono-ubiquitination, and the molecular weight of the ubiquitinated WSTF PHD_EL5 RING finger is approximately 14569 Da.

E3s with cooperating UbcH5b. In addition, mono-ubiquitination of the K14R mutant was not observed in the reaction system. Taken together, these data indicate that Lys14 of the WSTF PHD_EL5 RING finger is essential for mono- and poly-ubiquitination under the present conditions. The spatial location and structural features of Lys14 in the WSTF PHD_EL5 RING finger at the atomic level will be examined later via structural modeling methods.

Structural model of the artificial RING finger. As no structure of the artificial RING finger is available, homology modeling was performed using the program I-TASSER, and the structure of the WSTF PHD_EL5 RING finger was predicted (Supplementary PDB file 1). The built structure has a good C-score of 1.11 and a TM-score of 0.87 for the top model. The C-score indicates high quality of the model predicted by I-TASSER. The appropriate templates (EL5 RING finger (PDB code: 2L0B); FANCL (PDB code: 3K1L); praja-1 RING finger (PDB code: 2K1L);) were automatically collected for the building calculation by I-TASSER. The sequence identities of the whole template chains with the query sequence for EL5 RING finger, FANCL, and praja-1 RING finger are 45, 36, and 28%, respectively. The resulting three-dimensional structure of the WSTF PHD_EL5 RING finger possessed one α-helical region (α1) and two β-sheets regions (β1 and β2), indicative of the typical cross-brace zinc finger as shown in Figure 4. The structure has a groove for the E2-binding site consisting of α1, L1, and L2 regions, which are always present in E3 RING fingers. The quality of the structure was checked using the program PROCHECK. In a Ramachandran plot of the WSTF PHD_EL5 RING finger, non-glycine residues in the most favored regions and the additional allowed regions were located within 85.4% and 14.6%, respectively. No residue of the structural model presented in this study exists in the disallowed region. The Connolly surface calculated using Discovery Studio 2.1 illustrated that positively charged residues gather at the molecular surface, and thus, the structure of the WSTF PHD_EL5 RING finger exhibits an amphipathic character under the present conditions.

The structure-function relationships of the WSTF PHD_EL5 RING finger provide insight into the structural properties responsible for functional ubiquitination. The structural features of the WSTF PHD_EL5 RING finger were analyzed from the standpoint of its structural similarities with the WSTF PHD and EL5 RING fingers in Figure 4. The WSTF PHD_EL5 RING finger was superimposed over the backbone (N, Cα, C') atoms of the WSTF PHD finger with rms deviations of 6.16 Å (Ala1–Thr47) and 2.26 Å (Ala1–Leu30), whereas the comparison with the structure of the EL5 RING finger yielded an rms deviation of 2.81 Å (Ala1–Thr47). The structure of the WSTF PHD_EL5 RING finger is similar to that of the WSTF PHD finger in the residues before the helical region. However, the structure of the artificial RING finger as a whole resembles that of the EL5 RING finger in that it has the indispensable helical region for E3 activity.

Possible docking poses. The structural docking of the WSTF PHD_EL5 RING finger and the UbcH5b–ubiquitin conjugate (PDB code: 3A33) was calculated using ZDOCK and Discovery Studio 2.1. The possible binding poses were obtained as 1000 structures contacting Lys63 of UbcH5b, which is considered important in UbcH5b–E3 binding. Figure 6A presents a best-fit superposition of the ensemble of the three lowest energy structures (Supplementary PDB file 2). The rms deviation is 1.58 Å for the backbone (N, Cα, C') atoms in all residues of the WSTF PHD_EL5 RING finger and the UbcH5b–ubiquitin conjugate. Lys63 of UbcH5b forms a hydrogen bond with Ser36 of the artificial RING finger. Regarding Lys residues for accepting ubiquitin in the WSTF PHD_EL5 RING finger in the lowest energy structure, Lys4 and Lys8...
The WSTF PHD_EL5 RING finger is shown in magenta. Computational results were obtained using software programs from Accelrys Software Inc.

The artificial WSTF PHD_EL5 RING finger was created via backbone atoms for the (A) WSTF PHD and (B) EL5 RING fingers. Ribbon diagrams illustrating a trace of the backbone atoms are used for visualization.

Discussion

The WSTF PHD_EL5 RING finger is shown in magenta. Computational results were obtained using software programs from Accelrys Software Inc.

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The WSTF PHD_EL5 RING finger was superimposed over the backbone of the artificial WSTF PHD_EL5 RING finger. The WSTF PHD_EL5 RING finger was superimposed over the backbone atoms for the (A) WSTF PHD and (B) EL5 RING fingers. Ribbon diagrams illustrating a trace of the backbone atoms are used for visualization.

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**CD spectroscopy.** The CD experiments were conducted on a JASCO J-805 spectropolarimeter after calibration using d-camphor-10-sulfonate. A 1-mm path length quartz cell was used for 25 μM samples. Spectra were obtained at room temperature from 200 to 250 nm under the conditions of a bandwidth of 1 nm and a data pitch of 1 nm at a scan speed of 50 nm/min. Each CD spectrum was the average of four scans. After subtraction of the solvent spectrum, the CD data were collected by transforming the CD signal into mean residue molar ellipticity.

**In vitro substrate-independent ubiquitination.** The WSTF PHD_EL5 RING finger (molecular weight, 5287 Da) and its five mutants were used as E3 enzymes in substrate-independent ubiquitination reactions. The ubiquitination assays were performed in 50-μl reaction volumes of ubiquitination buffer (20 mM Tris-HCl (pH 6.9), 5 mM Mg-ATP, 1 mM dithiothreitol, 20 U/ml inorganic pyrophosphatase (Sigma, St. Louis, MO, USA), 50 μM ZnCl2) including 2.5 μM biotinylated ubiquitin, 0.1 μM human recombinant E1 (His-tagged), and 2.5 μg UbcH5b as E2 (His-tagged). Biotinylated ubiquitin (molecular weight, approximately 9300 Da), human E1, and UbcH5b were purchased from Enzo Life Sciences (Farmingdale, NY, USA). The mixtures also contained the WSTF PHD_EL5 RING finger or its five mutants at a concentration of 20 μM. The reaction solutions were incubated at 37 °C for 60 min with gentle agitation, and the reaction was stopped by adding non-reducing sodium dodecyl sulfate (SDS) sample buffer. The obtained samples (10 μl) were subjected to SDS-PAGE (10–20%) and transferred to a polyvinylidene difluoride (PVDF) membrane. To detect biotinylated ubiquitin, the membrane was reacted with streptavidin-horseradish peroxidase solution (Vectastain ABC Elite Kit; Vector Laboratories, Burlingame, CA, USA), and then enhanced chemiluminescence (ECL; GE Healthcare, Buckinghamshire, UK) was used as a western blotting detection reagent according to the manufacturer’s protocol. The emitted signals were detected using a Luminescent Image Analyzer (LAS-3000; Fujifilm, Tokyo, Japan).

**Protein structure modeling.** The structure modeling was performed using the I-TASSER server (as “Zhang Server”), which consists of multiple threading alignments and iterative template fragment assembly simulations. The I-TASSER program was ranked as the best server in the Critical Assessment of protein Structure Prediction experiments. The server generates the most accurate structural predictions.
via a state-of-the-art process. The quality of the predicted structures was evaluated by a confidence score (C-score), and a C-score of $\geq -1.5$ denotes a correct fold. The template modeling score (TM-score) describes the topological similarity between the predicted model and the native structure. A TM-score of $\geq 0.5$ indicates protein pairs with similar folds as well as a more accurate model prediction. The amino acid sequence of the WSTF PHD EL5 RING finger (Figure 1) was subjected to the I-TASSER server, and the obtained structure was validated using PROCHECK. The program Discovery Studio 2.1, San Diego: Accelrys Software Inc., was used to calculate the Connolly surface and prepare drawings of the structures.

**ZDOCK calculations.** The docking calculation was performed using the program ZDOCK 3.0.1.5, 36. ZDOCK utilizes a fast Fourier transform algorithm to improve the performance for searching in a translational space, and it is an initial-stage rigid-body molecular docking program. The precision of the docking structure calculated by ZDOCK was proven in the Critical Assessment of Prediction of Interactions Challenge. The available structure from the I-TASSER server was used to calculate the docking poses, and the obtained structure was subjected to energy minimization using the Smart Minimizer algorithm (Max steps 200, RMS gradient 0.01) in Discovery Studio 2.1.13, 14. The resulting top three models were used as appropriate candidates.

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**Acknowledgments**
This work was supported by Takeda Science Foundation and JSPS KAKENHI Grant Number 26430147.

**Author contributions**
K.M. designed and performed all the experiments. K.M. wrote the manuscript text and prepared all the figures. All authors reviewed the manuscript.

**Additional information**
Supplementary information accompanies this paper at http://www.nature.com/scientificreports/

**Competing financial interests:** The authors declare no competing financial interests.

**How to cite this article:** Miyamoto, K. Structural model of ubiquitin transfer onto an artificial RING finger as an E3 ligase. Sci. Rep. 4, 6574; DOI:10.1038/srep06574 (2014).

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