Stepwise multipolyubiquitination of p53 by the E6AP-E6 ubiquitin ligase complex

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The human papillomavirus (HPV) oncoprotein E6 specifically binds to E6AP (E6-associated protein), a HECT (homologous to the E6AP C terminus)-type ubiquitin ligase, and directs its ligase activity toward the tumor suppressor p53. To examine the biochemical reaction in vitro, we established an efficient reconstitution system for the polyubiquitination of p53 by the E6AP-E6 complex. We demonstrate that E6AP-E6 formed a stable ternary complex with p53, which underwent extensive polyubiquitination when the isolated ternary complex was incubated with E1, E2, and ubiquitin. Mass spectrometry and biochemical analysis of the reaction products identified lysine residues as p53 ubiquitination sites. A p53 mutant with arginine substitutions of its 18 lysine residues was not ubiquitinated. Analysis of additional p53 mutants retaining only one or two intact ubiquitination sites revealed that chain elongation at each of these sites was limited to 5–6-mers. We also determined the size distribution of ubiquitin chains released by en bloc cleavage from polyubiquitinated p53 to be 2–6-mers. Taken together, these results strongly suggest that p53 is multipolyubiquitinated with short chains by E6AP-E6. In addition, analysis of growing chains provided strong evidence for step-by-step chain elongation. Thus, we hypothesize that p53 is polyubiquitinated in a stepwise manner through the back-and-forth movement of the C-lobe, and the permissive distance for the movement of the C-lobe restricts the length of the chains in the E6AP-E6-p53 ternary complex. Finally, we show that multipolyubiquitination at different sites provides a signal for proteasomal degradation.
linked short poly-Ub chains via stepwise reactions. We hypothesize that p53 is polyubiquitinated in a stepwise manner through the back-and-forth movement of the C-lobe, and the permissive distance for the movement of the C-lobe restricts the length of the chains in the E6AP-E6-p53 ternary complex.

Results

A reconstitution system for the polyubiquitination of p53 by the E6AP-E6 complex in vitro

The biochemical reactions of polyubiquitination by HECT-type Ub ligases were analyzed by establishing a reconstitution system for the polyubiquitination of p53 by E6AP-E6 in vitro (Fig. 1 and Fig. S1). Remarkably, co-expression of E6AP and E6 in Escherichia coli cells and their subsequent purification as an E6AP-E6 complex yielded a highly active preparation. Standard reactions were performed at 30 °C for 10 min, and the reaction products were detected by Western blotting (Fig. 1). In this system, efficient ubiquitination of his-p53 and self-ubiquitination of E6AP were detected as slower migrating forms in the presence of E1, E2 (UBCH5c or UBCH7), E6, and Ub, as reported previously (Fig. 1A) (1, 2, 5, 23–25).

To confirm the formation of thiol linkages between E6AP and Ub (10), assays were performed for 30 s without his-p53 to avoid Ub transfer from E6AP–Ub to his-p53, and the products were analyzed under nonreducing and reducing conditions by Western blotting with anti-E6AP antibody (Fig. 1B). The results clearly showed E2-dependent formation of E6AP–Ub in the absence of E6 (lane 4), as indicated by the sensitivity of the complex to a reducing agent (lane 8). In the presence of E6, many bands larger than E6AP–Ub were detected (lane 2). Treatment with the reducing agent resulted in the disappearance of several products; however, some products were still detectable (lane 6), suggesting that bands detected under nonreducing conditions were a mixture of self-ubiquitinated E6AP and self-ubiquitinated E6AP with an additional Ub via thiol linkage. These results demonstrated that the reconstitution system efficiently generated E6AP–Ub.

Polyubiquitination in a stable complex consisting of E6AP, E6, and p53

The effect of the concentration of E6AP-E6 on p53 ubiquitination was examined (Fig. 2A). Although the amounts of polyubiquitinated products increased in a manner dependent on E6AP-E6 concentration, relatively large products were detectable even in the presence of low E6AP-E6 concentrations. This result suggested that once E6AP-E6 binds to his-p53, it successively transfers many Ub molecules without dissociation (i.e., E6AP-E6 might form a stable complex with p53). To examine complex formation, his-E6AP, E6, and p53 were co-produced in E. coli. Then his-E6AP was purified in the presence of 1 M NaCl by nickel affinity, and fractionated by gel filtration chromatography to separate his-E6AP-E6 from the ternary complex consisting of his-E6AP, E6, and p53. Each fraction was analyzed again by gel filtration (Fig. 2B). The three proteins, his-E6AP, E6, and p53, co-eluted together at 600–700 kDa of apparent molecular mass, which was considerably larger than the mass of his-E6AP-E6 at 170–200 kDa. We analyzed the intensity of each protein component in the peak fractions from the gel images of Fig. 2B. The calculated stoichiometries of each subunit in the his-E6AP-E6-p53 and his-E6AP-E6 complexes were close to 1:1:1 and 1:1, respectively (Table 1). The ligase activity of the ternary complex was confirmed by a ubiquitination assay with the additional introduction of E1, UBCH5c, and Ub (Fig. 2C). These results suggest that the stable binding of E6AP-E6 to p53 ensures that each p53 molecule is ubiquitinated processively.

Effects of E2 concentration to E6AP trans-thiolation and p53 ubiquitination

Next, the effect of E2 concentration was examined (Fig. 3). The results showed that the concentration of E2 strongly affected the size of the products during a 10-min incubation. Both UBCH5c and UBCH7 supported the ubiquitination of his-p53 at similar concentrations (Fig. 3, A and B). N-terminal histidine-tagged UBCH5c (hisUBCH5c) was inefficient (Fig. 3C), as an ~17-fold higher concentration of hisUBCH5c than UBCH5c was required to obtain similar levels of ubiquitination. We also tested two specific mutants of UBCH5c, S22R (design-
nated as UBCH5cSR), which is deficient in self-assembly of E2–Ub conjugates and therefore inefficient with RING-type ligases (26, 27), and P61A/F62A (designated as UBCH5c2A), which has reduced affinity for E6AP (28–31). Unlike the RING-type ligases, UBCH5cSR supported p53 ubiquitination by E6AP-E6 (Fig. 3D). By contrast, UBCH5c2A showed a clear defect in p53 ubiquitination with ~2000-fold reduced affinity (Fig. 3E). We excluded the possibility that defects in hisUBCH5c and UBCH5c2A were attributed to inefficient E2–Ub formation catalyzed by E1, because both hisUBCH5c and UBCH5c2A were charged as well as UBCH5c and UBCH5cSR for 30 s (Fig. 3F).

Then we analyzed the subsequent reaction step, E6AP trans-thiolation (Fig. 3G). The reactions were performed for 30 s in the absence of E6 to prevent subsequent Ub transfer to E6AP itself (see Fig. 1B). The results showed that UBCH5c and UBCH5cSR supported E6AP–Ub formation at 50 nM (Fig. 3G, lanes 2 and 3). By contrast, hisUBCH5c was ineffective at 50 nM but effective at 1600 nM (lanes 5 and 6), and UBCH5c2A was apparently defective even at 1600 nM (lane 4). These results indicated a positive correlation between the efficiency of p53 ubiquitination and E6AP–Ub formation, suggesting that trans-thiolation is a critical step in determining the rate of p53 ubiquitination.

### Kinetics of ubiquitination reactions

The reaction mechanism was further analyzed by examining the time course of the reaction (Fig. 4A). The result showed that the size of ubiquitinated his-p53 increased in a time-dependent manner, reaching the maximum level at 30 min. Unanchored
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A

| α-p53 | UBCH5c (nM) |
|-------|-------------|
|       | 0.1 0.2 0.4 0.8 1.6 3 | 0 3 6 13 25 50 100 |

B

| α-p53 | UBCH7 (nM) |
|-------|-------------|
|       | 0 3 13 25 50 100 |

C

| α-p53 | hisUBCH5c (nM) |
|-------|----------------|
|       | 0 50 200 400 800 |

D

| α-p53 | UBCH5c SR (nM) |
|-------|----------------|
|       | 3 6 13 25 50 100 |

E

| α-p53 | UBCH5c 2A (nM) |
|-------|----------------|
|       | 0 50 200 400 800 |

F

| α-UBCH5c |
|---------|
| Non-reducing + E1 |
| Reducing |

G

| α-E6AP |
|---------|
| Non-reducing |
| Reducing |

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Ub chains were generated after 15 min and increased considerably after 60 min. Hyperubiquitination of E6AP was initiated at 10 min and reached a maximum level at 60 min. Monoubiquitination of UBCH5c was observed at later time points (Fig. 4A).

The purified E6AP-E6-p53 complex (Fig. 2B) showed similar patterns (Fig. S2A).

If polyubiquitination proceeds processively on each his-p53 molecule, a reduced concentration of E6AP should not affect the rate of the chain elongation. To examine this, we analyzed the effect of a lower E6AP-E6 concentration (8 nM, 4-fold lower than the standard concentration) on the time course of polyubiquitination (Fig. 4B). As expected, the rates of chain elongation were essentially identical to those of the standard concentration (compare top panels of Fig. 4, A and B). Importantly, large amounts of unmodified his-p53 remained even after a 150-min incubation that resulted in almost all E6AP being self-ubiquitinated (Fig. 4B), indicating that his-p53 was present in excess over E6AP-E6. This result supports the notion that each his-p53 molecule undergoes processive polyubiquitination (i.e. once an E6AP-E6 molecule binds to a his-p53 molecule, the his-p53 molecule is processively polyubiquitinated by the E6AP-E6 molecule).

By contrast, the E2 concentration strongly affected the rate of ubiquitination of his-p53 (Fig. 3). Thus, the time course was performed under a lower UBCH5c concentration (12.5 nM, 4-fold lower than the standard condition) (Fig. 4C). The result showed that the size of ubiquitinated his-p53 increased at a slower rate and reached the maximum level at 60 min; additionally, all other reactions were also delayed. Consistent with the pattern of his-p53-ubiquitination, unanchored Ub chains were generated after 60 min, and hyper-self-ubiquitination of E6AP was now detectable after 60 min. Even after 150 min, E6AP ubiquitination was still partial. Accumulation of unanchored Ub chains and monoubiquitination of UBCH5c were also delayed beyond 150 min (Fig. 4C).

It has been established that E6AP specifically generates Lys48-linked Ub chains (11, 12, 32). To confirm this in our reconstitution system, a time course of the reactions was performed. A, the reaction was started in a 250-μl mixture under standard conditions, and 15-μl aliquots were withdrawn at the indicated times. B, the reaction was performed as in A except for the use of a 4-fold lower E6AP-E6 concentration. C, the reaction was performed as in A except for the use of a 4-fold lower E2 concentration. D, the reaction was performed as in A except for the use of the UbK48R mutant instead of WT Ub. The reaction products were visualized by Western blotting with the indicated antibodies. Note that because of the presence of cross-reacting material (indicated by the asterisk) in the commercial UbK48R preparation (D), it was not possible to determine whether or not di-Ub was generated in the reaction.
formed with a Ub mutant, UbK48R (Fig. 4D). In contrast to the reactions performed with WT Ub (Fig. 4A), the size of ubiquitinated p53 increased to ~110 kDa in the early time points, and then gradually increased to fractions of ~150 kDa together with minor fractions of considerably higher molecular mass (>250 kDa). Such restriction was also observed in the self-ubiquitination of E6AP (Fig. 4D). Similar results were obtained with methylated Ub, but the higher-molecular mass species (>250 kDa) were not detectable (Fig. S2B). Because Ub is methylated on amino groups and thus unable to form poly-Ub chains, the ubiquitinated products formed by methylated Ub are definitely (multi-)monoubiquitinated forms (33). Thus, the higher-molecular mass species (>250 kDa) detected with UbK48R (Fig. 4D), but not detected with methylated Ub, are most likely polyubiquitinated forms. These results suggested that E6AP predominantly forms Lys48 linkages and potentially other linkage(s) inefficiently and that p53 was multiubiquitinated at multiple sites (33), as estimated from the size of 110–150 kDa.

Mass spectrometry analysis of Ub chains

The Ub-linkage types in these reactions were also determined by quantitative MS-based analysis called Ub-AQUA (absolute quantitation)/PRM (parallel reaction monitoring) (34). The reaction products (>150 kDa) detected at 10 min for WT Ub and at 30 min for UbK48R were subjected to Ub-AQUA/PRM. In the WT Ub reaction, most Ub molecules were found in Lys48 linkages (49%) or unmodified (50%) (Table 2). Shotgun MS analysis identified 14 lysine residues of p53 as the ubiquitination targets. Then the additional mutant p5312KR, which contained an additional K351R mutation (Fig. 6A), was ubiquitinated in a manner similar to that of the p5314KR mutant (Fig. 6C), confirming the result of MS analysis that Lys351 was not a target (Fig. 5). The p5314+2KR mutant, which contained the additional K132R and K139R mutations (Fig. 6A), exhibited a slight reduction of ubiquitination with WT Ub, and only two targets were detected with UbK48R (Fig. 6D). Finally, the p5314+4KR mutant, which contained the additional K101R and K120R mutations (Fig. 6A), exhibited no apparent ubiquitination (Fig. 6E). These results suggested that in addition to the 14 lysine residues detected by MS analysis, four additional lysine residues are potential targets for ubiquitination.

To verify that the ubiquitination sites determined by MS analysis were indeed ubiquitinated, two major ubiquitination sites, Lys305 and Lys319 (see Fig. 5), were selected and reversed to lysine residues (Fig. 6A), and the resulting mutants, p5313(1)+4KR and p533(2)+4KR, were analyzed. The results with UbK48R confirmed that both mutants had one target for ubiquitination (Fig. 6, F and G, bottom panels). In the reaction with WT Ub, chain elongation was limited to 5–6-mers (Fig. 6, F and G, top panels) despite the fact that these lysine residues are efficient target sites. Then the additional mutant p5312+4KR, in which both Lys305 and Lys319 were reversed to lysine residues (Fig. 6A), was analyzed. The results with UbK48R demonstrated that the two target sites of the p5312+4KR mutant could be simultaneously ubiquitinated, and no preference between the two target sites was observed, because the relative amounts of the two monoubiquitinated forms, distinguishable as two closely migrating doublet bands, were constant over the time course of the reaction (Fig. 6H, bottom). In the presence of WT Ub, the number of ubiquitin molecules attached to the p5312+4KR mutant was also limited to approximately the sum of those in the single site mutants, p5313(1)+4KR and p533(2)+4KR.

**Table 2**

Uquitin-AQUA/PRM analysis of ubiquitin fragments with GG modifications in lysine residues

| Ubiquitin | TITLE6 | Totala | Lys6(GG) | Lys11(GG) | Lys14(GG) | Lys16(GG) | Lys18(GG) | Lys21(GG) | Lys34(GG) | Lys36(GG) | Lys38(GG) | Lys39(GG) | Lys41(GG) | M’S(GG) | Unmodifiedd |
|-----------|--------|--------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|
| WT        | 234.4  | 236.1  | 0.04     | 1.65     | ND       | 0.003    | 0.07     | 115.2    | 0.08     | ND       | 25.3     | 24.0     | 0.08     | ND       |
| K48R      | 62.4   | 65.5   | 0.15     | 3.05     | ND       | 0.002    | 0.01     | NA       | 0.09     | ND       | 37.6     | 36.0     | 0.11     | ND       |

a Three independent reaction products were analyzed as described previously (33), and the averages are shown.
b The amounts of the TITLE (TITLEVESSDTIDNVLK) fragment were determined as internal controls.
c Total ubiquitin levels were assessed as the sum of TITLE, Lys11(GG), and Lys37(GG) fragments. Because the TITLE fragment is between Lys11 and Lys37, generation of these fragments is mutually exclusive.
d The amounts of unmodified ubiquitin were calculated by subtraction of the amount of total GG-modified fragments from that of total ubiquitin.

\[ \text{Amounts are shown in fmol, and the percentage for each fragment is shown in parentheses.} \]
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These results suggested that E6AP-E6 is inefficient at generating long Ub chains, and the high-molecular-weight products generated with WT p53 were a consequence of multiple polyubiquitination with relatively short Ub chains.

### Multiple polyubiquitination and stepwise elongation

To demonstrate that WT p53 is multipolyubiquitinated with short chains, we decided to use a deubiquitinating enzyme, USP7, to cleave off and release preferentially substrate-attached Ub chains from ubiquitinated p53. USP7 is ideal for this purpose because it releases unanchored Ub chains from polyubiquitinated substrates through selective digestion of substrate-Ub linkages (35). We focused on Ub chains at early time points of the time course experiments when the size of ubiquitinated hsp53 was constantly increasing (Fig. 4A). The reaction products were visualized by Western blotting with anti-p53 antibody.

**Figure 6. Analysis of p53 mutants.** *A*, schematic representation of mutation sites in p53. Respective domains are described according to a previous report (52). Open circles, positions of lysine residues. Closed circles, positions of KR mutations. *B–H*, reconstitution of ubiquitination reactions with the indicated mutant complexes and WT Ub (top panels) or UbK48R (bottom panels). Reactions were performed under standard conditions with fraction 12 of gel filtration chromatography (Fig. S3) as shown in Fig. 2C and Fig. S2A. The reaction products were visualized by Western blotting with anti-p53 antibody.

(D, 14+2KR) These results suggested that E6AP-E6 is inefficient at generating long Ub chains, and the high-molecular-weight products generated with WT p53 were a consequence of multiple polyubiquitination with relatively short Ub chains.

**Figure 5. Ubiquitination sites in hsp53 determined by shotgun MS analysis.** *A* and *B*, reaction products with WT Ub (*A*) or with UbK48R (*B*) were subjected to MS analysis. Peptide fragments with G-G modifications detected by MS analysis are shown as black bars with numbers in parentheses. The numbers indicate the number of fragments detected. The modification sites in the fragments are indicated by G. Peptide fragments not covered by MS analysis are enclosed by open boxes. Lysine residues marked by open circles indicate that ubiquitination was detected, those marked by closed circles indicate that ubiquitination was not detected, and those marked by open squares indicate that the ubiquitination status is unknown.

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Reactions with 50 nM UBCH6c
3 min products 6 min products 7 min products

α-p53
p33
p33-Ub

α-Ub

Reactions with 12.5 nM UBCH6c
10 min products 20 min products 30 min products

α-p53
p33
p33-Ub

α-Ub

Relative total amounts by chain length (mer)

3 min products 5 min products 7 min products

Relative total amounts by chain length (mer)

10 min products 20 min products 30 min products

Ub**
Ub6
Ub7
Ub8
Ub3
Ub2
Ub1

Ub8
Ub7
Ub6
Ub5
Ub4
Ub3
Ub2
Ub1

E

F

G

Ubiquitination (7 min)
Ub + + + + + + +
F-Ub - - - - - - -

After addition of F-Ub (min)
0 0.5 1 2 3 5 6 7 8 10

α-p53
p33
p33-Ub

α-Ub

α-p53
p33
p33-Ub

α-Ub

Incubation time with USP7 (min)
0 0 20 40 80 120 0 20 40 80 120

α-p53

α-Ub

Ub2
Ub3
Ub4
Ub5
Ub6
Ub7
Ub8

Ub2
Ub3
Ub4
Ub5
Ub6
Ub7
Ub8
size distribution of the chains could be addressed at earlier time points. To compare the patterns of the chain distribution, the products after 20-min digestion were quantified (Fig. 7C). The result showed that the size of chains increased gradually in a time-dependent manner (Fig. 7C), with most chains distributed between dimers and tetramers at 3 min and dimers and pentamers at 5 and 7 min (Fig. 7C). We also examined chain lengths in the presence of a lower concentration of UBC75c (Fig. 7B), which resulted in slow chain elongation (Fig. 4C). The reaction products at 10, 20, and 30 min (Fig. 4C) were subjected to digestion by hispUSP7 (Fig. 7B), because the size of ubiquitinated p53 was equivalent to that at 3, 5, and 7 min when a high UBC75c concentration was employed (Fig. 4, compare A and C). The data obtained after 20 min of digestion were quantified and are shown in Fig. 7D. The results were slightly different from those obtained with a high E2 concentration (Fig. 7C). The Ub chains showed a peak of trimers at all time points (Fig. 7D). The results obtained under both conditions indicated the presence of multipolyubiquitination with relatively short dimer to hexamer chains at the early stages of the reactions. The continuous increase in the total size of ubiquitinated hisp53 during early time points (Fig. 4, A and B) suggested that this was due to an increase in the number of chains. As a control, we performed the reaction with UbK48R. Consistent with the results of MS analysis mentioned above (Table 2), Ub ladders were not generated by USP7 treatment when UbK48R was used in the reactions (Fig. S4A), suggesting that p53 mainly underwent multipolyubiquitination with UbK48R.

Next, we examined whether the chains were elongated in a stepwise fashion. First, hisp53 was partially ubiquitinated using a low Ub concentration (0.3 μM) for 5 min until the Ub molecules were exhausted (Fig. 7E, lanes 1–6) and Fig. S4 (B and C). Then the reaction was chased by the addition of a large excess of N-terminally FLAG-tagged Ub (FLAGUb) (Fig. 7E, lanes 7–11). We assumed that Ub-FLAGUb hybrid chains would be generated if p53 is polyubiquitinated via stepwise reactions, whereas hybrid chains would not be generated if p53 is polyubiquitinated by en bloc reactions only. Whether the Ub-FLAGUb hybrid chains were generated or not could be determined by making comparisons of the molecular sizes of the released chains. To obtain a standard for Ub-FLAGUb hybrid chains, polyubiquitination reactions were performed with Ub-FLAGUb, or a mixture of both (Fig. 7F, lanes 1–3), and the chains generated were released by hispUSP7 (Fig. 7F, lanes 4–6). As shown in Fig. 7F, hybrid chains with corresponding numbers of ubiquitin molecules (lane 5) migrated between corresponding Ub-only chains (lane 4) and FLAGUb-only chains (lane 6) and were clearly separated from each other. Next, polyubiquitinated p53 obtained before and after the addition of FLAGUb (Fig. 7E) was subjected to hispUSP7 digestion, and the sizes of the released chains were compared with those of the standard (Fig. 7G). The result revealed multiple bands corresponding to hybrid chains and FLAGUb-Ub-only chains. Those were distributed from dimer to pentamer and larger chains. This result clearly indicated that respective chains of different lengths were elongated by stepwise reactions.

Behavior of p53 mutants in human cells

It is important to determine the characteristics of p53 KR mutants in vivo to understand how the fate of p53 and its downstream targets are affected by KR mutations. We therefore examined E6-dependent degradation, subcellular distribution, and transactivation activity of the mutants. N-terminally FLAG-tagged p53 (FLAGp53) KR mutants were transiently expressed in p53-null Saos-2 cells with or without E6 expression. Then protein levels of the p53 mutants after treatment with or without MG132 were analyzed by Western blotting. Expression of FLAGp53 and endogenous p21 and their subcellular distributions were determined by immunofluorescence staining and subsequent automated fluorescence microscope analysis. Endogenous p21 expression was also analyzed by Western blotting. The results are summarized in Table 3.

As reported previously (36), E6-induced proteasome-dependent degradation of WT FLAGp53 clearly occurred in vivo (Fig. 8A). We observed that the FLAGp534KR mutant was susceptible to degradation. By contrast, the nonubiquitinated mutant, FLAGp534+4KR, was resistant to degradation when it was co-expressed with E6. Interestingly, the other mutants containing one or two efficient ubiquitination sites in their C-terminal regions did not show degradation (Fig. 8A). Immunofluorescence staining with two independent anti-p53 antibodies (Fig. S5) confirmed these results.

The subcellular distributions of the mutants are shown in Fig. S6. Two mutants, FLAGp534+4KR and FLAGp5312+4KR, were predominantly localized to the nucleus, but the other mutants were equally distributed between the nucleus and cytoplasm. The subcellular distributions of the mutants were not affected by E6 co-expression or by MG132 treatment (Fig. S6). This result suggested that the resistance of the FLGp534+4KR mutant to E6 degradation was due to its nonubiquitinated state.

Figure 7. Analysis of the elongation of Ub chains. A and B, determination of chain length distribution. Reaction products obtained under standard conditions for 3, 5, and 7 min (A) or with 4-fold lower E2 concentrations for 10, 20, and 30 min (B) were digested with hispUSP7 for the indicated times. C and D, the signal intensities of the respective chains at 20 min of digestion for A and B were divided by the number of Ub molecules in the chains, and the calculated values (relative molar amounts of the respective chains) were plotted in C and D, respectively. E, two-step chain elongation reaction. The time course performed with a low amount of Ub (7.5 pmol) was followed by the addition of a large excess of hispUb (174 pmol). F, generation of Ub-FLAGUb hybrid chains. Reaction products obtained under the standard ubiquitination condition for 7 min with Ub-FLAGUb, or a mixture of both (lanes 1–3) were digested with USP7 for 80 min (lanes 4–6). G, evidence of stepwise chain elongation. Reaction products before (5-min incubation with Ub, lane 6 in E) and after the addition of FLAGUb (additional 3-min incubation, lane 10 in E) were digested with hispUSP7 for the indicated times. The product of the reaction performed with Ub and FLAGUb (lane 5 in F) was loaded on the side as a standard (S, lane 11). F-Ub, FLAGUb. Reaction products were visualized by Western blotting with the indicated antibodies.

| p53          | E6-dependent degradationa | Subcellular distributionb | Transactivationc |
|--------------|--------------------------|---------------------------|-----------------|
| WT           | +                        | Nucleus                   | +               |
| 14KR         | +                        | Nucleus                   | +               |
| 14+4KR       | –                        | Nucleus/cytoplasm         | –               |
| 13(1)+4KR    | –                        | Nucleus/cytoplasm         | –               |
| 13(2)+4KR    | –                        | Nucleus/cytoplasm         | –               |
| 12+4KR       | –                        | Nucleus                   | –               |

Table 3 Characteristics of the p53 mutants in vivo

a Data are shown in Fig. 8A and Fig. S5.

b Data are shown in Fig. S6.

c Data are shown in Fig. (B–D).
mutant to degradation was not caused by a difference in its subcellular distribution.

The transactivation activities of the mutants were determined by examining their ability to induce endogenous p21 expression. p21 expression was clearly detected in cells transfected with the WT or FLAGp5314KR expression plasmid, and the p21 signals exclusively co-localized with cells expressing FLAGp53 (Fig. 8, A and C). Importantly, co-transfection with the E6 protein itself (Fig. 8A). By contrast, none of the other mutants showed transactivation activity (Fig. 8, B−D), probably because of defects in their DNA-binding activities caused by additional mutations in the DNA-binding domain of p53 (Fig. 6A). Because the transactivation-defective mutants were all resistant to degradation, it is impossible to exclude the possibility that the defect in their DNA-binding activity was somehow related to their increased stability.

Discussion

Specific properties of the E6AP-E6 complex

In the present study, we used recombinant E6AP-E6 purified from E. coli simultaneously overproducing both proteins. The binding affinity of E6 to E6AP, which is estimated as 1 nM (37), is high enough to allow its isolation as a stable complex, even after multiple steps of chromatography. The co-purification circumvented the difficulty associated with the purification of the E6 protein itself (38−40) and enabled the generation of highly active E6AP-E6.

We estimated that the E6AP-E6 complex analyzed by gel filtration in the presence of 300 mM or 1 mM NaCl was 170−200 kDa. This was smaller than the calculated molecular mass of the tetramer, (E6AP-E6)2, at 244 kDa and larger than that of the heterodimer at 122 kDa. Previously, it was demonstrated that the ligase activity of E6AP is mediated by dynamic intermolecular interactions (37), which are observed even in the presence of E6, because individual E6AP-E6 complexes in a multimer are exchangeable (23, 24).

The K_d value of the oligomerization of E6AP has been estimated in the order of magnitude of 10 μM (37). Because of the weak interaction, the multimers are sensitive to protein concentration, pH, and ionic strength (37). Similarly, the K_d value of the dimerization of E6, 290 μM (41), is too high to form a stable complex under the gel filtration conditions used. Taken together with our observation, these reports suggest that the E6AP-E6 complex may have formed a heterodimer under the gel filtration conditions used in the present study and eluted at a higher apparent molecular mass probably because it has a nonglobular shape.
We showed that UBCH5c and UBCH7 similarly supported polyubiquitination of p53 (Fig. 3, A and B). A mutation at the interface of the interaction with E6AP severely decreased E6AP–Ub formation (28–31). In addition, the N-terminal extension of UBCH5c interfered with E6AP–Ub formation, suggesting that the structure of the N terminus is crucial for interaction with E6AP. By contrast, a mutant (UBCH5cSR) that interferes with the E2–Ub interaction (26, 27) did not exhibit any defect. These results indicated that the ability of each E2 to mediate p53-ubiquitination was associated with their capacity for trans-thiolation of E6AP, suggesting that the sole function of E2 is the trans-thiolation of E6AP. Previously, UBCH5c, UBCH7, and UBCH8 were reported as cognate E2s; however, the relative efficiencies of these E2s differ between reports (28, 42–45). We suggest that these differences could be due to differences in their N-terminal sequences resulting from the addition of tags for purification.

In the reconstitution experiments, polyubiquitination of p53 and E6AP, generation of unanchored Ub chains, and monoubiquitination of UBCH5c were observed; however, these reactions did not occur simultaneously. Analysis of the time course of the reactions revealed that polyubiquitination of p53 mainly occurred during early time points, followed by polyubiquitination of E6AP after the apparent saturation of p53 polyubiquitination. After that, unanchored Ub chains and monoubiquitinated UBCH5c were generated. The observed saturation of polyubiquitination of p53 and E6AP implies that the lengths of poly-Ub chains attached to p53 and E6AP were somehow recognized and restricted (discussed below). The results of the time course experiments indicated that p53 is a better target for ubiquitination than E6AP, suggesting that the thiol-linked Ub at the catalytic cysteine of E6AP is preferentially transferred to p53. This molecular mechanism could explain the inhibition of self-ubiquitination of E6AP by p53 observed previously (23). We also suspect that the generation of unanchored Ub chains and monoubiquitination of UBCH5c are secondary reactions, occurring after the saturation of E6AP self-ubiquitination.

Previously, E6AP was reported to have the potential to form thiol-linked Ub dimers on its catalytic cysteine (12). The authors showed that the reaction was severely limited under low E2 concentrations, such as 50 nm, the concentration employed in the present study (12). Consistently, we were unable to detect the thiol-linked Ub dimers on E6AP and rarely detected free Ub dimers under reducing conditions. Therefore, we suggest that en bloc transfer reactions of preformed Ub chains (12) were a minor reaction at relatively low E2 concentrations.

**A mechanism to ensure processive polyubiquitination on each p53 molecule**

Processive ubiquitination is important for efficient polyubiquitination of target proteins (6). In the present study, we demonstrated that hE6AP-E6 forms a stable ternary complex with p53 (Fig. 2). The measured stoichiometry of the components in the ternary complex was close to 1:1:1, which is consistent with the reported structure of the E6/E6AP/p53 complex (46). If the hE6AP-E6 dimer binds to each p53 molecule in a p53 tetramer, the molecular weight would be 600,000, which is close to the apparent molecular mass of 600–700 kDa of the complex eluted from the gel filtration column. In the ternary complex, hE6AP-E6 could ubiquitinate all p53 molecules in the tetramer (Fig. S2). Time course experiments with different E6AP-E6 concentrations (Fig. 4, A and B) showed that polyubiquitination was processive on each p53 molecule, strongly suggesting that stable binding of E6AP-E6 to p53 ensures processive p53-ubiquitination on each p53 molecule.

**A mechanism for Ub transfer to the distal ends of preformed Ub chains**

In the present study, we demonstrated that E6AP-E6 builds multiple poly-Ub chains on p53 through stepwise elongation, as suggested previously (11). We estimated that 7–12 lysine residues in p53 are efficient targets for ubiquitination, and the chain length tended to be restricted to relatively short chains composed of dimers to hexamers. We did not detect any preference for extending existing chains over priming new ones, suggesting that chain elongation was distributive. Recently, French et al. (21) reported a mechanism of Ub chain synthesis by another HECT-type Ub ligase, WWP1, which generates Lys63-linked short Ub chains of up to 6-mers via sequential addition; however, the enzyme was inefficient in further chain elongation and instead expanded the chains by adding branched linkages. This is similar to the activity of E6AP in the present study, suggesting that these two ligases share a common catalytic mechanism of ubiquitination. The C-lobe of the HECT domain is connected by a flexible linker to the N-lobe where E2–Ub binds and has the potential to travel a distance of 40 Å (17, 29). The flexible C-lobe may be able to move around a three-dimensional volume within its reach of at least a radius of 40 Å and potentially transfer Ub to any lysine residues in that area. Therefore, we suggest that the permissive distance for the movement of the C-lobe essentially restricts the length of the chains. The saturation of polyubiquitination revealed by the time course experiments (Fig. 4A) may reflect a situation in which all of the available lysine residues within the three-dimensional volume are ubiquitinated.

The properties of E6AP-E6, which transfers Ub onto multiple lysine residues of p53, indicate an intrinsic lower specificity for site selection. By contrast, the transfer of Ub to the Ub moiety attached to p53 was strictly specific to the Lys48 residue. These observations raise the question of how the Lys48 residue is specified and/or other lysine residues in Ub are rejected during chain elongation reactions. Lys48 linkage formation could be determined by the specific interaction between the C-lobe of E6AP and the acceptor Ub (11, 19). Further study is needed to elucidate the molecular mechanism underlying linkage determination.

**Implications of polyubiquitination at multiple sites for p53 degradation**

We found that one of the p53 KR mutants, p5314KKR, was susceptible to E6-dependent degradation (Fig. 8A), suggesting that polyubiquitination of p5314KKR at not more than four lysine residues (Lys101, Lys120, Lys132, and Lys139) provides an adequate signal for efficient proteasomal degradation. By contrast, polyubiquitination of the p5312+4KKR mutant at two lysine resi-
dues (Lys$^{305}$ and Lys$^{319}$) did not induce degradation (Fig. 8A), suggesting that polyubiquitination at those lysine residues is not sufficient for proteasomal degradation. These results are consistent with a model derived from single-molecule kinetic analysis of model substrates by the proteasome in vitro, in which multiple ubiquitination with short ubiquitin chains (diubiquitin is sufficient) provided a configuration of chains that efficiently directed the substrate into the translocation channel of the proteasome (47). Although we do not have direct evidence for multipolyubiquitination of p53 in vivo, the results suggest that the combination of polyubiquitin chains at the four sites of p53$^{4KR}$, but not at the two sites of p53$^{12 + 4KR}$, provided the configuration for degradation by the proteasome.

So far, we have no evidence to suggest a defined order of multipolyubiquitination. Rather, the results suggest that selection of different lysine residues during priming and each step of elongation is random, at least in vitro. Therefore, we suggest that random ubiquitination by HECT-type Ub ligases occurs at multiple sites with short Ub chains and that the resulting configuration of Ub chains is particularly suited for the initiation of degradation of p53 (47). Further analysis with more p53 KR mutants with different combinations of ubiquitination sites may provide a more complete picture of the optimal combination of ubiquitination sites required for proteasomal degradation.

**Experimental procedures**

**Proteins**

The Ub mutant, Ub$^{K48R}$, and methylated Ub were purchased from Boston Biochem (UM-K48R and U-501, respectively). The expression plasmids for intact E6AP (isoform II), UBCH5c, and UBCH7 were constructed in pET-20b(+). The genes encoding E6 of human papillomavirus type 16 (48) and p53 were respectively cloned into pACYCDuet-1 (Novagen) and (DE3) harboring pET20-FLAG-Ub, a pET20-Ub derivative (49). For N-terminally histidine-tagged proteins, the genes encoding p53 and UBCH5c were cloned into pET-15b, and E6AP was cloned into pET-28a(+).

Recombinant human proteins were produced in *E. coli* BL21 (DE3) at 15 °C by the addition of isopropyl β-D-thiogalactopyranoside at 0.2 mM, and purified by chromatography at 4 °C using columns from GE Healthcare. Purification of the respective mutants was performed as described for the WT proteins unless otherwise indicated. E1, Ub, and hisE6AP were purified as described previously (35, 50). FLAG-Ub was expressed in BL21 (DE3) harboring pET20-FLAG-Ub, a pET20-Ub derivative (50), and purified, after ammonium sulfate fractionation of cell lysates, by HiTrap Phenyl FF (high sub), HiTrap Heparin HP, and HiTrap Q FF chromatography. E6AP was expressed in BL21 (DE3) harboring pET20-E6AP and purified using the columns HiTrap SP HP, HiTrap Heparin HP, HiTrap Q HP, and Superdex 200. The E6AP-E6 complex was co-expressed in BL21 (DE3) harboring pET20-E6AP and pACYC-E6 and purified as described for E6AP. The hisE6AP-E6 complex was co-expressed in BL21 (DE3) harboring pET28-hisE6AP and pACYC-E6 and purified using the columns Ni$^{2+}$-charged HiTrap Chelating HP and Superdex 200. UBCH5c was expressed in BL21 (DE3) harboring pET20-UBCH5c and purified using the columns HiTrap DEAE FF, HiTrap SP FF, HiTrap Heparin HP, and Superdex 200. UBCH7 was expressed in BL21 (DE3) harboring pET20-UBCH7 and purified as described for UBCH5c. hisUBCH5c was expressed in BL21 (DE3) harboring pET15-hisUBCH5c and purified using the columns Ni$^{2+}$-charged HiTrap Chelating HP, HiTrap SP XL, and Superdex 200. hisp53 was expressed in BL21 (DE3) harboring pET15-hisp53 and purified using Ni$^{2+}$-charged HiTrap Chelating HP. A ternary complex of hisE6AP, E6, and p53 was expressed in BL21 (DE3) harboring pET28-hisE6AP, pACYC-E6, and pGMB-p53 and purified using the columns Ni$^{2+}$-charged HiTrap Chelating HP and Superdex 200. The intensities of the respective protein bands were measured by ImageJ software from a gel image obtained by scanning the wet SDS-polyacrylamide gel stained by Coomassie Brilliant Blue. Protein concentrations were determined using the Bio-Rad protein assay with BSA (Bio-Rad) as the standard.

**Ubiquitination assays**

The standard reaction mixture (25 μl) contained 20 mM HEPES-NaOH (pH 7.5), 50 mM NaCl, 0.02 mg/ml BSA, 1 mM DTT, 5 mM MgCl$_2$, 1 mM ATP, hisp53 (1 pmol as a tetramer), E1 (0.85 pmol), UBCH5c (1.25 pmol), E3 (0.8 pmol), and Ub (174 pmol). Reaction mixtures were prepared on ice and then incubated at 30 °C for 10 min unless otherwise indicated. The reactions were terminated by the addition of SDS-sample buffer.

**Western blotting**

Products were analyzed by Western blotting. Anti-E6AP (GeneTex, UBE3A antibody, GTX10487 or in-house-generated anti-E6AP serum raised in rabbits against the peptide KKG-PRVDPLETLEGVTLDLC), anti-p53 (Calbiochem, anti-p53 (Ab-6) mouse mAb (DO-1), OP43), anti-UBCH5c (Ab Frontier, anti-UBE2D3 (4C1-1E3), LF-MA10362), anti-Ub (Santa Cruz Biotechnology, Inc., Ub (P4D1), sc-8017), and anti-FLAG (Sigma-Aldrich, monoclonal anti-FLAG M2, F 1804) were used. Signals were detected with a Chemi-Lumi One L kit (Nacalai Tesque, 07880-70) using ImageQuantTM LAS 4000 Mini Biomolecular Imager (GE Healthcare) and analyzed using Image) 1.48v software (National Institutes of Health). When “reducing” or unless otherwise indicated, the samples were treated with SDS-sample buffer containing 280 mM β-mercaptoethanol or 100 mM DTT. When the samples were not treated with any reducing agent, they were described as “nonreducing.”

**E2-charging assays**

The reaction mixture (25 μl) containing 20 mM HEPES-NaOH (pH 7.5), 50 mM NaCl, 0.02 mg/ml BSA, 1 mM DTT, 5 mM MgCl$_2$, 1 mM ATP, E2 (10 pmol), and Ub (174 pmol) was preincubated at 30 °C for 1 min. Reactions were started by the addition of E1 (0.85 pmol) and terminated by the addition of SDS-sample buffer with or without reducing agents after a 30-s incubation at 30 °C.

**E3-charging assays**

The reaction mixture (25 μl) containing 20 mM HEPES-NaOH (pH 7.5), 50 mM NaCl, 0.02 mg/ml BSA, 1 mM DTT, 5
mm MgCl₂, 1 mm ATP, E1 (0.85 pmol), E2 (1.25 pmol), and Ub (174 pmol) was preincubated at 30 °C for 1 min. Reactions were started by the addition of E3 (0.8 pmol) and terminated by the addition of SDS-sample buffer with or without reducing agents after a 30-s incubation at 30 °C.

**Analysis of polyubiquitin chains by digestion with USP7**

For digestion of the reaction products with **his**USP7, DTT was introduced at 5 mm after the ubiquitination reactions were terminated by the addition of EDTA at 20 mm. Digestion was performed with 500 ng of **his**USP7 per 10 μl of reaction mixture at 30 °C for the indicated times.

**Ub-AQUA/PRM**

MS/MS-based quantification of Ub peptides by PRM was performed as described previously (34). To minimize nonspecific adsorption of peptides, Protein LoBind tubes (Eppendorf) and ProteoSave MS vials (AMR Inc.) were used. Ubiquitination reactions were performed under standard conditions except for the absence of BSA for 10 min with the WT or for 30 min with UbK48R. The reaction products were separated by 4–12% NuPAGE BisTris gels (Life Technologies) with a short run (3 cm). The gels were stained with Bio-Safe Coomassie Stain (Bio-Rad). After the gels were extensively washed with Milli-Q water (Millipore), the gel region above 150 kDa was excised, cut into 1-mm³ pieces, destained for 1 h with 1 ml of 50 mM ammonium bicarbonate (AMBC), 30% acetonitrile (ACN), with agitation, and then further washed for 1 h with 1 ml of 50 mM AMBC, 50% ACN. Finally, a 100% ACN wash was performed to ensure complete gel dehydration. Trypsin solution (Promega, 20 ng/μl in 50 mM AMBC, 5% ACN) was subsequently added to the gel pieces at approximately equivalent volumes followed by incubation on ice for 30 min. After the addition of another small volume of trypsin solution, the gel samples were incubated at 37 °C overnight.Digests were extracted by the addition of 100 μl of 50% ACN, 0.1% TFA for 1 h with shaking. The peptides were recovered into fresh Eppendorf tubes, and an additional extraction step was performed with 70% ACN, 0.1% TFA for 30 min. The extracted peptides were concentrated using a SpeedVac, added to the AQUA peptides (Table S1), and oxidized with 0.05% H₂O₂, 0.1% TFA for 12 h at 4 °C. The peptides were analyzed in targeted MS/MS mode on a Q Exactive mass spectrometer coupled with an EASY-nLC 1000 liquid chromatograph and nanoelectrospray ion source (Thermo Scientific). The mobile phases were 0.1% formic acid (FA) in water (solvent A) and 0.1% FA in 100% ACN (solvent B). Peptides were directly loaded onto a C18 analytical column (ReproSil-Pur 3 μm, 75-μm inner diameter and 12-cm length, Nikkyo Technos) and separated using a 90-min three-step gradient (0–10% solvent B for 5 min, 10–30% for 70 min and 30–80% for 5 min) at a constant flow rate of 300 nl/min. Ionization was performed with a liquid junction voltage of 1.8 kV and capillary temperature of 250 °C. The Q Exactive was operated by the Xcalibur software in the target MS/MS mode, with an Orbitrap resolution of 70,000 at m/z 200, target automatic gain control values of 1 × 10⁶, maximum ion fill times of 200 ms, an isolation window of 2.0 m/z, and fragmentation by HCD with normalized collision energies of 28. Raw data were processed using PinPoint software version 1.3 (Thermo Scientific).

**Shotgun MS analysis**

The tryptic digests described above were also subjected to shotgun MS analysis. Separation and elution from the column were achieved using a 40-min two-step gradient (0–35% solvent B for 35 min and 35–100% for 5 min) at a constant flow rate of 300 nl/min. The Q Exactive was operated in the data-dependent MS/MS mode, using Xcalibur software, with survey scans acquired at a resolution of 70,000 at m/z 200. The top 10 most abundant isotope patterns with charge 2–5 were selected from the survey scans with an isolation window of 2.0 m/z and fragmented by HCD with normalized collision energies of 28. The maximum ion injection times were 60 ms for both survey and MS/MS scans, and the automatic gain control values were set to 3 × 10⁶ and 5 × 10⁵ for the survey and MS/MS scans, respectively. Ions selected for MS/MS were dynamically excluded for 10 s. Proteome Discoverer software (version 1.3, Thermo Scientific) was used to generate peak lists. The MS/MS spectra were searched against a Swiss-Prot database (version 2012_10 of the UniProtKB/Swiss-Prot protein database) supplemented with the amino acid sequences of recombinant proteins using the SEQUEST search engine. The precursor and fragment mass tolerances were set to 10 ppm and 20 milli mass units, respectively. Methionine oxidation, deamidation, and diglycine modification of lysine and cysteine side chains were set as variable modifications for database searching. Peptide identification was filtered at a 1% false discovery rate, and high-confidence peptides are shown in Fig. 5.

**Cell culture, transfection, and Western blotting**

Human osteoblast-like Saos-2 cells were maintained in minimum essential medium Eagle, α modification (Sigma-Aldrich) supplemented with 10% fetal bovine serum and l-glutamine. Plasmid DNAs (pcDNA-FLAG-tagged-p53-WT (51) or -mutant plasmid DNAs with either pcAGGS-empty or -E6 plasmid DNAs) were introduced into Saos-2 cells using LipofectAMINE3000 (Thermo Fisher Scientific) following the manufacturer’s instructions. Briefly, cells were plated in 24-well plates 18 h before transfection and then incubated with the plasmid DNAs/LipofectAMINE3000 mixture (0.1 μg of pcDNA-FLAG-p53s and 0.5 μg of pcAGGS-E6 or empty vector per well) for 6 h, after which the medium was replaced with fresh medium, and the cells were incubated for another 24 h. The proteasome inhibitor MG132 (final concentration 7.5 μM) (Thermo Fisher Scientific) was added to the wells 6 h before harvesting the cells. Western blotting was performed using EzLabel FluoroNeo (ATTO), EzRun (ATTO), and EzFastBlot (ATTO) following the manufacturer’s instructions. Briefly, whole-cell lysates were prepared with the fluorescent labeling reagent and electrophoresed in a 5–20% gradient gel (e-PAGE, ATTO). Following transfer, membranes were subjected to Western blotting using an anti-p53 antibody (DO-1, 1:1000, Thermo Fisher Scientific) or an anti-p21 antibody (6B6, 1:1000, BD Pharmingen), followed by a goat anti-mouse IgG (H + L) secondary antibody, horseradish peroxidase (1:1000, Thermo Fisher Scientific). The chemiluminescent signals are shown in Fig. 5.
were detected by a Luminata Forte (Thermo Fisher Scientific) and ImageQuant LAS 4000 mini (GE Healthcare).

**Immunostaining and automated fluorescence microscope analysis**

Plasmid DNAs were introduced into Saos-2 cells using LipofectAMINE3000 as described above. Briefly, cells were plated in 384-well plates 18 h before transfection and then incubated with plasmid DNAs/LipofectAMINE3000 mixture (0.02 µg of pcDNA-FLAG-p53s and 0.1 µg of pcAGGS-E6 or empty vector per well) for 6 h, after which the medium was replaced with fresh medium, and the cells were incubated for another 24 h. The proteasome inhibitor MG132 (final concentration 7.5 µM) (Thermo Fisher Scientific) was added to the wells 6 h before 4% paraformaldehyde (Nacalai Tesque) fixation. After Triton X-100 (0.5%) treatment, double immunofluorescence staining was performed using an anti-p53 antibody (FL-393, 1:500, Santa Cruz Biotechnology), an anti-p21 antibody (6B6, 1:1000, BD Pharmingen), followed by a rabbit IgG(H + L) secondary antibody (Alexa Fluor 555, 1:1000, Thermo Fisher Scientific) or a mouse IgG(H+L) cross-adsorbed secondary antibody (Alexa Fluor 488, 1:1000, Thermo Fisher Scientific). After Hoechst 33342 (10 µg/ml, Thermo Fisher Scientific) staining, confocal fluorescent Image stacks (five planes, 2.5-µm intervals) were acquired using Opera Phenix (PerkinElmer Life Sciences) with a ×20 water immersion objective lens. All stacked images were analyzed using the Harmony High Content Imaging and Analysis Software (PerkinElmer Life Sciences), and the area of each nucleus/cytoplasm/cell from the signal of Hoechst 33342 staining and the intensity of each fluorescence per area was calculated. The histo-box plots (Fig. S5) and scatter plots (Fig. S6) were generated using SpotFire (TIBCO).

**Author contributions**—Y. M. and C. M. conceived the project. Y. M. designed and performed biochemical analyses. Y. S., N. A., and K. T. performed MS analyses. H. K. designed and performed biochemical analyses. Y. M., and C. M. wrote the paper.

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