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# EZH2 Generates a Methyl Degron that Is Recognized by the DCAF1/DDB1/CUL4 E3 Ubiquitin Ligase Complex

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## SUMMARY

**Ubiquitination plays a major role in protein degradation. Although phosphorylation-dependent ubiquitination is well known for the regulation of protein stability, methylation-dependent ubiquitination machinery has not been characterized. Here, we provide evidence that methylation-dependent ubiquitination is carried out by damage-specific DNA binding protein 1 (DDB1)/cullin4 (CUL4) E3 ubiquitin ligase complex and a DDB1-CUL4-associated factor 1 (DCAF1) adaptor, which recognizes monomethylated substrates. Molecular modeling and binding affinity studies reveal that the putative chromo domain of DCAF1 directly recognizes monomethylated substrates, whereas critical binding pocket mutations of the DCAF1 chromo domain ablated the binding from the monomethylated substrates. Further, we discovered that enhancer of zeste homolog 2 (EZH2) methyltransferase has distinct substrate specificities for histone H3K27 and nonhistones exemplified by an orphan nuclear receptor, RORα. We propose that EZH2-DCAF1/DDB1/CUL4 represents a previously unrecognized methylation-dependent ubiquitination machinery specifically recognizing "methyl degron"; through this, nonhistone protein stability can be dynamically regulated in a methylation-dependent manner.**

## INTRODUCTION

Ubiquitination is one of the posttranslational modifications which regulate not only signaling processes leading to degradation of short-lived regulatory proteins, but also act as recognition signal which can activate, or inactivate proteins within a signaling cascade (Pickart, 2004). The covalent attachment of ubiquitin occurs on lysine residues where E1 ubiquitin activating enzymes, the E2 ubiquitin-conjugating enzymes and the E3 ubiquitin ligases work in concert (Ciechanover et al., 1982; Hershko, 1983). Among E3 ligase family members, there are cullin family in vertebrates (CUL1, 2, 3, 4A, 4B, 5 and 7) and each cullin allows interaction with specific adapters by acting as a scaffold. CUL4 family members including CUL4A and CUL4B are distinct from other cullins in that they utilize more than 50 WD40-containing adapters referred to as DCAFs (also known as VprBPs) conferring substrate specificity (Angers et al., 2006; He et al., 2006; Higa et al., 2006; Jin et al., 2006).

Apart from ubiquitination, methylation is another posttranslational modification which also occurs on lysine residue. Among many lysine methyltransferases, EZH2 is a SET domain-containing protein that exhibits histone methyltransferase activity with specificity to histone H3K27 methylation, and forms a polycomb group repressive complex 2 (PRC2) with EED and SUZ12 (Cao et al., 2002; Jones et al., 1998; Kuzmichev et al., 2004). Importantly, EZH2 has been proposed to have an oncogenic activity in that it is often deregulated in a number of cancer types (Cao et al., 2011; Chang et al., 2011), and aggressive breast and prostate cancers exhibit high levels of EZH2 which correlates with poor prognosis (Kleer et al., 2003; Varambally et al., 2002).

Although nuclear receptors have a variety of different functions to regulate numerous processes by switching transcription on and off by recruiting a complex of coregulatory proteins (Atkins et al., 1999; Glass and Rosenfeld, 2000), there is increasing evidence that some orphan nuclear receptors play a critical role in tumor suppression. Orphan nuclear receptors are ligand-activated transcription factors, for which no cognate ligands have been identified (Blumberg and Evans, 1998; Giguère, 1999), and one such orphan nuclear receptor includes retinoic acid-related orphan nuclear receptor α (RORα) which acts as an inhibitor of colon cancer growth by transrepressing canonical Wnt/β-catenin signaling (Lee et al., 2010). DNA damage-induced RORα is involved in the positive regulation of p53 stability leading to increased apoptosis (Kim et al., 2011). RORα has been shown

to reduce the migratory and invasive abilities of androgen-independent prostate cancer cells, such as DU145 cells (Moretti et al., 2002). Together, these findings suggest a tumor suppressive role of RORα.

Here, we provide the evidence that methylation-dependent ubiquitination of RORα is carried out by DCAF1/DDB1/CUL4 E3 ubiquitin ligase complex. We identify DCAF1 as a direct adaptor linking DDB1/CUL4 to RORα by specifically recognizing monomethylated RORα. Our studies reveal that the chromo domain in DCAF1 functions as a monomethyl-specific reader by molecular modeling and binding affinity studies. These findings suggest a previously unrecognized methyl degron pathway where nonhistone protein stability is dynamically regulated by methylation. Further, We present physiological data in conjunction with biochemical data strongly supporting the oncogenic role of EZH2 by facilitating RORα methylation-dependent degradation, thereby inhibiting the tumor suppressive role exerted by RORα.

## RESULTS

### EZH2 Methyltransferase-Mediated Monomethylation of RORα at K38

Although some methyltransferases have been shown to act on transcription factors and histone modifiers as well as histones in the nucleus, the possibility of there being distinct substrate specificity for histones and nonhistone proteins for the same methyltransferase has not been extensively studied. Given not much is known about the nonhistone substrates of EZH2 thus far, we intended to identify and compare their substrate specificity with histone substrates, and explore their underlying mechanisms. We computationally screened for proteins having similar amino acid sequence to the region in histone H3K27 methylated by EZH2, namely the amino acid sequence "R-K-S" (Figure 1A). From this screening, 627 proteins came out as a "hit" and we considered 469 proteins which were annotated and curated from The Reference Sequence (RefSeq) database. The Database for Annotation, Visualization and Integrated Discovery (DAVID) Bioinformatics Resource (http://david.abcc.ncigcrf.gov/) provides a rapid means to reduce large lists of genes into functionally related groups of genes to help unravel the biological content captured by high throughput technologies. Therefore, we utilized DAVID to perform functional annotation and clustering of these proteins. There were several categories of protein function, and we looked closely into 52 proteins which are involved in transcriptional regulation. We selected an orphan nuclear receptor RORα (Giguère et al., 1994; Hamilton et al., 1996; Kim et al., 2011; Lee et al., 2010) out of three nuclear receptors that were present in this group. The R-K-S sequence was located within the N-terminal domain of RORα and thus speculated to serve as an acceptor site for methylation (Figure 1A). Therefore, we hypothesized that the histone-like sequence in RORα might allow RORα K38 methylation by H3K27 histone methyltransferase exemplified by EZH2, and generated a K38A mutant in which a lysine residue was replaced by an alanine to abrogate lysine methylation. Coimmunoprecipitation assay revealed that K38A mutation abrogated RORα methylation, suggesting that K38 is the methyl-acceptor site of RORα (Figure 1B).

The association of EZH2 with RORα at the endogenous level was confirmed by coimmunoprecipitation analysis (Figure 1C). To assess whether RORα is methylated by EZH2 and to determine the methylation status of RORα, peptides bearing amino acids 31-40 of RORα (LNQESARKSE) were in vitro methylated by EZH2 and analyzed by mass spectrometry. The nonmethylated RORα wild-type (WT) peptide had its main peak at 1161.6 Da, while the monomethylated peptides appeared at 1215 Da, with the 38-Da mass of $K^+$ incorporated during the peptide sample preparation after the histone methyltransferase assay with EZH2 (Figure 1D). On the other hand, there was no detectable alteration in the methylation state of RORα K38A by EZH2 (Figure 1D). Together, these data indicate that EZH2 induced the addition of one methyl group to the K38 site.

Since EZH2 has a histone substrate specificity of di- and trimethylation of H3K27, the unexpected finding of monomethylation of RORα, a nonhistone substrate, allowed us to explore whether EZH2 has distinct substrate specificities for histones and nonhistone proteins. Therefore, we generated RORα K38 mono-, di-, or trimethyl-specific antibodies, while ensuring that they had no cross-reactivity with the nonmethylated RORα peptide (Figure 1E). Although EZH2 is capable of generating mainly di- and trimethylated histone H3K27 (Figure 1F), only monomethylation of RORα was detected at K38 site by the ectopic expression of EZH2 (Figure 1G). Immunoprecipitation assay confirmed that RORα K38A mutant failed to be recognized by anti-RORα monomethyl-specific antibody (Figure 1H). Further, EZH2 knockdown by specific siRNA or miR101 caused a marked decrease in the monomethylation of RORα (Figure 1I). Based on these results, we conclude that EZH2-dependent monomethylation occurs on RORα K38.

### EZH2-Mediated RORα Methylation Destabilizes RORα

We then attempted to address the functional consequence of the EZH2-mediated RORα lysine methylation. We compared the protein expression levels of RORα in WT and EZH2-deficient ($Ezh2^{-/-}$) mouse embryonic fibroblasts (MEFs). Interestingly, RORα protein levels were significantly higher in $Ezh2^{-/-}$ MEFs as compared to WT MEFs (Figure 2A). Reconstitution of EZH2 WT or H689A mutant (MT) of EZH2, a catalytically inactive mutant of EZH2 revealed that RORα protein level was reduced only in EZH2 WT-reconstituted $Ezh2^{-/-}$ MEFs (Figure 2B). Under these conditions, however, the mRNA levels of RORα were not affected (Figure 2C), suggesting that EZH2 influences RORα protein stability. Therefore, we further examined an inverse correlation between EZH2 and RORα protein expression. The half-life of endogenous RORα was significantly increased by EZH2 knockdown with the treatment of the protein synthesis inhibitor, cycloheximide (CHX) (Figure 2D). To examine whether the enzymatic activity of EZH2 is directly required for the regulation of RORα protein stability, we monitored the half-life of RORα by overexpressing EZH2 WT or EZH2 MT. The overexpression of EZH2 MT extend the half-life of RORα in cells treated with CHX (Figure 2E), suggesting that the methyltransferase activity of EZH2 is required for the regulation of RORα protein stability.

In order to determine whether the ubiquitin-proteasome pathway is responsible for RORα degradation and to further examine whether the introduction of EZH2 augments RORα ubiquitination

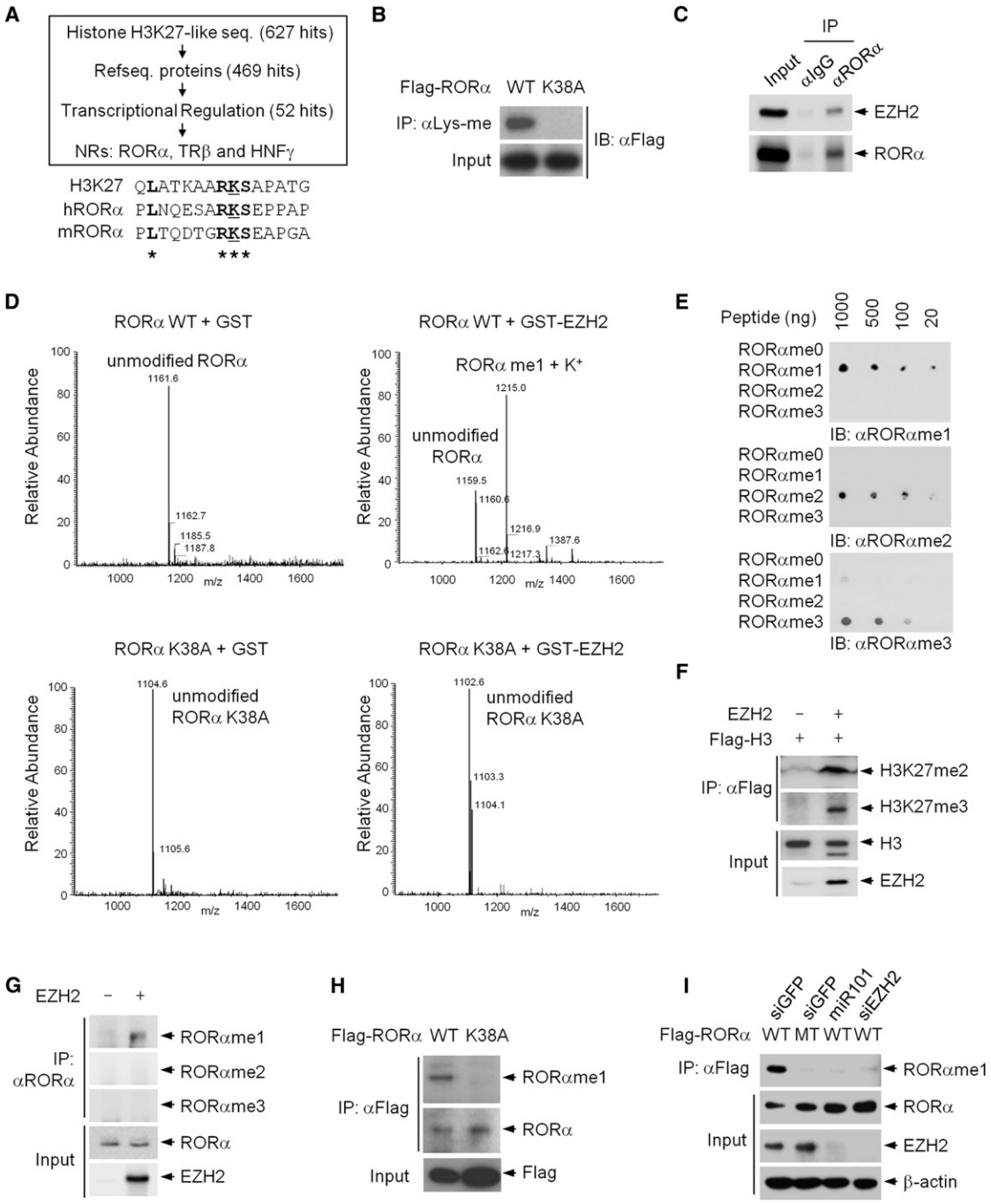


**Figure 1. EZH2-Mediated Monomethylation of RORα at K38**

(A) Screening process of histone H3K27-like sequence-containing proteins. N-terminal domain of RORα containing histone H3K27-like sequence: *Homo sapiens* (h) and *Mus musculus* (m) forms of RORα are aligned.

(B) Coimmunoprecipitation assay was conducted with an anti-lysine methyl antibody, followed by immunoblot with anti-FLAG antibody.

(C) Coimmunoprecipitation assay was performed to detect the interaction between the endogenous RORα and EZH2 in HEK293 cells treated with MG132.

(D) Mass spectrometric analysis of RORα WT and K38A following a methyltransferase assay using either GST or GST-EZH2.

(E) The specificity of antibodies against mono-, di-, or trimethyl RORα K38 was assessed by dot blot analysis.

(F) FLAG-histone H3 was immunoprecipitated with anti-FLAG antibody in the presence or absence of EZH2.

(G) Immunoprecipitation assay was performed with anti-RORα antibody in the absence or presence of EZH2 with MG132 treatment.

(H) FLAG-RORα WT or K38A mutant was immunoprecipitated with anti-FLAG antibody, followed by immunoblot with anti-RORαme1 antibody.

(I) Indicated siRNAs were transfected with FLAG-RORα WT and K38A mutant. After immunoprecipitation with anti-FLAG antibody, monomethylation level of RORα was detected by anti-RORαme1 antibody.

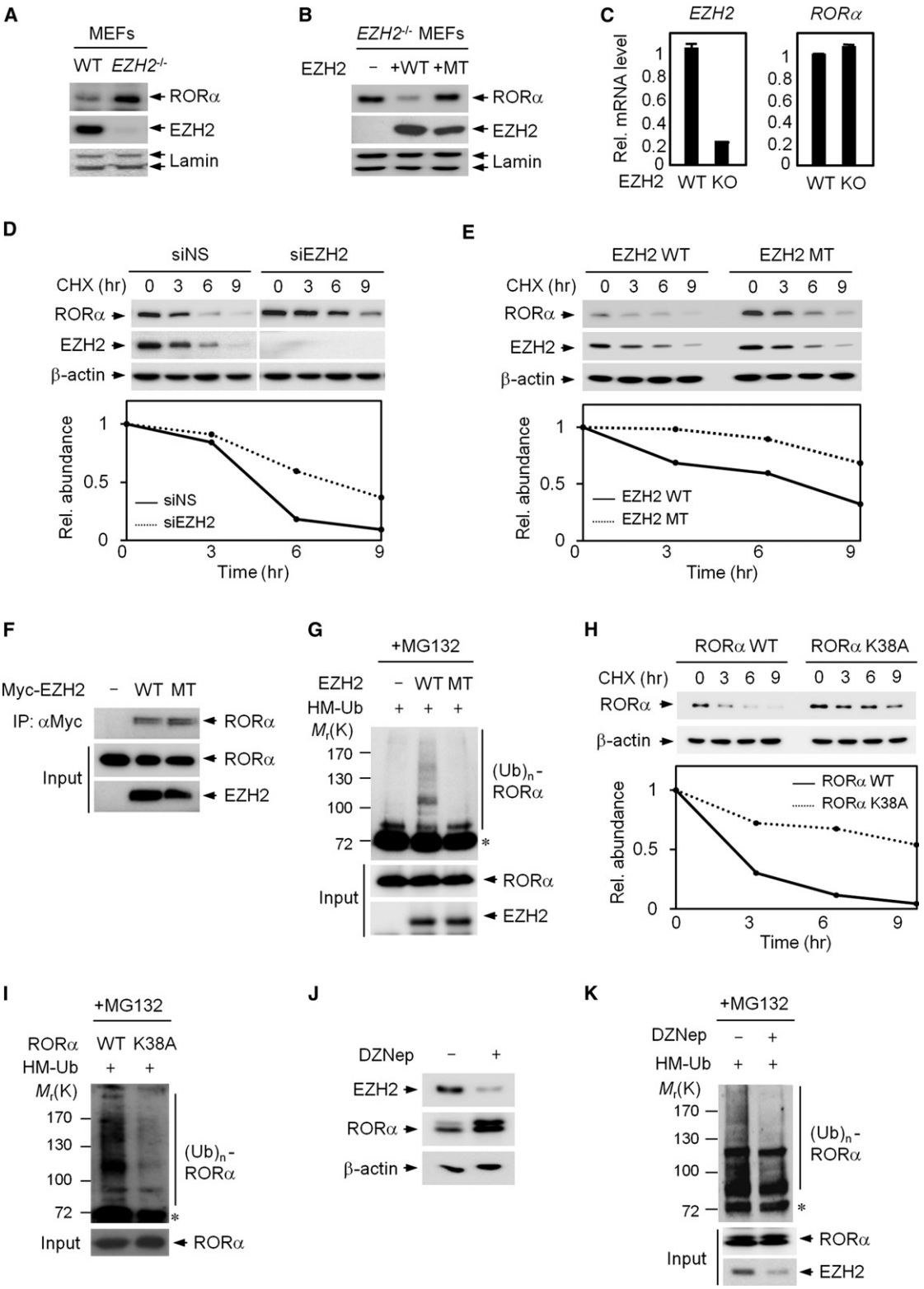


**Figure 2. EZH2-Dependent RORα Methylation Destabilizes RORα**

(A) EZH2 and RORα protein levels in WT and *Ezh2*-deficient (KO) MEFs were compared.

(B) *Ezh2* KO MEFs were reconstituted with either WT or a catalytically inactive mutant (MT) of EZH2, and changes in RORα protein levels were detected.

(C) mRNA levels of *EZH2* and *RORα* were measured in WT and *Ezh2* KO MEFs. Values are expressed as mean ± s.d. for three independent experiments.

by allowing increased RORα methylation, we performed RORα ubiquitination assay with EZH2 in the presence of a 26S proteasome inhibitor, MG132. Although both EZH2 WT and EZH2 MT associated with RORα (Figure 2F), only EZH2 WT was able to increase RORα ubiquitination (Figure 2G), indicating that the ability of EZH2 to enhance RORα ubiquitination requires the methyltransferase activity of EZH2. Moreover, RORα K38A mutation also markedly stabilized its protein levels (Figure 2H), suggesting that RORα degradation relies on K38 methylation. To further examine whether K38 methylation directly affects RORα ubiquitination, we examined ubiquitination of RORα WT and RORα K38A in the presence of MG132. Indeed, RORα K38A mutation led to a marked reduction in RORα ubiquitination compared to RORα WT (Figure 2I). Next, we examined whether EZH2 small molecule inhibitor, exemplified by DZNep (Tan et al., 2007), rescues the EZH2-mediated degradation of RORα. The treatment of DZNep led to EZH2 protein level reduction and increased RORα protein level (Figure 2J). We compared RORα ubiquitination levels in the presence and absence of DZNep, and observed reduced RORα ubiquitination with the treatment of DZNep (Figure 2K). Together, these results indicate that K38 monomethylation by EZH2 is a prerequisite for RORα degradation through the ubiquitin-proteasome pathway.

## Identification of DCAF1 Selectively Recognizing Monomethylated Substrates

We hypothesized that certain substrate receptor proteins, possessing a methyl-recognition domain such as a chromo domain (Cavalli and Paro, 1998; Fischle et al., 2003; Kim et al., 2006), might be required for linking monomethylated substrates to ubiquitin-dependent degradation machinery. Therefore, we looked for RORα-interacting proteins by affinity chromatography in the presence of MG132 to block ubiquitin-dependent degradation pathway, followed by liquid chromatography mass spectrometry/mass spectrometry (LC-MS/MS) analysis (Figure 3A). Intriguingly, DDB1 and DCAF1 were identified as RORα-binding proteins which were previously reported to be components of a CUL4-containing complex (Angers et al., 2006; Higa et al., 2006; Jin et al., 2006). In mammals, two closely related types of CUL4, CUL4A and CUL4B have been identified, and the CUL4 E3 ligase differs from other cullin family members in that it employs the WD40-like repeat-containing protein DDB1 as an adaptor (He et al., 2006; Hu et al., 2008; Wang et al., 2006). The association of DCAF1 and DDB1 with RORα was further confirmed by immunoblot analysis from eluates using specific antibodies (Figure 3B).

Although posttranslational modifications such as phosphorylation, glycosylation, and proline hydroxylation is required to generate specific degron for the recognition of cullin-based E3 ligases (Ivan et al., 2001; Westbrook et al., 2008; Wu et al., 2007), the molecular basis of recognition of methylated substrates has not been reported. To examine the possibility that DCAF1 serves as a substrate receptor possessing a methyl-binding domain for the recognition of methylated RORα and thereby subsequent ubiquitination and degradation, we examined the interaction between DCAF1 with RORα in the presence of EZH2 WT or MT. Indeed, the introduction of EZH2 WT, but not EZH2 MT, significantly enhanced the binding of RORα to DCAF1 (Figure 3C). The association between RORα and DCAF1 appeared to be methylation-dependent, as only RORα WT was able to interact with DCAF1, while RORα K38A exhibited significantly reduced interaction (Figure 3D). In accordance to the methylation-dependent binding of RORα to DCAF1, immunoprecipitation assays were performed with anti-RORα K38 monomethyl-specific antibody. EZH2 knockdown significantly reduced RORα WT binding to DCAF1 and DDB1 while RORα K38A mutant failed to show significant interaction (Figure 3E). Together, these data suggest that EZH2-dependent RORα methylation is crucial for the association with DCAF1.

Next, we examined whether DCAF1 directly affects RORα protein stability. Indeed, the protein level of RORα in DCAF1-deficient MEFs (*Dcaf1*$^{-/-}$) was much higher than that in WT MEFs (Figure 3F). In order to further examine whether DCAF1 serves as a functional substrate receptor linking methylated substrates to subsequent ubiquitination-dependent degradation pathway, we performed RORα ubiquitination assay. The increased DCAF1 expression enhanced RORα ubiquitination (Figure 3G). We next examined whether DCAF1 and EZH2 combine to control RORα stability via ubiquitin-proteasome pathway. Indeed, introduction of EZH2 drastically increased RORα ubiquitination, whereas the knockdown of DCAF1 led to a significant decrease in RORα ubiquitination (Figure 3H). DCAF1 knockdown significantly reduced the binding of RORα to CUL4B (Figure 3I). Based on the finding of DDB1 as a RORα-binding protein by affinity chromatography (Figure 3A), we examined whether DDB1 knockdown also affects RORα binding to CUL4B as DCAF1 does. DDB1 knockdown significantly decreased the binding of RORα to CUL4B (Figure 3I). We then performed an in vivo ubiquitination assay to examine whether DDB1 and EZH2 combine to control RORα ubiquitination in the absence or presence of DDB1. Consistently, introduction of DDB1 increased RORα ubiquitination, and knockdown of EZH2 led to a significant decrease (Figure 3J). Together, these data indicate that DCAF1 and DDB1 are required for EZH2-dependent RORα ubiquitination.

Since CUL4A and CUL4B E3 ubiquitin ligases were reported to employ DDB1 as an adaptor, we examined the mutual binding

(D) and (E) HEK293 cells were transfected with the indicated siRNAs (D) and EZH2 WT/MT expression constructs (E), and samples were collected after cycloheximide (20 μg/ml) treatment at the indicated time to determine the protein levels of RORα.
(F) Binding affinity of EZH2 WT or MT with RORα was assessed.
(G) Protein extracts from HEK293 cells cotransfected with FLAG-RORα, HisMax-ubiquitin, and EZH2 WT or MT were subjected to pull-down with $Ni^{2+}$-NTA beads.
(H) The half-life of RORα WT and K38A mutant was compared in HEK293 cells.
(I) Ubiquitination of RORα was determined after transfection with FLAG RORα WT or K38A mutant.
(J) Immunoblot analysis was performed with or without DZNep (1 μM).
(K) RORα ubiquitination assay was conducted in the absence or presence of DZNep (1 μM, 24 hr).

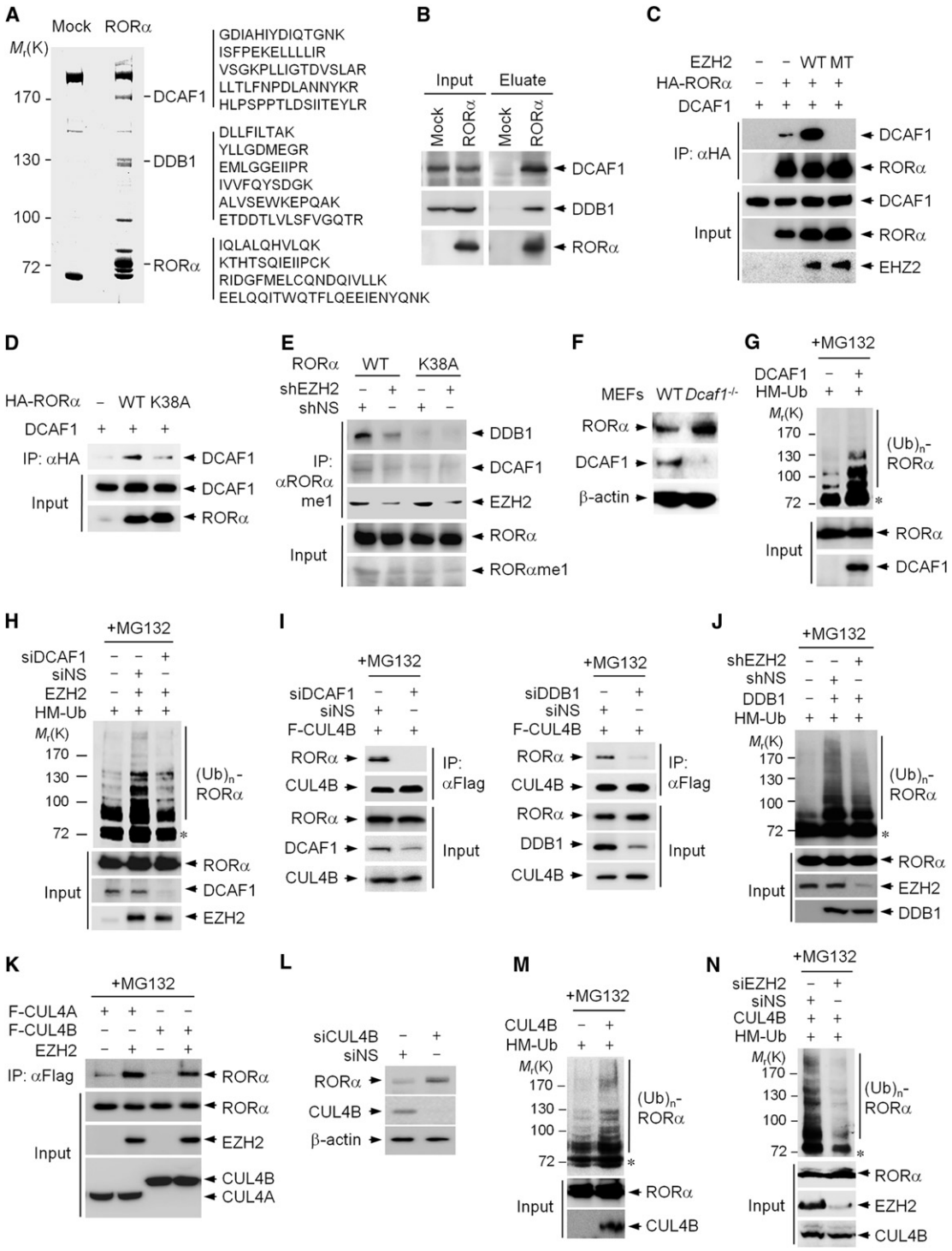


**Figure 3. Identification of DCAF1 Selectively Recognizing Monomethylated Substrates**

(A) RORα-interacting proteins were purified from HEK293 cells stably expressing FLAG-RORα in the presence of MG132 by coimmunoprecipitation with anti-FLAG antibody. Bound proteins were resolved by SDS-PAGE and prepared for LC-MS/MS analysis.

(B) The binding of DCAF1 or DDB1 with RORα was detected from the eluate by immunoblot analysis.

(C) Coimmunoprecipitation of DCAF1 and RORα in HEK293 cells treated with 20 μM of MG132 for 4 hr.

(D) Binding affinity of RORα WT or K38A mutant with DCAF1 was assessed in HEK293 cells expressing indicated constructs treated with MG132.

(E) Coimmunoprecipitation assay was performed to detect EZH2-dependent interaction of endogenous DCAF1 or DDB1 with RORα using anti-RORαme1 antibody in the presence of MG132.

of RORα to CUL4A and CUL4B. RORα bound to both CUL4A and CUL4B and the introduction of EZH2 enhanced the binding as assessed by coimmunoprecipitation (Figure 3K). Based on the nuclear localization of CUL4B compared to the cytoplasmic localization of CUL4A (Zou et al., 2009), we tested one of the CUL4 paralogues, CUL4B for RORα ubiquitination and protein stability assays. CUL4B knockdown significantly increased RORα protein levels (Figure 3L). To confirm whether CUL4B is responsible for RORα ubiquitination and EZH2-dependent RORα methylation is a prerequisite for CUL4B-dependent ubiquitination, we introduced CUL4B and EZH2 shRNAs and performed RORα ubiquitination assay. Overexpression of CUL4B led to a marked increase in RORα ubiquitination (Figure 3M), and this increase was almost completely abolished by EZH2 knockdown (Figure 3N). Taken together, our data strongly demonstrate that EZH2-mediated methylation of RORα triggers ubiquitination of RORα, and DCAF1 serves as a substrate receptor along with DDB1 for the recognition of methylated RORα and its subsequent CUL4-dependent ubiquitination and degradation.

## Molecular Basis for the Recognition of Methylated Substrates by DCAF1

We then examined whether the putative chromo domain of DCAF1 (cDCAF1) could recognize a monomethyl substrate. It has been reported that the hydrophobic amino acids within the binding pocket of the chromo domain are important for its function (i.e., the recognition of methylated substrates) (Fischle et al., 2003; Nielsen et al., 2002). Chromo domains of HP1, Pc, and MRG15 contain four hydrophobic amino acid residues within the binding pocket (highlighted with yellow boxes in Figure 4A) (Messmer et al., 1992; Pardo et al., 2002; Platero et al., 1995). Based on the model structure of the cDCAF1 bound to RORα peptides, as well as the sequence alignment, Y563, Y578, P580, and Y584 amino acids of DCAF1 were proposed to be key residues forming a substrate recognition pocket that can fit into the methyl-ammonium group of K38 of RORα peptide (Figure 4B). However, unlike the hydrophobic binding pockets of the chromo domain of Pc or HP1, which recognize trimethyl histone H3, DCAF1 possesses a smaller binding pocket, which cannot accommodate for the trimethyl group of histone H3 (Figure 4B). These results allowed us to propose that DCAF1 functions as a putative monomethyl reader. Therefore, in order to examine whether cDCAF1 directly recognizes the monomethyl peptide, we performed an in vitro peptide binding assay of WT and mutant cDCAF1 to the peptides derived from nonmethyl RORα, monomethyl RORα, nonmethyl histone H3K27, and trimethyl histone H3K27. WT cDCAF1 could bind only to the monomethyl RORα peptide, whereas the mutation of amino acid residues in the critical binding pocket of cDCAF1 (Y563A, Y578A, P580A, and Y584A) ablated the binding of cDCAF1 to the monomethyl RORα peptide (Figure 4C). In parallel, GST-pull-down assay confirmed that cDCAF1 selectively binds to monomethyl RORα, but not nonmethyl RORα (Figure 4D). Together, these in vitro binding assay results strongly suggest that cDCAF1 can specifically recognize monomethyl RORα, but not trimethyl histone H3K27, by utilizing the hydrophobic binding pocket in the chromo domain. These results demonstrate that DCAF1 discerns only monomethylation and specifically links methylation to ubiquitination-dependent degradation.

To further clarify whether the ability of DCAF1 to read monomethylation is due to its pocket size, we have explored a strategy to enlarge its pocket size, similar to that of Pc or HP1. From the sequence alignment and modeling analysis, it was predicted that the insertion of both Lys (K) (between Y578 and G579) and Asn-Thr (NT) residues (between Y584 and W585) could enlarge the pocket size (highlighted with yellow boxes in Figure 4E). Indeed, the insertion of both K and NT led to a decrease in the ability of DCAF1 to bind the monomethyl RORα peptide with an increase in the ability to recognize the trimethyl histone H3 peptide (Figures 4F and 4G). On the other hand, the insertion of either K or NT did not affect the binding of DCAF1 to monomethyl RORα peptides (Figure 4G). Based on in vitro peptide binding assay results as well as modeling structures, we identify DCAF1 as the monomethyl reader, having a relatively small binding pocket that specifically accommodates for monomethyl substrate, but not di- or trimethyl substrates. Together, these results demonstrate that DCAF1 discerns monomethylated substrates possessing a "methyl degron" and specifically functions to link monomethylation to ubiquitin-dependent degradation machinery. Based on these findings, we suggest a previously unrecognized regulatory molecular mechanism by which EZH2-DCAF1/DDB1/CUL4 serves as a methylation-specific E3 ubiquitin ligase complex that plays a crucial role in dynamic regulation of nonhistone protein stability.

## Recognition of a Methyl Degron by DCAF1

To test the possibility that a histone-like sequence within RORα acts as a molecular determinant required or even sufficient for the recognition by DCAF1, we prompted to interchange the RORα histone-like sequence (aa 29-47) with that of the histone H3 sequence (aa 18-36) to generate a RORα/H3 chimeric protein (Figure 5A). Although the RORα/H3 chimeric protein bound to EZH2 as well as RORα WT (Figure 5B), it exhibited almost complete loss of binding ability to DCAF1 unlike RORα WT (Figure 5C). Furthermore, in contrast to RORα WT, neither RORα

(F) DCAF1 and RORα protein levels were compared between WT and *Dcaf1*-deficient MEFs.
(G) The increased RORα ubiquitination by DCAF1.
(H) The enhanced RORα ubiquitination by EZH2 was decreased by DCAF1 knockdown.
(I) Knockdown of DCAF1 or DDB1 decreased the binding of CUL4B to RORα.
(J) Increased RORα ubiquitination by DDB1 was decreased by knockdown of EZH2.
(K) HEK293 cells were transfected with FLAG-CUL4A or CUL4B, and coimmunoprecipitation assay was performed using anti-FLAG antibody in the absence or presence of EZH2, followed by immunoblot analysis using anti-RORα antibody.
(L) Knockdown of CUL4B increased RORα protein levels.
(M) Ubiquitination of RORα was increased by CUL4B.
(N) Increased RORα ubiquitination by CUL4B was significantly decreased by knockdown of EZH2.

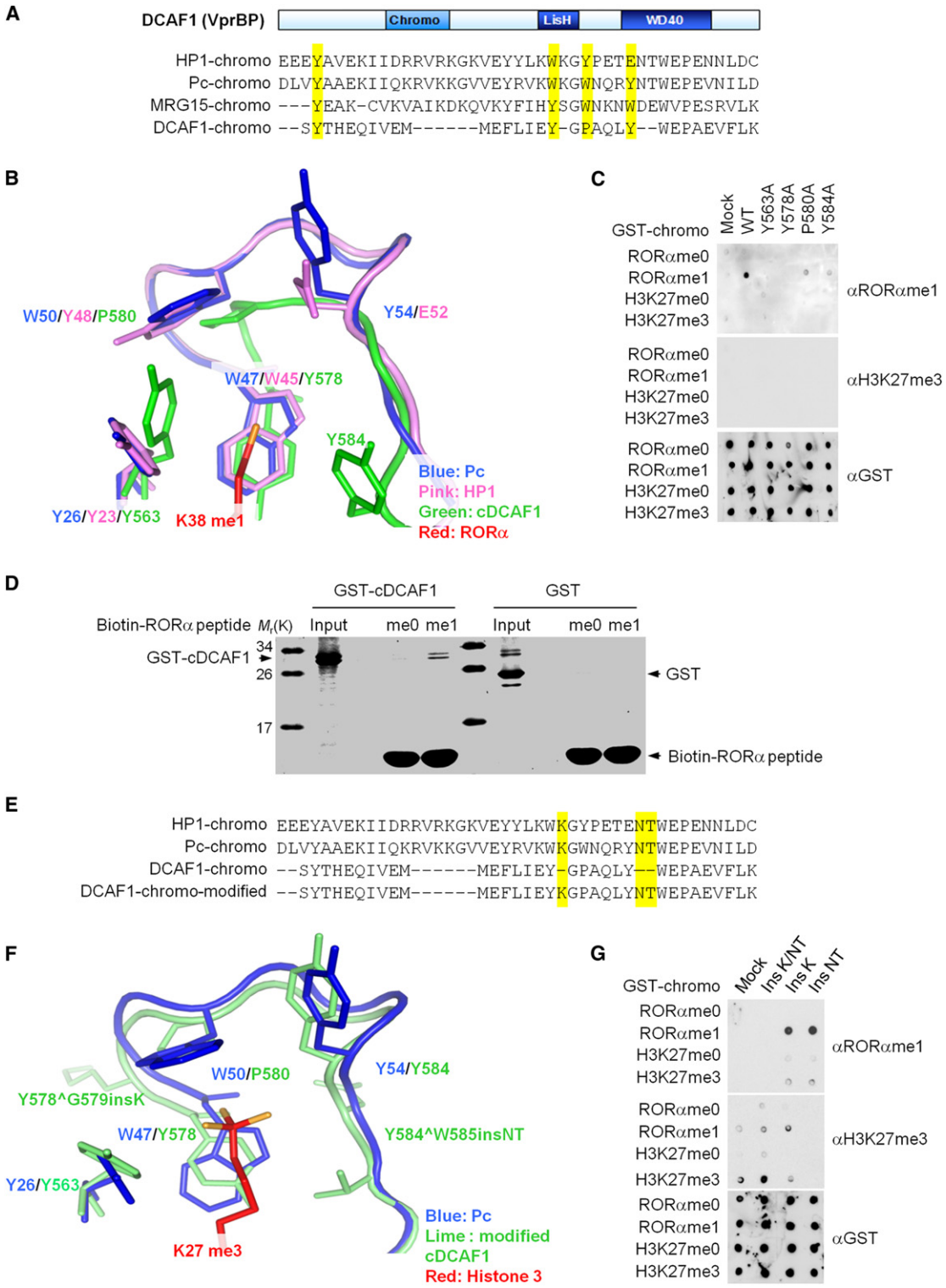


**Figure 4. Molecular Basis for the Recognition of Methylated Substrates by DCAF1**

(A) Sequence comparison between the chromo domains from HP1, Pc, and MRG15, and the putative cDCAF1. Strictly conserved aromatic residues are highlighted in yellow boxes.

(B) The model of cDCAF1 (green) bound to RORα peptide (red) is superimposed with the chromo domains of Pc (blue) and HP1 (pink). Four hydrophobic residues (Y563, Y578, P580 and Y584) in the cDCAF1 and overlapped residues in the other proteins are presented in the stick models and labeled. The methylated K38 of RORα peptide is also drawn in a stick model. Methyl group in K38 is shown orange in color.

K38A nor the RORα/H3 chimeric protein exhibited comparable binding to the cDCAF1 (Figure 5D).

Because DCAF1 failed to interact with the RORα/H3 chimeric protein, we speculated that the RORα/H3 protein might be trimethylated by EZH2 similar to the histone H3K27, preventing it from being recognized by DCAF1 and subsequent ubiquitination-dependent degradation. Indeed, the RORα/H3 chimeric protein can now be trimethylated by EZH2, as revealed by its detection by H3K27me3 antibodies, but not by RORαme1 antibodies (Figure 5E). Further, in contrast to RORα WT protein, the RORα/H3 chimeric protein failed to show an increase in ubiquitination upon the introduction of EZH2 WT (Figure 5F). Our results confirm that the replacement of the 19 aa histone-like sequence within RORα by the corresponding histone H3 sequences, is sufficient to restore trimethylation. Therefore, the ability of DCAF1 specifically recognizing the monomethylated substrates and thus linking to the DDB1/CUL4B E3 ubiquitin ligase complex for degradation is apparently conferred by the methylation status.

To further test the possibility that a histone-like sequence within RORα functions as a methyl degron recognized by DCAF1 for subsequent ubiquitin-dependent degradation, we have inserted RORα histone-like sequence (aa 29-47) to the histone H3 sequence (aa 18-36) to generate a H3/RORα chimeric protein (Figure 5G). The H3/RORα chimeric protein was able to interact with EZH2 in a similar fashion to histone H3 (Figure 5H), but intriguingly, it acquired the binding ability to DCAF1 unlike histone H3 (Figure 5I). Indeed, the H3/RORα chimeric protein can now be monomethylated by EZH2, as revealed by its detection with anti-RORα K38 monomethyl-specific antibodies similar to RORα WT (Figure 5J). Furthermore, unlike histone H3 proteins, the H3/RORα chimeric protein showed increased ubiquitination in EZH2 activity-dependent manner (Figure 5K). EZH2ΔSET mutant, which is deficient of enzymatic activity, significantly attenuated ubiquitination of the H3/RORα chimeric protein. Together, these data confirm that the histone-like sequence within RORα functions as a methyl degron which is subject to DCAF1 binding and subsequent ubiquitin-dependent degradation.

## Methylation-Dependent Ubiquitination of RORα Leads to the Transcriptional Repression of RORα Target Genes

Next, we examined the functional consequence of EZH2-dependent methylation and subsequent degradation of RORα on the transcription of RORα-dependent target genes. Introduction of EZH2 WT, but not EZH2 MT, attenuated RORα-dependent activation of RORE-luciferase activity (Figure 6A). Compared to RORα WT, RORα K38A was able to further increase RORE-luciferase activity (Figure 6A). Similarly, EZH2 knockdown resulted in potentiating the transcriptional activity of RORα (Figure 6B). Intriguingly, RORα K38A mutant possessed more potent transcriptional activity compared to RORα WT in the absence of MG132, whereas in the presence of MG132, this difference was abolished (Figure 6C). Consistent with the ubiquitination data shown in Figure 3H, DCAF1 knockdown led to the increased transcriptional activity of RORα (Figure 6D). In parallel, immunoblot analysis confirmed that the increased RORE-luciferase activity is correlated with increased RORα protein levels (Figure 6D). These data indicate that EZH2-dependent methylation and DCAF1-mediated, ubiquitin-dependent degradation of RORα affects transcription of RORα-dependent target genes.

As *p21* is one of the well-known RORα target genes with a functional RORE on the promoter (Steinman et al., 1998), and to examine whether *p21* transcript level is affected by EZH2, we performed ChIP assays on *p21* promoter in MCF7 breast cancer cells. ChIP assay further confirmed that EZH2 knockdown increased RORα recruitment concomitant with increased RNA polymerase II recruitment (Figure 6E). *GADD45* promoter containing RORE was also examined, and similar results were observed (Figure 6E). Together, these data indicate that EZH2-dependent methylation triggers ubiquitin-dependent degradation of RORα, leading to the transcriptional repression of RORα target genes.

## Functional Consequence of Methylation-Dependent RORα Ubiquitination

Given that DCAF1 is responsible for RORα degradation, it is reasonable to expect that there must be a physiologically relevant function that underlies these intriguing findings. In breast and prostate cancers, elevated EZH2 protein levels have been observed suggesting that EZH2 may be acting as an oncogene, and therefore might be used as a marker of cancer diagnosis (Bachmann et al., 2006; Kleer et al., 2003; Varambally et al., 2002). In order to determine whether RORα protein levels ultimately alter the cell's ability to transform, we have taken several approaches in regulating RORα protein levels. First, overexpression of RORα in MCF7 breast cancer cells led to a significant reduction in colonies, compared to vector control in soft agar (Figure 7A). Second, the knockdown of DCAF1 also led to a marked reduction in the number of colonies in soft agar by stabilizing RORα protein levels (Figure 7B). Moreover, DZNep

(C) Binding of the GST-DCAF1 WT and chromo domain mutants (Y563A, Y578A, P580A, and Y584A) to the RORα K38me0, RORα K38me1, H3K27me0, and H3K27me3 peptides was determined by in vitro binding assay.
(D) Biotinylated RORα K38me0 and me1 peptides were immobilized onto streptavidin-conjugated Sepharose beads. GST and GST-DCAF1 chromo fusion proteins were added to the beads and the bound fraction was subjected to immunoblot analysis.
(E) Protein sequence alignment of the chromo domains of HP1, Pc, DCAF1, and modified DCAF1. Inserted amino acid sequences are highlighted by yellow boxes in the sequence alignment.
(F) The modeled chromo domain of modified DCAF1 (lime) bound to the trimethyl ammonium group of Lys is compared with the chromo domain of Pc (blue). Y578^G579insK indicates a Lys residue inserted between Y578 and G579 and Y584^W585insNK represents an Asp and Thr residues inserted between Y584 and W585. Methyl groups in histone K27 are shown in red.
(G) Binding of chromo domains of the K/NT-inserted, K-inserted, or NT-inserted mutants of DCAF1 with the indicated peptides was measured by in vitro binding assay.

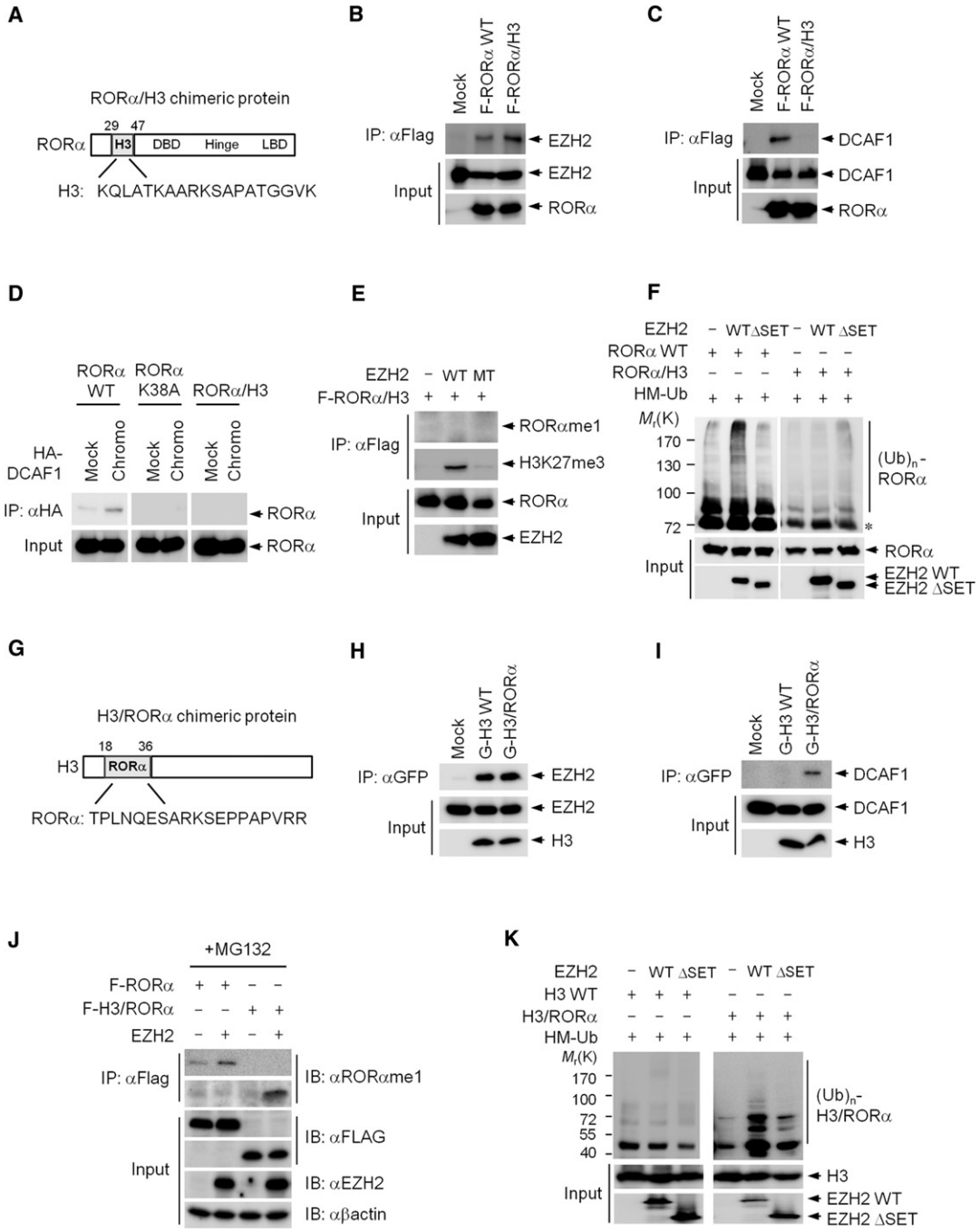


**Figure 5. Recognition of a Methyl Degron by DCAF1**

(A) Schematic representation of RORα/H3 chimeric protein.

(B) and (C) Cell extracts were immunoprecipitated with anti-FLAG antibody followed by immunoblot analysis against anti-EZH2 (B) or anti-DCAF1 (C) antibodies.

(D) Cells were transfected with RORα WT, K38A, or RORα/H3 chimeric mutant in the absence or presence of HA-DCAF1 chromo domain. Immunoprecipitation assay was performed with anti-HA antibody, and the bound RORα was detected by immunoblot analysis with anti-FLAG antibody.

(E) RORαme1 and H3K27me3 levels were detected with anti-RORαme1 antibody and anti-H3K27me3 antibody, respectively, after immunoprecipitation against anti-FLAG antibody.

(F) Ubiquitination of RORα or RORα/H3 chimeric proteins was assessed by anti-FLAG antibody.

(G) Schematic representation of H3/RORα chimeric protein.

(H) and (I) Cell extracts were immunoprecipitated with anti-GFP antibody followed by immunoblot analysis against anti-EZH2 (H) or anti-DCAF1 (I) antibodies.

(J) EZH2 overexpression led to the increased monomethylation of H3/RORα chimeric proteins.

(K) Ubiquitination of histone H3 and H3/RORα chimeric proteins was determined using anti-FLAG antibody.

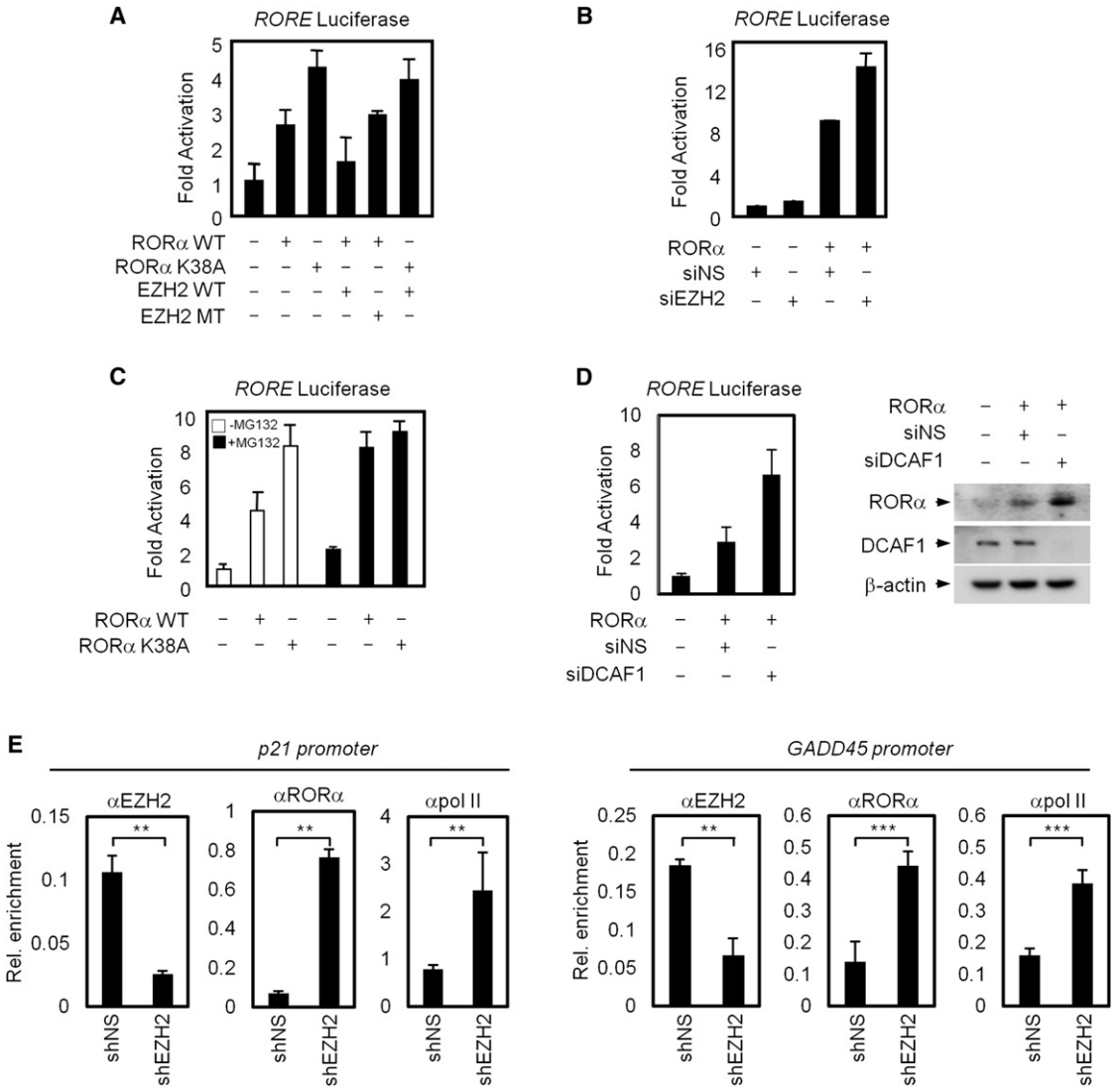


**Figure 6. Effects of Methylation-Dependent RORα Ubiquitination on Transcription of RORα-Dependent Genes**

(A–D) 5X RORE-luciferase reporter plasmid was transfected into 293T cells with indicated plasmids and siRNAs. Luciferase activity was measured at 48 hr after transfection and normalized by β-galactosidase activity. Values are expressed as mean ± s.d. for three independent experiments. MG132 (20 μM) was treated for 24 hr. Immunoblot analysis was performed to detect RORα protein levels.

(E) ChIP assays were performed on the *p21* or *GADD45* promoters in the presence of control shRNA or EZH2 shRNA in MCF7 cells. *P* value is shown from Student's t test analysis. **p < 0.05 and ***p < 0.005. Values are expressed as mean ± s.d. for three independent experiments.

treatment (2 μM) of MCF7 breast cancer cells also had an inhibitory effect on cellular transformation (Figure 7C). Together, these transformation assay results strongly support our inverse correlation between EZH2 and RORα protein levels conferred by methylation-dependent ubiquitination of RORα.

The inverse correlation between EZH2 and RORα was found in breast tumor tissue specimens compared to the normal counterpart. RORα protein levels were very low in tumors exhibiting high levels of EZH2 (Figure 7D), strongly supporting the biological importance of these findings. Indeed, reduced RORα and elevated EZH2 protein levels in the tumor samples compared to normal samples were statistically significant (Figure 7E). Out of 43 breast cancer patient specimens, there were 30 cases where EZH2 protein levels were higher in the tumor samples compared to normal samples. Within these 30 cases, 25 tumor specimens showed markedly reduced RORα protein levels, supporting the idea that elevated levels of EZH2 in the tumors might facilitate RORα degradation.

## DISCUSSION

In this manuscript, we report monomethylation can be used to mark proteins for degradation by the DCAF1/DDB1/CUL4 E3 ubiquitin ligase complex. A previously unrecognized region of

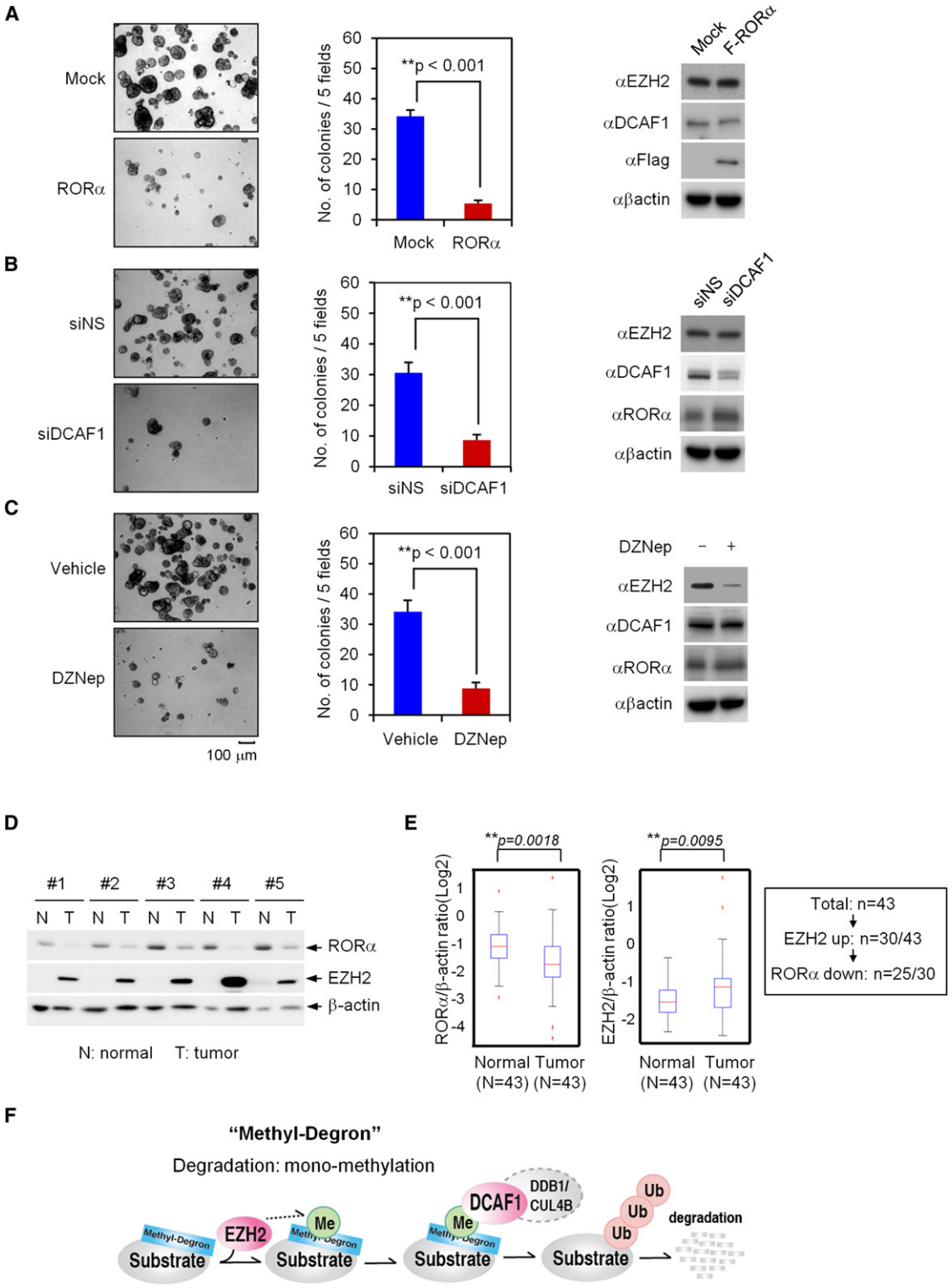


**Figure 7. Functional Consequence of the Methylation-Dependent RORα Ubiquitination**

(A–C) Anchorage-independent growth of MCF7 breast cancer cells stably expressing empty vector or RORα (A) or transfected with control siRNA or DCAF1 siRNA (B) and cells treated with vehicle or DZNep (2 μM) (C) in soft agar. Representative images are shown for each group. Immunoblot analysis was performed to detect each protein level. Statistical analysis was performed comparing each set of colonies with their control using Student's t test. Error bars represent SEM; n = 5.

(D) Immunoblot analysis of RORα and EZH2 in human breast tumor tissue samples (T) along with matched normal tissue samples (N).

DCAF1 behaves as a chromo domain with a restricted pocket size such that only monomethylated substrate can bind. We propose that EZH2 can monomethylate the lysine on a RKS histone-like sequence on RORα leading to its subsequent ubiquitination through the chromo domain of DCAF1. Understanding the mechanism by which a monomethylated lysine can act as a mark for protein degradation is important as it suggests a posttranslational modification that can modulate protein turnover and gene expression. Although a number of posttranslational modifications such as phosphorylation and glycosylation have all been shown to be required to generate a specific degron for the recognition by cullin E3 ligases, our study is the report of recognition of methylated substrates. Here, we show the presence of such a methyl lysine binding domain in DCAF1, a substrate receptor for a CUL4 E3 ligase. We have established a molecular basis of methyl degron recognition by DCAF1/DDB1/CUL4 E3 ubiquitin ligase complex, and uncovered an unidentified regulatory signaling pathway (Figure 7F): Monomethylated substrates recognized by DCAF1/DDB1/CUL4 complex, leading to protein degradation, whereas di- or trimethylated substrates such as histone H3 escape from DCAF1/DDB1/CUL4-dependent degradation.

Given that RORα degradation is triggered by DCAF1 binding, we can expect that there are physiologically important functions that underlie these findings. Increased EZH2 expression has been often observed in breast and prostate cancers suggesting EZH2 as a possible candidate for a diagnostic marker (Bachmann et al., 2006; Kleer et al., 2003; Varambally et al., 2002). We found that there is an inverse correlation between RORα and EZH2 where protein levels of RORα is reduced and EZH2 is increased in breast tumor tissue specimens suggesting a biological importance of these findings in the cancer setting. We suspect that a well-known oncogenic function of EZH2 might be augmented by methylation-dependent degradation of tumor suppressive proteins such as RORα via the DCAF1-mediated degradation pathway, therefore providing an attractive avenue for the development of powerful anticancer therapeutics.

Further, the identification of methyl degron-containing proteins on a genome-wide level by systemic approaches will further extend the understanding of the methylation-dependent ubiquitination function. Our genome-wide screening study has identified RORα as a nonhistone substrate of EZH2, and we have elucidated the molecular mechanism by which RORα methylation leads to its ubiquitination. The existence of methylation-dependent ubiquitination on nuclear receptors and transcription factors suggests a possibility that may add another layer of complexity to epigenetic regulation. Further functional analysis of methyl degron-containing proteins will likely to shed light on many biological functions of methylation-dependent ubiquitination in different physiological context.

## EXPERIMENTAL PROCEDURES

### Antibodies

The following commercially available antibodies were used: RORα (Santa Cruz Biotechnology), FLAG (Sigma), EZH2 (BD Biosciences), Xpress (Invitrogen), DDB1, CUL4B, DCAF1, H3K27me2, H3K27me3, and methyl-Lys antibodies (Abcam). RORαK38me1, RORαK38me2, and RORαK38me3 antibodies were generated by Abmart (China).

### Ubiquitination Assay

Cells were transfected with combinations of plasmids including HisMax-ubiquitin. After incubation for 48 hr, cells were treated with MG132, lysed in buffer A (6 M guanidinium-HCl, 0.1 M $Na_2HPO_4/NaH_2PO_4$, 0.01 M Tris-Cl [pH 8.0], 5 mM imidazole, and 10 mM β-mercaptoethanol), and incubated with $Ni^{2+}$-NTA beads (QIAGEN) for 4 hr at room temperature. The beads were sequentially washed with buffer A, buffer B (8 M urea, 0.1 M $Na_2PO_4/NaH_2PO_4$, 0.01 M Tris-Cl [pH 8.0], and 10 mM β-mercaptoethanol), and buffer C (8 M urea, 0.1 M $Na_2PO_4/NaH_2PO_4$, 0.01 M Tris-Cl [pH 6.3], and 10 mM β-mercaptoethanol). Bound proteins were eluted with buffer D (200 mM imidazole, 0.15 M Tris-Cl [pH 6.7], 30% glycerol, 0.72 M β-mercaptoethanol, and 5% SDS), and subject to immunoblot analysis.

### In Vitro Peptide Binding Assay

GST-tagged DCAF1 chromo domain WT and its mutants bound to Glutathione-Sepharose beads were prepared. The beads were mixed with 100 μg of the bovine serum albumin and 15 μg of the RORαK38me0, RORαK38me1, H3K27me0, or H3K27me3 peptides in 1 ml of binding buffer (10 mM $Na_2HPO_4$, 2.7 mM KCl, 1.8 mM $KH_2PO_4$, and 140 mM NaCl, pH 7.4) supplemented with 1 mM phenylmethylsulfonyl fluoride and 2 mM dithiothreitol followed by incubation at room temperature for 2 hr. After washing with the binding buffer, the protein with bound peptide was eluted by 6.8 mg/ml of Glutathione in Tris-HCl (pH 8.0) buffer and visualized by dot blot analysis.

### Cell Transformation Assay

Anchorage-independent growth of MCF7 breast cancer cells stably expressing empty vector or RORα or cells transfected with control siRNA or DCAF1 siRNAs or treated in the presence and absence of DZNep (2 μM) was determined by analyzing colony formation in soft agar. Cells ($10^5$) were placed in DMEM media containing 0.4% noble agar containing 10% FBS for 3 weeks in 5% $CO_2$, and colonies exhibiting greater than 100 μM were counted and analyzed.

## SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and Supplemental References and can be found with this article online at http://dx.doi.org/10.1016/j.molcel.2012.09.004.

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(E) Statistical analysis of the ratio of RORα or EZH2 to β-actin in 43 human breast tumor samples compared to matched normal counterpart calculated by paired Student's t test. Data are presented as whisker graphs, showing the median and the distribution of 50% (bar) and 99.3% (whisker) of all specimens examined. $^{**}p < 0.05$ and $^{***}p < 0.005$. Shown is a summary of protein expression analysis from the human breast tumor specimens.
(F) Proposed model of DCAF1 serving as a methyl reader that specifically recognizes methyl degron, thereby allowing DDB1/CUL4-dependent ubiquitination and subsequent degradation of RORα by the 26S proteasome.

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