Long noncoding RNA *UPAT* promotes colon tumorigenesis by inhibiting degradation of UHRF1

Kenzui Taniue[a], Akiko Kurimoto[a,b], Hironobu Sugimasa[a], Emiko Nasu[a], Yasuko Takeda[a], Kei Iwasaki[a], Takeshi Nagashima[c], Mariko Okada-Hatakeyama[d], Masaaki Oyama[e], Hiroko Kozuka-Hata[e], Masaya Hiyoshi[f], Joji Kitayama[f], Lumi Negishi[a], Yoshihiro Kawasaki[a], and Tetsu Akiyama[a,1]

[a]Laboratory of Molecular and Genetic Information, Institute of Molecular and Cellular Biosciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo, 113-0032, Japan; [b]Oncology Research Laboratories, Daiichi Sankyo Co., Ltd, 1-2-58 Hiromachi, Shinagawa-ku, Tokyo, 140-8710, Japan; [c]Department of Cell Proliferation, United Center for Advanced Research and Translational Medicine, Graduate School of Medicine, Tohoku University, 2-1 Seiryo-machi, Aoba-ku, Sendai, Miyagi, 980-0872, Japan; [d]Laboratory for Integrated Cellular Systems, RIKEN Center for Integrative Medical Sciences (IMS-RCAI), Tsurumi-ku, Yokohama, Kanagawa, 230-0045, Japan; [e]Medical Proteomics Laboratory, Institute of Medical Science, The University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo, 108-8639, Japan; and [f]Department of Surgical Oncology, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo, 113-8655, Japan

Edited by Kevin Struhl, Harvard Medical School, Boston, MA, and approved December 11, 2015 (received for review January 16, 2015)

Many long noncoding RNAs (lncRNAs) are reported to be dysregulated in human cancers and play critical roles in tumor development and progression. Furthermore, it has been reported that many lncRNAs regulate gene expression by recruiting chromatin remodeling complexes to specific genomic loci or by controlling transcriptional or posttranscriptional processes. Here we show that an lncRNA termed *UPAT* [ubiquitin-like plant homeodomain (PHD) and really interesting new gene (RING) finger domain-containing protein 1 (UHRF1) Protein Associated Transcript] is required for the survival and tumorigenicity of colorectal cancer cells. *UPAT* interacts with and stabilizes the epigenetic factor UHRF1 by interfering with its β-transducin repeat-containing protein (TrCP)–mediated ubiquitination. Furthermore, we demonstrate that UHRF1 up-regulates *Stearoyl-CoA desaturase 1* and *Sprouty 4*, which are required for the survival of colon tumor cells. Our study provides evidence for an lncRNA that regulates protein ubiquitination and degradation and thereby plays a critical role in the survival and tumorigenicity of tumor cells. Our results suggest that *UPAT* and UHRF1 may be promising molecular targets for the therapy of colon cancer.

long noncoding RNA | UPAT | UHRF1 | ubiquitination | tumorigenicity

Among the RNA products transcribed from the mammalian genome are numerous long noncoding RNAs (lncRNAs)—that is, RNAs longer than 200 nucleotides with little or no protein-coding potential (1, 2). Many lncRNAs are expressed in a developmentally regulated and cell type-dependent manner (3, 4). Increasing evidence suggests that lncRNAs play critical roles in a diverse set of biological processes, including proliferation, differentiation, embryogenesis, neurogenesis, and stem cell pluripotency (5, 6).

It has been reported that many lncRNAs regulate gene expression by recruiting chromatin remodeling complexes to specific genomic regions (2). It has also been shown that many lncRNAs regulate transcription by modulating the activity of transcriptional regulators (1, 6–8). lncRNAs also regulate various posttranscriptional processes, including splicing, transport, translation, and degradation of mRNA. Furthermore, recent studies have shown that a number of lncRNAs play critical roles in tumor development and progression.

UHRF1 [ubiquitin-like plant homeodomain (PHD) and really interesting new gene (RING) finger domain-containing protein 1] is an epigenetic factor that consists of multiple domains (9). UHRF1 regulates transcription by regulating DNA methylation and histone modification. UHRF1 also possesses E3 ubiquitin ligase activity and ubiquitinates histones and DNA methyltransferase 1 (DNMT1), thereby regulating the chromatin structure and stability of DNMT1 (10, 11). UHRF1 plays key roles in multiple biological processes, including proliferation and development. Furthermore, UHRF1 is overexpressed in various tumors, including colon, breast, bladder, prostate, and lung cancers, and plays a critical role in the proliferation and survival of tumor cells (9).

In the present study, we attempted to identify lncRNAs critical for the tumorigenicity of colon tumor cells by performing RNA-sequencing (RNA-seq) analysis of the colon cancer cell line CCSC#P and a subclone that exhibits drastically decreased tumorigenicity, CCSC#11. We have found that an lncRNA termed *UPAT* (UHRF1 Protein Associated Transcript) is down-regulated in CCSC#11 and is required for the tumorigenicity of CCSC#P. We further show that *UPAT* interacts with UHRF1. Moreover, we show that *UPAT* interferes with the ubiquitination and degradation of UHRF1 and thereby plays a critical role in determining the survival and tumorigenicity of colorectal tumor cells. We also demonstrate that UHRF1 is required for the up-regulation of *Stearoyl-CoA desaturase 1* (*SCD1*) and *Sprouty 4* (*SPRY4*), which play critical roles in the survival of colon tumor cells.

Results

***UPAT* Is Required for the Tumorigenicity of Colon Tumor Cells.** We established single cell-derived subclones from the colon cancer cell line CCSC#P by limiting dilution and examined their tumorigenicity. Out of 34 clones examined, two of these, termed CCSC#11 and #22, exhibited drastically decreased tumorigenicity compared with CCSC#P when implanted s.c. into immunocompromised mice (Fig. 1*A*). To investigate the mechanisms underlying this decreased tumorigenicity, we performed RNA-seq analysis of CCSC#P and #11 cells (Dataset S1). We selected two lncRNAs, NR_015379

Significance

Many long noncoding RNAs (lncRNAs) play critical roles in tumor development. Here we show that an lncRNA termed *UPAT* [ubiquitin-like plant homeodomain and really interesting new gene finger domain-containing protein 1 (UHRF1) Protein Associated Transcript] is required for the tumorigenicity of colorectal cancer cells. *UPAT* interacts with and stabilizes the epigenetic factor UHRF1 by interfering with its ubiquitination and degradation. Furthermore, the UHRF1–*UPAT* axis up-regulates *Stearoyl-CoA desaturase 1* and *Sprouty 4*, which are required for the survival of colon tumor cells. Our study provides evidence for an lncRNA that regulates protein ubiquitination and degradation and thereby plays a critical role in the survival and tumorigenicity of tumor cells. Our results suggest that *UPAT* and UHRF1 may be promising molecular targets for the therapy of colon cancer.

Author contributions: K.T. and T.A. designed research; K.T., A.K., H.S., E.N., Y.T., K.I., M.O., H.K.-H., M.H., J.K., L.N., and Y.K. performed research; K.T., T.N., and M.O.-H. performed bioinformatic analyses; K.T., A.K., H.S., and T.A. analyzed data; and K.T. and T.A. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Data deposition: The sequence reported in this paper has been deposited in the NCBI Sequence Read Archive (SRA), www.ncbi.nlm.nih.gov/sra (accession no. DRA004047).

[1]To whom correspondence should be addressed. Email: akiyama@iam.u-tokyo.ac.jp.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1500992113/-/DCSupplemental.

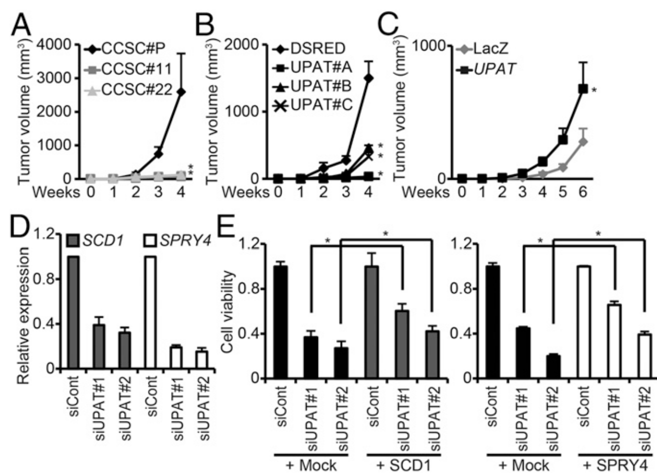

Fig. 1. *UPAT* is required for the tumorigenicity of colon cancer cells. (*A*) CCSC#P, CCSC#11, or CCSC#22 cells were injected s.c. into nude mice and assessed for tumor growth. Results are expressed as the mean ± SEM ($n = 6$). *$P < 0.05$. (*B*) CCSC#P cells infected with a lentivirus expressing an sh*UPAT* were injected into nude mice. Results are expressed as the mean ± SEM ($n = 6$). *$P < 0.05$. (*C*) CCSC#11 cells infected with a lentivirus expressing *UPAT* were injected into nude mice. Results are expressed as the mean ± SEM ($n = 6$). *$P < 0.05$. (*D*) qRT-PCR analysis of *SCD1* and *SPRY4* expression in HCT116 cells transfected with siRNA targeting *UPAT*. Results are expressed as the mean ± SEM ($n = 3$). (*E*) Viability of HCT116 cells transfected with SCD1 (*Left*) or SPRY4 (*Right*) and/or siRNA targeting *UPAT* was assessed by Cell Titer-Glo assays. Results are expressed as the mean ± SEM ($n = 3$). *$P < 0.05$.

and NR_002773, down-regulated in CCSC#11 cells (Fig. S1*A*) and examined the effects of knocking down these genes on the survival of HCT116 and CCSC#P cells. The most significant reduction in the viability of HCT116 and CCSC#P cells was achieved by knockdown of an lncRNA, NR_002773, termed *UPAT* (Fig. S1 *B* and *C*), which is encoded by the pseudogene of the amine oxidase copper containing 3 (AOC3) gene (nucleotides 239–1732; 94% homology) (12–14) (Fig. S1 *D–F*). *UPAT* is also homologous to AOC2 (239–1261; homology 76%) but not to AOC1. Quantitative (q)RT-PCR analysis revealed that *UPAT* expression was up-regulated in highly tumorigenic colon cancer cell lines compared with weakly tumorigenic colon cancer cell lines and normal cell lines (Fig. S1*G*). Subcellular fractionation and qRT-PCR analysis revealed that *UPAT* was present in the nucleus (Fig. S1*H*).

To clarify the significance of UPAT in colorectal tumorigenesis, we infected HCT116 and CCSC#P cells with a lentivirus expressing an shRNA targeting UPAT (shUPAT) and examined their tumorigenicity. When transplanted into nude mice, the growth of these cells was significantly retarded compared with cells infected with a control lentivirus (Fig. 1*B* and Fig. S2 *A* and *B*). Moreover, we found that CCSC#11 cells infected with a lentivirus expressing UPAT exhibited increased tumorigenicity and colony formation in soft agar compared with CCSC#11 cells infected with control virus (Fig. 1*C* and Fig. S2 *C* and *D*). In addition, Cell Titer-Glo assays revealed that knockdown of UPAT by siRNA (siUPAT) caused a significant reduction in the growth of HCT116, CCSC#P, and DLD-1 cells but not of normal keratinocyte HaCaT cells in vitro (Fig. S2 *E* and *F*). Knockdown of UPAT by antisense oligonucleotide also resulted in the decreased growth of HCT116 cells (Fig. S2 *G* and *H*). Furthermore, Annexin V assays showed that knockdown of UPAT induced apoptosis of HCT116 but not HaCaT cells (Fig. S2 *E* and *I*). In contrast, knockdown of the AOC family of genes, AOC1~3, did not cause a reduction in the growth of HCT116 and CCSC#P cells (Fig. S2 *J–L*). Moreover, knockdown of UPAT did not affect the expression of AOC3 in either HCT116 or CCSC#P cells (Fig. S2*M*). These results suggest that UPAT, but not the AOC family of genes, may be required for the survival and tumorigenicity of colorectal cancer cells.

To study the role of UPAT in colorectal cancer cells, we investigated the gene expression profiles of HCT116 cells in which UPAT expression had been suppressed by siRNA. RNA-seq analyses revealed that rat sarcoma viral oncogene homolog (RAS)-, cadherin 1 (CDH1)-, and hypoxia-related genes are regulated in UPAT knockdown cells (Dataset S2). From these genes, we selected 25 genes that were down-regulated > twofold and examined whether knockdown of any of them could cause apoptotic death in HCT116 cells. We found that siRNA knockdown of SCD1, SPRY4, phosphoglucomutase 1 (PGM1), or G protein-coupled receptor, class C, group 5, member A (GPRC5A) resulted in a marked increase in apoptotic cell death (Fig. S2 *N–U*). We also performed qRT-PCR analyses and confirmed that knockdown of UPAT by siRNA or antisense oligonucleotides resulted in decreased expression of these four genes (Fig. 1*D* and Fig. S2 *V–X*). Furthermore, we found that overexpression of either SCD1 or SPRY4 partially restored the growth of HCT116 cells in which UPAT had been knocked down (Fig. 1*E*). Moreover, we found that UPAT, SPRY4, and SCD1 mRNA expression was higher in colorectal tumors than in adjacent normal tissues (Fig. S2*Y*). These results suggest that UPAT-mediated up-regulation of these genes is involved in the survival of colon cancer cells. In addition, treatment of HaCaT cells with EGF did not significantly affect the expression levels of UPAT (Fig. S2*Z*).

***UPAT* Is Associated with UHRF1 in Colon Cancer Cells.** Many lncRNAs have been shown to exert their biological function by forming complexes with proteins (1, 6–8). We therefore performed RNA pull-down assays to identify proteins that potentially could associate with *UPAT*. Nuclear extracts from HCT116 cells were incubated with biotinylated sense or antisense *UPAT* RNA generated in vitro, and proteins precipitated with streptavidin beads were resolved by SDS/PAGE. A band that specifically coprecipitated with sense *UPAT* was excised and subjected to liquid chromatography–mass spectrometry (Fig. S3*A* and Dataset S3). Among the proteins identified, we selected nuclear proteins whose molecular weight were 88–92 kDa, which narrowed down our screen to five candidate proteins (Fig. S3*B*). We then examined whether these proteins precipitate with *UPAT* (RIP analysis) and from this obtained UHRF1. The association of UHRF1 with *UPAT* sense RNA, but not *UPAT* antisense or *ASBEL* [antisense ncRNA in the ANA/BTG3 (three) locus] RNA (15), was also confirmed by immunoblotting analysis with anti-UHRF1 antibody (Fig. 2*A*). To further verify this result, we performed RNA immunoprecipitation (RIP) analysis with anti-UHRF1 antibody using lysates from HCT116 cells. qRT-PCR analysis of the immunoprecipitates revealed that UHRF1 was associated with endogenous *UPAT*, but not with *GAPDH* mRNA, *U1* small nuclear RNA, *ASBEL*, or urothelial cancer associated 1 (*UCA1*) (16, 17) (Fig. 2*B*). In addition, RIP analysis with anti-HA antibody using lysates from HCT116 cells transfected with HA-tagged UHRF1 revealed that exogenously expressed UHRF1 was also associated with *UPAT* but not with antisense *UPAT*, *GAPDH* mRNA, *U1* small nuclear RNA, *ASBEL*, *UCA1*, or *AOC3* mRNA (Fig. 2*C* and Fig. S3 *C* and *D*). These results suggest that *UPAT* is associated with UHRF1 in colon cancer cells.

RIP assays using a series of UHRF1 deletion mutants revealed that a small region (amino acids 636–670) was required for the association of UHRF1 with *UPAT* (Fig. 2 *D–G* and Fig. S3*E*). Furthermore, we showed that a fragment consisting of amino acids 636–736 [termed the UBR (*UPAT*-binding region)] is sufficient to bind *UPAT*. We also attempted to delineate the region in *UPAT* that binds UHRF1 and found that both the 5′ and 3′ regions were required (Fig. 2 *D* and *H*).

It has been shown that UHRF1 plays a key role in the proliferation and survival of tumor cells (9). Indeed, Cell Titer-Glo assays revealed that knockdown of UHRF1 by siRNA caused a significant reduction in the growth of HCT116 and CCSC#P cells (Fig. 2 *I* and *J* and Fig. S3 *F* and *G*). Annexin V assays showed that knockdown of UHRF1 resulted in a marked

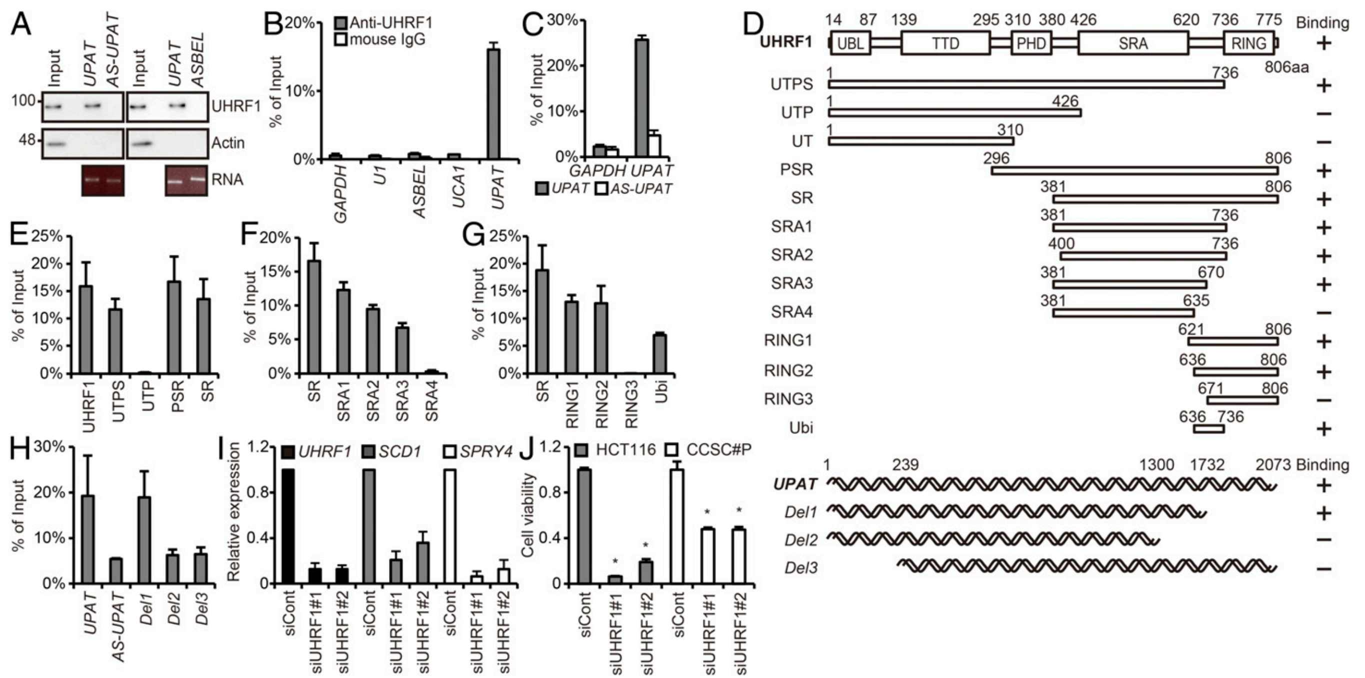

Fig. 2. *UPAT* is associated with UHRF1 in colon cancer cells. (*A*) Nuclear extracts from HCT116 cells were incubated with biotinylated sense, antisense *UPAT* (*Left*), or *ASBEL* (*Right*) generated in vitro, and proteins were precipitated with streptavidin beads and subjected to immunoblotting analysis with anti-UHRF1 or anti-Actin antibody. *AS-UPAT*, in vitro-transcribed antisense *UPAT*. Actin was used as a negative control. (*B*) Lysates from HCT116 cells were subjected to immunoprecipitation with anti-UHRF1 antibody or anti-mouse IgG antibody followed by qRT-PCR analysis to detect *UPAT* mRNA. *GAPDH* mRNA, *U1* small nuclear RNA, *ASBEL*, and *UCA1* were used as negative controls. Results are expressed as the mean ± SEM ($n = 3$). (*C*) Lysates from HCT116 cells transfected with sense (*UPAT*) or antisense (*AS-UPAT*) *UPAT* and HA-UHRF1 were subjected to immunoprecipitation with anti-HA antibody followed by RT-PCR analysis to detect *UPAT* mRNA. *AS-UPAT* and *GAPDH* were used as negative controls. Results are expressed as the mean ± SEM ($n = 3$). (*D*) Schematic representation of the UHRF1 protein (*Upper*) and *UPAT* (*Lower*). Mutants used in RIP (Fig. 2 *E–H*), immunoprecipitation (Fig. 3*E* and Fig. S4*H*), and ubiquitination (Fig. 4*A* and Fig. S5*B*) assays are also shown. (*E–G*) Lysates from HCT116 cells transfected with *UPAT* along with wild-type, mutant HA-UHRF1 (*E*), or mutant Flag-UHRF1 (*F* and *G*) were subjected to immunoprecipitation with anti-HA (*E*) or anti-Flag (*F* and *G*) antibody followed by RT-PCR analysis to detect *UPAT* and *U1* small nuclear RNA. Results are expressed as the mean ± SEM ($n = 3$). See also Fig. S3*E*. (*H*) Lysates from HCT116 cells transfected with wild-type or mutant *UPAT* and HA-UHRF1 were subjected to immunoprecipitation with anti-HA antibody followed by RT-PCR analysis to detect *UPAT* mRNA. Results are expressed as the mean ± SEM ($n = 3$). (*I*) qRT-PCR analysis of *UHRF1*, *SCD1*, and *SPRY4* expression in HCT116 cells transfected with siRNA targeting UHRF1. Results are expressed as the mean ± SEM ($n = 3$). (*J*) Viability of HCT116 and CCSC#P cells transfected with siRNA targeting UHRF1 was assessed by Cell Titer-Glo assays. Results are expressed as the mean ± SEM ($n = 3$). $^*P < 0.05$.

increase in the apoptotic death of HCT116 cells (Fig. S3*H*). Moreover, we found that UHRF1 mRNA expression was higher in colorectal tumors than in adjacent normal tissues (Fig. S3*I*). These results raise the possibility that UHRF1 associated with UPAT is involved in the growth and survival of colon tumor cells.

***UPAT* and UHRF1 Epigenetically Up-Regulate SPRY4.** We next examined whether UHRF1 is involved in the up-regulation of SCD1, SPRY4, PGM1, and/or GPRC5A in HCT116 cells. We found that knockdown of UHRF1 resulted in decreased expression of the SCD1 and SPRY4 mRNAs (Fig. 2*I* and Fig. S3*G*). By contrast, knockdown of UHRF1 did not affect the expression of PGM1 or GPRC5A (Fig. S3*J*). Chromatin immunoprecipitation (ChIP) assays using anti-UHRF1 antibody revealed that UHRF1 was associated with the *SPRY4* but not the *SCD1* promoter region (Fig. S3*K*). Furthermore, knockdown of *UPAT* significantly reduced the association of UHRF1 with the *SPRY4* promoter region (Fig. S3*K*). These results suggest that UHRF1 directly transactivates SPRY4 and that *UPAT* is required for this transactivation. By contrast, SCD1 may not be a direct target of UHRF1 but rather is up-regulated indirectly downstream of UHRF1 and *UPAT*. In addition, we found that knockdown of UPAT did not affect the stability of *SCD1* mRNA (Fig. S3*L*).

Covalent modifications of DNA and histones can influence transcriptional activity and thereby regulate cell proliferation, survival, and tumorigenesis (18, 19). To elucidate the mechanisms underlying UHRF1-mediated transactivation of *SPRY4*, we performed (hydroxymethylated) methylated DNA immunoprecipitation [(h)MeDIP] analyses using anti–5-hydroxymethylcytosine (5hmC) or anti–5-methylcytosine (mC) antibody. We detected 5hmC and 5mC in the intragenic regions of the *SPRY4* locus (Fig. S3 *M–O*). Knockdown of either UHRF1 or *UPAT* resulted in decreases in 5hmC levels but not in 5mC levels in the *SPRY4* locus. Furthermore, knockdown of tet methylcytosine dioxygenase 1 (TET1), an enzyme that catalyzes the oxidation of 5mC to 5hmC, led to a decrease in the expression of *SPRY4* (Fig. S3*P*). These results suggest that TET1-mediated methyl hydroxylation of the *SPRY4* gene is required for the expression of *SPRY4*.

It has recently been reported that knockdown of UHRF1 leads to a dramatic decrease in DNMT1 (20). Indeed, qRT-PCR and immunoblotting analyses revealed that knockdown of *UPAT* resulted in a drastic decrease in the levels of DNMT1 protein, but not mRNA, in HCT116 cells (Fig. S3*Q*). Consistent with this result, dot blot analysis showed that *UPAT* knockdown caused a decrease in the levels of 5mC (Fig. S3*R*).

We also investigated whether UPAT regulates histone modification but found that knockdown of UPAT did not affect the levels of H3K4Me3, H3K9Me3, H3K27Me3, or H3K36Me3 (Fig. S3*S*). On the other hand, we found that knockdown of UPAT led to increases in the phosphorylation of Histone H2AX-Ser-216 and H3-Ser10, which are markers of the DNA damage response (Fig. S3*T*). This is consistent with a previous report showing that UHRF1 depletion caused the activation of the DNA damage response pathway (21).

UPAT Interferes with β-TrCP1– and β-TrCP2–Mediated Ubiquitination and Degradation of UHRF1. It has been reported that the stability of UHRF1 is regulated by proteasome-mediated degradation (11,

PNAS

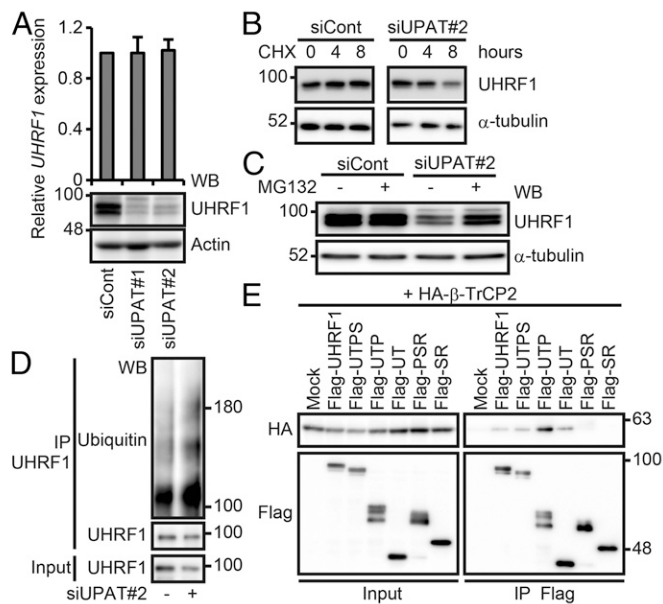

Fig. 3. *UPAT* stabilizes UHRF1 protein by interfering with its ubiquitination and degradation. (*A*, *Upper*) qRT-PCR analysis of *UHRF1* expression in HCT116 cells transfected with siRNA targeting *UPAT*. Results are expressed as the mean ± SEM ($n = 3$). (*A*, *Lower*) Cell lysates were subjected to immunoblotting analysis with anti-UHRF1 or anti-Actin antibody. Actin was used as a loading control. (*B*) HCT116 cells transfected with siRNA targeting *UPAT* were treated with CHX for the indicated times and then subjected to immunoblotting analysis with anti-UHRF1 or anti–α-tubulin antibody. α-tubulin was used as a loading control. (*C*) HCT116 cells transfected with siRNA targeting *UPAT* were cultured in the presence or absence of MG132 and then subjected to immunoblotting analysis with anti-UHRF1 or anti–α-tubulin antibody. α-tubulin was used as a loading control. (*D*) Lysates from HCT116 cells that had been transfected with siRNA targeting *UPAT* and treated with MG132 were subjected to immunoprecipitation with anti-UHRF1 antibody followed by immunoblotting analysis with anti-ubiquitin or anti-UHRF1 antibody. (*E*) Lysates from 293FT cells transfected with HA-tagged β-TrCP2 along with empty vector (Mock) or mutants of UHRF1 were immunoprecipitated (IP) with anti-Flag antibody followed by immunoblotting analysis with anti-HA or anti-Flag antibody.

22–24). We therefore investigated whether UPAT is involved in the regulation of UHRF1 expression levels in HCT116 cells. qRT-PCR and immunoblotting analyses revealed that knockdown of UPAT by siUPAT or antisense oligonucleotides resulted in a drastic decrease in the levels of UHRF1 protein but not mRNA (Fig. 3*A*). We found that knockdown of UPAT reduced the stability of UHRF1 protein in HCT116 cells treated with cycloheximide (CHX) (Fig. 3*B* and Fig. S4*A*). We also found that treatment of cells with the proteasome inhibitor MG132 inhibited the decrease in the UHRF1 protein levels caused by knockdown of UPAT (Fig. 3*C* and Fig. S4*B*). Furthermore, we found that knockdown of UPAT resulted in increased ubiquitination of UHRF1 (Fig. 3*D* and Fig. S4*C*). In addition, RNA-seq analyses of HCT116 cells in which UPAT or UHRF1 expression was suppressed revealed that the expression profile of UHRF1 knockdown cells closely resembled that of UPAT knockdown cells (*P* values = 2.24E-22 for up-regulated genes and 3.71E-21 for down-regulated genes) (Fig. S4*D* and Dataset S4). Taken together, these results suggest that UPAT stabilizes UHRF1 protein by interfering with its proteasome-mediated ubiquitination and degradation.

To identify the ubiquitin ligase targeting UHRF1 in colorectal cancer cells, we immunoprecipitated UHRF1 from HCT116 cell lysates and analyzed the coprecipitated proteins by liquid chromatography–mass spectrometry (Fig. S4*E* and Dataset S3). In agreement with a recent report (24), we identified peptides corresponding to two paralogues of the F-box protein β-TrCPs, β-TrCP1/β-transducin repeat containing E3 ubiquitin protein ligase (BTRC) and β-TrCP2/F-box and WD repeat domain containing 11 (FBXW11), in addition to peptides derived from known UHRF1 binding proteins, including tripartite motif containing 28 (TRIM28) (25) and histone deacetylase 1 (HDAC1) (26) (Dataset S3). Pull-down assays using lysates from 293FT cells transfected with UHRF1 along with β-TrCP1 or β-TrCP2 confirmed that UHRF1 coprecipitated with β-TrCP1 or β-TrCP2 (Fig. S4*F*). Furthermore, we found that UHRF1 coprecipitated with exogenously expressed β-TrCP1 or β-TrCP2, but not with the other F-box proteins tested, FBXW2, 4, 5, 7A, or 8 (Fig. S4*G*). Pull-down assays using a series of UHRF1 deletion fragments revealed that a fragment (amino acids 1–282) containing the ubiquitin-like (UBL) and tandem tudor (TTD) domains (Fig. 2*D*), which have been reported to be histone-binding domains, was required for interaction with β-TrCP1 and β-TrCP2 (Fig. 3*E* and Fig. S4*H*).

The above results suggest that β-TrCP1 and β-TrCP2 are ubiquitin ligases targeting UHRF1 for degradation. Indeed, overexpression of β-TrCP1 or β-TrCP2 resulted in the ubiquitination of UHRF1 in 293FT cells (Fig. S5*A*). In the absence of exogenous β-TrCP1 or β-TrCP2, ubiquitination of UHRF1 was not observed, suggesting that UHRF is not self-ubiquitinated. Furthermore, β-TrCP1– or β-TrCP2–induced ubiquitination of UHRF1 was barely detected when UPAT was overexpressed (Fig. S5*A*). In contrast, ubiquitination of UHRF1 was not affected by overexpression of UPAT-Del2 or -Del3 (Fig. 2*D*), which is unable to associate with UHRF1 (Fig. 4*A* and Fig. S5*B*). We also found that knockdown of UPAT barely induced the degradation of UHRF1 in cells transfected with an siRNA targeting β-TrCP1 or β-TrCP2 (Fig. 4*B* and Fig. S5*C*). Consistent with this, overexpression of β-TrCP1 or β-TrCP2 resulted in decreased expression of UHRF1 protein in HCT116 cells (Fig. S5*D*). Furthermore, overexpression of UPAT restored β-TrCP2–induced degradation of UHRF1 (Fig. 4*C* and Fig. S5*E*). On the other hand, overexpression of UPAT moderately inhibited the interaction between UHRF1 and β-TrCP1 or β-TrCP2 (Fig. S5*F*). Taken together, these results suggest that β-TrCP1and β-TrCP2 mediate the ubiquitination and degradation of UHRF1 and that UPAT interferes with β-TrCP1– and β-TrCP2–mediated ubiquitination of UHRF1.

Consistent with the finding that UPAT inhibits β-TrCP1– and β-TrCP2–mediated degradation of UHRF1, we found that growth suppression of HCT116 cells by siUPAT could be partially rescued by overexpression of UHRF1 (Fig. 4*D*).

UHRF1 Is Ubiquitinated at Lys-663. Because amino acids 636–670 of UHRF1 were required for its interaction with UPAT (Fig. 2 *D*–*G*), we hypothesized that UPAT may inhibit ubiquitination of this region of UHRF1. We generated mutant derivatives of UHRF1s in which Lys-639, -657, -659, -661, or -663 were replaced with Arg (UHRF1-K639R, -K657R, -K659R, -K661R, or -K663R; Fig. S5*G*) and examined their ubiquitination of each by transfecting these together with β-TrCP1 and β-TrCP2 and ubiquitin into 293T cells. We found that ubiquitination was normal for all fragments except UHRF1-K663R, whose ubiquitination was attenuated (Fig. 4*E* and Fig. S5*H*). Moreover, overexpression of β-TrCP2 did not induce the degradation of UHRF1-K663R (Fig. 4*F*). These results suggest that UHRF1 may be ubiquitinated at Lys-663 by β-TrCP1 and β-TrCP2.

Discussion

A screen for genes required for the tumorigenicity of colon tumor cells identified the lncRNA UPAT, which is encoded by a pseudogene of the AOC3 gene. We found that UPAT, but not AOC3, is required for the survival and tumorigenicity of colon tumor cells. Moreover, we found that UPAT interacts with UHRF1 and interferes with its β-TrCP1– and β-TrCP2–mediated ubiquitination and degradation. Consistent with previous reports (27–31), we confirmed that UHRF1 plays a critical role in the growth and survival of colon tumor cells. Thus, our findings suggest that UPAT-mediated stabilization of UHRF1 is critical for the proliferation and tumorigenicity of colon tumor cells.

In line with the above notion, RNA-seq analyses of HCT116 cells in which UPAT or UHRF1 expression was suppressed

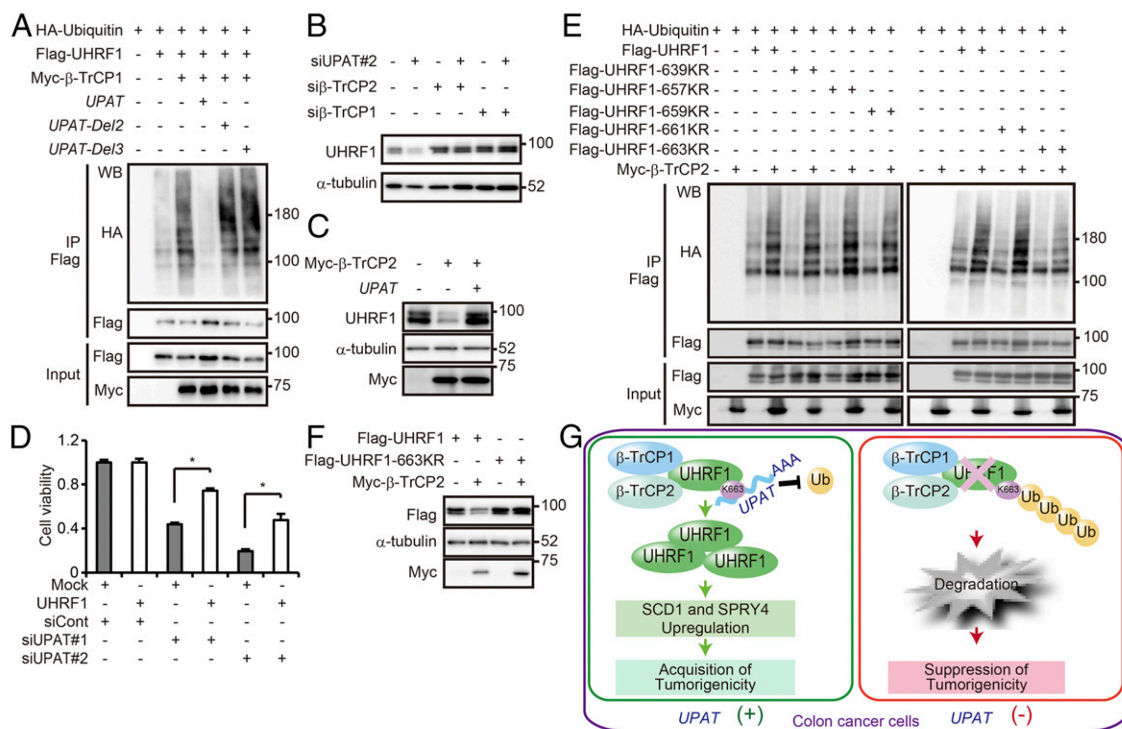

Fig. 4. *UPAT* inhibits β-TrCP1– and β-TrCP2–mediated polyubiquitination of UHRF1. (*A*) Lysates from 293FT cells that had been transfected with the indicated expression constructs and treated with MG132 were subjected to immunoprecipitation with anti-Flag antibody followed by immunoblotting analysis with anti-HA, anti-Flag, or anti-Myc antibody. See also Fig. S5*B*. (*B*) Lysates from HCT116 cells transfected with siRNA targeting β-TrCP1 or β-TrCP2 and/or siUPAT were subjected to immunoblotting analysis with anti-UHRF1 or anti–α-tubulin antibody. α-tubulin was used as a loading control. (*C*) Lysates from HCT116 cells that had been transfected with Myc-tagged β-TrCP2 and/or *UPAT* were subjected to immunobotting analysis with anti-UHRF1, anti–α-tubulin, or anti-Myc antibody. α-tubulin was used as a loading control. (*D*) Viability of HCT116 cells transfected with UHRF1 and/or siRNA targeting *UPAT* was assessed by Cell Titer-Glo assays. Results are expressed as the mean ± SEM (n = 3). *$P < 0.05$. (*E*) Lysates from 293FT cells transfected with the indicated expression constructs and treated with MG132 were immunoprecipitated (IP) with anti-Flag antibody followed by immunoblotting analysis with anti-HA, anti-Flag, or anti-Myc antibody. (*F*) Lysates from 293FT cells that had been transfected with the indicated expression constructs were subjected to immunoblotting analysis with anti-Flag, anti–α-tubulin, or anti-Myc antibody. α-tubulin was used as a loading control. (*G*) *UPAT* binds to UHRF1 and interferes with its β-TrCP1– and β-TrCP2–mediated polyubiqutination. UHRF1 stabilized by *UPAT* up-regulates SCD1 and SPRY4, which are required for the survival of colon cancer cells.

revealed that the expression profile of UHRF1 knockdown cells closely resembled that of UPAT knockdown cells (P values = 2.24E-22 for up-regulated genes and 3.71E-21 for down-regulated genes). However, we found many genes that are regulated by UPAT in an UHRF1-independent manner. Thus, UPAT may also have important target molecules other than UHRF1.

Our RNA-seq analyses revealed that RAS-, CDH1-, and hypoxia-related genes are down-regulated in *UPAT* knockdown cells. Indeed, our qRT-PCR analysis showed that siRNA knockdown of *UPAT* resulted in a marked decrease in SCD1, SPRY4, PGM1, and GPRC5A expression. Furthermore, we found that *UPAT*-mediated up-regulation of these genes is required for the survival of colon cancer cells. Consistent with our results, SCD1, the main enzyme involved in the synthesis of monounsaturated fatty acids, has been shown to be required for cancer cell proliferation, survival, transformation to cancer (32), and cancer spheroids propagation (33). The Sprouty family of proteins, key regulators of ERK signaling, has been shown to be able to function as negative or positive regulators of tumor development and/or progression in a cell type-dependent manner (34). PGM1 is known to be induced under hypoxic conditions and promotes cancer cell survival (35). In addition, it has been shown that GPRC5A is a modifier of breast cancer risk in breast cancer (BRCA)-mutation carriers and GPRC5A inactivation negatively affects BRCA1-mediated DNA repair (36). We found that UHRF1 is involved in the up-regulation of *SCD1* and *SPRY4* but not of *PGM1* and *GPRC5A*. Thus, *UPAT* may also inhibit apoptotic cell death by mechanisms other than UHRF1 protein stabilization. We further showed that UHRF1 is associated with the *SPRY4* but not the *SCD1* promoter region. Thus, UHRF1 may directly transactivate *SPRY4*. We also found that UHRF1 increases 5hmC levels in the *SPRY4* gene and thereby enhances its expression. It remains to be clarified how UHRF1 increases the level of 5hmC but not 5mC. It also remains to be investigated whether the epigenetic function of UHRF1 requires the formation of a UHRF1–*UPAT* complex.

The β-TrCP E3 ubiquitin ligases, β-TrCP1 and β-TrCP2, are known to play critical roles in the regulation of diverse biological processes, including cell cycle progression, differentiation, and various signal transduction pathways (37). β-TrCPs have been shown to ubiquitinate a number of important proteins, including β-catenin, cell division cycle 25 (Cdc25), RE1-silencing transcription factor (REST), mouse double minute 2 (Mdm2), and IκBβ (37). Furthermore, it has recently been reported that UHRF1 is ubiquitinated by the β-TrCP E3 ubiquitin ligases and degraded by the proteasome and that this process is accelerated in response to DNA damage (24). Consistent with these results, we found that β-TrCP1 and β-TrCP2 mediate the ubiquitination and degradation of UHRF1. Moreover, we found that *UPAT* interferes with the β-TrCP1– and β-TrCP2–mediated ubiquitination and degradation of UHRF1. In line with the results of Chen et al., our pull-down assays showed that β-TrCP1 and β-TrCP2 bind to amino acids 1–282 of UHRF1, which contain the UBL and TTD domains. Furthermore, we found that β-TrCP1 and β-TrCP2 ubiquitinates Lys-663 of UHRF1. Intriguingly, our RIP assays revealed that *UPAT* interacts with the UBR domain of UHRF1 (amino acids 636–736), which contains Lys-663. These results raise the possibility that *UPAT* may inhibit ubiquitination of UHRF1 by

masking its ubiquitination site, Lys-663, but not by competing with β-TrCP1 and β-TrCP2 for binding to UHRF1.

It has recently been reported that the lncRNA HOX transcript antisense RNA (HOTAIR) functions as a scaffold that enhances E3-mediated ubiquitination and degradation of substrate proteins (38). It has also been shown that lincRNA-p21, originally identified as a p53-inducible lncRNA that mediates p53-induced apoptosis in mouse cells (39), is a hypoxia-responsive lncRNA that plays a critical role in the regulation of hypoxia-enhanced glycolysis by inhibiting von Hippel–Lindau (VHL)-mediated hypoxia inducible factor 1 alpha (HIF1α) ubiquitination (40). Thus, although lncRNAs are best known to regulate transcription by recruiting chromatin remodeling complexes to specific genomic regions (2), our findings, together with these earlier findings, indicate that there is a class of lncRNAs that regulate protein ubiquitination and degradation.

In summary, we have shown that the lncRNA *UPAT* alleviates apoptotic cell death by interfering with β-TrCP1– and β-TrCP2–mediated ubiquitination and degradation of UHRF1 (Fig. 4*G*). Our findings suggest that the UHRF1–*UPAT* axis may be a promising molecular target for colon cancer therapies.

Materials and Methods

Details are provided in *SI Materials and Methods*.

qRT-PCR Analysis. Total RNA was isolated using the Total RNA Isolation kit (MACHEREY-NAGEL) and treated with DNase I (TAKARA). One microgram RNA was reverse transcribed using PrimeScript RT Master Mix (TAKARA, RR036A). qRT-PCR analysis of cDNA was performed on a LightCycler 480 (Roche Applied Science) using Syber Green PCR mastermix (Applied Biosystems). Prior to fold-change calculation, the values were normalized to the signal generated from *β-actin* mRNA. Primer sequences are listed in Dataset S5.

Clinical Samples. Following written consent, resected colon cancer specimens were obtained from patients who underwent surgical treatment at the Department of Surgical Oncology, The University of Tokyo Hospital as approved by the Institutional Review Board.

ACKNOWLEDGMENTS. This work was supported by the Research Program of Innovative Cell Biology by Innovative Technology (Integrated Systems Analysis of Cellular Oncogenic Signaling Networks), Grants-in-Aid for Scientific Research on Innovative Areas (Integrative Research on Cancer Microenvironment Network), the Project for Development of Innovative Research on Cancer Therapeutics, and the Takeda Science Foundation and in part by the Global Centers of Excellence Program (Integrative Life Science Based on the Study of Biosignaling Mechanisms), Ministry of Education, Culture, Sports, Science and Technology, Japan.

1. Ulitsky I, Bartel DP (2013) lincRNAs: Genomics, evolution, and mechanisms. *Cell* 154(1):26–46.
2. Rinn JL, Chang HY (2012) Genome regulation by long noncoding RNAs. *Annu Rev Biochem* 81:145–166.
3. Amaral PP, Mattick JS (2008) Noncoding RNA in development. *Mamm Genome* 19(7-8):454–492.
4. Mercer TR, et al. (2008) Noncoding RNAs in long-term memory formation. *Neuroscientist* 14(5):434–445.
5. Flynn RA, Chang HY (2014) Long noncoding RNAs in cell-fate programming and reprogramming. *Cell Stem Cell* 14(6):752–761.
6. Batista PJ, Chang HY (2013) Long noncoding RNAs: Cellular address codes in development and disease. *Cell* 152(6):1298–1307.
7. Lee JT (2012) Epigenetic regulation by long noncoding RNAs. *Science* 338(6113):1435–1439.
8. Cech TR, Steitz JA (2014) The noncoding RNA revolution-trashing old rules to forge new ones. *Cell* 157(1):77–94.
9. Bronner C, Krifa M, Mousli M (2013) Increasing role of UHRF1 in the reading and inheritance of the epigenetic code as well as in tumorogenesis. *Biochem Pharmacol* 86(12):1643–1649.
10. Du Z, et al. (2010) DNMT1 stability is regulated by proteins coordinating deubiquitination and acetylation-driven ubiquitination. *Sci Signal* 3(146):ra80.
11. Qin W, Leonhardt H, Spada F (2011) Usp7 and Uhrf1 control ubiquitination and stability of the maintenance DNA methyltransferase Dnmt1. *J Cell Biochem* 112(2):439–444.
12. Chassande O, Renard S, Barbry P, Lazdunski M (1994) The human gene for diamine oxidase, an amiloride binding protein. Molecular cloning, sequencing, and characterization of the promoter. *J Biol Chem* 269(20):14484–14489.
13. Imamura Y, et al. (1997) Human retina-specific amine oxidase (RAO): cDNA cloning, tissue expression, and chromosomal mapping. *Genomics* 40(2):277–283.
14. Salmi M, Jalkanen S (1992) A 90-kilodalton endothelial cell molecule mediating lymphocyte binding in humans. *Science* 257(5075):1407–1409.
15. Yanagida S, et al. (2013) ASBEL, an ANA/BTG3 antisense transcript required for tumorigenicity of ovarian carcinoma. *Sci Rep* 3:1305.
16. Wang F, Li X, Xie X, Zhao L, Chen W (2008) UCA1, a non-protein-coding RNA up-regulated in bladder carcinoma and embryo, influencing cell growth and promoting invasion. *FEBS Lett* 582(13):1919–1927.
17. Wang X-S, et al. (2006) Rapid identification of UCA1 as a very sensitive and specific unique marker for human bladder carcinoma. *Clin Cancer Res* 12(16):4851–4858.
18. Goldberg AD, Allis CD, Bernstein E (2007) Epigenetics: A landscape takes shape. *Cell* 128(4):635–638.
19. Sasaki H, Matsui Y (2008) Epigenetic events in mammalian germ-cell development: Reprogramming and beyond. *Nat Rev Genet* 9(2):129–140.
20. Rothbart SB, et al. (2012) Association of UHRF1 with methylated H3K9 directs the maintenance of DNA methylation. *Nat Struct Mol Biol* 19(11):1155–1160.
21. Tien AL, et al. (2011) UHRF1 depletion causes a G2/M arrest, activation of DNA damage response and apoptosis. *Biochem J* 435(1):175–185.
22. Felle M, et al. (2011) The USP7/Dnmt1 complex stimulates the DNA methylation activity of Dnmt1 and regulates the stability of UHRF1. *Nucleic Acids Res* 39(19):8355–8365.
23. Ma H, et al. (2012) M phase phosphorylation of the epigenetic regulator UHRF1 regulates its physical association with the deubiquitylase USP7 and stability. *Proc Natl Acad Sci USA* 109(13):4828–4833.
24. Chen H, et al. (2013) DNA damage regulates UHRF1 stability via the SCF(β-TrCP) E3 ligase. *Mol Cell Biol* 33(6):1139–1148.
25. Quenneville S, et al. (2011) In embryonic stem cells, ZFP57/KAP1 recognize a methylated hexanucleotide to affect chromatin and DNA methylation of imprinting control regions. *Mol Cell* 44(3):361–372.
26. Unoki M, Nishidate T, Nakamura Y (2004) ICBP90, an E2F-1 target, recruits HDAC1 and binds to methyl-CpG through its SRA domain. *Oncogene* 23(46):7601–7610.
27. Li X-L, Xu J-H, Nie J-H, Fan S-J (2012) Exogenous expression of UHRF1 promotes proliferation and metastasis of breast cancer cells. *Oncol Rep* 28(1):375–383.
28. Unoki M, et al. (2009) UHRF1 is a novel molecular marker for diagnosis and the prognosis of bladder cancer. *Br J Cancer* 101(1):98–105.
29. Unoki M, et al. (2010) UHRF1 is a novel diagnostic marker of lung cancer. *Br J Cancer* 103(2):217–222.
30. Sabatino L, et al. (2012) UHRF1 coordinates peroxisome proliferator activated receptor gamma (PPARG) epigenetic silencing and mediates colorectal cancer progression. *Oncogene* 31(49):5061–5072.
31. Babbio F, et al. (2012) The SRA protein UHRF1 promotes epigenetic crosstalks and is involved in prostate cancer progression. *Oncogene* 31(46):4878–4887.
32. Igal RA (2010) Stearoyl-CoA desaturase-1: A novel key player in the mechanisms of cell proliferation, programmed cell death and transformation to cancer. *Carcinogenesis* 31(9):1509–1515.
33. Noto A, et al. (2013) Stearoyl-CoA desaturase-1 is a key factor for lung cancer-initiating cells. *Cell Death Dis* 4:e947.
34. Masoumi-Moghaddam S, Amini A, Morris DL (2014) The developing story of Sprouty and cancer. *Cancer Metastasis Rev* 33(2-3):695–720.
35. Pelletier J, et al. (2012) Glycogen synthesis is induced in hypoxia by the hypoxia-inducible factor and promotes cancer cell survival. *Front Oncol* 2:18.
36. Sokolenko AP, et al. (2014) High prevalence of GPRC5A germline mutations in BRCA1-mutant breast cancer patients. *Int J Cancer* 134(10):2352–2358.
37. Frescas D, Pagano M (2008) Deregulated proteolysis by the F-box proteins SKP2 and beta-TrCP: Tipping the scales of cancer. *Nat Rev Cancer* 8(6):438–449.
38. Yoon J-H, et al. (2013) Scaffold function of long non-coding RNA HOTAIR in protein ubiquitination. *Nat Commun* 4:2939.
39. Huarte M, et al. (2010) A large intergenic noncoding RNA induced by p53 mediates global gene repression in the p53 response. *Cell* 142(3):409–419.
40. Yang F, Zhang H, Mei Y, Wu M (2014) Reciprocal regulation of HIF-1α and lincRNA-p21 modulates the Warburg effect. *Mol Cell* 53(1):88–100.
41. Kozuka-Hata H, et al. (2012) Phosphoproteome of human glioblastoma initiating cells reveals novel signaling regulators encoded by the transcriptome. *PLoS One* 7(8):e43398.
42. Ficz G, et al. (2011) Dynamic regulation of 5-hydroxymethylcytosine in mouse ES cells and during differentiation. *Nature* 473(7347):398–402.
43. Weber M, et al. (2005) Chromosome-wide and promoter-specific analyses identify sites of differential DNA methylation in normal and transformed human cells. *Nat Genet* 37(8):853–862.