Recognition and Suppression of Transfected Plasmids by Protein ZNF511-PRAP1, a Potential Molecular Barrier to Transgene Expression

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Nonviral vectors present considerable advantages over viral counterparts in gene transfer. However, the poor expression efficiency of the transfected genes poses a challenge for their use in gene therapy, primarily due to the inability of these vectors to overcome various barriers, including the biological barriers. Here, we report that ZNF511-PRAP1 may be involved in the recognition and inactivation of transfected plasmids. ZNF511-PRAP1 is induced by transfection of plasmid DNA and suppresses the transcription of transfected plasmids. It binds directly to the p21 promoter in transfected plasmids but not the endogenous counterpart. Similarly, ZNF511-PRAP1 suppresses the expression of the green fluorescent protein reporter gene on transiently transfected plasmids but not an integrated red fluorescence reporter gene with the same cytomegalovirus (CMV) promoter. Therefore, ZNF511-PRAP1 is able to differentiate between exogenous/nonintegrated and endogenous/integrated DNA. The suppression by ZNF511-PRAP1 is independent of DNA methylation and can be abolished by trichostatin A (TSA) treatment and knockdown of HDAC2 and/or ZNF511-PRAP1. Furthermore, ZNF511-PRAP1 interacts directly with HDAC2. Our results revealed that transfected plasmids are recognized by ZNF511-PRAP1 and suppressed by a repressor complex comprising ZNF511-PRAP1 and HDAC2 and suggest that ZNF511-PRAP1 could play a role as a potential molecular barrier in nonviral transgene expression.

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INTRODUCTION

The success of gene therapy depends on delivering genes to target cells *in vivo*.[1–3] Methods to transfer genes fall into two classes: viral and nonviral delivery systems. Nonviral vectors such as cationic lipids and polymers can potentially have considerable advantages, including decreased cytotoxicity, better control of the molecular composition, flexibility in the size of target genes, and lower immunogenicity compared to viral analogues.[1,2,4] However, the efficiency of gene delivery and expression by these nonviral methods is significantly lower compared with viral systems. The inefficiency is primarily due to the inability of these vectors to overcome various barriers.[3,5,6] These physical, chemical, and biological obstacles are present throughout the gene delivery and expression pathway: from lipoplexes formation to nuclear localization of plasmid and its transcription in the nucleus.[6–9] The biological barrier, such as innate immune responses, has been extensively studied. It can be activated by nonviral vectors to levels comparable to or even higher than viral vectors[10–12] and is mediated by transmembrane Toll-like receptor 9 signaling pathway.[13,14] Furthermore, a recent study has revealed that the low efficiency of expression in nonviral systems arises from the low transcription efficiency in the nucleus.[15] Therefore, understanding the mechanisms underlying the suppression of transcription of exogenous DNA introduced into the cell is important to develop newer strategies to improve the efficiency of nonviral gene delivery and expression.[2,3,16]

In this study, we report that ZNF511-PRAP1 is a potential molecular barrier to transgene expression. It can recognize and suppress the transcription of genes in transfected plasmids. *ZNF511-PRAP1* was identified during the cloning of *PRAP1* (proline-rich acidic protein 1), a gene highly expressed in epithelial cells of the intestine, kidney, liver, and cervix and involved in the regulation of cell fate.[17] *ZNF511-PRAP1* is a chimeric transcript that incorporates both *ZNF511* and *PRAP1*, and encodes a fusion protein between the N-terminus of the zinc finger protein ZNF511 and the entire PRAP1 protein. We provide evidence that ZNF511-PRAP1 is induced by plasmid DNA transfections, binds directly to and suppresses the transcriptional activities of exogenous promoters present in the plasmids, but not endogenous counterparts, through a mechanism that involves histone deacetylase activity and HDAC2.

RESULTS

Identification of *ZNF511-PRAP1* by 5′ RACE

The *ZNF511-PRAP1* transcript was identified through a 5′RACE strategy using RNA from HCT116, a colon cancer cell line, as template (**Figure 1**). The primers used for the amplifications are listed in **Table 1**. The open reading frame of *ZNF511-PRAP1* has 930 nucleotides and 8 exons (GenBank accession no: EU937518). The first exon of *ZNF511-PRAP1* comprises the entire second exon of *ZNF511* and sequences immediately upstream; the second to

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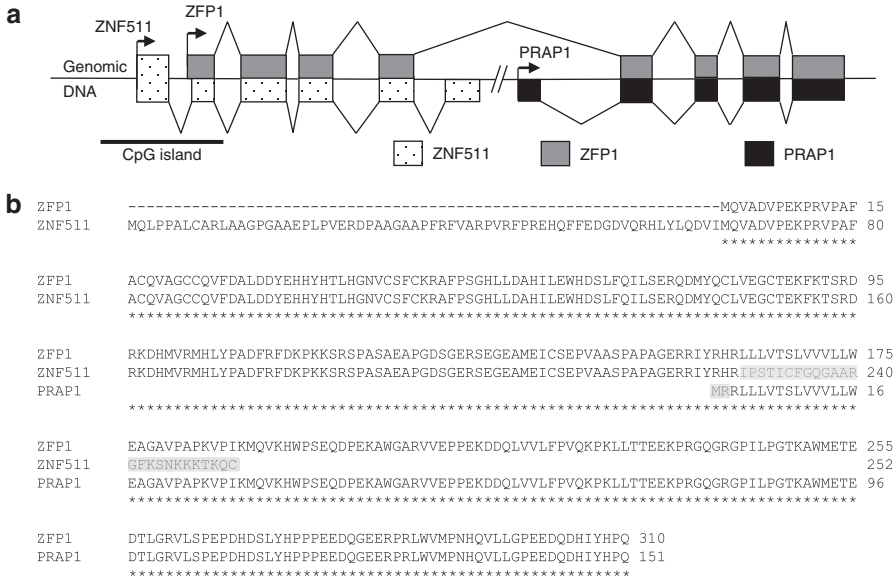

Figure 1 Map of the *ZNF511-PRAP1* locus and amino acid comparison. (**a**) Genomic structure of *ZNF511-PRAP1* identified by 5′ RACE of *PRAP1*. Boxes indicate exons. The *ZNF511*, *ZNF511-PRAP1* and *PRAP1* transcripts are outlined. (**b**) Amino acid comparison of ZNF511, ZNF511-PRAP1 and PRAP1. Asterisks (*) indicate amino acids identical to either ZNF511 or PRAP1; grayed amino acids are not present in ZNF511-PRAP1. ZFP1: ZNF511-PRAP1.

fourth exons are identical to the third to fifth exons of *ZNF511*;[18] the remaining four exons are identical to the second to fifth exons of *PRAP1* (**Figure 1a**). Therefore, the transcript was named as *ZNF511-PRAP1*, which was approved by the HGNC (The HUGO Gene Nomenclature Committee). Of the 310 amino acids encoded, 161 amino acids at N-terminus are identical to those of ZNF511, while the 148 amino acids at C-terminus are identical to those of PRAP1. The single amino acid at position 162 is common to both ZNF511 and PRAP1 (**Figure 1b**). The intergenic region between the two genes is about 35 kb. Hence, *ZNF511-PRAP1* is a chimeric transcript of *ZNF511* and *PRAP1*.

ZNF511-PRAP1 represses the transcription of promoters present in transfected plasmids but not endogenous promoters

We tested the hypothesis that ZNF511-PRAP1 may function as a transcription factor since it was detected in the nuclear fraction of cells by western blotting (**Figure 2a**) and contains zinc finger domains which are present in many transcription factors. *ZNF511-PRAP1* was fused to the GAL4 expression vector and the construct (p31GAL4ZFP1) was co-transfected into HCT116 cells together with the luciferase expression vector (pGBDluc) containing 5 GAL4 binding sites in its promoter. Luciferase assays showed that ZNF511-PRAP1 repressed the reporter gene expression of pGBD-luc (*Firefly* luciferase) in a dose-dependent manner with increasing concentrations of *ZNF511-PRAP1* plasmids (**Figure 2b**, left). It was noted that ZNF511-PRAP1 also suppressed the expression of *Renilla* luciferase to a similar level as *Firefly* luciferase, despite the absence of GAL4 binding sites at the promoter region of the *Renilla* luciferase plasmid (pRL-TK) (**Figure 2b**, middle). This suggests that ZNF511-PRAP1 could bind to promoters present in the plasmids in a nonsequence specific manner. Real-time reverse transcriptase (RT)-PCR analysis of *Renilla* mRNA expression showed that ZNF511-PRAP1 repressed the activity of *Renilla* at the transcriptional level (**Figure 2b**, right). The promoters driving the *Firefly* and *Renilla* luciferase plasmids are SV40 (Simian virus 40, with GAL4 binding sites at 5′ upstream region) and TK (Thymidine kinase**,** without GAL4 binding sites), respectively. The overexpression of *ZNF511-PRAP1* (p31ZFP1) was also able to repress *Firefly* luciferase activity in the absence of GAL4 binding sites in HCT116, as well as HeLa, HepG2 and Hep3B (**Figure 2c**). Similarly, overexpression of *ZNF511-PRAP1* could suppress the cytomegalovirus (CMV) promoter driving the expression of the green fluorescent protein, GFP (**Figure 2d**, left). The suppression of GFP expression was confirmed by fluorescent microscopy which showed fewer fluorescent cells in cells co-transfected with p31ZFP1 (**Figure 2d**, right) compared to pcDNA3.1 controls (**Figure 2d**, middle). The results suggest that ZNF511-PRAP1 is able to bind to promoter sequences in a nonsequence specific manner to suppress gene transcription.

To confirm that ZNF511-PRAP1 suppresses the transcription of transfected plasmids in a promoter dependent but nonsequence specific manner, we cotransfected p31ZFP1 with luciferase reporter plasmids containing p21, HDAC1, c-MYC or cyclin A2 (CA2) promoters. The luciferase activities driven by all these promoters were suppressed to a level of about 50–60% of controls (**Figure 3a**, upper panel).

Given that ZNF511-PRAP1 was able to repress all exogenous promoters examined, we proceeded to determine whether ZNF511-PRAP1 could also suppress the corresponding endogenous promoters in the cell. This was done by determining the effect of ZNF511-PRAP1 overexpression on the endogenous *p21*, *HDAC1*, *c-MYC* and *CA2* mRNA levels. To ensure that only cells containing the ZNF511-PRAP1 overexpression plasmid was examined, we cloned p31ZFP1 or pcDNA3.1 into the *GFP* expression vector (pEGFP-C2) so that *ZNF511-PRAP1* and *GFP*

Table 1 Sequences of oligonucleotides used in this study

Usage	Gene	Name	Sequence (5′→3′)
5′ RACE	*ZFN511-PRAP1*	PRAP1RACE1	GGT CCT GCT CTG AGG GCC AGT GTT
		PRAP1RACE3	CCC TGT CCA GAA GCC GAA ACT CT
pcDNA3.1	*ZFN511-PRAP1* ORF cloning	PRAP1AcF1	CCG GAA TTC GCC ACC ATG CAG GTG GCC GAC
		PRAP1AcR1	GCT CTA GAC TAC TGG GGG TGG TAG
p3xFlag-CMV		ZFPFH3F	T AAG CTT ACC ATG CAG GTG GCC GAC GTG
		ZFPRB1	GCC CCT GGA TCC CTA CTG G
GAL4 vector	*ZFN511-PRAP1*	ZFPRAPF1	GAATCCATGCAG GTGGCCGACGTG
		ZFPRAPR2	GAATTCCTGGAGCCCTACTGGGG
EMSA	*p21*	p21pGelshift1	TTGCCTTTG TTGACATTAG CTTGCCCTTC AGTTGC
		p21pGelshift2	GCAACTGAAGGGCAAGCTAATGTCAACAAAGGCAA
	LUC	LucGelshift1	CTGAATACAA ATCACAGAAT CGTCGTATGC AGTGA
		LucGelshift2	TCACTGCATACGACGATTCTGTGATTTGTATTCAG
	pGL3	pGL3Gelshift1	AACTGTTGGG AAGGGCGATC GGTGCGGGCC TCTTC
		pGL3Gelshift2	GAAGAGGCCCGCACCGATCGCCCTTCCCAACAGTT
ChIP	*LUC*	LUCF	CTGTGTGTGAGAGGTCC
		LUCR	CTTGACTGGCGACGTA
	p21	p21F	AAC TCG GCC AGG CTC AGC
		p21Rg	TCG GTG CCT CGG CGA ATC
		p21Rp	GTC GAC GGT ATC GAT AAG C
Bisulfite sequencing	*SV40* promoter	SV40BGS1	TTG YGA TTT GTA TTT TAA TTA GTT
		SV40BGS2	TTC CAT AAT AAC TTT ACC AAC AA
Duplex siRNA	*HDAC2*	siHDAC2A	UUU AGC AUG ACC UUU GAC UGU UAG A
		siHDAC2C	ACU AGG UUG AUA CAU CUC CAU CAC C
	ZFN511-PRAP1	siZFP	CUA CAG ACA UAG GCU CCU CC
Real-time RT-PCR	*HDAC1*	HDAC1LCF	AACTGGGGACCTACGG
		HDAC1LCR	ACTTGGCGTGTCCTT
	p21	P21LCF	ATTAGCAGCGGAACAAGGAGTCAGACAT
		P21LCR	CTGTGAAAGACACAGAACAGTACAGGGT
	ZFN511-PRAP1	PRAP1AF1	CGG ACT TCC GGT TTG ATA AGC
		P1AE4R1	TG CTG GGA CTG CAC CTG CCT CCC
	c-Myc	cMycF	AAC ACC CGA GCA AGG ACG
		cMycR	ATA CGG CTG CAC CGA GTC
	Cyclin A2	CA2F	CGC CTG CTA GCA TTG CAG C
		CA2R	GGA GGA ACG GTG ACA TGC

Abbreviations: ChIP, chromatin immunoprecipitation; EMSA, electrophoretic mobility shift assay; ORP, open reading frame; RT, reverse transcriptase; siRNA, small interfering RNA.

are expressed in separate cistrons. This allowed for the use of GFP expression as a marker of ZNF511-PRAP1 overexpression. HCT116 cells transfected with p31-GFP or p31ZFP1-GFP were sorted for GFP expression by flow cytometry. RNA or protein was extracted from an equal number of sorted cells. The overexpression of ZNF511-PRAP1 in HCT116 cells was not able to suppress the endogenous expression of p21, HDAC1, c-MYC or CA2 mRNA, as determined by quantitative real-time RT-PCR (**Figure 3a**, middle panel), nor p21 and HDAC1 protein levels, as determined by western blot analysis (**Figure 3a**, lower panel). The expression of several other endogenous proteins was also examined to confirm that ZNF511-PRAP1 overexpression did not significantly affect endogenous protein expression (**Figure 3b**). Hence, the suppression of transcription by ZNF511-PRAP1 appears to be specific for transfected plasmids and does not apply to endogenous promoters. The overexpression of ZNF511-PRAP1 was confirmed by western blotting (**Figure 3c**).

ZNF511-PRAP1 recognizes and binds to plasmid DNA in a nonsequence specific manner

The above results showed that ZNF511-PRAP1 suppresses expression of promoters on transfected plasmids but not endogenous promoters. We examined the interaction of ZNF511-PRAP1 and plasmid DNA in gel shift assays. Our results showed that ZFN511-

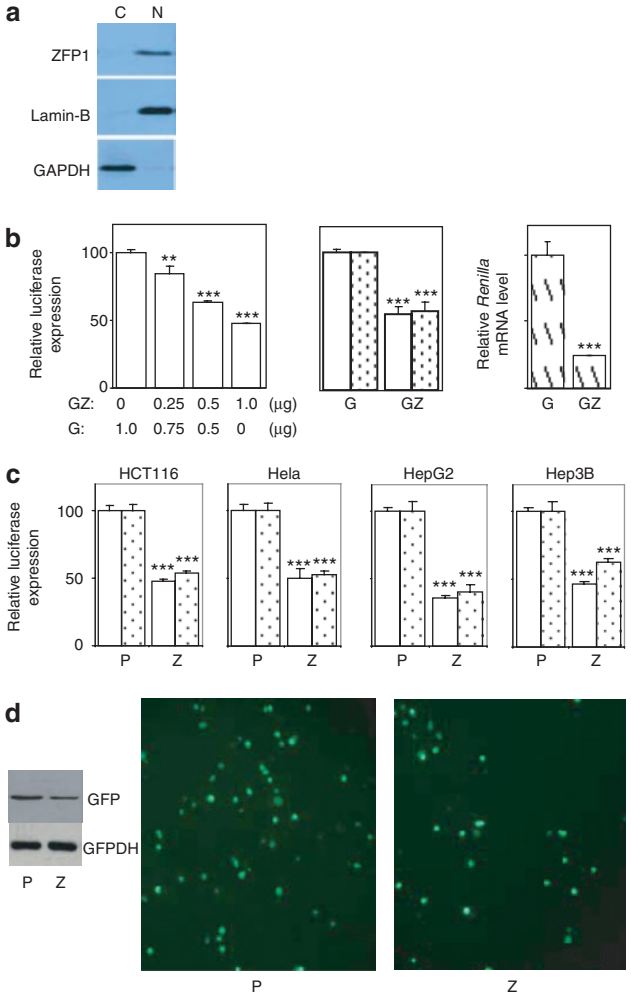

Figure 2 ZNF511-PRAP1 suppresses the transcriptional activities of promoters in transfected plasmids in various cell lines. (**a**) Nucleus localization of ZNF511-PRAP1 in HeLa. (**b**) Repression of *Firefly* luciferase by ZNF511-PRAP1 increased with increasing amounts of ZNF511-PRAP1 expression plasmids in HCT116 (left), ZNF511-PRAP1 repressed both the *Firefly* and *Renilla* luciferase activities (middle) and the mRNA of *Renilla* was suppressed by ZNF511-PRAP1 (right). (**c**) The luciferase activities were also suppressed by overexpression of p31ZFP1 in HCT116, HeLa, HepG2 and Hep3B. (**d**) Green fluorescent protein (GFP) was suppressed by ZNF511-PRAP1 (left). The fluorescent cells were fewer in cells co-transfected with p31ZFP1 (right) compared to pcDNA3.1 controls (middle). Experimental differences were tested for statistical significance using the two-tailed *t*-test. *P* value of <0.05 was considered to be significant. $^{**}P < 0.01$. $^{***}P < 0.001$. G: p31GAL4, GZ: p31GAL4ZFP1, P: pcDNA3.1, Z: p31ZFP1; ZFP1: ZNF511-PRAP1. Open rectangle, *Firefly* luciferase activity, filled rectangle, *Renilla* luciferase activity. C, cytoplasm, N, nucleus. The *Firefly* and *Renilla* luciferase activities were measured as relative luminescence units (RLU) and normalized to total protein content. The relative expression level is expressed as a percentage of the standardized luciferase activity from the sample transfected with ZNF511-PRAP1 divided by that from the respective control vector. Representative results of at least two separate experiments are shown with the mean ± SEM of relative activities. Each experiment was performed in triplicates.

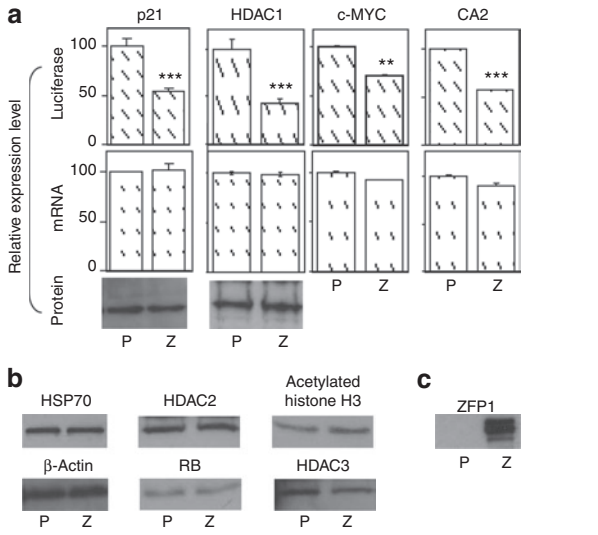

Figure 3 ZNF511-PRAP1 does not suppress the transcriptional activities of endogenous promoters. (**a**) *Firefly* luciferase activity assay of p21, HDAC1, c-MYC and cyclin A2 (CA2) promoters (upper panel), their endogenous expression by real-time reverse transcriptase-PCR (middle panel) and western blotting of p21 and HDAC1 (lower panel) with equal number of transfected cells sorted with green fluorescent protein. (**b**) Western blot of other proteins with equal number of transfected cells. (**c**) ZNF511-PRAP1 overexpression. P, pcDNA3.1; Z, p31ZFP1; ZFP1, ZNF511-PRAP1.

PRAP1 formed DNA-protein complexes with p21 DNA fragment (**Figure 4a**, upper panel), suggesting that ZFN511-PRAP1 is able to bind to this promoter sequence. We also observed binding of *in vitro* translated ZFN511-PRAP1 and the other labeled DNA fragments from either the backbone of pGL3 plasmid (**Figure 4a**, middle panel) or the luciferase coding region (**Figure 4a**, lower panel). Our results show that ZFN511-PRAP1 binds to DNA fragments in a nonsequence specific manner.

Next, we determined whether ZNF511-PRAP1 binds to the transfected and endogenous p21 promoter using chromatin immunoprecipitation. Cross-linked DNA-protein complexes from cells transfected with p21 promoter plasmid (WWP-LUC) and either p31ZFP1 or pcDNA3.1 were co-immunoprecipitated with the PRAP1 antibody. Specific primers were designed to amplify either the exogenous p21 promoter on the plasmid or the endogenous p21 promoter on genomic DNA. Unique primers were used to distinguish between endogenous and transfected p21 promoters. The upper and middle panels in **Figure 4b** show that the target was amplified only from exogenous p21, but not from the endogenous p21 promoter in cells transfected with ZNF511-PRAP1. The results indicate that ZNF511-PRAP1 binds only to exogenous but not endogenous p21 promoters. Results also indicate that ZNF511-PRAP1 was able to bind to the luciferase coding region (**Figure 4b**, lower panel), hence the amplification of the coding region. To confirm the ability of ZNF511-PRAP1 to differentiate between exogenous and endogenous DNA, we established HCT116 cells to stably express the red fluorescence gene under the CMV promoter. These cells have integrated the pDsRed2-C1 plasmid into their genomes. The stable cells were then transiently transfected with pEGFP-C2 and pcDNA3.1 or p31ZFP1. These constructs contained the CMV promoter driving GFP and ZNF511-PRAP1 expression. The GFP expression was suppressed in the p31ZPF1-transfected cells (**Figure 4c**, lower panel). In contrast, the CMV promoters driving the red fluorescent gene that have integrated into the genome were not suppressed, hence the expression of the red fluorescence gene was unaffected (**Figure 4c**,

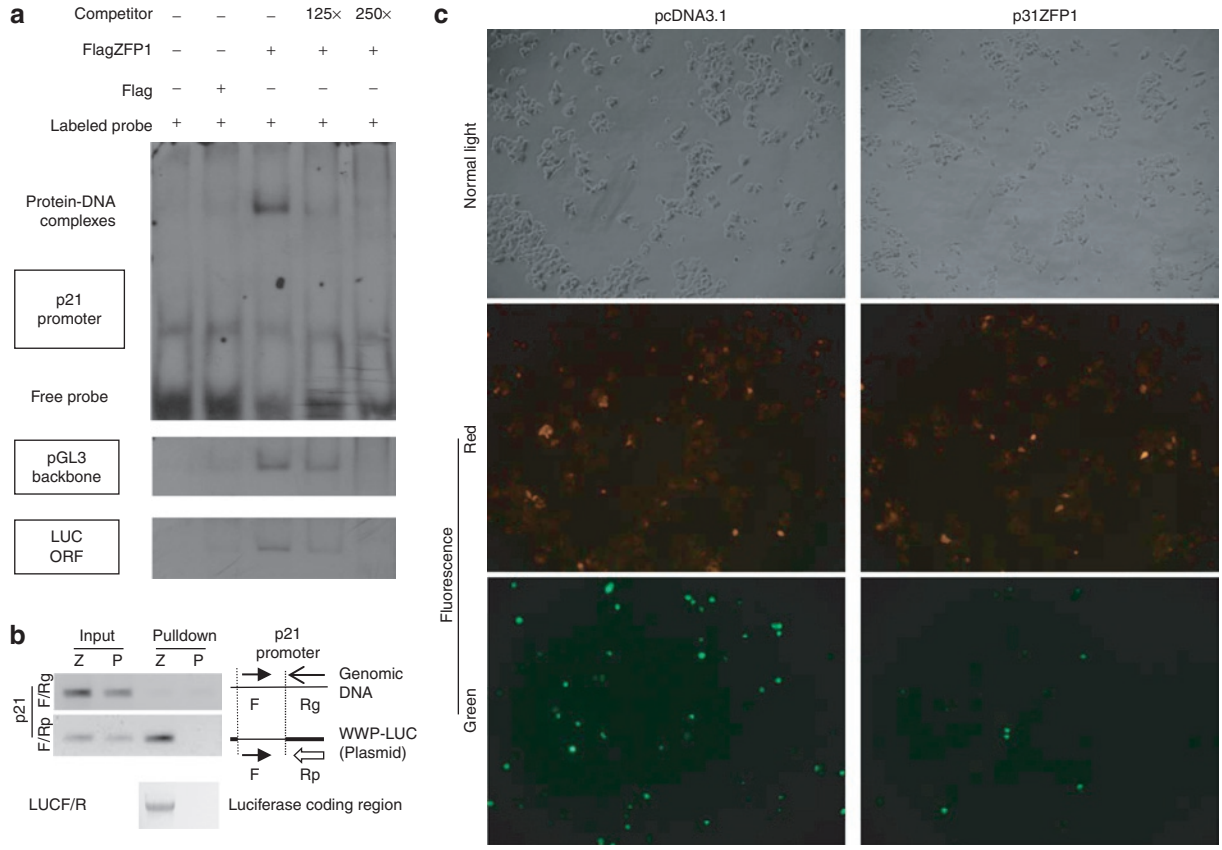

Figure 4 ZNF511-PRAP1 binds to transfected plasmids. (**a**) Electrophoretic mobility shift assay (EMSA) assay showing exogenous ZNF511-PRAP1 bound to randomly selected DNA fragments at p21 promoter (upper panel), pGL3 backbone (middle panel) and luciferase coding region (lower panel) on plasmids. Name in box indicates the DNA used in EMSA. (**b**) Chromatin immunoprecipitation assay showing exogenous ZNF511-PRAP1 bound to p21 promoter on plasmid but not on genomic DNA (upper and middle panel), and to luciferase coding region in transfected plasmids (lower panel). The primers used to specifically amplify p21 promoter fragments on either genomic or plasmid DNA are shown in the right panel. F, common forward primer, Rg: reverse primer for specific amplification of p21 promoter on genomic DNA; Rp, reverse primer for specific amplification of p21 promoter on plasmid; P, pcDNA3.1, Z, p31ZFP1. (**c**) ZNF511-PRAP1 suppresses green fluorescent protein (*GFP*) gene expression in transiently transfected plasmids but does not affect the expression of integrated red fluorescence gene within the same cells although these two genes are driven by the same cytomegalovirus promoters.

middle panel). The data strengthens the hypothesis that ZNF511-PRAP1 is able to differentiate between "endogenous/integrated" and "exogenous/nonintegrated" promoters.

If ZNF511-PRAP1 is part of the cells' response to the introduction of foreign DNA, we may expect the expression of *ZNF511-PRAP1* to be regulated by the transfection of plasmid DNA. Preliminary experiments in HCT116 cells using primers that specifically amplify the *ZNF511-PRAP1* transcript showed that transfection reagent alone could not induce *ZNF511-PRAP1* expression while transfection with a mixture of transfection reagent and plasmid induced a fivefold increase in expression compared to untransfected controls, as quantified by real-time RT-PCR (data not shown). Further plasmid dose–response experiments showed that *ZNF511-PRAP1* was maximally induced by plasmid/transfection reagent at a concentration of 2 μg/10 cm^2 culture area (**Figure 5a**, upper panel) at 2 days post-transfection. This was confirmed by real-time RT-PCR (**Figure 5a**, lower panel). A time-course analysis showed that plasmid DNA transfection increased *ZNF511-PRAP1* expression from day 2, peaking at day 3, with the effect sustained for about a week after transfection (**Figure 5b**).

Suppression of transcription by ZNF511-PRAP1 involves HDAC2

It has been recognized that DNA methylation is able to silence transfected plasmids in mammalian cells.[19,20] To test the involvement of DNA methylation in the transcriptional suppression by ZNF511-PRAP1, HCT116 cells were cotransfected with *Renilla* expression plasmid (pRL-SV40) and p31ZFP1 or pcDNA3.1 and treated with 5-aza-2′-deoxycytidine (5-Aza-dC) to demethylate the plasmids. pRL-SV40 was used since it has been shown that *in vitro* methylated SV40 early promoter suppresses the transient expression of reporter genes in transfected cells[21] and *de novo* methylation of SV40 promoter has been detected in transfected cells.[19] As shown in **Figure 6a,** 5-Aza-dC treatment increased *Renilla* luciferase activity to the same extent (3.4-fold) in both control- and ZNF511-PRAP1-transfected cells. Further, bisulfite DNA sequencing did not demonstrate a difference in the DNA methylation pattern in the CpG sites within the SV40 promoter in both sets of cells (**Figure 6b**). To confirm the result, small interfering RNA (siRNA) specific against ZNF511-PRAP1 (siZFP) was used to knock down the plasmid-induced endogenous ZNF511-PRAP1. Consistent with its suppression function, ZNF511-PRAP1

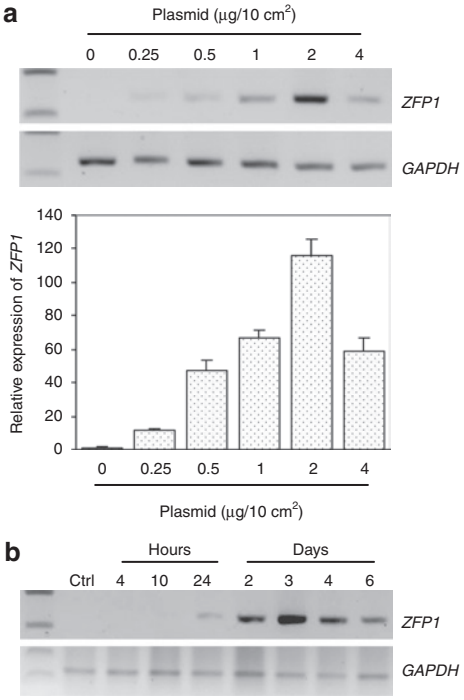

Figure 5 ZNF511-PRAP1 is induced by transfected plasmids. (**a**) Expression of endogenous *ZNF511-PRAP1* mRNA in response to transfections of varying concentrations of plasmid DNA, 2 days post-transfection. Upper panel: conventional reverse transcriptase (RT)-PCR; lower panel: real-time RT-PCR. Expression of *ZNF511-PRAP1* was normalized to that of *GAPDH*. (**b**) Time course of endogenous ZNF511-PRAP1 induction using plasmid DNA at 2 μg/10 cm² culture area. ZFP1, ZNF511-PRAP1.

knockdown increased the *Renilla* luciferase activity in siZFP compared to SCR (2.7-fold). Again 5-Aza-dC treatment increased luciferase activity to the almost equal extent (3.4- and 3.5-fold) in both SCR- and siZFP-transfected cells (**Figure 6c**). Hence, although there are methylation dependent transcriptional suppression mechanisms in the cells, the suppression by ZNF511-PRAP1 appears to be independent of DNA methylation.

To determine whether other epigenetic regulatory mechanisms are involved in the suppression of transcription by ZNF511-PRAP1, cells transfected with ZNF511-PRAP1 siRNA and plasmids were treated with a histone deacetylation inhibitor, TSA. As shown in **Figure 6d,e**, ZNF511-PRAP1 knockdown increased the both *Firefly* (**Figure 6d**) and *Renilla* (**Figure 6e**) luciferase activities, compared to SCR. TSA treatment accentuated the increase in luciferase activity after knockdown of ZNF511-PRAP1, suggesting that TSA treatment abrogated the suppression of transcription by ZNF511-PRAP1. To examine whether HDAC1 and HDAC2 were involved in the suppression of transcription by ZNF511-PRAP1, cells were treated with HDAC1- and HDAC2-specific siRNAs. HDAC2-specific siRNAs consistently increased the luciferase activities to the same degree as knockdown of ZNF511-PRAP1 (**Figure 6f**), but not HDAC1-specific siRNAs (data not shown). Combined knockdown of HDAC2 and ZNF511-PRAP1 had a small additive effect on the *Firefly* luciferase activity (**Figure 6f**). Similar results were observed with *Renilla* luciferase activity (data not shown). These results suggest that HDAC2 and ZNF511-PRAP1 may work together to suppress transfected plasmids. Other factors may also be involved in this suppression by ZNF511-PRAP1 since the *Renilla* and *Firefly* luciferase activities were only partially restored after HDAC2 knockdown compared to TSA treatment (**Figure 6d–f**). Therefore, we hypothesized that this suppression is mediated by a repression complex containing ZNF511-PRAP1, HDAC2, and probably other transcriptional corepressors.

ZNF511-PRAP1 interacts with HDAC2

To confirm the involvement of HDAC2 in the suppression of transfected plasmids by ZNF511-PRAP1, co-immunoprecipitation experiments were performed to determine the interactions between ZNF511-PRAP1 and HDAC2. HCT116 cells were transfected with Flag-tagged ZNF511-PRAP1 (pFlagZFP1) expression plasmids. Whole-cell extracts were then subjected to immunoprecipitation using anti-Flag M2 affinity gel. Western blotting showed that HDAC2 was detected in the IP samples with ZNF511-PRAP1 overexpression (**Figure 6g**, upper panel), indicating that Flag-tagged ZNF511-PRAP1 can interact with HDAC2. HDAC1 was not detected in these samples (data not shown). In the reciprocal experiment, anti-HDAC2 antibody was used to immunoprecipitate proteins in whole-cell lysates after overexpression of Flag-tagged ZNF511-PRAP1. Flag-tagged ZNF511-PRAP1 was detected in the HDAC2-immunoprecipitated complex (**Figure 6h**). The results suggest that HDAC2 interacts directly with ZNF511-PRAP1.

DISCUSSION

In the present study, we show that *ZNF511-PRAP1* is a chimeric transcript resulting from intergenic splicing. Intergenic splicing or transcription-induced chimeras generate transcripts that span two consecutive, independent genes in mammalian cells and is a mechanism to increase protein complexity.[22,23] Typically, such chimeric transcripts begin at the promoter or first exon of the upstream gene and end at the termination point of the downstream gene. The intergenic region of ZNF511-PRAP1 is about 35 kb and is spliced out of the transcript as an intron, so the resulting fused transcript possesses exons from two different genes. Other patterns, such as alternative exon splicing,[24] *trans*-splicing,[25] alternative transcription start sites,[26] and alternative polyadenylation transcription termination sites[27] have also been increasingly recognized as mechanisms to increase protein diversity. The discovery of these alternative gene splicing mechanisms explains why protein complexity in humans cannot be directly extrapolated from the number of genes.[24]

The function of ZNF511-PRAP1 protein appears to be related to the recognition and suppression of foreign DNAs in cells. This is supported by our results showing the binding and suppression of transfected plasmid DNA but not endogenous genomic DNA. The ability of ZNF511-PRAP1 to bind to DNA in a nonsequence specific manner allows ZNF511-PRAP1 to suppress transcription of a wide range of different genes. Other examples of nonsequence specific DNA binding proteins include p53,[28] RAG1[29] in animal and Filamentous Flower protein from *Arabidopsis thaliana*.[30] It is interesting to note that the Filamentous Flower protein also has a zinc finger domain at the N-terminal and a proline-rich domain at the C-terminal, similar to that of ZNF511-PRAP1. Physiologically, this function may be important for host defense, especially for

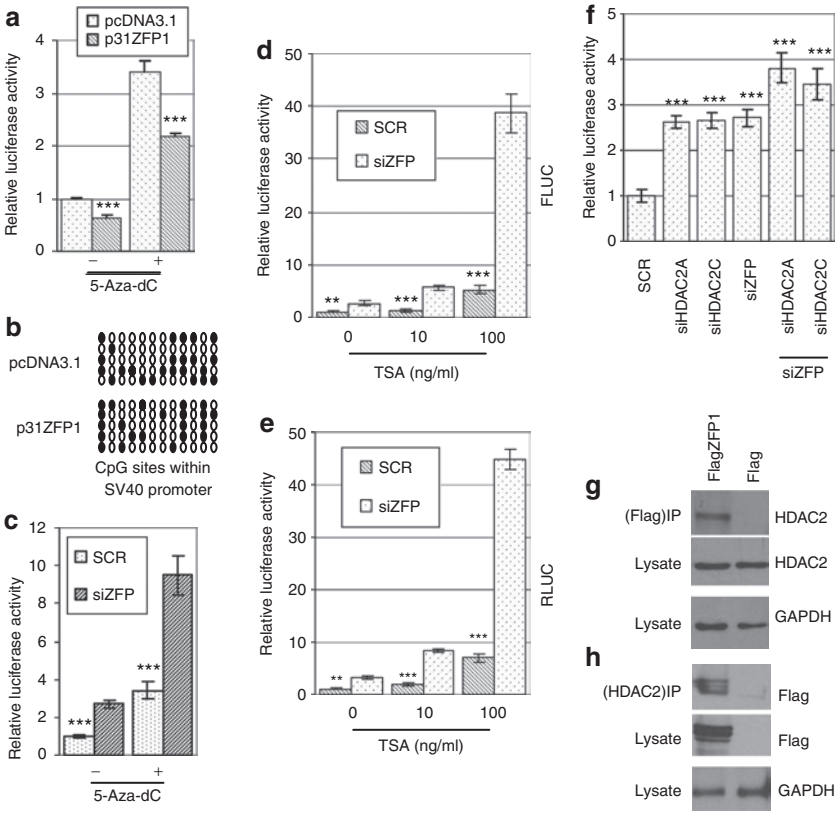

Figure 6 Involvement of HDAC2 in the suppression of ZNF511-PRAP1 in transfected plasmids. (**a**) Effect of 5-aza-2′-deoxycytidine (5-Aza-dC) treatment on cells transfected with either pcDNA3.1 or p31ZFP1. (**b**) The methylation status of CpG sites within the SV40 promoter in cells transfected with either p31ZFP1 or pcDNA3.1. (**c**) Relative luciferase activity after 5-Aza-dC treatment in cells knocked down with SCR (scramble) or small interfering RNA specific for ZNF511-PRAP1 (siZFP). (**d**,**e**) Relative luciferase activity after trichostatin A (TSA) treatment in cells knocked down with SCR or siZFP. (**f**) Relative luciferase activities in cells after HDAC2 and ZNF511-PRAP1 individual or combined knockdown. (**g**) Co-IP assay. Flag-tagged ZNF511-PRAP1 (pFlagZFP1) was transfected into HCT116 cells. FlagZFP1 was co-immunoprecipitated (co-IP) with anti-Flag. The immunoprecipitate and total cell lysates were subjected to immunoblotting with anti-HDAC2 antibody. (**h**) ZNF511-PRAP1 was co-immunoprecipitated by anti-HDAC2 antibody. Anti-HDAC2 IP prepared from HCT116 cells expressing Flag-ZNF511-PRAP1 was analyzed by western blotting using anti-Flag antibody.

epithelial cells which constantly interact with the environment. Therefore, ZNF511-PRAP1 may represent a new class of proteins that can recognize foreign DNA and suppress its transcription in a nonsequence specific manner. The ZNF511-PRAP1 protein may be part of a sensing system that detects and inactivates a wide variety of foreign DNA introduced into cells. This finding has important implications for transgene expression. A recent study suggests that low efficiency of expression in nonviral delivery system arises mainly from the low transcription efficiency in nucleus rather than from a difference in intracellular trafficking compared to viral delivery systems.[2,15] Therefore, the efficiency of transgene expression may be increased by knocking down the expression of the ZNF511-PRAP1 protein.

The mechanism of transcription suppression by ZNF511-PRAP1 involves deacetylation of histone proteins. Recent studies have shown that transfected plasmids are transcriptionally repressed in a TSA-sensitive manner[31] and that all four core histones H2A, H2B, H3, H4, linker histone H1, and intermediate levels of nucleosomal assembly are found in transiently transfected plasmids.[32,33] Hence, the deacetylation of proteins attached on the transfected plasmids may be involved in the process of transcriptional repression of transfected plasmids by ZNF511-PRAP1. Interestingly, knocking down HDAC2 appeared to increase luciferase activity in plasmid-transfected cells. This suggests that HDAC2 may be required for ZNF511-PRAP1 to suppress gene transcription. Consistent with this hypothesis, we show that HDAC2 interacts directly with ZNF511-PRAP1. Therefore, similar to the knockdown of ZNF511-PRAP1, the knocking down of HDAC2 or HDAC inhibitor treatment could also be used to increase the expression level of transgenes in nonviral delivery system. In viral gene transfer systems, various studies have shown that HDAC inhibitors treatment prior to adenovirus infection can increase the efficiency of adenoviral transgene expression in cancer cell lines[34,35] and mice.[36,37] The improvement of efficiency in transgene expression is thought to be associated with increased expression of Coxsackie adenovirus receptor, integrin and acetylated histone H3.[34,35] However, it may also be through repressing the ZNF511-PRAP1/HDAC2 complex because ZNF511-PRAP1 is also induced by virus infection (data not shown).

Although DNA methylation has been shown to be an important mechanism to suppress transfected plasmids,[19,20,38] the suppression by ZNF511-PRAP1 does not appear to involve DNA methylation. Our results showed that 5-Aza-dC treatment increased *Renilla* luciferase activity to the same extent (3.4-fold) in both control- and ZNF511-PRAP1-transfected cells. Similarly, although 5-Aza-dC treatment increased luciferase activities in

the cells, it did not significantly change the effect of ZNF511-PRAP1 knockdown. Further, bisulfite DNA sequencing did not demonstrate a difference in the DNA methylation pattern in the CpG sites within the SV40 promoter in both sets of cells. Hence, although there are methylation dependent transcriptional suppression mechanisms in the cells, the suppression by ZNF511-PRAP1 appears to be independent of DNA methylation.

In summary, we have identified a chimeric transcript *ZNF511-PRAP1*, which encodes a protein that recognizes and represses the transcription of transgenes in mammalian cells. ZNF511-PRAP1 is induced by the transfection of plasmid DNA, binds directly to exogenous plasmid DNA in a nonsequence specific manner and suppresses transcription through a mechanism that involves protein deacetylation and interaction with HDAC2, independent of DNA methylation. Disruption of ZNF511-PRAP1 and/or HDAC2 by siRNA knockdown and TSA treatment can increase the expression of transgenes. Therefore, ZNF511-PRAP1 may play a role as a potential intracellular barrier to transgene expression.

MATERIALS AND METHODS

Cell culture and cell sorting. HCT116, Hep3B, HepG2, and HeLa were purchased from American Type Culture Collection (Manassas, VA). HCT116 was cultured in McCoy's modified medium (Sigma-Aldrich Corporation, St. Louis, MO), and Hep3B, HepG2 and HeLa in Dulbecco's modified Eagle's medium (Sigma), supplemented with 10% fetal bovine serum. To sort for GFP positive live cells, HCT116 cells were transfected with appropriate plasmids. Forty-eight hours after transfection, cells were trypsinized, spun and resuspended in staining medium supplemented with propidium iodide (2 ng/ml, final concentration) for live-dead discrimination. Cell sorting was carried out on a FACS Vantage (Becton Dickinson, Franklin Lakes, NJ) cytometer.

RNA extraction, real-time RT-PCR and 5′ RACE. Total cellular RNAs were extracted using an RNeasy Mini Kit (Qiagen, GmbH, Hilden, Germany) according to the manufacturer's instruction. Real-time RT-PCR was performed as previously described.[39] 5′RACE (rapid amplification of complementary DNA ends) was performed using the GeneRacer kit (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's instructions.

Reporter plasmids and expression vectors. The WWP-LUC plasmid containing the p21 promoter and the Del-1 plasmid containing c-MYC promoter were gifts from Dr Vogelstein (Johns Hopkins, Baltimore, MD);[40,41] the plasmids containing the promoters of cyclin A2 and HDAC1 were gifts from Drs Birrer (National Institutes of Health, Rockville, MD),[42] and Seiser (University of Vienna, Vienna, Austria),[43] respectively. *Renilla* luciferase gene (*Rluc*) expression plasmids pRL-TK or pRL-SV40 containing TK-*Rluc*, or SV40-*Rluc* (Promega, Madison, WI), pDsRed2-C1, pEGFP-C2 (BD Biosciences, Clontech, Palo Alto, CA), pcDNA3.1 (+) and pCR4-Topo (Invitrogen) were also used.

The open reading frame of ZFN511-PRAP1 was amplified by PCR from pCR4-Topo containing the full-length ZFN511-PRAP1 and then cloned into the *EcoR* I and *Xba* I sites of pcDNA3.1 (Invitrogen) to generate p31ZFP1, or cloned into the *Hind* III and *Bam* HI sites of p3xFlag-CMV (Sigma) to generate pFlagZFP1, respectively. To fuse ZFN511-PRAP1 into GAL4, the N-terminal coding sequence (1–147 amino acids) of GAL4 was cloned into pcDNA31 by restriction sites *Hind* III and *EcoR* I to generate p31GAL4. ZFN511-PRAP1 was amplified by PCR and cloned into the *EcoR* I site of the plasmid p31GAL4 to generate p31GAL4ZFP1. A plasmid containing 5× GAL4 binding sites was also constructed by inserting the *Kpn* I-*Nhe* I fragment from pG5luc (Promega) into the same site of pGL3-control (Promega) to generate pGBDluc. To generate a plasmid to express both *ZFN511-PRAP1* and *GFP* in separate cistrons, pEGFP-C2, pcDNA3.1 and p31ZFP1 were digested with *Bgl* II and dephosphorylated with shrimp alkaline phosphatase. pEGFP-C2 was ligated to pcDNA3.1 or p31ZFP1, transformed into Escherichia *coli* and selected with both ampicillin and kanamycin to generate expression cassettes p31-GFP or p31ZFP1-GFP to express GFP alone or GFP and ZFN511-PRAP1, respectively.

Plasmid and siRNA transfections and drug treatments. Transfection of plasmid was carried out using Lipofectamine 2000 (Invitrogen) and Opti-MEM (Gibco-BRL, Gaithersburg, MD) according to the manufacturers' instruction. To examine the effect of 5-Aza-dC (Sigma) on the suppression of luciferase by overexpression of *ZFN511-PRAP1*, 5 μmol/l 5-Aza-dC was added after 5 hours of plasmid transfection. Cells were harvested 43 hours after drug treatment. To examine the effect 5-Aza-dC or TSA (Sigma) on luciferase activity by ZFN511-PRAP1 knockdown, 5-Aza-dC or TSA was added to a final concentration of 5 μmol/l (5-Aza-dC) or as indicated (TSA) after 24 hours of plasmid and siRNA transfection. Cells were harvested 48 hours after drug treatment for luciferase assay. Luciferase assays were carried out using Dual-Luciferase Assay Kit (Promega) on a Varioskan Flash multimode reader (Thermo Fisher Scientific, Waltham, MA). The total protein content was determined using the Bradford Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA).

Stable clones and fluorescence images. Cells in a 6-well plate were transfected with 1 μg pDsRed2-C1 (BD Biosciences) using Lipofectamine 2000 (Invitrogen). Forty-eight hours post-transfection, cells were trypsinized and plated in 10 cm dishes to establish stable clones. Colonies that survive after a 2–3-week selection in 600 μg/ml G418 were isolated under fluorescence microscope. Stable cells in six-well plates were transfected with 10 ng plasmid pEGFP-C2 and 1 μg pcDNA3.1 or p31ZFP1. Cells were examined at normal light, green or red fluorescence with an inverted fluorescence microscope (Axiovert 40 CFL, Zeiss, Thornwood, NY) and live cell images were recorded with a digital camera (PowerShot A640, Canon, Ohta-ku, Tokyo, Japan).

Chromatin immunoprecipitation. Chromatin immunoprecipitation was conducted using ChIP Assay Kit (Upstate, Lake Placid, NY) according to the protocol provided.

Induction of ZFN511-PRAP1 by transfection of plasmid DNA. HCT116 cells with confluency at 50% or below were trypsinized and plated on the day before transfection so that cell confluency is ~20–30% on the day of transfection.

Plasmid DNA extraction from mammalian cells, bisulfite treatment and sequencing. Plasmid DNA was isolated from mammalian cells using DNeasy Blood and Tissue Kit (Qiagen) using the provided protocol. The SV40 promoter specific primers were used to amplify the DNA fragment, which was then cloned and sequenced.

In vivo *protein interaction and immunoprecipitation.* HCT116 cells were transiently co-transfected in 10 cm dishes with 4 μg ZFN511-PRAP1 expression vector pFlagZFP1 or vector control. Forty-eight hours after transfection, protein extracts were prepared in either 1 ml ice-cold RIPA buffer (1× PBS, 1% NP40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate) for immunoprecipitation of HDAC2 protein, or ice-cold lysis buffer (50 mmol/l Tris HCl, pH7.4, 150 mmol/l NaCl, 1 mmol/l EDTA, 1% TRITON X-100) for pull-down using anti-Flag M2 Affinity Gel (Sigma). After that, standard methods were followed.

Electrophoretic mobility shift assay. ZFN511-PRAP1 was synthesized using the TnT T7 Quick Coupled Transcription/Translation system (trial size) according to the manufacture's protocol (Promega). Briefly, 2 μg of the plasmid pFlagZFP1 or Flag empty vector was used as DNA template in a final volume of 50 μl, and coupled transcription/translation reaction was conducted at 30 °C for 90 minutes. Electrophoretic mobility shift assay were

performed with Dig Gel Shift Kit 2nd generation (Roche Applied Science, Penzberg, Germany) according to the protocol provided. Briefly, the DIG-3′-end-labeled probes were incubated with a total of 5 µl of reticulocyte lysate containing newly synthesized ZFN511-PRAP1 for 20 minutes at room temperature. In competition experiments, a 125- or 250-fold excess of unlabeled oligonucleotides was added to the reaction mixture. DNA-protein complex was separated on 7% polyacrylamide native gels and blotted to Hybond-N^+ membrane (Amersham Biosciences, Sunnyvale, CA).

Statistical analysis. Experimental differences were tested for statistical significance using the two-tailed *t*-test. *P* value of <0.05 was considered to be significant.

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