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Suppression of Enhancer Overactivation by a RACK7-Histone Demethylase Complex

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Summary

Regulation of enhancer activity is important for controlling gene expression programs. Here we report that a biochemical complex that contains a potential chromatin reader, RACK7 and the

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ACCESSION NUMBERS

All data generated in this study are deposited at GEO database (GSE71327). The mESC H3K4me1 and H3K27Ac raw ChIP-seq data were downloaded from GEO (GSM594577 and GSM594578) (Creyghton et al., 2010).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures can be found online.

Author contributions

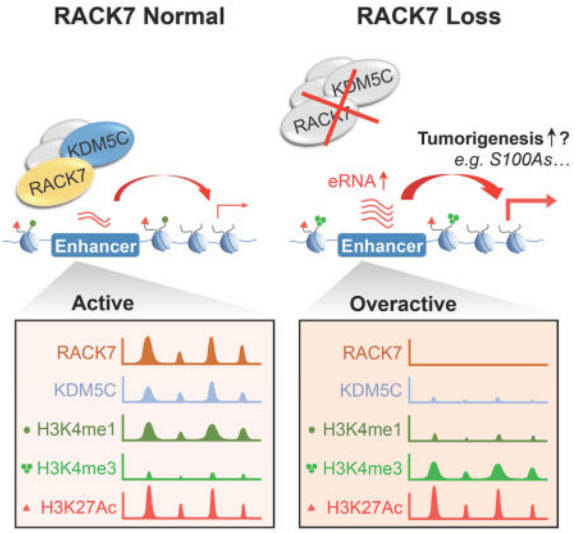
H.S. and W.X carried out most of the experiments and the bioinformatics analyses in this manuscript. R.G. purified protein complex and carried out co-IP, pull-down assays. B.R. and C.H. carried out some of the ChIP analyses. Z.W. and L.Z. prepared the recombinant proteins. L.G. and F.W. provided additional insights on bioinformatics analyses. X.H., H.Z. and Z.S. helped in data analyses of tumor related phenotypes. P.Y contributed in mass spectrometry analysis. F.L. and Y.S. directed all the experiments with input from Y.G.S.. F.L. and Y.S. conceived the project and co-wrote the manuscript with H.S.

Conflict of Interest Statement

F.L. is a shareholder of Constellation Pharmaceuticals, Inc. Y.S. is a co-founder of Constellation Pharmaceuticals, Inc., as well as a member of its scientific advisory board. F.L. and Y.S. are consultants of Active Motif, Inc.

histone lysine 4 tri-methyl (H3K4me3)-specific demethylase KDM5C occupies many active enhancers, including almost all super-enhancers. Loss of RACK7 or KDM5C results in overactivation of enhancers, characterized by the deposition of H3K4me3 and H3K27Ac, together with increased transcription of eRNAs and nearby genes. Furthermore, loss of RACK7 or KDM5C leads to de-repression of S100A oncogenes and various cancer-related phenotypes. Our findings reveal a RACK7/KDM5C-regulated, dynamic interchange between histone H3K4me1 and H3K4me3 at active enhancers, representing an additional layer of regulation of enhancer activity. We propose that RACK7/KDM5C functions as an enhancer “brake” to ensure appropriate enhancer activity, which, when compromised, could contribute to tumorigenesis.

Graphical Abstract



Introduction

Enhancers, which are important regulatory elements controlling gene expression, exist in primed, poised and active states, decorated by unique sets of chromatin modifications (Calo and Wysocka, 2013). For instance, H3K4me1 is associated with all three states of enhancers, which is primarily mediated by the MLL3/4 methyltransferases (Lee et al., 2013). H3K4me1 alone marks primed enhancers and when presents with H3K27me3 or H3K27Ac, the combinatorial histone modifications further define the poised (H3K4me1/H3K27me3) and active (H3K4me1/H3K27Ac) states of enhancers, respectively (Calo and Wysocka, 2013). Conversion of poised enhancers to active ones is a critical step in many biological processes including embryonic stem (ES) cell differentiation (Buecker et al., 2014). Mechanistically, this conversion involves acetylation of histone H3K27 by the histone acetyltransferases, p300/CBP, and the action of Mediator/Pol2, which help increase local transcription (Calo and Wysocka, 2013) and produce enhancer RNAs (eRNAs) (Kim et al., 2010; Wang et al., 2011). eRNAs are believed to regulate Pol2 pause/release (Lam et al., 2014) and therefore is another landmark of active enhancers. More recently, a subset of active enhancers with unusually high levels of H3K27Ac and Mediator binding is identified and termed super-enhancers (Hnisz et al., 2013; Loven et al., 2013; Pott and Lieb, 2015; Whyte et al., 2013).

Super-enhancers are usually composed of multiple putative active enhancers in close genomic proximity and possess stronger enhancer activities (Pott and Lieb, 2015). However, once activated, it's unclear whether active enhancers including super-enhancers are subject to further activation, and if so, what the functional significance may be.

Emerging evidence suggests that dysregulation of enhancers may lead to deleterious consequences including cancer. For instance, enzymes that are involved in modifying the chromatin landscapes of enhancers, such as the histone methyltransferases MLL2, MLL3, MLL4, the H3K4me2/3-specific demethylase KDM5C, the acetyltransferases CBP/p300, as well as the histone H3 variant, H3.3, have been found to be frequently mutated in various cancers (Blair et al., 2011; Ford and Dingwall, 2015; Lan and Shi, 2015; Rasmussen and Staller, 2014; Wang et al., 2013). Consistently, recent cancer genome sequence efforts have also identified genetic mutations of enhancers and super-enhancers, which regulate the expression of oncogenes and tumor suppressors (Fredriksson et al., 2014; Melton et al., 2015). Collectively, these functional and genomic investigations highlight the importance of enhancers and their regulation, which, when compromised, can lead to tumorigenesis.

Here we report the identification of a chromatin complex containing two putative tumor suppressors, RACK7 (Receptor for Activated C-Kinase 7, aka ZMYND8), a potential chromatin binder initially identified as activated Protein Kinase C binding protein (Fossey et al., 2000), and the histone demethylase KDM5C (Lan et al., 2008; Mosammaparast and Shi, 2010), which co-occupy a large set of active enhancers including almost all super-enhancers, and function as general negative regulators of enhancers. We show that RACK7 interacts with KDM5C and loss of RACK7 significantly impairs KDM5C localization to active enhancers, indicating that RACK7 is important for KDM5C recruitment to enhancers. Genetic ablation of either RACK7 or KDM5C in the breast cancer cell line, ZR-75-30, leads to hyper-activation of target enhancers, characterized by an increase of H3K4me3, a decrease of H3K4me1, and an elevated eRNA level at RACK7-bound enhancers. The RACK7 null ZR-75-30 cells also display increased anchorage independent growth, migration and invasion abilities in vitro, as well as enhanced tumor growth in a mouse xenograft model. Likewise, KDM5C loss leads to similar increases of cell migration and invasion, further supporting the idea that RACK7 and KDM5C work together to regulate cellular processes relevant to tumorigenesis. Collectively, our findings reveal that active enhancers are subject to negative regulation, and that RACK7 and KDM5C together act as a "brake" of active enhancers by controlling the dynamics between H3K4me1 versus H3K4me3 at active enhancers. Loss of such an enhancer surveillance mechanism can lead to altered cell behaviors, which may contribute to tumorigenesis.

Results

RACK7 primarily binds active enhancers

RACK7 is a close homolog of BS69/ZMYND11 with a similar domain architecture (Figure 1A). Recently, others and we uncovered an unexpected role of BS69 as a histone H3.3K36me3 specific reader, which regulates transcriptional elongation and mRNA processing (Guo et al., 2014; Wen et al., 2014), but much less is known about RACK7. Importantly, sporadic RACK7 somatic mutations (likely inactivating mutations) have been

identified in a number of cancers including breast cancer, suggesting a potential role for RACK7 in tumorigenesis.

To understand the function and mechanism of action of RACK7 in chromatin regulation, we first determined the chromatin landscape of RACK7 bound genomic locations including H3K4me1, H3K4me2, H3K4me3, H3K27me3, H3K36me3 and H3K27Ac, which mark specific chromatin states and regulatory regions in ZR-75-30, a ductal carcinoma type breast cancer cell line with a relatively high level of RACK7 expression. We found a total of 14,663 RACK7 binding events and bioinformatics analyses identified a strong overlap of the RACK7 binding events with H3K4me1 and H3K27Ac (*P* values<2.2e-16), two histone modifications, which together, mark active enhancers. Specifically, 73.2% (10,728/14,663) of the RACK7 peaks are positive for both H3K4me1 and H3K27Ac (Figure 1B, *P* value<2.2e-16), and 9,231 of them overlap with candidate active enhancers (excluding TSS ±1kb) (Calo and Wysocka, 2013; Plank and Dean, 2014). Reciprocally, we identified 28,961 candidate active enhancers in the ZR-75-30 cells, and significantly, 30.4% (8,807) of them are bound by RACK7, suggesting that RACK7 may be a general regulator of active enhancers. We further confirmed that the majority of RACK7 bound putative enhancer peaks (97.2%, 8,558/8,807) are also bound by p300, which is another hallmark for active enhancers (Figure S1A and S1B). In addition to active enhancers, approximately 1/4 of RACK7 peaks are localized to promoter regions. We also found that 51.6% (5,558/10,766) and 26.0% (4,089/15,778) of putative active enhancers (H3K4me1 and H3K27Ac, excluding TSS±1kb) were bound by RACK7 in MCF7 and mESC cells, respectively (data not shown), indicating that RACK7 likely acts as an general regulator of active enhancers in multiple cell lines.

RACK7 binds majority of super-enhancers

Super-enhancers are a newly identified enhancer type, which consists of multiple enhancers in close genomic proximity (Hnisz et al., 2013; Pott and Lieb, 2015; Whyte et al., 2013). They are marked by a high level of H3K27Ac and Mediator, likely controlling genes important for cell type specification (Hnisz et al., 2013; Loven et al., 2013). The fact that RACK7 binds active enhancers promoted us to further investigate whether it also binds super-enhancers. Using ROSE algorithm mainly based on the ranking of H3K27Ac intensities (Loven et al., 2013), we identified a total of 776 super-enhancers in ZR-75-30 and essentially all of the super-enhancers (99.2%, 770/776) are bound by RACK7 (Figure 1C). Importantly, RACK7 binding of super-enhancers is not limited to one cell line or cell type. We found that RACK7 also binds almost all super-enhancers in MCF7 (100%, 90/90) and mESC cells (92.4%, 536/580) (Figure 1C). Taken together, these findings indicate that majority of super-enhancers are bound and possibly regulated by RACK7.

RACK7 interacts with histone H3 lysine 4 demethylase KDM5C

To gain mechanistic insight into RACK7 function at enhancers, we next set out to identify RACK7 interacting proteins using a HeLa-S cell line stably expressing FLAG-HA-RACK7 (Figure S1C and S1D). Mass spectrometry analysis identified association of several zinc finger (ZNF) proteins, ZNF532, ZNF592 and ZNF687, with RACK7, consistent with the previously published reports (Kloet et al., 2014; Malovannaya et al., 2011). In addition to the

ZNF proteins, RACK7 also associates with multiple factors involved in chromatin regulation, including KDM5C (aka SMCX), a histone H3K4me2/3-specific demethylase, as well as components of the histone H3K4me1/2-specific demethylase LSD1 repressor complex, including LSD1, BHC80, RCOR1/2, HDAC1/2 and CHD4 (Figure1D, S1E and S1F). We next confirmed the interaction between the endogenous RACK7 and KDM5C by reciprocal co-immunoprecipitation in ZR-75-30 cells (Figure 1E). In vitro pull-down assay using recombinant RACK7 and KDM5C purified from insect cell further suggested that the interaction is likely to be direct (Figure 1F).

Genome-wide RACK7 co-localization with KDM5C and the requirement of RACK7 for the recruitment of KDM5C to active enhancers

The interaction of RACK7 and KDM5C promoted us to investigate whether these proteins co-localize genome-wide. Consistently, our KDM5C ChIP-seq analysis in ZR-75-30 cells identified 8,487 peaks at both intergenic regions and promoters, similar to those of RACK7 (Figure S1G). Venn diagram analysis of their ChIP-seq peaks and UCSC tracks showed a significant overlap between KDM5C and RACK7 binding events (Figure 1G, *P* value<2.2e-16). Specifically, more than two thirds of the KDM5C peaks (68.3%, 5,799/8,487) also show RACK7 binding, and (58.8%, 3,412/5,799) of these co-bound events are localized to active enhancers. Consistent with the finding of RACK7 binding of majority of super-enhancers, we found KDM5C co-binds with RACK7 on (88.7%, 688/776) super-enhancers. Additionally, RACK7 and KDM5C share a very similar distribution pattern across an average active enhancer and super-enhancer unit (Figure S1H left, *P* value=2.49e-118, and right, *P* value=6.31e-195).

Although the enzymology of KDM5C as a histone H3K4me3 demethylase is well understood (Lan et al., 2008; Lan and Shi, 2015; Mosammaparast and Shi, 2010), an important question that remains unanswered is how it is recruited to its target regions. We next asked whether RACK7 might play a recruiting role for KDM5C to chromatin. Supporting this idea, we observed a significant global reduction of KDM5C chromatin association in a RACK7 deleted ZR-75-30 cell line (KO1) generated using CRISPR (Figure 1H and 1I, *P* value=0). This change was further confirmed at three selected enhancers by ChIP-qPCR in KO1 and another independently generated KO cell line (KO2) using a different guide RNA (Figure 1J and S1I). Since the KDM5C protein level was unaffected in the RACK7 KO cells (Figure S1I), the reduction of KDM5C chromatin association in the absence of RACK7 is likely due to an impaired recruitment.

Taken together, our findings uncovered a connection between RACK7 and the histone demethylase KDM5C and raised the possibility that RACK7 may function together with KDM5C to regulate histone H3K4 methylation levels at their target genomic regions.

RACK7 or KDM5C loss leads to increased H3K4me3 and decreased H3K4me1 levels at active enhancers

To further determine whether and how RACK7 regulates chromatin modification landscape, especially H3K4 methylation, at its target regions, we carried out ChIP-seq analyses of all three states of H3K4 methylation, i.e., H3K4me1, H3K4me2 and K3K4me3, and H3K27Ac,

respectively, in the parental as well as the RACK7 KO1 cells (Figure 2A). Upon RACK7 loss, we identified a significant and consistent increase of H3K4me3 at 53.0% (4,668/8,807, >1.5 fold) of RACK7-bound enhancers, and 68.7% (3,206/4,668) of them showed a decrease of H3K4me1. When analyzed more quantitatively, we observed a strong association of H3K4me3 gain and H3K4me1 loss at the active enhancers with a higher RACK7 binding intensity in the parental cells (Figure S2A, *P* value=0), including super-enhancers (Figure 2B, left, *P* value=6.49e-65; right, *P* value=2.95e-7), suggesting that RACK7 directly impacts these chromatin modification changes. Importantly, these changes were also seen in another breast cancer cell line, MCF7 (Figure S2B and S2C), again suggesting this is not a ZR-75-30 specific mechanism. In contrast to H3K4me3 and H3K4me1, the change of H3K4me2 is much milder, and in some cases not significant (Figure 2A). We also observed the same H3K4me3 gain and H3K4me1 loss on 4,731 RACK7 bound intergenic enhancers (Figure S2D), excluding the possibility that these changes are due to the activation of intronic alternative promoters, which occur only in intragenic regions (Kowalczyk et al., 2012). Such alterations were shown in UCSC tracks (Figure 2C) and can be readily confirmed by ChIP-qPCR analyses in both RACK7 KO cell lines (Figure 2D). Consistent with the idea that RACK7 mainly induces enhancer-specific H3K4 methylation alterations, loss of RACK7 had no effect at RACK7-bound promoters and on global H3K4 methylation levels (Figure 2A bottom panel, and Figure S2E). Importantly, reintroduction of a full-length, wildtype RACK7 expression construct into RACK7 KO1 cells restored RACK7 binding as well as H3K4me3 level (Figure 2E, 2F, S2F and S2G), albeit partially, suggesting that the alterations of H3K4 methylation at these enhancers are specific and are a consequence of the loss of RACK7.

Since KDM5C is an H3K4me3 demethylase capable of removing one or two methyl groups from tri-methylated lysine H3K4, generating H3K4me2 and H3K4me1, we wished to determine whether RACK7 negatively regulates the H3K4me3 level through KDM5C at enhancers. Consistent with this hypothesis, we observed a significant increase of H3K4me3 at 3 of 3, selected RACK7-bound enhancers when KDM5C is abrogated (Figure 2G); however, RACK7 recruitment was only slightly affected in the absence of KDM5C (Figure S2H and S2I). These findings support the idea that RACK7 recruits KDM5C, which in turn modulates H3K4 methylation at target enhancers.

RACK7 or KDM5C loss leads to an increased eRNA transcription

Recent studies have also shown that enhancers themselves are broadly transcribed, resulting in the production of enhancer-derived RNAs (eRNAs) (De Santa et al., 2010; Kim et al., 2010; Koch and Andrau, 2011; Lam et al., 2014). Given that transcription is generally associated with H3K4me3 (Vermeulen et al., 2007; Wysocka et al., 2006), we therefore wished to determine whether the switch from H3K4me1 to H3K4me3 in the absence of RACK7 also influenced local transcription of eRNAs at target enhancers. Interestingly, our data discovered a significant elevation of eRNA production from RACK7 bound enhancers, including super-enhancers, in the RACK7 KO cells, especially among the top 2,000 RACK7 bound enhancers (Figure 3A, Upper part, Figure 3B, *P* value=2.65e-26). Such alterations were shown in UCSC tracks and readily confirmed by subsequent RT-qPCR analyses (Figure 3C and 3D). At the same time, we also observed a very similar increase of eRNA

transcription at RACK7 bound enhancers (3 out of 3) in the KDM5C KO cells (Figure 3E). Importantly, as a control, we did not observe overt changes of eRNA production from those enhancers that lack RACK7 binding (Figure 3A, Lower part).

RACK7 or KDM5C loss leads to an increased transcription of the surrounding genes

Enhancers are known to activate gene transcription both at proximal and distal regions, and emerging evidence suggests that eRNAs activate gene(s) in their vicinity through regulation of the transcription machineries, such as Mediator and RNA polymerase II at gene promoters (Koch and Andrau, 2011; Lai et al., 2013; Zabidi et al., 2015). We next asked whether the increase in eRNAs in the RACK7 KO cells is coincident with increased transcriptional activity of the surrounding genes. We performed mRNA sequencing (mRNA-seq) using mRNA samples isolated from the parental ZR-75-30 and the RACK7 KO1 cells, and identified a significant increase of the steady levels of mRNAs transcribed from the very next genes linked to the top 2,000 RACK7 bound enhancers (Figure 4A, left, *P* value=0.039) and the 770 RACK7 bound super-enhancers (Figure 4B, *P* value=5.08e-6). Importantly, this effect is specifically associated with RACK7 bound enhancers since we found no apparent changes from the very next genes linked to the 2,000 enhancers with the lowest RACK7 binding intensity as a control (Figure 4A, Right, *P* value=0.487). Such alterations were shown in UCSC tracks and readily confirmed by subsequent RT-qPCR analyses using the very next genes linked to 3 selected, RACK-targeted enhancers as examples (Figure 4C and 4D). Consistently, we also observed a similar increase of transcription of the same target genes in the KDM5C KO cells (Figure 4E). Interestingly, we did not observe overt changes of transcript levels driven by the top 2,000 RACK7 bound promoters in the RACK7 null cells (Figure S2J), suggesting RACK7 functions mainly at enhancers. Using an established, enhancer activity reporter assay (Koues et al., 2015; Outchkourov et al., 2013), we were able to confirm that 10 out of 10 candidate active enhancers bound by RACK7 and KDM5C indeed possess enhancer activities and most, if not all, of them are regulated by RACK7 and KDM5C (Figure S3). Together with the finding discussed in Figure 3, we conclude that RACK7 or KDM5C loss both lead to hyper-activation of these enhancers and higher transcriptional activities.

RACK7 and KDM5C KO cells have enhanced tumorigenic capability

As discussed earlier, RACK7 mis-sense, non-sense and frame-shift mutations have been identified in a variety of cancers, suggesting that RACK7 may function as a tumor suppressor. Interestingly, inactivating mutations of KDM5C have also been reported in various cancers (Dalgliesh et al., 2010; Hakimi et al., 2013). Thus, loss of RACK7 and KDM5C may impact tumorigenesis.

To address this hypothesis, we first asked whether RACK7 regulates genes important for tumorigenesis by analyzing the RNA-seq data, which identified 452 differentially expressed genes, with 270 up- and 182 down-regulated (FPKM >= 1 in either control or RACK7 KO, change of expression >= 2 folds and *P* value < 0.05 by Cuffdiff program), respectively, upon RACK7 deletion. We focused our subsequent analyses only on the up-regulated genes since our findings (discussed earlier) identified RACK7 as a negative regulator of enhancers, and therefore genes that are up-regulated upon RACK7 loss are likely to be direct targets of

RACK7. Supporting this, we found significantly more enrichment of RACK7 binding at the enhancers near the differentially up-regulated genes than the differentially down-regulated genes (Figure S4A, $P = 0.0001$). Using GO analysis, we found those differentially up-regulated genes enriched in the categories of cell adhesion (Figure S4B), suggesting that RACK7 may regulate tumorigenesis through regulating cell adhesion. Interestingly, although RACK7 or KDM5C does not regulate proliferation in the regular 2D cell culture (Figure S4C and S4D), using several 3D-based assays, we found that RACK7 KO cells have enhanced tumorigenic phenotypes, including increased anchorage independent growth, invasion and migration abilities, which can be fully suppressed by re-introduction of RACK7 (Figure 5A–C). Utilizing a mammary fat pad xenograft model, we further demonstrated that loss of RACK7 also affected tumorigenesis in vivo (Figure 5D), which is consistent with the increased anchorage independent growth of RACK7 null cells observed in the soft agar assay (Figure 5A). Consistently, KDM5C KO cells pheno-copied the RACK7 KO cells in the invasion and migration assays (Figure 5E and 5F).

Finally, supporting our in vitro finding that tumor cells with a lower RACK7 level have higher invasion and migration capabilities, RACK7 expression in breast cancer patients with invasive ductal carcinoma (IDC) is lower than those with ductal carcinoma in situ (DCIS) (Figure 5G). Collectively, these findings suggest that RACK7 and KDM5C suppress tumor cell invasion and migration possibly through regulating enhancer functions.

RACK7 regulates tumor progression in part through the S100A family oncogenes

Dys-regulated expression of multiple members of the S100A family is a common feature of human cancers, with each type of cancer showing a unique S100A protein profile or signature (Bresnick et al., 2015). Emerging evidence suggests that these proteins actively contribute to tumorigenic process such as metastasis and angiogenesis in vivo (Bresnick et al., 2015). Most family members (such as *S100A1-S100A14, S100A16*) are encoded in two tandem clusters within a 2 Mb region on chromosome 1q21 (Figure 6A). Interestingly, in ZR-75-30, the *S100A* genes in Cluster I are actively transcribed, while all genes in Cluster II are silent based on our mRNA profiling. We found that RACK7 and KDM5C only occupy regions marked by H3K4me1 and H3K27Ac in Cluster I (Figure 6A), suggesting that RACK7 and KDM5C bind active enhancers in Cluster I but not silent enhancers in Cluster II. We also found that Cluster I contains a super-enhancer that shows binding of both RACK7 and KDM5C (Figure 6A). Upon RACK7 loss, many *S100A* members in Cluster I, especially S100A4, displayed a significant increase in their expression, while Cluster II genes were not affected (Figure 6A–C). Consistently, we also observed increased eRNA production from the RACK7 bound regions, supporting that these are active enhancers (Figure 6A, shadow areas). Importantly, *S100A4* expression in IDC is also higher than DCIS from the same 6 patients (GDS2046) described in Figure 5G (Figure 6D). These findings raise the possibility that RACK7 may regulate tumor cell migration and metastasis through regulating the expression of S100A family members, such as S100A4.

To address the possibility that elevated S100A family members are in part responsible for the enhanced tumorigenic activities of the RACK7 KO1 cells, we asked whether inhibition of S100A4 expression in the RACK7 KO1 cells suppresses the tumor phenotypes.

Significantly, genetic ablation of *S100A4* by and largely reduced the invasion and migration abilities of the RACK7 KO1 cells (Figure 6E and 6F). We noticed that the effect of S100A4 deletion on invasion was partial (compare Figure 6F right, upper part, with Figure 5B and 5C), which could be explained by the fact that RACK7 suppresses the expression of multiple *S100A* genes. Supporting this hypothesis, treatment of RACK7 KO1 cells with a small molecule inhibitor, Calcimycin, reported to primarily suppress the level of S100A4 (Bresnick et al., 2015; Sack et al., 2011), but also affect S100A2 and S100A6 functions by influencing their intracellular localization (Mueller et al., 1999), resulted in a greater reduction of the invasion ability (Figure 6E and Figure 6F table, lower part). These data suggest that RACK7 suppresses cell invasion and migration potential at least partially through repressing *S100A* family of oncogenes.

Discussion

The salient finding of this study is that activities of active enhancer are being fine-tuned by negative regulators (e.g., RACK7 and KDM5C), and loss of such regulation leads to overactive enhancers and super-enhancers, represented by a higher level of H3K4me3 and increased eRNA transcription.

H3K4me3 and H3K4me1 at enhancers

H3K4me1 is considered a chromatin mark for enhancers, which, when combined with H3K27Ac, further defines active enhancers. Importantly, loss of RACK7 or KDM5C leads to a reduced H3K4me1 and an elevated H3K4me3, revealing an otherwise unappreciated dynamic interchange between H3K4me1 and H3K4me3 at active enhancers, including super-enhancers, regulated by a distinct biochemical complex. Consistently, low levels of H3K4me3 at active enhancer associated with increased enhancer activity have been noted by previous studies (Chen et al., 2015; Cheng et al., 2014; Pekowska et al., 2011). It remains to be determined whether RACK7/KDM5C localization to active enhancers is a regulated event or whether inclusion of such a negative regulatory complex is an integral part of active enhancer assembly.

Although MLL2/3/4 are mainly responsible for the genesis of H3K4me1 at enhancers, our data suggest that at active enhancers the histone H3K4me2/3-specific demethylases such as KDM5C may also contribute to the formation of H3K4me1. Of note, KDM5C regulation of enhancers in mES cells has been reported recently (Outchkourov et al., 2013). However, in contrast to our findings, enhancers have been shown to be positively regulated by KDM5C in that study. The basis for this difference is unknown at the present time. It may be due to the fact that the two studies were done in different cellular contexts (mESCs versus human breast cancer cells). Regardless, both studies suggest dynamic interchange between H3K4me1 and H3K4me3 at enhancers involving the histone demethylase KDM5C.

KDM5C and LSD1 in regulating enhancer functions

Recently, another histone demethylase, LSD1, has been shown to bind essentially all active enhancers in mESCs (Whyte et al., 2012). LSD1 becomes active during differentiation to decommission enhancers that need to be shut down for the differentiation programs. Unlike

LSD1, which is there for future repression, KDM5C is actively engaged in dampening the activities of its bound enhancers in ZR-75-30 cells. When KDM5C is genetically ablated, active enhancers become even more active. Interestingly, LSD1 and its complex components are also associated with RACK7 (Figure S1E, S1F and (Malovannaya et al., 2011)). However, our preliminary results (not shown) suggest that LSD1 in ZR-75-30 cells is probably not actively involved in the regulation of RACK7/KDM5C target enhancers. It remains to be determined whether LSD1 functions similarly as KDM5C in somatic cells to dampen rather than decommission active enhancers, and whether RACK7 plays a recruitment role of LSD1 to a distinct set of enhancers for their regulation.

RACK7 and KDM5C in cancer and other human diseases

Recent cancer genome sequencing efforts have uncovered genetic mutations in a large number of chromatin regulators. Many of these factors are involved in enhancer regulation, such as H3.3, MLL2/3, MLL4, KDM5C and CBP/p300, suggesting enhancer mis-regulation may play an important role in tumorigenesis. However, the mechanistic insights of how these mutations affect enhancer activity and tumorigenesis are still lacking. Importantly, inactivating mutations (non-sense and frame-shift) of RACK7, (9.5%, COSMIC) and KDM5C (21.3%, COSMIC) have been reported in a variety of cancer types. RACK7 was found with a high mutation frequency (17/91, 19%) in MMR (Mismatch repair)-deficient colorectal cancers (Park et al., 2002), and KDM5C mutations are found in ~5% renal cell carcinoma (Dalgliesh et al., 2010; Niu et al., 2012), supporting their proposed tumor suppressor roles. Interestingly and consistently, RACK7 interacts with the activated PKC beta (hence the alternate name, PRKCBP1, for RACK7), which has recently been shown to function as a tumor suppressor in various cancer types (Antal et al., 2015).

We show that loss of RACK7/KDM5C results in enhancer overactivation and a concomitant increase in tumorigenic abilities of the affected cells, raising the possibility that RACK7/KDM5C regulates tumorigenesis through modulating enhancer activity. The identification of S100A oncogenes as downstream effectors further supports this idea. It will be interesting to investigate in the future whether this connection also exists in other types of cancers bearing RACK7/KDM5C or PKC mutations, and if so, whether pharmacological inhibition of S100A4 would provide a therapeutic strategy for these cancers.

In addition to their roles in cancer, RACK7 is mutated in epilepsy, while KDM5C are frequently mutated in X-linked mental retardation (Goncalves et al., 2014; Veeramah et al., 2013). Our findings suggest that loss of RACK7 and KDM5C may similarly result in enhancer mis-regulation in these pathological contexts.

Modulation of enhancer activity as a therapeutic strategy

Recent cancer epigenetics drug discovery efforts have provided a successful example of pharmacological inhibition of super-enhancers, which are often hijacked by cancer. Inhibition of BRD4, a component of super-enhancers, resulted in a dramatic decrease of *c-MYC* expression and an impact on cancer cell proliferation (Jung et al., 2015; Loven et al., 2013; Shi et al., 2013). Our finding of RACK7 and KDM5C regulation of super-enhancers suggests that the cancer phenotypes caused by loss of RACK7 and KDM5C might be in part

due to overactivation of certain super-enhancers, such as the one located in the S100A Cluster I. These finding raise the interesting possibility that enhancer inhibitors, such as BRD4 inhibitors, when applied to cancer bearing RACK7 or KDM5C mutations, may be particularly beneficial therapeutically. Our data also suggest that the development of small molecule inhibitors of the histone methyltransferase(s) (yet to be identified) responsible for enhancer H3K4me3 formation may represent an independent new therapeutic strategy, specifically targeting cancers with enhancer mis-regulation.

Experimental Procedures

Tandem Affinity Complex Purification and Gel-filtration

Complex purification was performed as published (Ogawa et al., 2002). Gel-filtration chromatography analysis was performed using Superose 6 HR 10/30 (Amersham).

Generation of Knockout cell lines

CRISPR-Cas9 targeting was carried out as previously described (Maeder et al., 2013).

Chromatin Immunoprecipitation (ChIP) and ChIP-Seq

ChIP assays were carried out as previously described (Lan et al., 2007). The precipitated DNA samples were analyzed using real time PCR and prepared for deep sequencing according to manufacture's guidelines (Illumina) and previous study (Guo et al., 2014).

Nascent RNA-seq and mRNA-seq

Nascent RNA-seq and mRNA-seq were carried out according to manufacture's guidelines (C-10365, Life Technologies) and previous study (Guo et al., 2014).

Definition of Active Enhancers, Intergenic Enhancers and Super-Enhancers

Active enhancers are defined as H3K4me1/H3K27Ac co-binding sites excluding TSS ± 1kb. Intergenic enhancers were identified as active enhancers excluding gene regions (TSS-1kb to TES). Super-enhancers are identified using ROSE which stitches constituent enhancers within 12.5kb (excluding TSS ± 2kb) and ranks the enhancers by input-subtracted signal of H3K27Ac (Loven et al., 2013). Active enhancers and super-enhancers are assigned to genes whose TSSs are the nearest to the center of the enhancers.

Heatmap Analyses

Heatmaps were generated by Cluster 3.0 and Java Treeview (1.1.6r4). Each analyzed genomic region was evenly divided into 400 windows, and the sums of the signal densities in each window were used to generate the heatmap.

Correlation Analyses of Genome-wide Sequencing Data

All ChIP-seq and Nascent RNA-seq experiments were performed using samples from two biological replicates, and the Pearson correlation coefficients were calculated using "Multiple wiggle files correlation" program from Cistrome Analysis Pipeline with default parameters (Liu et al., 2011). For mRNA-seq, three biological replicates were carried out,

and the Pearson correlation coefficients of FPKMs were calculated by Corrcoef in MATLAB (R2009a) (Table S1). All replicates of ChIP-seq, RNA-seq and Nascent RNA-seq data were validated individually for all the analyses, and Replicate #1 were used for figure presentation in Figure 1–4, S1 and S2.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

1. RACK7 binds a large set of active enhancers including almost all super-enhancers
2. RACK7 recruits KDM5C to enhancer regions and negatively regulates enhancer H3K4me3
3. Loss of RACK7 or KDM5C results in further activation of their target enhancers
4. RACK7 exerts tumor suppressive function in part by repressing S100A oncogenes

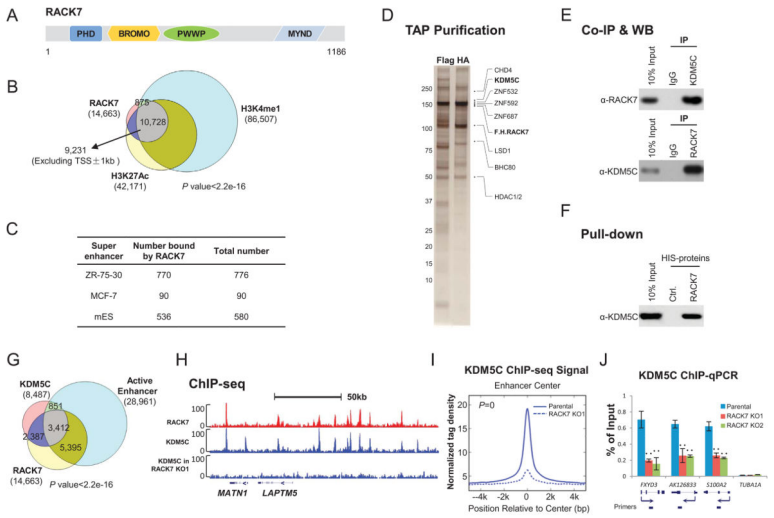

Figure 1. Identification of RACK7 and KDM5C interaction and their binding events at active enhancers and super-enhancers

(A) Schematic representation of the domain architecture of RACK7 protein.

(B) Venn diagram analysis of ChIP-seq peaks of RACK7, H3K4me1 and H3K27Ac. *P* value by Pearson's Chi-squared test.

(C) Total numbers of super-enhancers and RACK7 bound super-enhancers in ZR-75-30, MCF-7 and mES cells.

(D) Tandem affinity purified FLAG-HA-RACK7 (F.H.RACK7) protein complex was resolved and visualized by silver staining.

(E) Reciprocal immunoprecipitation between endogenous RACK7 and KDM5C.

(F) In vitro pull-down between recombinant RACK7 and KDM5C proteins.

(G) Venn diagram analysis shows the overlap between RACK7 and KDM5C co-bound regions and active enhancers defined by H3K4me1 and H3K27Ac, *P* value by Pearson's Chi-squared test.

(H) UCSC tracks showing RACK7 and KDM5C ChIP-seq signals in parental ZR-75-30 and KDM5C ChIP-seq in RACK7 KO1 cells at a select genomic location.

(I) KDM5C recruitment to chromatin in the parental and the RACK7 KO1 cells examined by the genome-wide analyses of ChIP-seq signals

(J) KDM5C recruitment to chromatin in parental ZR-75-30 and the RACK7 KO cells confirmed by ChIP-qPCR. q-PCR Data are represented as mean ± SD from three biological replicates, * $P < 0.05$; ** $P < 0.01$, T test.

See also Figure S1, Table S1 and S2

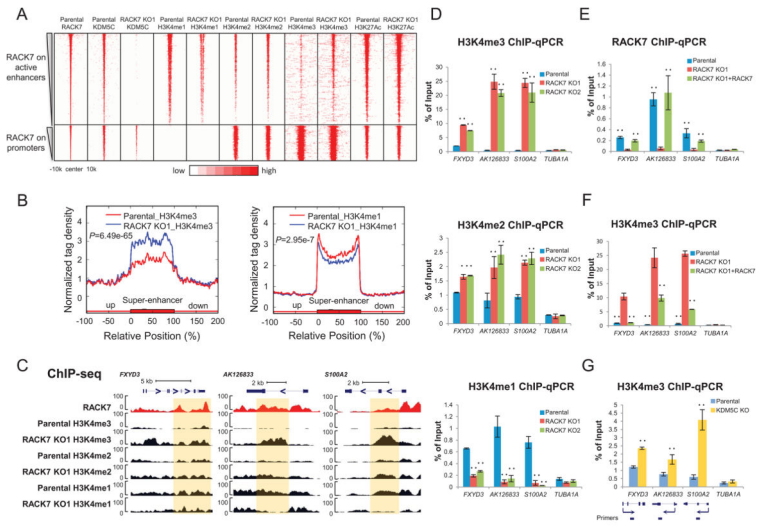

Figure 2. RACK7 and KDM5C suppress H3K4me3 at active enhancers and super-enhancers

(A) Heatmap analyses of ChIP-seq signals of RACK7, KDM5C and select histone modifications in the parental and RACK7 KO1 ZR-75-30 cells, ranked by RACK7 ChIP-seq signals in parental ZR-75-30 cells. All ChIP-seq signals are displayed from −10 kb to +10 kb surrounding the center of each annotated RACK7 peak.

(B) Normalized levels of H3K4me3 and H3K4me1 at the RACK7 bound super-enhancers in the parental and the RACK7 KO1 ZR-75-30 cells. *P* values by ANOVA test.

(C–D) H3K4 methylation states at three representative enhancers in parental ZR-75-30 and RACK7 KO cell lines, shown by ChIP-seq snapshots (C) and confirmed by ChIP-qPCR (D).

(E–F) RACK7 binding and H3K4me3 level at three select enhancers examined by ChIP-qPCR in the parental, RACK7 KO1 and RACK7 KO1 containing a rescuing, wildtype RACK7 transgene.

(G) H3K4me3 levels at three selected enhancers in parental ZR-75-30 and KDM5C KO cells.

In all panels, q-PCR Data are represented as mean ± SD from three biological replicates. * $P < 0.05$; ** $P < 0.01$, T test.

See also Figure S2, Table S1 and S2

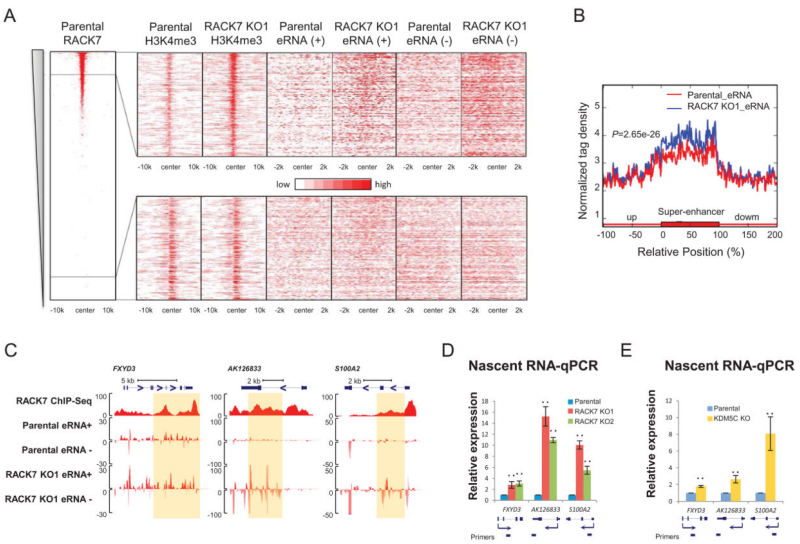

Figure 3. RACK7 or KDM5C loss leads to an increase of eRNA production

(A) Heatmap analyses of H3K4me3 ChIP-seq and nascent RNA-seq data from the parental and RACK7 KO1 ZR-75-30 cells ranked by RACK7 ChIP-seq signals at all enhancers in the parental ZR-75-30 cells. Nascent RNA-seq signals are displayed from −2 kb to +2 kb and ChIP-seq signals are displayed from −10 kb to +10 kb surrounding the centers of the annotated RACK7 peaks. Sense and antisense stands are indicated by "+" and "−".

(B) Comparison of eRNA levels from RACK7 bound super-enhancers between the parental and RACK7 KO1 ZR-75-30 cells, as assessed by nascent RNA-seq. *P* value by ANOVA test.

(C) Snapshots showing eRNA increases at all three select RACK7 bound enhancers in RACK7 KO1 cells.

(D–E) Nascent RNA RT-qPCR confirmation of eRNA increase in the RACK7 KO (D) and KDM5C KO (E) cells from the same three select enhancers shown in 3C. All q-PCR data are represented as mean ± SD from three biological replicates. * $P < 0.05$; ** $P < 0.01$, T test.

See also Figure S2, Table S1 and S2

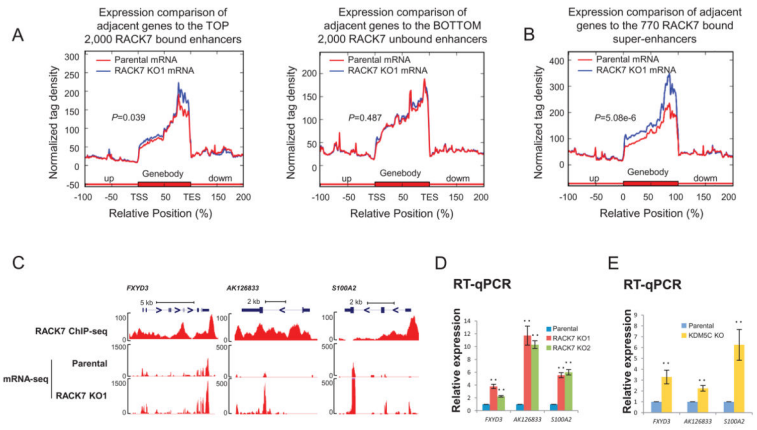

Figure 4. Loss of RACK7 or KDM5C leads to hyper-activated enhancers

(A) Expression comparison (using mRNA-seq data) of the adjacent genes to the RACK7 bound and unbound enhancers (Left: top 2,000; and right: bottom 2,000) between the parental and RACK7 KO1 ZR-75-30 cells. *P* values by ANOVA test.

(B) Expression comparison (using mRNA-seq data) of the adjacent genes to the RACK7 bound super-enhancers between the parental and RACK7 KO1 ZR-75-30 cell lines. *P* value by ANOVA test.

(C) Snapshots showing mRNA increases from the nearest genes of the three select RACK7 bound enhancers in RACK7 KO1 cells.

(D–E) RT-qPCR confirmation of the expression changes of the three select target genes in the parental, RACK7 KO (C) and KDM5C KO (D) cells. Total mRNA samples were used. All q-PCR data are represented as mean ± SD from three biological replicates. * $P < 0.05$; ** $P < 0.01$, T test.

See also Figure S2, S3 and Table S1, S2

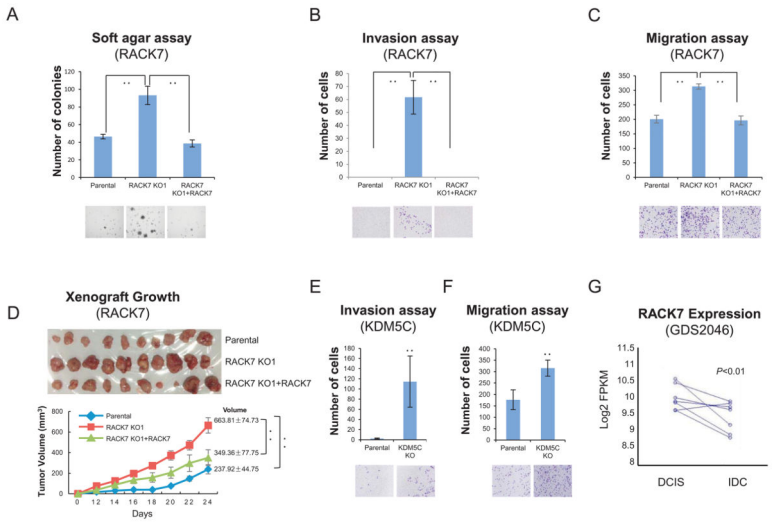

Figure 5. Loss of RACK7 or KDM5C promotes tumorigenic potential of ZR-75-30 cells

(A–C) In vitro soft agar, invasion and migration assays examining the anchorage independent growth, invasion and migration abilities of the parental, RACK7 KO1 and the RACK7 KO1 cells with a rescuing, wildtype RACK7 transgene.

(D) Xenograft growth (24 days, n=10) analysis of the parental, RACK7 KO1 and the RACK7 KO1 cells with a rescuing, wildtype RACK7 transgene. Quantifications of tumor volume (Upper) and representative images of the tumors (Lower) are shown. Error bars represent SEM of the mean, $^{**}P < 0.01$, T test.

(E–F) In vitro invasion and migration assays examining the invasion and migration abilities of the parental and KDM5C KO ZR-75-30 cells.

(G) RACK7 expression is significantly lower in IDC than the paired DCIS tumors from 6 breast cancer patients (GDS2046) (Schuetz et al., 2006).

In panel (A–C, E and F), all data are represented as mean ± SD from three biological replicates, $^{**}P < 0.01$, T test.

See also Figure S4

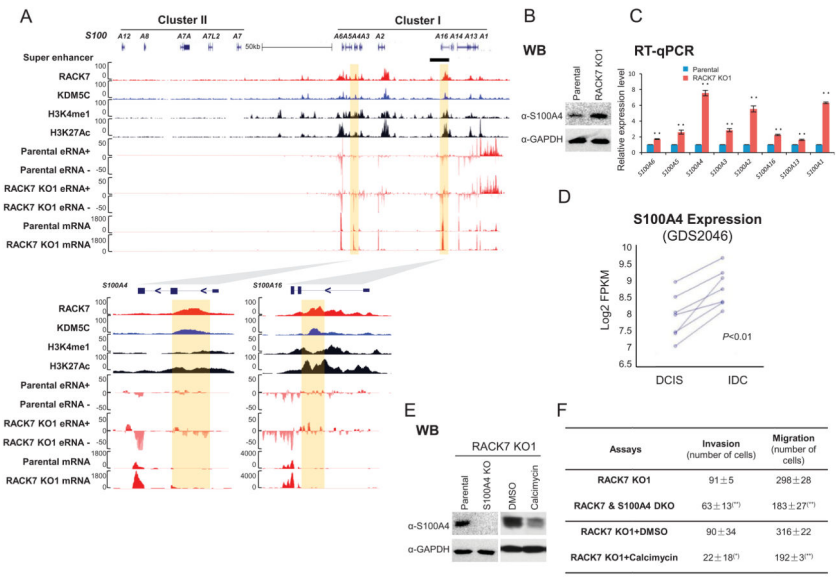

Figure 6. RACK7 suppresses tumorigenesis in part through repressing the S100A family of oncogenes

(A) ChIP-seq profiles of RACK7, KDM5C, H3K4me1 and H3K27Ac in ZR-75-30, and nascent RNA-seq and mRNA-seq profiles in the parental and RACK7 KO1 cells at S100A oncogene cluster. S100A4 and S100A16 are selected as examples to show increased eRNA and mRNA expression in the RACK7 KO1 cells (Shadow).

(B) S100A4 protein levels in the parental and the RACK7 KO1 cells examined by Western blotting.

(C) RT-qPCR showing the expression of multiple S100A oncogenes in the RACK7 KO1 cells compared to the parental ZR-75-30 cells.

(D) S100A4 expression is significantly higher in IDC than the paired DCIS tumors from the same dataset shown in Figure 5G (GDS2046).

(E) S100A4 protein levels were examined by Western blotting in the RACK7 KO1 cells with indicated treatment.

(F) The tumorigenic abilities of the RACK7 KO1 cells with the indicated treatments were examined and showed by the numbers of the invaded and migrated cells. In panel C, all data are represented as mean ± SD from three biological replicates, * $P < 0.05$ and ** $P < 0.01$, T test.