The PRMT7-dependent methylation of shank2 modulates invasion-proliferation switching during breast cancer metastasis

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Abstract

Invasiveness of cancer cells is associated with proliferation inhibition in multiple types of cancers. Here, we identified the pivotal roles of Arginine methyltransferase PRMT7 in promoting invasion and attenuating proliferation of breast cancer cells. PRMT7 exerted its functions through binding to the scaffold protein shank2 to induce the di-methylation of shank2 at R240. Shank2 R240 methylation exposed ANK domain by disrupting its SPN-ANK domain blockade. Moreover, shank2 R240 methylation rendered recruitment of FAK that elicited the FAK auto-phosphorylation, which consequently augmented the shank2-dependent migration and invasion of breast cancer cells. On the other hand, the shank2 R240 methylation impeded proliferation of breast cancer cells by antagonizing the Ras-Raf binding via tethering the mono-ubiquitinated H-Ras. These findings characterize the PRMT7-dependent shank2 methylation as a key player in mediating reciprocal switching between invasion and proliferation, also point to the value of shank2 R240 methylation as a target for tumour metastasis treatment strategies.

65 Introduction

66 Metastasis is the leading cause of cancer-associated death (Lambert et al., 2017). Tumour
67 cells confers a metastatic phenotype by controlling the balance between cell proliferation and
68 cell motility. It is conventionally thought that metastasis develops at least partly as a function of
69 tumour growth. Indeed, tumour size is an unfavourable prognostic marker for many kinds of
70 tumours such as squamous cell carcinoma, glioma, prostatic cancer, melanoma and breast
71 cancer, implying metastasis develops as a result of tumour cell suppresses proliferation
72 capability but increases invasion potential (Gao et al., 2005; Kemper et al., 2014; Patsos et al.,
73 2010; Shiwarski et al., 2014; Whittle et al., 2015). Studies of invasive tumour cell populations
74 have shown that motile cells upregulate genes that support invasion while attenuating
75 proliferation. In glioma, EphB2 overexpression promotes migration and inhibits proliferation
76 of glioma cells by binding and activating FAK (Wang et al., 2012). In prostatic cancer, hypoxia
77 reduces SNPH expression resulting increase in ROS inhibits tumour cell proliferation, while
78 promoting increased FAK-dependent tumour cell migration and invasion (Caino et al., 2017).
79 In basal cell carcinomas, p16^{INK4a} was up-regulated at the invasive front of the majority of basal
80 cell carcinomas with infiltrative growth patterns, followed by ceased proliferation (Svensson et
81 al., 2003). In melanoma, c-Jun was reported critical for inflammation-induced dedifferentiation
82 and played as a key driver of the transition between proliferative and invasive state
83 (Riesenberg et al., 2015). In breast cancer, invasiveness of breast cancer cells is associated
84 with growth arrest due to p21^{CIP1} upregulation (Qian et al., 2013). Similarly, snail causes the
85 loss of epithelial markers, upregulates mesenchymal markers, and impairs cell proliferation in
86 breast cancer (Vega et al., 2004). However, the molecular mechanism behind the switch
87 between proliferative and invasive states during breast cancer metastasis remains unclear.

88 During invasive cell migration, the cytoskeleton polarizes by actin polymerization via
89 scaffold proteins and additional cytoskeletal linker proteins, leading to integrin clustering and
90 activation of FAK to execute locally controlled proteolysis, and to form a leading protrusion
91 (Caino et al., 2017; Hamidi and Ivaska, 2018; Wang et al., 2012). Scaffolding proteins shanks
92 are localized in cytoplasm and play important roles in regulating cortical cytoskeleton (Lee et
93 al., 2010; Naisbitt et al., 1999; Sala et al., 2001; Sheng and Kim, 2000; Tu et al., 1999). The

shank family of master scaffolding proteins include three members, i.e., shank1, 2 and 3. Shank family proteins have been linked to syndromic and idiopathic autism spectrum disorder, as well as to schizophrenia and intellectual disability (Lim et al., 1999; Mei et al., 2016; Monteiro and Feng, 2017; Yoon et al., 2017). Mutations in the genes encoding shank family proteins (shank1, 2 and 3) often result in marked behavioural phenotypes in mice (Mameza et al., 2013; Schmeisser et al., 2012; Won et al., 2012), include an increase in repetitive routines, altered social behaviour and anxiety-like phenotypes. Recent studies have also linked shank family proteins to cancer cell invasion. Shank1 and shank3 were reported to inhibit cell spreading, migration and invasion in cancer cells through sequestering active Rap1 and R-Ras (Lilja et al., 2017). However, the precise roles of shank2 in tumour progression have not been investigated.

Arginine methyltransferase 7 (PRMT7) is a type II methyltransferase catalysing symmetric dimethylation of arginine residues on both histone and non-histone substrates (Bedford and Clarke, 2009; Blanc and Richard, 2017; Lee et al., 2005). Others and our previous study have linked PRMT7 to breast cancer progression. Our previous work reported that PRMT7 promotes metastasis of breast carcinoma cells by triggering EMT (epithelial-mesenchymal transition) program (Yao et al., 2014). We also found that PRMT7 was automethylated at residue R531 which potentiates the PRMT7-induced EMT program and metastasis (Geng et al., 2017). PRMT7 can also upregulates the expression of matrix metalloproteinase 9 to promote metastasis of breast cancer (Baldwin et al., 2015). On the other hand, our previously report suggested that PRMT7 impaired breast cancer cell proliferation endowing the pro-migratory and invasive properties (Yao et al., 2014). However, the molecular mechanisms behind the PRMT7-orchestrated reciprocal switch between proliferative and invasive states, particularly its significance to metastatic cancers, must be fully elucidated for a full understanding of cancer progression.

This study aimed to explore the molecular mechanisms underlying the PRMT7-orchestrated switch between proliferative and invasive states during breast cancer metastasis. By using mass spectrometry (MS) analysis, we identified shank2 as a new

substrate of PRMT7. We further unravelled that methylation of shank2 at R240 by PRMT7 reinforced breast cancer cell migration and inhibited cell proliferation by triggering FAK phosphorylation and H-Ras mono-ubiquitination. Collectively, these findings represent one mechanistic explanation for how PRMT7 regulates breast cancer cell metastasis by mediating reciprocal switching between invasion and proliferation, and may provide potential clues for tumour metastasis treatment strategies.

WITHDRAWN
see manuscript DOI for details

144

145 Results

146 Shank2 interacts with PRMT7 and is a substrate for PRMT7-mediated arginine 147 methylation

148 To explore the mechanism of PRMT7 action in tumour metastatic progression, we used the
149 Flag-tagged PRMT7 fusion protein as a bait in mass spectrometry to pick up the possible
150 regulatory factors that are directly regulated by PRMT7 (data not shown). Among these
151 PRMT7-interacting proteins, we chose shank2 as a target for a more detailed study, since it
152 plays a critical role in cytoskeleton organization. Although shank1 and shank3 have been
153 reported to inhibit cancer cell migration, whether shank2 plays a role in cancer progression is
154 unknown. We first conducted a co-immunoprecipitation assay to confirm the binding between
155 PRMT7 and shank2 *in vitro* (Figure 1A,B). We then incubated the purified GST-tagged
156 PRMT7 with shank2 protein, and the results showed that they could directly interact with each
157 other (Figure 1C). To map the shank2 regions that bind PRMT7, we expressed a series of
158 truncated GST-shank2 (Figure 1D), and we detected that the shank2 ANK, PDZ and SAM
159 domains were responsible for the interaction between PRMT7 and shank2 (Figure 1E). By
160 using *in vivo* methylation assay, we found that overexpression of PRMT7 resulted in
161 increased symmetric arginine di-methylation of shank2 (Figure 1F). Meanwhile, shank2
162 di-methylation was reduced upon PRMT7 depletion, and the reduced di-methylation was
163 restored by the reconstituted expression of WT PRMT7 (PRMT7 WT) but not of mutant
164 PRMT7 at its enzymatic domain (PRMT7 Mut) (Figure 1G,H, Figure 1—figure supplements 1).
165 To further confirm PRMT7 directly methylates shank2, we used purified GST-PRMT7 and
166 Flag-hank2 for *in vitro* methylation assay followed by incubation with SAM (the methyl donor).
167 Apparently, incubation of recombinant PRMT7 and shank2 gave rise to a remarkable
168 increase in methylation of shank2 in the presence of SAM, while shank2 methylation was
169 undetectable in the absence of SAM or PRMT7 (Figure 1I). These results suggest that
170 PRMT7 physically interacts with and methylates shank2.

Arginine methylation of shank2 is upregulated in clinical breast cancer samples

Previous reports showed that the expression of shank2 in head and neck cancer tissues was higher than that in adjacent tissues, and this upregulation was correlated with the survival rate and prognosis of patients (Qin et al., 2016). According to the Human Protein Atlas database, the expression of shank2 in breast cancer samples is higher than that in the adjacent tissues (Figure 2A). We also observed that shank2 mRNA was highly expressed in human breast tumours (Figure 2B). To investigate the clinical relevance of shank2 methylation, a direct western blot analysis of breast cancer tumours (T) and their adjacent normal tissues (N) was performed, and the results demonstrated that shank2 was overexpressed in human breast cancer tumours, and was remarkably hypermethylated in cancerous tissues, with a corresponding activation of shank2 (Figure 2C). This analysis revealed a positive correlation between shank2 di-methylation and neoplastic tissue. Together, these data indicate that upregulated shank2 expression and its elevated di-methylation level are relevant indicators of breast cancers.

Shank2 R240 methylation potentiates cancer cell migration and invasion

To further identify the methylation site of shank2 in breast cancer cells, we performed mass spectrometric analysis of methylation of shank2 in malignant breast cancer cell MDA-MB-231 that overexpressed Rat HA-shank2 (a gift from Dr. Min Goo Lee). We detected that the arginine 240 residue (R240) of shank2 was di-methylated (Figure 3A). Using sequence homology comparison, we found that the R240 residue is evolutionarily conserved (Figure 3B). Then we constructed a shank2 mutation with R240 residue replaced by lysine (shank2 R240K) to test the effect of shank2 R240 methylation. We found that shank2 di-methylation was decreased in shank2 R240K, whereas shank2 WT did not affect the di-methylation level (Figure 3C,D). We then overexpressed increasing doses of PRMT7 in HEK293T cells, and we found that shank2 R240 di-methylation level was increased accordingly (Figure 3E,

Figure 3—figure supplements 1). These results indicate that PRMT7 physically interacts with shank2 to methylate the R240 of the protein.

Next, we intended to study the effects of shank2 di-methylation on breast cancer cells. Out of 6 cell lines analysed, the human triple negative breast cancer cells (BT549 and MDA-MB-231) exhibited high shank2 di-methylation levels, whereas normal mammary epithelial cell (MCF10A), luminal-type breast cancer cell (MCF7, BT474) and HER-2 positive cell (T47D) showed relatively low shank2 di-methylation levels (Figure 3F, Figure 3—figure supplements 2A). Furthermore, we analysed the expression profile of EMT-associated markers, and found that the epithelial marker ZO-1 was increased, while the mesenchymal markers Fibronectin and N-cadherin were decreased upon shank2 depletion; meanwhile, Fibronectin and N-cadherin expression was restored by reconstituted expression of shank2 WT but not by shank2 R240K (Figure 3G, Figure 3—figure supplements 2B-D). In addition, MMP2/MMP9 activation and the migration/invasion capabilities of MDA-MB-231 cells were also inhibited by shank2 depletion, and in both cases the inhibition was abrogated by reconstituted expression of shank2 WT but not by shank2 R240K (Figure 3H-K, Figure 3—figure supplements 2E-F). Together, these results strongly support the assumption that shank2 di-methylation is a crucial factor controlling EMT, migration and invasion characteristics of breast cancer cells.

R240 methylation disturbs SPN-ANK domain blockade of shank2

To explore the mechanism of shank2 function in breast cancer cell migration and invasion, we studied the conformational changes of wild-type shank2 (WT) and its mutant (R240K) by using molecular dynamics simulations. As shown in Figure 4A, the SPN domain in shank2 R240K mutant needs more energy to reach an “open” state than that in shank2 WT, according to the free energy profile. The trajectory for the window with the global minimum free energy (the distance between SPN and ANK is 31.5 Å, the SPN and ANK domains were represented in different colours in Figure 4B) in WT and the one (the distance between SPN and ANK is 30 Å) in R240K were analysed. The average number of hydrogen bonds between

the two domains was 0.96 ± 0.05 in each frame of WT. The most hydrogen bonds were formed in N100/R227 and E119/K260 for the locations of these residues (Figure 4C). After R240 was mutated to K, two domains formed 1.57 ± 0.02 hydrogen bonds in D97/K229, Y140/H192, N100/R227, Y140/N190 and S256/E119 for the locations of mentioned residues (Figure 4D). Thus, structural analyses suggest that shank2 R240K mutants not only possess a more stable energy fluctuation, but also form more hydrogen bonds, causing the loss of appropriate space for insertion of partner proteins.

Shank2 R240 methylation promotes cancer cell migration through activating FAK/cortactin signalling

A previous study reported that shank3 SPN domain interacts with the ANK domain in an intramolecular manner, thereby restricting access of either Sharpin or α -fodrin (Won et al., 2012). To further figure out which shank2-bound proteins might be affected by shank2 structural changes, we used mass spectrometry to find the possible regulatory factors. Our mass spectrometric data showed that shank2 could interact with proteins in integrin signalling pathway, including β 1-integrin, Talin, FAK, EEA1 and cortactin. To test this, we conducted Co-IP assays to determine that Talin, FAK and shank2 were in the same complex in MDA-MB-231 cells (Figure 5A, Figure 5—figure supplements 1A). We also found that PRMT7 promoted the co-precipitation of Talin/FAK/cortactin with shank2 (Figure 5B,C). Meanwhile, the PRMT7 enzymatic activity mutation led to attenuated formation of shank2/Talin/FAK/cortactin complex (Figure 5—figure supplements 1B,C). Also, we showed that the formation of protein complex of shank2, Talin, FAK and cortactin was significantly increased by reconstituted expression of shank2 WT but not shank2 R240K (Figure 5D,E, Figure 5—figure supplements 1D). Consistent with this finding, the interaction between Talin and FAK was enhanced by reconstituted expression of shank2 WT but not shank2 R240K (Figure 5F, Figure 5—figure supplements 1E-G). Together, our data suggest that the shank2 R240 di-methylation is in favour of the assembly of a multi-protein complex to facilitate tumour development.

We next determined whether the PRMT7-induced breast cancer cell migration was shank2-dependent, as deficiency of shank2 attenuated the precipitation of Talin/FAK/cortactin with shank2 in MCF7 cells overexpressing PRMT7 (Figure 5G). Depletion of FAK expression or use of FAK inhibitor impaired the binding of cortactin to shank2 (Figure 5H, Figure 5—figure supplements 1H). Previous studies reported that FAK can activate AKT and cortactin to promote tumour cell migration and invasion (Eke et al., 2012; Hamidi and Ivaska, 2018; Tomar et al., 2012; Wang et al., 2011). As expected, FAK, AKT and cortactin were activated by reconstituted expression of shank2 WT but not shank2 R240K (Figure 5I,J, Figure 5—figure supplements 1I-L, Figure 5—figure supplements 2A-D). Similarly, decreased expression of shank2 inhibited FAK, cortactin and AKT activities in MCF7 cells overexpressing PRMT7 (Figure 5K, Figure 5—figure supplements 3A-F). Collectively, these findings provide evidence that shank2 di-methylation can potentiate tumour metastasis through FAK-mediated cortactin activation.

The PRMT7-mediated shank2 R240 methylation inhibits breast cancer cell proliferation by enhancing H-Ras mono-ubiquitination

Activation of the Ras oncogenic pathway is a common event in tumorigenesis (Karnoub and Weinberg, 2008). It has been reported that shank3 could interact with wild type H-Ras, and constitutively active G12V mutant form of H-Ras (Lilja et al., 2017). Our mass spectrometric analysis found that shank2 also bind to H-Ras. To validate this, we first investigated shank2 interact with H-Ras WT (Figure 6A,B). Existing data suggest that Ras family members can undergo reversible mono- and di-ubiquitination modifications and thus to orchestrate tumorigenic properties (Baietti et al., 2016; Bigenzahn et al., 2018; Steklov et al., 2018; Xu et al., 2010). Noticeably, our mass spectrometric searching for shank2-interacting proteins identified a list of E3 ligase for Ras, including Rabex5 and RIN1. Co-IP assays showed that PRMT7 promoted the association of shank2 with H-Ras (Figure 6C,D). We further demonstrated that the interaction between shank2 and H-Ras was significantly increased by reconstituted expression of shank2 WT but not shank2 R240K (Figure 6E). Of note, the

PRMT7-dependent methylation did not affect Rabex5 and RIN1 binding to shank2 (Figure 6C-E). Moreover, in MCF7 cells, suppression of shank2 or overexpression of shank2 R240F, a methylation mimic, but not shank2 WT, dramatically increased H-Ras binding ability to Rabex5 and RIN1 (Figure 6F). Consistently with this observation, in vitro His pulldown assay showed that PRMT7 knockdown decreased and shank2 knockdown increased H-Ras mono-ubiquitination in HEK293T cells (Figure 6G,H), whereas overexpression of WT shank2 or shank2 R240F caused accumulation of mono-ubiquitinated H-Ras rather than shank2 R240K (Figure 6I, Figure 6—figure supplements 1A-C). These observations implicate that di-methylated shank2 interacts with mono-ubiquitinated H-Ras.

To determine whether shank2 is involved in regulation of H-Ras activation, we examined the H-Ras activity in MDA-MB-231 cells using GST Ras binding domain of Raf-1 (GST-Raf-RBD) to pull down GTP-H-Ras from cell lysates. As expected, the basal and EGF-induced H-Ras activity was largely increased by reconstituted expression of R240K shank2 but not of shank2 WT (Figure 6J,K, Figure 6—figure supplements 1D-G). In line with this finding, Ras-induced downstream ERK1/2 activation, as well as breast cancer cell growth, was promoted by reconstituted expression of R240K shank2 but not of shank2 WT (Figure 6L,M, Figure 6—figure supplements 1H-K). Collectively, we conclude that shank2 R240 methylation functions to inhibit H-Ras activity, and to attenuate the H-Ras-dependent tumorigenesis via promoting H-Ras mono-ubiquitination.

Shank2 R240 methylation promotes breast cancer metastasis and inhibits tumour growth

The above findings intrigued us to test the functions of shank2 in the pathological progression of breast cancer. To assess the biological effect of shank2 *in vivo*, we examined whether shank2 di-methylation affects tumour growth and metastasis in xenograft mouse models. We subcutaneously transplanted equal amounts of MDA-MB-231-shank2 depletion cells with reconstituted expression of shank2 WT or shank2 R240K into the mammary fat pads of BALB/c mice. The results demonstrated that the tumour size and tumour weight were

significantly decreased by reconstituted expression of shank2 WT but not shank2 R240K (Figure 7A-C, Figure 7—figure supplements 1A-C). In contrast, lung metastasis and the number of metastatic pulmonary nodules were dramatically increased by reconstituted expression of shank2 WT but not shank2 R240K (Figure 7D,E, Figure 7—figure supplements 1D,E). To investigate the role of shank2 in tumour growth *in vivo*, we subcutaneously transplanted equal numbers of vector- or shank2-knockdown MCF7 cells into the mammary fat pads of BALB/c mice. As expected, suppression of shank2 in MCF7 cells strongly inhibited tumour growth *in vivo* (Figure 7—figure supplements 1F-H). Our observations indicate that loss of shank2 in tumour cells dramatically inhibits tumour growth in MCF7 cells; and lowered shank2 di-methylation reduces metastasis of MDA-MB-231 cells *in vivo*.

Discussion

The invasive ability of cancer cells is correlated with the attenuation of cell proliferation in a number of cancer cell lines, tumour models, and at the invasive front of human melanoma, colorectal and breast cancer (Gil-Henn et al., 2013; Hur et al., 2016; Patsialou et al., 2015). PRMTs, such as PRMT1, CARM1, PRMT5, PRMT6 and PRMT7, tend to be upregulated during cancer progression including breast cancer (Bao et al., 2018; Gao et al., 2016; Geng et al., 2017; Rengasamy et al., 2017; Yao et al., 2014). Nevertheless, the roles and mechanisms of arginine methylation in regulation of cell reciprocal switch between proliferation and metastasis are yet to be elucidated. Previously we reported that PRMT7 attenuates breast cancer cells proliferation during the process of metastasis (Yao et al., 2014). In this study, we report a new substrate of PRMT7, shank2, which is di-methylated at the residue arginine 240 by PRMT7. Shank2 methylation directs the switch of breast cancer cells in their proliferation and metastasis status. In particular, shank2 R240K methylation disturbs SPN-ANK domain blockade of shank2 (Figure 4C,D), promotes the binding of FAK and cortactin to shank2 (Figure 5E), further potentiates tumour metastasis through FAK-mediated cortactin activation. Meanwhile, methylated shank2 inhibits breast cancer cell proliferation by binding mono-ubiquitinated H-Ras and inhibiting the interaction between Ras and Raf (Figure 6I,J).

For the first time, we have proposed a model for the arginine methylation-modified shank2 in regulating the switch between proliferation and metastasis (Figure 7F). Shank2 R240 methylation may also affect the function of other reported shank2 binding proteins such as Sharpin, GKAP, NMDAR and mGluR. If these shank2 binding proteins are involved in shank2 R240 methylation-mediated switch between proliferation and metastasis needs further investigation.

Scaffold proteins shank1, 2, 3 are able to bind and interact with a wide range of proteins including actin binding proteins and actin modulators, such as cortactin, RICH2, and PIX (Lee et al., 2010; Sheng and Kim, 2000). Shank1, 2, 3 have been reported to affect the structure and function of the neural circuits and to alter behaviour, but their roles in tumorigenesis are far from illuminated. Recent studies have reported that shank1 and shank3 act as negative regulators of integrin activity by binding H-Ras, thereby inhibiting migration and invasion of MDA-MB-231 cells (Lilja et al., 2017). In contrast to shank1 and shank3, the present study reveals that shank2, especially the methylated shank2, is correlated with high level of incidence of breast cancer (Figure 2C). Our data demonstrate that methylated shank2 promotes breast cancer metastasis (Figure 7D,E). We also identify the interaction between shank2 and H-Ras from mass spectrometry analysis. Particularly, shank2 binds to H-Ras and promotes interaction between Ras and Raf. In contrast, the R240-methylated shank2 protein binds to and triggers H-Ras mono-ubiquitination, antagonizes the Ras-Raf binding and blocks the downstream ERK1/2 signalling pathway (Figure 6L), further impedes proliferation of breast cancer cells (Figure 6M). Similar to shank3, our mass spectrometry results suggested that shank2 also interacted with integrin. Integrin-binding FAK has been reported to be localized to EEA-positive endosomes (Wang et al., 2012). Meanwhile, Rabex5 and RIN1 can also be localized in the endosome to direct H-Ras mono-ubiquitination and to promote endosome trafficking and fusion (Baietti et al., 2016), suggesting that the action of the shank2 binding and promoting H-Ras mono-ubiquitination may rely on the endosomal integrin. However, whether shank2 and shank3 modulate the different function of H-Ras through integrin signalling pathway on the endosome remains to be further studied. Collectively, the above results have

made us to believe that shank2 may have a parallel function to that of shank3 and may be a potential target of breast cancer therapy.

From a clinical perspective, in patients successfully treated for primary tumours, cancer cells that have disseminated to other sites can be dormant and viable, but not proliferating (Lambert et al., 2017). Two particular mechanisms permits dormant cancer cells to embark upon a low rate of proliferation, i.e., upregulation of p38 phosphorylation and downregulation of ERK1/2 phosphorylation, which in turn leads to the arrest in G0/G1 phase of cell cycle and quiescence (Bragado et al., 2013). It has been proven that PRMT7 is a regulator for muscle oxidative metabolism by methylating p38 protein to promote p38 phosphorylation and to regulate PGC-1 α expression (Jeong et al., 2016). Similarly, our data suggest that PRMT7 plays a negative role in Ras activation, and subsequently suppresses ERK1/2 activation (Figure 6L), and this is presumably the mechanism that switches tumour cells from proliferation into a dormancy state. Our data implicate that the PRMT7-mediated shank2 methylation may be a critical mediator of dormancy tumour. Shank2 R240 methylation and its downstream effectors could also be targeted to reduce the risk of cancer recurrence in breast cancer patients.

In conclusion, data presented in this report outline a working model in which PRMT7 regulates breast tumour metastasis and growth by methylation of shank2, a new substrate of PRMT7. Specifically, shank2 methylation enforces tumour metastasis, while it simultaneously diminishes tumorigenesis via triggers H-Ras mono-ubiquitination. Therefore, we propose that the PRMT7-mediated methylation of shank2 R240 modulates the reciprocal switching between invasion and proliferation through binding to phosphorylation of FAK/cortactin and mono-ubiquitination of H-Ras (Figure 7F). As a key player in breast cancer development, methylated shank2 may be a potential therapeutic target.

Materials and methods

Cell culture

MCF10A, MCF7, MDA-MB-231, BT549 and HEK293T cell lines were purchased from ATCC, where all the cell lines were characterized by DNA finger printing and isozyme detection. Cells were immediately expanded and frozen such that they could be revived every 3 to 4 months. MCF10A cells were cultured as previously described (Geng et al., 2017). BT549 cells were cultured in RPMI-1640 medium supplemented with 10% FBS (ExCell Bio). MDA-MB-231 cells were cultured in L15 medium with 10% FBS at 37°C without CO₂; HEK293T and MCF7 cells were cultured in DMEM containing 10% FBS.

Antibodies and plasmids

The following antibodies were used: antibodies against E-cadherin, N-cadherin, vimentin, fibronectin, β -catenin (BD Biosciences), β -actin (Sigma-Aldrich), MMP2, MMP9 (GeneTex), PRMT7 (Upstate Biotechnology), PRMT7 (Santa Cruz Biotechnology), PRMT7 (cell signaling technology), Dimethylation (Abcam), Flag (Abmart) and HA (Abmart). H-Ras, shank2, p-FAK, FAK, p-AKT, AKT, p-ERK1/2, ERK1/2, p-Talin, Talin, ZO-1, ubiquitination (cell signaling technology), K,N,H-Ras, p-cortactin, and cortactin (Millipore), Rabex5 (Santa Cruz Biotechnology), RIN1 (Abcam). Flag-cortactin expression vector was a gift from Dr. Alpha S. Yap (Division of Cell Biology and Molecular Medicine, The University of Queensland, Australia), HA-shank2 expression vector was a gift from Dr. Min Goo Lee (Department of Pharmacology, Pusan National University, Pusan, Korea), pcDNA3-HA-shank2, pWPXLD-Flag-shank2, pWPXLD-Flag-PRMT7, pWPXLD-H-Ras, pcDNA3-His-ub. Additionally, shshank2#1 and shshank2#2 oligonucleotides were designed and cloned into lentiviral RNAi system pLKO.1. The sequences of shRNAs, which were designed to target human genes, were described below.

Target shank2#1: GCTTTGGCCCTCTAGAATACA.

Target shank2#1: GGAGTTAGTCAAAGCACAAAG.

Western blotting and Immunoprecipitation

Western blotting and Immunoprecipitation was performed as described previously (Geng et al., 2017).

RNA extraction, reverse transcription and real-time RT-PCR

Total RNA was extracted using Trizol reagent (TaKaRa, Dalian, China), according to the manufacturer's instructions. RT-PCR was performed using the Access RT-PCR System from

Promega. Real-time PCR was done using SYBR Green Realtime PCR Master Mix (TOYOBO, Osaka, Japan) on a LightCycler 480 Real Time PCR system (Roche).

The β -actin was used as an internal control. The sequences of the primers used in this study are described below.

β -actin: 5'-GAGCACAGAGCCTCGCCTTT-3' and 5'-ATCCTTCTGACCCATGCCCA-3'.

shank2:5'-CGGGTAATCCTCCCAAATCA-3' and 5'-CTTTATCCCGGCGTTTCATC-3'.

Lentiviral production and infection

These experiments were essentially performed as described previously (Geng et al., 2017).

Wound healing, cell invasion and migration assays

These experiments were essentially performed as described previously (Qian et al., 2013).

MTT assay

The procedure was performed basically as described previously (Gil-Henn et al., 2013).

Glutathione S-transferase pull-down assay

Briefly, GST, GST-PRMT7 protein expressions were induced by adding 0.1 mmol/L IPTG at 25°C for 8 hours with shaking, and then purified on glutathione-Sepharose4B according to the manufacturer's instructions (GE Healthcare).

Affinity purification of Flag-tagged protein

HEK293T cells were transfected with pWPXLD-3xflag-PRMT7 plasmid, using the PEI reagent according to the manufacturer's instructions. After transfection for 48 h, cells were harvested and lysed in the buffer A containing protease inhibitor cocktail tablet (Roche). Total cell extracts were incubated with anti-Flag affinity gel (Biotool, Kirchberg, Switzerland) for 3 h at 4°C, and the immunoprecipitates were washed 3 times with 13 Tris-buffered saline buffer [50 mM Tris-HCl (pH 7.4) and 150 mM NaCl]. Finally, the bound proteins were eluted with 3xflag peptide (ApexBio, Houston, TX, USA) for 1 h at 4°C.

In vitro shank2 methylation assay

pWPXLD-3xflag-shank2 (5 μ g) was incubated with GST-PRMT7 in the presence of S-adenosyl-methionine (SAM; 15 Ci/mmol; PerkinElmer, Waltham, MA, USA) at 30°C for 1 h. The reaction was stopped by adding loading buffer followed by SDS PAGE.

Structure analysis

The shank2 (NP_036441.2) structure was homology modeled via SWISS-MODEL web server based on the template of PDB ID 5G4X with the identity of 69%. The wild type shank2 model and the mutant one (Arg240Lys) were solvated in ~32,000 TIP3P water molecules with 150 mM NaCl in a 116×116×80 Å box. Both systems were built and pre-equilibrated with the CHARMM program using the CHARMM36 force field. The systems were equilibrated for 25ns using the NAMD2.12 program package under the periodic orthorhombic boundary conditions applied in all directions with the time step of 2 fs. The NPT ensemble was used for both simulations with pressure set to 1 atm and temperature to 310.15 K. Long-range electrostatic interactions were treated by the particle mesh Ewald (PME) algorithm. Non-bonded interactions were switched off at 10-12 Å.

MS analysis

HA-shank2 protein was purified from HA-shank2- MDA-MB-231 cells and was then resolved by 10% SDS-PAGE. After Coomassie brilliant blue staining, the band corresponding to HA-shank2 was excised for liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis performed in the Institute of Biophysics (Chinese Academy of Sciences, Beijing, China).

Breast cancer specimen collection

Human breast tumour specimens were obtained from the Second Hospital of Jilin University, China

Animal studies

MDA-MB-231-Vector, MDA-MB-231-shshank2, MDA-MB-231-shshank2 with shank2 WT or shank2 R240K (2×10^6) cells were injected into the mammary fat pads of 5-week old female BALB/c nude mice. Tumor height and weight were measured with a caliper every 2-3 days. Two months later, the mice were sacrificed and lungs fixed in formalin before embedded in paraffin using the routine procedure. Hematoxylin and eosin (H&E) staining was performed on sections from paraffin-embedded tissues. For bioluminescence imaging, mice were injected with 150 mg/kg D-luciferin (GoldBio) 10 minutes before imaging. Following anesthesia, images were collected using the Night OWL LB 983 Spectrum Imaging System. For tumorigenesis test, MCF7-Vector or MCF7-shshank2 cells were injected into the mammary fat pads of 5-week old

female BALB/c nude mice. Mice were sacrificed when tumors reached maximum allowed size (15 mm in diameter) or when signs of ulceration were evident. Tumor height and weight were measured with a caliper every 2-3 days. Then, the primary tumor weight and volume were recorded. All animal experiments were approved by the Animal Care Committee of the Northeast Normal University, China.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism 5 software. Statistical parameters and methods are reported in the Figures and the Figure Legends. Unless specified, comparisons between groups were made by unpaired two-tailed Student's *t*-test. A value of $p < 0.05$ was considered statistically significant.

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Yingqi Liu: Conceptualization; Data curation; Formal analysis; Supervision; Validation; Investigation; Visualization; Writing—original draft; Project administration; Xiaoqing Liu: Conceptualization; Formal analysis; Investigation; Lingxia Liu: Resources; Validation; Yibo Wang: Resources; Software; Funding acquisition; Validation; Visualization; Lu Peng: Conceptualization; Formal analysis; Jiayuan Liu: Conceptualization; Formal analysis; Lingling Li: Conceptualization; Formal analysis; Lian zhang: Resources; Formal analysis; Validation;

Guannan Wang: Resources; Software; Formal analysis; Validation; Visualization; Hongyuan Li: Resources; Validation; Dongxu Liu: Project administration; Baiqu Huang: Conceptualization; Resources; Formal analysis; Funding acquisition; Project administration; Writing—review and editing; Jun Lu: Resources; Formal analysis; Supervision; Funding acquisition; Project administration; Writing—review and editing; Yu Zhang: Formal analysis; Supervision; Funding acquisition; Validation; Writing—original draft; Project administration; Writing—review and editing.

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Figure 1. PRMT7 methylated shank2. Immunoprecipitation and immunoblotting analyses were performed with the indicated antibodies. **(A)** HEK293T cells were co-transfected with Flag-PRMT7 and/or HA-shank2 plasmids. **(B)** Endogenous shank2 or PRMT7 was IP from MDA-MB-231 cells lysate, with anti-shank2 or anti-PRMT7 antibody, and the binding of PRMT7 and shank2 was examined by western blot. **(C)** Purified bacterially expressed GST or GST-PRMT7 was incubated with MDA-MB-231 cells lysate. GST-pull-down assay showing direct interaction between shank2 and recombinant PRMT7. **(D)** Diagram of the domains in shank2 protein. The schematics of the GST-shank2 expression plasmid, as well as domains and truncated mutants. **(E)** shank2 truncated mutants were incubated with

MDA-MB-231 cells lysate. GST-pulldown assay showing direct interaction between PRMT7 and recombinant shank2. **(F)** HEK293T cells were transfected with increasing amounts of Flag-PRMT7 expression plasmids. **(G)** MDA-MB-231 with or without stable expression of the indicated PRMT7 shRNA or a control shRNA. **(H)** MDA-MB-231 cells with or without expressing PRMT7 shRNA and with or without reconstituted expression of WT PRMT7 or PRMT7 enzymatic activity mutant. **(I)** Flag-tagged shank2 was expressed in HEK293T cells. Flag-purified shank2 protein was incubated with SAM and with or without purified bacterially expressed PRMT7. Methylation of recombinant shank2 protein was determined by western blot.

Figure 2. Di-methylation of shank2 in human breast cancers. (A) Analysis of shank2 gene expression in human tissues using the Human Protein Atlas data. **(B)** Comparison of shank2 expression level between non-tumorigenic and tumorigenic mammary cell lines by RT-PCR. P values were calculated by unpaired, two-tailed Student's-tests. **(C)** Comparison

of PRMT7, shank2 and di-methylated shank2 levels between breast cancer tissues and adjacent normal tissues using western blot analysis. Data are based on the analysis of independent samples (n=18). Blots were quantified and normalized to β -actin. shank2 methylation of immunopurified shank2 was determined and normalized to shank2 protein.

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Figure 3. PRMT7 methylated shank2 at R240 promoted migration and invasion of breast cancer cells. (A) Immunoprecipitation and immunoblotting analyses were performed with the indicated antibodies. The purified shank2 from MDA-MB-231 cells transfected with HA-shank2 was analysed for methylation by mass spectrometry. The shank2 R240 residue in fragmentation of KAARMRN was methylated. The Mascot score was 27.28, and the expectation value was 3.74E-04. (B) Alignment of the consensus shank2 sequences between different species near the R240 methylation site. (C) MDA-MB-231 cells with or without expressing shank2 shRNA and with or without reconstituted expression of WT shank2 or shank2 R240K mutant. (D) Flag-tagged shank2 WT or shank2 R240K was transfected into MDA-MB-231 cells. Flag-purified shank2 WT or shank2 R240K proteins were

incubated with purified bacterially expressed PRMT7 and SAM. Methylation of shank2 protein was determined by western blot. **(E)** HEK293T cells were co-transfected with increasing amounts of Flag-PRMT7 and shank2 WT or shank2 R240K expression plasmids. **(F)** shank2 was IP from indicated breast cancer cell lines lysate, and input lysates and IP samples were analysed using anti-methylation, anti-shank2 and anti-PRMT7 antibodies, as indicated. **(G)** MDA-MB-231 cells expressing shank2 shRNA and reconstituted expression of shank2 WT or shank2 R240K mutant. Western blot was performed with the indicated EMT marker antibodies. **(H)** MDA-MB-231 cells with or without stable expression of the indicated shank2 shRNA were transfected with shank2 WT or shank2 R240K mutant. Assessment of MMP2 and MMP9 enzymatic activities by gelatin zymography. **(I)** MDA-MB-231 cells expressing shank2 shRNA and reconstituted expression of shank2 WT or shank2 R240K mutant. Cell mobility was determined by wound healing assays. Scale bars: 100 μ m. **(J and K)** MDA-MB-231 cells expressing shank2 shRNA and reconstituted expression of shank2 WT or shank2 R240K mutant. Cell migration (J) and Invasion (K) were determined by transwell assays. (n=3, independent experiments). Scale bars: 50 μ m.

Figure 4. R240 methylation impairs SPN-ANK domain interaction of shank2

(A) Potential of mean forces for the conformational changes of SPN and ANK in the wild type (WT, black line) and mutant (R240K, red line) of shank2. **(B)** Definition of the SPN domain (coloured in green) and the ANK domain (coloured in cyan) in shank2. The location of R240 was represented with sticks. **(C)** Key residues forming hydrogen bonds between SPN

764 and ANK in shank2 WT. R240 can form hydrogen bonds with H236 in ANK (coloured in
765 magenta). **(D)** Key residues forming hydrogen bonds between SPN and ANK in shank2
766 R240K. K240 can still form hydrogen bonds with H236 in ANK (coloured in magenta).

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787 **Figure 5. Shank2 R240 methylation was necessary for FAK and cortactin activation.**

788 Immunoprecipitation and immunoblotting analyses were performed with the indicated
789 antibodies. **(A)** Endogenous Talin, FAK and shank2 were IP from MDA-MB-231 cells, with
790 anti-Talin, anti-FAK and anti-shank2 antibodies, and the binding of Talin, FAK and shank2

was examined by western blot. **(B)** MDA-MB-231 with or without stable expression of the indicated PRMT7 shRNA or a control shRNA. **(C)** MCF7 cells expressing the indicated Flag-Vector or Flag-tagged PRMT7. **(D and F)** MDA-MB-231 cells with or without expressing shank2 shRNA and with or without reconstituted expression of WT shank2 or shank2 R240K mutant. **(E)** MCF10A cells with reconstituted expression of shank2 WT, shank2 R240K or shank2 R240F (arginine to phenylalanine) mutant. **(G)** MCF7 cells with reconstituted expression of PRMT7 and with or without stable expression of the indicated shank2 shRNA. **(H)** MDA-MB-231 with or without stable expression of the indicated FAK shRNA or a control shRNA. **(I)** Phosphorylated and total FAK, cortactin and AKT were determined in MDA-MB-231 cells with or without expressing shank2 shRNA and with or without reconstituted expression of WT shank2 or shank2 R240K mutant. Western blot was performed with the indicated antibodies. **(J)** Phosphorylated and total FAK, cortactin and AKT were determined in MCF10A cells co-transfected with shank2 and FAK plasmids. Western blot was performed with the indicated antibodies. **(K)** Phosphorylated and total FAK, cortactin and AKT were determined in MCF7 cells MDA-MB-231 cells with PRMT7 and with or without stable expression of the indicated shank2 shRNA. Western blot was performed with the indicated antibodies.

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Figure 6. Shank2 R240 methylation impeded breast cancer proliferation by enhancing

H-Ras mono-ubiquitination. Immunoprecipitation and immunoblotting analyses were performed with the indicated antibodies. **(A)** Endogenous Ras and shank2 were IP from MDA-MB-231 cells lysate, with anti-Ras or anti-shank2 antibody, and the binding of endogenous Ras and shank2 was examined by western blot. **(B)** HEK293T cells were co-transfected with H-Ras and/or HA-shank2 plasmids. **(C)** MDA-MB-231 with or without stable expression of the indicated PRMT7 shRNA or a control shRNA. **(D)** MCF7 cells expressing the indicated Flag-Vector or Flag-tagged PRMT7. **(E)** MDA-MB-231 cells with or without expressing shank2 shRNA and with or without reconstituted expression of WT shank2 or shank2 R240K mutant. **(F)** MCF7 cells with or without expressing shank2 shRNA and with or without reconstituted expression of WT shank2 or shank2 R240F (arginine to phenylalanine) mutant. **(G)** HEK293T cells were co-transfected with His-tagged Ub with or without stable expression of the indicated PRMT7 shRNA. **(H)** HEK293T cells were co-transfected with His-tagged Ub with or without stable expression of the indicated shank2 shRNA. **(I)** HEK293T cells were co-transfected with H-Ras and His-Ub together with shank2 WT or shank2 mutants. **(J)** MDA-MB-231 cells with or without expressing shank2 shRNA and with or without reconstituted expression of WT shank2 or shank2 R240K mutant, cells treated with EGF (100 ng/mL) for the indicated time intervals. GST pull down with recombinant Raf1 RBD and input lysates and pull down samples were analysed using anti-Ras, anti-GST and anti-shank2 antibodies, as indicated. **(K)** MDA-MB-231 cells were treated same as **(J)**. Western blot was performed with the indicated antibodies. **(L)** Phosphorylated and total ERK1/2 was determined in MDA-MB-231 cells with or without expressing shank2 shRNA and with or without reconstituted expression of WT shank2 or shank2 R240K mutant. Western blot was performed with the indicated antibodies. **(M)** MTT assay in MDA-MB-231 cells with or without expressing shank2 shRNA and with or without reconstituted expression of WT shank2 or shank2 R240K mutant for cell counting. Data represent the mean $\pm$ SD of three independent experiments. * P < 0.001, Student's t test.

852 **tumour proliferation. (A)** Representative images of BALB/c mice transplanted with equal
853 numbers of MDA-MB-231 cells with or without expressing shank2 shRNA and with or without
854 reconstituted expression of WT shank2 or shank2 R240K mutant. **(B)** Equal numbers of

MDA-MB-231 cells with or without expressing shank2 shRNA and with or without reconstituted expression of WT shank2 or shank2 R240K mutant were transplanted into mammary fat pads of BALB/c mice, and tumour growth was monitored at the indicated times. Data are present as means $\pm$ SEM; n=6 tumours in each group. * p <0.05, ** p <0.01, Student's t test. **(C)** Equal numbers of MDA-MB-231 cells with or without expressing shank2 shRNA and with or without reconstituted expression of WT shank2 or shank2 R240K mutant were transplanted into mammary fat pads of BALB/c mice, and tumour weight was determined after transplantation. Data are present as means $\pm$ SEM; n=6 tumours in each group. * p <0.05, ** p <0.01, Student's t test. **(D and E)** Equal numbers of MDA-MB-231 cells with or without expressing shank2 shRNA and with or without reconstituted expression of WT shank2 or shank2 R240K mutant were transplanted into the mammary fat pads of BALB/c mice, and lung metastasis of the primary tumour was determined. Normal lung tissue was stained with yellow India ink, whereas tumour nodules remained white. The gross appearance of the lungs and the tumour nodules on the lungs were examined. Tumours were paraffin embedded and stained with H&E 12 days after transplantation. Data are presented as means $\pm$ SEM; n=6 tumours in each group. * p <0.05, ** p <0.01, Student's t test. **(F)** Schematic of methylated shank2-regulated FAK phosphorylation and H-Ras mono-ubiquitination. PRMT7 methylates shank2 at R240, resulting in binding of phosphorylated FAK and mono-ubiquitinated H-Ras to shank2. Methylated shank2 inhibits H-Ras activation for ERK1/2 dependent tumour cell proliferation and enhances FAK, AKT and cortactin activation, which in turn promotes cell migration and invasion.

Figure 1—figure supplement 1. Shank2 methylation dependent in PRMT7.

Immunoprecipitation and immunoblotting analyses were performed with the indicated antibodies. **(A)** MDA-MB-231 with or without stable expression of the indicated PRMT7 shRNA or a control shRNA. Western blot was performed with the indicated antibodies. **(B)** MDA-MB-231 cells with or without expressing PRMT7 shRNA and with or without reconstituted expression of PRMT7 WT or PRMT7 R531K mutant.

Figure 3—figure supplement 1. Shank2 R240 methylation did not dependent on PRMT5.

Immunoprecipitation and immunoblotting analyses were performed with the indicated antibodies. (A) HEK293T cells were co-transfected with control pcDNA3.1 or PRMT5 plasmids. (B) HEK293T cells were co-transfected with increasing amounts of Flag-PRMT5 and shank2 WT or shank2 R240K expression plasmids.

Figure 3—figure supplement 2. Shank2 was involved in breast cancer cells EMT. (A)

shank2 mRNA expression in 18 tumor tissues and paired adjacent normal mammary by quantitative real-time polymerase chain reaction (qRT-PCR). The logarithmic scale of $2^{-\Delta\Delta Ct}$ was used to measure the fold-change. β -actin was used as an internal reference. **(B)** BT549 cells with or without expressing shank2 shRNA and with or without reconstituted expression of shank2 WT or shank2 R240K mutant. Western blot was performed with the indicated EMT marker antibodies. **(C)** MDA-MB-231 with or without stable expression of the indicated shank2 shRNA or a control shRNA. Western blot was performed with the indicated EMT marker antibodies. **(D)** MCF-10A cells expressing the indicated HA-tagged shank2. Western blot was performed with the indicated EMT marker antibodies. **(E)** MDA-MB-231 cells transfected with stable expression of the indicated PRMT7 shRNA or a control shRNA, and MCF-10A cells expressing the indicated HA-tagged shank2. Assessment of MMP2 and MMP9 enzymatic activities by gelatin zymography. **(F)** Stem-like $CD44^{high}/CD24^{low}$ profile in MDA-MB-231 cells transfected with stable expression of the indicated PRMT7 shRNA or a control shRNA, and MCF-10A cells expressing the indicated HA-tagged shank2.

Figure 5—figure supplement 1. Shank2 was essential for the interaction and activity between talin and FAK.

Immunoprecipitation and immunoblotting analyses were performed with the indicated antibodies. **(A)** shank2 truncated mutants were incubated with MDA-MB-231 lysates. GST-pulldown assay showing direct interaction between Talin, FAK and recombinant

shank2. **(B)** MDA-MB-231 cells with or without expressing PRMT7 shRNA and with or without reconstituted expression of WT PRMT7 or PRMT7 enzymatic activity mutant. **(C)** MDA-MB-231 cells with or without expressing PRMT7 shRNA and with or without reconstituted expression of PRMT7 WT or PRMT7 R531K mutant. **(D)** BT549 cells with or without expressing PRMT7 shRNA and with or without reconstituted expression of PRMT7 WT or PRMT7 R531K mutant. **(E)** MDA-MB-231 with or without stable expression of the indicated shank2 shRNA or a control shRNA. **(F)** HEK293T cells were transfected with increasing amounts of Flag-PRMT7 expression plasmids. **(G)** MCF-7 cells with reconstituted expression of PRMT7 and with or without stable expression of the indicated shank2 shRNA. **(H)** MDA-MB-231 cells was treated with FAK inhibitor GSK2256. **(I)** Phosphorylated and total FAK, cortactin and AKT were determined in MDA-MB-231 cells with or without stable expression of the indicated shank2 shRNA or a control shRNA. **(J)** Phosphorylated and total FAK, cortactin and AKT were determined in MCF-7 cells expressing the indicated Flag-tagged PRMT7. **(K)** Phosphorylated and total FAK and AKT were determined in MCF-10A cells expressing the indicated HA-tagged shank2. **(L)** Phosphorylated and total Talin were determined in MDA-MB-231 cells with or without stable expression of the indicated shank2 shRNA or a control shRNA.

Figure 5—figure supplement 2. Shank2 R240F activated FAK and cortactin. (A)

Phosphorylated and total FAK, cortactin and AKT were determined in MCF-10A cells co-transfected with shank2 and cortactin. Western blot was performed with the indicated antibodies. **(B)** MCF-10A cells co-transfected with shank2 and FAK. Assessment of MMP2 and MMP9 enzymatic activities by gelatin zymography. **(C)** MCF-10A cells co-transfected with shank2 and cortactin. Assessment of the MMP2 and MMP9 enzymatic activities by gelatin zymography. **(D)** BT549 cells with or without expressing shank2 shRNA and with or without reconstituted expression of shank2 WT or shank2 R240K mutant. Flow cytometric analysis of annexin V-FITC/PI staining was conducted to examine cell death.

Figure 5—figure supplement 3. Shank2 was necessary for PRMT7 induced-EMT. (A)

MCF-7 cells with reconstituted expression of PRMT7 and with or without stable expression of the indicated shank2 shRNA. Western blot was performed on cell lysates with the indicated EMT marker antibodies. **(B)** Wound healing assays of MCF-7 cells with reconstituted expression of PRMT7 and with or without stable expression of the indicated shank2 shRNA. Scale bars: 100 μm. **(C)** MCF-7 cells with reconstituted expression of PRMT7 and with or without stable expression of the indicated shank2 shRNA. Assessment of the MMP2 and MMP9 enzymatic activities by gelatin zymography. **(D and E)** Migration assays (D) and Invasion assays (E) in MCF-7 cells with reconstituted expression of PRMT7 and with or without stable expression of the indicated shank2 shRNA. **(F)** MDA-MB-231 cells was treated with or without GSK2256 for cell counting. Data represent the mean ± SD of three independent experiments. * $P < 0.01$, Student's t test.

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Figure 6—figure supplement 1. Un-methylated shank2 enhanced Ras binding to Raf independent of FAK and cortactin activation. Immunoprecipitation and immunoblotting analyses were performed with the indicated antibodies. **(A)** MDA-MB-231 cells was treated with or without U0126. **(B and C)** HEK293T cells were transiently expressed the indicated plasmids. **(D)** MDA-MB-231 cells was treated with EGF for 24 hr. **(E)** GTP-bound Ras was pulled down from MDA-MB-231 cells with or without stable expression of the indicated shank2 shRNA or a control shRNA. **(F)** GTP-bound Ras was pulled down from MDA-MB-231 cells with or without stable expression of the indicated PRMT7 shRNA or a control shRNA. **(G)** GTP-bound Ras was pulled down from MCF-7 cells expressing the indicated Flag-tagged PRMT7. **(H)** Phosphorylated and total ERK1/2 were determined in MDA-MB-231 cells with or without stable expression of the indicated shank2 shRNA or a control shRNA. **(I)** Phosphorylated and total ERK1/2 were determined in MCF-10A cells expressing the indicated HA-tagged shank2. **(J)** Phosphorylated and total ERK1/2 were determined in MDA-MB-231 cells with or without stable expression of the indicated PRMT7 shRNA or a control shRNA. **(K)** Phosphorylated and total ERK1/2 were determined in MCF-7 cells expressing the indicated Flag-tagged PRMT7. **(L)** MCF-10A cells expressing the indicated HA-tagged shank2 for cell counting. Data represent the mean $\pm$ SD of three independent experiments. *** $P < 0.001$, Student's t test.

Figure 7—figure supplement 1. Shank2 promoted tumour growth. (A) Equal numbers of MDA-MB-231 cells with or without stable expression of the indicated shank2 shRNA or a control shRNA were transplanted into mammary fat pads of BALB/c mice, and tumor growth was monitored at the indicated times. Data are present as means $\pm$ SEM; n=6 tumors in each group. *** p <0.001, Student's t test. **(B and C)** Equal numbers of MDA-MB-231 cells with or without stable expression of the indicated shank2 shRNA or a control shRNA were transplanted into mammary fat pads of BALB/c mice, and tumor volume (B) and weight (C) was determined after transplantation. Data are present as means $\pm$ SEM; n=6 tumors in each group. *** p <0.001, Student's t test. **(D and E)** Equal numbers of MDA-MB-231 cells with or without stable expression of the indicated shank2 shRNA or a control shRNA were transplanted into the mammary fat pads of BALB/c mice, and lung metastasis of the primary tumor was determined. Normal lung tissue was stained with yellow India ink, whereas tumor nodules remained white. The gross appearance of the lungs and the tumor nodules on the lungs were examined. Tumors were paraffin embedded and stained with H&E 12 days after transplantation. Data are presented as means $\pm$ SEM; n=6 tumors in each group. * p <0.05,

1035 ** $p < 0.01$, Student's t test. **(F)** Bioluminescence images of BALB/c mice transplanted with
 1036 equal numbers of MCF-7 cells with or without stable expression of the indicated shank2
 1037 shRNA or a control shRNA. **(G and H)** Equal numbers of MCF-7 cells with or without stable
 1038 expression of the indicated shank2 shRNA or a control shRNA were transplanted into BALB/c
 1039 mice, and tumor volume (G) and weight (H) was determined after transplantation. Data are
 1040 present as means $\pm$ SEM; $n=6$ tumors in each group. * $p < 0.05$, ** $p < 0.01$, Student's t test.

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