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Original Paper

The Interaction between Histone Lysine Methyltransferase GLP and EZH2

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Abstract

Recent studies have implicated that H3K9 lysine methyltransferases (KMTs) and polycomb repressive complex2 (PRC2) act in a cross-talk. However, a connection between GLP, one of the well-known H3K9 KMTs and the EZH2, one of the core members of PRC2, has not been established. Here we not only demonstrated that there was interaction between the GLP and the EZH2 proteins in vivo, but also provided evidence that the activity of EZH2 was effected by down-regulation of GLP. In light of these observations, western blotting, co-immunoprecipitation and qRT-PCR were performed to explore in GLP siRNA KGN cells. The specific interaction between GLP and EZH2 was demonstrated with co-immunoprecipitation. Our results also indicate that the decreased level of GLP participate in the modulation of EZH2 function in vivo.Taken together, our findings identified that an unanticipated interplay between the two histone lysine methyltransferases, which is implicated in regulating of a subset of developmental genes.

Keywords

lysine methyltransferases, cross-talk, GLP, EZH2

1. Introduction

The highly similar euchromatic methyltransferases G9a-related protein GLP (also known as EuHMT1), which is form a heteromeric complex with G9a (also known as EuHMT2) dependent on their SET domain-containing proteins (Su(var)3-9 Enhancer of zeste Trithorax, SET domain proteins) (Collins & Cheng, 2010; Tachibana, Ueda, Fukuda, Takeda, Ohta, Iwanari, Sakihama, Kodama, Hamakubo, & Shinkai, 2005). In Mammal cells, GLP is the only one closely related homolog of G9a and which is 63% identical to G9a, is directly involved in the mono-and di-methylation of lysine 9 on histone H3 (H3K9me1 and H3K9me2) at euchromatic regions (Tachibana, Ueda, Fukuda, Takeda, Ohta, Iwanari, Sakihama, Kodama, Hamakubo, & Shinkai, 2005; Tachibana, Sugimoto, Nozaki, Ueda, Ohta, Ohki, Fukuda, Takeda, Niida, Kato, & Shinkai, 2002). In cells lacking GLP, the steady-state levels of G9a is

decreased. However, GLP content was unaffected in G9a−/− ES cells (Tachibana, Ueda, Fukuda, Takeda, Ohta, Iwanari, Sakihama, Kodama, Hamakubo, & Shinkai, 2005). Interesting, G9a is more stable in the G9a/GLP heteromeric complex than when it is expressed alone but the converse apparently does not apply to GLP. *In vitro*, H3K9me1 and H3K9me2 levels were significantly reduced after G9A or GLP knockout, however, the level of H3K9me1 and H3K9me2 cannot be further reduced after the double knockout of G9a and GLP. Therefore, G9a generally does not compensate for the loss of GLP histone methyltransferase function *in vitro*, and vice versa (Kleefstra, Brunner, Amiel, Oudakker, Nillesen, Magee, Genevi ève, Cormier-Daire, van Esch, Fryns, Hamel, Sistermans, de Vries, & van Bokhoven, 2006; Kleefstra, Smidt, Banning, Oudakker, Van Esch, de Brouwer, Nillesen, Sistermans, Hamel, de Bruijn, Fryns, Yntema, Brunner, de Vries, & van Bokhoven, 2005; Tachibana, Sugimoto, Fukushima, & Shinkai, 2001).

Enhancer of zeste homologue 2 (EZH2) is the core member and catalytic subunit of the complex PRC2 (polycomb repressive complex 2), which is directly responsible for the deposition of me2 and me3 of H3K27 (H3K27me2/3) through its SET domain (Pasini, Bracken, Hansen, Capillo, & Helin, 2007; Cao, Wang, Wang, Xia, Erdjument-Bromage, Tempst, Jones, & Zhang, 2002). Inhibited EZH2 methyltranferase activity is not associated with the levels of other two noncatalytic subunits of PRC2 core complex: Suppressor-of-Zeste 12 (Suz12), embryonic ectoderm development (Eed) (Qu, Lu, Jiang, Chi, & Zhang, 2016; O'Meara & Simon, 2012). In the mouse oocytes with depletion of EZH2, the expression of H3K27me3 and H3K9me2 were greatly inhibited ic ectoderm development (Eed) (Qu, Lu, Jiang, Chi, & Zhang, 2016). H3K9 and H3K27 lysines are insert into a similar peptide motif, namely Alanine-Arginine-Lysine-Serine (ARKS), have implicated their modification by common enzymes (Mozzetta, Pontis, & Ait-Si-Ali, 2015).

PRC2/EZH2 play important role in induced pluripotency in different somatic cells, however, G9a/GLP and H3K9 methylation correlate with impeded the reacquisition of pluripotency and an efficient cellular reprogramming (Chen, Liu, Liu, Qi, Wei, Yang, Liang, Chen, Chen, Wu, Guo, Zhu, Zhao, Peng, Zhang, Chen, Li, Li, Wang, & Pei, 2013). Recent findings suggest that, these two major lysine methyltransferases (KMTs) families emerged in favor of a functional crosstalk (Mozzetta, Pontis, & Ait-Si-Ali, 2015; Mozzetta, Pontis, Fritsch, Robin, Portoso, Proux, Margueron, & Ait-Si-Ali, 2014). However, a direct evidence of a functional interplay between GLP and EZH2 *in vivo* is still lacking. Our results suggested that GLP interacts functionally with EZH2. Co-immunoprecipitation (Co-IP) experiments analyse show that a crosstalk between GLP and EZH2.

2. Materials and Methods

2.1 Cell Culture and RNA Interference

Human granulosa-like tumor cell line (KGN cells) was seeded in 6-well plates at $5 \times 10_6$ cells/well and allowed to adhere 24 hours. The cells were then transfected with siRNA. The siRNAs directed against GLP and the scrambled control siRNA were synthesised by Invitrogen biotechnology. The sequence

are listed in the Supplementary Table 1. siRNA transfection was performed using Lipofectamine™ RNAiMAX Transfection Reagent (Invitrogen, USA) according to the manufacturer's instructions.

2.2 Protein Extraction and Western Blot Analysis

The protein extraction and Western blotting were performed according to Kit instructions (Biyuntian, China). Briefly, KGN cells were homogenized and pooled, and equal amounts of protein per sample and ten μl of Multi-Coloured Standard markers (Biyuntian, China) were separated by 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels and transferred to a polyvinylidene fluoride (PVDF) membrane (Amersham Biosciences, USA) at 100 V for 90 min, which was then blocked in 1% w/v BSA (Sigma, USA) in 0.1% PBS-Tween. Membranes were incubated with the specific antibodies against GLP (1:2000; R&D), EZH2 (1:1000; Cell Signaling Technology), H3K27me2 (1:1000; Cell Signaling Technology), H3K27me3 (1:1000; Abcam), or GAPDH (1:5000; Cell Signaling Technology), respectively, at 4℃overnight. Membranes were then incubated with alkaline phosphatase-linked secondary antibody (1:5000; Santa Cruz) at room temperature for 50 min. Protein bands were exposed and developed by Thermo Scientific™ Pierce ECL Plus Substrate (Rockford, USA) through Image Quant LAS 4000 (Waukesha, USA).

2.3 Co-immunoprecipitation (Co-IP)

Co-immunoprecipitation experiments (Co-IP) were examined according Kit instructions (abs,China). Briefly, 10μl of antibodies directed against the GLP or EZH2 was added to precleaned KGN cells lysates and incubated at 4℃overnight. Nonspecific mouse IgG was used as controls. Immune complexes were added 5μl protein A and 5μl protein B and incubated for an additional 3 hours at room temperature. The resulting immobilized immune complexes were pelleted by centrifugation at 12000×g for 1 min, washed four times with 0.5 ml 1×wash buffer. The bound protein was boiling in 30μl 1×SDS loading buffer for 5 min, centrifugation at 14000×g for 1 min and Keep the supernatant. Immunoprecipitated complexes were detected by Western blot analysis as described above.

2.4 Quantitative Real Time RT-PCR (qRT-PCR)

qRT-PCR was performed with SYBR Premix Ex Taq (Takara Biotechnology, China) for 40 cycles using the Applied Biosystems (Foster City, USA) StepOnePlus™ Real-Time PCR System. The primer pairs used are listed in Supplementary Table 2. GAPDH was used as a reference gene.

2.5 Statistical Analysis

All experiments were repeated three times. Data from the RT-qPCR experiments were analyzed with paired t-tests, $P \leq 0.05$ were considered significant.

3. Results

3.1 Loss of GLP in the KGN Cells

The human granulosa-like tumor cell line KGN was used for assessing the effects of GLP siRNA. siRNA that specifically target GLP were transiently transfected into KGN cells, 24h later, were collected for tested. The results showed that downregulation of GLP has no apparent defects for the

cellular morphology (Figure 1a). qRT-PCR and western blotting were perform to detect the knocking down efficiency of GLP siRNA.The knocking down efficiency of GLP siRNA is very effective ($P < 0.05$) (Figure 1b,c) and the third siRNA strand (one of three siRNA strands, knocking down efficiency is 77.32%±0.04) will be used to predicted and confirmed the function of GLP.

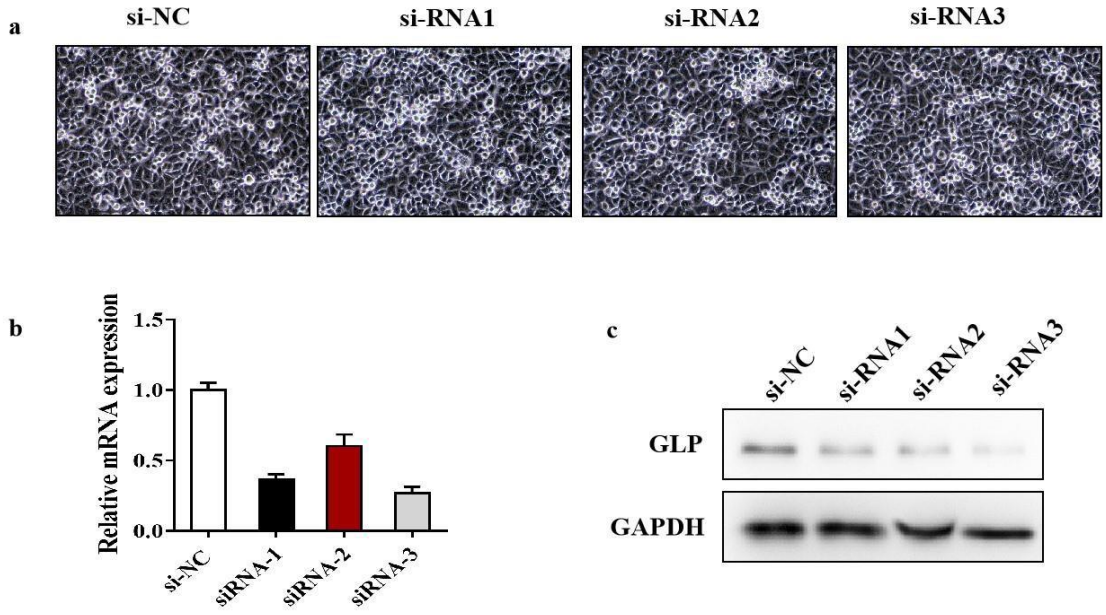

Figure 1. Each of the three distinct siRNAs that specifically target GLP were transiently transfected into KGN cells. **a.** 24 hours after transfection, the cellular morphology was observed. **b.** GLP mRNA expression was determined by qRT-PCR at 24 hours after transfection. Bars represent means ± standard deviations (SD) of data from ≥3 independent experiments. **c.** GLP protein expression was determined by western blotting at 24 hours after transfection. The level of GAPDH was used as an internal standard to verify equal loading of proteins. si-NC: transfected with control siRNA, si-GLP: transfected with GLP siRNA.

3.2 GLP Interacts with EZH2 in the KGN Cells

To elucidate if GLP and EZH2 interacted in the intracellular environment, we performed Co-IP experiments. Total lysates were subjected to Co-IP with anti-GLP or anti-EZH2 antibodies in order to obtain endogenous either GLP or EZH2 from normal level (si-NC, transfected with control siRNA) and downregulation level of GLP (si-GLP, transfected with GLP siRNA) KGN cells. Subsequently, the immunocomplexes were analyzed by western blotting analyses with an anti-GLP antibody to detect the interacted complexes of EZH2/GLP. We observed that: (1) in the anti-GLP immunoprecipitates, a major single EZH2-immunoreactive band was revealed in si-NC and si-GLP KGN cells (Figure 2a); (2) in the anti-EZH2 immunoprecipitates, a major single GLP-immunoreactive band was revealed in si-NC and si-GLP KGN cells (Figure 2b). The GLP band was recognized lighter in the si-GLP KGN cells lysates immunoprecipitates consistent with the observation in direct western blotting analyses (Figure

1c). The EZH2 immunoreactive band was revealed little lighter in the si-GLP compare to si-NC KGN cells.

Thus, this experiment provides strong evidence that there was crosstalk between GLP and EZH2.

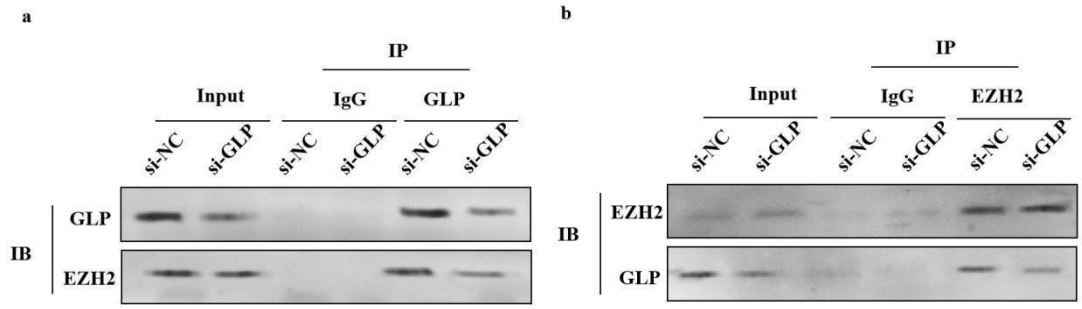

Figure 2. GLP interacts with EZH2 in vivo. Whole tissue extracts isolated from normal level or downregulation level of GLP KGN cells, were immunoprecipitated with either anti-GLP or anti-EZH2 antibodies. **a.** In the anti-GLP immunoprecipitates, a major single EZH2-immunoreactive band was revealed in si-NC and si-GLP KGN cells. **b.** In the anti-EZH2 immunoprecipitates, a major single GLP- immunoreactive band was revealed in si-NC and si-GLP KGN cells. IP: immunoprecipitation, IB: immunoblotting.

3.3 Loss of GLP Weaken K3K27me2/3 Modification in KGN Cells

In the case of illuminate there was crosstalk between GLP and EZH2, western blotting experiment was performed in the si-GLP KGN cells. Preimplantation embryos lacking Ezh2 showed markedly reduced modification of H3K27 me2 and H3K27 me3 in our previous experiment (Huang, Wang, Ma, Sun, Zhou, Zhu, & Liu, 2014). Loss of GLP weaked the EZH2 modification in si-GLP KGN cells (Figure 2a,b), whether those H3K27 me2 and H3K27 me3 level would be affected? western blotting analyses in si-GLP KGN cells have shown that the modification of H3K27 me2 and H3K27 me3 protein expression were all significantly decrease (Figure 3), which is in line with our previous studies.

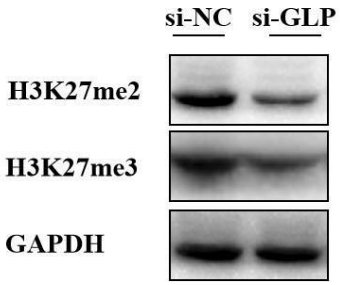

Figure 3. A negative correlation between GLP and H3K27me2/3 expression in KGN cells. Protein samples were isolated from KGN cells at 24 hours after transfected with either si-NC or si-GLP. Total cell lysate was subjected to western blotting analysis of H3K27me2 and H3K27me3 expression. The level of GAPDH was used as an internal standard to verify equal loading of proteins.

3.4 Loss of GLP Causes Up-regulation of EZH2 Target Genes

As important epigenetic regulators, PcG proteins bind to target gene promoters and direct post-translational modifications through H3K27, thereby silencing gene expression(8). The reduction of GLP levels by siRNA decrease EZH2, therefore, increased the expression of Tumor protein p53 (TP53)(2.13±0.17) and its downstream effector P21 mRNA (1.94±0.13) expression ($P<0.05$) in KGN cells (Figure 4), has been consistent with previous reports (Gao, Li, Qiu, Zhu, Pan, Zhao, Wei, Shi, Jin, & Xue, 2017). Similarly, the knockdown of GLP by distinct siRNA clearly increased Kruppel-like factor 2 (KLF2)(2.24±0.14) , cell cycle suppressor INK/ARF(3.28±0.31), DNA damage repair protein RAD51 mRNA (1.74±0.47) expression ($P < 0.05$), but BMPR1B slight decrease expression (0.84±0.16)($P>0.05$), which are all the directed of EZH2 target genes (Figure 4). In conclusion, our findings strongly suggest that there was crosstalk between GLP and EZH2.

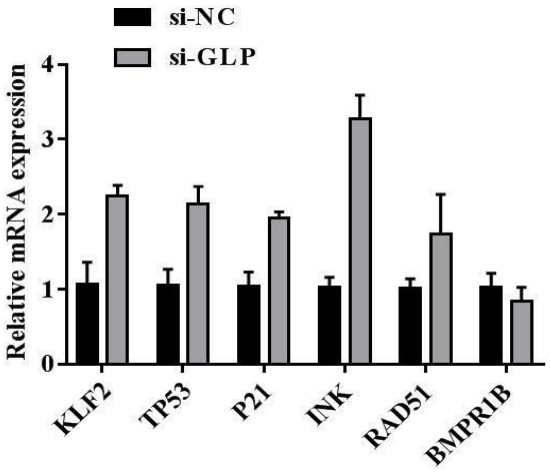

Figure 4. GLP impact EZH2 target genes expression in KGN cells. The mRNA levels of KLF2, TP53, P21, INK, RAD51and BMPR1B were determined by qRT-PCR in KGN cells transfected with si-NC or GLP siRNA at 24 hours after transfection. Bars represent means ±SD of data from ≥3 independent experiments.

4. Discussion

In the present study, we have demonstrated that GLP protein link a functional crosstalk with EZH2 in KGN cells. Furthermore, GLP not only affect the activity of histone methylation of EZH2, but also suggesting affect the target genes expression of EZH2.

PRC2 controls all forms of H3K27 methylation: directly involved in the transcribed genes distribution of H3K27me1 to promote gene transcription; H3K27me2 which is the main activity of PRC2, is involved in protective function by inhibiting the firing of non-cell type-specific enhancers; H3K27me3 is mostly associated to CpG island, exert a cell- specific maintenance of epigenetic silencing (Ferrari, Scelfo, Jammula, Cuomo, Barozzi, Stützer, Fischle, Bonaldi, & Pasini, 2014; Mendenhall, Koche, Truong, Zhou, Issac, Chi, Ku, & Bernstein, 2010). The SET domain- containing protein of PRC2 is

EZH2, which is the catalytic subunit and exert H3K27me3 (Ku, Koche, Rheinbay, Mendenhall, Endoh, Mikkelsen, Presser, Nusbaum, Xie, Chi, Adli, Kasif, Ptaszek, Cowan, Lander, Koseki, & Bernstein, 2008; Simon & Kingston, 2013). G9a/GLP has been claimed co-localize with PRC2 at promoters and CpG islands which seems with a potential common recruiting factor (Mozzetta, Pontis, Fritsch, Robin, Portoso, Proux, Margueron, & Ait-Si-Ali, 2014). Using the GLP siRNA KGN cells, we have demonstrated that the GLP protein is able to interact with the EZH2 protein in vivo.

PcG involved in regulated induced pluripotency gene expression with H3K27me3-dependent or independent manner (Ku, Koche, Rheinbay, Mendenhall, Endoh, Mikkelsen, Presser, Nusbaum, Xie, Chi, Adli, Kasif, Ptaszek, Cowan, Lander, Koseki, & Bernstein, 2008). The expression of TP53, a typical tumor suppressor, and its downstream effector P21, are regulated in an H3K27me3-independent manner by PcG and with upregulation in multiple tumors (Mozzetta, Pontis, Fritsch, Robin, Portoso, Proux, Margueron, & Ait-Si-Ali, 2014; Huang, Wang, Ma, Sun, Zhou, Zhu, & Liu, 2014). Kruppel-like factor 2 (KLF2), cell cycle suppressor INK/ARF, DNA damage repair protein RAD51 and BMP Receptor1-Beta (BMPR1B) were all direct target gene of EZH2 which increase expression in many cancers (Taniguchi, Jacinto, Villanueva, Fernandez, Yamamoto, Carmona, Puertas, Marquez, Shinomura, Imai, & Esteller, 2012; Chang & Hung, 2012). Therefore, loss of GLP with EZH2 activity decreased may be related to avoid tumourigenesis in normal cells and which will ultimately become to be a way with cancer prevention and remission.

Overall, our results indicate that deregulation of GLP with subsequent blocking EZH2 expression led to decrease the modification of H3K27me3 with up-regulation of EZH2 targeted genes expression.

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Supplementary Table 1. **siRNA Sequences of GLP**

Gene	Target sequence	Sequence
GLP-siRNA1	GACCAATGGGTCTTGTGAA	F:5'-ACCAAUGGGUCUUGUGAA dTdT-3'
		R:5'-UUCACAAGACCCAUUGGUdTdT-3'
GLP-siRNA2	AGTTCGAGAAGCTAGAGAT	F:5'-AGUUCGAGAAGCUAGAGAU-3'
		R:5'-AUCUCUAGCUUCUCGAACUdTdT-3'
GLP-siRNA3	AGGGTACATGGAAGTTTCT	F:5'-AGGGUACAUGGAAGUUTCU-3'
		R:5'-AGAAACUUCCAUGUACCCUdTdT-3'

Footnotes: F, Forward; R, Reverse.

Supplementary Table 2. The Primer Pairs for qRT-PCR.

Gene name	Sequence
GLP	F: GCTGTGTGAAAACCGAGCTG
	R: TCCGCTATCCGAGTTAGTGTG
KLF2	F:CTACACCAAGAGTTCGCATCTG
	R:CCGTGTGCTTTCGGTAGTG
TP53	F:CAGCACATGACGGAGGTTGT
	R:TCATCCAAATACTCCACACGC
P21	F:TGTCCGTCAGAACCCATGC
	R:AAAGTCGAAGTTCCATCGCTC
INK	F:GATCCAGGTGGGTAGAAGGTC
	R:CCCCTGCAAACTTCGTCCT
RAD51	F:CAACCCATTTCACGGTTAGAGC
	R:TTCTTTGGCGCATAGGCAACA
BMPR1B	F:CTTTTGCGAAGTGCAGGAAAAT
	R:TGTTGACTGAGTCTTCTGGACAA
GAPDH	F: GCACCGTCAAGGCTGAGAAC
	R: TGGTGAAGACGCCAGTGGA