科技部補助

大專學生研究計畫研究成果報告

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＊ 計 畫 ＊
＊ ： 斑馬魚 PRMT8 N 端結合蛋白的確認與分析 ＊
＊ 名 稱 ＊
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中華民國 104 年 03 月 25 日

利用 yeast two hybrid 和 pull down assay 來證明 ependymin(EPD) 與 zebra fish prmt8 N 端(ZF8N) 之間的結合

Abstract:

Protein arginine methyltransferase (PRMT)8 is a vertebrate-restricted paralogue of PRMT1 with an extra N-terminal sequence. Its expression is restricted to the brain as well as neuron cells and is the only PRMT with tissue-restricted expression pattern. We has already constructed a $(His)_6$-tagged zebrafish Prmt8 N-terminus 96 amino acid recombinant protein expression plasmid. First, we use the $(HIS)_6$-ZF8N on beads to pull down the zebra fish brain extract and we also found some interesting putative interacting candidates identified by mass spectrometry. We found Ependymin(EPD) as a target which is one of the candidate proteins; therefore, we constructed a GST-EPD to do a pull-down assay with (HIS)6-ZF8N on beads in order to prove their interaction initially. Second, we wanted to use yeast two-hybrid (Y2H) assay which is a well-established technique that has been developed as a useful method for understanding protein interactions. The purpose of this study is to find some PRMT8 related binding protein and preliminarily understand the interactome of PRMT8.

Itroduction:

PRMT8 is a paralogue of PRMT1 in vertebrates and is the only tissue-restricted PRMT with its expression only appears in brain and neuron cells. It is also the only PRMT known to be membrane associated; PRMT8 is attached to the plasma membrane because of N-terminal myristoylation (Lee et al., 2005a). The results may implicate a putative upstream role of PRMT8 in certain signaling pathways. However, dominant nuclear localization of PRMT8 was observed within mouse central nervous neurons by a PRMT8-specific antibody (Kousaka et al., 2009), arguing against the plasma membrane localization of PRMT8. Initiation from the third AUG and no myristoylation of PRMT8 were proposed (Kousaka et al., 2009). In this way, the possibility that PRMT8, like PRMT1 and most other PRMTs, participates in epigenetic histone modification increases.

PRMT1 and PRMT8 share high sequence identity and the major difference is that PRMT8 has an extra N-terminal sequence for about 76 of the amino acids found in humans; however, removal of the N-terminal domain by truncation or proteolysis results in activation (Sayegh et al., 2007). Recently, a number of PRMT8-binding proteins were identified in cultured cells of probable neuronal origin (Pahlich et al., 2008). The proline-rich sequence in the N-terminus has been shown to bind to SH3 domain of PRMT2 and Fyn, pIc and p85, but the binding did not alter the methyltransferase activity. High sequence identity as well as conserved substrate preference of PRMT8 and PRMT1 indicate similar catalytic activity and implicate putative redundancy. For example, specific association and modification of Ewing sarcoma (EWS) by PRMT8 has been reported (Kim et al., 2008). However, EWS is also an excellent substrate of PRMT1.

Beside, automethylation of two argininyl resides in the N-terminus has been identified and can reduce PRMT8 activity by increasing the Km of AdoMet (Dillon et al., 2013). Therefore, we would like to analyze the specific interactome of PRMT8. We would like to find the interacting protein of zebra fish PRMT8 N via pull-down assay, and confirm their interaction via yeast two hybrid.

Materials and methods:

Zebra fish embryo extract and brain extract

24, 48, 72 hr embryo extract

1. The chorions of 24, 48 hr embryos were removed by immersing in 1 mg/ml pronase on shaker of 75 rpm for 5~10 min, in a petri dish. The embryos were transferred into 1.5 ml microcentrifuge tubes, and the chorions of 24, 48 hr embryos were removed as much as possible with a pipette.
2. The yolks of 24, 48, 72 hr embryos were removed with 1 ml deyolk buffer, and they they were removed as much as possible with a pipette.
3. The 24, 48, 72 hr embryos and brain were washed with wash buffer as well as subjected to brief centrifugation three times, and supernatant were discarded. The pellets were added cold lysis buffer(0.15 M NaCl, 0.1 M Tris-HCl ph 7.5, 5％ glycerol, 1％ Triton-X 100, protease inhibitor complete-mini, 1mM PMSF, 1 mM DTT), and homogenized with a pestle. They were collected by centrifugation at 12,000 rpm for 20 minutes in 4℃ the total extracts were collected in supernatant.

Construction

GST-EPD fusion protein

We used polymerase chain reaction (PCR) to amplify EPD with primers EPD-BamHI and EPD-EcoRI. Zebra fish brain genomic DNA as template and KAPA DNA polymerase that offers the highest PCR success. To improve PCR amplicons we used proofreading polymerase with blunt ends which with an 3'adenine-overhangs post-amplification (A-tailing) is necessary before TA colning. Subsequently, TA colning reaction was set up and composed of 10x buffer A, B, TA vector and T4 DNA ligase, incubating for 2 hours at 22 ℃. Finally, TA plasmid was transformed into *E. coli*, and selected from LB agar plate containing ampicillin.

TA-EPD and pGEX4T1 was digested with BamHI and EcoRI as well as cloned into pGEX4T1 to yield GST-EPD.

$(HIS)_6$-ZF8N

Colony PCR

The bacteria from selected colonies were directly picked up a small amount of culture with a sterile pipette and suspended in PCR solution composed of 10x buffer, 2.5 Mm dNTP, 1 mM F', 1 mM R', template and Taq. PCR reaction was performed at 95℃ for 5 min and followed by 25 cycles of 95℃ for 30 sec, 55℃ (depend on primer) for 30

sec and 72℃ for 1 min. After the final extension at 72℃ for 5~10 min, PCR mixture was chilled at 4℃. Finally, the PCR amplicons were analyzed with 1% agarose gel electrophoresis.

Purification

GST-EPD fusion protein

E. coli (BL21) transformed with pGEX4T1-EPD were induced at OD600 of 0.6~0.8 by adding isopropylthiogalactoside (IPTG, a final concentration of 1 mM) and incubated at 37℃ for 4 hr. The harvested cells were lysed by sonication in lysis buffer (1 mM DTT and lysozyme in PBS). The lysed cells were centrifuged at 12,000 rpm for 20 min at 4℃. The supernatant were binding with Glutatione Sepharose for 1 hr, and flowing through purification column. GST fusion proteins were washed with 10 ml PBS, and eluted with 6 ml of 10 mM glutationnin 50 mM Tris-HCL, pH 8.0, and collected in 1 ml fractions.

The harvested cells were lysed by sonication in 20 ml lysis buffer (NaH2PO4 50 mM, NaCl 300mM, pH 8). The lysed cells were centrifuged at 12000 rpm for 20 min at 4 ℃. The supernatant were binding with Ni-NTA for 1 hr, and flowing through purification column. (HIS)6 fusion proteins were washed with wash buffer (NaH2PO4 50 Mm, NaCl 300 mM, Imidazole 20 mM, pH 8), and eluted with elution buffer (NaH2PO4 50 Mm, NaCl 300 mM, Imidazole 250 mM, pH 8). Finally, the purified protein were stored at -20 ℃.

(HIS)6-ZF8N

Pull-down assay

$(HIS)_6$-ZF8N on beads pull down 24, 48, 72 hr embryo extract and brain extract

The total extract of embryo and brain were 200~400 mg to be mixed with (HIS)6-ZF8N on Ni-NTA beads and Ni-NTA beads in 1.5 ml tube overnight. The proportion of extract and ZF8N were 10:1 and 20:1. After binding overnight, the mixtures were centrifugated at 3000 rpm for 3 minutes which discarded the supernatant. Subsequently, the pellets were washed with 400 ml PBS and collected by centrifugation for three times. Finally, the pellet were suspended with 100 ml PBS for a later analysis of the ZF8N related binding protein with 1% agarose gel electrophoresis.

$(HIS)_6$-ZF8N on beads pull down GST-EPD

The GST-EPD were 200~400 mg to be mixed with $(HIS)_6$-ZF8N on Ni-NTA beads and Ni-NTA beads in 1.5 ml tube overnight. The proportion of extract and ZF8N were 10:1 and 20:1. After binding overnight, the mixtures were centrifugated at 3,000 rpm for 3 minutes, and the supernatants were discarded. Subsequently, the pellet were washed with 400 ml PBS and collected by centrifugation for three times. Finally, the pellet were suspended with 100 ml PBS for a later analysis of their interaction in western blot.

Yeast two hybrid

Construction

Construction of AD, AD-EPD, AD-ZF8N

The pACT2 vector expressed Gal4 activation domain (AD). Therefore, pact2, GST-EPD and $(HIS)_6$-ZF8N were digested with BamHI and EcoRI, and EPD and ZF8N cloned into pact2 vector to yield AD-EPD and AD-ZF8N.

Construction of BD, BD-EPD, BD-ZF8N

The pGBKT7 vector expresses Gal4 DNA binding domain (BD). At first, we used polymerase chain reaction (PCR) to change the restriction sites with EPD-F'EcoRI, EPD-R'BamHI, ZF8N-F'EcoRI and ZF8N-R'BamHI. GST-EPD and $(HIS)_6$-ZF8N were used as templates that were amplified by PCR. Subsequently, the blunt ends of 3'A tail could be ligated to TA vector, and TA-EPD(F'EocRI, R'BamHI) as well as TA-ZF8N(F'EocRI, R'BamHI) were yielded. Therefore, pGBKT7, TA-EPD(F'EocRI, R'BamHI) and TA-ZF8N(F'EocRI, R'BamHI) were digested with EcoRI, BamHI, EPD and ZF8N cloned into pGBKT7 vector to yield BD-EPD and BD-ZF8N.

Yeast transformation

The cells of Y187 and AH109 strains were spread out with an inoculation loop on YAPD agar plate and grown overnight in the incubator at 30℃. A selected single colony from YAPD agar plate was precultured in 50 ml YAPD medium overnight on the shaker at 200 rpm and in the incubator at 30℃. After 12~16 hours growth, the culture was up to stationary phase, given OD600 of 1.2~1.6. The culture was diluted in fresh 50 ml YAPD medium to OD600 of 0.15 and grown for additional 4.5 hours with same conditions. Until the culture of OD600 of 0.6, cells were collected by centrifugation at 3000 rpm for 5 minutes. The culture was washed once with 10 ml sterile water and collected by centrifugation again. The pellet was centrifugated at 3,000 rpm for 5 minutes and was resuspended in 1 ml LiAc/TE buffer (100 mM lithium acetate, 10 mM Tris-HCl and 1 mM EDTA). Salmon sperm DNA (10 mg/ml) was used as a DNA carrier and denatured at 95℃ for 10 minutes and chilled immediately on ice at least 10 minutes. At least 100 ng transform target of AD and BD of plasmid DNA and 10 μl DNA carrier were premixed on ice, and respectively added to 100 μl Y187 and AH109 competent cell in 1.5 ml microcentrifuge tube. Subsequently, 40% PEG 3350, LiAc, TE) was added to this mixture and briefly mixed well, and incubated on shaker at 200 rpm and at 30℃ for 30 minutes. Transformation mixtures were took a heat-shock of 15 minutes at 42℃ in a water bath. After spun down briefly and removed supernatant, the pellet was resuspended in 150 μl sterile water and plate onto appropriated selection medium agar plate such us AD in SC-L plate and BD in SC-W plate. The plates were incubated at 30℃ for 2 days, and colonies appeared.

Yeast mating

Single colony was picked up with toothpick, and smeared in the SC-L (AD) or SC-W (BD) plate for two days. After incubating for two days, they will cultured at 30℃ with shaking in 2 ml SC-L or SC-W medium for two days. Subsequently, the pellet of cells were collected by centrifugation, and mixed with 1.5 ml YAPD medium. The

mating of cells were mixed with 0.5 ml AD and BD, and incubated on shaker at 200 rpm and at 30℃ for 4 hours. After spun down briefly and removed supernatant, the pellet was resuspended in 150 μl sterile water and plate onto in SC-L-W plate for two days. If the mating of cells succeeded, they would be smeared in the SC-L-W-H plate for two days to inspect the activation of reporter gene.

Spot dilution plate assay

BD-EPD~AD-ZF8N and BD-EPD~AD in SC-L-W-H plate were picked up and incubated in SC-L-W-H medium on shaker at 200 rpm at 30℃ overnight. The cells were diluted to OD600 of 1(107 cells),and they were also diluted to 106、105、104、103、102 cells which respectively grow in SC-L-W-H、SC-L-W-H 5 mM 3-AT、SC-L-W-H 10 mM 3-AT、SC-L-W-H 25 mM 3-AT、SC-L-W-H 50 mM 3-AT plate.

Results:

Identification of putative interacting proteins of zebrafish PRMT8

In order to measure the amount of $(HIS)_6$-ZF8N, we used BSA as a standard template. We load 2、5、10、16 μg BSA and half of $(HIS)_6$-ZF8N in SDS PAGE (Figure 1A). The final concentration of $(HIS)_6$-ZF8N was estimated as 2.325 μg/μl. The zebra fish brain extract and embryo extract at the stage of 24、48、72 hour were followed with SDS-PAGE (Figure 1B). $(HIS)_6$-ZF8N pull-down assay was performed with zebra fish brain extract and embryo extract. $(HIS)_6$-ZF8N on Ni bead and Ni bead were incubated with zebra fish brain extract and embryo extract. Therefore, we found some putative ZF8N binding protein in the zebrafish brain, and we chose ZF brain 55、45、40、37、35、30 kDa (a、b、c、d、e、f) to be identified by mass spectrometry.

The result was identified by mass spectrometry (table 1), and we would like to choose 55 (tubulin, beta, 2), 45 (actin cytoplasmic 2), 35 (ependymin precursor) kDa as candidate to identify their interaction further. At first, we choose enendymin (EPD) as a target to confirm their interaction.

Putative interaction between PRMT8 N-terminus with ependymin determined by pull-down assay

In this study, we would like to confirm the interaction between ZF8N and EPD by a pull-down assay. Hence, we looked up the sequence of ependymin (NM_131005.2) in NCBI database. The full coding region of ependymin mRNA sequence is from 36 to 686 (Figure 2A). We acquired the total mRNA from the zebra fish brain. To prepare the ependymin coding sequence template, we used polymerase chain reaction (PCR) to amplify the template with primers. After transformation, Successful ligation of ependymin cDNA with TA vector was examined by colony PCR (Figure 3A). We then examined the plasmid by cleavage with two restriction enzyme sites of BamHI and EcoRI (Figure 3B) and further confirmed the sequence of this construction by a commercial company.

We then used the BamHI and EcoRI restriction enzyme to digest the insert of TA-EPD and ligate it to pGEX4T1 to construct a plasmid to express GST-EPD (Figure 3C). Consequently, we conducted colony PCR and DNA sequencing of the plasmid expressing GST-EPD (Figure 3D).

When we compared after induction with before induction, we could find that ependymin can be induced by IPTG. However, most of EPD precipitate at pellet and few EPD suspend at supernatant. As a result, the elution just eluted few EPD but a lot of GST (Figure 4).

$(HIS)_6$ pull-down assay was performed with purified recombinant proteins $(HIS)_6$-ZF8N and GST-EPD. Because purification of GST-EPD had multiple bands, we could not confirm specific interaction between ZF8N and EPD (Figure 5). We thus would like to confirm their interaction by yeast two hybrid.

Putative interaction between PRMT8 N-terminus with ependymin determined by yeast two hybrid analyses

The plasmids are constructed for yeast two hybrid analyses. We chose pACT2 activation domain and pGBKT7 binding domain. We constructed pACT2-EPD, pACT2-ZF8N (Figure 6A), pGBKT7-EPD and pGBKT7-ZF8N (Figure 6B) for yeast two hybrid system.

Among the hybrid of BD ~ AD、 BD ~ AD-EPD、BD ~ AD-ZF8N、BD-EPD ~ AD、BD-EPD ~ AD -ZF8N 、BD-ZF8N ~ AD 、BD-ZF8N ~ AD-EPD, BD-EPD~AD-ZF8N and BD-EPD~AD can grow in a SC-L-W-H plate (Figure 7A). However, the signal sequence of EPD that should be cleaved for the mature protein was present in the EDP constructs that may interfere with the results. Therefore, we wanted to delete the signal peptide of ependymin and confirm their interaction. Although we could find that all of the mating type can grow in SC-L-W-H plate (Figure 7B), the false positive also appeared at BD-EPD~AD in SC-L-W-H plate (Figure 7C). Moreover, we used spot dilution plate assay to compare their binding intensity and obtained similar results (Figure 8). Although we couldn’t confirm EPD as a ZF8N binding protein, we were able to use yeast two system to check the other related binding protein of ZF8N such as tubulin, beta, 2 and actin cytoplasmic 2.

Conclusion:

At first, we would like to use $(HIS)_6$ pull-down assay to find the related binding proteins of ZF8N. $(HIS)_6$-ZF8N pull-down assay was performed with zebra fish brain extract and embryo extract. Although we just sent interacting signals from ZF brain with the molecular mass of 55、45、40、37、35、30 kDa (a、b、c、d、e、f) mass spectrometry, there are signals of the same molecular mass ZF embryo extract 55、45、35 kDa at the 24、48 and 72 hpf stage. Therefore, we wanted to inspect three related binding proteins first. We chose one of related binding proteins as a target and $(HIS)_6$ pull-down assay was performed with purified recombinant proteins $(HIS)_6$-ZF8N and GST-EPD. Because the

purification of GST-EPD was not pure, we could not find the specific binding of EPD. Furthermore, there is a signal peptide which is a short hydrophobic peptide present at the N-terminal EPD. It is possible the presence of sequence lead to the presence of most of EPD precipitated in pellets.

In this study, we would like to confirm the interaction between ZF8N and EPD in yeast two hybrid systems. We constructed AD-ZF8N, AD-EPD, BD-ZF8N and BD-EPD as experiment materials. BD-ZF8N ~ AD-EPD, BD-EPD~AD-ZF8N are the experimental group and BD ~ AD, BD ~ AD-EPD, BD ~ AD-ZF8N, BD-EPD ~ AD, BD-ZF8N ~ AD are the control. Although BD-EPD~AD-ZF8N could activate the reporter gene and grow in the SC-L-W-H plate, BD-ZF8N ~ AD-EPD could not. However, BD-EPD~AD which is one of the control group would activate the reporter gene without the activation of AD. To determine whether the signal peptide of EPD could affect the activation of reporter gene, we used site-Directed Mutagenesis to delete the signal peptide of EPD. In the same way, BD-EPD~AD also activate the reporter gene directly. Whether AD could bind to EPD or activate reporter gene without BD. It might be the over expression of EPD (data did not show). We also observed the intensity of reporter gene. We compared BD-EPD~AD-ZF8N to BD-EPD~AD, and we cannot find the difference between two group. Although BD-EPD~AD-ZF8N could activate the reporter gene and grow in SC-L-W-H plate, BD-ZF8N ~ AD-EPD could not. Because stereoscopic structure of ZF8N is blocked by DBD, it might not be like the structure of full length PRMT8. In the future, we want to construct human PRMT8 N at N terminal of DBD, and screening with human brain cDNA library. At the end, we want to find more PRMT8 related binding protein as well as inspect their interaction in culture cell.

Reference:

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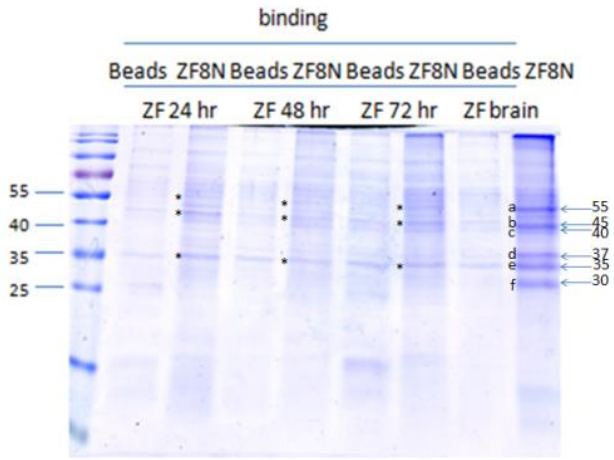

Figure 1. ZF PRMT8 N pull down with the embryo and brain extract of ZF

We used the $(HIS)_6$-ZF8N on beads to pull down the zebra fish brain extract and embryo extract at the stage of 24、48、72 hours. There were some related binding proteins at ZF 24、48、72 embryo extract (*) as well as brain extract(a、b、e) and those position are at 55、45、35 kDa. Finally, we identified the related binding proteins at ZF brain 55、45、40、37、35、30 kDa (a、b、c、d、e、f.) by mass spectrometry.

Table 1. the related binding proteins of ZF PRMT8 N

(a) 55 kDa

GI ID	Protein name	Mass	Score	Matches	Sequences	% Cov
37681963	tubulin, beta, 2	49754	1457	52	19	66 %
2599500	alpha-tubulin	49922	932	35	16	51 %

(b) 45 kDa

GI ID	Protein name	Mass	Score	Matches	Sequences	% Cov
449310793	actin, cytoplasmic 2	41726	1162	40	12	48 %
50344802	beta-actin-like protein 2	41979	693	23	6	35 %
6636384	alpha-cardiac actin	41969	686	22	5	23 %
62955473	actin, gamma-enteric smooth muscle	41890	685	22	5	23 %
41054651	isocitrate dehydrogenase [NADP], mitochondrial	50365	542	20	13	44 %

(c) 40 kDa

GI ID	Protein name	Mass	Score	Matches	Sequences	% Cov
449310793	actin, cytoplasmic 2	41726	665	22	9	42 %
6636384	alpha-cardiac actin	41969	393	11	4	19 %
50344802	beta-actin-like protein 2	41979	391	11	4	24 %
41054736	internexin neuronal intermediate filament protein, alpha	64712	211	12	8	15 %
23452398	class I beta tubulin	49794	137	5	2	9 %
31581593	glutamine synthetase 1	41586	117	5	3	16 %

(d) 37 kDa

GI ID	Protein name	Mass	Score	Matches	Sequences	% Cov
47085833	glyceraldehyde 3-phosphate dehydrogenase 2	36084	1059	36	9	46 %
449310793	actin, cytoplasmic 2	41726	305	10	5	22 %
50344802	beta-actin-like protein 2	41979	216	5	2	13 %
18858633	ependymin precursor	24456	163	5	2	28 %
2599500	alpha-tubulin	49922	130	8	5	16 %

(e) 35 kDa

GI ID	Protein name	Mass	Score	Matches	Sequences	% Cov
18858633	ependymin precursor	24456	460	14	3	28 %
28277811	Bactin2	41655	191	7	5	29 %
23452398	class I beta tubulin	49794	191	5	3	8 %
2599500	alpha-tubulin	49922	145	5	3	10 %

(f) 30 kDa

GI ID	Protein name	Mass	Score	Matches	Sequences	% Cov
18858633	ependymin precursor	24456	449	15	3	28 %
27545251	ADP/ATP translocase 2	32742	358	21	9	38 %
449310793	actin, cytoplasmic 2	41726	172	6	5	28 %
56269288	Ywhab1 protein	32355	142	4	2	14 %

A

Danio rerio ependymin (epd), mRNA

NCBI Reference Sequence: NM_131005.2

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aagtgtctgg acctgaagaa agttaaagag caacgatgca tacagtcaag ctgctctgtg
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B

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ORIGIN
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      661 acagcttctt cgacctgttc cacgactgaa gaaaacacac aagatgacct gcaaactgcc
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      961 aaaaaa
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Figure 2: Gene sequence of ependymin

(A) The full length of ependymin mRNA sequence is from 36 to 689.

(B) The signal peptide sequence of ependymin is from 36 to 95.

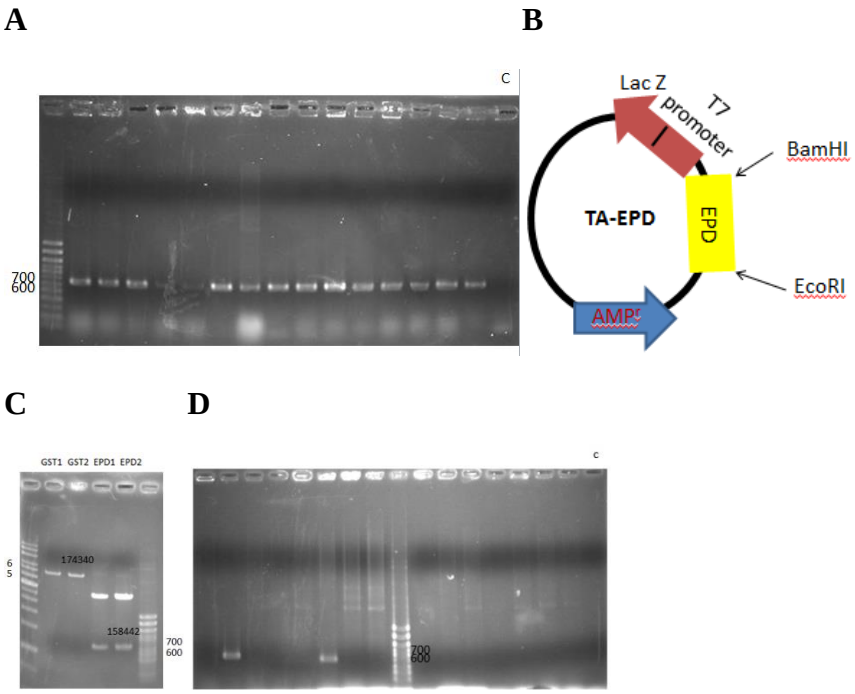


Figure 3.Colning GST-EPD

(A) colony PCR TA-EPD, 654 bp

(B) Schematic diagram of TA-EPD

(C) pGEX4T1(4950 bp) and TA-EPD (654 bp) were cut by BamHI and EcoRI

(D) colony PCR GST-EPD, 654 bp

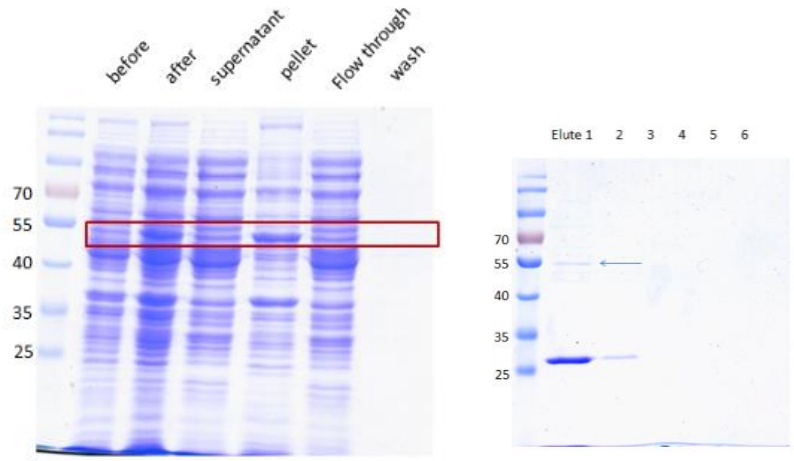


Figure 4. GST-EPD Purification

Most of the proteins were in the pellet.

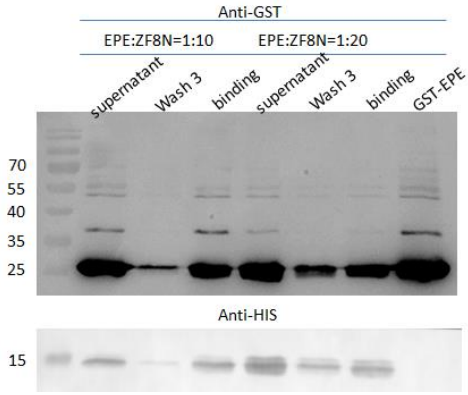


Figure 5. $(HIS)_6$-ZF8N on beads pull down GST-EPD

In order to observe the protein-protein interaction, we used $(HIS)_6$-ZF8N on beads to pull down GST-EPD. However, the interaction between ZF8N and GST were stronger than GST-EPD, because the amount of GST protein was much higher than GST-EPD.

A **B**

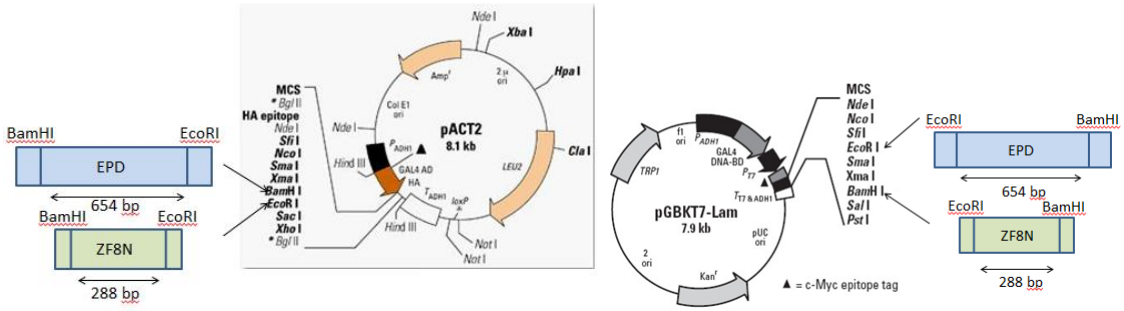
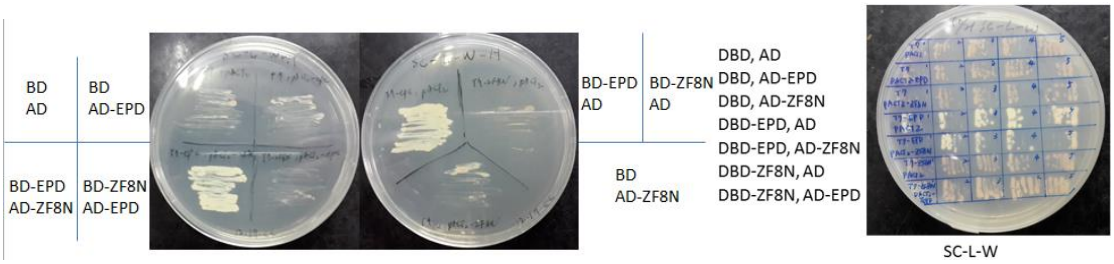


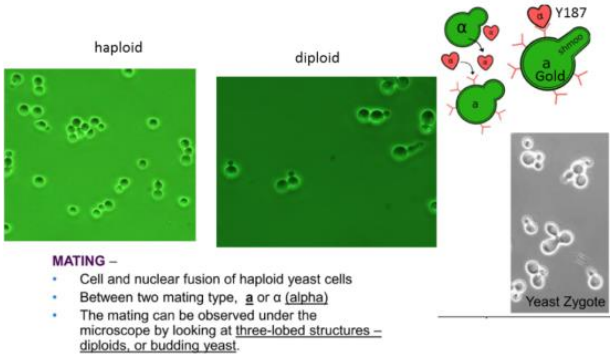
Figure 6. the vector for yeast two hybrid

(A) pACT2 is a shuttle vector that replicates autonomously in both E. coli and S. cerevisiae, which confers ampicillin resistance in E. coli. pACT2 also contains the LEU2 nutritional gene that allows yeast to grow on limiting synthetic media. Hence, we chose EcoRI and BamHI to insert EPD and ZF8N.

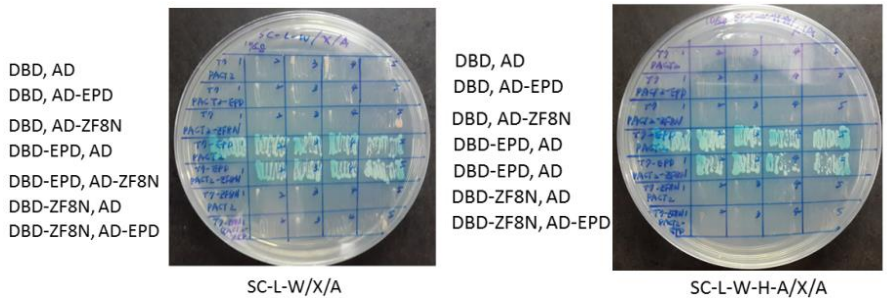
(B) pGBKT7 replicates autonomously in both *E. coli* and *S. cerevisiae* from the pUC and 2 μ ori, respectively. The vector carries the Kana for selection in *E. coli* and the TRP1 nutritional marker for selection in yeast. Yeast strains containing pGBKT7 exhibit a higher transformation efficiency than strains carrying other DNA-BD domain vectors, so we chose EcoRI and BamHI to insert EPD and ZF8N.

A B





C



D

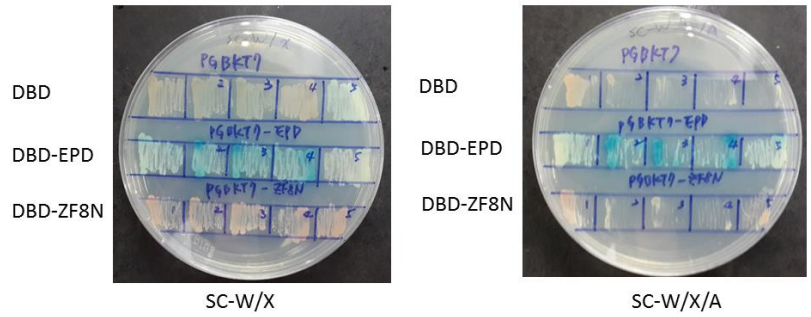


Figure 7: Application of YTH system between EPD and ZF8N

(A) In order to observe the interaction between EPD (full length) and ZF8N, we used yeast two hybrid system. Among BD ~ AD、 BD ~ AD-EPD、BD ~ AD-ZF8N、BD-EPD ~ AD 、BD-EPD ~ AD -ZF8N 、BD-ZF8N ~ AD 、BD-ZF8N ~ AD-EPD, BD-EPD~AD-ZF8N and BD-EPD~AD could grow in SC-L-W-H plate.

(B) We changed the EPD without signal peptide.BD ~ AD 、BD ~ AD-EPD 、BD ~ AD-ZF8N、BD-EPD ~ AD、BD-EPD ~ AD-ZF8N、BD-ZF8N~ AD、BD-ZF8N~ AD-EPD can grow in SC-L-W plate, so the mating was successful.

(C) BD-EPD~AD-ZF8N and BD-EPD~AD could grow and turn blue in SC-L-W/X/A

and SC-L-W-H-A/X/A plate

(D) BD-EPD could grow and turn blue in SC-W/X and SC-W /X/A plate.

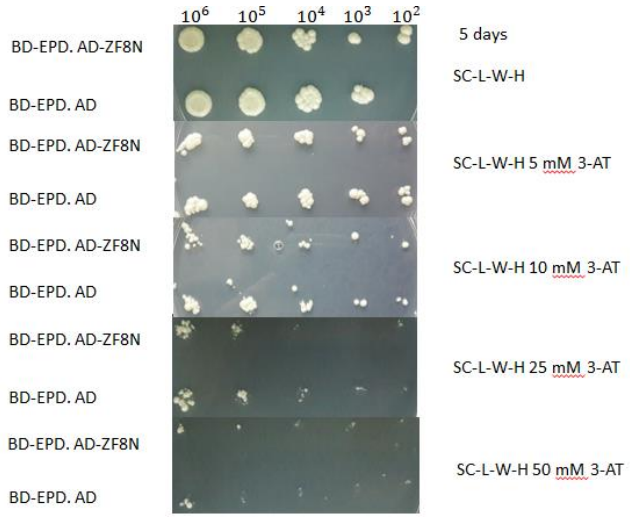


Figure 8: Spot dilution plate assay

BD-EPD~AD-ZF8N and BD-EPD~AD which are 10^6、10^5、10^4、10^3、10^2 cell respectively grow in SC-L-W-H、SC-L-W-H 5 mM 3-AT、SC-L-W-H 10 mM 3-AT、SC-L-W-H 25 mM 3-AT、SC-L-W-H 50 mM 3-AT plate.