Rational design of novel anticancer small-molecule RNA m6A demethylase ALKBH5 inhibitors

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I. ALKBH5 as related to cancer.

Table S1. ALKBH5 and cancer.

| Cancer type                        | Subtype                                                                 | m6A target                                                                                           | ALKBH5 function                           | Reference |
|------------------------------------|-------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------|-------------------------------------------|-----------|
| Non-small cell lung cancer (NSCLC) | Human lung cancer and normal lung specimens collected in Affiliated Hospital of Binzhou Medical College | Ubiquitin-conjugating enzyme E2C (UBE2C) overexpression enhances tumor cell growth and colony formation in malignant transformation in vitro and in vivo (Okamoto, Y. et al. Cancer Res. 63, 4167–4173 (2003)). ALKBH5-induced epitranscriptional activation of UBE2C was identified as essential mechanism governing upstream regulation of UBE2C. ALKBH5 is highly expressed in lung cancer cells and Alkbh5 knockout significantly decreased UBE2C expression in NSCLC. | Cancer promoter                           | ¹         |
| Cancer type                  | Tumor types                                      | Subcutaneous model details                                                                 | ALKBH5 effects                                                                                                                                       | Notes                                                                                      |
|------------------------------|--------------------------------------------------|---------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------|
| Lung adenocarcinoma          | Lung cancer cell lines A549 and NCI-H522 (ATCC©). Subcutaneous A549 animal (mice) model | ALKBH5 affects the proliferation and invasion of lung adenocarcinoma cells under intermittent hypoxia (IH) by downregulating m6A modification on FOXM1 (Forkhead box M1) mRNA and by promoting FOXM1 expression. ALKBH5 knockout inhibits the growth of lung adenocarcinoma cells under IH. | Cancer promoter                                                                                                                                     |
| Non-small cell lung cancer (NSCLC) | Female BALB/c athymic (NU/NU) nude mice injected with $5 \times 10^6$ lung cancer cells Lung adenocarcinoma cell line A549, Lung large cell carcinoma cell line H1299, Lung squamous cell carcinoma cell lines Calu6 and H520. | The results suggest that m6A demethylase ALKBH5 inhibits tumor growth and metastasis by reducing YTHDFs-mediated YAP (transcription regulator Yes-associated protein) expression and inhibiting miR-107/LATS2-mediated YAP activity in NSCLC. Further, ALKBH5 inhibited tumor growth and metastasis *in vivo* by reducing the expression and activity of YAP. | Cancer suppressor                                                                                                                                     |
| Non-small cell lung cancer    | $2 \times 10^6$ A549 cells stably transfected with shRNA-ALKBH5 injected into the flank of male athymic BALB/c nude mice Lung adenocarcinoma cell line A549, | Results revealed that ALKBH5 was ectopically up-regulated in the NSCLC tissue and cells, and closely correlated with the poor prognosis. Functionally, ALKBH5 promoted the proliferation and reduced apoptosis of NSCLC cells in vitro, and knockdown of ALKBH5 repressed the tumor growth *in vivo*. Mechanistically, RIP-Seq revealed that ALKBH5 targeted the TIMP3, Moreover, | Cancer promoter                                                                                                                                     |
| Cell Line Type | Cell Line Details | ALKBH5 Function | Cancer Promoter |
|---------------|-------------------|-----------------|----------------|
| Lung large cell carcinoma | H1299, H460, SK-MES-1, H460. | ALKBH5 repressed TIMP3 mRNA stability and protein production. | |
| Lung squamous cell carcinoma | MV4-11, MOLM-13, THP-1, MonoMac 6, NOMO-1, U-937, THP-1, MLL-AF9 leukemia model | ALKBH5 is specifically required for maintaining the function of acute myeloid leukemia (AML) stem cells but not normal hematopoietic stem cells and reveal KDM4C-ALKBH5-AXL signaling axis in AML development and maintenance. KDM4C Regulates ALKBH5 expression by modulating chromatin accessibility. | Cancer promoter |

**Acute myeloid leukemia (AML)**

- Adult acute myeloid leukemia, cell lines MV4-11, MOLM-13 (DSMZ); Acute monoblastic/monocytic leukemia; cell lines THP-1 (ATCC©).
- Murine MLL-AF9 leukemia model

ALKBH5 is aberrantly overexpressed in AML that correlates with poor prognosis in AML patients. ALKBH5 is required for the development and maintenance of AML and self-renewal of leukemia stem/initiating cells (LSCs/LICs) but not essential for normal hematopoiesis. Mechanistically, ALKBH5 exerts tumor-promoting effects in AML by post-transcriptional regulation of its critical targets such as TACC3, a prognosis-associated oncogene in various cancers. | Cancer promoter |
| **leukemia with PML-RARA cell line NB-4 (DSMZ).**
| **NSGS mouse, C57BL/6 mice, B6.SJL-tprc<sup>a</sup>Pepc<sup>b</sup>/BoyCrCrl mice**

| **Hepatocellular carcinoma (HCC)**
| Two HCC cohorts from First Affiliated Hospital of Zhejiang University.
| HCC cell lines Huh7, MHCC97H, HCCLM3, Hep3B, PLC/PRF/5,
| Hepatoblastoma cell lines HepG2,
| Human papillomavirus-related endocervical adenocarcinoma cell lines SMCC7721 and BEL7402.
| ALKBH5 was down-regulated in HCC, and decreased ALKBH5 expression was an independent prognostic factor of worse survival in HCC patients. Functionally, ALKBH5 suppressed the proliferation and invasion capabilities of HCC cells in vitro and in vivo. Mechanistically, ALKBH5-mediated m6A demethylation led to a post-transcriptional inhibition of LY6/PLAUR Domain Containing 1 (LYPD1), which could be recognized and stabilized by the m6A effector IGF2BP1.

| **Renal carcinoma**
| Clear cell renal carcinoma, patient tissue samples within the framework of the Biobank at the Centre for Integrated ALKBH5 mRNA, as well as ALKBH5 and FTO protein expressions, was significantly downregulated in ccRCC compared to normal tissue and most of the other studied tumor entities.

| **Cancer suppressor**

| **Cancer suppressor**
Renal carcinoma

Patient samples from the Department of Urology of the First Affiliated Hospital of Nanjing Medical University. Clear cell renal cell carcinoma cell lines Caki-1, 769P, 786-0, (CCCAS). Papillary renal cell carcinoma cell lines Caki-2, ACHN (CCCAS).

ALKBH5 was highly expressed in both RCC tumor tissues and cell lines. Stable overexpression ALKBH5 could promote the cell proliferation, colony formation, cell migration and cell invasion of renal cell carcinoma cells in vitro and promote tumor growth in vivo. In contrast, ALKBH5 knocked down inhibited cell proliferation, colony formation, migration and invasion of renal cell carcinoma cells in vitro.

Based on TCGA database analysis, AURKB (Aurora Kinase B) gene was predicted highly expressed in RCC and a potential target of ALKBH5.

Colon cancer

Het116, RKO, SW620 and HCT8 human colon cancer cell lines (CCCAS).

ALKBH5 was downregulated in human colon cancer tissues, where its decreased expression significantly correlated with distant metastasis and American Joint Committee on Cancer (AJCC) stage. Overexpression of ALKBH5 inhibited colon cancer cells invasion in vitro and metastasis in vivo. These results indicated that ALKBH5 significantly inhibits tumor progression and serves as a potential therapeutic target for colon cancer.

Oral squamous cell carcinoma

Tongue squamous cell carcinoma cell lines H357, SCC-9, SCC-4 (Sigma Aldrich)

A genetic (shRNA) or pharmacological (ketorolac salt) inhibition of DDX3 (RNA helicase) reduced CSC population by suppressing the expression of FOXM1 and NANOG. It was also found that m6A demethylase ALKBH5 is directly regulated by
| Tumor Type                      | Experiment Details                                                                                                                                                                                                                                                                                                                                                                                                                                                                 | Cancer promoter |
|--------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------|
| Esophageal squamous cell carcinoma | OE21 cells were stably transfected with sh-ALKBH5 (#1), sh-ALKBH5 (#2) or sh-scramble as negative control subcutaneously into the back of male athymic BALB/c nude mice. ESCC cell lines TE1, TE5 and TE9 (RIKEN BRC Cell Bank, Japan), OE21 (ATCC©). Analysis of tissue microarray of the tumors in 177 ESCC patients showed that higher expression of m6A demethylase ALKBH5 correlated with poor prognosis. ALKBH5 knockdown delayed progression of cell cycle and accumulated the cells to G0/G1 phase. Expression of CDKN1A (p21) was significantly up-regulated in ALKBH5-depleted cells, and m6A modification and stability of CDKN1A mRNA were increased by ALKBH5 knockdown. Depletion of ALKBH5 substantially suppressed tumor growth of ESCC cells subcutaneously transplanted in BALB/c nude mice. |
| Glioblastoma (GBM)             | Alkbh5 knockout mice Human glioma cell lines Hs683, SW1783 (ATCC©). Elevated RNA m6A demethylase ALKBH5 in glioblastoma stem-like cells enhances self-renewal and tumorigenesis through regulation of FOXM1. The lncRNA antisense to FOXM1 promotes the interaction of ALKBH5 with FOXM1 nascent transcript that contribute to chemoresistance. The m6A methylation of FOXM1 and NANOG mRNA are tightly regulated by m6A demethylase ALKBH5 (Zhang C, et al, Proc Natl Acad Sci USA 113:E2047–2056 (2016); Zhang S et al, Cancer Cell 31(591–606):e596 (2017). |
|                                | 12                                                                                                                                                                                                                                                                                                                                                                                                                                                                               | 13              |
| Cancer Type                          | Cell Lines/Details                                                                 | RNA Modification \nEffect                                                                 | Therapeutic Target |
|-------------------------------------|------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------|-------------------|
| Human GBM cell lines \nLN229, U87MG \n(ATCC©) \nHuman GBM U251MG cells \n(Sigma) | RNA, leading to demethylation and elevated expression of FOXM1.                   |                                                                                          |                   |
| Cervical cancer                      | 286 pairs of samples from patients with primary cervical cancer. \nPapillomavirus-related cervical squamous cell carcinoma cell line SiHa (ATCC©). | Reducing m6A level via manipulating the m6A regulators FTO and AlkBH5 expression promoted cervical cancer cell proliferation. Increasing m6A level significantly suppressed tumor development both in vitro and in vivo. Our results showed that the reduced m6A level is tightly associated with cervical cancer development and m6A mRNA methylation might be a potential therapeutic target in cervical cancer. | Cancer promoter   |
| Ovarian cancer                       | Ovarian serious cystadenocarcinoma cell lines SKOV3, HEY and OVCAR3 (ATCC©). \nPapilloma-virus related endocervical adenocarcinoma cell line HO8910 (ATCC©). \nEndometrial cancer cell line (Ishikawa) (ATCC©). | NANOG expression increased after mRNA demethylation, consequently enhancing the aggressiveness of ovarian cancer cells. In conclusion, highly expressed TLR4 activated NF-κB pathway, up-regulated ALKBH5 expression and increased m6A level and NANOG expression, all contributing to ovarian carcinogenesis. | Cancer promoter   |
| Tissue Type                  | Sample Details                                                                                     | Findings                                                                                                      | Gene Role                  |
|-----------------------------|---------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------|-----------------------------|
| Pancreatic adenocarcinoma   | The expression level of m6A-related genes was correlated with the immune microenvironment of pancreatic cancer. Specifically, both arm-level gain and deletion of ALKBH5 decreased the infiltration of CD8+T cells (P <0.05 and P <0.01, respectively). | Both cancer promoter and suppressor                                                                    |
| Pancreatic cancer           | 42 PC tissues and adjacent normal tissues collected from PC patients.                              | ALKBH5 loss characterized the occurrence and poor clinicopathological manifestations in patients with PC. Overexpression of ALKBH5 reduced tumoural proliferative, migrative, invasive activities *in vitro* and ameliorated tumour growth *in vivo*, whereas ALKBH5 knockdown facilitated PC progression. Mechanistically, ALKBH5 posttranscriptionally activated PER1 by m6A demethylation in an m6A-YTHDF2-dependent manner. | Cancer suppressor          |
| Osteosarcoma                | 70 pairs of OS and adjacent normal tissues were collected from OS patients.                        | ALKBH5-mediated m6A modification of PVT1 contributes to osteosarcoma tumorigenesis. PVT (Plasmacytoma variant translocation 1) is a well-known oncogenic long noncoding RNA (lncRNA). | Cancer promoter            |
| Osteosarcoma                | Osteosarcoma cell lines LM7, SaOS2, HOS, U2OS, MG63 and 143B                                        | ALKBH5-based m6A demethylation suppressed osteosarcoma cancer progression through m6A-based direct/indirect regulation of YAP. | Cancer suppressor          |
Table S2. ALKBH5 related target genes in cancer.

| Cancer                        | ALKBH5 related gene | Molecular target                                                                                   | Proposed mechanism of action                                                                                                                                                                                                 | Reference |
|-------------------------------|---------------------|---------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------|
| Non-small cell lung cancer (NSCLC) | *UbcH10* (human chromosome 20q13.12) | Ubiquitin-conjugating enzyme E2C (*UBE2C*), one of ubiquitination enzymes catalyzing degradation of proteins into smaller polypeptides, amino acids, and ubiquitins in 26S proteasome, is involved in carcinogenesis via regulating cell cycle, apoptosis, and transcriptional process, in which *UBE2C* was upregulated and correlated with poorer overall survival (OS) and progression-free survival of NSCLC patients | It was found that ALKBH5 is highly expressed in lung cancer cells, and more, *Alkbh5* knockout significantly decreased *UBE2C* expression in NSCLC. This study first reports the epigenetic and epitranscriptional regulation of *UBE2C* in lung carcinogenesis. | 17        |
| Non-small cell lung cancer (NSCLC) | *Yap* (human chromosome 11q22.1) | Yes-associated protein 1, also known as YAP, YAP1 or YAP65, is a protein that acts as a transcriptional regulator by activating the transcription of genes involved in cell proliferation and suppressing apoptotic genes. YAP1 is inhibited in the Hippo signaling pathway which allows the cellular control of organ size and tumor suppression. | YAP expression is negatively correlated with ALKBH5 expression and plays an opposite role in the regulation of cellular proliferation, invasion, migration, and epithelial mesenchymal transition (EMT) of NSCLC cells. Furthermore, ALKBH5 decreased YAP activity by regulating miR-107/LATS2 axis in an HuR-dependent manner. Further, ALKBH5 inhibited tumor growth and metastasis in vivo by reducing the expression and activity of YAP. | 3         |
| Tissue                        | Gene Information                          | Description                                                                 | Reference(s) |
|-------------------------------|--------------------------------------------|-----------------------------------------------------------------------------|--------------|
| Non-small cell lung cancer (NSCLC) | **Timp3** (human chromosome 22q12.3) | Metalloproteinase inhibitor 3 (TIMP3) belongs to the tissue inhibitor of metalloproteinases gene family. These proteins are inhibitors of the matrix metalloproteinases, a group of peptidases involved in degradation of the extracellular matrix (ECM). | 4            |
|                                | MeRIP-Seq revealed that ALKBH5 targeted the TIMP3, therefore, it might remove the methylation of TIMP3 at its transcript. Western blotting and RT-PCR revealed that ALKBH5 overexpression induced the decreased expression of TIMP3 protein, while the ALKBH5 silencing up-regulated the TIMP3 mRNA level. | 4            |
| Lung adenocarcinoma | **FOXM1** (human chromosome 12p13.33) | Forkhead box M1 (FOXM1) protein is a member of the FOX family of transcription factors. It has been demonstrated that FOXM1 is a tumor inducer due to its function in the proliferation, invasion and chemoresistance of lung cancer. | 2            |
|                                | ALKBH5 promotes FOXM1 mRNA translation by reducing the m6A level of FOXM1 mRNA under intermittent hypoxia (IH). FOXM1 is a pivotal target for ALKBH5 in lung adenocarcinoma under IH. | 2            |
| Acute myeloid leukemia (AML) | **KDM4C** (human chromosome 9p24.1) **AXL** (human chromosome 19q13.2) | Lysine-specific demethylase 4C (KDM4C) is a trimethylation-specific demethylase, converting specific trimethylated histone residues to the dimethylated form. Tyrosine-protein kinase receptor UFO (AXL) is a cell surface receptor tyrosine kinase that is a key facilitator of immune escape and drug-resistance by cancer cells, leading to aggressive and metastatic cancers. | 5            |
|                                | KDM4C regulates ALKBH5 expression by increasing chromatin accessibility to MYC and Pol II. ALKBH5 affects mRNA stability of AXL in an m6A-dependent manner. | 5            |
| Cancer Type                           | Gene          | Description                                                                                                                                                                                                                                                                                                                                 | Reference |
|--------------------------------------|---------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------|
| Acute myeloid leukemia (AML)         | TACC3         | Transforming acidic coiled-coil-containing protein 3 (TACC3) gene expression is upregulated in some cancer cell lines.                                                                                                                                                                                                                       | 6         |
|                                      | ALKBH5        | ALKBH5 exerts tumor-promoting effects in AML by post-transcriptional regulation of its critical targets such as TACC3, a prognosis-associated oncogene in various cancers.                                                                                           |           |
| Hepatocellular carcinoma (HCC)       | LYPD1         | Ly6/PLAUR domain-containing protein 1 (LYPD1) is a member of the Lynx family of neurotransmitter receptor-binding proteins.                                                                                                                                                                                                                  | 7         |
|                                      | ALKBH5        | ALKBH5, characterized as a tumor suppressor, attenuates the expression of LYPD1 via an m6A-dependent manner in HCC cells.                                                                                                                                                                                                                  |           |
| Renal carcinoma                      | AURKB         | Aurora B kinase (AURKB) is a protein that functions in the attachment of the mitotic spindle to the centromere. AURKB promotes cell cycle and survival of cancer cells.                                                                                                                                                                               | 9         |
|                                      | ALKBH5        | ALKBH5 may play a carcinogenic role in renal cell carcinoma by stabilizing AURKB mRNA in a m6A-dependent manner.                                                                                                                                                                                                                       |           |
| Oral squamous cell carcinoma (OSCC)  | DDX3          | ATP-dependent RNA helicase DDX3X is involved in many different types of cancer.                                                                                                                                                                                                                                                      | 11        |
|                                      | ALKBH5        | DDX3 regulates FOXM1 and NANOG by up-regulating ALKBH5 in chemoresistant OSCC.                                                                                                                                                                                                                                                        |           |
| Esophageal squamous cell carcinoma   | CDKN1A        | Cyclin dependent kinase inhibitor 1A (CDKN1A) mediates the tumor suppressor protein p53-dependent cell cycle G1 phase arrest in response to a variety of stress stimuli.                                                                                                                                                                      | 12        |
|                                      | ALKBH5        | Expression of CDKN1A (p21) was significantly up-regulated in ALKBH5-depleted cells, and m6A modification and stability of CDKN1A mRNA were increased by ALKBH5 knockdown. Furthermore,                                                                                                                                 |           |
| Tumor Type          | Gene(s)                           | Description                                                                                             | Notes                                                                 |
|---------------------|-----------------------------------|--------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------|
| Glioblastoma (GBM)  | FOXM1 (human chromosome 12p13.33) | Forkhead box M1 (FOXM1) protein is a member of the FOX family of transcription factors. It has been demonstrated that FOXM1 is a tumor inducer. | Depletion of ALKBH5 substantially suppressed tumor growth of ESCC cells subcutaneously transplanted in BALB/c nude mice. |
| Ovarian cancer      | TLR4 (human chromosome 9q33.1)    | Toll-like receptor 4 (TLR4) is a member of the toll-like receptor (TLR) family, which plays a fundamental role in pathogen recognition and activation of innate immunity. | TLR4, a molecular functioning in tumour microenvironment demonstrated the same expression trend as AlkBH5. |
|                     | NANOG (human chromosome 12p13.31) | Homeobox protein NANOG is a transcriptional factor that helps embryonic stem cells (ESCs) maintain pluripotency by suppressing cell determination factors.[5] Several types of cancer are associated with NANOG. | In the ovarian cancer cells co-cultured with M2 macrophages, the expression of ALKBH5 and TLR4 increased. It was also verified that TLR4 up-regulated ALKBH5 expression via activating NF-κB pathway. The m6A-Seq and m6A MeRIP, showed that NANOG served as a target in ALKBH5-mediated m6A modification. NANOG expression increased after mRNA demethylation, consequently enhancing the |
### Pancreatic cancer (PC)

**PER1** (period circadian protein homolog 1) is important to the maintenance of circadian rhythms in cells, and may also play a role in the development of cancer.

ALKBH5 serves as a PC suppressor by supporting the posttranscriptional activation of PER1 through m6A abolishment.

### Osteosarcoma (OS)

**PVT1** (Plasmacytoma Variant Translocation 1) is a long non-coding RNA gene. Overexpression of PVT1 is associated with many cancers in human through dysregulation of certain different genes in different cancers.

This study suggests that ALKBH5-mediated m6A modification of PVT1 contributes to OS tumorigenesis.

| II. ALKBH5 protein synthesis. |
|--------------------------------|
| **Stage I:** cloning of cDNA sequences into the pFastBac1 recombination vector |
| Cloned cDNAs were ordered from and synthesized by GENEWIZ ([https://www.genewiz.com/en-GB/](https://www.genewiz.com/en-GB/)). All the open reading frames were codon optimized for expression in *Spodoptera frugiperda Sf9* tissue culture cells and were designed to contain an additional N-terminal strep-II affinity tag MASAWSHPQFEKSG. The cDNAs were subcloned between BamHI and HindIII restriction sites in the multicloning site of the pFastBac1 plasmid vector and initial verification of the resulting plasmid clones was carried out with restriction enzyme analysis. The coding regions of all plasmid constructs that were picked for subsequent baculovirus construction were then also fully sequenced. |
| **Stage II:** construction of baculoviruses expressing the desired proteins |
| Transfer of the expression cassettes from pFastBac1 vectors to the baculovirus genomic DNA was carried out using Bac-to-Bac protocol and reagents (Thermo Fisher Scientific). Resulting genomic DNA preparations of recombinant baculoviruses were then transfected into the Sf9 cells using the 007 transfection reagent (Icosagen) and following the protocol provided by manufacturer. The resulting P1 generation baculoviruses were passed through two additional amplification rounds (P2-P3) to obtain virus quantities and titers that would be sufficient for subsequent large-scale protein expression experiments. Preliminary small-scale protein purification tests to verify the successful expression were carried out with 50 μl of |
Strep-Tactin XT beads (IBA GmbH) from the extracts of the infected Sf9 cells used in P3 viral amplification round. In these experiments, the scaled down version of the same protocol was used as in the following large scale purification experiments (Appendix I).

Stage III: large scale protein expression and purification

1L of suspension culture of Sf9 cells at concentration $2 \times 10^6$ cells/ml was infected with the high titer P3 generation virus at approximate MOI (multiplicity of infection) 5. Cells were harvested after three days and the Strep-Tactin XT affinity chromatography was carried out as described in protocols in Appendix I.

ALKBH5 eluted from the Strep-Tactin XT column with much higher protein concentrations, enabling to use these fractions straight for the following Superdex 200 chromatography without any additional concentration step. The Superdex 200 columns were developed with HEPES pH 7.6 / 150mM NaCl / 10% glycerol storage buffer, which is also the storage buffer for the final purified ALKBH5 preparation.

Details of ALKBH5 purification

![Coomassie stained SDS-PAGE gel](image)

**Figure S1.** The image of Coomassie stained SDS-PAGE gel with Strep-Tactin XT affinity chromatography fractions. Fraction 4 from this step (500 μl) was injected into the 24ml Superdex 200 column.
Figure S2. Superdex 200 chromatogram of the ALKBH5 protein (280nm trace in blue, 260nm in red)

Figure S3. The protein gel of Superdex 200 fractions. Fractions A11 and A12 were pooled for the final purified AlkBH5 protein prep. Molecular weight standards co-elute in following fractions: 67kD (BSA)–A10; 150kD (alcohol dehydrogenase) –A8; 443kD (apoferritin) –A5; void–A2.

Appendix I: Protein purification methods

Cell lysis protocol
Collect baculovirus infected Sf9 cells by centrifugation and wash once with ice-cold PBS.

Resuspend cell pellets in lysis buffer (25 mM HEPES-KOH pH 7.6 / 0.02% Tween-20 / 10% glycerol / 15mM KCl / 1mM DTT / 0.2 mM PMSF / Roche protease inhibitors cocktail without EDTA). Use 1ml per each 15cm dish in small scale test purification experiments, or 60ml for the cells from 1L culture in the large scale purification experiments.

Flash freeze the cell suspension in liquid nitrogen and store at -70°C.

Strep-Tactin XT purification protocol

Volumes are for large scale purification experiments from 1L of culture; preliminary purification tests were carried out with the similar scaled down version of the protocol.

All steps on ice or at 4°C

Thaw the cell suspension and homogenize in 40 ml Dounce grinder, 15 strokes with tight (‘B’) pestle.

Transfer to SS34 tubes and add 62.7μl of 4M KCl per each ml of homogenate, 250 mM final concentration. Incubate for 10 min.

Clear by centrifugation (10min 10 krpm 4ºC, transfer to clean tube, then one more time 16 krpm 10 min).

Load the cleared extract on 1ml Strep-Tactin XT Superflow column pre-equilibrated with 250-Cbuffer (25mM HEPES-KOH pH7.6 / 250mM KCl / 0.02% Tween-20 / 10% glycerol / 0.2mM PMSF / 1mM DTT).

Wash column 3 times with 250-C buffer and 2 times with storage buffer SB (25mM HEPES-NaOH pH7.6 / 150 mM NaCl / 10% glycerol).

Elute with SB buffer supplemented with 50mM biotin, collect 10 x 0.5ml factions and take samples for SDS-PAGE analysis.

Appendix II: Protein sequence

ALKBH5

```
MASAWSHPQFEKSGMPERSD YEEQLQKKEQ EARKVKGIR QMRLFSQDEC AKIEARIDEV VSRAEKGLYNEHTVDRAPLR NKYFFGEQYT YGAQLQKRGPFQERLYPPGD VDEIPEWVHQ LVQIKLVEHRSVFEGFVNSA VINDYQPGGC IVSHVDPHI FEPPIVSVSF FSDSALCFGCF KFQFKPIRSPEVLSLPVRR GSVTVLSGYA ADEITHCIRPF QDIKERRVII LRKRLDAP RLETKSLSSSVLPPSYASDR LSGNNRDPAL KFKRSHRKAD FADAHPRIL EMDKEENRSGTVLPHRRGFSSENVWRK SYESSEDCSE AAGSPARKVK MRH
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ALKBH5 (66-292)

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PERSD YEEQLQKKEQ EARKVKGIR QMRLFSQDEC AKIEARIDEV VSRAEKGLYNEHTVDRAPLR NKYFFGEQYT YGAQLQKRGPFQERLYPPGD VDEIPEWVHQ LVQIKLVEHRSVFEGFVNSA VINDYQPGGC IVSHVDPHI FEPPIVSVSF FSDSALCFGCF KFQFKPIRSPEVLSLPVRR GSVTVLSGYA ADEITHCIRPF QDIKERRVII LRKRLDAP R
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Appendix III: Plasmid map

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