The XLMR gene PHF8 encodes a histone H4K20/H3K9 demethylase and regulates zebrafish brain and craniofacial development

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Supplemental Information

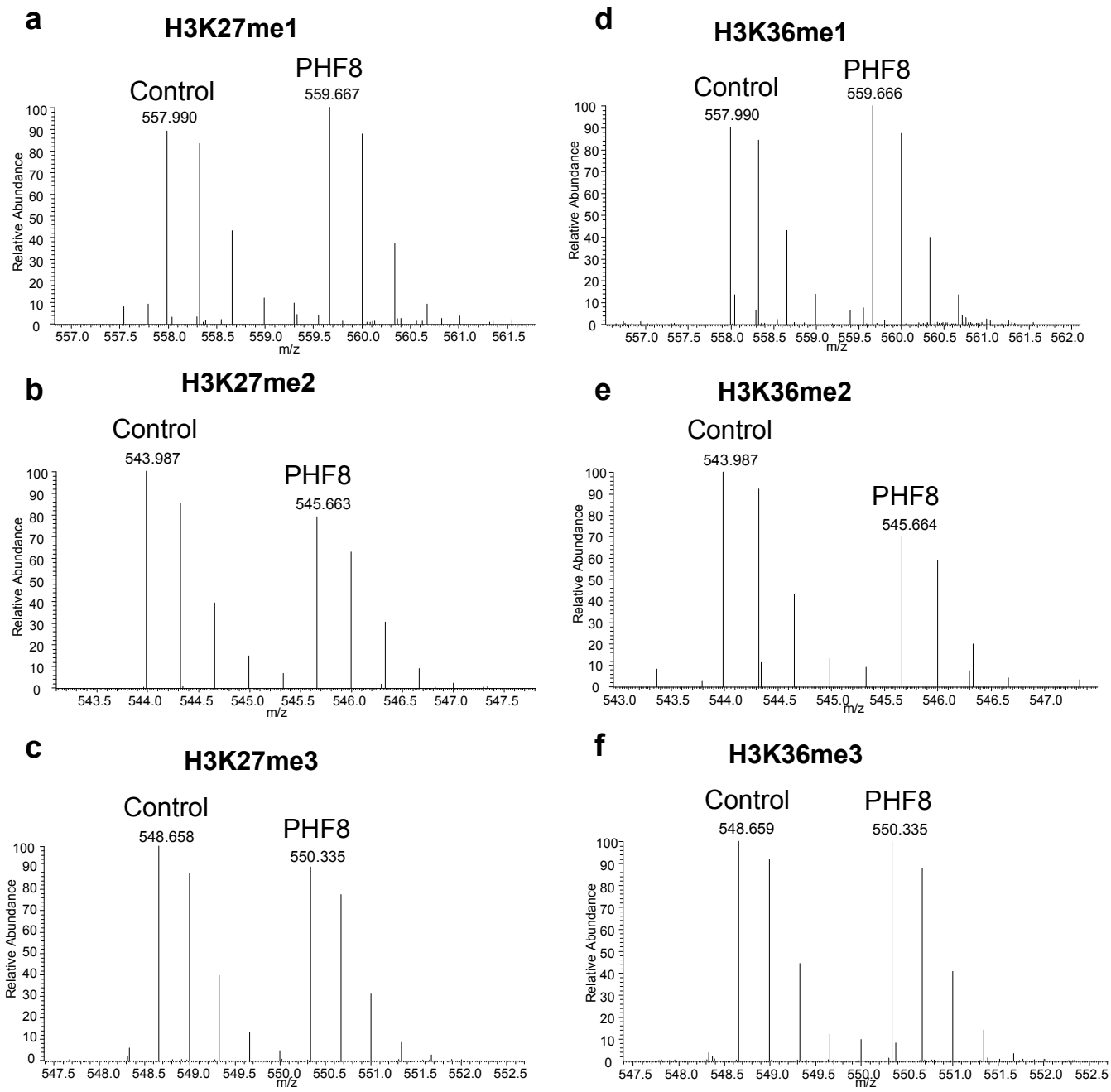
Merged Supplemental Figure 1 to 15 and Supplemental Tables 1, 2, 5 and 7, 8, 9.

Supplemental Table 3. ChIP-seq results of PHF8 binding events around TSS, xls file

Supplemental Table 4. ChIP-chip results of PHF8 binding events around TSS, xls file

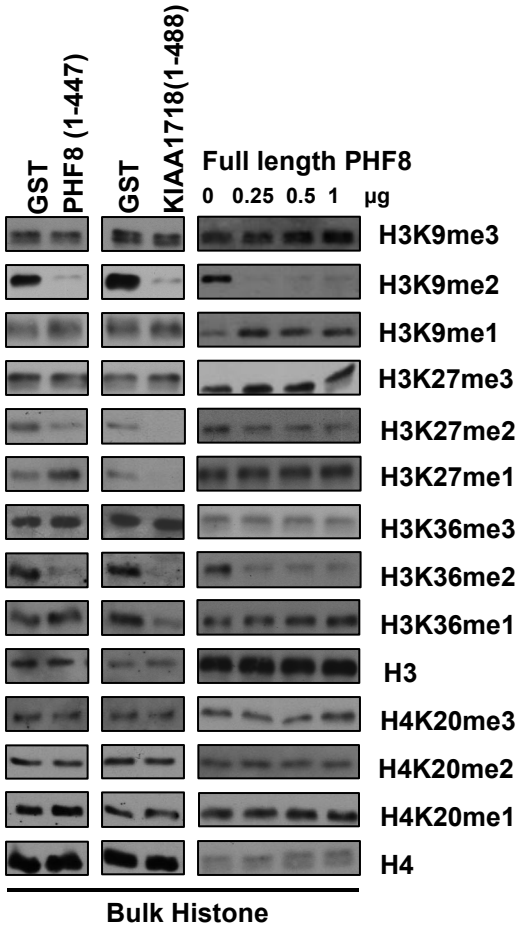
Supplemental Table 6. Gene expression microarray data (Phalanx) from HeLa cells and IPA Analysis, xls file

Supplemental Figure 1



Supplemental Figure 1. Mass spectrometry analysis of selected histone peptides from control (GST) and PHF8 (GST-PHF8(1-447)) *in vitro* reacted HeLa cell nucleosomes. Nucleosome samples from the control and PHF8 demethylation reaction were chemically isotopically labeled with D0-propionyl and D5-propionyl, respectively. This labeling induces a 5 Da mass shift between the two samples, which is observed as a 2.5 m/z shift for doubly charged peptides. Shown are m/z ranges depicting peptides from H3K27me1(a), H3K27me2 (b), H3K27me3 (c), H3K36me1 (d), H3K36me2 (e) and H3K36me3 (f). Moderate decreases for H3K27me2 and H3K36me2 were observed.

Supplemental Figure 2



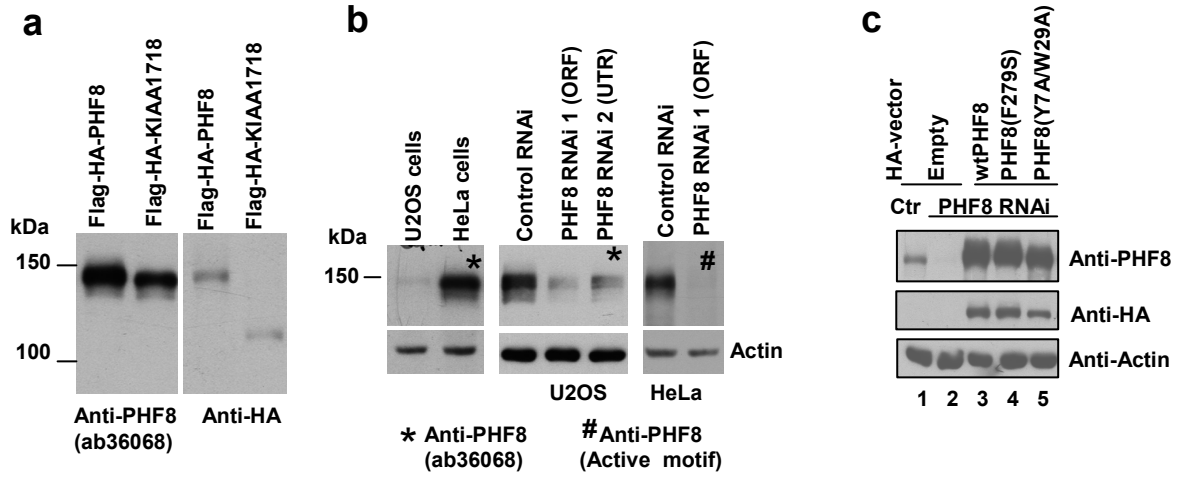
Supplemental Figure 2. PHF8 and KIAA1718 display demethylase activities on bulk histone. In vitro demethylation assays on bulk histones (0.6μg) were performed with GST-fused portions of wtPHF8 and KIAA1718 as well as full length PHF8 purified from insect Sf9 cells. Western blots were performed with indicated antibodies.

Supplemental Table 1. PHF8 and KIAA1718 In vitro demethylase activities

	PHF8		KIAA1718	
	Histone	Nucleosome	Histone	Nucleosome
H3K9me2	√	√	√	√
H3K9me1	-	√	-	√
H3K27me2	√	√	√	√
H3K27me1	-	-	√	-
H3K36me2	√	-	√	-
H3K36me1	-	-	√	-
H4K20me2	-	-	-	-
H4K20me1	-	√	-	√

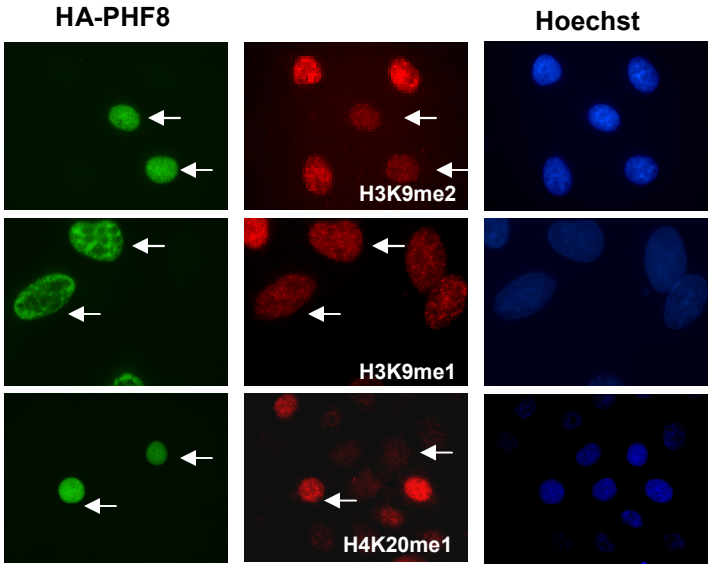
PHF8 and KIAA1718 did not show demethylase activities on tri-methylated H3K9, 27, 36 and H4K20.

Supplemental Figure 3



Supplemental Figure 3. PHF8 antibody validation and PHF8 expression in the rescue experiments. a. U2OS cells were transfected with Flag-HA-PHF8 or KIAA1718, followed with Western blot using anti-PHf8 (Ab36068) or Anti-HA antibodies. **b**. PHF8 protein level were examined in U2OS or HeLa cells (left). PHF8 RNAi efficiency was monitored in both U2OS (middle) and HeLa (right) cells with indicated antibodies. **c**. Experiment was described in Figure 3c. Western blot was performed with anti-HA, anti-PHF8 and anti-actin antibodies.

Supplemental Figure 4



Supplemental Figure 4. Investigation of over-expressed PHF8 on global levels of histone H4K20me1 and H3K9me1/2. HA-PHF8 was transfected into U2OS cells, immunofluorescence was performed with anti-HA (polyclonal for the H3K9me2 panel, monoclonal for the H3K9me1 and H4K20me1 panels), H3K9me2 (Ab1220), H3K9me1(Ab8898) and H4K20me1 (ab9051) antibodies. The H4K20me1 signal varies from cell to cell (high and low levels).

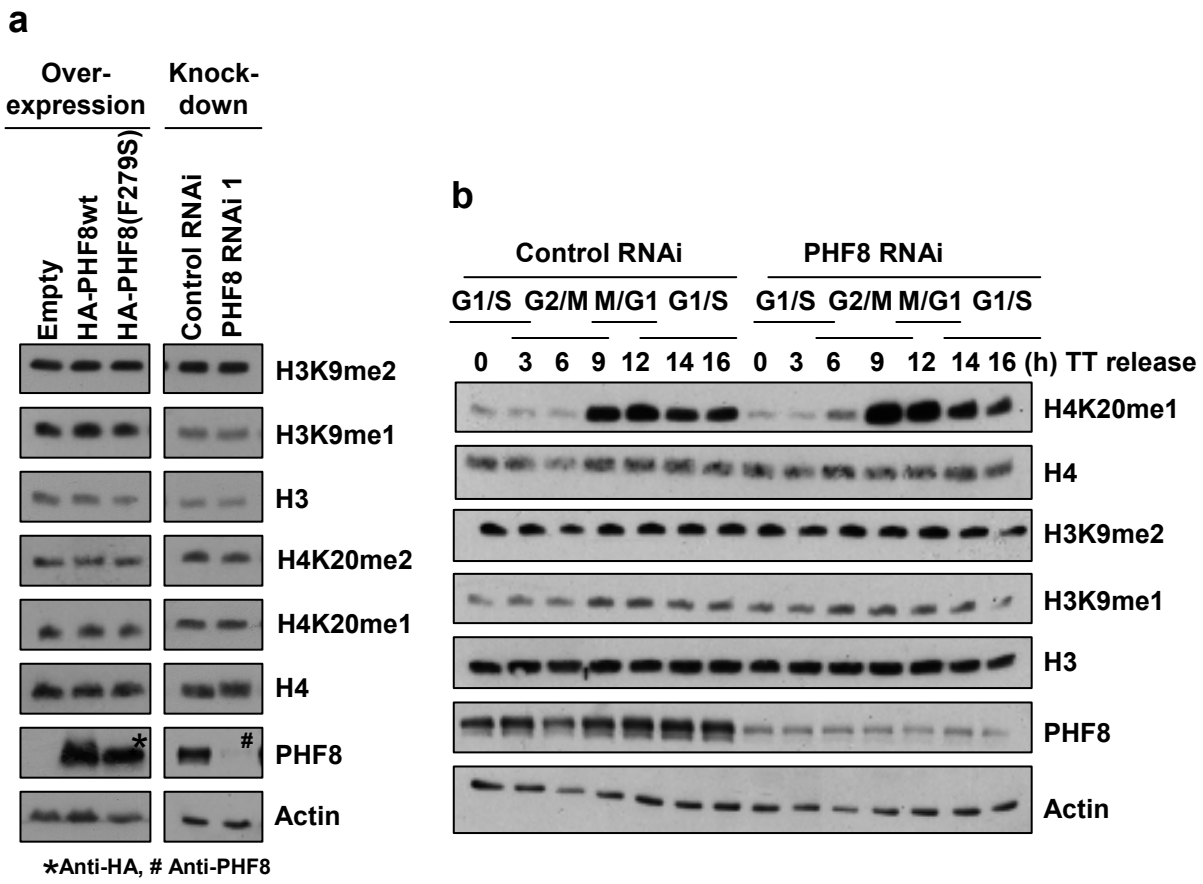
Supplemental Table 2a. Over-expression of PHF8 decreased H3K9me2 in U2OS cells

	No change	Decrease	Increase
H3K9me3	97.5% (195/200)	1% (2/200)	1.5%(3/200)
H3K9me2	2% (3/152)	90.8% (138/152)	7.2% (11/152)
H3K9me1	80% (140/175)	5.7% (10/175)	13.8% (25/175)
H3K27me3	88% (184/209)	2.4% (5/209)	9.6% (20/209)
H3K27me2	78.6% (88/112)	18.7% (21/112)	2.7% (3/112)
H3K27me1	89.6% (69/77)	3.8% (3/77)	6.6% (5/77)
H3K36me3	93.3% (125/134)	3% (4/134)	3.7% (5/134)
H3K36me2	89.3% (126/141)	7% (10/141)	3.5% (5/141)
H3K36me1	84.2% (128/152)	6.6 (10/152)	9.2 (14/152)

Supplemental Table 2b. Over-expression of PHF8 slightly reduced H4K20me1 level in U2OS cells

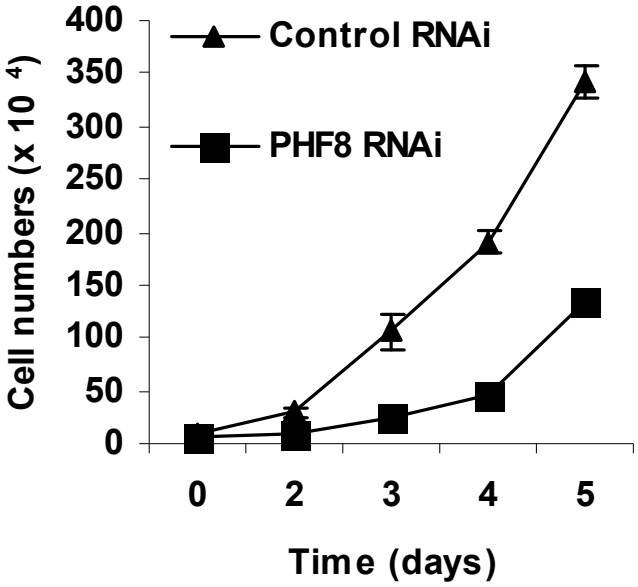
H4K20me1	Low level	High level
Mock	67.2% (133/198)	32.8% (65/198)
wtPHF8	80.4% (218/271)	19.6% (53/271)
PHF8(F279S)	69% (200/290)	31% (90/290)
PHF8(Y14A,W29A)	66% (162/246)	34% (84/246)

Supplemental Figure 5



Supplemental Figure 5. PHF8 impacts global H4K20me1 in a cell cycle dependent manner. a. HeLa cells were either transfected with an empty vector, HA-PHF8wt or HA-PHF8 (F279S) (left panel) or stably expressing control or PHF8 shRNA (right panel) were lyzed with 2% SDS. Western blot were carried out to determine histone modifications. **b**. Double thymidine (TT) block was performed in HeLa cells stably expressing control or PHF8 shRNA. Cells were released, collected at indicated time points and lyzed with 2% SDS. Western blot were carried out with indicated antibodies.

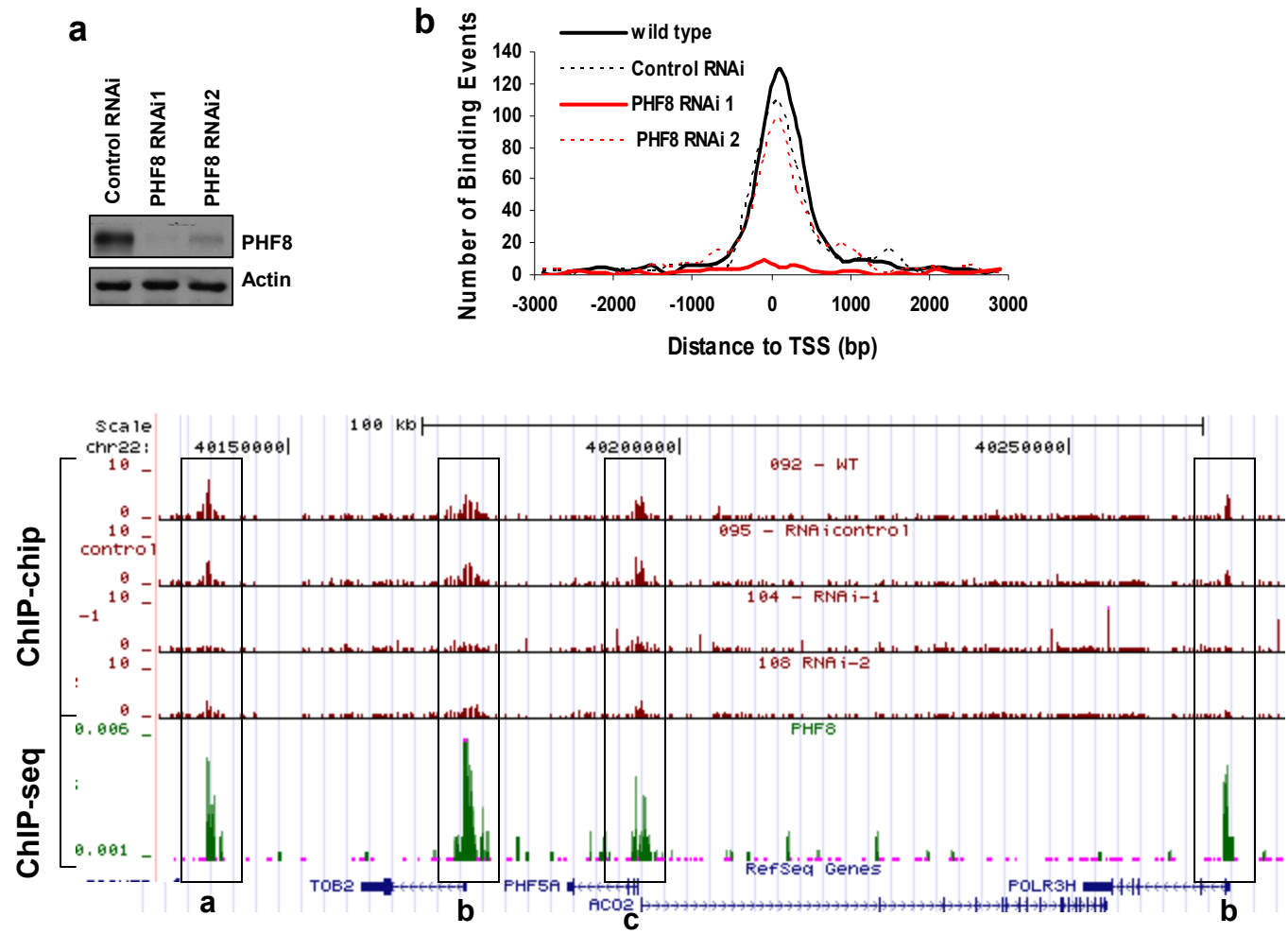
Supplemental Figure 6



Supplemental Figure 6. Knockdown of PHF8 impairs HeLa cell proliferation.

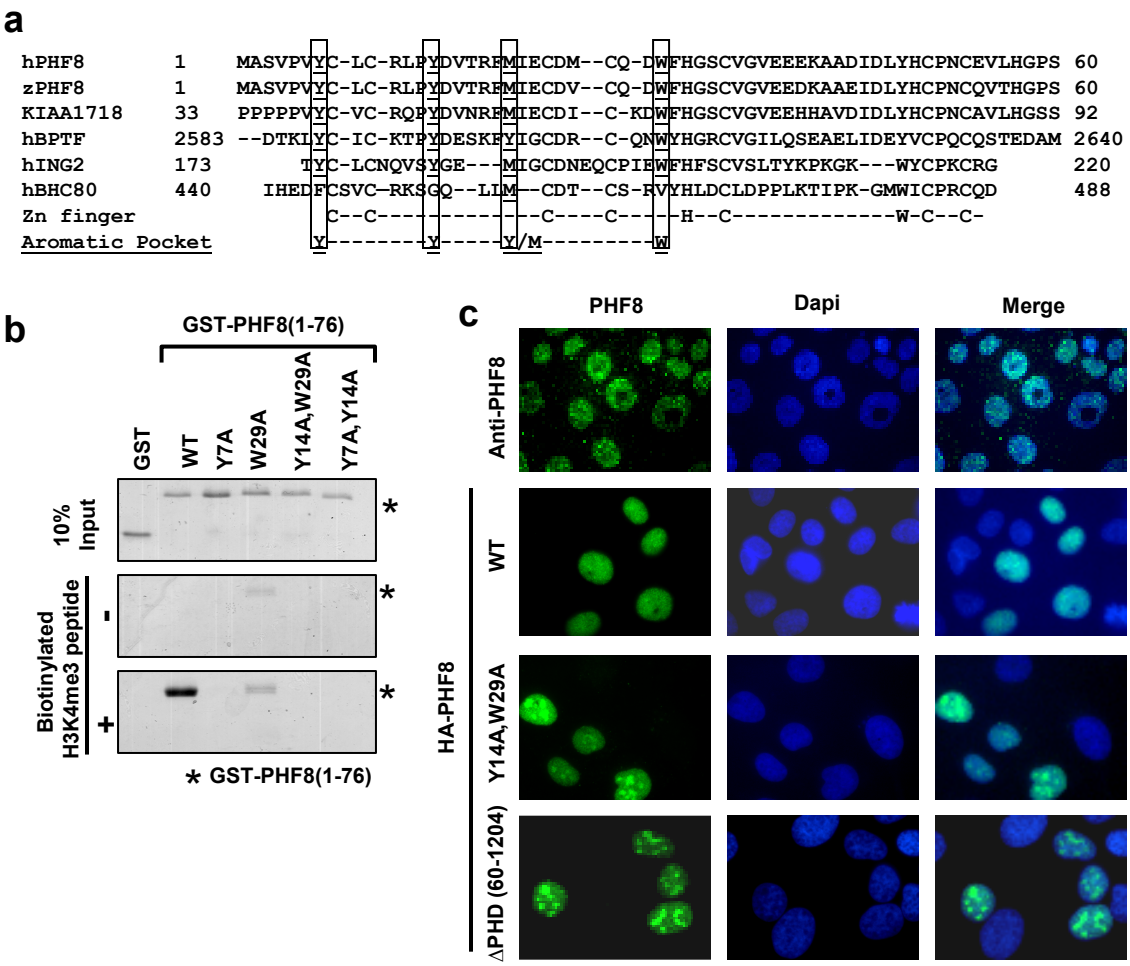
HeLa cells stably expressing control or PHF8 shRNA were seeded at $1x10^4$ cells in triplicate 60mm plates. Cells were trypsinized and counted at indicated time points. Standard deviation bars were obtained from the triplicate counts.

Supplemental Figure 7



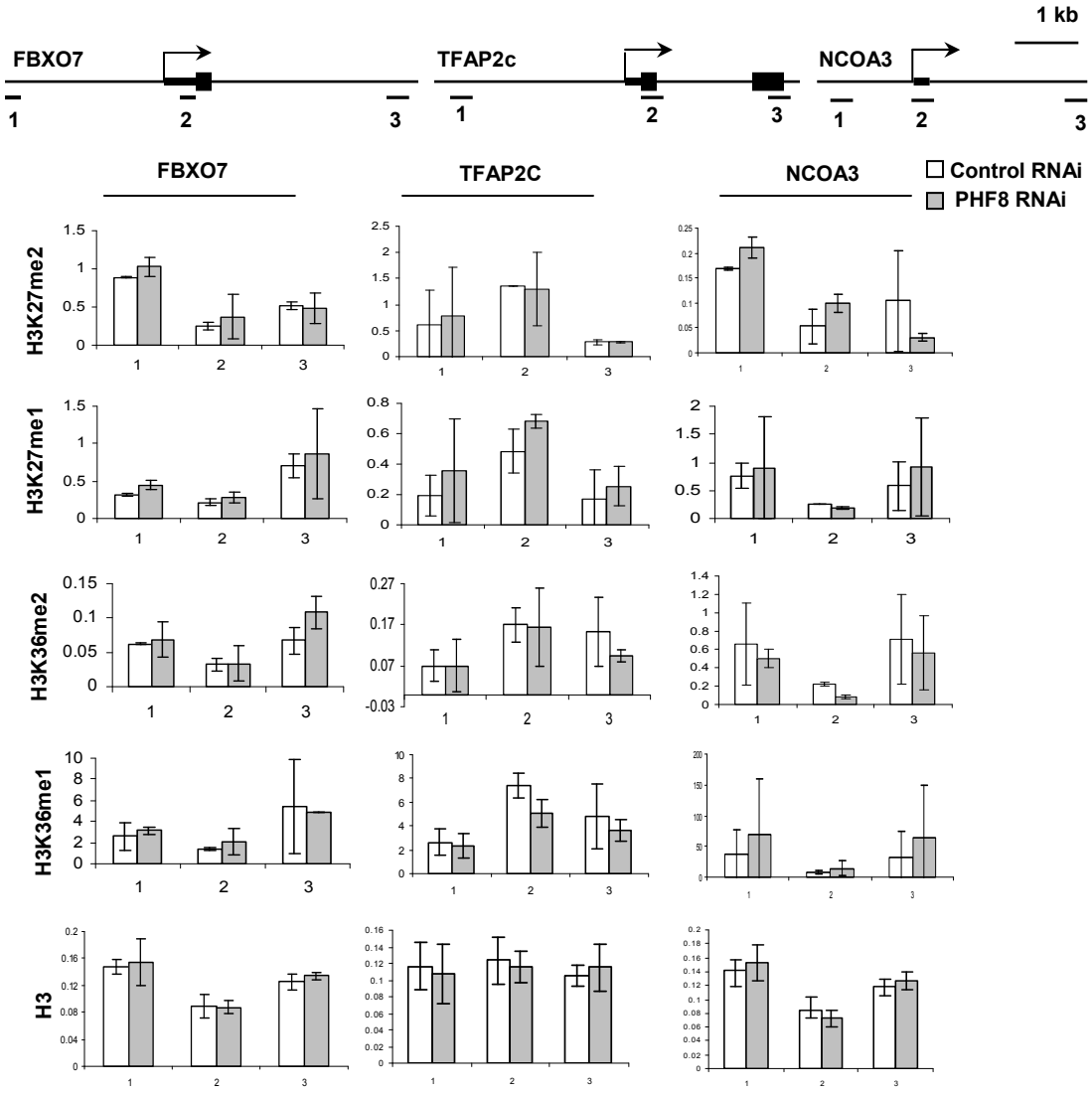
Supplemental Figure 7 ChIP-chip and ChIP-seq idenitified PHF8 located near transcription start sites (TSS) a. PHF8 RNAi efficientcy was monitored by Western blot. **b**. Distribution of PHF8 binding events. The binding events from four ChIP-chip experiments were plotted. The data show that majority of binding events are within -0.5 to +1kb of TSS and PHF8 RNAi1 abolished majority of the binding events. **c**. ChIP-chip and ChIP-seq data were compared at a representative genomic region. PHF8 binding events were detected at intergenic region (a), near TSS of RefSeq benes (b), as well as region between two transcripts (c).

Supplemental Figure 8



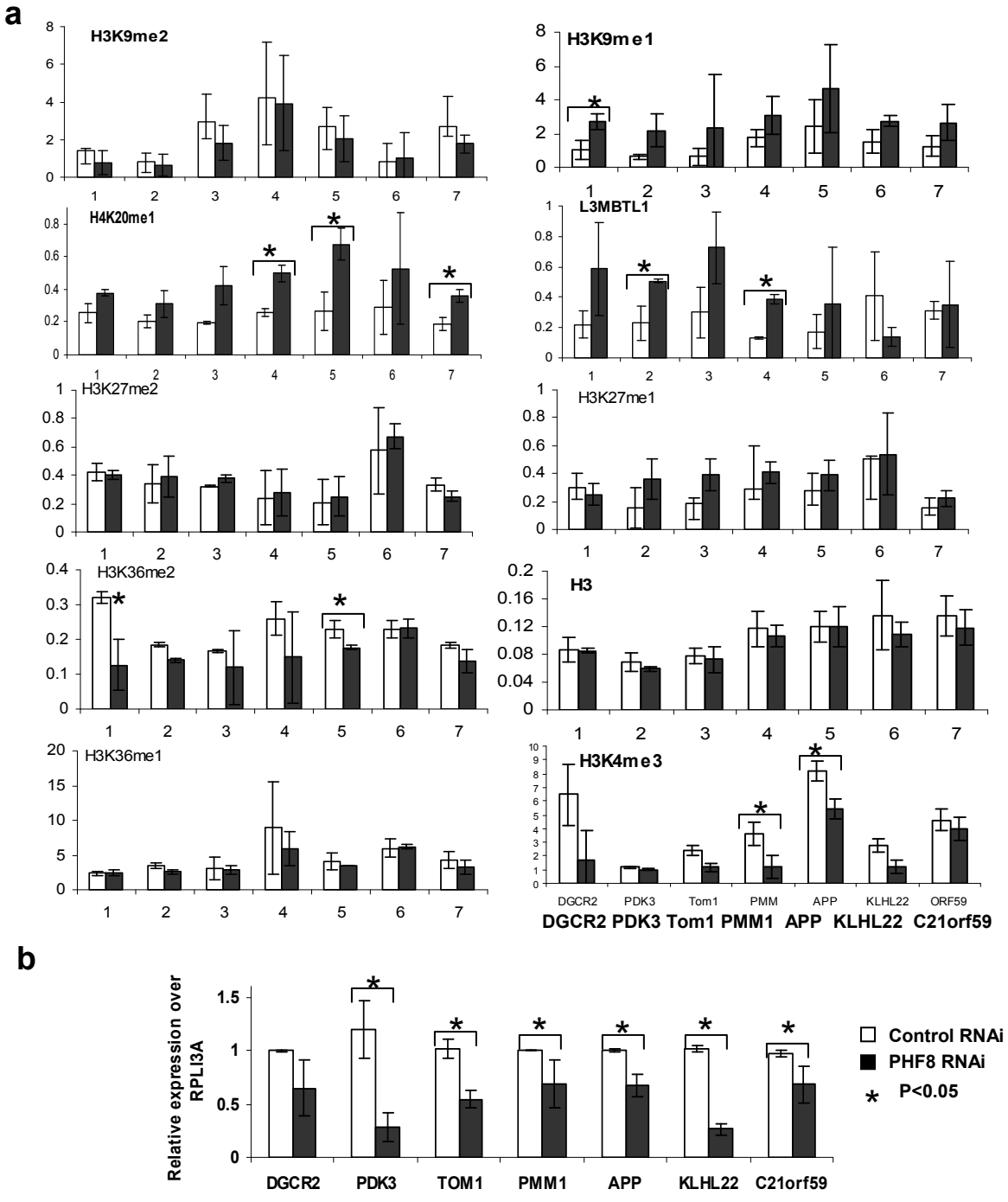
Supplemental Figure 8. The aromatic pocket of PHF8 PHD finger is important for H3K4me3 binding and is required for PHF8 proper localization. a. Alignment of PHD fingers from human (h) and zebrafish (z)PHF8, KIAA1718, BPTF, ING2 and BHC80. Aromatic pocket amino acids are highlighted with rectangular boxes. **b.** The PHD finger of PHF8 binds H3K4me3. Wild type or mutant GST-PHF8 (1-76) proteins were incubated with indicated biotinylated histone peptides. The bound GST-PHF8 was pulled down by streptavidin agarose and visualized by Commassie blue staining. Input and bound GST-PHF8 (1-76) are indicated by asterisks (*). **c.** Immunofluorescence was performed using anti-PHF8 (Ab36068) or anti-HA antibody in U2OS cells or U2OS cells transfected with indicated plasmids.

Supplemental Figure 9

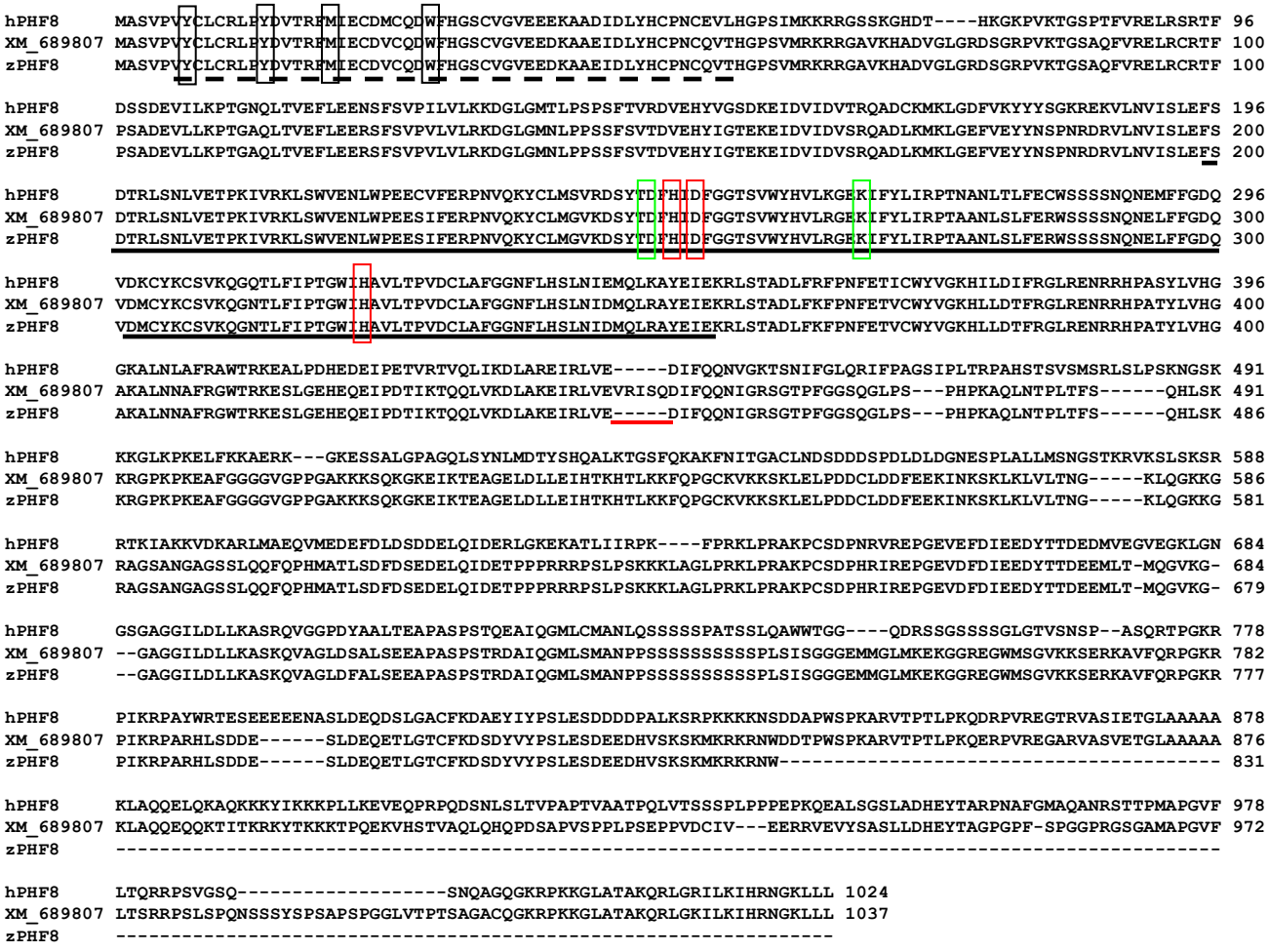


Supplemental Figure 9. Knockdown of PHF8 has no impact on H3K27me2/1 and H3K36me2/1 at TBXO7, NCOA3, TFAP2C loci. ChIP-qPCR was performed with indicated antibodies. Amplicon 2 represents the PHF8 binding locus. The standard deviations were obtained from three independent experiments. T-Test show that the p values are less than 0.05 comparing control and PHF8 RNAi, indicating no significant changes of these modifications in response to PHF8 knockdown.

Supplemental Figure 10

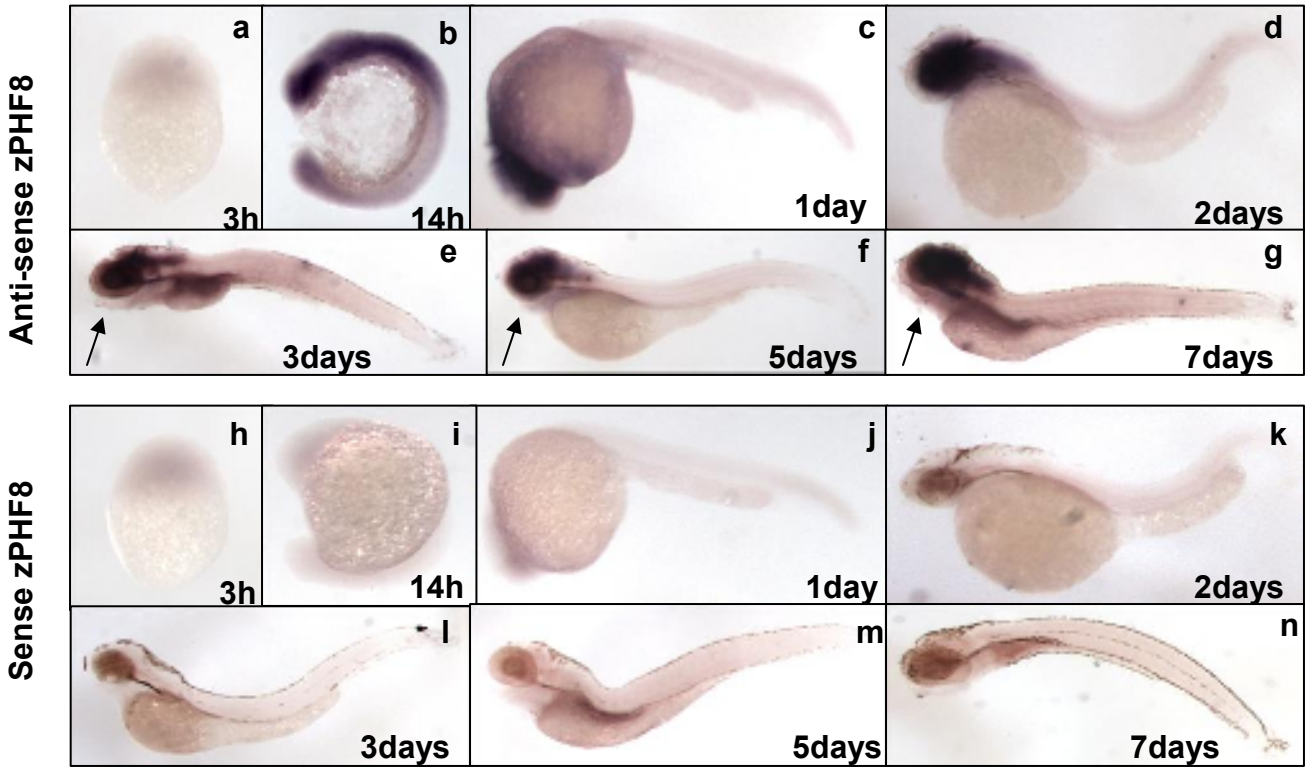


Supplemental Figure 10. Knockdown of PHF8 results in increase of H4K20me1, H3K9me1 and L3MBTL1; and reduced target gene expression and H3K4me3. a. Conventional ChIP assays with indicated antibodies were performed using HeLa cells stably expressing control or PHF8 shRNA. The amplicon spans PHF8 binding sites. **b**. mRNA expression of seven predicted PHF8 direct target genes was measured by real time PCR in control and PHF8 RNAi HeLa cells. Data are presented as % of input. Standard deviation bars were obtained from three independent experiments. p values from T-Test are shown.



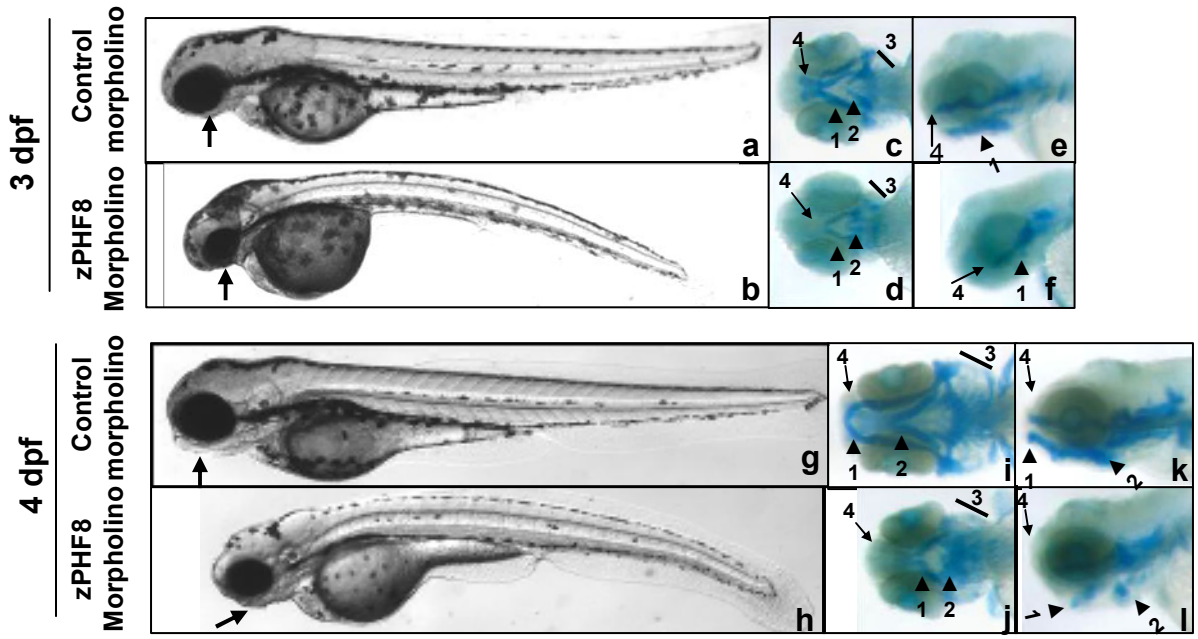
Supplemental Figure 11. Comparison of human and zebrafish PHF8. The human PHF8 sequence was taken from nm_015107, predicted zebrafish PHF8 (XM_689807) was retrieved from Ensembl. zPHF8 shown above was cloned by RT-PCR using zebrafish RNA. The PHD finger was underlined with dotted line, the aromatic residues are boxed. The JmjC domain is underlined with the Fe(II)-binding residues (red box) and α KG-binding (green box) residues highlighted. The zPHF8 clone we have isolated has five amino acids missing compared with XM_689807 (red line) and is 204 amino acids shorter at the c-terminus.

Supplemental Figure 12



Supplemental Figure 12. PHF8 expression in Zebrafish. RNA in situ hybridization of whole mount zebrafish embryos was performed using sense and anti-sense zPHF8 probes. Panels a through g represents the antisense PHF8 probe hybridized to embryos at different developmental stages post fertilization. Panels h through n represent the sense control PHF8 probe hybridized to embryos at the same as those used for the antisense probe. Arrows point to the weak staining of the jaw not present in panels h through n.

Supplemental Figure 13



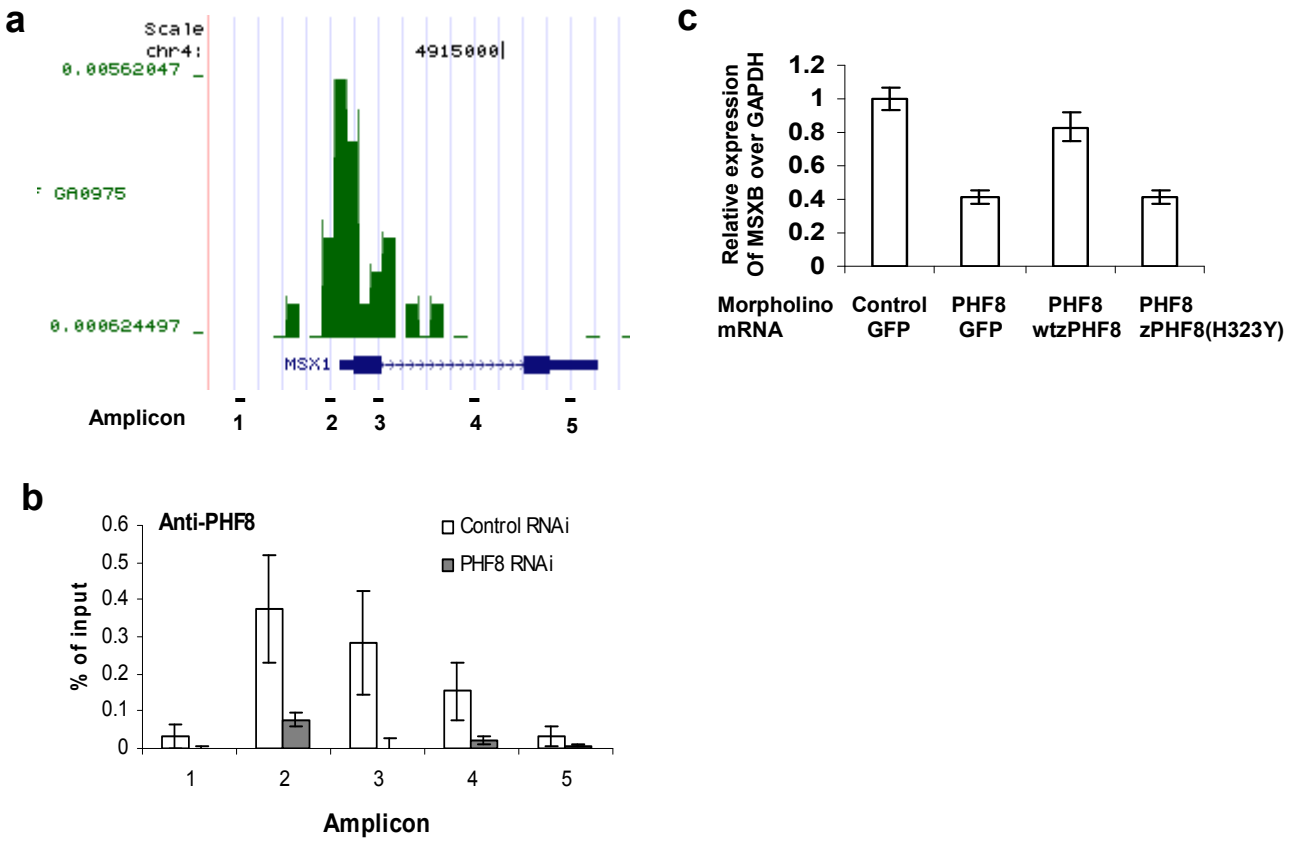
Supplemental Figure 13. Zebrafish embryos were injected at the 1 cell stage with 250uM of control or zPHF8 morpholino (MO). At 3 and 4 days post fertilization (dpf), PHF8 MO embryos displayed craniofacial developmental abnormalities including stunted lower jaw (compare panels b with a , h withg). Alcian Blue staining of the embryos at the same stages shows stunted 1st pharyngeal arch (1), inverted 2nd pharyngeal arch (2), deformed or absent 3rd through 5th pharyngeal arches (3). and delayed ethmoid plate (4) (compare panels d,f with c,e; j,l with i,k).

Supplemental Table 5. Numbers of embryos analyzed in the zebrafish experiments

Morpholino (MO) + mRNA	Craniofacial analysis				Apoptosis
	24 hpf	3 dpf	4 dpf	7 dpf	30 hpf
Control MO	30[a]/32[b]	20/23	27/31	30/32	35/36
zPHF8 MO	37/42	26/32	28/34	35/42	30/38
zPHF8 MO + wt zPHF8				33/43	25/34
zPHF8 MO + zPHF8 (H323Y)				31/39	31/37
zPHF8 MO + zMSXB				26/40	27/39

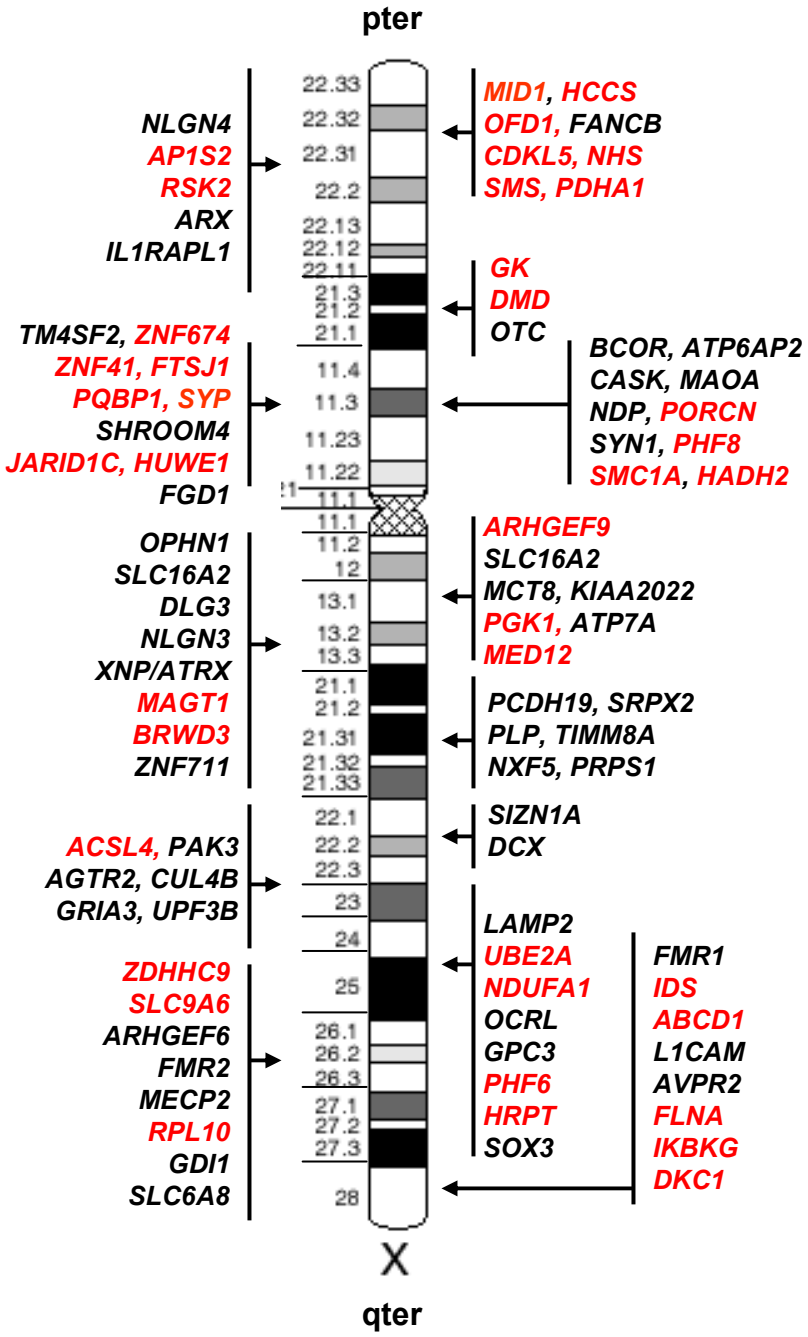
a: Numbers of zebrafish embryos with the phenotype(s) described in the text.
b: Total zebrafish embryos counted

Supplemental Figure 14



Supplemental Figure 14. PHF8 targets MSX1 and regulates MSXB expression in Zebrafish. a. PHF8 peaks around the TSS of MSX1 gene were identified by ChIP-seq in HeLa cell. **b**. Conventional ChIP with PHF8 antibody followed by real time PCR confirmed PHF8 binding to MSX1 in HeLa cells. Five amplicons are shown in a. The standard deviation was obtained from three independent experiments. **c.** Zebrafish embryos injected at the 1 cell stage with 250uM PHF8 ATG morpholino alone or with 200ng wt or mutant PHF8 mRNA were allowed to develop for 48hrs prior to harvesting and mRNA purification and reverse transcription. These samples were then subjected to RT-real time PCR**.** GAPDH was use as an internal control**.** Standard deviation bars were obtained from three independent experiments.

Supplemental Figure 15



Supplemental Figure 15. PHF8 direct target genes covers XLMR genes. A total of 90 XLMR genes are listed. Genes mutated in Non-syndromic and syndromic XLMR are shown on the left and right, respectively. 40 potential PHF8 direct target genes identified by ChIP-seq or ChIP-chip are in red.

Supplemental Table 7. Antibodies

Antibody	Source; Cat. No.	Application
Anti-H3K4me3	Upstate; 07-473	WB[a], ChIP[b]
Anti-H3K9me1	Abcam; ab8896	WB, IF[c], ChIP
Anti-H3K9me2	Abcam; ab1220	WB, IF, ChIP
Anti-H3K27me1	Upstate; 07-448	WB, IF, ChIP
Anti-H3K27me2	Upstate; 07-452	WB, IF
Anti-H3K27me2	Active motif; 39245	ChIP
Anti-H3K27me3	Upstate; 07-449	WB, IF
Anti-H3K36me1	Abcam; ab9048	WB, IF, ChIP
Anti-H3K36me2	Upstate; 07-369	WB, IF, ChIP
Anti-H3K36me3	Abcam; ab9050	WB, IF
Anti-histone H3	Abcam; ab1791	WB, ChIP
Anti-H4K20me1	Abcam; ab9051	WB, IF, ChIP
Anti-H4K20me2	Upstate; 07031	WB
Anti-H4K20me3	Active motif; 39180	WB
L3MBTL1	Active motif; 39182	ChIP
Anti-H4	Abcam; ab31827	WB
Anti-HA	Covance; MMS-101P	WB, IF
Anti-PHF8	Abcam; ab36068	WB, IF, ChIP
Anti-PHF8	Active motif[e]	WB

a, WB: Western blot; b, ChIP: Chromatin immunoprecipitation

c, IF: immunofluorescence

Supplemental Table 8. RT-real time PCR primers (5'-3')

Genes	Forward primer	Reverse primer
FBXO7	cgagacaatactgtcagagttcaagaca	ggtgtgagttgacgatggcagga
NCOA3	ctgcatccatctatcagtcc	actgctgccattcatgtgca
APP	gtacacatccattcatcatggt	ggtctagttctgcatctgctca
PMM1	caggacagcttcgacaccatc	cgtgtcctgaggagacacca
TFAP2C	gcagctaggaagaacatgcta	gtatgttcgtctccaagactg
Tom1	caccggcaacctctcatc	agaggcctccagagactgc
DGCR2	cctacacggcatacaagtac	ccggagtaatgcaccttcact
C21orf59	gtactatcacagaagacaaga	catcagctggtgaacttcatct
KLHL22	gcaaccctcctcaacaagctgtatgtga	gacagatgaccactgtccag
PDK3	ctgtattccatggaaggcgt	gcttcaggcgtggtcttgt
PHF8 (3'UTR)	gccttctccactgaggagcaggta	ctcctcatcctgccttccagctct
RPL13A	cctggaggagaagaggaaagaga	ttgaggacctctgtgtatttgtca

Supplemental Table 9. ChIP-real time PCR primers (5'-3')

Genes	Amplicon	Forward primer	Reverse primer
FBXO7	Amp1*	gtctagcggcttcccataca	cagtgaggaatggtgccagga
	Amp2	cctaaggcttctcagagcaga	ccagtacttactccctgtgtg
	Amp3	ggtagacagatcatagacaagta	cctcttcatcattccagtctca
TFAP2C	Amp1	cagctttggctagtgttgct	gcagcttgaacaagctgtct
	Amp2	cagacgcctggtcaccgtga	gcactcacctcgcagtcct ctt
	Amp3	cctcaccagatggacgaggtgcag	aggtgctggtcgtcgacatt
NCOA3	Amp1	cagaagtgagtggaagagaa	gtcagtctctgtccctcctg
	Amp2	ggctgagcggcgagtttccg	cctcctttatctccactcac
	Amp3	agtactgttgtacgtgagac	ccagtcaagaagacattagt
KLHL22#		gaggaatgagggtaggaccgaggca	gatcgccaaggctcagtgagtc
C21ORF59		gtccgctggaggatggctgga	cgcacttcttcgttcaggttga
APP		ggaaaaccgaaaacgcagcg	gaacgcagggaaaagcgagg
PMM1		gcaggaaggagcgcgtcctctgcct	gtgtccacggtcacgctgct
Tom1		ctggcggttgctgtcagctgatt	ggagagctacacgccagcctcct
DGCR2		tgcctgaggttgcagccgagagtgt	ggaccgtgccaagcggagggtca
PDK3		ccagccagaggcggagctga	gctgcctctgtgacgtttgt

* Amp (Amplicon)1: about 1.1~2.5kb upstream of TSS, Amp 2: PHF8 binding site, Amp 3: 1.5~3.2 kb downstream of TSS. # For the additional seven genes, one amplicon was chosen at PHF8 binding sites.