ATXR5 and ATXR6 are novel H3K27 monomethyltransferases are required for chromatin structure and gene silencing

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ATXR5     MATWNASSPAASPCSSRRRTKAPARRPSSESPPPRKMKSMAEIMAKSVPV 50
ATXR6                  MVAVRRRRTQASNPRSEPP------------------ 19
P.patens1
P.patens2

ATXR5     VEQEEEEDEDSYSNVTCEKCGSGEGDDELLLCDKCDRGFHMKCLRPIVVR 100
ATXR6     -QHMSDHDSDSDWDTVCEECSSGKQPAKLLLCDKCDKGFHLFCLRPILVS 68
P.patens1                             MLLCDRCDRGYHMYCLSPILPT 22
P.patens2 -VVWTNGPASAYSLTLCEECKGGDSAEQMLLCDQCDRGFHMFCLSPILVS 50
consensus                 CE C SG      LLCD CDRGFHM CL PILV

ATXR5     VPIGTWLCVDCSDQRPVR-----RLSQKKILHFFRIEKHTHQTDKLELSQ 145
ATXR6     VPKGSWFCPSCSKHQIPK---SFPLIQTKIIDFFRIKR-SPDSSQISSSS 114
P.patens1 VPLDDWFCPKCSQSSHVQGIAEFPKVQKKIVDFFRIQKPSPFTAELKCVE 72
P.patens2 IPPGDWICPHCSKSTIAH---EFLMVQKKIVDYFRIQNLLPSKSVTEAIE 97
consensus VP G W CP CS            F    QKKI DFFRI     P

          Nuclear localization signal
ATXR5     EETRKRRRSCSLTVKKRRRKLLPLVPSEDPDQRLAQMGTLASALTALGIK 195
ATXR6     DSIGKKRKKTSLVMSKKKRRLLPYNPSNDPQRRLEQMASLATALRASNTK 164
P.patens1 TRKRRRPSGGSLCLQKKSRRLLPYVPCAEPQRRLEQMASLATALTSIGVE 122
P.patens2 RKKRKKQSAFSFCPPKRGRRLLPYVPTIDPQRRLKQMASLATALTSIGVQ 147
consensus     RK      SL    K  RRLLPYVP   DPQRRL QMASLATALT   G

ATXR5     YSDGLNYVPGMAPRSANQSKLEKGGMQVLCKEDLETLEQCQSMYRRGECP 245
ATXR6     FSNELTYVSGKAPRSANQAAFEKGGMQVLSKEGVETLALCKKMMDLGECP 214
P.patens1 FTDSLSYG--LAPRSANRAENEKGGMQVMSKEDKATLDLCKKMCSHGEWP 170
P.patens2 FSDSLSYR--YAPRTANRAAHEKGGMREMPRDDKEAFDKCKAMCKSGLWQ 195
consensus FSD L Y     APRSAN A  EKGGMQV  KED ETL   CK M    GE P

                              SET domain
ATXR5     PLVVVFDPLEGYTVEADGPIKDLTFIAEYTGDVDYLKNREKDD-CDSIMT 294
ATXR6     PLMVVFDPYEGFTVEADRFIKDWTIITEYVGDVDYLSNREDDYDGDSMMT 264
P.patens1 PLMVTHDSRQGFVVEADGNIKDLTIIAEYTGEVDYMRCREHDS-GNSIMG 219
P.patens2 PLTVAYDMRQGFVVEADEDIKDMTFIAEYTGEVDYMCCRHYDS-GNSIMG 244
consensus PL V   D    GF VEAD   IKD T IAEYTG VDY    RE D   G SIM

ATXR5     LLLSEDPSKTLVICPDKFGNISRFINGINNHNPVAKKKQNCKCVRYSING 344
ATXR6     LLHASDPSQCLVICPDRRSNIARFISGINNHSPEGRKKQNLKCVRFNING 314
P.patens1 LLFSDDPAKELVICPDRCGNIARFVSGINNHSPEGRKKQNVRCVRYNIDG 269
P.patens2 LLFSDDPIKELVICPDKRSNIARFLSGINNHTEEGRKKQNVRCVRYSING 294
consensus LL S DP K LVICPD    NIARF SGINNH PEGRKKQN   CVRY ING

ATXR5     ECRVLLVATRDISKGERLYYDYNGYEHEYPTHHFL 379
ATXR6     EARVLLVANRDISKGERLYYDYNGYEHEYPTEHFV 349
P.patens1 EARAILVAIRDIPKGERLYYDYNAYQTEYPTKHFV 304
P.patens2 EARVILIAMRDILKGERLYYDYNAYYTEYPTQHFV 329
consensus EARV LVA RDI KGERLYYDYN Y   EYPT HFV
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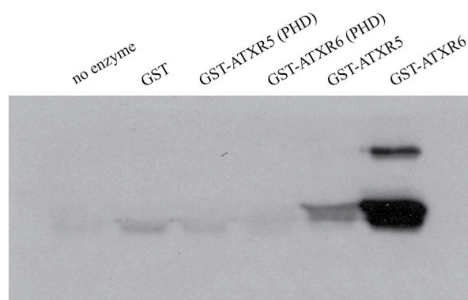
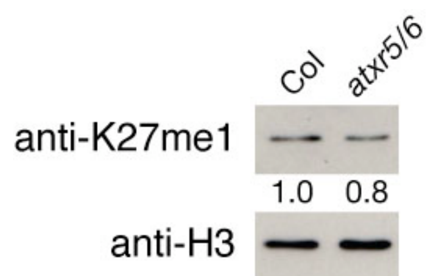
Supplementary Fig. 1. Alignment and functional domains of ATXR5, ATXR6, and homologous proteins (XP_001784397.1 and XP_001776886.1, National Center for Biotechnology Information) from *Physcomitrella patens*.



Supplementary Fig. 2. GST alone and the PHD domains from ATXR5 and ATXR6 show no H3K27 monomethyltransferase activity. The products of ATXR5 and ATXR6 HMT assays were analyzed by Western blot using anti-H3K27me1.



Supplementary Fig. 3. Western blot analysis of H3K27me1. Quantization was performed using ImageJ.

Table S1. PCR primers and conditions.

Technique	Primer	Sequence (5' to 3')	Reaction Conditions
RT-PCR			
ATXR5 (P1)	712-ATXR5-Fn	TCAATGGCTGAGATAATGGCG	95°C (3 min); 35 cycles of 95°C (30 sec), 55°C (30 sec), 72°C (30 sec)
	858-ATXR5-R	TCTGAGATAACCTTCTTACA	
ATXR5 (P2)	647-5g09790-F	CCATTGGAACTTGGCTTTGTGTC	95°C (3 min); 35 cycles of 95°C (30 sec), 60°C (30 sec), 72°C (30 sec)
	648-5g09790-R	AATAGGACCATCTGCTTCAACTGTG	
ATXR5 (P3)	856-ATXR5-F	TCCGCTTGAAGGTTACACAG	95°C (3 min); 35 cycles of 95°C (30 sec), 60°C (30 sec), 72°C (30 sec)
	857-ATXR5-R	TTCTTAGCAACCGGATTGTG	
ATXR6 (P1)	312-24330-F	CATCAGATCCCTAAATCTTTCCCTC	95°C (3 min); 35 cycles of 95°C (30 sec), 60°C (30 sec), 72°C (30 sec)
	392-5g24330-GatR212	TTCACCGAGGTCCATCATTTTCTTGCA	
ATXR6 (P2)	860-ATXR6-F	GGAGGCATGCAGGTTCTATC	95°C (3 min); 35 cycles of 95°C (30 sec), 60°C (30 sec), 72°C (30 sec)
	861-ATXR6-R	TTCCTCCCTTCTGGTGAGTG	
UBQ	190-UBQF	GATCTTTGCCGGAAAACAATTGGAGGATGGT	95°C (3 min); 26 cycles of 95°C (30 sec), 55°C (30 sec), 72°C (30 sec)
	191-UBQR	CGACTTGTCATTAGAAAGAAAGAGATAACAGG	
RT-PCR[4]			
Ta3	1128-Ta3-F	GATTCTTACTGTAAAGAACATGGCATTGAGAGA	RT-PCR: 95°C (3 min); 44 cycles of 95°C (30 sec), 55°C (30 sec), 72°C (45 sec)
	1129-Ta3-R	TCCAAATTTCCTGAGGTGCTTGTAACC	
CACTA-like	1126-CACTA-like-F	GGCTAGCTGTCCGACTCAATGACCT	RT-PCR: 95°C (3 min); 40 cycles of 95°C (30 sec), 55°C (30 sec), 72°C (45 sec)
	1127-CACTA-like-R	CAGACATCCTTTCCTTCAGCTTAGC	
TSI	1113-TSI-F	GAACTCATGGATACCCTAAAATAC	RT-PCR: 95°C (3 min); 37 cycles of 95°C (30 sec), 55°C (30 sec), 72°C (45 sec)
	1114-TSI-R	CTCTACCCTTTGCACTCATGAATC	
ChIP QPCR			

Ta3	QPCR-Ta3-F	AAGAGAGCTGGCAGAAGCAG TTGA	QPCR: 95°C (10 min); 40 cycles of 95°C (15 sec), 58°C (30 sec), 72°C (15 sec)
	QPCR-Ta3-R	ACGCCCTTTACCTTGACCTCC TTT	
CACTA-like	QPCR-CACTA-F	TGTGTGGAAGGGTCTTGTGGA CTT	QPCR: 95°C (10 min); 40 cycles of 95°C (15 sec), 58°C (30 sec), 72°C (15 sec)
	QPCR-CACTA-R	AACTTACATGTTTGCGGGCAC GAG	
TSI	QPCR-TSI-F	ATCCAGTCCGAAGAACGCGA ACTA	QPCR: 95°C (10 min); 40 cycles of 95°C (15 sec), 58°C (30 sec), 72°C (15 sec)
	QPCR-TSI-R	TCACTTGTGAGTGTTCGTGAG GTC	
Actin	ChIP-actin-F	CGTTTCGCTTTCCTTAGTGTT AGCT	QPCR: 95°C (10 min); 40 cycles of 95°C (15 sec), 58°C (30 sec), 72°C (15 sec)
	ChIP-actin-R	AGCGAACGGATCTAGAGACT CACCTTG	

SUPPLEMENTAL METHODS

Protein expression and purification

For the expression and purification of GST-ATXR5$_{PHD}$, GST-ATXR6$_{PHD}$, GST-ATXR5$_{PHD\text{-}SET}$ and GST-ATXR6$_{PHD\text{-}SET}$, *E. coli* BL21 DE3 cells were transformed with the appropriate pGEX-6P expression plasmid. A starter culture of 30 ml was inoculated and grown overnight at 37°C. The volume of the culture was then increased to 400 ml and ZnCl was added to a final concentration of 50 µM. The culture was shaken for 1 h before adding IPTG to a concentration of 0.1 mM. Induction was allowed to proceed overnight at 20°C. After centrifugation of the culture, the bacterial pellet was resuspended in 20 ml of cold lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05% NP-40). The cells were broken by treatment with 1 mg/ml of lysozyme for 15 min on ice, followed by sonication (Branson Sonifier 250, output 3, 2 x 2 min). The cell lysate was then centrifuged at 18,000 g. The supernatant was mixed with 400 µl of washed 50% glutathione sepharose beads (GE Life Sciences) and rotated at 4°C for 1 h. The beads were then washed once with lysis buffer, twice with elution buffer (50 mM Tris-HCl pH 7.5), and resuspended in 400 µl of elution buffer containing 10 mM of reduced glutathione (Sigma). Elution was performed with rotation, overnight at 4°C. Beads were then pelleted by centrifugation and the supernatant was collected. DTT and glycerol were added to a final concentration of 20 mM and 10%, respectively.

Expression and purification of the plant histone 3.1 proteins (wild-type and H3K27A) were performed as previously described[1]. After purification, proteins were dialyzed overnight into 1 L of buffer A (50 mM Tris-HCl pH 8.0, 1 mM DTT, 1 mM EDTA and 300 mM NaCl). The dialysis was initiated at room temperature for 3 h before transfer to 4°C. Buffer A was replaced the next day and the dialysis was allowed to proceed for another 4 h. Note that the two plant H3 proteins (wild-type and H3K27A) used in the histone methyltransferase assay have a higher molecular weight than the human homolog due to the C-terminal V5 epitope-6xHis tag used for purification of the proteins.

Immunofluorescence and FISH

Nuclei isolation from mature leaves was performed as described[2]. Fixed nuclei were dried onto coverslips, post-fixed for 5 min in cold methanol and rehydrated for 5 min in TBS (20 mM Tris, pH 7.5, 100 mM NaCl). Nuclei were blocked in Abdil (TBS, 0.1% Triton X-100, 2% BSA, and 0.1% NaN_3) for 30 min at 37˚C, incubated with primary antibodies for 1 h (anti-H3K27me1, 1 µg/ml; anti-H3K27me2, 0.5 µg/ml; anti-H3K9me2, 2 µg/ml; or anti-H3K27me3, 2 µg/ml) and washed in TBS-Tx (TBS, 0.1% Triton X-100). The nuclei were then incubated either with anti-rabbit Alexa Fluor 488 (1 µg/ml) (Invitrogen) or anti-mouse Alexa Fluor 594 (1 µg/ml) (Invitrogen), and washed in TBS-Tx. All antibodies were diluted in Abdil. Coverslips were mounted in Vectashield mounting medium with DAPI (Vector Laboratories).

FISH was performed as described [3] with the following modifications. The centromeric 180-bp repeats were amplified from the pARR20-1 clone using the following primers: pARR20-1F 5'-ATCCTCTAGAGTCGACCTGCA-3' and pARR20-1R 5'-TTCCCAGTCACGACGTTGTAA. The resulting PCR product was labeled with digoxigenin (DIG) using a DIG Nick translation kit according to the manufacturer's protocol (Roche). Leaf nuclei were isolated and fixed onto coverslips as descrived above, followed by post-fixation in 2% formaldehyde in PBS for 5 minutes. Coverslips were hybridized with the labeled probes for 18 hours at room temperature. For detection of DIG-labelled probes, coverslips were incubated with anti-DIG-fluorescein (1 g/ml) (Roche), washed in TBS-Tx, and incubated with anti-Fluorescein (0.4 g/ml) (Roche). After washing in TBS-Tx, coverslips were incubated with anti-rabbit Alexa Fluor 488 (1 µg/ml)

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(Invitrogen). All antibodies were diluted in Abdil and incubated for 30 minutes at 37ºC. Coverslips were mounted in Vectashield mounting medium with DAPI (Vector Laboratories). Nuclei were imaged on a Nikon 90i microscope equipped with a 100X 1.4 Plan Apo VC objective (Nikon). Digital images were obtained using a Photometrics Coolsnap HQ cooled CCD camera. The camera, shutters, and filter wheels were controlled by MetaMorph software (Molecular Devices). Z-series optical sections through each nucleus were obtained at 0.5 m steps. All images were taken with the same exposure time and scaled identically. Images were deconvolved (blind iterative deconvolution) using AutoDeblur 9.3 software (AutoQuant Imaging). At least 80 nuclei were examined from each genotype in each experiment.

Real-time PCR

Precipitated DNA was quantified by real-time PCR using an MxPro3000 qPCR system (Stratagene) with Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen). Relative quantities were determined by using a comparative Ct method as follows: Relative quantity $= 2^{(-((\text{Ct GOI unknown} - \text{Ct normalizer unknown}) - (\text{Ct GOI calibrator} - \text{Ct normalizer calibrator})))}$, where GOI is gene of interest. Actin was used as the normalizer. At least two different biological samples were used for each ChiP experiment.

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