H4K20me1 plays a dual role in transcriptional regulation during regeneration and axis patterning of *Hydra*

Akhila Gungi[1], Mrinmoy Pal[1], Shagnik Saha[2], and Sanjeev Galande[1,2,3]

[1] Department of Biology, Indian Institute of Science Education and Research, Pune, India

[2] Centre of Excellence in Epigenetics, Department of Life Sciences, Shiv Nadar University, Delhi-NCR, India

[3] To whom correspondence should be addressed

Running title: Activation-repression duality of H4K20me1

Keywords: H4K20me1, transcriptional regulation, Wnt signaling, developmental patterning, SETD8, Hydra

Address for correspondence

Department of Biology

Indian Institute of Science Education and Research

Dr. Homi Bhabha Road, Pune 411008, India

Tel: 91-20-25908060, Fax: 91-20-20251566

Email: sanjeev@iiserpune.ac.in; sanjeev.galande@snu.edu.in

ABSTRACT

The evolution of the first body axis in the animal kingdom and an extensive ability to regenerate makes *Hydra*, a Cnidarian, an excellent model system to understand the underlying epigenetic mechanisms. We identify that SETD8 is critical for regeneration, and H4K20me1 colocalizes with transcriptional activation machinery locally at the β-catenin bound TCF/LEF binding sites on the promoters of head-associated genes, marking an epigenetic activation node. Contrastingly, genome-wide analysis of the H4K20me1 occupancy revealed a negative correlation with transcriptional activation. We propose H4K20me1 as a general repressive histone mark in Cnidaria and describe its dichotomous role in transcriptional regulation in *Hydra*.

INTRODUCTION

Post-translational modifications (PTMs) of proteins are major regulators of cellular functions (Deribe et al. 2010) with nuclear histone PTMs being prominent context-specific players in transcriptional regulation. Although the methylation of histone H4 was one of the first histone PTM discovered (DeLange et al. 1969), the modifiers responsible are recent discoveries. While several enzymes are known to deposit the di- and tri-methyl marks on H4K20 — SUV4-20H1 and SUV4-20H2 being the predominant ones (Schotta et al. 2008), there is only one known monomethyltransferase — SETD8 (KMT5A or Pr-SET7) (Oda et al. 2009). SETD8 has many functions in cells and acts by disrupting signaling pathways (Ke et al. 2014), regulating transcription factors (Choi et al. 2020), altering the chromatin accessibility around the promoters of genes (Myers et al. 2020), and preventing both oncogene-induced and replicative cellular senescence by suppressing nucleolar and mitochondrial activity (Tanaka et al. 2017). SETD8 is involved directly in the Wnt/β-catenin signaling pathway in mammalian cells, *Drosophila* larvae, and zebrafish (Li et al. 2011). SETD8 is required for the activation of Wnt target genes by interaction with TCF4 during the development of zebrafish and the wings of *Drosophila* (Li et al. 2011; Yu et al. 2019). The methylation of H4 is highly evolutionarily conserved and exists in three states as mono-, di-, and trimethylation. While the mono- (H4K20me1) and di-methylated (H4K20me2) H4K20 are involved in DNA replication and DNA damage repair, the trimethylated H4K20 (H4K20me3) is a mark of silenced heterochromatic regions (Schotta et al. 2004; Schotta et al. 2008). The function of H4K20me1 is elusive with reports of activation (Kapoor-Vazirani and Vertino 2014; Lv et al. 2016; Shoaib et al. 2021) and repression (Nishioka et al. 2002; Tjalsma et al. 2021) of transcription.

To understand the role of H4K20me1 in transcriptional regulation of developmental processes including axis patterning and regeneration, *Hydra* serves as an excellent model organism.

Hydra belongs to the phylum Cnidaria which is the phylum that innovated a body axis during the evolution of multicellular organisms. It also harbours extensive powers of regeneration which require the recapitulation of the axis patterning gene regulatory networks to be formed *de novo*. Both embryonic and regenerative *de novo* axis patterning is regulated by the head organizer Wnt/β-catenin signaling pathway. While multiple studies have investigated the molecular networks underlying the axis patterning (Bode 2011; Gufler et al. 2018; Reddy et al. 2019; Vogg et al. 2019; Moneer et al. 2021; Unni et al. 2021), there are few that study the epigenetic regulators of gene expression (Reddy et al. 2020). We identify that the H4K20me1 writer SETD8 is critical for Wnt-triggered regeneration and that both the enzyme and the target modification are regulated by the Wnt pathway. We also identify dual modes of transcriptional regulation by H4K20me1 which acts to activate transcription at promoters downstream to specific signaling pathways. Additionally, at the level of the whole genome, it excludes activation associated features allowing us to describe the presence of a repressive histone mark for the first time in Cnidaria.

RESULTS AND DISCUSSION

SETD8 governs the regeneration gene regulatory network in Hydra

In *Hydra*, upon amputation, the site of injury triggers a wound healing response and cascading molecular changes resulting in the morphological features of head regeneration as depicted in Fig. 1A. Upon a screen using pharmacological inhibitors (Fig. S1), we identified a significant role for SETD8 in head and foot regeneration (Fig. 1B). Inhibition of SETD8 led to a transient reduction of the target H4K20me1 histone modification corroborating the 12-hour delay observed in head regeneration (Fig. 1C, 1D, Fig. S2, S3). Foot regeneration is also severely impacted following the inhibition of SETD8 (Fig. 1E). We investigated the process of head regeneration in-depth to dissect the physiological role played by SETD8. The earliest step of

regeneration, wound healing, is morphologically not affected by the inhibition of SETD8 (Fig. 1F). To understand further molecular dynamics, we performed 3'mRNA sequencing on the regenerating tips of polyps treated with UNC0379 after different times of regeneration. This revealed various Wnt signaling components, apoptosis-related genes, Jun-kinase, and many insulin signaling components are dysregulated upon inhibition of SETD8 (Fig. 1G). Brachyury has been established as a bonafide head specification marker among the various genes that are part of the Wnt signaling pathway and are direct targets of the TCF7L2 transcription factor (Technau and Bode 1999). The formation of the head organizer by expression of various patterning transcription factors like *Brachyury* is dramatically altered when the histone methyltransferase is inhibited (Fig. 1H, Fig. S4). To decipher the mechanisms underlying transcriptional regulation by SETD8, we characterized the target histone modification, H4K20me1. This modification is present in *Hydra* and can be detected by available antibodies against the mammalian histone mark allowing us to study its dynamics. H4K20me1 is present all across the body column of *Hydra* but has a slightly higher localization near the hypostome and the foot of the polyp (Fig. 1I). These are two regions of the polyp enriched in differentiating cells and wherein most patterning events occur. The increased occurrence of this histone mark indicates a putative role in these processes.

SETD8 has been co-opted in the Wnt signaling network of Hydra

In *Hydra*, an active Wnt/β-catenin signaling pathway is required in the early stages of regeneration of both the head and the foot (Gufler et al. 2018). To understand the role SETD8 has in the patterning of the oro-aboral axis of *Hydra*, we activated the Wnt signaling pathway using GSK-3β inhibitor Alsterpaullone (ALP) (Fig. 2A) leading to the whole polyp turning into a head (Fig. 2B). SETD8 in *Hydra* is upregulated at both mRNA and protein levels upon

activating the Wnt signaling pathway (Fig. 2C, 2D). Further, it also physically interacts with β-catenin in the nucleus, as evident by its immunoprecipitation with active β-catenin in *Hydra* (Fig. 2E). Inhibition of SETD8 clearly rescues the ectopic tentacle phenotype indicating its role in regulating the Wnt signaling pathway (Fig. S5). Similar to other systems where the SETD8 methyltransferase has been studied in the context of organismal development (Huang et al., 2021; Li et al., 2011), in *Hydra*, where the axis patterning processes are continuously active, SETD8 interacts with the β-catenin in the nucleus and possibly has a role in transcriptional regulation. In a previous study, we identified a direct target of β-catenin named *Margin*, a homeodomain-containing transcription factor, (Reddy et al. 2019). Upon ectopic activation of the Wnt signaling pathway, we observed an enhanced occupancy of H4K20me1 (Fig. 2F) on the promoter regions of *Margin* and *Setd8.*

H4K20me1 is a global repression associated histone modification

To elucidate the role of this unique modification in the regeneration of *Hydra*, we performed ChIP-sequencing for H4K20me1 in Wnt-activated polyps (Fig. 3) and regenerating tips at five time points (Fig. 4). The occupancy of H4K20me1 was plotted on gene bodies which are regions comprised of the coding sequence (CDS) and 2 Kb of genomic DNA on either ends of the CDS (Fig. 3A, Fig. 3B, Fig. 4A). Using the gene ontology (GO) terms that are associated with important physiological processes, we plotted the relative enrichment of genes in the four k-means clusters. While many signaling pathway genes displayed an occupancy of the H4K20me1 mark around the gene bodies, the Hippo signaling pathway had the least number of gene bodies with this modification (Fig. 3C, Fig. 4B). Since the role of H4K20me1 in transcriptional regulation is ambiguous or context-dependent, to understand the same we used the publicly available datasets on regenerating *Hydra* and correlated our data with those. We

also identified the regulation of transcription by this histone mark in the context of regeneration. We observed that at all time points, the occupancy of H4K20me1 is negatively correlated with that of the activation associated histone marks such as H3K27ac, H3K9ac, H3K4me2, and H3K4me3 (Fig. 3D, Fig. 4C). Investigation of the global occupancy of H4K20me1 upon ectopic activation of the Wnt signaling pathway revealed that H4K20me1 excludes sites occupied by the transcription activation associated histone marks studied in a previous report (Reddy et al. 2020). During regeneration, the H4K20me1 occupancy does not drastically change across time points in a global manner (Fig. 4A). This occupancy is differentially regulated on many genes associated with the different cellular and physiological processes. The readout of active transcription is the resulting gene expression, and this is mediated by the opening of the chromatin. The available Assay for Transposase-Accessible Chromatin using sequencing (ATAC-seq) data allows us to investigate the chromatin accessibility during regeneration and the RNA sequencing data is the final readout for gene regulation. In addition to the histone modifications, the occupancy of H4K20me1 is also negatively correlated with the opening of the chromatin and successful transcription (Fig. 4D–F). The occurrence of H4K20me1 extends downstream of the promoters and into gene bodies indicating an association with transcriptional elongation. H4K20me1 occupancy is known to be associated with the elongation of rapidly transcribing genes in mammalian cells (Vakoc et al. 2006; Veloso et al. 2014). Contrastingly, in senescent cells and neurons, H4K20me1 occupancy seems to prevent elongation (Wang et al. 2015; Tanaka et al. 2017). Thus, the effect of H4K20me1 occupancy on transcription appears to be highly context specific and regulated in a cell type specific and location specific manner.

We have identified the molecular mechanism of action of SETD8 during the head organizer function via the Wnt pathway. Activation of the Wnt signaling pathway leads to the interaction

of SETD8 and nuclear translocated β-catenin at the TCF binding regions of the genome and culminates in transcriptional activation in *Hydra*, as seen for both the validated targets *Margin* and *Setd8* (Fig. 5A). While SETD8 is conserved in flies and humans, the enzyme and the target H4K20me1 have distinct roles in the eye development of *Drosophila* (Crain et al. 2022). The transient decrease in the target mark in *Hydra* and the marked phenotype observed also point towards such a role in the Cnidarian. The role of SETD8 and H4K20me1 in transcriptional regulation is not well established. In zebrafish and *Drosophila*, H4K20me1 is positively correlated with transcription in a few developmental contexts involving the Wnt signaling pathway (Li et al. 2011; Huang et al. 2021). This has also been observed in global high-resolution profiling done in human T cells (Barski et al. 2007). We observe this positive correlation with transcriptional regulation in the Wnt/β-catenin signaling network in *Hydra* and a reduction specifically in the Wnt-mediated differentiation potential of the *Hydra* cells for regeneration of head and foot. A contrasting role for this modification has also been identified using *in vitro* studies involving the L3MBT1 protein which is critical of transcriptional repression (Kalakonda et al. 2008). During the progression of cell cycle stages, the SETD8 protein is necessary for maintaining chromatin condensation and acts via the PCNA axis (Abbas et al. 2010). This is important to maintain the repressed state of transcription to allow mitosis to move forward and is additionally facilitated by the reduction in the levels of the demethylase PHF8 in the prophase of cell division (Karachentsev et al. 2005; Liu et al. 2010). However, in the context of *Hydra* head regeneration, where there is a necessity for various signaling pathways to interact with each other, the trend indicates that H4K20me1 is negatively correlated with the transcription of genes. The regeneration process involves various cellular processes, one of which is the reduced cellular proliferation and increased trans-differentiation during the morphallactic regeneration of *Hydra*. Therefore, it is possible that the H4K20me1

mark, at a global scale, shows an association with reduced transcription, similar to its role in cell cycle regulation.

Therefore, the dual mode of action of this mark is more specific than we previously were able to decipher. The dual nature of this histone mark has been recently also described in studies conducted in cancer cells where the enzyme SETD8 is necessary for recruiting the transcription activation machinery while the mark itself is a prerequisite for the subsequent di- and tri-methylation and compaction of the chromatin (Kapoor-Vazirani and Vertino 2014). The specificity is at TF binding sites, and a deeper investigation of the TCF binding sites displayed distinct modes of H4K20me1 occupancy at these motifs in response to Wnt signaling activation. We have, therefore, identified a putative repressive mark in *Hydra*, H4K20me1. The typically pericentric H4K20me2 and me3 marks appear to have a transcriptional regulatory role in this early eumetazoan which evolved to be restricted only to pericentromeric heterochromatinization in higher animals. The unique physiology of *Hydra* enables studying and visualizing the different modes of action for the histone post-translational modification, H4K20me1, and understanding its dual nature in transcriptional regulation (Fig. 5B).

MATERIALS AND METHODS

Hydra culture

A clonal culture of *Hydra vulgaris* Ind-Pune was maintained at 18°C in Hydra medium (HM) (100 µM KCl, 100 µM $MgSO_4.7H_2O$, 1 mM $CaCl_2.2H_2O$, 1 mM Tris (pH-8.0), 1 mM NaCl) using standard protocols described previously (Horibata et al. 2004). *Hydra* polyps were fed with freshly hatched *Artemia nauplii* larvae daily and cleaned 6–8 h post-feeding.

Inhibitor treatments

The details of the inhibitors used, and the treatment regime as shown in Fig. 1B are provided in the Supplementary Information.

Regeneration assays

Hydra polyps were treated with target inhibitors for 8 h and amputated. Following amputation, the inhibitor treatment was continued till the target time points and assayed for head and foot regeneration. The detailed methods are given in the Supplementary Information.

Whole-mount in situ hybridization on regenerating tips

Hydra polyps were treated with target inhibitors for 8 h and decapitated. Following decapitation, the inhibitor treatment was continued till the target time points of 1 hour post amputation (hpa), 2 hpa, 4 hpa, and 8 hpa. The polyps were then processed for whole mount *in situ* hybridization as described previously (Reddy et al., 2020) and the detailed method is given in the Supplementary Information.

RNA sequencing

The polyps were decapitated, and the regenerating tips were collected at 0 hpa, 3 hpa, 8 hpa, 12 hpa, and 24 hpa. RNA was isolated using Trizol and used to perform 3’ mRNA sequencing

using the QuantSeq 3' mRNA-Seq Library Prep Kit FWD for Illumina (Lexogen, Austria) according to the manufacturer's instructions.

Chromatin immunoprecipitation (ChIP)

Two thousand *Hydra* polyps per time point were decapitated, and the regenerating tips were collected at 0 hpa, 3 hpa, 8 hpa, 12 hpa, and 24 hpa to perform ChIP. Two thousand *Hydra* polyps per treatment condition were used for ALP treatment and fixed for ChIP. ChIP was performed as described previously (Reddy et al., 2020) and the detailed method is given in the Supplementary Information.

ChIP-Seq library preparation and sequencing

A total of 1 ng of purified ChIP-ed DNA for each sample was used for library preparation using Ultra II DNA kit (NEB) as per the manufacturer's instructions. Library concentration was determined using Qubit (Thermo Scientific, USA), and average fragment size was estimated using DNA HS assay on Bioanalyzer 2100 (Agilent Technologies, USA) before pooling libraries at equimolar ratio. 1.5 pM of the denatured libraries were used as an input to obtain sequencing reads using Nextseq 550 (Illumina) at IISER Pune. The detailed protocol is provided in the Supplementary Information.

ChIP-Seq Analysis

The quality of the fastq files was checked using FastQC (Andrews 2010), and the quality control (QC) data was consolidated using MultiQC (Ewels et al. 2016). The fastq files were aligned to the latest assembly of the *Hydra* genome using the bowtie2 aligner (Langmead and Salzberg 2012). The sam files were converted to bam files and indexed using samtools (Li et al. 2009). The sorted bam files were used to generate bigwig files using deeptools (Ramirez et al. 2016). The bam files for the H4K20me1 ChIP samples were normalized against the

respective input bam file for each timepoint. The combinatorial occupancy matrices were generated using the computeMatrix tool and the plots were generated using the plotHeatmap tool in the deeptools package. The detailed code is provided in the Supplementary Information.

Further detailed methods are provided in the Supplementary Information and the raw data will be provided upon request to the reviewers.

ACKNOWLEDGEMENTS

We thank all Galande Lab members and especially Ankita Sharma for the discussions. We thank Ankita Sharma for help with the sequencing runs. We thank Amarendranath Soory for the discussions and feedback. The work was supported by internal grants from Indian Institute of Science Education and Research, Pune and Shiv Nadar University (Delhi, NCR) to SG. AG was supported by fellowship from the CSIR. SS is supported by fellowship from the CSIR.

Author contributions

AG and SG conceived the project and wrote the manuscript. AG, MP, and SS performed experiments. AG performed the ChIP-Seq, ATA-seq and transcriptome analyses. AG and SG interpreted the data. SG supervised the project. All authors read and approved the final manuscript.

REFERENCES

Abbas T, Shibata E, Park J, Jha S, Karnani N, Dutta A. 2010. CRL4(Cdt2) regulates cell proliferation and histone gene expression by targeting PR-Set7/Set8 for degradation. *Mol Cell* **40**: 9-21.

Andrews S. 2010. FastQC: a quality control tool for high throughput sequence data. Babraham Bioinformatics, Babraham Institute, Cambridge, United Kingdom.

Barski A, Cuddapah S, Cui K, Roh TY, Schones DE, Wang Z, Wei G, Chepelev I, Zhao K. 2007. High-resolution profiling of histone methylations in the human genome. *Cell* **129**: 823-837.

Bode H. 2011. Axis formation in hydra. *Annu Rev Genet* **45**: 105-117.

Choi DK, Kim YK, Park SW, Lee H, Lee S, Kim SA, Kim SJ, Lee J, Kim W, Min SH et al. 2020. The histone lysine methyltransferase SETD8 regulates angiogenesis through HES-1 in human umbilical vein endothelial cells. *Sci Rep* **10**: 12089.

Crain AT, Klusza S, Armstrong RL, Santa Rosa P, Temple BRS, Strahl BD, McKay DJ, Matera AG, Duronio RJ. 2022. Distinct developmental phenotypes result from mutation of Set8/KMT5A and histone H4 lysine 20 in Drosophila melanogaster. *Genetics*.

DeLange RJ, Fambrough DM, Smith EL, Bonner J. 1969. Calf and pea histone IV. II. The complete amino acid sequence of calf thymus histone IV; presence of epsilon-N-acetyllysine. *J Biol Chem* **244**: 319-334.

Deribe YL, Pawson T, Dikic I. 2010. Post-translational modifications in signal integration. *Nat Struct Mol Biol* **17**: 666-672.

Ewels P, Magnusson M, Lundin S, Kaller M. 2016. MultiQC: summarize analysis results for multiple tools and samples in a single report. *Bioinformatics* **32**: 3047-3048.

Gufler S, Artes B, Bielen H, Krainer I, Eder MK, Falschlunger J, Bollmann A, Ostermann T, Valovka T, Hartl M et al. 2018. beta-Catenin acts in a position-independent regeneration response in the simple eumetazoan Hydra. *Dev Biol* **433**: 310-323.

Horibata Y, Sakaguchi K, Okino N, Iida H, Inagaki M, Fujisawa T, Hama Y, Ito M. 2004. Unique catabolic pathway of glycosphingolipids in a hydrozoan, Hydra magnipapillata, involving endoglycoceramidase. *J Biol Chem* **279**: 33379-33389.

Huang J, Gujar MR, Deng Q, S YC, Li S, Tan P, Sung WK, Wang H. 2021. Histone lysine methyltransferase Pr-set7/SETD8 promotes neural stem cell reactivation. *EMBO Rep* **22**: e50994.

Kalakonda N, Fischle W, Boccuni P, Gurvich N, Hoya-Arias R, Zhao X, Miyata Y, Macgrogan D, Zhang J, Sims JK et al. 2008. Histone H4 lysine 20 monomethylation promotes transcriptional repression by L3MBTL1. *Oncogene* **27**: 4293-4304.

Kapoor-Vazirani P, Vertino PM. 2014. A dual role for the histone methyltransferase PR-SET7/SETD8 and histone H4 lysine 20 monomethylation in the local regulation of RNA polymerase II pausing. *J Biol Chem* **289**: 7425-7437.

Karachentsev D, Sarma K, Reinberg D, Steward R. 2005. PR-Set7-dependent methylation of histone H4 Lys 20 functions in repression of gene expression and is essential for mitosis. *Genes Dev* **19**: 431-435.

Ke X, Xing B, Yu B, Yu X, Majnik A, Cohen S, Lane R, Joss-Moore L. 2014. IUGR disrupts the PPARgamma-Setd8-H4K20me(1) and Wnt signaling pathways in the juvenile rat hippocampus. *Int J Dev Neurosci* **38**: 59-67.

Langmead B, Salzberg SL. 2012. Fast gapped-read alignment with Bowtie 2. *Nat Methods* **9**: 357-359.

Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, Marth G, Abecasis G, Durbin R, Genome Project Data Processing S. 2009. The Sequence Alignment/Map format and SAMtools. *Bioinformatics* **25**: 2078-2079.

Li Z, Nie F, Wang S, Li L. 2011. Histone H4 Lys 20 monomethylation by histone methylase SET8 mediates Wnt target gene activation. *Proc Natl Acad Sci U S A* **108**: 3116-3123.

Liu W, Tanasa B, Tyurina OV, Zhou TY, Gassmann R, Liu WT, Ohgi KA, Benner C, Garcia-Bassets I, Aggarwal AK et al. 2010. PHF8 mediates histone H4 lysine 20 demethylation events involved in cell cycle progression. *Nature* **466**: 508-512.

Lv X, Han Z, Chen H, Yang B, Yang X, Xia Y, Pan C, Fu L, Zhang S, Han H et al. 2016. A positive role for polycomb in transcriptional regulation via H4K20me1. *Cell Res* **26**: 529-542.

Moneer J, Siebert S, Krebs S, Cazet J, Prexl A, Pan Q, Juliano C, Bottger A. 2021. Differential gene regulation in DAPT-treated Hydra reveals candidate direct Notch signaling targets. *J Cell Sci* **134**.

Myers JA, Couch T, Murphy Z, Malik J, Getman M, Steiner LA. 2020. The histone methyltransferase Setd8 alters the chromatin landscape and regulates the expression of key transcription factors during erythroid differentiation. *Epigenetics Chromatin* **13**: 16.

Nishioka K, Rice JC, Sarma K, Erdjument-Bromage H, Werner J, Wang Y, Chuikov S, Valenzuela P, Tempst P, Steward R et al. 2002. PR-Set7 is a nucleosome-specific methyltransferase that modifies lysine 20 of histone H4 and is associated with silent chromatin. *Mol Cell* **9**: 1201-1213.

Oda H, Okamoto I, Murphy N, Chu J, Price SM, Shen MM, Torres-Padilla ME, Heard E, Reinberg D. 2009. Monomethylation of histone H4-lysine 20 is involved in chromosome structure and stability and is essential for mouse development. *Mol Cell Biol* **29**: 2278-2295.

Ramirez F, Ryan DP, Gruning B, Bhardwaj V, Kilpert F, Richter AS, Heyne S, Dundar F, Manke T. 2016. deepTools2: a next generation web server for deep-sequencing data analysis. *Nucleic Acids Res* **44**: W160-165.

Reddy PC, Gungi A, Ubhe S, Galande S. 2020. Epigenomic landscape of enhancer elements during Hydra head organizer formation. *Epigenetics Chromatin* **13**: 43.

Reddy PC, Gungi A, Ubhe S, Pradhan SJ, Kolte A, Galande S. 2019. Molecular signature of an ancient organizer regulated by Wnt/beta-catenin signaling during primary body axis patterning in Hydra. *Commun Biol* **2**.

Schotta G, Lachner M, Sarma K, Ebert A, Sengupta R, Reuter G, Reinberg D, Jenuwein T. 2004. A silencing pathway to induce H3-K9 and H4-K20 trimethylation at constitutive heterochromatin. *Genes Dev* **18**: 1251-1262.

Schotta G, Sengupta R, Kubicek S, Malin S, Kauer M, Callen E, Celeste A, Pagani M, Opravil S, De La Rosa-Velazquez IA et al. 2008. A chromatin-wide transition to H4K20 monomethylation impairs genome integrity and programmed DNA rearrangements in the mouse. *Genes Dev* **22**: 2048-2061.

Shoaib M, Chen Q, Shi X, Nair N, Prasanna C, Yang R, Walter D, Frederiksen KS, Einarsson H, Svensson JP et al. 2021. Histone H4 lysine 20 mono-methylation directly facilitates chromatin openness and promotes transcription of housekeeping genes. *Nat Commun* **12**: 4800.

Tanaka H, Takebayashi SI, Sakamoto A, Igata T, Nakatsu Y, Saitoh N, Hino S, Nakao M. 2017. The SETD8/PR-Set7 Methyltransferase Functions as a Barrier to Prevent Senescence-Associated Metabolic Remodeling. *Cell Rep* **18**: 2148-2161.

Technau U, Bode HR. 1999. HyBra1, a Brachyury homologue, acts during head formation in Hydra. *Development* **126**: 999-1010.

Tjalsma SJD, Hori M, Sato Y, Bousard A, Ohi A, Raposo AC, Roensch J, Le Saux A, Nogami J, Maehara K et al. 2021. H4K20me1 and H3K27me3 are concurrently loaded onto the inactive X chromosome but dispensable for inducing gene silencing. *EMBO Rep* **22**: e51989.

Unni M, Reddy PC, Pal M, Sagi I, Galande S. 2021. Identification of Components of the Hippo Pathway in Hydra and Potential Role of YAP in Cell Division and Differentiation. *Front Genet* **12**: 676182.

Vakoc CR, Sachdeva MM, Wang H, Blobel GA. 2006. Profile of histone lysine methylation across transcribed mammalian chromatin. *Mol Cell Biol* **26**: 9185-9195.

Veloso A, Kirkconnell KS, Magnuson B, Biewen B, Paulsen MT, Wilson TE, Ljungman M. 2014. Rate of elongation by RNA polymerase II is associated with specific gene features and epigenetic modifications. *Genome Res* **24**: 896-905.

Vogg MC, Beccari L, Iglesias Olle L, Rampon C, Vriz S, Perruchoud C, Wenger Y, Galliot B. 2019. An evolutionarily-conserved Wnt3/beta-catenin/Sp5 feedback loop restricts head organizer activity in Hydra. *Nat Commun* **10**: 312.

Wang J, Telese F, Tan Y, Li W, Jin C, He X, Basnet H, Ma Q, Merkurjev D, Zhu X et al. 2015. LSD1n is an H4K20 demethylase regulating memory formation via transcriptional elongation control. *Nat Neurosci* **18**: 1256-1264.

Yu Y, Liu L, Li X, Hu X, Song H. 2019. The histone H4K20 methyltransferase PR-Set7 fine-tunes the transcriptional activation of Wingless signaling in Drosophila. *J Genet Genomics* **46**: 57-59.

FIGURES

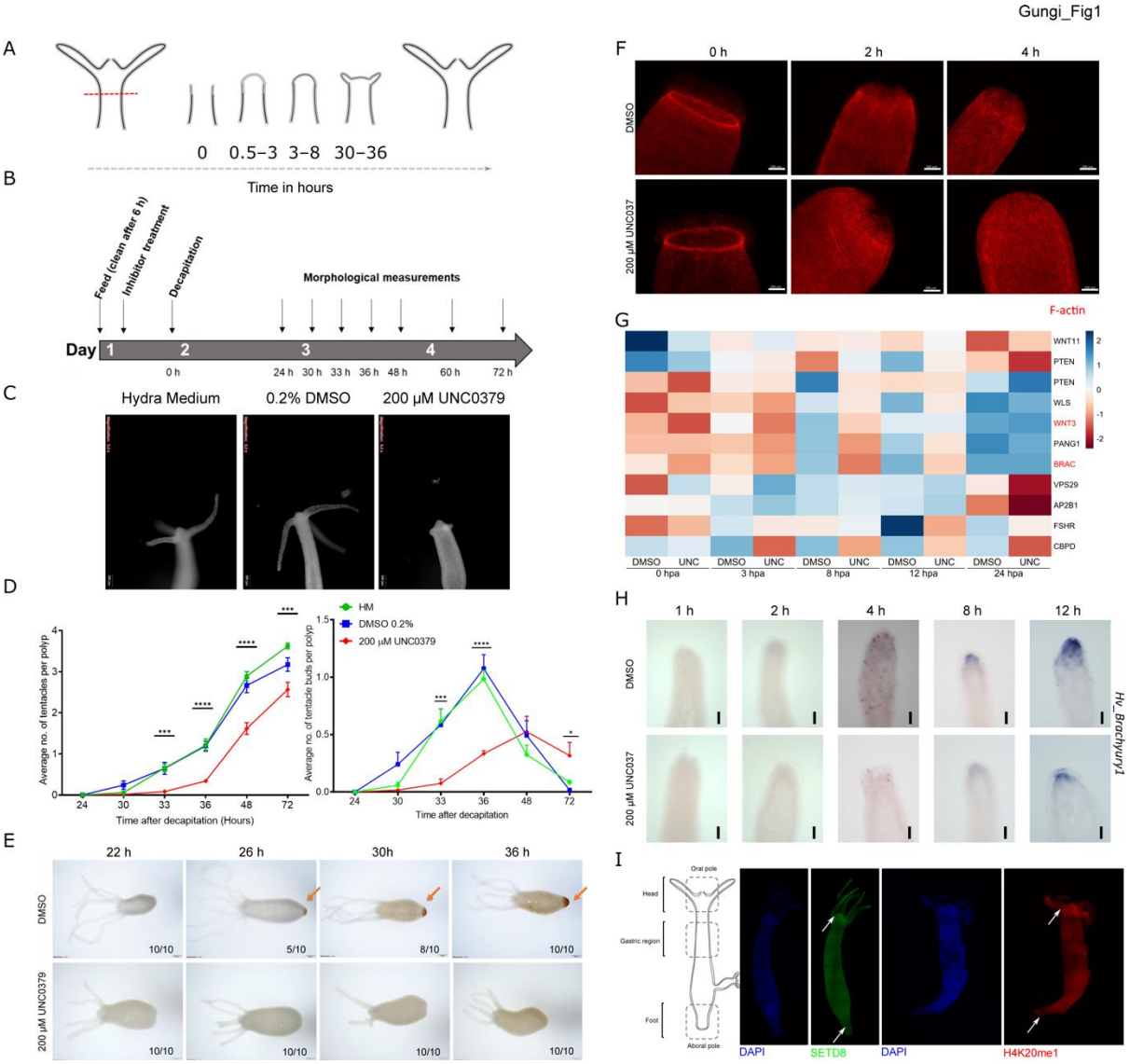

Fig. 1: SETD8 activity is necessary for regeneration in Hydra. A) Kinetics of gross morphological changes during Hydra head regeneration. B) Regime for the chemical inhibitor treatment and head regeneration assay. C) Polyps decapitated and fixed at 72 hours post amputation (hpa) with and without treatment with the SETD8 inhibitor, UNC0379. D) The graph depicts the average number of tentacles (left) and the number of tentacle buds per polyp (right) at each time point post decapitation during the course of regeneration. (N = 5, n = 25, *- P <0.5, ***- P < 0.001, ****- P <0.0001). E) A regeneration time course for observing the process of foot regeneration following amputation. Polyps fixed at 22 h, 26 h, 30 h, and 36 hpa were subjected to peroxidase-staining assay using DAB resulting in a brown precipitate at the regenerated foot. Upon treatment with the inhibitor UNC0379, foot regeneration is severely impaired. Scale bar measures 200 µm. F) Wound healing during regeneration. The regenerating polyps at early time points are stained with phalloidin to observe the filamentous actin structures. The top row depicts control polyps, and the bottom row depicts the polyps treated with SETD8 inhibitor. Scale bar measures 200 µm. G) Differential expression of Wnt signaling associated genes during head regeneration upon inhibition of SETD8. The plot depicts the

expression in control and treated regenerating tips at each time point. Genes with a log2 fold change cut-off of ±0.58 and a P value of 0.01 have been depicted. H) Whole mount RNA in situ hybridization against the *Brachyury1* gene in regenerating polyps with and without inhibitor treatment. The blue stain depicts the expression pattern of the *Brachyury1* gene and shows that the inhibitor treatment impairs the expression of *Brachyury1*. Scale bar measures 100 μm. I) The schematic of the Hydra polyp on the left depicts the three different regions of the polyp. The right panels depict polyps stained with α-SETD8 and α-H4K20me1 antibodies in red and green, respectively. The nuclei have been stained with DAPI as a counterstain.

Gungi_Fig2

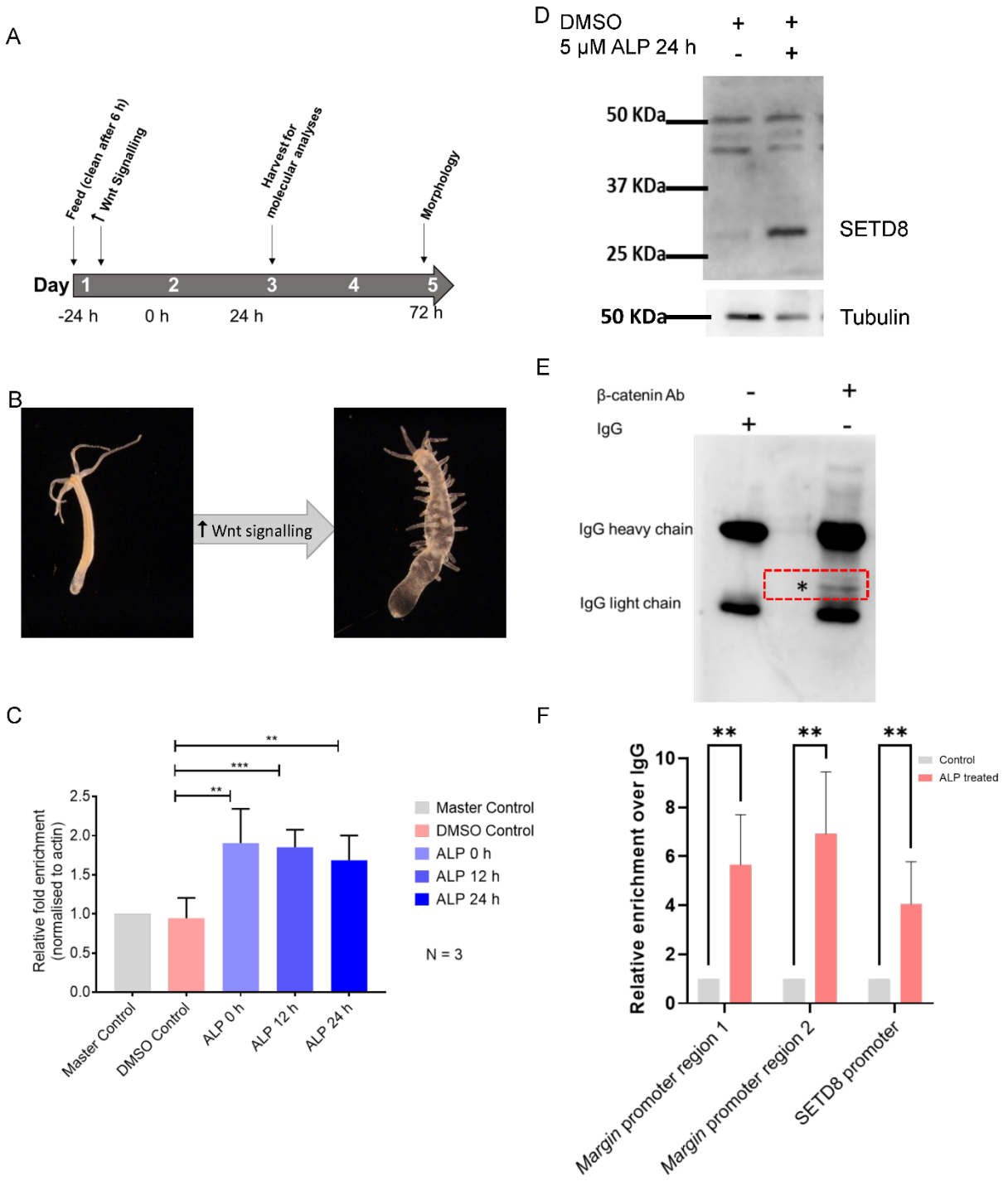

Fig. 2: SETD8 is regulated by the Wnt/β-catenin signaling pathway and also is part of the transcriptional machinery with β-catenin A) The treatment regime used to activate the Wnt signaling pathway using ALP and study the level of SETD8. B) Following activation of Wnt signaling in Hydra the entire polyp turns into a head, and ectopic tentacles are seen all over the body column. C) The levels of SETD8 mRNA were investigated using qRT-PCR and show that the expression of SETD8 is upregulated following ALP treatment. D) The level of SETD8 protein was studied using Western blot and this also shows the increased level upon activation

of the Wnt signaling pathway. The size of Hydra SETD8 is 32.2 KDa and the molecular weight ladder is indicated on the left of the gel. E) Western blot showing the co-immunoprecipitation of SETD8 upon pulldown using a α-βcatenin antibody. The red box depicts the Hydra SETD8 which is 32.2 KDa in size and the asterisks mark the SETD8 band in the lysates following immunoprecipitation. F) The occupancy of H4K20me1 was assayed using ChIP followed by q-PCR and plotted. The occupancy in the ALP treated samples has been normalized to that in the DMSO control samples. (**- P = 0.0073, Two-way ANOVA with Šídák's multiple comparisons test).

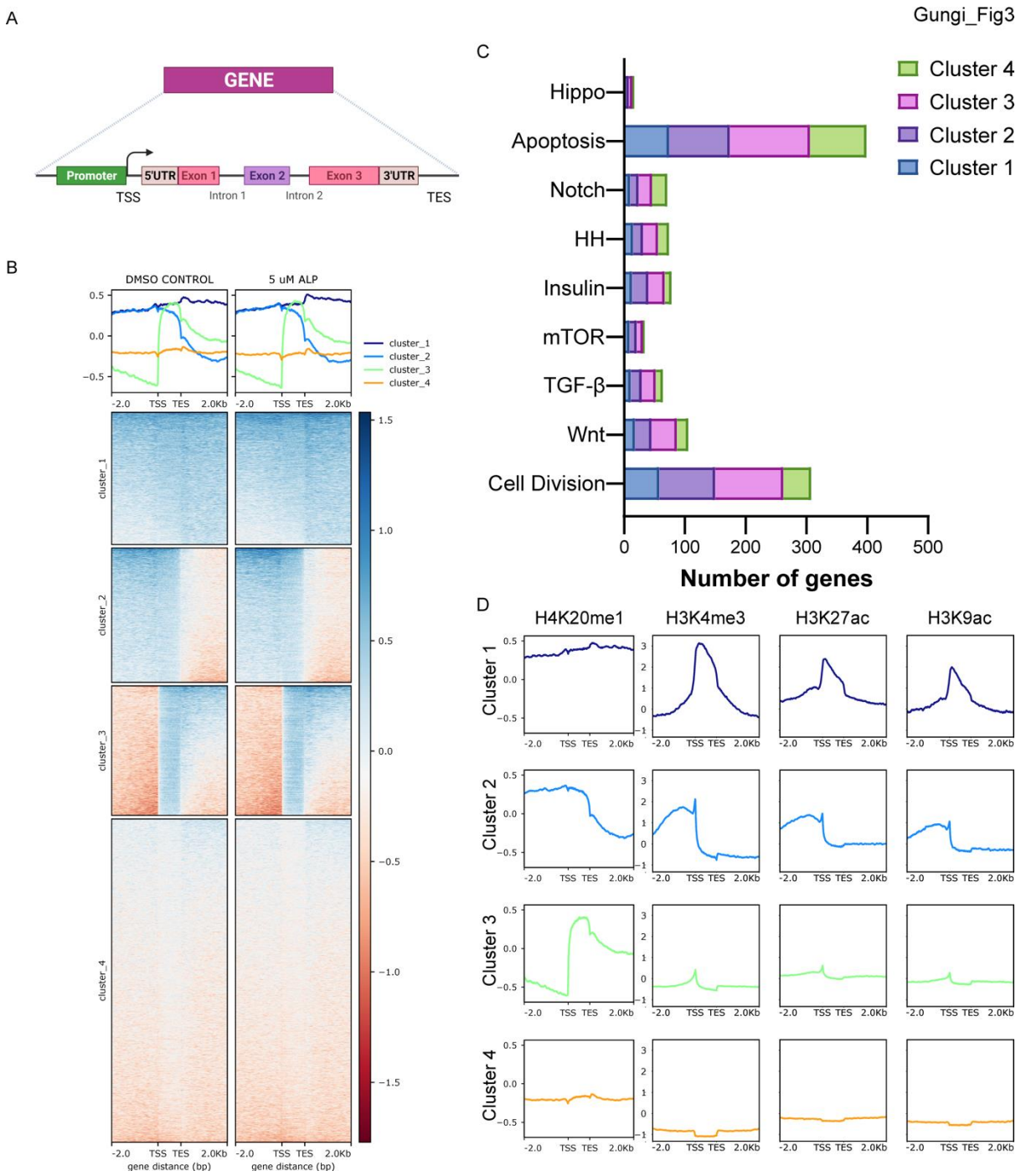

Fig. 3: H4K20me1 occupancy is modestly increased globally on the gene bodies in Hydra upon activation of the Wnt/β-catenin signaling pathway. A) A schematic of gene body that is used for H4K20me1 occupancy analyses. B) H4K20me1 ChIP signal on gene bodies of DMSO control and ALP treated (24h) polyps separated into 4 clusters. The occupancy on each gene body is shown in the clustered heatmaps (bottom panel). The profile plots (top panel) show the average ChIP signals of each cluster. C) Representation of pathways in the four clusters obtained based on the distribution of H4K20me1. D) The four clusters obtained based on the occupancy of H4K20me1 on gene bodies have been used to understand the occupancy of the three activation associated histone marks: H3K4me3, H3K27ac, and H3K9ac in control polyps.

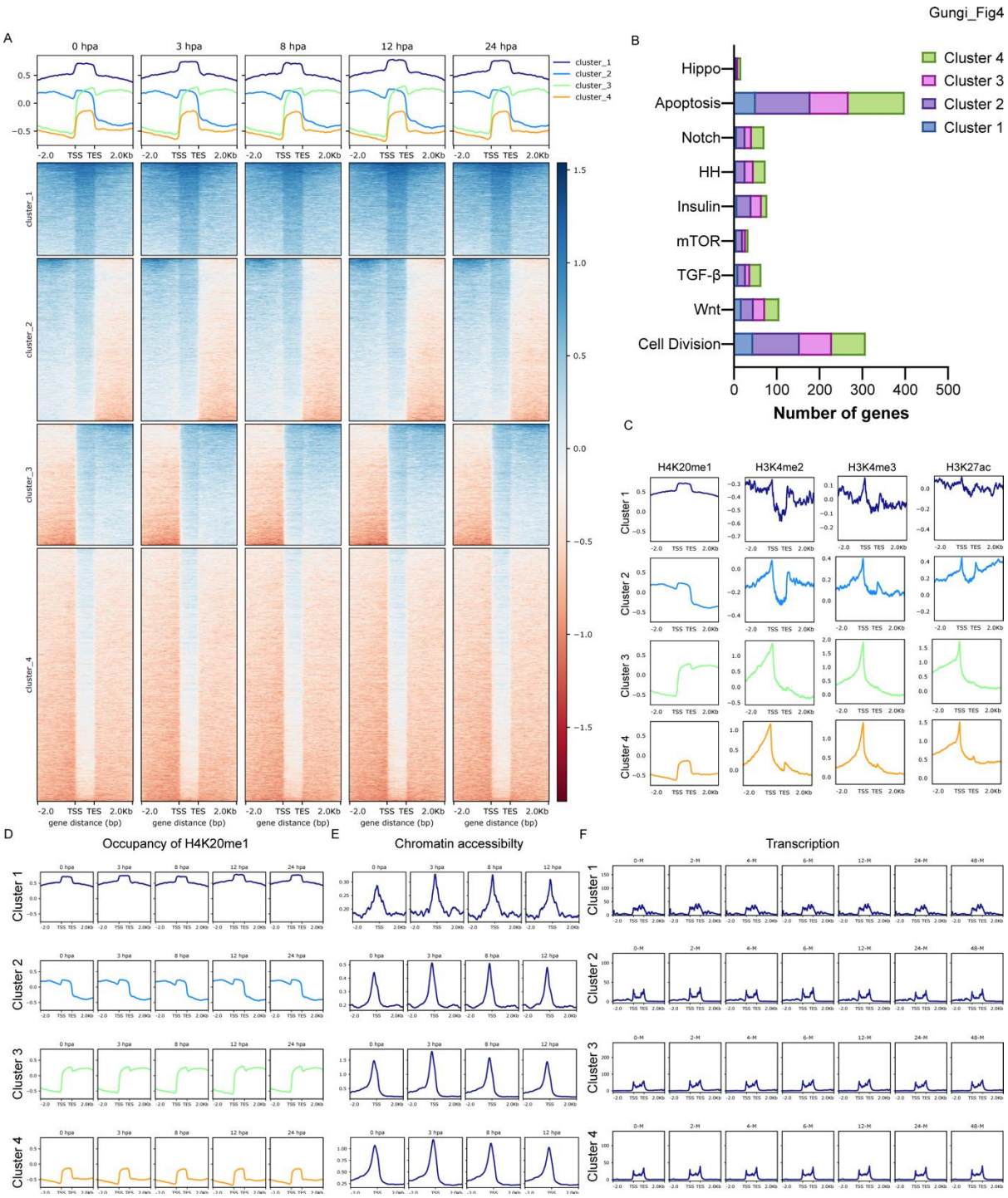

Fig. 4: H4K20me1 occupancy during head regeneration indicates a negative association with activation associated histone modifications, open chromatin, and active transcription. A) The occupancy of H4K20me1 on gene bodies across different regenerating time points following head amputation. B) Representation of pathways in the four clusters obtained based on the occupancy of H4K20me1 during regeneration. C) The four clusters obtained based on the occupancy of H4K20me1 on gene bodies were used to investigate the occupancy of the three activation associated histone marks: H3K4me2, H3K4me3, and H3K27ac at the 0 hpa timepoint. D–F) The four clusters have been used to interrogate the chromatin accessibility and the transcription of the respective genes. The ATAC-seq reads, and the RNA-seq reads during a regeneration time course have been plotted for the four clusters

separately and represent the openness of the genomic regions and the resultant transcription from those regions, respectively.

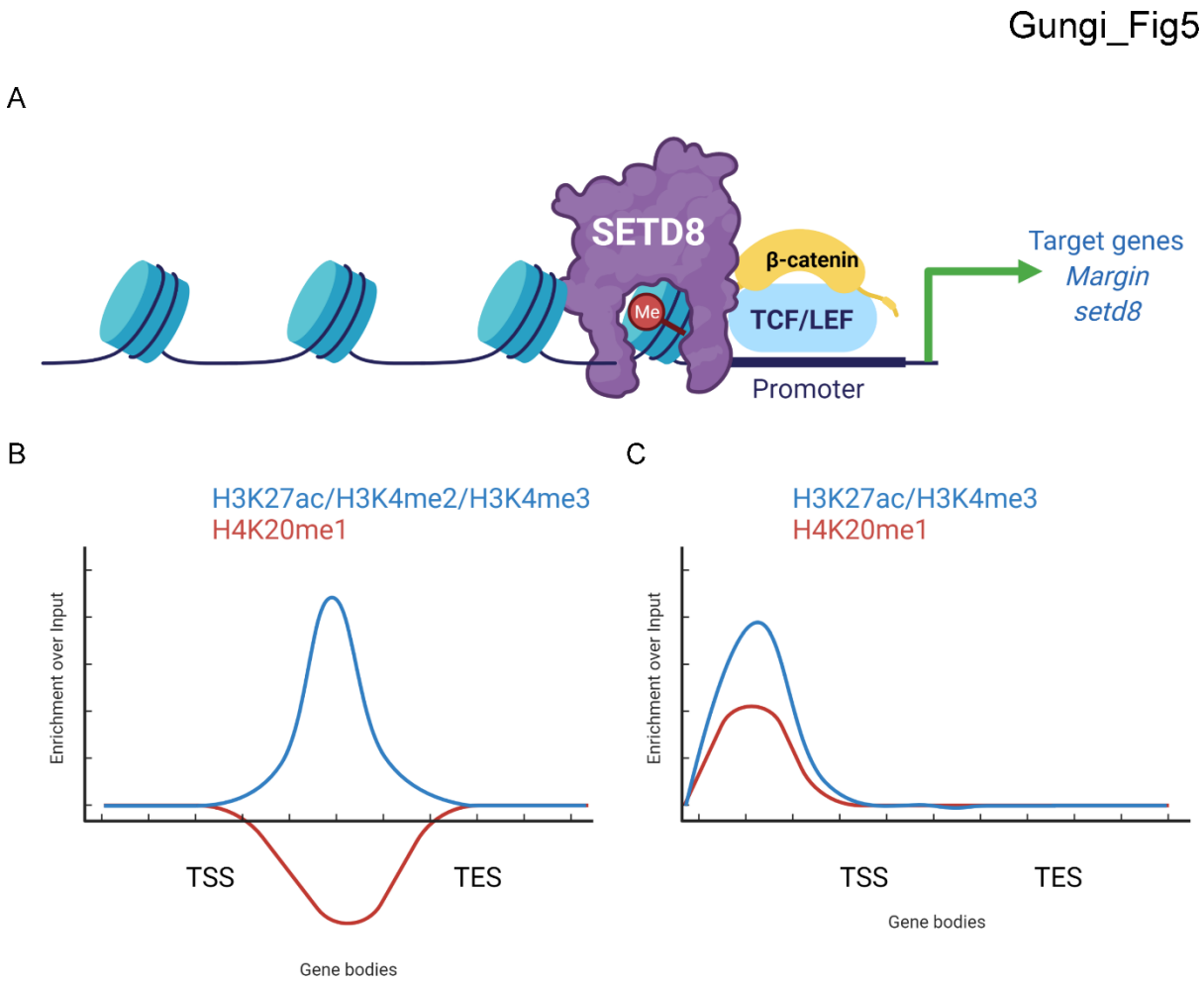

Fig. 5: Transcriptional regulation by SETD8 and H4K20me1 A) Molecular mechanism of action of SETD8 in *Hydra*. Upon activation of the Wnt signaling pathway, β-catenin is translocated to the nucleus where it interacts with the TCF7L2 transcription factor to activate the transcription of target genes. The effective activation of the signaling pathway is correlated with SETD8 interacting with β-catenin at the promoters of the target genes and depositing the H4K20me1 mark on the nucleosomes. B) Based on our global analysis of gene bodies, during regeneration and ectopic activation of Wnt signaling, the occupancy of H4K20me1 is negatively correlated with the occupancy of the activation associated histone marks. C) Contrastingly, based on our localized analysis on the promoters of highly upregulated genes in the context of Wnt signaling mediated axis patterning, H4K20me1 acts as an activator of gene expression and its occupancy coincides with that of the activation associated histone marks.