Epigenetic analyses of the planarian genome reveals conservation of bivalent promoters in animal stem cells.

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Abstract

Background

Planarian flatworms have an indefinite capacity to regenerate due to a population of pluripotent adult stem cells (neoblasts). Previous studies have suggested that they have features in common with both pluripotent mammalian embryonic stem cells and germ line stem cells. However, little is known about the importance of epigenetic regulation in these cells, which is likely to be crucial for neoblast biology and regeneration. We set out to develop analytical and experimental tools for planarians to allow the study of epigenetic marks in neoblasts and allow direct comparison of this model system with other animals.

Results

We developed an optimized ChIP-seq protocol for planarian neoblasts that allowed us to generate genome wide profiles for H3K4me1, H3K4me3 and H3K27me3. These were found to correlate as expected with genome wide expression profiles from analyses of planarian RNA-seq data. We found that many genes that are silent in neoblasts and then switch in post-mitotic progeny during differentiation have both H3K4me3 and H3K27me3 at promoter regions and are therefore bivalent. Further analysis suggested that bivalency is present at hundreds of loci in the pluripotent neoblast population.

Conclusions

We confirm that epigenetic regulation is key to neoblast biology and that bivalent promoters are not confined to vertebrate lineages, but may be a conserved feature of animal stem cells. Our work further establishes planarian neoblasts as a powerful model system for understanding the epigenetic regulation of pluripotency and regeneration.

Background

The potential use of stem cells in regenerative medicine has driven research into exploring the molecular mechanisms that govern stem cell potency, maintenance and differentiation. Despite this, we clearly still need to better understand the fundamental regulatory processes underpinning stem cell function, preferably using *in vivo* model systems. Highly regenerative planarians can be considered as a relatively simple stem cell study system that offers a large pool of adult stem cells called neoblasts (NBs). These cells are the driving force behind the almost limitless capacity to regenerate [1-3]. With a simple bilaterian anatomy, the ability to study gene function using RNAi [4] and a growing list of well-defined markers [2,5-12], planarians make for a powerful model system for studying stem cell processes. There is growing evidence for the deep conservation of molecular regulation in stem cells across metazoans, particularly from work with planarians [10,13-19]. This suggests research on planarian NBs can also lead to new insights relevant to mammals.

Independent studies from multiple groups have characterized the transcriptome of NBs, using both bulk approaches [10,13,15,20,21] and single cell sampling and sequencing approaches [22-24]. The characterization of the transcriptome of NBs has relied on approaches that facilitate separation of NBs from the rest of the cells in the body or the targeted removal of NBs. This is followed by differential expression analysis to identify genes that have enriched NB expression. The most widely used of these approaches has involved the development of protocols for Fluorescence Activated Cell Sorting (FACS) that sort dissociated cells on the basis of nuclear to cytoplasmic ratio [25-27]. This approach results in three distinct cell populations, an immediately radiosensitive population of >2N cells representing dividing cells in S/-phaseG2/M NBs (called the X1 compartment), a population of ≤2N cells that is partially radiosensitive and, over a longer time frame, containing G1 NBs and also transient undifferentiated post-mitotic progeny (called the X2 compartment), and a radio-resistant population of post-mitotic differentiated cells (called the Xins compartment) (see Figure 1A for summary). By sequencing these cell populations in bulk [9,10,13,21,24,28], performing single cell multiplex PCR analysis [24] or single cell sequencing [22,23] many groups have contributed to characterizing the NB transcriptome. These studies have identified novel NB markers, described gene expression heterogeneity in the NB population and found other cell type specific markers.

Another approach has been to compare expression profiles of whole animals with and without NBs, where NBs have been removed by ionizing radiation to kill all cycling cells [20] or by RNAi of a gene [15] to rapidly deplete NBs. These two approaches, combined with subsequent functional analyses, have allowed the development of more detailed models of the dynamics of NB proliferation, self-renewal and differentiation during regeneration and homeostasis [7,9,29-36]. One important feature that these studies highlight are chromatin-modifying factors involved in epigenetic regulation, the expression of which is enriched in planarian NBs [10,13,15].

A regulatory mechanism of key importance that mammalian embryonic stem cells (ESCs) and germ line stem cells (GSCs) share is histone modification-based bivalent control of promoters [37-42]. Bivalent promoters are characterized by the simultaneous presence of the repressive mark H3K27me3 and the activation mark H3K4me3 around transcriptional start sites (TSS) [37,38]. This bivalent promoter configuration is commonly seen on genes 'poised' for activation upon stem cell commitment and differentiation. In ESCs this is thought to allow pluripotency and the capacity to sensitively respond to developmental signals to achieve rapid differentiation when required. Bivalent promoters may achieve this by suppressing the formation of active RNA polymerase II complexes on one hand (hence 'poised'), and on the other, not allow other less easily reversible suppressive regulatory mechanisms, like DNA methylation, to silence genes [40,41]. Bivalent promoters have been described in mammals [37-39,42] and in zebrafish [43]. However, genome wide examples of promoter bivalency have so far not been found in invertebrates, for example no evidence for widespread bivalency has been observed in early *Drosophila* embryos [44]. These data support the interpretation that bivalency may be vertebrate specific, however more invertebrates clearly need to be studied and "it remains unclear how universal bivalent domains are across species" [43]. Given the role bivalency is thought to play in regulating pluripotency, planarian NBs are a logical place to look for bivalency in invertebrates.

To ask whether bivalency is present in NBs, we needed to combine transcriptomic and epigenetic analyses in the context of the genome. We first identified expressed loci on the genome and annotated transcribed regions using all available planarian RNA-seq data. We define the proportion of expression of every locus in X1, X2 and Xins FACS sorted cell populations allowing us to robustly identify genes silenced or expressed at very low levels in NB that are then actively transcribed during differentiation. We next

107 developed a robust ChIP-seq protocol for use with X1 NBs and demonstrate clear
108 correlation of conserved epigenetic marks and gene expression based on the
109 distribution of Histone 3 Lysine 4 mono-methylation (H3K4me1), tri-methylation
110 (H3K4me3) and Histone 3 Lysine 27 tri-methylation (H3K27me3) marks. This revealed
111 many bivalent promoters containing both H3K4me3 and H3K27me3 at similar levels,
112 particularly at loci that go on to greatly increase their expression in post-mitotic progeny
113 after asymmetric NB division. This provides strong evidence that this method of
114 epigenetic regulation may in fact be conserved in animal stem cells. Overall, our work
115 provides an essential annotation framework to study coding and non-coding loci in the
116 genome, establishes a robust approach for ChIP-seq in NBs of *S. mediterranea* and
117 reveals the potential for broad conservation of bivalent promoters in animal stem cells.
118

119 **Results**

120 **Establishing a genome wide annotation of transcribed loci in *S. mediterranea***

121 A growing number of different transcriptome studies have characterized the RNA-seq
122 profiles of NBs and other planarian cells by sequencing either FACS-sorted cell
123 populations (Figure 1A) or animals depleted of stem cells [9,10,13,15,20,21], but these
124 have not been integrated. We performed a comparison of the results of four published
125 transcriptomic studies, and found very poor overlap between those genes defined as
126 having enriched expression in NBs (Figure 1B, Additional File 1).

127 To address this, we used the rapidly growing collection of publicly available *S.*
128 *mediterranea* transcriptome sequence data to define a set of genome annotations on the
129 current assembly of the asexual strain to serve as a basis for comparing transcriptional
130 and epigenetic. This annotation then served as a basis for regulation across different
131 planarian research projects. Distinct from previous annotations of the genome [45,46],
132 this annotation includes all transcribed elements present across all available RNA-seq
133 datasets (Additional file 2). As we have integrated all available RNA-seq data, our
134 annotation should be particularly useful for describing potential non-coding RNAs and
135 protein coding genes expressed at low levels, as these may not have been discovered
136 by individual studies with limited numbers of reads and/or reliant on homology of protein
137 coding exons.

138 Our annotation is markedly different from the current available annotation [46] of the *S.*
139 *mediterranea* asexual genome sequence. We annotated more than 11,000 potential new

140 protein coding loci that are expressed at similar overall levels to previously annotated
141 protein coding genes that were also present in our annotation. These new annotations
142 were enriched for less well conserved proteins that may not be predicted by homology
143 based annotation. A total of 6,300 existing annotations were not present in our
144 expression driven annotation and further analysis of these MAKER specific genes shows
145 that they generally have no or very low potential expression within the 164 RNA-seq
146 libraries used for our annotations. (Additional File 2)
147 In summary, our annotation on the current planarian genome assembly shows regions of
148 active transcription detected by current RNA-seq and transcriptome data, defines many
149 more protein coding regions than currently available annotations and highlights a large
150 number of non-coding transcribed loci. Additionally, it facilitates a consistent comparison
151 specifically between *bona fide* transcriptional activity and the presence of post-
152 translational histone modifications (ChIP-seq), allowing the relationship between
153 epigenetic regulation and gene expression to be studied.
154
155 **A genome wide expression profile of FACS-sorted cell populations.**
156 The ability to use FACS is a powerful experimental tool for working with *S. mediterranea*,
157 providing convenient access to NBs and other cell populations. A growing number of
158 studies have produced RNA-seq data for the different FACS cell populations that can be
159 differentiated by nuclear to cytoplasmic ratio [9,10,13,21,24]. Given the discrepancies
160 we uncovered between different studies that have taken this approach (Figure 1B,
161 Additional File 1), we decided to reanalyze these datasets and newly available FACS
162 RNA-seq data in public databases. Hierarchical clustering of the normalized expression
163 values of each of these libraries revealed a rough congruence between different FACS
164 cell populations (Additional File 3), and revealed greater heterogeneity among the X1
165 samples than within the X2 and Xins samples. For example, some X1 samples clustered
166 with X2 samples (Additional File 3).
167 These inconsistencies are potentially biases introduced by variation in the underlying
168 biological or technical conditions. To mitigate against technical differences affecting
169 absolute expression values we transformed absolute expression values into proportional
170 expression values for each FACs compartment (Figure 1A, Additional File 4). For each
171 locus, we divided each of the three X1, X2, Xins expression values by the sum of
172 expression of all compartments for that locus (Additional File 4), obtaining a proportional

173 expression value for a total of 27,206 annotated loci that had at least 10 reads mapped
174 in at least one FACS RNA-seq library, 18,010 of these are likely to be protein coding.
175 The advantage of this transformation is that instead of using independent absolute
176 expression values of the various samples, we can use the relationship among the three
177 cell populations in each sample. Given similar FACS gating settings, results should be
178 more consistent between labs despite any technical variations that affect absolute
179 expression levels. Hierarchical clustering of these proportional values showed a
180 consistent clustering of FACS samples by cell type, with good separation between
181 clusters (Additional File 4). We then combined all available FACS RNA-seq data to
182 reach one set of proportional expression values for each locus in our annotation. This
183 gave us a new robust expression metric to compare every transcriptional unit in the
184 genome across FACS cell compartments.
185 In order to achieve a visual representation of the data, we simply used a line to
186 represent each gene, colored according to the proportion of its total expression in each
187 of the three cell compartments. This allowed us to create genome wide expression
188 spectra as an intuitive visualization and analysis tool. For example, we can sort all genes
189 according to the proportion of their expression in X1 (S/G2/M NBs) (Figure 2A), X2 (G1
190 NBs and stem cell progeny) (Figure 2B) and Xins (post-mitotic differentiated cells)
191 (Figure 2C).
192 We used this approach to define which genes were expressed in each FACs
193 compartment, dividing all genes expressed in FACS RNA-seq data into classes of
194 enrichment (Figure 2D, Additional File 5). We confirmed our analysis by checking for the
195 enrichment classes of genes previously described as being expressed in NBs (X1 and
196 X1/X2 classes), in stem cell progeny (X2 and X2/Xins classes) and in differentiated cells
197 (Xins class, Figure 3A-I, Additional File 5). In addition, we performed Gene
198 Ontology(GO) enrichment analyses. We also identified enriched expression for genes
199 not previously called as differentially expressed due to low levels of absolute expression
200 in individual studies. An example of this is *Smed-tert*, the gene encoding the protein
201 subunit of telomerase [47] that is amongst the most enriched X1 genes by proportional
202 expression but does not appear in previous individual studies because of low absolute
203 expression (Figure 3A). We also used the ESCAPE database of human pluripotency
204 factors [48] and found 233 best reciprocal hits to *S. mediterranea.* Looking at the

205 expression of these genes we found them to be enriched in the X1/X2 expression
206 category (Additional File 5).
207 Taken together our analyses, using all publicly available data, define the transcribed loci
208 whose expression can be detected in planarian FACS compartments. As well as
209 defining absolute levels of relative expression, we represent data by proportion of
210 expression in each FACS compartment. This allows us to generate expression spectra
211 highlighting loci expressed disproportionately in G2/M stem cells, loci expressed
212 throughout the cell cycle, loci with most of their expression in transient differentiating
213 post-mitotic cells and those expressed mainly in post-mitotic differentiated cells. As our
214 annotations and expression data are in the context of the genome assembly these data
215 can be integrated with ChIP-seq data.
216
217 **Expression spectra are supported by RNA-seq of RNAi phenotypes and single cell**
218 **sequencing analyses**
219 As an independent confirmation of our annotation and expression data we re-examined
220 previously published RNA-seq after RNAi datasets and single cell RNA-seq data. For a
221 selection of genes described as being required for stem cell progeny maintenance
222 (Additional File 6) we visualized the RNA-seq profiles in relation to the defined FACS
223 expression categories (Figure 2E) and observed that down-regulation of highly enriched
224 X2 category genes was the main feature of both S*med-mex3* and *Smed-zfp1* RNAi
225 datasets (Additional File 6). From this data, it is straightforward to conclude that both
226 *Smed-mex-3* and *Smed-zfp-1* have a collective effect on many genes that normally
227 switch on in NB progeny as they differentiate and leave the cell cycle, and this correlates
228 with the phenotypic effects of RNAi in both cases causing a depletion in stem cell
229 progeny as stem cell differentiation fails [9,24]. This approach to analyzing RNA-seq
230 data is useful for identifying patterns in the global effects of RNAi.
231 Recently, two planarian single cell transcriptomic studies have also been used to define
232 expression profiles of single stem cells and other cell types [22,23]. These have helped
233 to reveal heterogeneity of expression profiles in planarian stem cells and provide
234 persuasive evidence for the existence of cycling NB cells that might be committed to
235 particular lineages [23,24]. We re-mapped available single cell RNA-seq data [22,23] to
236 identify the the top one thousand genes ranked by expression for each cell type defined
237 by these two studies. We looked at the position of these genes along expression spectra

238 sorted by X1 proportion (Additional File 6). The single cell data analyzed in this way
239 follows patterns we would expect and independently validates our proportional
240 expression spectra. For different NB populations defined by single cell studies (sigma,
241 gamma, zeta and head X1) we saw enrichment of genes in the X1 and X1/X2 categories
242 (Additional File 6). Differentiated cell types were enriched for genes in the Xins category.
243 However, all differentiated cell-types, with the exception of the 'epidermis II' class [22],
244 have an enrichment of genes in the X2 category as these genes are amongst those with
245 highest absolute expression in all non-NB cell types, and thus appear amongst the top
246 1,000 expressed genes in single cell RNA-seq data.
247 Overall, our annotation and expression analysis is congruent and compatible with
248 independent data from RNAi coupled RNA-seq experiments and single cell sequencing
249 data, further validating the success of our approach.
250
251 **An optimized ChIP-seq protocol reveals H3K4me3 levels at TSSs in cycling cells**
252 **correlate with gene expression in NBs.**
253 We next wished to combine our new genome annotation with predicted transcriptional
254 start sites (TSSs) of expressed loci by cell compartment expression with NB derived
255 epigenetic data. Research into epigenetic mechanisms in planarians is still very much in
256 its infancy. Previous work characterized loss of function phenotypes of members of the
257 NuRD complex [32,33,49,50], COMPASS and COMPASS-like families [51,52] and
258 established a lack of endogenous DNA methylation in the *S. mediterranea* genome [32].
259 With respect to monitoring epigenetic marks, some of the effects of *mll1/2* and *set1*
260 RNAi on the H3K4me3 mark of active transcription have been previously reported [52]).
261 We noted that in this study, the total number of ChIP-seq reads from ~1 million X1
262 sorted planarian NBs were at relatively low numbers compared to those from *Drosophila*
263 S2 'carrier' cells, which were added at 10x excess to X1 NBs (Additional File 7). These
264 data suggested to us that ChIP-seq experiments with FACS sorted NBs might be very
265 technically challenging.
266 In order to begin to study epigenetic regulation of NBs, we first developed an optimized
267 protocol for ChIP-seq on FACS sorted X1 cells for H3K4me3 mark. Relatively high levels
268 of H3K4me3 have been shown to be broadly characteristic of active promoters [53,54].
269 We found we were able to generate 13-26 million high quality *S. mediterranea* uniquely
270 mapped reads using 150,000-200,000 X1 cells per immunoprecipitation, 5-7 times less

271 starting material compared to the a previous planarian ChIP-seq study [52]. We
272 therefore used *Drosophila* S2 cells to act as a spike-in control for normalization of any
273 technical replicate differences in immunoprecipitation across samples [55,56].
274 Sequencing X1 sorted cells, we observed high average H3K4me3 peaks around the
275 TSSs of genes categorized as X1 and X1/X2 enriched, indicative of high expression in
276 NBs (Figure 4A, Additional File 8). Conversely, we saw much less H3K4me3 at the
277 promoters of Xins enriched gene. These results validate our planarian ChIP-seq method
278 and confirm that our annotation is useful for studying global correlations between
279 epigenetic marks and gene expression in the context of the genome. We saw
280 intermediate levels of H3K4me3 in the X2 enriched compartment (Figure 4A, Additional
281 File 8), which includes both NBs and recent post-mitotic progeny. A finer grained look at
282 the X2 compartment revealed that genes with the highest proportion of X2 expression
283 had lower levels of H3K4me3 in X1 cells (Figure 4B, Additional File 8), indicative of
284 enriched expression in post mitotic progeny rather than cycling cells of the X1
285 compartment (see also Figure 3E). The presence of H3K4me3 at X2 enriched gene
286 promoters as a whole is, however, higher than that observed in genes enriched for
287 expression in the differentiated Xins cell compartment (Figure 4B). These observations
288 are broadly in agreement with previous findings from X1 cells [52] using the previously
289 available annotations [45,46].
290 A base by base correlation analysis of ChIP-seq signal across the promoter region to
291 proportional expression in the X1/X2/Xins FACS compartments shows a positive
292 correlation between X1 proportional expression and levels of H3K4me3 deposition from
293 near the TSS region to ~1kb downstream (Figure 4C). On the other hand, there is a
294 negative correlation between H3K4me3 deposition and the proportion of Xins expression
295 across the same region (Figure 4C). Thus, higher H3K4me3 ChIP-seq signal in X1 cells
296 tends to reflect higher gene expression in X1 cells and lower H3K4me3 signal reflects
297 lower expression in X1 cells and higher expression in the Xins compartment. We also
298 looked at a individual loci of genes known to be expressed in NB and found them all to
299 have relatively high levels of H3K4me3 and low levels of suppressive marks (Figure 4D).
300 Overall, the patterns we observe across the genome are consistent with what would be
301 expected with H3K4me3 being an activating mark. Additionally, it broadly validates our
302 annotation of transcribed loci, our assignment of proportional expression values for each

303 locus to FACS compartments and our method of ChIP-seq using relatively small
304 numbers of starting cells.
305
306 **Levels of the repressive histone marks H3K27me3 and H3K4me1 at TSSs also**
307 **correlate with gene expression in NBs.**
308 With an optimized ChIP-seq protocol, we decided to investigate two additional key
309 histone modifications, the repressive mark H3K27me3 important for the assessment of
310 bivalency [37,57] and H3K4me1 which has also recently been implicated as a repressive
311 mark at promoter regions, mediated by the MLL3/4 family of histone methyltransferases
312 [58].
313 We performed ChIP-seq on these two marks in X1 cells and observed ChIP-seq profiles
314 consistent with these marks being associated with repression of gene expression in
315 NBs. At loci enriched for X1 expression we observed low levels of H3K27me3 around
316 the TSS and higher signal for loci with enriched expression in the Xins FACS
317 compartment. (Figure 5A, Additional File 8). A positive correlation is observed around
318 the TSS and 1 kb downstream between the levels of H3K27me3 and expression in the
319 Xins compartment (figure 5B). This fairly broad domain of H3K27me3 is consistent with
320 previous studies in mammals [59,60] A negative correlation at the TSS is observed
321 between H3K27me3 signal and genes enriched for X1 expression (Figure 5B). Overall,
322 this pattern is the opposite to that observed for H3K4me3.
323 ChIP-seq to detect distribution of the H3K4me1 mark revealed a different pattern to that
324 of either H3K4me3 or H3K27me3. Rather than clear differences in the amount of
325 H3K4me1 signal between loci with different FACS expression profiles, we observed a
326 clear shift in the position of signal peaks (Figure 5C). Loci with a high proportion of
327 expression in the Xins FACS compartment have high levels of H3K4me1 close to the
328 TSS in X1 cells. Conversely, loci that are expressed in cycling cells (X1 and X1/X2
329 enriched) have peaks of H3K4me1 signal on average ~1kb downstream of the TSS.
330 Thus, the peak of H3K4me1 shifts away from the TSS for genes that are actively
331 expressed and is consistent with observations of a previous study looking at H3K4me1
332 levels at promoters in mammalian cells [58]. The Spearman correlation of H3K4me1
333 signal and FACS proportional expression confirms these observations, showing a
334 positive correlation close to the TSS for Xins enriched loci and a negative correlation for
335 X1 enriched loci (Figure 5D). The relationship between H3K4me1 and X1 enriched loci

336 is positive further downstream, at which position, we therefore conclude, this
337 modification does not broadly exert a repressive effect (Figure 5D). We noticed that for
338 X2 enriched genes H3K4me1 signal had two distinct peaks, one around the TSS and the
339 other downstream. This suggests two populations of loci, one with raised levels of
340 H3K4me1 near the TSS suggesting repression, and the another further downstream
341 suggesting an absence of repression involving H3K4me1 (Figure 5D). One clear
342 possibility is that the repressive peak near the TSS might be for genes that are off in
343 NBs and only switch in in post-mitotic progeny, while the other peak represents X2
344 enriched genes that are expressed in cycling NBs. We also checked individual loci of
345 genes known to be expressed in differentiated cells, and found they all had relatively
346 high levels of repressive marks at or near the predicted TSS and low levels of H3K4me3
347 (Figure 5 E). Conversely genes known to be expressed in NBs had low levels of
348 repressive marks (Figure 4D).
349 A common method of analyzing ChIP-seq data is to perform a cluster analysis on
350 coverage profiles to observe whether groups of similar profiles are enriched for a
351 biological function [61]. While this blind approach to analyzing ChIP-seq profiles can
352 sometimes yield interesting results when manually checking cluster members, it is often
353 the case that the broad biological interpretation of clusters is vague due to low-resolution
354 third party classifications such as gene ontology. Instead of a blind approach, here we
355 have used proportional expression to categorize loci into distinct groups to observe
356 broad trends in the ChIP-seq data. Taken together, our work demonstrates that the
357 dynamics between states of promoter histone methylation are distinct between loci
358 grouped by expression dynamics, and in agreement with previously studied roles of
359 these marks described in mammalian cells [53,54,57-60]. The congruity of our
360 annotation data, expression analysis and ChIP-seq datasets validates our framework for
361 studying epigenetic regulation in NBs. As well as the genome wide analyses presented
362 here, it will now be possible to look at the epigenetic regulation of individual planarians
363 genes or sets of genes of interest in different experimental and environmental conditions
364 using ChIPseq data.
365
366 **Evidence for the conservation of bivalent promoter regulation in pluripotent**
367 **animal stem cells**

368 Having validated our epigenetic analysis and demonstrated conservation of activating
369 and suppressive marks we next investigated whether promoter of bivalency could be a
370 regulatory mechanism in NBs. Bivalent promoters were originally observed at genomic
371 loci for genes that were not expressed or expressed at very low levels in mouse ESCs
372 [37], and were surprising because they contain both activating H3K4me3 and repressive
373 H3K27me3 marks. This state is associated with the presence of RNA polymerase in a
374 poised state and may allow rapid transcriptional responses to incoming signals to
375 differentiate, at which point histone marks at bivalent promoters resolve so one of the
376 two marks becomes dominant, resulting in activation or suppression of expression
377 [40,41]. Bivalent promoters have since been described in various stem cells of different
378 developmental origin and potency [39,42]. While they have been described outside of
379 mammals in zebrafish [43], they have not so far been found in any invertebrates,
380 suggesting they may be a novel epigenetic regulatory feature of vertebrates. Our ChIP-
381 seq data from FACS sorted cells makes it possible to detect potential bivalent promoters
382 in NBs if they are present in these cells. We reasoned loci that have relatively low
383 expression in the X1 fraction and are up-regulated during differentiation and highly
384 enriched in post-mitotic progeny (high X2 expression) may be good candidates for
385 regulation by bivalent promoters in NBs.
386 We analysed the ChIP-seq signal as a continuous dataset by transforming the coverage
387 profile into percent coverage by dividing the coverage at each base by the maximum
388 coverage in the entire dataset. For each FACS category, we took the top one thousand
389 most enriched loci and plotted the percent coverage profiles of H3K4me3, H3K4me1,
390 and H3K27me3 to observe potential bivalency across all these loci. For the top one
391 thousand X1 enriched loci, we see the expected profiles of a high H3K4me3 peak and
392 low H3K27me3 peak (Figure 6A). We observe the opposite pattern for the top one
393 thousand Xins enriched loci (Figure 6B). For the top one thousand X2 enriched loci,
394 which are enriched for expression in post-mitotic progeny, we see similar percent
395 coverage peaks for both H3K4me3 and H3K27me3 across these 1000 genes, consistent
396 with potential bivalency in NBs at many of these promoters (Figure 6C).
397 As an independent source of validation, we also extracted all genes that were
398 significantly down-regulated more than 2-fold after *Smed-mex3*(RNAi), which blocks the
399 production of post-mitotic progeny. The ChIP-seq profile of these genes in X1 cells
400 shows a similar profile to that of the top one thousand X2 enriched loci and is also

401 indicative of potential bivalency (Figure 6D). Genes expressed in the X2 compartment
402 may stay on as cells differentiate so that they have an X2/Xins expression profile, some
403 of these genes may also have bivalent promoters. Analysis of this gene sets also
404 showed suggested some of these loci may be bivalent in X1 NBs (Figure 6E)
405 One caveat of our analysis so far is the possibly that bivalent ChIP signals represent
406 underlying cell heterogeneity in the sampled X1 cell population [40]. While we know that
407 cycling NB have some heterogeneity in gene expression that can describe subclasses
408 with different lineage commitment [24], our focus on promoters of genes that are only
409 upregulated upon differentiation and not expressed in NBs makes it unlikely the patterns
410 we observe represent heterogenous epigenetic regulation in the NB population..
411 Similarly, given that our analysis identified bivalency across large numbers of promoters
412 it is also possible that our observation is the result of genes that have mostly one or
413 other mark in NBs leading to an average profile that appears bivalent profile when many
414 genes are looked at simultaneously. To rule this possibility out we looked at the
415 correlation (Pearson) between H3K4me3 and H3K27me3 and observed the distribution
416 of correlations for the top 500 ranked amongst X1, X2 and loci with reduced expression
417 in *Smed-mex3*(RNAi) (Figure 6F). For X2 and *Smed-mex3* RNAi category loci, we
418 observe a high density of well correlated H3K4me3 and H3K27me3 profiles indicating
419 similar paired signals for these marks across the TSS, and indeed closer inspection of
420 individual genes confirms this to be the case (Figure 7). For X1 enriched loci, we see a
421 less correlation, with many negatively correlated loci compared to the top X2 enriched
422 genes and *Smed-mex3* RNAi loci. This analysis suggests that many 100s of loci are in
423 fact bivalent with respect to H3K4me3 and H3K27me3 in planarian NBs.
424 Overall, our data demonstrate the presence of bivalency at promoters in NBs. This
425 suggests that this mechanism of gene regulation may be conserved amongst animals
426 rather than confined to vertebrates [37,62]. It seems likely that the need to have both
427 embryonic and, where appropriate, adult stem cells, capable of sensitive regulatory
428 decisions and responses to incoming signals may have arisen very near the origin of
429 multicellularity. Our work suggests that the evolution of bivalent promoters, arising
430 earlier than previously thought, may have been an important component of achieving
431 stem cell flexibility.
432
433 **Conclusion**

434 While there have been successful attempts in the model species *S. mediterranea* to
435 integrate transcriptome data from different sources to improve overall representation and
436 annotation [45,46,63-65], different FACS expression datasets from different experiments
437 and laboratories have not been integrated to improve the quality of gene expression
438 profiles across these cell compartments. Additionally, many previous approaches
439 quantifying gene expression have focused on using assembled transcriptomes without
440 the context of a genome assembly. This means that linking these RNA-seq based
441 expression datasets directly to epigenetic or transcription factor based regulation using
442 ChIP-seq is not possible. The goal of our work here was to address these deficits by
443 combining transcriptome and epigenetic approaches to describe the landscape of
444 epigenetic regulation at promoter regions in NBs in the context of expression level data.
445 Our analyses validate our annotation, trancriptome analysis and ChIP-seq protocol and
446 provide clear demonstration of the existence of bivalent promoters in cycling NBs. Our
447 analysis is particularly sensitive for detecting genes that switch on after NB
448 differentiation, due to the structure of the transcriptome and epigenetic datasets
449 available for analysis. Future work can now use planarians as a model for understanding
450 how this mode of regulation works, and the similarities and differences with vertebrates.
451 The discovery that bivalent promoters exist outside of vertebrates adds to the growing
452 body of evidence that suggests a deeper conservation of stem cell biology amongst
453 animals then previously appreciated. Previously, endogenous genome stability
454 mechanisms, splicing and post-transcriptional regulatory mechanisms have all been
455 shown to be important for NB function [17,28,66]. Additionally, a number of proteins
456 involved in epigenetically mediated gene regulation have also been shown to be
457 essential to maintain NB function [10,13,15]. Particularly the previously described cases
458 of MBNF/CELF mediated splicing regulation [17] and PIWI mediated genome stability
459 [19], these represent deeply conserved processes that likely mediated stem cell function
460 in an ancestral animal. Our work suggests that bivalent promoters represent yet another
461 major conserved mechanism and this regulatory process is not, as previously thought,
462 vertebrate specific. As well as demonstrating bivalency, our work, through establishment
463 of an annotation framework and a robust ChIP-seq protocol for NBs, will allow the use of
464 planarians as a model for epigenetic regulation of stem cell function. For example, the
465 accessibility of the NB population should allow identification of regulatory targets of

466 chromatin modifying enzymes responsible for pluripotency, self-renewal and
467 proliferation.
468
469 **Materials and methods**
470
471 **Data Sources for this study**
472 The NCBI Project accession number for ChIP-seq data produced in this study is
473 PRJNA338116. All accession numbers for previously published RNA and ChIP-seq data
474 used in the study are listed in Additional file 9.
475
476 **Flow cytometry**
477 A modified version of a planarian FACS protocol [27] was used. The modifications were:
478 a 35 µm mesh filter was used instead of 100 µm, staining with Hoechst and calcein was
479 performed simultaneously rather than sequentially and the centrifugation-wash step was
480 omitted. We used Hoechst 34580 instead of Hoechst 33342. A FACS Aria III machine
481 equipped with a violet laser was used for cell sorting. BD FACSDiva and FlowJo
482 software were used for analyses and setting cell population gates.
483
484 **ChIP-seq**
485 For each experimental replicate 600,000-700,000 planarian X1 cells (enough for Chip-
486 seq of 3 histone marks and an input control sample) were FACS-sorted (using 3-day
487 regenerates) in PBS and pelleted at 4 ºC. The pellet was re-suspended in nuclei
488 extraction buffer (0.5% NP40, 0.25% Triton X-100, 10mM Tris HCl pH 7.5, 3mM CaCl2,
489 0.25mM sucrose, 1mM DTT, 1/10th Phosphatase Cocktail Inhibitor 2 (Sigma Aldrich),
490 1/10th Phosphatase Cocktail Inhibitor 3 (Sigma Aldrich)). This was followed by
491 formaldehyde fixation, that was stopped with 2.5M glycine. The pellet was re-suspended
492 in SDS lysis buffer (1% SDS, 50mM Tris HCl pH 8, 10mM EDTA) and incubated on ice.
493 ChIP dilution buffer (0.1% SDS, 1.2mM EDTA, 16.7mM Tris HCl pH 8, 167mM NaCl,
494 1/1000th Phosphatase Cocktail Inhibitor 2, 1/1000th Phosphatase Cocktail Inhibitor 3,
495 1mM DTT) was added in a 2.3:1 ratio to the sample. Samples were sonicated and 1/10th
496 volume 10% Triton X-100 was added. Samples were pelleted at 4 ºC and the
497 supernatant kept for further processing. Test de-crosslinkling was performed on 1/8th

498 volume of the chromatin solution to verify the DNA fragment range following sonication
499 was 100-500 bp.
500 Protein A-covered Dynabeads (Thermofisher) were used for immunoprecipitation (IP).
501 The amount of reagent used was in a 1:2 ratio to the amount of chromatin used per IP.
502 The Dynabeads were first pre-blocked with 0.5% BSA/PBS and re-suspended in 0.5%
503 BSA/PBS (2.5 times their original volume) containing 7 µg of antibody per IP. ChIP
504 grade antibodies used were anti-H3K4me3 (rabbit polyclonal; Abcam; ab8580), anti-
505 H3K4me1 (rabbit polyclonal; Abcam; ab8895), anti-H3K27me3 (mouse monoclonal;
506 Abcam; ab6002).
507 After overnight incubation of the Dynabeads at 4 ºC, they were washed 3 times with
508 0.5% BSA/PBS and re-suspended in 0.5% BSA/PBS, matching their original volume.
509 1/4th of the total chromatin was used for each IP, leaving a final 1/8th for input control
510 libraries. The IP was done on a rotating wheel overnight at 4 ºC.
511 Post-IP washes were done 6 times with RIPA buffer (50mM HEPES-KOH pH 8, 500mM
512 LiCl2, 1mM EDTA, 1% NP40, 0.7% Sodium Deoxycholate, cOmplete protease inhibitors
513 – 1 tablet per 50 ml). Beads were then washed in TE/NaCl (50mM NaCl in TE) and re-
514 supended in Elution Buffer (50mM Tris HCl pH 8, 10mM EDTA, 1% SDS). Proteins were
515 separated from the beads via 15-minute incubation at 65 ºC on a shaking heat block
516 (1400 rpm). Supernatant and input samples underwent overnight heat-based de-
517 crosslinking at 65 ºC. RNaseA (0.2 µg/ml) and Proteinase K (0.2 µg/ml) were used for 1
518 hour each in order to remove residual RNA and protein. DNA was purified with
519 phenol:chloroform extraction and ethanol precipitation. DNA was re-suspended in TE
520 and quantified with Qubit ds DNA HS kit (Thermo Fisher Scientific). The NEBNext Ultra
521 II (NEB) kit was used for library preparation. Manufacturer's instructions were followed.
522 Library clean-up was performed with Becton Coulter AMPureXP beads. Libraries were
523 quantified with Qubit, Agilent Bioanalyzer and using a KAPA Library Quantification
524 qPCR kit. Libraries were sequenced on an Illumina NextSeq machine.
525
526 **Comparison of previous NB transcriptomes**
527 Independently assembled transcriptomes were downloaded from four previous
528 publications [10,14,20,21]. Transcripts enriched in NBs were extracted based on the
529 classifications provided in respective publications' supplementary information. A
530 clustering of these sequences was done by running CAP3 [67] on all transcripts and

531 then extracting transcript groups that assembled. Detailed methods are recorded in an
532 IPython notebook (Additional File 9).
533
534 **Reference assembly and annotations**
535 Transcript sequences from previously assembled transcriptomes (Oxford, Dresden,
536 SmedGD Asexual, SmedGD Unigenes) and known genes were downloaded from
537 SmedGD [46], PlanMine [63] and NCBI. These sequences were mapped to the
538 SmedGD Asexual 1.1 genome with GMAP [68]. PASA [69] was then used to consolidate
539 the annotations. An independent reference assembly was also performed on 164
540 available RNA-seq libraries with HISAT2 [70] for mapping and StringTie [71] for
541 assembly. PASA consolidated annotations and StringTie reference assembly were
542 merged together with StringTie.
543 To remove redundancy from the annotations we first calculated an intron jaccard
544 similarity score (intersection of introns / union of introns) for all overlapping transcripts.
545 Pair-wise jaccard similarity scores of 0.9 or more were kept and used to create a graph
546 of similar annotations. Maximal-cliques were extracted from this graph as clusters of
547 redundant annotations. From these cliques, we chose one transcript to be the
548 representative by prioritizing transcript length, ORF length and BLAST homology. Strand
549 information was assigned to each transcript by using strand specific RNA-seq libraries,
550 BLAST homology, and longest ORF length. We ran TransDecoder (utilizing Uniprot and
551 PFAM for coding evidence) [72] to identify protein coding transcripts. Detailed methods
552 are recorded in an IPython notebook (Adddional File file 9). The genome annotations are
553 made available here as a gtf file (Additional File 10).
554
555 **Proportional expression value generation**
556 Kallisto [73] was used to pseudo-map RNA-seq libraries from four datasets [9,13,24],
557 accession: PRJNA296017, generating estimated counts and TPM values for each
558 transcript. Sleuth [74] was used to calculate a normalization factor for each library. For
559 each locus, the TPM values of member transcripts were summed to generate a loci TPM
560 value and then normalized accordingly.
561 Not all datasets contained all three X1/X2/Xins populations. The Reddien [24] and
562 Sanchez (accession: PRJNA296017) datasets only had two of the three populations. In
563 order to consolidate proportional expression values among all four datasets, pair-wise

564 ratios were first calculated for each dataset (X1:X2, X1:Xins, X2:Xins) using normalized
565 TPM values. These ratios were then averaged across the datasets.
566 Using two of the three ratios, we can calculate a predicted third ratio (i.e., given X1:X2
567 and X1:Xins, we can calculate X2:Xins). We then correlate the calculated proportion with
568 the actual proportion and kept the pairs of actual proportions (in this case, X1 and Xins)
569 that had the best correlation with the calculated proportion. Detailed methods are
570 recorded in an IPython notebook (Additional File 9).
571
572 **Single cell RNA-seq analysis**
573 Single cell RNA-seq data were downloaded from short-read archive [22,23]. Reads were
574 pseudo-mapped with Kallisto and the TPM values were used for down-stream analysis.
575 Cell types of each RNA-seq library were previously defined in the respective publications
576 by both FACS (X1/X2/Xins) and by gene markers.
577 The top 1,000 expressed loci from each cell type cluster were used for generating the
578 spectrum density figure and ternary plots.
579
580 **ChIP-seq mapping and track generation**
581 Biological triplicate ChIP-seq data from planarian X1 cells for each of three histone
582 marks considered was analyzed in conjunction with *D. melanogaster* S2 spike-in cells,
583 used for downstream between IP replicate normalization. The trimmed reads were
584 mapped to both *S. mediterranea* asexual 1.1 genome (SmedGD, [46]) and *D.*
585 *melanogaster* r6.10 genome [75] with BWA mem 0.7.12 [76]. Only uniquely mapping
586 reads were considered further. Paired reads that map to both species were also
587 removed. Picard tools 1.115 was used to remove duplicate reads. Reads were
588 separated into the sets that mapped to *D. melanogaster* and *S. mediterranea*
589 respectively so that numbers of mapped reads could be used for downstream
590 normalization calculations. For each paired or single mapped read, coordinates
591 representing the 100bp at the center of the sequenced fragment were parsed and
592 written to a BED file.
593 To generate coverage tracks in bedgraph format, the bedtools' genomecov function was
594 used. A normalization factor was calculated using the number of mapped reads
595 corresponding to the *D. melanogaster* spike-in [55,56]. A scaling factor for the input
596 ChIP-seq libraries was calculated using the DeepTools [61] python API that utilizes the

SES method [77]. Mean normalized coverage was calculated for each sample and input. The normalized input coverage was then subtracted from the normalized sample coverage to generate the final coverage track for downstream visualization and analysis. The normalization process is detailed in an IPython notebook (Additional file 7)

To calculate correlation of ChIP-seq coverage to proportional expression, two vector of values were used for a group of loci. The first vector is the proportional expression and the second vector is the coverage at a position in the 5kb region of the loci. A spearman correlation was performed on both vectors yielding a correlation value for the assayed position. This correlation value was calculated for every non-overlapping 50 base pair window in the 5kb region around the TSS.

For bivalency, a percent coverage was used instead of the absolute normalized coverage. This was generated by calculating the maximum coverage across all 5kb regions around assay loci. Each absolute coverage value across the loci is then divided by the maximum coverage resulting in a percent coverage.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and material

All data produced in this study is in the form ChiPseq data submitted under The NCBI Project accession number PRJNA338116.

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

AA conceived designed the study. DK led and performed all data analysis with help from YM and AA. YM led the acquisition of all experimental data, with help from AGL. SH and YM optimized the ChIP-seq protocol. DK, YM and AA wrote the manuscript.

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845 transform. Bioinformatics. Oxford University Press; 2010;26:589–95.

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847 for ChIP-seq. Stat Appl Genet Mol Biol. 2012;11:Article9.

848

849 **Figure legends**

850 Figure 1. Planarian FACs compartments and analysis of currently available neoblast
851 transcriptome datasets.
852 A) Schematic of FACS cell populations and their relationship to stages in the cell cycle,
853 stem cell progeny and differentiated cells. B) Venn diagram describing the overlaps from
854 four independently assembled transcriptomes and genes described as being enriched in
855 neoblasts, produced by the Aboobaker, Pearson, Rajewsky and Graveley labs
856 respectively.
857
858 Figure 2. Proportional transformation of gene expression values in planarian FACS
859 compartments.
860 Spectrum of genes sorted by X1 (A), X2 (B), and Xins (C) proportion of expression
861 where each vertical line in the spectrum represents one expressed loci. The proportion
862 of dark blue, light blue, and orange corresponds proportions of expression in the X1, X2,
863 and Xins FACS compartments respectively. D) A table presenting colour-coded
864 classification groups according to proportional expression in different FACS populations
865 of cells based on the detailed analysis of proportional expression (Additional Files 4 and
866 5).
867
868 Figure 3. Gene categories based on proportional expression values.
869 Previously described planarian genes are marked in expression profile following panels
870 displaying a gene category. A) Genes with 50% or more X1 proportional expression. B)
871 Genes with 50% or more Xins proportional expression. C) Gene ontology (GO)
872 enrichment of X1 enriched genes showing terms mainly associated with cell division. D)
873 GO enrichment of Xins genes showing terms associated with, for example, the
874 extracellular matrix. E) Genes with 50% or more X2 proportional expression. F) Genes
875 with the of sum X1 and X2 proportional expression more than or equal to 75% and in
876 neither falling into X1 nor X2 categories. Gene names in blue and red are not
877 characterised *S.mediterranea* genes. Blue gene names are genes associated with
878 methyltransferase activity according to GO. Red names are genes associated with

879 mRNA processing according to GO. G) GO enrichment for X1/X2 genes from F)
880 showing enrichment of terms involved in RNA and ribosomal processes. H) Genes with
881 sum X2 and Xins proportional expression more than or equal to 75% and in neither X2
882 nor Xins categories. I) Genes that are expressed in roughly similar proportions among
883 X1, X2, and Xins cells.
884
885
886 Figure 4. ChIP-seq analysis of H3K4me3 in planarian neoblasts.
887 A) ChIP-seq profile of 5kb around predicted transcriptional start sites (TSS) for
888 H3K4me3 histone marks in X1 cells. B) The amount of H3K4me3 signal decreases with
889 increasing proportional expression in the X2 compartment, indicative of expression
890 becoming limited to post-mitotic progeny rather than NBs. C) The correlation for each 50
891 bp window across the 5kb region around TSS to the X1 (dark blue), X2 (light blue), and
892 Xins (orange) proportional expression value. A positive correlation value means that the
893 higher the ChIP-seq signal, the higher the proportional expression value. A negative
894 correlation means that the lower a ChIP-seq signal, the higher the proportional
895 expression value. D) Example ChIP-seq profiles of individual planarian neoblast genes.
896
897 Figure 5. ChIP-seq analysis of repressive marks in planarian neoblasts.
898 A) ChIP-seq profile of 5kb around transcriptional start sites (TSS) for H3K27me3 in X1
899 cells B) The correlation between H3K27me3 ChIP-seq signal with the X1 (dark blue), X2
900 (light blue), and Xins (orange) proportional expression value for each 50 bp window
901 across the 5kb region around TSS. A positive correlation value means that the higher
902 the ChIP-seq signal, the higher the proportional expression value. A negative correlation
903 means that the lower a ChIP-seq signal, the higher the proportional expression value. C)
904 ChIP-seq profile of 5kb around transcriptional start sites (TSS) for H3K4me1 in X1 cells
905 D) The correlation between H3K4me1 ChIP-seq signal with the X1 (dark blue), X2 (light
906 blue), and Xins (orange) proportional expression value for each 50 bp window across
907 the 5kb region around TSS. E) Example ChIP-seq profiles of individual planarian genes
908 expressed in differentiated cells.
909
910 Figure 6. Bivalency of activation and repressive histone marks signal and shifting of
911 H3K4me1 signal. For figure A-D, the mean ChIP-seq profiles shown were transformed

912 into percent coverage (y axis) by dividing each coverage value by the max coverage
913 among all loci. The percent coverage of genomic region around TSS (x axis) was
914 plotted. A) H3K4me3 and H3K27me3 profile of the top 1000 ranked X1 enriched genes.
915 B) H3K4me3 and H3K27me3 profile of the top 1000 ranked Xins enriched genes. C)
916 H3K4me3 and H3K27me3 profile of the top 1000 ranked X2 enriched genes. D)
917 H3K4me3 H3K27me3 profile of genes down-regulated after *Smed-mex3* RNAi. E)
918 H3K4me3 and H3K27me3 profile of X2/X2ins enriched genes with less than 10% X1
919 proportional expression. F) The distribution of correlations between H3K4me3 signal
920 5KB around TSS and H3K27me3 signal 5KB around TSS for X1, X2, and *Smed-mex3*
921 RNAi loci.
922
923 Figure 7. ChIP-seq profiles of high ranked X2 genes focusing on annotated transcription
924 factors as examples. H3K4me3 and H3K27me3 profiles are displayed for the 5kb region
925 surrounding the transcription start site of each loci.
926
927

928 **Additional files**

929 Additional File 1. PDF Format.
930 Bar chart showing the number of neoblast transcripts from each of the dataset (blue), as
931 well as the number of neoblast transcripts that have at least one other match in another
932 dataset (red).
933
934 Additional file 2. PDF Format.
935 Asexual genome annotation workflow and metrics. A) A schematic of the workflow used
936 to annotate the asexual genome. This process involved utilizing previously *de novo*
937 assembled transcripts and available RNA-seq datasets. PASA (Program to Assemble
938 Spliced Alignments) was used to create a merged reference assembly. HISAT2 was
939 used for mapping RNA-seq data to the genome and StringTie for defining transcripts.
940 The two sets of reference assemblies (from consolidated transcriptomes and from RNA-
941 seq data) were merged with StringTie and filtered for redundancies, resulting in a final
942 annotation set. B) Proportion of the annotations from the final annotation set that are

943 likely coding (with TransDecoder evidence) and non-coding (no TransDecoder
944 evidence). Proportion of loci without TransDecoder evidence but with a BLAST hit to the
945 non-redundant (NR) protein database (e-value <= 1e-5) is also shown. C) A comparison
946 of the new annotation in this study with MAKER annotations available on *Schmidtea*
947 *mediterranea* Genome Database (SmedGD) showing cumulative percentages of
948 annotations at a range of expression value thresholds for SmedGD MAKER annotations
949 as a whole, for the 'Oxford' annotations as a whole, for SmedGD MAKER exclusive
950 annotations, and 'Oxford' exclusive annotations, and 'Oxford' coding annotations.
951
952 Additional File 3. PDF Format.
953 Mapping available FACS RNA-seq libraries to new annotations. A) FACS libraries from
954 the Rajewsky, Reddien, Pearson, and Sanchez labs were downloaded and mapped to
955 the annotations with Kallisto and normalized using Sleuth. Normalization was performed
956 for datasets within each lab. B) A hierarchical clustering of the FACS samples using
957 normalized transcripts per million (TPM). Wherever possible, a Short Read Archive Run
958 (SRR) ID is provided for the original dataset
959
960 Additional File 4. PDF Format.
961 Proportional transformation of expression values. Expression values were converted to
962 proportional expression values resulting in consistent clustering of samples.
963
964 Additional File 5. PDF Format.
965 The ternary plots within this PDF file describe subsets of expressed loci with each of the
966 three axis representing X1, X2, and Xins proportional expression. Dots represent loci,
967 which are categorized according to proportional expression. X1 is dark blue, X2 is light
968 blue, Xins is orange, X1/X2 is green, X2/Xins is red, X1/Xins is purple, and non-enriched
969 is grey.
970 The second page shows the best reciprocal hits to the ESCAPE database. Human
971 pluripotency factors were obtained from the ESCAPE database. Best reciprocal hits
972 were found between human and *S.mediterranea* genes. A) Ternary plot showing the
973 distribution of the *S.mediterranea* best reciprocal hits of human pluripotency factors. B)
974 Shows the same set of data but extracted from the proportional spectrum. C) Shows a
975 pie chart indicating the percentage of the 233 genes that belong to each category.

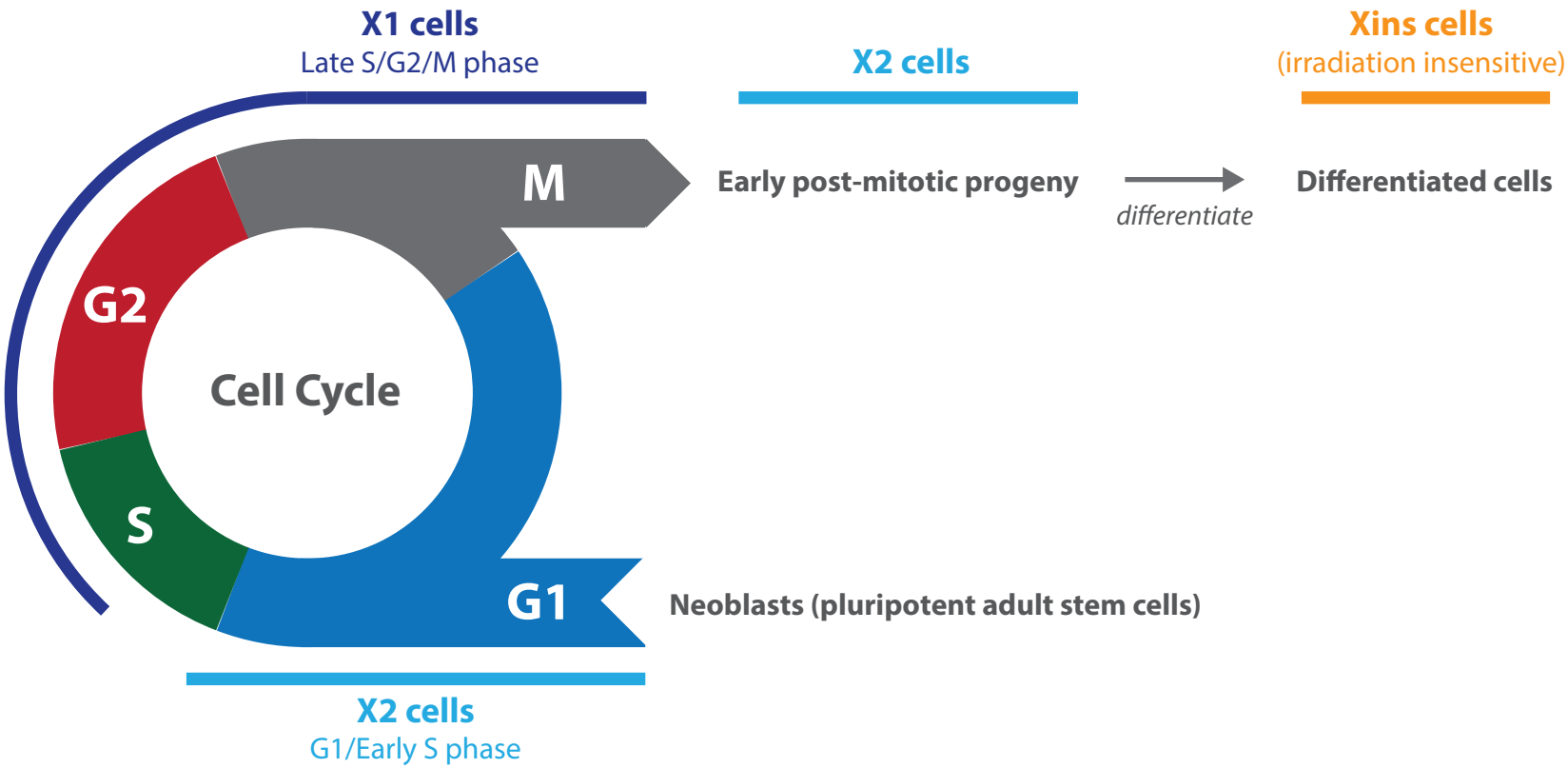
976 The third, fourth, fifth pages show ternary plots of loci with X1/X2, Xins, and X1 enriched
977 GO terms.
978
979 Additional File 6. PDF Format.
980 RNA-seq profiles of selected RNAi datasets. The first page of the PDF file shows RNA-
981 seq profiles divided into four segments representing genes enriched in X1, X2, Xins, and
982 X1/X2. The proportional values of each category are plotted as dark blue (X1
983 proportion), light blue (X2 proportion), and orange (Xins proportion) on the right of each
984 profile. The RNA-seq profile is displayed as red (up-regulated) and blue (down-
985 regulated) lines that are significantly differentially expressed with a p-value less than
986 0.05 and fold-change value < -1.5 (log2 fold-change –0.58) or > 1.5 (log2 fold-change
987 0.58). The length of each line represents the log2 fold-change. A) RNA-seq profile of
988 *Smed-mex3* RNAi performed on whole worms. B) RNA-seq profile of *Smed-zfp-1*
989 performed on X2 and X1 cells. C) RNA-seq time-course data for *Smed-CHD4* RNAi
990 performed on whole worms. D) RNA-seq time-course data for *Smed-p53* RNAi
991 performed on whole worms. E) RNA-seq data for *Smed-coe* RNAi performed on whole
992 worms.
993 The second page of the PDF file shows single-cell RNA-seq data. A) Transcripts per
994 million (TPM) values for RNA-seq libraries for each cell type were averaged and the top
995 one thousand genes extracted. Each row represents a cell type and the intensity of the
996 color represents the density of genes at the position on the proportional expression
997 spectra. B) Ternary plots of the cell types where the three axes represent X1, X2, and
998 Xins proportional expression.
999
1000 Additional File 7. PDF format.
1001 Summary of ChIP-seq mapping data from all available planarian ChIP seq data,
1002 demonstrating the improved data yield from the methods developed in the current study.
1003
1004 Additional File 8. PDF format.
1005 Genome wide ChIP signal presented for all genes in each proportional gene expression
1006 category, average profiles are presented above each genome wide plot.
1007
1008 Additional File 9. HTML format.

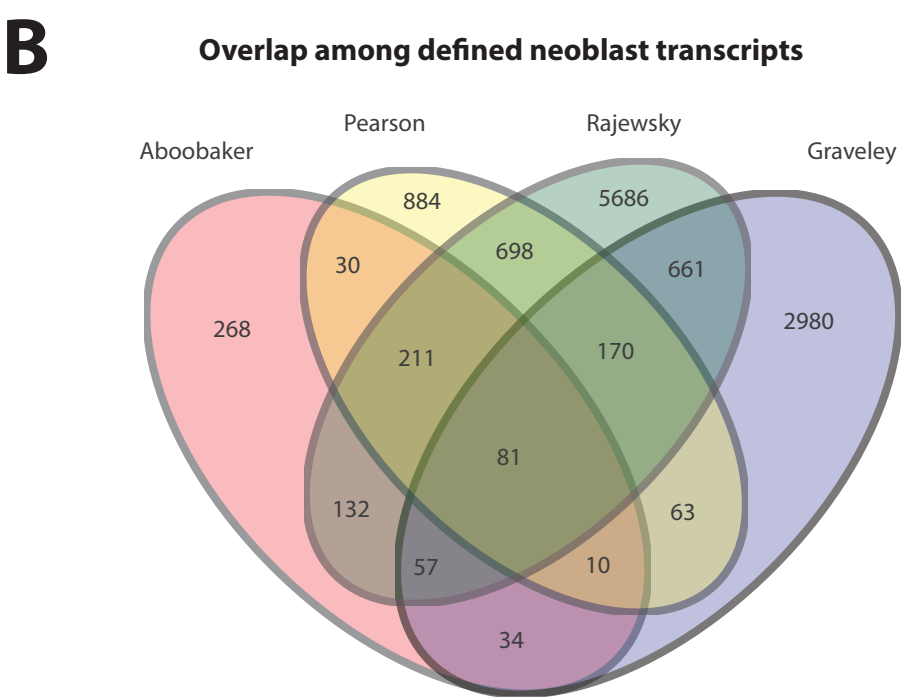
1009 Jupyter notebook of all analysis performed.

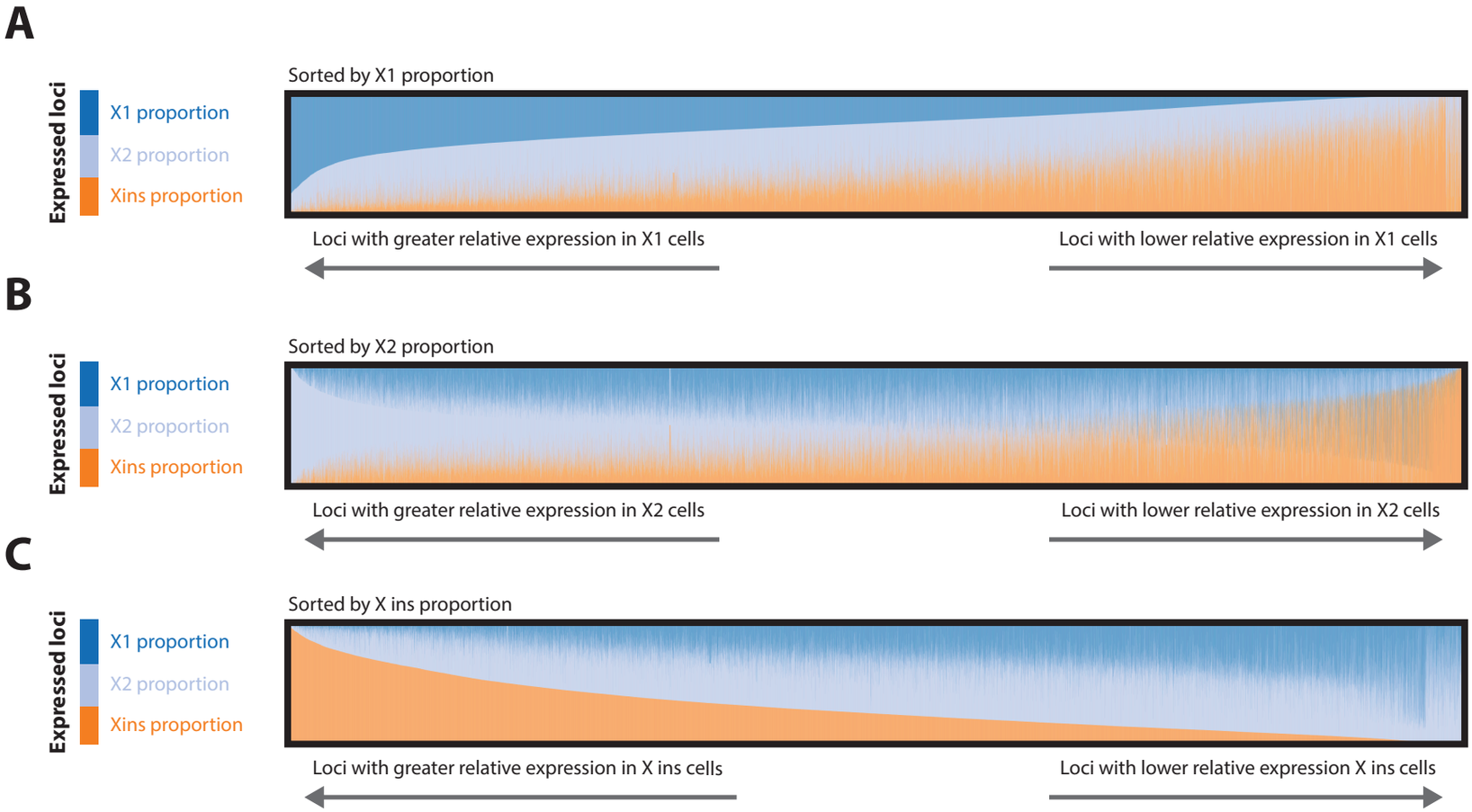
1010

1011 Addition File 10. ZIP format.

1012 GTF annotation file of the asexual genome


A
X1 cells
Late S/G2/M phase
X2 cells
Xins cells
(irradiation insensitive)
M
Early post-mitotic progeny
differentiate
Differentiated cells
G2
Cell Cycle
S
G1
Neoblasts (pluripotent adult stem cells)
X2 cells
G1/Early S phase


B
Overlap among defined neoblast transcripts
Aboobaker
Pearson
Rajewsky
Graveley
884
5686
30
698
661
268
2980
211
170
81
132
63
57
10
34



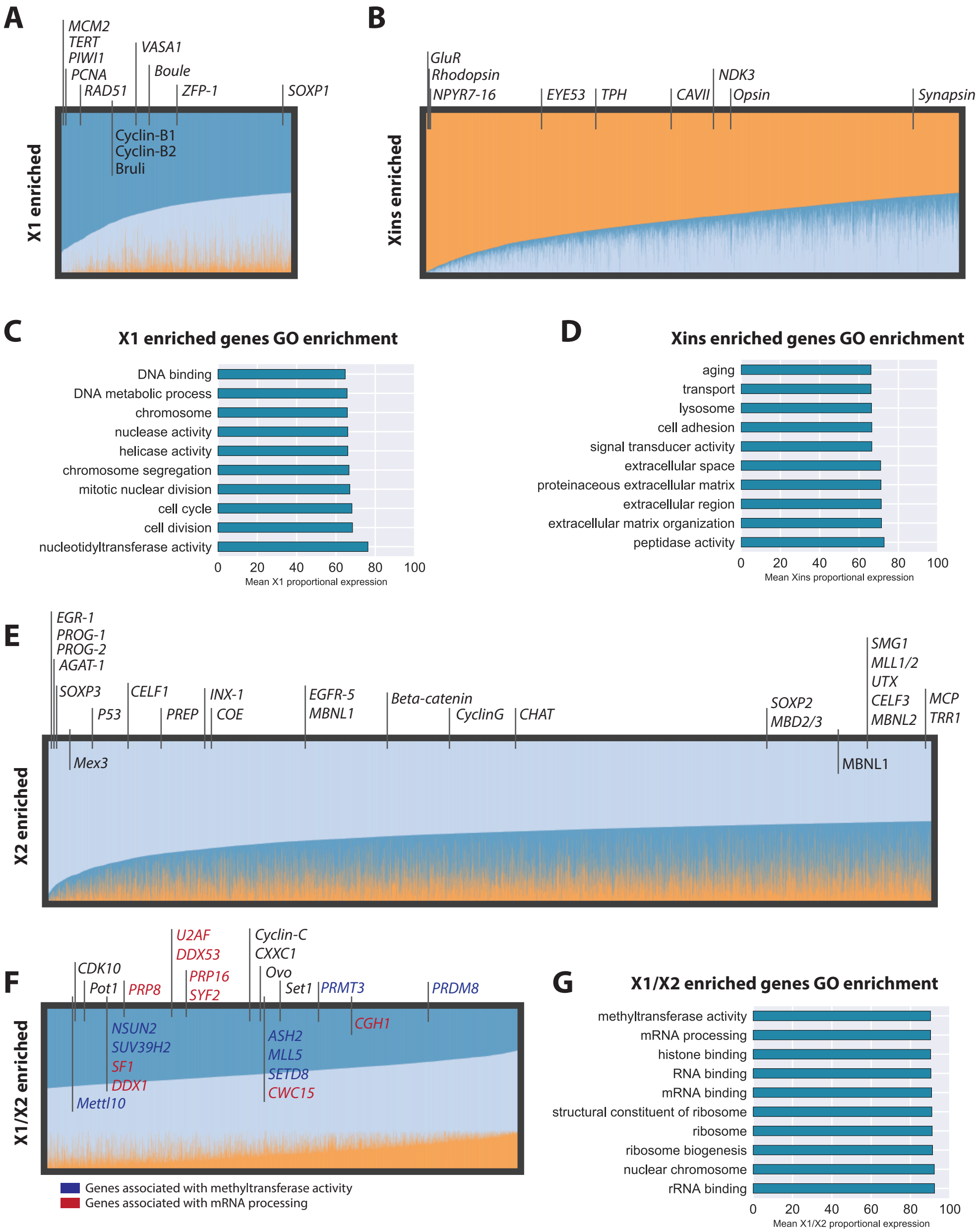
D

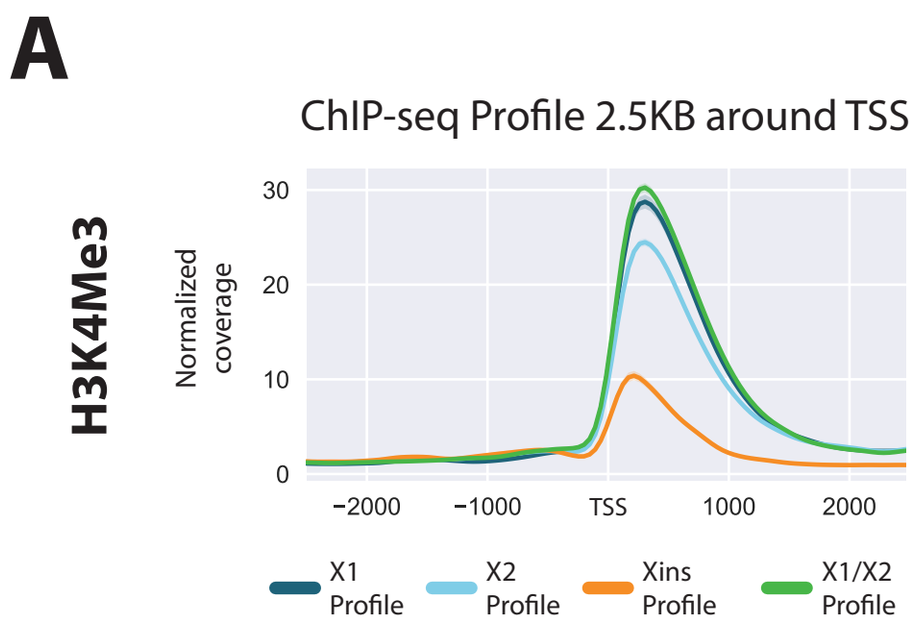
38,771 loci total

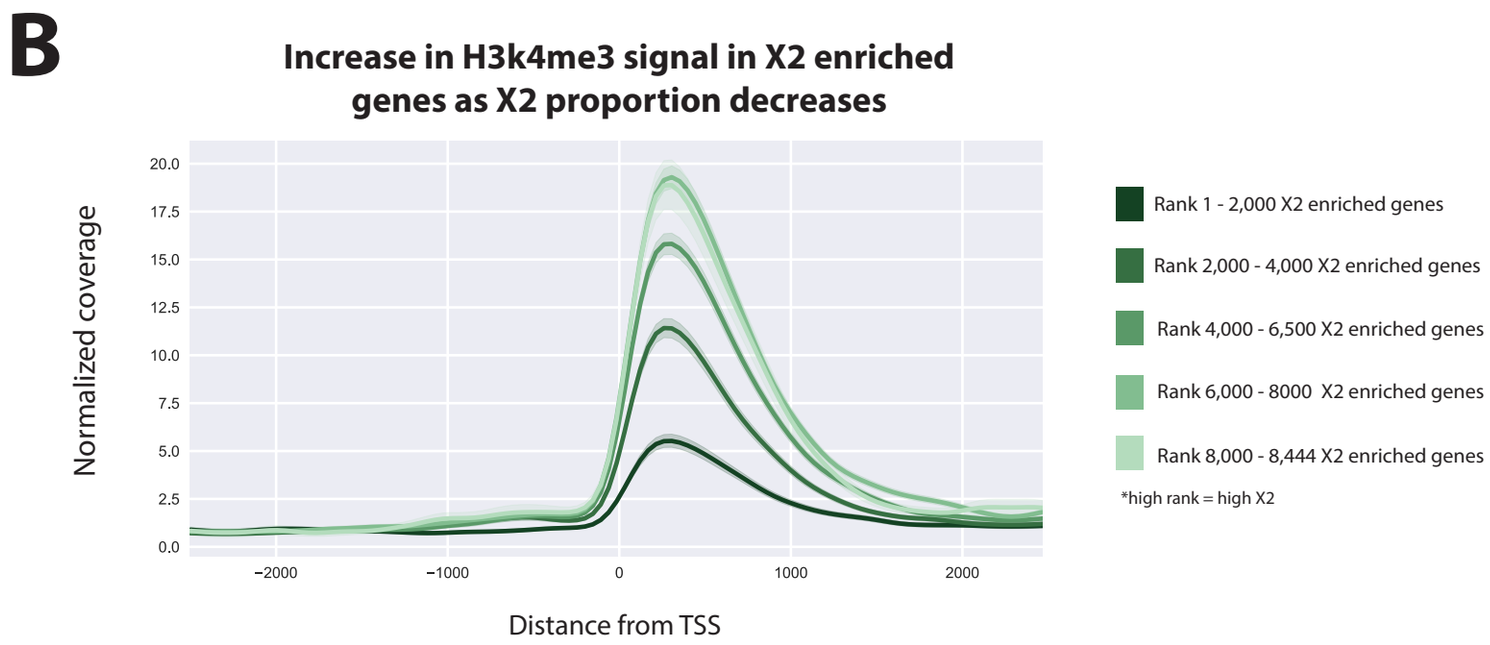
Categories defined by FACS RNA-seq data

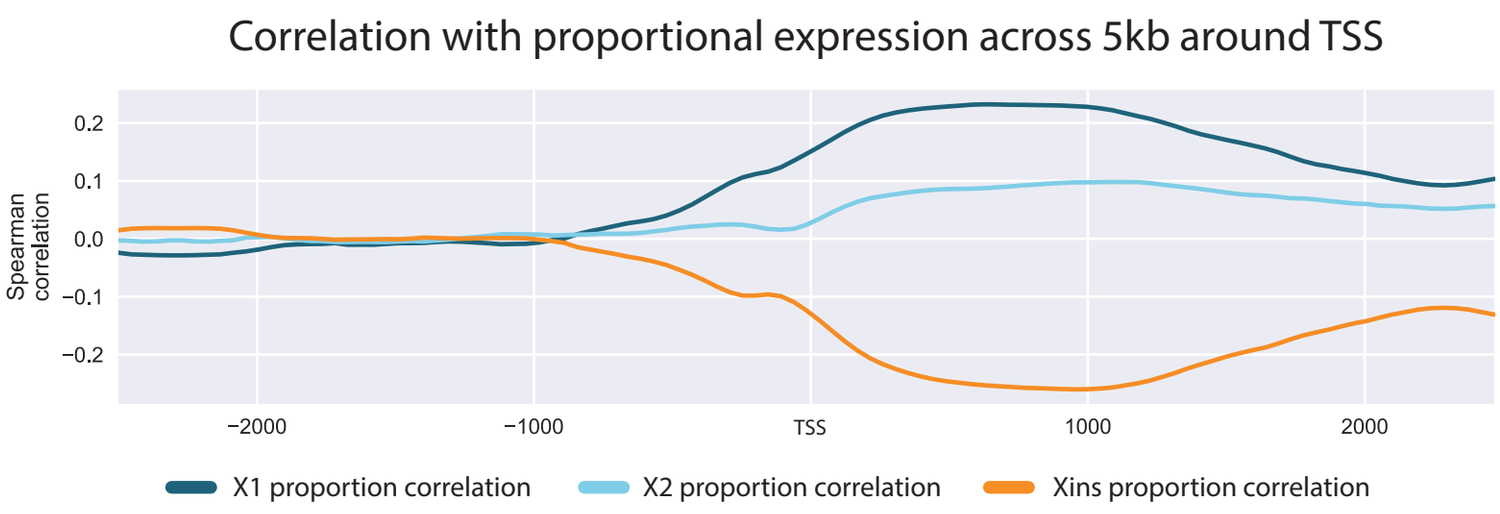
Category	Criteria	# Loci	# Coding* loci (% of category)
X1 enriched	X1 proprtional expression >= 50%	2,253	1,544 (68%)
X2 enriched	X2 proprtional expression >= 50%	8,444	4,781 (57%)
Xins enriched	Xins proprtional expression >= 50%	5,119	3,887 (76%)
X1/X2 enriched	X1 + X2 proprtional expression >= 75% and not in X1 enriched nor X2 enriched	4,538	3,107 (68%)
X2/Xins enriched	X2 + Xins proprtional expression >= 75% and not in X2 enriched nor Xins enriched	3,652	2,688 (74%)
X1/xins enriched	X2 + Xins proprtional expression >= 75% and not in X2 enriched nor Xins enriched	303	0 (0%)
unenriched	Remaining loci with roughly equal proportions among X1, X2, and Xins	2,897	2,003 (69%)
unclassified	Loci with # of reads less than 10 in all FACS RNA-seq libraries	11,565	3,762 (33%)

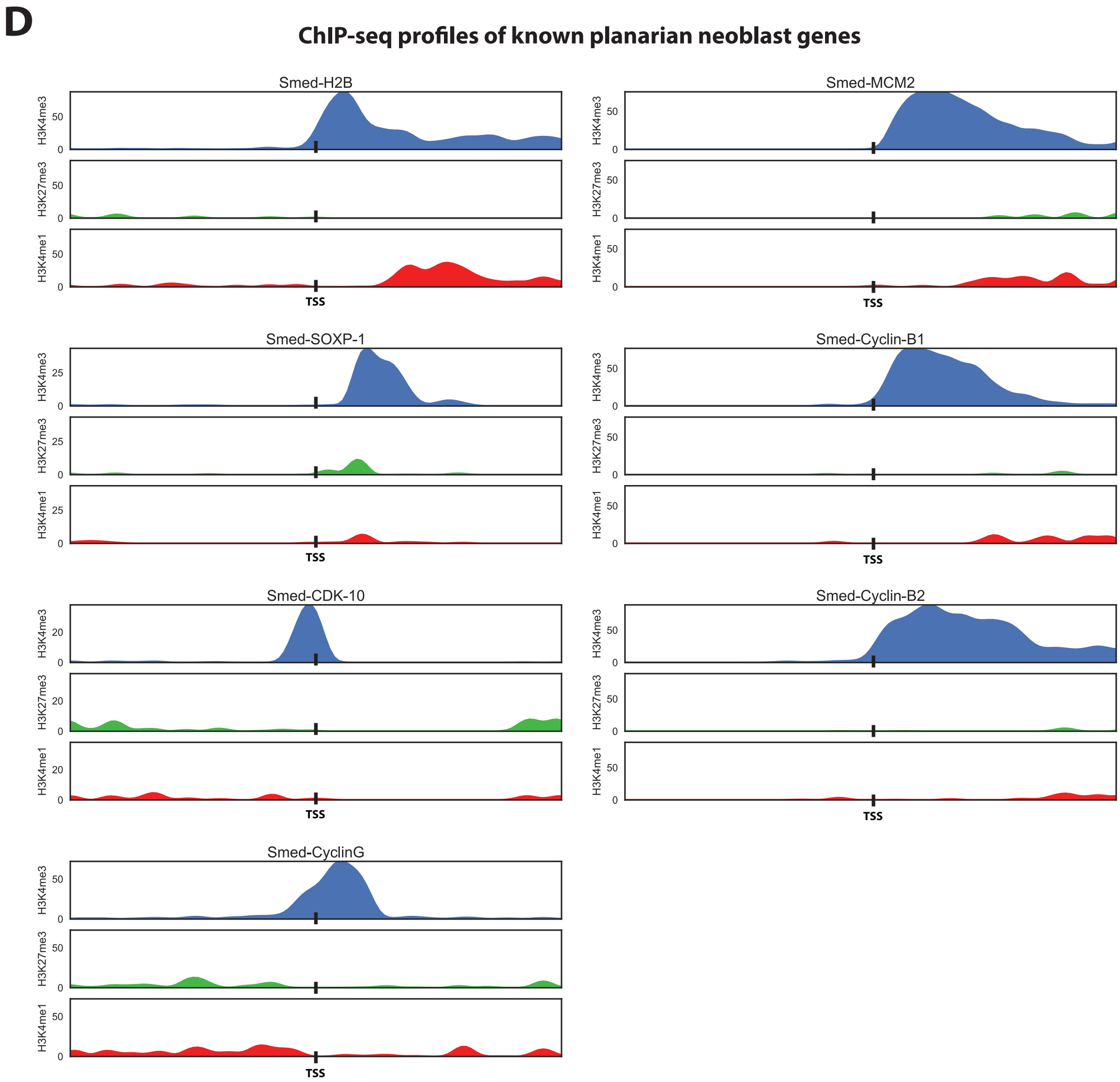
* Coding is defined by having TransDecoder evidence which includes homology (Uniprot, PFAM), hexamer frequency, and ORF length

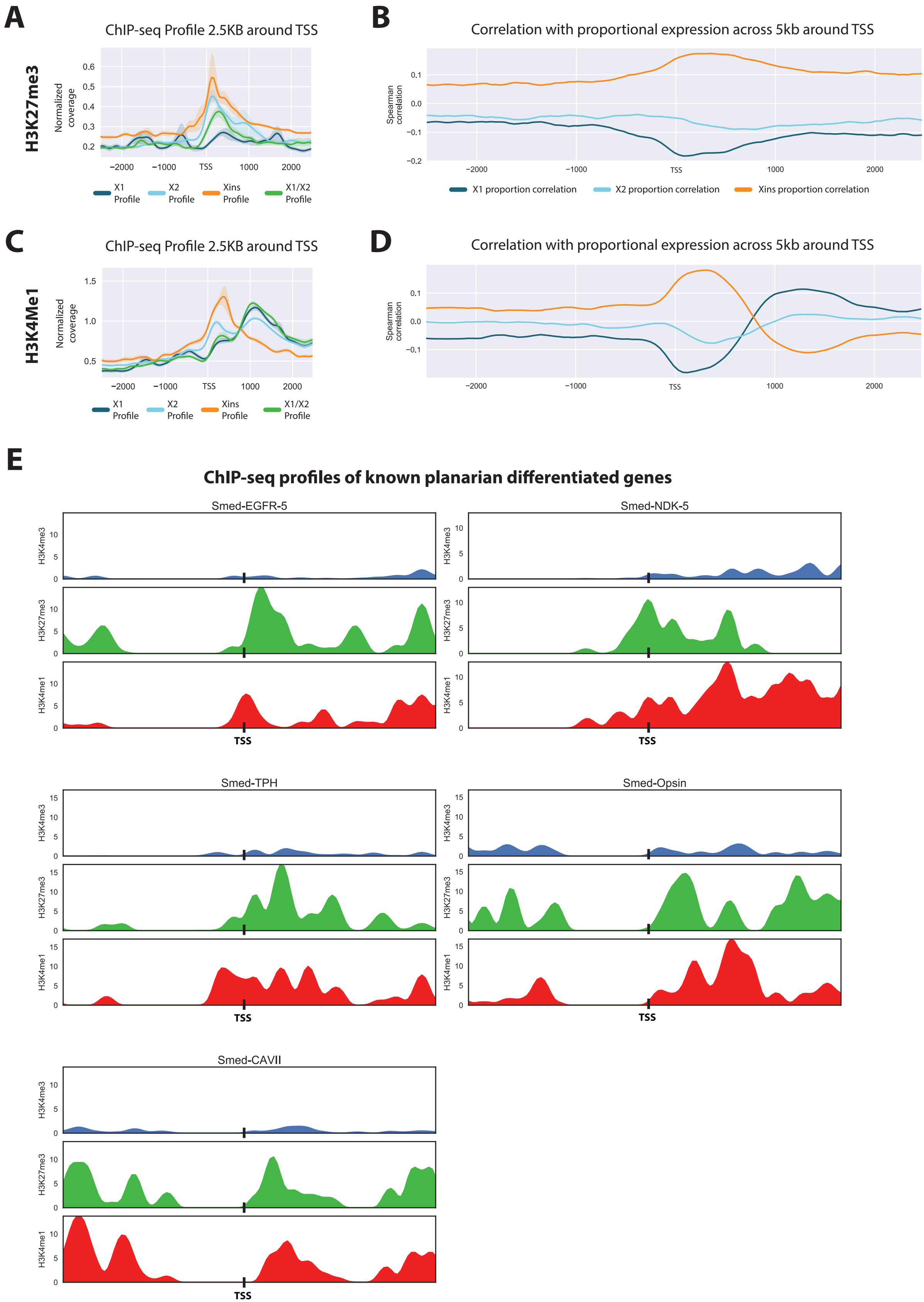

A
MCM2
TERT
PIWI1
PCNA
RAD51
VASA1
Boule
ZFP-1
SOXP1
Cyclin-B1
Cyclin-B2
Bruli
X1 enriched
B
GluR
Rhodopsin
NPYR7-16
EYE53
TPH
CAVII
NDK3
Opsin
Synapsin
Xins enriched
C
X1 enriched genes GO enrichment
DNA binding
DNA metabolic process
chromosome
nuclease activity
helicase activity
chromosome segregation
mitotic nuclear division
cell cycle
cell division
nucleotidyltransferase activity
0 20 40 60 80 100
Mean X1 proportional expression
D
Xins enriched genes GO enrichment
aging
transport
lysosome
cell adhesion
signal transducer activity
extracellular space
proteinaceous extracellular matrix
extracellular region
extracellular matrix organization
peptidase activity
0 20 40 60 80 100
Mean Xins proportional expression
E
EGR-1
PROG-1
PROG-2
AGAT-1
SOXP3
P53
Mex3
CELF1
PREP
INX-1
COE
EGFR-5
MBNL1
Beta-catenin
CyclinG
CHAT
SOXP2
MBD2/3
SMG1
MLL1/2
UTX
CELF3
MBNL2
MCP
TRR1
MBNL1
X2 enriched
F
CDK10
Pot1
PRP8
U2AF
DDX53
PRP16
SYF2
Cyclin-C
CXXC1
Ovo
Set1
PRMT3
PRDM8
NSUN2
SUV39H2
SF1
DDX1
Mettl10
ASH2
MLL5
SETD8
CWC15
CGH1
X1/X2 enriched
Genes associated with methyltransferase activity
Genes associated with mRNA processing
G
X1/X2 enriched genes GO enrichment
methyltransferase activity
mRNA processing
histone binding
RNA binding
mRNA binding
structural constituent of ribosome
ribosome
ribosome biogenesis
nuclear chromosome
rRNA binding
0 20 40 60 80 100
Mean X1/X2 proportional expression
H
CELF2
Glut4
MBNL3
NDK2
X2/Xins enriched
I
unenriched

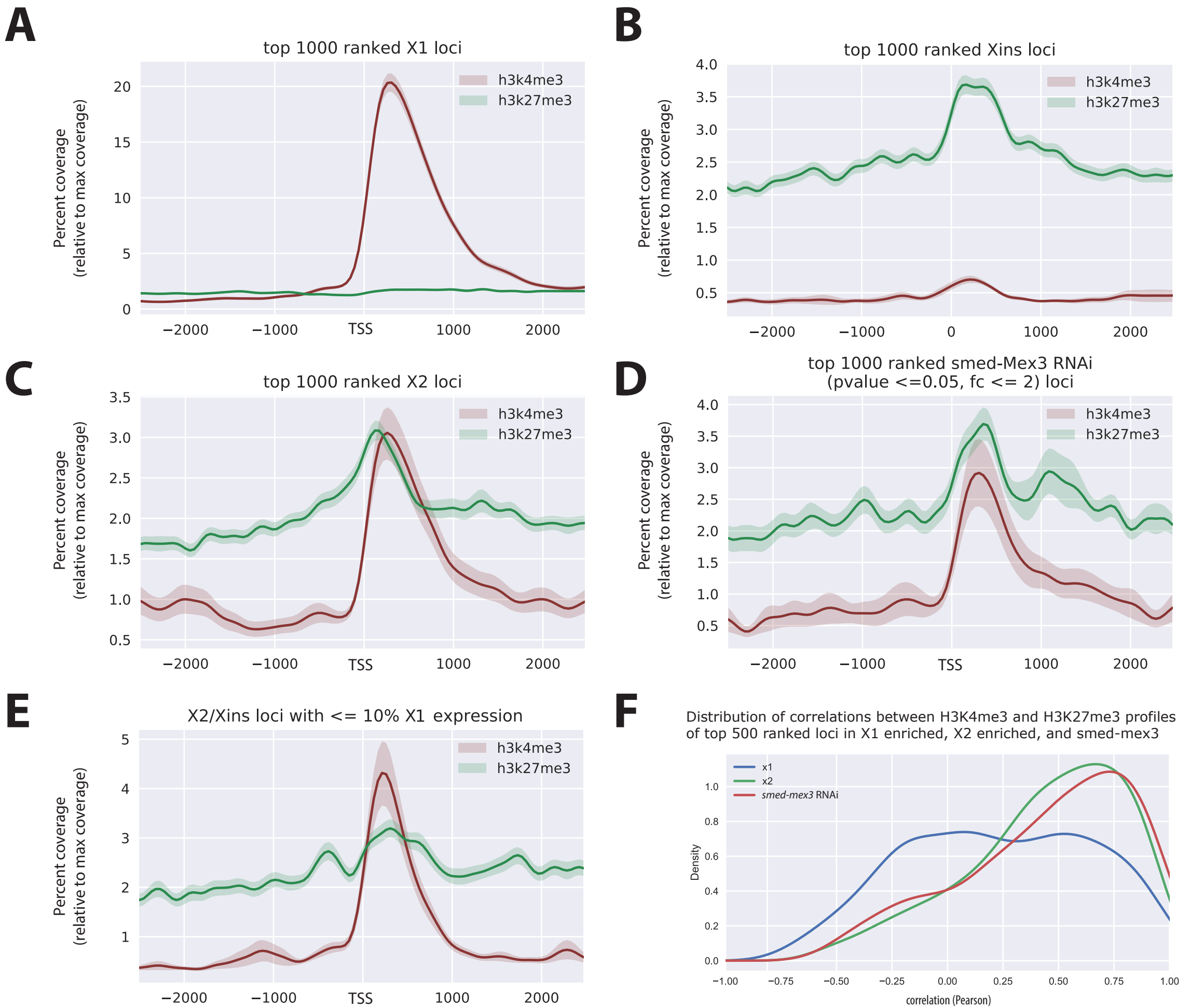
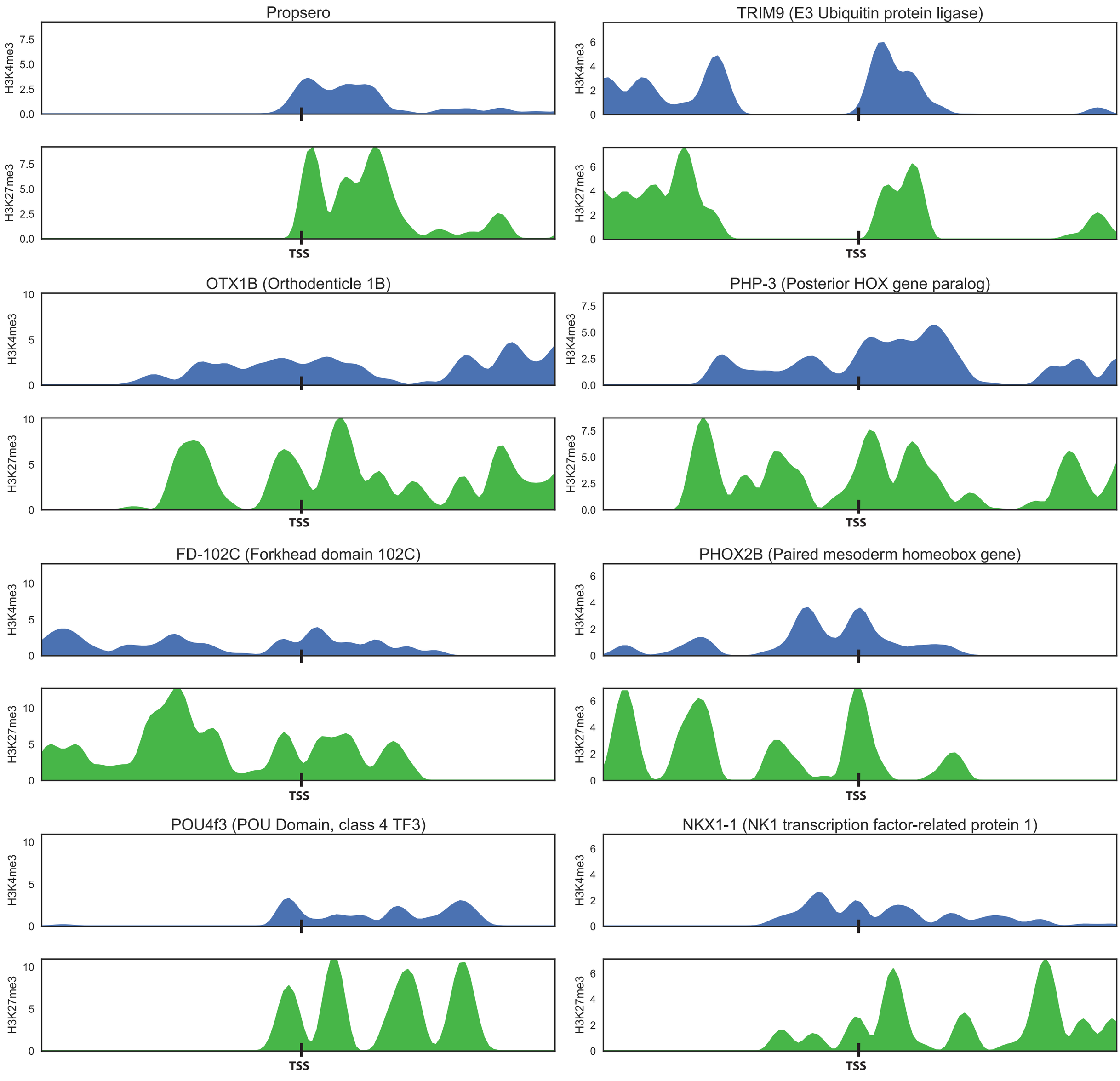

A
H3K4Me3
ChIP-seq Profile 2.5KB around TSS
Normalized coverage
30
20
10
0
−2000
−1000
TSS
1000
2000
X1 Profile
X2 Profile
Xins Profile
X1/X2 Profile


B
Increase in H3k4me3 signal in X2 enriched genes as X2 proportion decreases
Normalized coverage
20.0
17.5
15.0
12.5
10.0
7.5
5.0
2.5
0.0
−2000
−1000
0
1000
2000
Distance from TSS
Rank 1 - 2,000 X2 enriched genes
Rank 2,000 - 4,000 X2 enriched genes
Rank 4,000 - 6,500 X2 enriched genes
Rank 6,000 - 8000 X2 enriched genes
Rank 8,000 - 8,444 X2 enriched genes
*high rank = high X2


C
Correlation with proportional expression across 5kb around TSS
Spearman correlation
0.2
0.1
0.0
−0.1
−0.2
−2000
−1000
TSS
1000
2000
X1 proportion correlation
X2 proportion correlation
Xins proportion correlation


D
ChIP-seq profiles of known planarian neoblast genes
Smed-H2B
Smed-MCM2
Smed-SOXP-1
Smed-Cyclin-B1
Smed-CDK-10
Smed-Cyclin-B2
Smed-CyclinG
H3K4me3
H3K27me3
H3K4me1
TSS


A
ChIP-seq Profile 2.5KB around TSS
H3K27me3
Normalized coverage
0.6
0.5
0.4
0.3
0.2
−2000
−1000
TSS
1000
2000
X1 Profile
X2 Profile
Xins Profile
X1/X2 Profile
B
Correlation with proportional expression across 5kb around TSS
Spearman correlation
0.1
0.0
−0.1
−0.2
X1 proportion correlation
X2 proportion correlation
Xins proportion correlation
C
ChIP-seq Profile 2.5KB around TSS
H3K4Me1
1.5
1.0
0.5
D
Correlation with proportional expression across 5kb around TSS
E
ChIP-seq profiles of known planarian differentiated genes
Smed-EGFR-5
Smed-NDK-5
Smed-TPH
Smed-Opsin
Smed-CAVII
H3K4me3
H3K27me3
H3K4me1
TSS


A
top 1000 ranked X1 loci
h3k4me3
h3k27me3
Percent coverage
(relative to max coverage)
TSS
B
top 1000 ranked Xins loci
C
top 1000 ranked X2 loci
D
top 1000 ranked smed-Mex3 RNAi
(pvalue <=0.05, fc <= 2) loci
E
X2/Xins loci with <= 10% X1 expression
F
Distribution of correlations between H3K4me3 and H3K27me3 profiles
of top 500 ranked loci in X1 enriched, X2 enriched, and smed-mex3
x1
x2
smed-mex3 RNAi
Density
correlation (Pearson)

ChIP-seq profiles of high ranked X2 loci