The cohesin regulator Stag1 promotes cell plasticity through heterochromatin regulation.

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SUMMARY

Fundamental biological processes such as embryo development and stem cell control rely on cellular plasticity. We present a role for the cohesin regulator, Stag1 in cellular plasticity control via heterochromatin regulation. Stag1 localises to heterochromatin domains and repetitive sequences in embryonic stem (ES) cells and contains intrinsically disordered regions in its divergent terminal ends which promote heterochromatin compaction. ES cells express Stag1 protein isoforms lacking the disordered ends and fluctuations in isoform abundance skews the cell state continuum towards increased differentiation or reprogramming. The role for Stag1 in heterochromatin condensates and nucleolar function is dependent on its unique N-terminus. Stag1$^{N\Delta}$ ESCs have decompacted chromatin and reprogram towards totipotency, exhibiting MERVL de-repression, reduced nucleolar transcription and decreased translation. Our results move beyond protein-coding gene regulation via chromatin loops into a new role for Stag1 in heterochromatin and nucleolar function and offer fresh perspectives on its contribution to cell identity and disease.

KEY WORDS

Cellular plasticity, heterochromatin, repetitive elements, nucleolus, Stag1, translation

INTRODUCTION

Cellular populations consist of mixtures of cells across a continuum of states and such heterogeneity underlies responsiveness to changing conditions. Fundamental biological processes including embryo development, stem cell control and cancer rely on conversion between states. While transcriptional states are ever-increasingly well described (Wagner et al., 2016), the mechanisms that control the stability of a given state are less well understood. The chromatin landscape with its inherent dynamics (Cho et al., 2018; Finn et al., 2019; Nozaki et al., 2017; Ricci et al., 2015) and complex 3-dimensional (3D) organization (Bonev et al., 2017; Cardozo Gizzi et al., 2019; Dekker and Mirny, 2016; Nagano et al., 2017; Schlesinger and Meshorer, 2019) can act as a mechanism to support transcriptional heterogeneity (Mateo et al., 2019; Rodriguez et al., 2019) and the chromatin proteins that regulate this landscape may play key roles in cell plasticity (Meshorer et al., 2006).

Genomes are partitioned into distinct functional domains within the nucleus (Bickmore and van Steensel, 2013; Bonev and Cavalli, 2016). A prominent feature of nuclear organization is the compact heterochromatin that accumulates at the periphery of the nucleus, around the nucleolus and at distinct foci within the nucleoplasm (Guelen et al., 2008; Németh et al., 2010; Padeken and Heun, 2014; Quinodoz et al., 2018). Heterochromatin is formed at repetitive sequences, is tightly associated with repressive histone modifications like methylation of histone H3 at lysine 9 (H3K9me2,3), and a specific set of proteins, including Heterochromatin Protein 1 (HP1) that together condense chromatin to maintain repression (Allshire and Madhani, 2018). Further, sequestration of condensed chromatin into dynamic phase-separated condensates (Larson et al., 2017; Strom et al., 2017) contributes to heterochromatin-mediated silencing. Heterochromatin organization plays a key role in cell identity and is rapidly remodelled during ES cell differentiation and embryo development. Decompaction of heterochromatin and subsequent de-repression of repetitive elements drives reprogramming towards totipotent embryos, while progressive compaction is associated with terminal differentiation (Ahmed et al., 2010; Borsos and Torres-Padilla, 2016; Martin et al., 2006; Meshorer et al., 2006; Novo et al., 2016).

Cohesin is a ubiquitously expressed, multi-subunit protein complex that has fundamental roles in cell biology including sister chromosome cohesion, 3D chromatin topology and regulation of cell identity (Cuartero et al., 2018; Horsfield et al., 2007; Kline et al., 2018; Leiserson et al., 2015; Romero-Pérez et al., 2019; Viny et al., 2019). Much of our understanding of how cohesin contributes to cell identity has been studied in the context of its roles in protein-coding gene expression and 3D organization of interphase chromatin structure (Hadjur et al., 2009; Kagey et al., 2010; Mishiro and Tsutsumi, 2009; Misulovin et al., 2007; Parelho et al., 2008; Phillips-Cremins et al., 2013; Rao et al., 2014; Vietri Rudan et al., 2015; Wendt et al., 2008). Indeed, loss of cohesin and its regulators results in a dramatic loss of chromatin topology at the level of Topologically Associated Domains (TAD) and chromatin loops, with only modest changes to gene expression (Haarhuis et al., 2017; Rao et al., 2017; Schwarzer et al., 2017; Seitan et al., 2013; Sofueva et al., 2013; Wutz et al., 2017; Zuin et al., 2014). This suggests that cohesin's roles in development and disease extend beyond gene expression regulation and highlight the need to re-evaluate how cohesin regulators shape the structure and function of the genome.

The association of cohesin with chromosomes is tightly controlled by several regulators, including the Stromalin Antigen protein (known as Stag or SA), which has been widely implicated in cell identity regulation and disease development (Cuadrado et al., 2019; Lehalle et al., 2017; Leiserson et al., 2015; Soardi et al., 2017; Viny et al., 2019; Yuan et al., 2019). Stag proteins interact with the Rad21 subunit of cohesin and mediate its association with DNA and CTCF (Hara et al., 2014; Li et al., 2020; Orgil et al., 2015; Xiao et al., 2011). Mammalian cells have three Stag paralogs, Stag1, 2 and 3. These show >90% conservation of sequence in their central domain yet perform distinct functions (Canudas and Smith, 2009; Kojic et al., 2018; Remeseiro et al., 2012a; Winters et al., 2014). It is likely that the divergent N- and C-terminal regions provide functional specificity. For example, the N-terminus of Stag1 contains a unique AT-hook (Bisht et al., 2013) which is required for its preferential participation in telomere cohesion (Canudas and Smith, 2009). The underlying mechanisms by which Stag proteins and their divergent ends influence cell identity are still largely unknown.

Here we report that Stag1 is the dominant paralogue in ES cells and supports pluripotency by regulating heterochromatin organization. We discover that ES cells regulate the level of Stag1 protein and the proportion of its divergent N and C-terminal ends, which contain disordered regions. This naturally occurring Stag1 protein heterogeneity supports a continuum of functionally distinct cellular states within the population. Changing the balance in the levels of Stag1 isoforms leads to conversion between cell states, with the loss of the N-terminus favoring a reprogrammed, totipotent state and decompaction of heterochromatin condensates and the loss of the C-terminus priming cells towards exit from pluripotency through gene expression deregulation. These results define specialised, non-redundant roles for the divergent ends. Mechanistically, the N-terminus of Stag1 represses reprogramming to totipotent 2-cell-like (2C-L) cells by maintaining nucleolar structure and function. We uncover Nucleolin and Trim28 as direct interactors of Stag1 and show that cells selectively expressing Stag1 isoforms lacking the N-terminal AT-hook domain exhibit reduced nascent nucleolar transcription and a decrease in global translation. Our results take us beyond protein coding gene regulation via chromatin loops into a new role for Stag1 in the regulation of heterochromatin and nucleolar structure and function. Importantly, by identifying changes to translation control upon Stag1 loss in stem cells, we open a new perspective by which Stag proteins and cohesin regulation can impact cell identity and disease.

RESULTS

A functional change in cohesin regulation in cells of different potential.

We analyzed the expression levels of cohesin regulators in embryonic stem cells (ES) at different stages of pluripotency. During the transition between naïve (2i) and primed (EpiLC) pluripotency *in vitro*, levels of the core cohesin subunits Smc1 and Smc3 do not change, while Stag1 becomes downregulated and Stag2 becomes upregulated (Figures 1A, S1A, B). This was confirmed at the protein level, where we observe a 2-3-fold higher level of chromatin-associated Stag1 compared to Stag2 in naïve ES cells, while Stag2 levels are 5-10-fold higher in EpiLCs (Figures 1B, S1C). These results, together with similar observations (Cuadrado et al., 2019), identify Stag1 as the dominant paralog in naïve ES cells and suggest that a switch between Stag1 and Stag2 may represent a functionally relevant change in cohesin regulation at different stages of pluripotency.

Stag1 supports the naïve pluripotent state.

To investigate the functional importance of Stag1 in the regulation of pluripotency, we established a Stag1 RNA knockdown (KD, 'siSA1-SP', Methods) strategy using siRNAs. This resulted in a significant reduction of Stag1 at the mRNA and protein levels (4-5-fold, 8-10-fold, respectively), in both serum-grown (FCS) and naïve (2i) ES cells without affecting the cell cycle (Figures 1C, S1D, E). Using Nanog as a marker of naïve pluripotency, we observed a significant downregulation of Nanog mRNA and protein levels within 24hrs of Stag1 KD in all ES populations (Figures 1D, E, S1F), suggesting that Stag1 may be functionally required for pluripotency. Indeed, a global analysis of the ES transcriptome upon siRNA-mediated Stag1 KD revealed that 375 genes were up- and 205 genes were down-regulated by at least 2-fold (Figure 1F). Among the downregulated group were several genes known to have key roles in the maintenance of pluripotency, including Nanog, Tbx3, Esrrb, Klf2, Klf4, Prdm14, Tfcp2l1, Lefty2. Notably, we did not detect a change in expression of Oct4 or Sox2, and Zfp42 was minimally affected. Moreover, we also observed both an upregulation of genes associated with exit from the pluripotent state (Dppa3, Fgf5) as well as differentiation-specific genes such as Pou3f1

128 (Oct6) and Sox11 (Figure 1F). Single-gene analysis revealed consistent, albeit low fold-
129 change trends across our biological replicates, thus we used Gene Set Enrichment
130 Analysis (GSEA) (Mootha et al., 2003; Subramanian et al., 2005) to detect modest but
131 coordinate changes in the expression of groups of functionally related genes. This
132 revealed a robust gene signature of exit from pluripotency and enrichment for genes
133 associated with primed pluripotency upon Stag1 KD across all biological replicates
134 (Figures 1G, S1G).

135 The loss of the naïve transcriptional programme upon Stag1 KD suggests that ES
136 cells may require Stag1 for the maintenance of self-renewal. To test this, we plated cells
137 in self-renewal conditions at clonal density and determined the proportion of
138 undifferentiated cells upon Stag1 KD by measuring the area occupied by the colonies
139 with high alkaline phosphatase activity (AP+). In scrambled siRNA-treated controls, 52%
140 of plated cells retain their naïve state, identified by AP+ colonies (Figure S1H). However,
141 upon Stag1 KD, both the proportion of AP+ colonies and the area they occupy decreased
142 by 20%, indicating that ES cells have a reduced self-renewal ability in the absence of
143 Stag1 (Figures 1H, S1H). As independent validation of these results, we used
144 CRISPR/Cas9 to knock-in an mNeonGreen-FKBP12^{F36V} tag (Nabet et al., 2018) at the C-
145 terminus of both alleles of the endogenous Stag1 locus (SA1^{NG_FKBP}) in ES cells (Figures
146 1I, S1I-K). Upon dTAG addition, Stag1 protein is robustly degraded in SA1^{NG_FKBP} ES
147 clones (Figure 1I, S1K). As was observed with siRNA treatment, dTAG-mediated
148 degradation of Stag1 led to a 50% reduction of self-renewal potential (Figure 1J).
149 Together, our results are consistent with a requirement for Stag1 in the control of naïve
150 pluripotency and provide an opportunity to discover the mechanisms of Stag1 actions.

151

152 **STAG1 localizes to AT-rich heterochromatin.**

153 To begin to understand how STAG1 contributes to pluripotency, we investigated the
154 subcellular localization of endogenous STAG1. Live cell imaging of Hoechst-labelled
155 Stag1^{NG_FKBP} ES cells revealed the expected and predominant localisation of STAG1 in

156 the nucleus (Figures 2A, B). Interestingly, STAG1 was not uniformly distributed within the
157 nucleoplasm. In addition to a dispersed nucleoplasmic localisation pattern, we observed
158 STAG1 colocalization with Hoechst-dense regions. These included colocalization at large
159 Hoechst-dense foci (Figure 2A, top cell), within the interior of the nucleolus (Figure 2A,
160 top cell) and at the periphery of the nucleolus (Figure 2A, all cells). The mean intensity of
161 STAG1 (as measured by mNeonGreen signal) was significantly enriched within Hoechst-
162 dense foci compared to the whole nucleus, and the signal was sensitive to treatment with
163 dTAG (Figure 2B). We made similar observations of STAG1 localization at DAPI-dense
164 foci in cells expressing Dox-inducible GFP-tagged full-length Stag1 ($SA1^{FL}$-GFP) (Figure
165 S2A, B). We note that repressive heterochromatin domains are readily observed by
166 staining with AT-rich DNA dyes (ie. DAPI and Hoechst) and are organized around the
167 nucleolus, in discreet foci within the nucleoplasm, or tethered to the nuclear periphery
168 (Padeken and Heun, 2014; Quinodoz et al., 2018). Thus, the profile of STAG1 within ES
169 cells is consistent with its localization to AT-rich heterochromatin. Given the presence of
170 an AT-hook within STAG1 and the importance of heterochromatin regulation in
171 development, we investigated whether STAG1 may have a role in heterochromatin
172 structure.
173
174 **STAG1 interacts with heterochromatin proteins and repetitive DNA.**
175 Since STAG1 was localised to nuclear heterochromatin domains, we investigated
176 whether it was also interacting with heterochromatin proteins and bound to genomic
177 sequences known to form heterochromatin, such as repeats. Constitutive
178 heterochromatin is characterized by the binding of HP1α to H3K9me2/) and plays a
179 critical role in silencing of repetitive DNA elements (Allshire and Madhani, 2018) and
180 nuclear organization (Larson et al., 2017). The periphery of the nucleolus accumulates
181 marks of constitutive heterochromatin coincident with transcriptionally inactive rDNA
182 repeats. Nucleolin is a major nucleolar protein which controls the organization of
183 nucleolar chromatin, rDNA transcription and ribosome assembly and plays important
184 roles during development and in ES cells (Kresoja-Rakic and Santoro, 2019; Percharde
185 et al., 2018). We observed nuclear colocalization between STAG1 and HP1α in dox-

186 induced SA1^{FL}-GFP cells around DAPI-dense foci (Figures S2A, B). Further, using
187 chromatin coImmunoprecipitation (coIP), we show that STAG1 interacts with both HP1α
188 and Nucleolin in ES cells (Figure 2C, S2C).

189 Previous studies have analysed STAG1 binding profiles in mouse ES cells and
190 have primarily focused on its association with protein-coding genes (Cuadrado et al.,
191 2019). A thorough investigation of STAG1 binding to repetitive sequences has not been
192 conducted. Thus, we re-analysed STAG1 chromatin immunoprecipitation followed by
193 sequencing (ChIP-seq) experiments to calculate the proportion of STAG1 peaks that
194 overlapped genes (based on promoter and exon features), repeats (within the Repeat
195 Masker annotation) introns and intergenic regions not already represented by repeats or
196 genes. Of the 18,600 STAG1 peaks identified, the majority (76%) are bound to genomic
197 elements that are distinct from protein-coding genes with a significant proportion of
198 binding sites at repetitive elements and intergenic regions (Figure 2D). Together with the
199 localizations observed by microscopy, this suggests that the role of STAG1 in ES cells
200 may extend beyond protein-coding gene regulation. While STAG1 binding is not enriched
201 compared to all genomic repeats, we asked whether specific repeat families might be
202 enriched for STAG1 binding above random expectation (Deniz et al., 2020). We found
203 several repeat families to be significantly enriched for STAG1 peaks, including those
204 within the DNA transposon and Retrotransposon classes, both known to form constitutive
205 heterochromatin. Specifically, STAG1 was enriched at SINE B2-Mm2, (previously shown
206 to be enriched at TAD borders (Dixon et al., 2012)) and B3 elements, LINE1 elements
207 (L1Tf, L1A), and several LTR families, two of which have been previously shown to be
208 associated with CTCF (LTR41, LTR55) (Schwalie et al., 2013) (Figures 2E and S2D). The
209 enrichment of Stag1 at non-coding and repetitive sequences has not been previously
210 described and points to a novel role for STAG1 in the regulation of repeats in ES cells.
211

212 **Stag1 regulates nuclear organization of heterochromatin.**

213 Since H3K9me3 is the defining histone modification of silent heterochromatin, we
214 assessed the impact of STAG1 loss and overexpression on H3K9me3 in ES cells. While
215 global levels of H3K9me3 were unchanged upon Stag1 KD (Figure S2E),

216 immunofluorescence (IF) of H3K9me3 revealed changes to heterochromatin
217 organization. H3K9me3 foci displayed a greater variation in volume compared to
218 scrambled control treated cells, suggesting that Stag1 is required for proper H3K9me3
219 compaction (Figures 2F, G). We detected similarly variable changes to global chromatin
220 accessibility, as measured by DNase I digestion. In four out of six experiments, ES cells
221 treated with Stag1 siRNAs revealed a tendency towards increased accessibility, whereas
222 two experiments showed increased compaction (Figure 2H, S2F). On the other hand,
223 Dox-inducible STAG1 expression led to a dramatic condensation of H3K9me3 into large
224 nuclear puncta compared to non-induced cells (Figures 2I, J), where STAG1 could also
225 be seen to be colocalized with H3K9me3 in the condensate. This phenotype led us to
226 investigate whether STAG1 may have characteristics found in other proteins known to
227 play a role in heterochromatin phase separation(Larson et al., 2017). We used the
228 PONDR tool (Obradovic et al., 2003) to assess potential intrinsically disordered regions
229 (IDR) (Banani et al., 2017) within STAG1. STAG1 has an overall PONDR score of 0.4397,
230 and both the N-and C-terminal divergent regions contain sequences with a high
231 propensity for intrinsic disorder (Figure 2K). Interestingly, these coincide with known
232 STAG1 domains, most notably the N-terminal AT-hook. Together our results uncover a
233 novel role for STAG1 in forming or maintaining heterochromatin structures in ES cells and
234 suggest that the terminal ends may play important roles therein.
235
236 **Stag1 expression is highly regulated in ES cells.**
237 STAG1 levels are highest in naïve 2i-grown and lower in FCS-grown ES cells, a culture
238 condition that supports a mix of naïve and primed cells (Figure S1B). This prompted us
239 to investigate whether STAG1 is regulated at the transcriptional level. To address this,
240 we employed a series of approaches to comprehensively characterize Stag1 mRNAs and
241 discovered widespread regulation of Stag1 transcription in ES cells. First, we used RACE
242 (Rapid Amplification of cDNA Ends) to characterize the starts and ends of Stag1 mRNAs.
243 Using 5' RACE, we uncovered four novel alternative transcription start sites (TSS) in ES
244 cells; one located 50kb upstream of the canonical Stag1 TSS (referred to as 'SATS', and
245 previously identified in (Feng et al., 2016)) (Figures 3A, D, S3A), one between canonical

246 exon 1 and exon 2 (referred to as alternative exon 1 or altex1) (Figure 3D), one at exon
247 6, and one at exon 7 (Figures 3B, D, S3A, B (for increased exposure). Interestingly, the
248 novel TSS located at exon 7 (e7) was preceded by a sequence located *in trans* to the
249 Stag1 gene, carrying simple repeats and transcription factor binding sites (Figure S3C).
250 While the frequency of this alternative TSS was significantly lower than the other TSSs,
251 it was identified in multiple RACE replicates, indicating it may have important functions in
252 a subset of the ES population. We also discovered widespread alternative splicing in the
253 5' region of Stag1, with particularly frequent skipping of exons 2 and 3 (e2/3Δ) and exon
254 5 (e5Δ) (Figures 3D, S3A). Using 3' RACE, we detected an early termination site in intron
255 25 and inclusion of an alternative exon 22 introducing an early STOP codon, as well as
256 several 3'UTRs (Figures 3C, D, S3D).
257 Next, PCR- and Sanger sequencing-based clonal screening confirmed that the
258 newly discovered 5' and 3' ends represent true Stag1 transcript ends, validated the
259 existence of the e2/3Δ and e5Δ isoforms, and uncovered an isoform lacking exon 31
260 (e31Δ) (Figures 3D, S3E). To determine the complete sequences of the Stag1 transcript
261 isoforms and to use a non-PCR-based approach, we performed long-read PacBio Iso-
262 seq from 2i ES RNA. This confirmed the diversity of the Stag1 5' and 3'UTRs, the e31Δ
263 isoform, multiple TSSs, including SATS, and early termination events, including in i22 and
264 i25 (Figure S3F). Importantly, these transcripts all had polyA tails, in support of their
265 protein-coding potential. Finally, we validated and quantified the newly discovered
266 splicing events by calculating the frequency (percentage spliced in (PSI)) of exon splicing
267 in our and published RNA-seq data using VAST-tools, a previously developed
268 computational method (Tapial et al., 2017) (Figure 3E, Table S2). Together, these results
269 point to a previously unappreciated diversity of endogenous Stag1 transcripts in ES cells,
270 highlighting the importance of Stag1 regulation in stem cell populations.
271
272 **The chromatin landscape reflects Stag1 transcriptional regulation.**
273 Visual inspection of the genome topology around the *Stag1* locus in our existing 2i ES
274 and neural stem (NS) cell Hi-C datasets (Barrington et al.) revealed that the *STAG1* gene
275 undergoes significant 3-D reorganization as cells differentiate (Figure 3F). First, the entire

276 *STAG1* TAD switches from the active to the repressive compartment during
277 differentiation, in line with the decrease in Stag1 levels described above. Furthermore,
278 we observed several changes to the sub-TAD architecture which corresponded to the
279 newly discovered Stag1 TSSs and TTSs described above (Figure 3F, compare 'SA1
280 transcripts' track with Hi-C maps). We quantified these changes by designing site-specific
281 UMI-4C baits guided by the Hi-C topology. In naïve ES cells, the genomic region
282 containing the alternative SATS TSS has several Nanog binding sites and makes
283 numerous contacts with the *Stag1* gene body (Figure 3F, 'Nanog bait 1'). Meanwhile, in
284 NS cells, the SATS TSS is no longer active and becomes isolated away from the *STAG1*
285 gene body due to the reinforcement of the TAD border at the canonical promoter of *Stag1*
286 (Figure 3F, 'CTCF bait'). In addition, a cluster of low-occupancy CTCF binding sites within
287 the *Stag1* gene mark an ES-specific contact insulation point giving rise to a sub-TAD. We
288 note that the observed Stag1 early termination events align with these CTCF sites. These
289 results suggest that 3D chromatin topology may play a direct role in facilitating the
290 transcriptional output of *Stag1*.
291
292 **Multiple Stag1 protein isoforms are expressed in ES cells.**
293 Stag1 transcript diversity was intriguing because many of the events were either specific
294 to ES cells or enriched compared to MEFs and NSCs (Figure 3A, E, S3E) and further,
295 because the transcript variants are predicted to produce STAG1 protein isoforms with
296 distinct structural features and molecular weights (Figure 3D, G). For example, the
297 truncation of the N terminus (e2/3Δ, e5Δ, e6 TSS and e7 TSS), and thus loss of the AT
298 hook (amino acid 3-58), could impact STAG1 association with DNA. Meanwhile, C-
299 terminal truncated Stag1 isoforms (altex22, i25 end, e31Δ) could affect STAG1-cohesin
300 interactions. Interestingly, the evolutionarily conserved Stag-domain ('SCD', AA 296-381)
301 (Orgil et al., 2015), shown to play a role in CTCF interaction (Li et al., 2020), would be
302 retained in all the transcripts identified here. Importantly, these events would yield STAG1
303 isoforms lacking either the N- or C-terminal disordered regions and could thus impact the
304 ability of STAG1 to form condensate-like structures (Figure 3H).

305 Immunoprecipitation (IP) of endogenous STAG1 revealed multiple bands
306 corresponding to the predicted molecular weights for several protein isoforms and
307 identified by mass spectrometry to contain Stag1 peptides (Figure 3I and Table S3),
308 whose levels were reduced between naïve and primed cells (Figure S3G) and sensitive
309 to Stag1 KD, alongside the canonical, full-length isoform (Figure 3J). Treatment of
310 Stag1^{NG_FKBP} ES cells with dTAG followed by Western blotting of chromatin-associated
311 proteins with an antibody to the v5 tag further confirmed the sensitivity of the isoforms to
312 dTAG-mediated degradation, validating the presence of N-terminally truncated STAG1
313 protein variants (Figure 3K). Overall, our results indicate that complex transcriptional
314 regulation gives rise to multiple Stag1 transcripts and protein isoforms with distinct
315 regulatory regions and coding potential, the majority of which are expressed specifically
316 in naïve ESCs. Our discovery of such naturally occurring STAG1 isoforms highlights the
317 importance of STAG1 in ES cells and offers a unique opportunity to define the ES-specific
318 functions of the divergent N- and C-terminal ends of STAG1 in the context of pluripotency
319 gene expression and heterochromatin regulation.
320
321 **Skewing the abundance of Stag1 isoforms promotes transitions between cell**
322 **states.**
323 To study the functional consequences of STAG1 isoform expression changes on ES cells,
324 we took advantage of our detailed understanding of Stag1 transcript diversity to design
325 custom siRNA pools to selectively target, or retain, specific isoforms (Figure 4A).
326 Alongside the siRNAs from Figure 1 (SmartPool, SP), we designed siRNAs to specifically
327 target the SATS 5'UTR (esiSATS), the 5' end (siSA1-5p) or the 3' end (siSA1-3p) of Stag1
328 mRNA (see Methods). We anticipated that the KD panels would not completely abolish
329 all Stag1 transcript variants, but rather change the relative proportions, in effect skewing
330 the levels of the N- and C-terminal ends of Stag1. 3p siRNAs were expected to
331 downregulate full-length and N-term truncated isoforms and retain C-term truncated
332 isoforms. Meanwhile 5p siRNAs would specifically retain N-term truncated isoforms.
333 siRNAs to the 5p and 3p ends of Stag1 reduce full-length Stag1 mRNA and protein
334 with similar efficiency to SP KDs (Figures 4B, C, S4A), while esiSATS reduces Stag1 by

335 50-60%, indicating that the SATS TSS functions to enhance expression of Stag1
336 specifically in naïve ES cells. We confirmed that Stag1 isoform proportions were altered
337 upon siRNA treatment using RNA-seq, RACE and immunoprecipitation. RNA-seq reads
338 aligning to Stag1 in the different siRNA treatments were quantified to represent the
339 residual N-terminal, middle and C-terminal read proportions (Figure 4D). Residual reads
340 in the SP and 3p KDs aligned primarily to the N-terminus and were depleted from the C-
341 term. While the 5p KD had the least read retention in the N-terminus, supporting the
342 expectation that residual transcripts in the 5p KD have full C-terminal ends (Figure 4D).
343 These results were further supported by quantifying specific splicing events from the KD
344 RNA-seq datasets using VAST-tools (Figure S4B). In parallel, RACE was used to
345 validate changes to the proportions of Stag1 isoforms. 5' RACE in ES cells treated with
346 5p siRNA revealed downregulation of full-length Stag1 transcript while several N-terminal
347 truncated isoforms were upregulated compared to untreated cells (Figures 4E, S3A).
348 Similarly, transcripts terminating at the canonical 3' end of Stag1 are strongly reduced in
349 the SP and 3p siRNA KD samples and to a lesser extent in the 5p KD, while the transcript
350 terminating in i25 is substantially enriched upon 3p KD (Figure 4E). Immunoprecipitation
351 of STAG1 using an antibody which recognizes the N-terminus in Stag1^{NG_FKBP} ES cells
352 treated with dTAG reveals the enrichment of STAG1-CtermΔ isoforms (Figure 4F,
353 arrows). Thus, the siRNA panel used here provide us with a powerful tool to modulate the
354 proportion of the divergent ends of STAG1 in ES cells and study their potential roles in
355 cell fate regulation.

356

357 **The C-terminus of STAG1 has a specific role in maintenance of the naïve**
358 **pluripotent state.**

359 We analyzed pluripotency gene expression in the different KDs using RT-qPCR and RNA-
360 seq. RT-qPCR results suggested that 5p and 3p KDs may have differential effects on
361 Nanog expression in serum-grown ES cells (Figure S4C). Namely, there was a consistent
362 tendency towards Nanog downregulation in 3p KD while 5p KD had little effect on Nanog.

363 GSEA supported the differential effect of the SATS, 3p and 5p KDs on naïve and primed
364 pluripotency signatures. In support of STAG1 playing a role in pluripotency, reducing
365 Stag1 levels by targeting the ES-specific SATS promoter leads to downregulation of the
366 naïve pluripotency gene signature and upregulation of the primed signature (Figure 4G),
367 reminiscent of the phenotype from SP KD (Figure 1G). A similar, more prominent loss of
368 the naïve signature was observed in 3p KD, while the opposite was true for 5p KD cells
369 where the naïve signature was maintained (Figure 4G).
370 The distinct gene expression signatures of the 3p and 5p KDs are reflected in
371 differences in cellular phenotypes. Cells treated with 3p siRNAs exhibited a further loss
372 of self-renewal potential, consistent with the loss of the naïve pluripotency signature, with
373 20% of colonies exhibiting AP-staining compared to 30% of colonies in the SP KDs, and
374 reduction of the area occupied by AP+ colonies by 40-60% (Figures 4H, S4D). This was
375 not evident in the 5p KD, where the effect on self-renewal was similar to scrambled control
376 treated cells (Figures 4H, S4D). Interestingly, unlike siRNA to Stag1, esiSATS results in
377 a variable effect on self-renewal (ranging from between 5-35% reduction in AP+ area)
378 (Figure 4H), likely because the SATS TSS is expressed in the most naïve cells of the
379 population, the frequency of which varies significantly between FCS populations. Our
380 results further confirm the importance of STAG1 in self-renewal and point to a specific
381 role for the C-terminal of Stag1 in the maintenance of the naïve pluripotent state.
382
383 **The N-terminus of Stag1 regulates heterochromatin and conversion to totipotent**
384 **state.**
385 Both the N-termΔ and C-termΔ STAG1 isoforms lost prominent IDRs (Figure 3H), thus
386 we investigated the effect of these on heterochromatin structure. ES cells treated with 5p
387 siRNAs exhibited variable H3K9me3 foci volumes, similar to the effect of SP KD (Figure
388 2G), implicating the N-terminus in H3K9me3 condensation (Figures S5A, B). On the other
389 hand, H3K9me3 foci volumes in 3p KD cells were not significantly different from scr
390 control cells. DNase I digestion of chromatin further confirmed global changes to
391 chromatin accessibility upon 5p, but not 3p, KD whereby four of six experiments revealed
392 chromatin decompaction (Figure S5C). We built up on these results by generating ES

393 cell lines expressing dox-inducible SA1$^{e5\Delta}$-GFP or SA1$^{i25-\Delta C}$-GFP, as representative of the
394 N-terminal, AT-hookΔ and the C-termΔ groups respectively. H3K9me3 foci in SA1$^{i25-\Delta C}$-
395 GFP-expressing cells revealed increased condensation compared to control cells, with a
396 similar effect as SA1^{FL}-GFP. Meanwhile, the condensation of H3K9me3 foci in SA1$^{e5\Delta}$-
397 GFP-expressing cells was significantly attenuated from SA1^{FL}-GFP cells (Figures 5A, B).
398 Thus, while both the N- and C-termini of STAG1 play a role in heterochromatin structure,
399 loss of the basic N-terminus (which interacts with DNA via the AT-hook region(Bisht et
400 al., 2013)), significantly affects condensate structure, indicating its importance in
401 heterochromatin compaction.

402 Since STAG1 was bound to repeats, which play significant roles in ES cell fate
403 determination, and are involved in heterochromatin regulation, we also profiled the
404 expression of LINE-1, IAPEz and MERVL elements in our KD panel. Surprisingly, MERVL
405 elements were significantly derepressed in 5p KD, but not in SP or 3p KD conditions
406 (Figures 5C and S5D). The effect was specific to MERVL, we saw no significant change
407 to the expression of LINE1-T or IAPEz (Figure 5C), despite the enrichment of STAG1 at
408 L1T elements (Figure 2D). Re-activation of MERVL is a hallmark of a rare subpopulation
409 of totipotent cells (termed two-cell-like, 2C-L) which spontaneously arise in ES cell
410 cultures and exhibit unique molecular and transcriptional features including chromatin
411 decompaction, reminiscent of our 5p KD (Eckersley-Maslin et al., 2016; Ishiuchi et al.,
412 2015; Macfarlan et al., 2012). Indeed, changes to MERVL expression in the 5p KD was
413 accompanied by increased expression of additional hallmark 2C-L genes and chimeric
414 transcripts (Dux, Gm6763, AW822073 and Gm4981) in serum-grown and 2i cells (Figures
415 5D, S5E). We note that the effect of 5p KD on 2C-L genes is modest in serum-grown ES
416 cells and significantly stronger in naïve ES cells. Notably, all 2C-L genes analyzed
417 remained unchanged in 3p KD conditions with a modest but insignificant upregulation in
418 SP KD. Despite the modest but coordinated changes we observed by qRT-PCR of 2C-L
419 genes (Figure 5D), GSEA using a published 2C gene set (Percharde et al., 2018)
420 revealed a specific enrichment among the upregulated genes in 5p KDs that was not

421 observed in 3p KDs (Figure 5E, S5F). Together our results point to a specific role for the
422 N-terminus of STAG1 in totipotency regulation.

423 To investigate this further, we asked whether the loss of the N-terminus of STAG1
424 drives conversion of ES cells towards a totipotent 2C-L state. We obtained ES cells
425 expressing a dox-inducible *Dux-HA*-expression construct together with a MERVL-linked
426 GFP reporter (Hendrickson et al., 2017) and used flow cytometry to measure the number
427 of GFP-positive cells in our different Stag1 KD conditions (Figures 5F, G). Chaf1 is a
428 chromatin accessibility factor previously shown to support conversion of ES cells towards
429 totipotency (Ishiuchi et al., 2015). In support of the upregulation of the 2C gene set in 5p
430 KD cells, we observed an 8-9% increase in the proportion of GFP-positive cells in 5p KD
431 conditions compared to scramble treated controls, similar to the effect of Chaf1 KD
432 (Figures 5F, G). There was a modest increase in GFP+ cells upon SP KD and no effect
433 upon 3p KD. Chaf1 and 5p double KD had an additive effect on the proportion of GFP-
434 positive cells, suggesting that the two proteins function in complementary pathways for
435 conversion towards totipotency.

436

437 **A 2C-specific Stag1 promoter.**

438 Our results point to the N-terminus of Stag1 having a protective function in the conversion
439 of ES cells towards totipotency. Given the known role for increased chromatin
440 accessibility during conversion to 2C (Ishiuchi et al., 2015) and the importance of the N-
441 terminus in heterochromatin organization reported here, we propose that the Stag1 N-
442 terminus, and specifically the AT-hook within it, is involved in heterochromatin silencing
443 by forming or maintaining a condensed heterochromatin structure. This predicts that the
444 2C-L cells which spontaneously arise within the ES population could express Stag1NΔ
445 protein isoforms to support their preferred chromatin status and reinforce their cell state.
446 To formally address this, we induced *DuxHA*-expression in the MERVL-GFP ES cells and
447 performed 5' RACE on sorted GFP+ and GFP- cells (Figure 5H). We enriched several of
448 the previously identified N-term truncated Stag1 transcripts in the GFP+ population

449 including e2/3Δ and e5Δ isoforms. Importantly, we also identified a transcript starting at
450 e7, similar to the one previously found in 5p KD ES cells (Figure 3B, S3C). Remarkably
451 however, the sequence preceding the TSS in e7 in *Dux*-induced cells was an MT2-
452 MERVL element, creating a chimeric, LTR-driven transcript akin to others specifically
453 expressed in the 2C-L state (Figures 5H, I). Thus, the 2C-L state selectively expresses
454 an N-term truncated Stag1 isoform which in turn supports the maintenance or emergence
455 of that state.

456

457 **Stag1 regulates 2C fate via changes to nucleolar structure and function.**

458 As ES cells preferentially expressing STAG1NΔ isoforms led to conversion towards
459 totipotent cell states and STAG1 is localised to the nucleolus and interacting with
460 Nucleolin, we asked whether the STAG1-induced 2C-L state could be explained by the
461 association of STAG1 with the Nucleolin/Trim28 complex known to derepress Dux targets
462 (Percharde et al., 2018). While STAG1 proteins directly interact with Nucleolin as well as
463 Trim28 (Figures 5J), Dux itself is weakly or variably deregulated in Stag1 KDs (Figure
464 5D). Similarly, while MERVL is robustly derepressed upon 5p KD (Figures 5C, F), Stag1
465 protein is not enriched at MERVL elements (Figure 2D), suggesting that the observed
466 effects are unlikely to be via *Dux*-mediated derepression of targets and may be indirect.
467 Thus, we investigated whether the Stag1-induced 2C-L state could be explained by
468 changes to nucleolar structure or function. In this context, we examined the consensus
469 sequence of the rDNA locus and note that there are several SINE elements located within
470 the intergenic spacer (IGS). Analysis of Stag1 ChIP-seq alignments to this region is
471 complicated by the repetitive nature of the region, however there was evidence of
472 possible Stag1 binding to the B3 elements within the IGS, suggesting that Stag1 may
473 directly support nucleolar structure and function (Figure S6B).

474 We explored this hypothesis by investigating the effect of Stag1 loss on rRNA
475 transcription. ES cells were pulsed with 5-ethynyl uridine (EU) which becomes actively
476 incorporated into nascent RNA and enables detection of newly synthesized RNA either

477 spatially by immunofluorescence or globally by flow cytometry. Samples for
478 immunofluorescence were co-stained with an antibody to Nucleolin to quantify changes
479 in nascent rRNA transcription. Cells treated with scramble siRNA showed a distinct
480 nucleolar structure and the EU signal could be seen throughout the nucleus, with a strong
481 enrichment within the nucleolus as expected from rRNA expression (Figures 6A, B). A
482 significant reduction in nascent rRNA signal was observed in all KD conditions compared
483 to the si scr control. Interestingly, while the medians between the three siSA1 KDs were
484 not dramatically different, the effect of the 5p KD on rRNA signal distribution was
485 significantly different from the 3p KD, revealing different roles for the N and C-terminal
486 ends of Stag1 in nucleolar function. In parallel, we used flow cytometry to validate the
487 above and quantify the effect on global levels of transcription upon Stag1 loss. In support
488 of the immunofluorescence results, we observed a reduction in nascent transcription in
489 all siSA1 KDs compared to scrambled control (Figures 6C, D). SP and 5p KD reduced
490 nascent transcription by 39% and 47% respectively, the bulk of which is likely from the
491 observed change to rRNA expression. Meanwhile, cells treated with 3p siRNA exhibited
492 a 16% reduction in global transcription compared to scrambled controls, further implying
493 that the N- and C-terminal ends of Stag1 likely target different pools of newly synthesized
494 transcripts.

495 Finally, given the changes to nascent rRNA transcription and the known role for
496 translation in 2C-L state conversion (Eckersley-Maslin et al., 2016; Hung et al., 2013), we
497 measured effects on translation. Indeed, previous results showed a stronger effect of
498 Stag1 KD on Nanog protein compared to Nanog mRNA levels (Figures 1D, E). ES cells
499 were pulsed with L-homopropargylglycine (HPG), an amino acid analog of methionine
500 and flow cytometry was used to quantify the impact of Stag1 KD on global protein
501 synthesis. The proportion of cells that had reduced incorporation of HPG increased
502 significantly in the SP and 5p siRNA treated cells compared to scramble controls (32%
503 and 35% of si Scr) (Figures 6E, F). We did observe a mild affect on global nascent
504 translation in 3p KD treated cells (16% of si scr), although this was not significantly
505 different from scramble controls (Figures 6E, F). Overall, our work has uncovered a novel

506 role for Stag1 in translation control and proposes that Stag1 may act directly at the rDNA
507 locus or via Nucleolin to influence the regulation of rRNA expression. To our knowledge,
508 a role for Stag1 in translation regulation has not been previously described and offers a
509 completely new perspective on how Stag1 impacts cell fate, moving beyond its known
510 roles in gene expression by regulating chromatin loops.

511 **DISCUSSION**

512 Cell fate transitions during early development are accompanied by extensive
513 transcriptional and epigenetic reprogramming. In addition to changes in protein-coding
514 gene expression, repetitive sequences play an integral role in developmental
515 programmes (Jachowicz et al., 2017; Macfarlan et al., 2012; Percharde et al., 2018).
516 Repetitive sequences are spatially clustered into heterochromatin domains to facilitate
517 their regulation (Allshire and Madhani, 2018; Padeken and Heun, 2014). How cohesin
518 function, or Stag proteins contribute to heterochromatin or repeat regulation has not been
519 extensively studied in mammalian cells, despite the importance of both for cell identity.

520 While previous studies have implicated Stag proteins in cell fate decisions
521 (Cuadrado et al., 2019; Viny et al., 2019), they have primarily focused on the impact to
522 protein-coding gene regulation. By discovering a novel role for Stag1 in heterochromatin
523 structure, repetitive elements and translation control, we significantly expand our
524 understanding of Stag1 functions and thus deliver new insight into the mechanisms by
525 which Stag proteins and cohesin regulation impact cell identity and lead to disease.
526 Further, by comprehensively characterising naturally occurring Stag1 protein isoforms,
527 we identify distinct roles for the N- and C-termini of Stag1 which shed mechanistic light
528 on the reported distinct functions of the Stag paralogs (Cuadrado and Losada, 2020). For
529 example, the N-terminal containing AT-hook of Stag1 represses heterochromatin
530 condensation and its loss leads to global changes in chromatin compaction and
531 reprogramming of ES cells towards totipotent 2C-L cells. Our observation that the AT-
532 hook domain is required for heterochromatin compaction may explain the dominance of
533 Stag1 in stem cell populations since Stag2 does not have an AT-hook and
534 heterochromatin must be tightly regulated during early development.

535 We have characterized diverse transcription–regulatory events at the Stag1 locus
536 in stem cell populations giving rise to protein isoforms with distinct regulatory domains.
537 Importantly, this extensive Stag1 protein heterogeneity supports a continuum of cell
538 states within the population since experimentally induced imbalances in isoform
539 proportions skews cell fate probabilities. Using RACE in cells enriched for the 2C-L fate,

540 we find evidence for skewed expression of particular Stag1 isoforms, arguing that
541 individual ES cells may naturally predominantly express particular Stag1 protein isoforms.
542 Interestingly, the proportions of Stag1 isoforms likely originate in part from the stochastic
543 process of splicing (Fiszbein and Kornblihtt, 2017; Gabut et al., 2011; Salomonis et al.,
544 2010), which is itself further randomized by the fluctuating chromatin landscape (Mateo
545 et al., 2019; Rodriguez et al., 2019), acting as a feedback mechanism for further Stag1
546 diversity. Thus, the naturally occurring Stag1 isoforms described here may act as intrinsic
547 sources of noise that both directly and indirectly support cellular plasticity.

548 We propose that a balance of Stag1 protein isoforms is required for variation in
549 gene and repeat expression and thus, a continuum of cellular states. The N-terminal
550 containing AT-hook of Stag1 supports global chromatin compaction and heterochromatin
551 condensation, suggesting that it plays an important role in clustering repetitive sequences
552 into heterochromatin condensates for their regulation. Indeed, we observe dramatic
553 changes to rRNA expression in 5p KD cells where isoforms lacking the N-terminus are
554 specifically retained. Moreover, we note that our live cell microscopy also revealed
555 condensate-like structures within the nucleoplasm. It is possible that the C-terminus of
556 Stag1 regulates euchromatin condensate formation to support pluripotency gene
557 networks. Several lines of evidence support this. First, many of the genes that are
558 downregulated upon 3p KD are super-enhancer-associated genes which are known to
559 form condensate structures (Sabari et al., 2018; Whyte et al., 2013). Further, global
560 nascent transcription is reduced in 3p KD cells, although rRNA is not as affected as in the
561 5p KD, suggesting that the C-term of Stag1 may have a role in regulating a different
562 subset of nascent transcripts. Thus, the Stag1 protein heterogeneity discovered here
563 provides the necessary fluctuations in the levels and composition of the Stag1 disordered
564 regions to impact the stability of both euchromatin and heterochromatin condensates,
565 thereby supporting plasticity within the population.

566 Stag1 knockout (Stag1$^{\Delta/\Delta}$) ES cells give rise to mice which survive to E13.5
567 (Remeseiro et al., 2012b). At first this observation seems at odds with our report that
568 Stag1 is required for pluripotency. However, our observations may in fact explain why the

569 Stag1$^{\Delta/\Delta}$ mouse model does not exhibit early embryonic lethality. In this model, only the
570 5' region of Stag1 was targeted, meaning that the Stag1 isoforms lacking the N-terminus
571 may still be retained in the targeted ES cells. This is consistent with our results showing
572 that 5p KD cells have not lost their ability to self-renew nor is their pluripotency gene
573 signature affected. It further suggests that changes to heterochromatin may exist in these
574 cells.

575 The role for Stag1 in heterochromatin structure described here may be quite
576 general, and the fact that Stag1 binds to HP1a and repetitive sequences known to form
577 constitutive heterochromatin suggests that this is true. Indeed, the AT-hook domain of
578 Stag1 has been shown to be important for telomere cohesion (Bisht et al., 2013). Here
579 we have specifically uncovered a role for Stag1 in nucleolar structure and function in stem
580 cells. Transcriptionally inactive rDNA arrays are associated with the periphery of the
581 nucleolus and accumulate marks of constitutive heterochromatin while actively
582 transcribed rDNA loci are looped inside the nucleolus (Padeken and Heun, 2014). We
583 observe Stag1 at both the interior and periphery, suggesting that it may be involved in
584 multiple aspects of rDNA and nucleolar regulation. Stag1 is enriched at SINE elements
585 and we note that the intergenic spacer region of the rDNA consensus contains multiple
586 B3 and B2_Mm2 sequences, strongly suggesting that the changes in nascent rRNA
587 transcription are direct effects of Stag1 loss and implicates Stag1 in the formation or the
588 maintenance of the nucleolus. Indeed, CTCF has previously been implicated in nucleolar
589 structure by tethering insulators to the periphery (Yusufzai et al., 2004). As well as
590 impacting global translation, the change to nucleolar structure may also indirectly support
591 the emergence of the 2C state by destabilizing complexes such as Nucleolin/Trim28
592 which are required for the derepression of repeat elements (Percharde et al., 2018).

593 Finally, a role for Stag proteins or cohesin in translation regulation has, to our
594 knowledge, not been described before despite the fact that translation has important roles
595 in development (Buszczak et al., 2014). How Stag1 contributes to translation in ES cells
596 and whether it plays similar roles in other stem or progenitor populations are important
597 future questions. Our results offer new perspectives on how Stag proteins and cohesin
598 regulation contribute to cell identity during development and in disease.

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Author Contributions

D.P. and S.H. conceived the project. D.P. designed and performed all the experiments on ES cells with assistance from S.W. S.W. performed all protein analysis, generated the SA1-NG-FKBP ES cell line, performed the Spinning Disk microscopy and helped with the siRNA knockdown experiments. W.V. performed all bioinformatic analyses with the exception of the Stag1 enrichments at repeat elements, which was done by M.B. P.D. and S.P. provided advice on CRISPR targeting. D.P. and S.H. formatted all figures and wrote the manuscript with input from all authors.

Declaration of Interests

The authors declare no competing interests.

621 **Figure legends**

622 **Figure 1. STAG1 is required for pluripotency.**

623 (A) Log2 fold change of pluripotency factors and cohesin subunit gene expression
624 assessed by qRT-PCR during embryonic stem cell (ES) differentiation towards Epiblast-
625 like (EpiLC) cells. Multiple primer pairs were used for SA1 and SA2 mRNA. Data are
626 derived from two biological replicates.

627 (B) Whole cell protein extracts (WCL) from serum-grown (FCS) or naïve (2i) ES cells,
628 EpiLC and mouse embryonic fibroblasts (MEF) and analyzed by western blot (WB) for
629 levels of SA1, SA2 and Smc3.

630 (C) WB analysis of SA1 levels in WCL, cytoplasmic and chromatin fractions upon
631 treatment with scrambled siRNAs (siScr) or SmartPool SA1 siRNAs (siSA1) for 24hrs in
632 2i ES cells. Tubulin (Tub) and H3 serve as fractionation and loading controls.

633 (D) Relative expression of Nanog mRNA by qRT-PCR in 2i- (left panel, n=19) or FCS-
634 grown (right panel, n=20) ES cells after no (UT), si scr or si SA1 treatment. Whiskers and
635 boxes indicate all and 50% of values respectively. Central line represents the median.
636 Asterisks indicate a statistically significant difference as assessed using 2-tailed T-test. *
637 $p<0.05$, ** $p<0.005$, n.s., not significant.

638 (E) Mean Fluorescence Intensity (MFI) of Nanog protein assessed by
639 Immunofluorescence in 2i ES cells treated with si scr, a second siRNA control to
640 Luciferase (esi Luc) and si SA1. Cells were stained for Nanog, SA1 and counterstained
641 with DAPI. Quantifications and statistical analysis were done as above. Each treatment
642 was repeated a minimum of two times (n>100/condition). **** $p<0.0005$.

643 (F) Volcano plot displaying the statistical significance (-log2 p-value) versus magnitude of
644 change (log2 fold change) from RNA-sequencing data produced in FCS ES cells treated
645 with si scr or siSA1 for 24hrs (n=3). Vertical blue dashed lines represent changes of 2-
646 fold. Selected genes associated with pluripotency and differentiation have been
647 highlighted.

648 (G) Enrichment score (ES) plots from Gene Set Enrichment analysis (GSEA) using
649 curated Naïve or Primed gene sets (see Methods). Negative and positive normalized
650 (NES) values point to the gene set being over-represented in the top most down- or up-
651 regulated genes in SA1 KD ES cells, respectively. Vertical bars refer to individual genes
652 in the gene set and their position reflects the contribution of each gene to the NES.

653 (H) Area occupied by AP+ colonies in ES cells treated with si scr and si SA1 from three
654 independent biological replicates where n>50 colonies/condition were counted.

655 (I) CRISPR/Cas9 was used to knock-in a NeonGreen-v5-FKBP tag on both alleles of
656 endogenous Stag1 at the C-terminus ($SA1^{NG\text{-}FKBP}$). The resultant Stag1 protein is 42kDa
657 larger. Shown are known features of SA1 including the N-terminal AT-hook (AT) and the
658 stromalin conserved domain (SCD). WB analysis of SA1 levels in a targeted ES clone
659 after treatment with DMSO-only or dTAG for 16 hrs. Tubulin (Tub) serves as a loading
660 control.

661 (J) Analysis of the area of AP+ colonies as above in wildtype ES and in a $SA1^{NG\text{-}FKBP}$ ES
662 clone treated with DMSO-only or dTAG for 16 hrs. Data is represented as mean ± SEM
663 of three independent experiments and relative to the DMSO control.

664 See also Figure S1.

665

666 **Figure 2. STAG1 associates with AT-rich heterochromatin.**

667 (A) Live-cell Spinning Disk confocal images of three $SA1^{NG\text{-}FKBP}$ cells counterstained with
668 Hoechst. Arrows indicate notable regions of overlap of SA1 and Hoechst, including inside
669 the nucleolus and at Hoechst-dense foci (top cell), and at the nucleolar periphery (bottom
670 cells).

671 (B) Mean Intensity of SA1-NeonGreen signal from (A) within the whole nucleus or
672 Hoechst-dense foci as identified using Imaris in the $SA1^{NG\text{-}FKBP}$ clone treated with DMSO
673 or dTAG. n=270 cells/condition.

674 (C) co-Immunoprecipitation of endogenous SA1 with Smc3, HP1a and Nucleolin in ES
675 cells.

676 (D) Percentage of SA1 peaks (unique reads) at promoters, exons, transposable elements
677 introns and intergenic sequences.

678 (E) STAG1 ChIP-seq data (unique and multimapping reads) aligned to full-length Repeat
679 elements of the indicated SINE, LINE and LTR families.

680 (F) Confocal images of Immunofluorescence (IF) to SA1 and H3K9me3 in siscr or siSA1
681 treated ES cells, counterstained with DAPI.

682 (G) Imaris quantification of the volume of H3K9me3 foci from (F). Box plots and statistical
683 analysis were done as above. Each treatment was repeated twice (n>100/condition).
684 p<0.05.

685 (H) Global chromatin accessibility as detected by DNase I digestion of genomic DNA at
686 different concentrations from 200,000 cells/condition in siScr or siSA1 ES cells.

687 (I) Confocal images of IF to GFP and H3K9me3 in ES cells expressing full-length SA1
688 tagged with GFP (SA1-FL), counterstained with DAPI.

689 (J) Imaris quantification of the volume of H3K9me3 foci from (I). Box plots and statistical
690 analysis were done as above. n>50/condition. p<0.0005.

691 (K) PONDR (Predictor of Natural Disordered Regions) analysis of SA1 protein using VSL2
692 predictor at http://www.pondr.com showing consecutive stretches of disordered regions
693 corresponding to the N- and C-terminus of Stag1.

694 See also Figure S2.

695

696 **Figure 3. Diverse Transcription-Regulatory Control of Stag1 in ES cells.**

697 (A, B) 5' Rapid Amplification of cDNA ends (RACE) for SA1 in ES and EpiLC cells. Arrows
698 indicate bands which were cloned and sequenced. In A, red star indicates SATS TSS and
699 red arrow indicates canonical TSS. In B: red indicates full length Stag1 with both SATS
700 and can TSSs; dark blue indicates alternatively spliced variants, skipping of various exons
701 in 5' region; light blue indicates the TSSs at e6, e7.

702 (C) 3' RACE for SA1 in ES cells. Arrows indicate bands which were cloned and
703 sequenced. Red indicates canonical full-length end; green indicates end in i25.

704 (D) Top, schematic of the Stag1 gene annotation in mm10 and the identified TSS and
705 TTSs from RACE indicated. Bottom, aligned sequence clones from the PCR mini-screen
706 and their predicted impact on the SA1 protein, right. Green arrows and red bars within the
707 transcripts indicate start of the coding sequence and the TTS respectively. Shown also
708 are the regions which code for the AT hook and SCD domains.

709 (E) Percent Spliced In (PSI) calculations based on VAST-Tools analysis of RNA-seq from
710 multiple 2i (blue) and FCS (red) datasets (see Methods for details of libraries). Data are
711 shown relative to Neural Stem cell (NSC) frequencies to highlight the events that are ES-
712 specific.

713 (F) Genome topology at the Stag1 locus. Hi-C contact maps in ES (2i) and NS cells of
714 the 900kb region on chromosome 9 containing the Stag1 topologically associated domain
715 (TAD). TADs are denoted with a vertical line and as repressed (orange) or active (blue).
716 Shown also are tracks for Genes, Nanog and CTCF ChIP-seq as well as a track indicating
717 the directionality of CTCF binding sites (red, forward; blue, reverse). Aligned to the Gene
718 track are also the SA1 transcripts discovered above where red represents the
719 untranslated regions and blue the coding body. UMI-4C-seq viewpoints (asterisks on the
720 ChIP tracks) are positioned to the leftmost CTCF site ('CTCF bait') and to the Nanog site
721 40 kb upstream of the Stag1 canonical TSS ('Nanog bait'). For each bait, UMI information
722 for each cell type is shown as well as the comparative plots where red represents an
723 enrichment of contacts in ES compared to NS.

724 (G) Top, cartoon depicting functional domains within Stag1 protein, including the AT-hook
725 (aa 3-58); Stromalin conserved domain (SCD, aa 296-381) and the C-terminus. Bottom,
726 the predicted Stag1 protein isoforms based on transcript analysis with estimated sizes for
727 each isoform. Purple boxes in the 105kDa and 90kDa isoforms represent retained introns.

728 (H) PONDR tracks as before shown for the N-terminal truncated (top) and C-terminal
729 truncated (bottom) transcripts.

730 (I) Chromatin immunoprecipitation of SA1 from ES cells.

731 (J) WB analysis of SA1 isoforms in chromatin fractions from ES cells treated with siscr
732 and siSA1. H3 serves as a fraction and loading control.

733 (K) Chromatin immunoprecipitation for the v5 tag in $SA1^{NG\text{-}FKBP}$ ES cells treated with
734 DMSO-only or dTAG. Note, SA1 bands now run 42kDa higher due to the addition of the
735 tag.

736 See also Figure S3, Table S1 and Table S2.

737

738 **Figure 4. Fluctuations in the levels of the Stag1 isoforms skews cell fates.**

739 (A) Schematic of the siRNA pools used in this study. esiRNA SATS represents
740 'enzymatically-prepared' siRNAs (see Methods).

741 (B) Relative expression of SA1 mRNA by qRT-PCR in FCS ES cells after no treatment
742 (UT), or upon si scr, si SA1 SP, si 3p, si 5p or esi SATS treatment. Data is represented
743 as mean ± SEM, n=3. * $p<0.05$

744 (C) WB analysis of SA1 levels in FCS ES WCL upon treatment with the same siRNA
745 panel from (B). Tubulin serves as a loading control.

746 (D) RNA-seq reads (as transcripts per million, TPM) aligning to sectioned Stag1 in
747 datasets from the various siRNA pools and presented as relative to untreated ES RNA-
748 seq. N-terminal reads include SATS and exons 1-8, Mid reads are from exons 12-19 and
749 C-terminal reads include exons 20-25 and exons 26-34.

750 (E) Left, 5' and Right, 3' RACE for SA1 in ES cells treated with the siRNA panels. Arrows
751 indicate bands which were cloned and sequenced and colour-coded as before.

752 (F) Chromatin immunoprecipitation using an N-terminal Stag1 antibody in SA1$^{NG\text{-}FKBP}$ ES
753 cells treated with DMSO-only or dTAG for 16 hrs. Green arrow indicates residual C-
754 terminal truncated Stag1 isoforms.

755 (G) Enrichment score (ES) plots from GSEA using Naïve or Primed gene sets as in Figure
756 1G and RNA-seq data from the different siRNA treated ES cell samples.

757 (H) Area occupied by AP+ colonies in ES cells treated with the siRNA panel from three
758 independent biological replicates. n>50 colonies/condition were counted.

759 See also Figure S4.

760

761 **Figure 5. Stag1 N-terminus represses heterochromatin and promotes transition**
762 **towards totipotency.**

763 (A) Confocal images of IF to GFP and H3K9me3 in ES cells expressing SA1-FL-GFP,
764 SA1-exon5Δ-GFP or SA1-CtermΔ-GFP. Nuclei were counterstained with DAPI.

765 (B) Imaris quantification of the volume of H3K9me3 foci per cell from (A). Box plots and
766 statistical analysis were done as before. n>50/condition. ** $p<0.005$, ****$p<0.0005$.

767 (C, D) Relative expression of (C) repeat elements; MERVL, LINE1-T and IAPEz RNA
768 (n=8 biological replicates each) and (D) 2C-L genes; Gm6763 (n=6) and Dux (n=5) by
769 qRT-PCR in FCS ES cells after treatment with the siRNA panel. Boxes and whiskers as
770 before. * $p<0.05$, n.s, not significant as per 2-tailed T-test. NB. The p-value for Gm6763
771 expression in 5p KD vs UT = 0.07.

772 (E) Enrichment score (ES) plots from GSEA using a published 2C-L gene set (Methods)
773 and RNA-seq data from the 3p and 5p siRNA treated ES cell samples.

774 (F) Representative FACS analysis of the proportion of ES cells expressing a MERVL-
775 GFP reporter in the different siRNA treated cells and including siRNA to Chaf1 as a
776 positive control. Percentage of MERVL-GFP+ cells based on Flo-Jo analysis is shown in
777 red.

778 (G) Proportion of MERVL-GFP+ cells in the different siRNA conditions relative to the
779 siChaf1 positive control. Data is represented as mean ± SEM of four independent
780 experiments. * $p<0.05$, ** $p<0.005$, n.s, not significant as per 2-tailed T-test.

781 (H) 5' RACE for SA1 in Dux-HA, MERVL-GFP ES cells sorted for GFP+ cells. Arrows
782 indicate bands which were cloned and sequenced and colour-coded as before.

783 (I) Sequence of the 5'RACE product identifying a novel Stag1 TSS with direct splicing of
784 exon7 to MT2_MERVL element.

785 (J) Co-Immunoprecipitation of SA1 with Nucleolin and Trim28 in ES cells.

786 See also Figure S5.

787

788 **Figure 6. rRNA expression and nascent translation upon Stag1 loss.**

789 (A) Confocal images of IF to Nucleolin (Ncl) and Nascent RNA in ES cells treated with the
790 siRNA panel. Nuclei were counterstained with DAPI. Note the accumulation of nascent
791 RNA within the nucleolus.

792 (B) Imaris quantification from (A) of the mean intensity of nascent RNA (EU-488) within
793 the nucleoli, as defined by Nucleolin signal. Box plots and statistical analysis were done
794 as before. Data are from two biological replicates, $n>50$/condition, except for siSA1 5p
795 where $n>35$. ** $p<0.005$, ****$p<0.0005$ as per 2-tailed T-test.

796 (C, D) Global analysis of Nascent transcription by measuring EU-488 incorporation using
797 Flow cytometry. (C) representative Flo-Jo analysis of EU incorporation in ES cells treated
798 with the siRNA KD panel. (D) Quantification of the change in EU incorporation relative to
799 si Scr treated cells. Data are from two biological replicates *$p<0.05$, **$p<0.005$ as per 2-
800 tailed T-test.

801 (E, F) Global analysis of Nascent translation by measuring HPG-597 incorporation using
802 Flow cytometry. (E) Representative Flo-Jo analysis of HPG incorporation in ES cells
803 treated with the siRNA KD panel. (F) Quantification of the change in EU incorporation

804 relative to si Scr treated cells. Data are from four biological replicates *$p<0.05$, **$p<0.005$
805 as per 2-tailed T-test.

806 See also Figure S6.

807

808 **METHODS**

809 **Embryonic stem cell culture and siRNA-mediated knockdown.**

810 Male mouse E14 embryonic stem (ES) cells were cultured in serum (FCS) or naïve (2i) conditions.
811 Serum-cultured cells were grown on 0.1% gelatin-coated plates in GMEM, 10% FCS (Sigma),
812 NEAA, Na Pyruvate, 0.1 mM ßMercaptoethanol (BMe), Glutamax, and freshly added LIF
813 (1:10,000). 2i-cultured cells were grown on plates coated with Fibronectin, in
814 DMEM:F12/Neurobasal 1:1, KnockOut Serum Replacement, N2, B27, Glutamax, 1μM
815 PD0325901, 3μM CHIR9902, 0.1 mM BMe, and freshly added LIF as above. DuxHA/MERVL-
816 GFP cells were cultured in 2i conditions. siRNAs were purchased from Horizon Discovery
817 (previously Dharmacon) or Sigma (for 'enzymatically-derived' esiRNAs). siRNA knockdowns
818 (KDs) were performed for 24hr with the exception of those in Figure 5 which were performed for
819 72hr. Knockdowns were performed in 6-well plates where 200,000 cells were seeded for 72 hr
820 KDs, and 400,000 for 24 hr KD. 50pmol siRNAs were transfected using RNAiMax Lipofectamine
821 at the time of seeding, and after 48 hrs for 72hr timepoints. Two siRNA controls were used,
822 scrambled (scr) was D-001810-10 and Luciferase (esiLuc) control purchased from Sigma. siSA1
823 'SmartPool' (SP) was derived from equimolar ratios of commercial siRNAs (D-041989-02, -04, -
824 05, -06, -07, -08). siSA1 5p was a custom Duplex siRNA sequence
825 (AGGAGCAGGUCGUGGAAGAUU). siSA1 3p was derived from equimolar ratios of commercial
826 siRNAs J-041989-05, -07, -08. esiRNA to SATS was purchased from Sigma as a custom-made
827 product to the entire SATS 5'UTR (mm10 chr9:100,597,794-100,598,109).

828

829 **qRT-PCR analysis**

830 Total RNA was isolated using Monarch RNA prep kit (NEB). Reverse transcription was performed
831 on 0.5 μg DNase-treated total RNA using Lunascript RT (NEB) in 20μl reactions. qPCR was
832 performed using 2x SensiFAST SYBR No-ROX kit (Bioline) in 20 μl reactions using 1μl of RT
833 reaction as input and 0.4μM each primer.

834

835 **Alkaline Phosphatase (AP) assay and quantification**

836 Cells were seeded in 6 well plates and transfected with siRNAs at the time of plating as above.
837 After 24 hrs, cells were collected for RNA isolation and KD efficiency analyzed by qRT-PCR. Cells
838 from each condition were counted and 1,000 cells per well seeded into a new 6-well plate. Cells
839 were re-transfected after 48 hrs using 5 pmol of siRNAs. Cells were fed every day. Four days

840 after seeding cells at clonal density, the cells were assayed for alkaline phosphatase (AP)
841 expression using StemTAG Alkaline Phosphatase staining kit (Cell Biolabs CBA-300). AP stained
842 cells were imaged in 6-well plates using a M7000 Imaging System (Zeiss) with a 4X objective and
843 a Trans-illumination brightfield light source. For quantification, AP-high and AP-low colonies from
844 each condition were counted. Area occupied by AP-high colonies was also measured using
845 ImageJ, and plotted as fraction of total area of all colonies.
846
847 **RACE (Rapid Amplification of cDNA Ends) and PCR mini screen**
848 RACE was performed using GeneRacer kit (RLM RACE, Invitrogen L1500). 2μg of total RNA was
849 used as input. Final products were amplified by nested PCR, using Kapa 2x MasterMix. First PCR
850 was done in a 50μl reaction using 1μl RT as input, 25 cycles. DNA was purified using Qiagen
851 PCR Purification kit, and nested PCR was performed on a tenth of the first PCR for 30 cycles.
852 Viewpoint for 5'RACE was in exon 2 (Fig 3A) or exon 8 (Fig 3B) of Stag1. Viewpoint for 3'RACE
853 was in exon 23 (Fig 3C). RACE primer details can be found in Table S3. PCR products were
854 excised from the gel, A-tailed using Klenow exo- (NEB) and cloned into pCR4-TOPO vector
855 (Invitrogen). At least three clones were sequenced per PCR product. For the PCR Mini-Screen,
856 forward primers at either SATS or canonical 5' UTR were used with reverse primers either at the
857 end of Stag1 canonical coding sequence, or at the end of coding sequence in intron 25 (see Table
858 S3). PCR was performed using Kapa 2x MasterMix. DNA was excised from the gel, A tailed, and
859 cloned into pCR4-TOPO. At least six clones per PCR product were Sanger-sequenced.
860 Sequences from the PCR Mini-screen were aligned using Minimap2 (2.14-r884) in 'splice' mode
861 to ensure long read splice alignment (Fig 3D and S3A).
862
863 **PONDR Predictions**
864 Internally disordered regions were predicted using VSL2 predictor at http://www.pondr.com.
865
866 **CRISPR-Mediated Stag1 Knock-in Cell Line Generation**
867 The guide RNA targeting Stag1 3' terminal coding region was designed using Tagin Software
868 (http://tagin.stembio.org) and purchased from IDT. Lyophilised gRNA was rehydrated in RNA
869 duplex buffer (100μM). The single stranded oligodeoxynucleotides (ssODN) encoding
870 mNeonGreen (mNG)-V5-FKBP12^{F36V} and the left and right homology arms was designed using
871 the software tool ChopChop (https://chopchop.cbu.uib.no) and purchased as a High-Copy Amp-
872 resistant plasmid from Twist Bioscience. 2.2μl gRNA (100μM) was mixed with 2.2μl tracrRNA

873 ATTO 550nm (IDT) and annealed together. The RNA duplex was then incubated with 20μg S.p
874 Cas9 Nuclease V3 (IDT) for 10min at room temperature and stored on ice prior to transfection.
875 Linearised KI sequence was mixed with 100% DMSO and denatured at 95°C for 5min. The
876 ssODN was plunged immediately into ice. The RNP complex was mixed with confluent 2i-grown
877 ES cells re-suspended in P3 transfection buffer (Lonza) before being transferred to an
878 electroporation microcuvette well (Lonza). Transfection was performed using a 4D Amaxa
879 electroporator. Post-nucleofection, the cells were seeded into a fibronectin-coated 6 well plate
880 with fresh ES media. The media was changed daily for four days before being expanded into a
881 T75 flask. Confluent ES cells were FACS sorted for GFP+ population (BD FACS Aria Fusion Cell
882 Sorter) and sparsely seeded into 10 cm plates. Clones were manually picked into 96 well plates
883 and expanded for selection by v5 IF, genotyping and Sanger sequencing.
884
885 **Dox-inducible Stag1-GFP isoform cell lines**
886 Stag1 isoforms were cloned into pCW57.1 vector (Addgene 41393), modified using Gibson
887 assembly to include an EGFP tag at the 3'end of the Gateway cassette, using Gateway
888 recombination by LR clonase. For primers used to clone the isoforms see Supplementary Table
889 S3. Plasmids were transfected into 2i-grown ES cells using Lipofectamine 3000 and cells grown
890 in Puromycin-supplemented media (1μg/ml) for ten days to make stable lines. Isoform expression
891 was induced using 2μg/ml Doxycycline for 24 hrs, and the population enriched for GFP-positive
892 cells using FACS. For IF experiments, isoforms were induced by adding Dox for 48 hours.
893
894 **Protein Lysates, Fractionations and Western blotting.**
895 Whole cell lysates (WCL) were collected by lysis in RIPA buffer (150mM NaCl, 1% NP-40
896 detergent, 0.5% Sodium Deoxycholate, 0.1% SDS, 25mM Tris-HCl pH 7.4, 1mM DTT) and
897 sonicated at 4°C for x5 30 second cycles using Diagenode Bioruptor. Insoluble material was
898 pelleted and the supernatant lysate was quantified using BSA Assay (Thermo Scientific). For
899 cellular fractionations, a cellular ratio of $5x10^6$ cells/80μl buffer was maintained throughout the
900 protocol. Cells were re-suspended in Cell Membrane Lysis Buffer (0.1% Triton X, 10mM HEPES
901 pH 7.9, 10mM KCl, 1.5mM MgCl2, 0.34M sucrose, 10% glycerol, 1mM DTT), incubated on ice for
902 5min and centrifuged for 5min at 3700rpm to collect the cytoplasmic sample. The pellet was
903 washed and then re-suspended in Nuclear Lysis Buffer (3mM EDTA, 0.2mM EGTA, 1mM DTT)
904 and incubated on ice for 1 hr. Nuclear lysis was aided by sonication with a handheld homogeniser
905 (VWR) for 10sec at 10min intervals. The nucleoplasmic supernatant and chromatin pellet were

906 separated by centrifugation at 9000rpm for 10min at 4°C. The chromatin pellet was re-suspended
907 in 160μl 2X Laemmli Buffer (Bio-Rad). Equal volumes of each fraction were used for Western
908 Blotting (WB). Cytoplasmic and nucleoplasmic protein samples were diluted in 2X Laemmli Buffer
909 and boiled for 5min at 95°C, then loaded on a 4-20% SDS-PAGE gel (Bio-rad) or a 3-8% Tris
910 Acetate gel (Invitrogen). Proteins were wet transferred onto a PDVF membrane (Millipore) and
911 assessed for successful transfer with Ponceau Red (Sigma). The membrane was blocked with
912 10% milk and incubated with primary antibodies in 1% milk, 0.1% Tween-PBS overnight at 4°C.
913 Membranes were imaged with SuperSignal West Femto Maximum Sensitivity (Thermo) on an
914 ImageQuant.
915
916 **Chromatin Co-Immunoprecipitation (co-IP)**
917 Cells were re-suspended in 0.1% NP-40-PBS (1ml/1x10^7 cells) with 1X Protease Inhibitors
918 (Roche) and 1mM DTT, and centrifuged at 1500rpm for 2min at 4°C. The pellet was re-suspended
919 in Nuclear Lysis Buffer (3mM EDTA, 0.2mM EGTA, 1X Protease Inhibitors, 1mM DTT), vortexed
920 for 30sec before being incubated on a rotator for 30min at 4°C and centrifuged at 6500g for 5min
921 at 4°C to isolate the glassy chromatin pellet. This was re-suspended in High Salt Chromatin
922 Solubilisation Buffer (50mM Tris-HCl pH 7.5, 1.5mM MgCl2, 300mM KCl, 20% glycerol, 1mM
923 EDTA, 0.1% NP-40, 1mM Pefabloc, 1X Protease Inhibitors, 1mM DTT) with Benzonase (Sigma)
924 (6U/1x10^7) and incubated on rotator for 30min at 4°C. Chromatin was digested with 3x 10sec
925 sonication at 30% intensity with a Vibra-Cell probe. The supernatant was collected by
926 centrifugation at 1300rpm for 30min at 4°C, and then diluted to 200mM KCl concentration with no
927 KCL buffer. 30μl of Dynabeads (Invitrogen) were used per co-IP. Beads were washed 2x in
928 200mM KCl IP Buffer, re-suspended in IP Buffer with 10μg of the IP antibody, or an IgG-containing
929 serum to match the species of the IP antibody and placed on rotator for 5h at 4°C. Beads were
930 washed 3x in IP buffer and then incubated in 1mg chromatin lysate on a rotator overnight at 4°C.
931 The beads were washed, re-suspended in 2X Laemmli Buffer (Bio-Rad), boiled for 10min at 95°C
932 and used for WB as above.
933
934 **Immunofluorescence and Microscopy**
935 ES cells were cultured on fibronectin or gelatin-coated cover glass in 6-well plates. Cells were
936 fixed in 4% Paraformaldehyde for 5min and incubated in 0.1% Triton X-PBS for 10min before
937 being washed and blocked in 10% FCS-PBS for 20min. Primary antibodies were diluted in 10%
938 FCS, 0.1% Saponin (Sigma) and incubated overnight at 4°C. The next day, the cells were

939 incubated with an Alexa fluorophore-conjugated secondary antibody diluted in 10% FCS, 0.1%
940 Saponin for 1 hr at room temperature, washed and mounted on cover slides with ProLong
941 Diamond Antifade Mountant with DAPI (Invitrogen). Z-stacks imaging of fixed cells was done
942 using a LSM 880 confocal microscope (Zeiss) with a 63X oil objective. Analysis was performed
943 using Imaris 9.6 (Oxford instruments). Live cell imaging was performed using a 3i Spinning Disc
944 confocal microscope (Zeiss). Stag1-mNG-V5-FKBP12^{F36V} cells were seeded in an 8-chambered
945 coverglass (Lab-Tek II) and DMSO or dTAG (500nM) were added for 24hr before imaging.
946 Directly prior to imaging, cells were incubated with Hoechst 33342 (BD Pharmingen) for 45min,
947 and then replaced with fresh 2i ES media. Cells were imaged as confocal Z-stacks using DAPI
948 and GFP lasers with a 63X objective and 1.4 Numerical Aperture.
949
950 **Antibodies used in this study**

Protein	Catalogue No.	Company	Figure references
Stag1/SA1, N-term epitope	ab4455	Abcam	1B, C, I, S1C, K, 2C, S2C, E, 3J, S3G, 4C, F, 5J
Stag1/SA1, C-term epitope	ab4457	Abcam	2F, S5A, 3I
Stag2/SA2	A300-158A	Bethyl	1B, S1C
Smc3	ab9263	Abcam	1B, 2C
Nanog	ab70482	Abcam	1E, S1F
Tubulin (Tub)	T5168	Sigma	1C, 1I, S2E, 4C, S6A
Actin	Mab8929	Novus	S1C
H3	ab1791	Abcam	1C
v5	14-6796-82	Invitrogen	3K
HP1a	2616	Cell Signalling	2C, S2B, C
Nucleolin (Ncl)	ab22758	Abcam	2C, 5J, 6A, S6A
H3K9me3	ab8898	Abcam	2F, I, S2E, 5A, S5A
H3K4me3	ab8580	Abcam	S2E
Alexa488-anti-GFP (GFP)	A-21311	ThermoFisher	2I, S2A, B, 5A
Trim28	MA1-2023	ThermoFisher	5J

951
952 **Chromatin accessibility analysis by DNase I treatment.**
953 DNase I digestion was performed as in Huo et al (Mol Cell, 2020), with modifications. 200,000
954 cells per condition were resuspended in DNaseI digestion buffer (50mM Tris-HCl pH 7.5, 5mM

955 $MgCl_2$, 0.1 mM $CaCl_2$, 0.2% Triton X, 5mM Na butyrate, protease inhibitor) and incubated for 10
956 min at room temp. DNase I (50U/μl, ThermoFisher ES0523) was diluted in 1X DNase buffer and
957 added to cells to have the following Units/μl: 1.25, 2.5, 5, 7.5 and 10. Cells were incubated at 37C
958 for 10 min in the thermoblock, shaking at 1,000 rpm. To stop the digestion, 10μl 0.5M EDTA and
959 10ul 10% SDS was added and incubated 10-15min at room temperature. 400μl TE buffer was
960 added, followed by 10μl PureLink RNaseA (ThermoFisher, 20mg/ml, 12091021), and incubated
961 at 37C for 1hr. Proteinase K digestion was then performed by adding 100μg of Proteinase K and
962 incubating at 55C for 3hr to overnight. To isolate DNA, 30μl of 5M NaCl and 525 μl Isopropanol
963 was added, DNA precipitated at room temperature for 15 min, pelleted by high speed
964 centrifugation at 4C for 20min, dried, resuspended in 20μl of TE buffer and loaded on 1% agarose
965 gel. The gel was stained using SYBR Green.
966
967 **Nascent transcription and translation analysis**
968 For nascent transcription analysis, we used the Click-iT® RNA Alexa Fluor® 488 HCS Assay
969 (Invitrogen C10327). ES cells were labelled with 1mM EU for 45min at 37C in fresh ES
970 media. Cells were fixed in solution or onto coverslips with 3.7% paraformaldehyde and
971 permeabilised with 0.5% Triton-X solution. Cells were incubated with the Click-iT reaction
972 cocktail for 30min. Cells were then either processed further for Immunofluorescence as
973 per methods described above (directly to the blocking step) or analysed by flow cytometry
974 on a BD Fortessa X20. For the Nascent translation analysis, Click-iT™ HPG Alexa Fluor™ 594
975 Protein Synthesis Assay Kit (Invitrogen C10429) was used. Cells were pre-incubated in
976 Methionine-free media for 30 min in the 37C incubator before addition of L-homopropargylglycine
977 (HPG) at 50μM. Cells were incubated with HPG for 30 min, then collected, fixed, permeabilized,
978 and stained using Click-It reaction in low retention tubes. HPG incorporation was measured by
979 Flow Cytometry. FACS analysis (in Figures 5,6) was done with FloJo software (version 10.7.1).
980
981 **Next generation Sequencing and Analysis**
982 Genomic data generated in this study (RNA-seq, PacBio-seq and UMI4C-seq) was submitted to
983 GEO with the Accession GSE160390.
984
985 **RNA sequencing (RNA-seq) library preparation and sequencing**
986 ES cells were treated for 24hrs with siRNA pools to Stag1 (SA1) and two sets of control siRNAs,
987 scrambled (SCR) and Luciferase (Luc). There are three replicate sets for SP KD and two for the

988 siRNA pools (SATS, 3p, 5p). Total RNA was isolated using NEB Monarch RNA prep kit. 1μg of
989 total RNA was rRNA-depleted using NEBNext rRNA depletion kit (Human/Mouse/Rat). Libraries
990 were prepared from 10-50ng rRNA-depleted total RNA, depending on availability of material,
991 using NEBNext Ultra II directional RNAseq kit according to manufacturer's instructions using 8
992 cycles of PCR. All ESC FCS libraries were rRNA depleted and only the ESC 2i libraries were
993 PolyA-enriched before library prep. Two rounds of PolyA+ enrichment were performed. RNA-seq
994 libraries were sequenced on the Illumina HiSeq3000 platform, 75bp paired-end or single-end
995 reads. Reads were quality controlled using FASTQC. RNA-seq data was processed using the
996 RNA-seq Nextflow pipeline (v19.01.0), with the following parameters –aligner hisat2 –genome
997 mm10, with –reverse_stranded specified for paired-end samples. FeatureCounts output was
998 parsed through edgeR (v3.16.5) and DESeq2 (v1.14.1) to generate normalised expression
999 counts. The normalised counts for RNAseq (Figure 1) were calculated in edgeR. Low expressed
1000 genes were removed (rowSum cpm <2 across SCR and SA1SP replicates), normalisation factors
1001 were calculated using calcNormFactors and dispersions estimated using estimateDisp. The
1002 edgeR volcano plot statistics were calculated using the exactTest and topTags functions. To
1003 generate the normalised counts for RNAseq experiments required to calculate the log2FC GSEA
1004 ranked lists, the FeatureCounts output for all experiments was combined into a single table and
1005 read into DESeq2. A DESeq2 object was built using the function DESeqDataSetFromMatrix and
1006 estimation of size factors and dispersions were calculated using the DEseq function. Normalised
1007 counts were calculated using the 'counts' function. Low expressed genes (rowSum normalised
1008 count <10 across all samples) were removed.
1009
1010 **GSEA**
1011 Broad Institute GSEAPreranked (v4.0.3) was used to determine the enrichment of curated
1012 genesets within our RNA-seq data. For each sample a ranked list was generated with genes
1013 ranked in descending order by their log2FC value using normalised expression scores from
1014 DEseq2. Log2FC per gene was calculated between the KD and its respective SCR using the
1015 following calculation: Log2(normalised_counts KD +1) - log2(normalised_counts SCR +1). In the
1016 case of experiments with multiple KD replicates, the average log2 normalised count was used.
1017 Three gene sets were assayed in this study, 'naïve pluripotency', 'primed pluripotency' and '2C
1018 signatures'. The naïve and primed pluripotency gene sets were curated in-house from Fidalgo M
1019 et al. (CSC, 2016) where genes were selected if they had $\geq$2 fold change. The naïve and primed
1020 gene sets contained 661 and 580 genes respectively. The 2C signatures gene set (147 genes)

1021 was obtained from Percharde M et al. (Cell, 2018). Gene sets were classed as having significant
1022 enrichment if the p-value was ≤0.05 and the normalised enrichment score (NES) exceeded +/- 1.
1023
1024 **VAST-TOOLS**
1025 VAST-TOOLS was used to generate Percent Spliced In (PSI) scores, a statistic which represents
1026 how often a particular exon is spliced into a transcript using the ratio between reads which include
1027 and exclude said exon. Paired-end RNA-seq datasets were submitted to VAST-TOOLS (v2.1.3)
1028 using the Mmu genome (Tapial J et al, Gen Res 2017). Briefly, reads are split into 50nt words
1029 with a 25nt sliding window. The 50nt words are aligned to a reference genome using Bowtie to
1030 obtain unmapped reads. These unmapped reads are then aligned to a set of predefined exon-
1031 exon junction (EJJ) libraries allowing for the quantification of alternative exon events. The output
1032 was further interrogated using a script which searches all hypothetical EEJ combinations between
1033 potential donors and acceptors within Stag1. PSI scores could be obtained providing there was
1034 at least a single read within our RNAseq data that supported one of these potential events. Some
1035 datasets were combined to have enough reads for the analysis. See Table S1 for PSI values and
1036 names of RNA-seq libraries used for analysis in Figure 3E and S4B.
1037
1038 **Quantifying sectioned Stag1**
1039 Stag1 was split into 5 sections; SATS, e1-e8, e12-e19, e20-e25, e26-e34. Using Kallisto
1040 (v0.46.1), raw RNAseq reads were used to quantify each section of Stag1. Kallisto was run in
1041 quant mode, using the –rf-stranded parameter, outputting a TPM per Stag1 section. A line plot
1042 was generated showing TPM in relative to UT.
1043
1044 **PacBio library, sequencing and analysis**
1045 ES cells were cultured in naïve 2i conditions and PolyA-enriched mRNAs were hybridized to a
1046 custom Biotinylated oligonucleoltide probe set. Post-capture, mRNAs were amplified using the
1047 Clontech SMARTer PCR cDNA Synthesis Kit with 9 cycles and used in the SMRTbell library prep
1048 according to manufacturers instructions. The library was sequenced on the SMRTseq 2000
1049 platform. PacBio reads were processed through the SMRTLINK v8.0.0 IsoSeq3 pipeline. 403,995
1050 Circular consensus sequences (CCS) were generated using default parameters (--minPasses =
1051 1, --min-rq = 0.8, CCS Polish = No). Further refining through lima (removal of adapters and correct
1052 orientation of sequences), poly-A trimming and concatemer removal resulted in 265,106 full length

1053 non-chimeric (FLNC) reads. FLNC reads were aligned to the mm10 genome using Minimap2 with
1054 the following parameters (-ax splice, -uf, -k14).
1055
1056 **ChIP-seq Analysis**
1057 Previously published Stag1 Chromatin Immunoprecipitation-sequencing (ChIP-seq) datasets
1058 from ES 2i cells (GSE126659, only Replicate 1 and 2 libraries) were trimmed using trim_galore
1059 and aligned to mm10 using bowtie2. Peak detection was performed with MACS2 using uniquely
1060 reads (MAPQ≥2). Peaks were overlapped with genomic features in a hierarchical manner
1061 (promoters > exons > repeats > introns > intergenic), and overlap frequency was compared with
1062 a randomly shuffled version of the peaks. To identify repeat families enriched for STAG1 peaks,
1063 a previously described pipeline was used (Deniz O et al. Nat Comm, 2020) that compares family-
1064 levels overlap frequency with that observed in 1,000 permutations of random peak shuffling.
1065 Coverage profiles across specific TE families were generated using HOMER and including multi-
1066 mapping reads (MAPQ<2).
1067
1068 **UMI-4C library preparation.**
1069 $1x10^7$ cells were fixed at RT for 10min in 1% formaldehyde and fixation was quenched with 0.125M
1070 Glycine for 5min. Cells were then lysed on ice in 10ml Lysis Buffer (10mM NaCl, 10mM Tris-HCl
1071 pH 8.0, 0.25% NP40, protease inhibitor) for 30min, followed by 10 strokes of douncing using a
1072 tight pestle. Nuclei were pelleted, 8min 700 rcf, washed in 1ml 1.2X DpnII buffer in Protein LoBind
1073 tubes (Eppendorf) and resuspended in 500 μl 1.2X DpnII buffer. **15ul of 10% SDS** was added
1074 and incubated for 1hr at 37°C shaking at 650 rcf. **50ul of 20% TritonX** was added to quench the
1075 SDS and incubated for 15 min at 37°C with shaking. **750U of DpnII was added and incubated**
1076 **overnight at 37**°C **with interval shaking. The next morning, nuclei were** pelleted at 4°C by
1077 650 rcf for 5 min and resuspended in 500μl 1X DpnII buffer. 500U DpnII was added and incubated
1078 for an additional four hours. The nuclei were washed twice in 100 μl of 1X T4 Ligase Buffer and
1079 resuspended in 200 μl Ligase Buffer. **6ul of T4 DNA Ligase was added and incubated for 3hr**
1080 **at 16°C.** Nuclei were then pelleted, resuspended in 200 μl 1x fresh Ligase Buffer, 6μl of T4 DNA
1081 Ligase added, and incubated overnight at 16°C. Samples were treated with 20μl of ProtK (NEB
1082 Molecular Biology Grade), incubated for 3 hrs at 55°C and 5 hrs at 65°C to reverse crosslinks.
1083 Samples were treated with **RNase A** (PureLink, Invitrogen) for 1 hr at 37°C and DNA was
1084 extracted and precipitated overnight. For library preparation, 3x5μg of ligated DNA was sonicated
1085 using Covaris (10% duty cycle, intensity 5, cycle burst 200, 70sec). Samples were end-repaired

1086 using DNA PolII Klenow Large Fragment (NEB), A-tailed using Klenow (exo-) (NEB), and Illumina
1087 indexed adapters ligated using Quick DNA Ligase (NEB). Reactions were denatured at 95°C for
1088 3 min, placed on ice, and purified using 1.2X SizeSelect AmpPure beads to recover ssDNA.
1089 Libraries were amplified using GoTaq (Promega), with 20 cycles for PCR1 and 15 cycles for
1090 nested PCR2 on 50% material from 1st PCR. For custom UMI bait sequences, see Table S3.

1091

1092 **Hi-C and UMI-4C-seq analysis**

1093 Hi-C libraries were analysed as previously described (Barrington 2019). UMI-4C tracks were
1094 processed using the 'umi4cPackage' pipeline (v0.0.0.9000) (Schwartzman, O et al. Nat Meth
1095 2017). Briefly, raw reads are parsed through the UMI-4C pipeline, those reads containing the bait
1096 and padding sequence are retained and de-multiplexed. Reads lacking the padding sequence are
1097 considered non-specific and are removed from further analysis. Retained reads are split based
1098 on a match to the restriction enzyme sequence to create a segmented fastq file. The first 10 bases
1099 of read 2 are extracted and attached to the segments derived from each read pair. Mapping to
1100 mm10 is done with Bowtie2. Read pairs that have reverse complement segments are mapped to
1101 a restriction fragment ID, with the fragment ID, strand and distance from each end represented
1102 within a fragment-chain table. UMI filtering is used to determine the number of molecules
1103 supporting each ligation event. The resulting UMI-4C tracks are then imported into R, and data
1104 from multiple bait replicates can be merged by summing the molecule counts per ligated fragment,
1105 at which point contact intensity profiles and domainograms around the viewpoint can be
1106 generated (see Figure 3). The contact intensity profile represents the mean number of ligations
1107 within a genomic window, with the resolution of the contact intensity profile being determined by
1108 the window size (set to 15 here). The domainogram reports the mean contact per fend at a series
1109 of window sizes, a stacked representation of contact intensity values in increasing window sizes
1110 from 10 to 300 fragment ends, their colour can be used to identify peak locations. ES and NSC
1111 contact profiles were compared after normalisation to correct for bias (see Schwartzman et al for
1112 further details). For the compared profiles, the total molecule count for restriction fragment ends
1113 for each are calculated at three ranges around the viewpoint. One profile is selected as a
1114 reference and the second is scaled to the first using the ratio in total molecule counts between
1115 the two profiles as the scaling factor. Below the contact profile is the profile resolution indicator,
1116 which shows the number of fends required to include at least 15 UMI molecules. The darker the
1117 colour, the larger the window size required. The domainogram at the bottom represents the log2

ratio between the domainogram values of the compared profiles and highlights locations where ES has more contacts than NSC or vice versa.

1121 **Supplementary titles and legends.**

1122 **Figure S1. Stag1 is required for pluripotency, Related to Figure 1.**

1123 (A) Cartoon of the cohesin complex including the core trimer subunits of Smc1a, Smc3
1124 and Rad21 complexed with either Stag1 or Stag2.

1125 (B) Relative expression of Stag1 and Stag2 mRNA by qRT-PCR in 2i- or FCS-grown ES
1126 cells, EpiLCs and MEFs. Data is represented as mean ± SEM of two independent
1127 experiments and relative to Actin control expression.

1128 (C) WCL from naïve (2i) ES and EpiLCs, sorted for cells in the G1 phase and analyzed
1129 by WB for levels of SA1 and SA2. Actin serves as a loading control.

1130 (D) Relative expression of Stag1 mRNA by qRT-PCR in FCS- (left panel, n=20) or 2i-
1131 grown (right panel, n=19) ES cells upon treatment with si scr or si SA1. Whiskers and
1132 boxes indicate all and 50% of values respectively. Central line represents the median.

1133 (E) Cell cycle analysis of Hoechst-stained 2i ES cells after treatment with siScr or siSA1
1134 siRNAs for 24hrs. Shown are the percentages of cells in G1 or G2 phases. These are the
1135 same cells that were used for the RNA-sequencing experiments shown in Figure 1F.

1136 (F) MFI of Stag1 protein assessed by IF in 2i ES cells treated with si scr and si SA1. Cells
1137 were stained for SA1 and counterstained with DAPI. Data is from the second biological
1138 replicate (replicate 1 is in Fig 1E), n>100 cells/condition **** p<0.0005.

1139 (G) Enrichment score (ES) plots from GSEA using Naïve or Primed gene sets as in Figure
1140 1G and RNA-seq data from the other two siSA1 treated ES cell samples.

1141 (H) AP+ colonies in ES cells (purple), as a percentage of all colonies (pink and purple)
1142 treated with siscr and si SA1. Data are from three independent biological replicates and
1143 merged. See Fig 1H for the individual experiments.

1144 (I-K) CRISPR/Cas9 targeting of the C-terminus of endogenous Stag1. (I) Schematic of
1145 the targeted locus and location of primers used for genotyping. (J) Shown are four ES cell
1146 clones representing integration into both alleles, one allele and a wildtype clone. (K) IF
1147 using the v5 tag in a homozygote clonal line treated with dTAG for 16 hrs shows complete
1148 loss of the NeonGreen signal.

1149

1150

1151 **Figure S2. Stag1 associates with heterochromatin, Related to Figure 2.**

1152 (A, B) Confocal IF images in dox-inducible full-length SA1-GFP ES cells (SA1-FL-GFP)
1153 stained with (A) GFP and (B) HP1a. Nuclei were counterstained with DAPI. NB,
1154 colocalization of SA1-GFP with DAPI-dense foci and HP1a.

1155 (C) Replicate experiment for co-Immunoprecipitation of endogenous SA1 with Smc3,
1156 HP1a and Nucleolin in ES cells.

1157 (D) Enrichment of Stag1 ChIP at additional LTR and DNA transposon elements.

1158 (E) WCL from ES treated with the siRNA panel and analyzed by WB for levels of SA1,
1159 H3K9me3 and K3K4me3. Tubulin serves as a loading control.

1160 (F) Global chromatin accessibility as detected by DNase I digestion of genomic DNA in
1161 siScr or siSA1 ES cells. NB, In two of six biological replicates, we observed increased
1162 compaction upon SA1 loss, as is shown here.

1163

1164 **Figure S3. Transcription-Regulatory Control of Stag1 in ESC, Related to Figure 3.**

1165 (A) Aligned Stag1 transcript variants identified from 5'RACE in Figure 3A, B. Arrows refer
1166 to the bands on the RACE gels which were cloned and sequenced. NB, the diversity of
1167 skipping events that all result in a functional loss of the 5' end of Stag1.

1168 (B) Over-exposure of the 5'RACE gel shown in Figure 3B to better show the small RACE
1169 products.

1170 (C) Close-up of the 5' RACE sequence that identified a new TSS at exon 7 spliced directly
1171 to a sequence in trans carrying regulatory elements.

1172 (D) Close-up of the 3' RACE sequence that identified a new alternative TTS in intron 25
1173 (sequence shown in dark blue).

1174 (E) Initial PCR screen in ES 2i and MEFs using various combinations of forward (5')
1175 primers (SATS, canonical TSS, Alt exon 1 TSS) and reverse (3') primers (canonical TTS,
1176 Alt intron 25 TTS). NB. SATS is only expressed in ES cells; canonical, full-length Stag1
1177 is more expressed in ES compared to MEFs; and the alternative intron 25 TTS is most
1178 often expressed with a canonical TSS.

1179 (F) Top, Strategy for mRNA capture of cohesin genes for Pac-Bio long read RNA-
1180 sequencing. Bottom, full-length transcripts sequenced on the PacBio platform includes
1181 many isoforms already discovered using RACE and PCR cloning methods.

1182 (G) Chromatin Immunoprecipitation of SA1 in ES and EpiLCs.

1183

1184 **Figure S4. Fluctuations in Stag1 isoforms skews cell fates, Related to Figure 4.**

1185 (A) Relative expression of Stag1 mRNA by qRT-PCR in FCS- (leftmost and rightmost
1186 panels, n=7) or 2i-grown (middle panel, n=6) ES cells upon si scr or the si SA1 panel.
1187 Whiskers and boxes as before. NB, all siRNAs knockdown Stag1 levels to a similar extent
1188 with the exception of esiRNA SATS which reduced Stag1 by ~40-50%.

1189 (B) Percent Spliced In (PSI) calculations based on VAST-Tools analysis of RNA-seq from
1190 ES cells treated with the siRNA panel. Data are shown relative to untreated ES cells.

1191 (C) Relative expression of Nanog mRNA by qRT-PCR in FCS-grown ES cells upon si scr
1192 or the si SA1 panel (n=13). Whiskers and boxes as before. NB, the modest, but different
1193 influence of the 5p and the 3p KDs on Nanog levels.

1194 (D) AP+ colonies in ES cells (purple), as a percentage of all colonies (pink and purple)
1195 treated with the siRNA panel. Data are from two independent biological replicates and
1196 merged. See Fig 4H for the individual experiments.

1197

1198 **Figure S5. Stag1 N-terminus represses heterochromatin and reprograms cells,**
1199 **Related to Figure 5.**

1200 (A) Confocal IF images of GFP and H3K9me3 in ES cells treated with siRNA panel. Nuclei
1201 were counterstained with DAPI.

1202 (B) Imaris quantification of the volume of H3K9me3 foci per cell from (A). Box plots and
1203 statistical analysis were done as before. Data are from two biological replicates,
1204 n>50/condition. ****p<0.0005.

1205 (C) Global chromatin accessibility as detected by DNase I digestion of genomic DNA in
1206 siScr or siSA1 3p or 5p ES cells. NB. There was no effect on accessibility in the 3p KD
1207 but a consistent decompaction observed in the 5p KD.

1208 (D, E) Relative expression of (C) MERVL and (D) genes associated with the 2C-L state
1209 by qRT-PCR in 2i-grown ES cells after 72hr of treatment with si scr or the si SA1 panel.
1210 Data are represented as mean +/- SEM from n=3 biological replicates. NB, the effect of
1211 the 5p KD on the expression of these hallmark 2C-L genes/repeats is much more
1212 significant than in the FCS-grown ES cells shown in Figure 5C, D.

1213 (F) Enrichment score (ES) plots from GSEA using 2C gene sets as in Figure 5E and
1214 RNA-seq data from the different siRNA treated ES cell samples.

1215

1216 **Figure S6. Stag1 deregulates nascent rRNA expression, Related to Figure 6.**

1217 (A) WCL from ES cells treated with the siRNA panel and analyzed by WB for global levels
1218 of Nucleolin. Tubulin serves as a loading control.

1219 (B) Top, cartoon of the consensus Mus musculus ribosomal DNA (rDNA) (GenBank:
1220 BK000964.3), showing the ribosomal genes and the intergenic spacer (IGS) region which
1221 contains several SINE elements (Red, B2_Mm2; Green, B3). Bottom, Stag1 ChIP
1222 replicates aligned to this region. NB, possible Stag1 binding to the B3 elements in the
1223 IGS.

1224

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Figure 1.

Pezic et al.

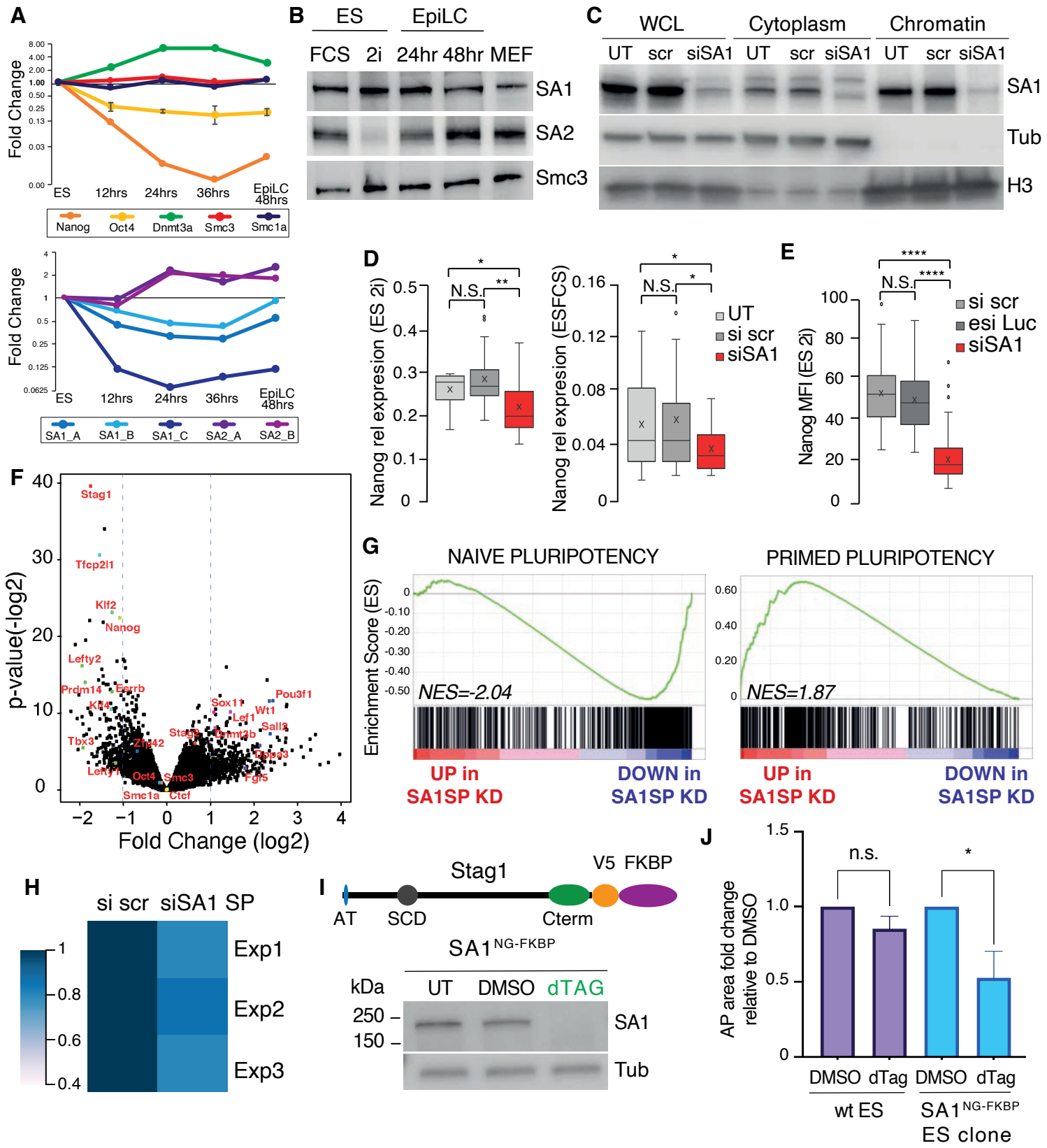
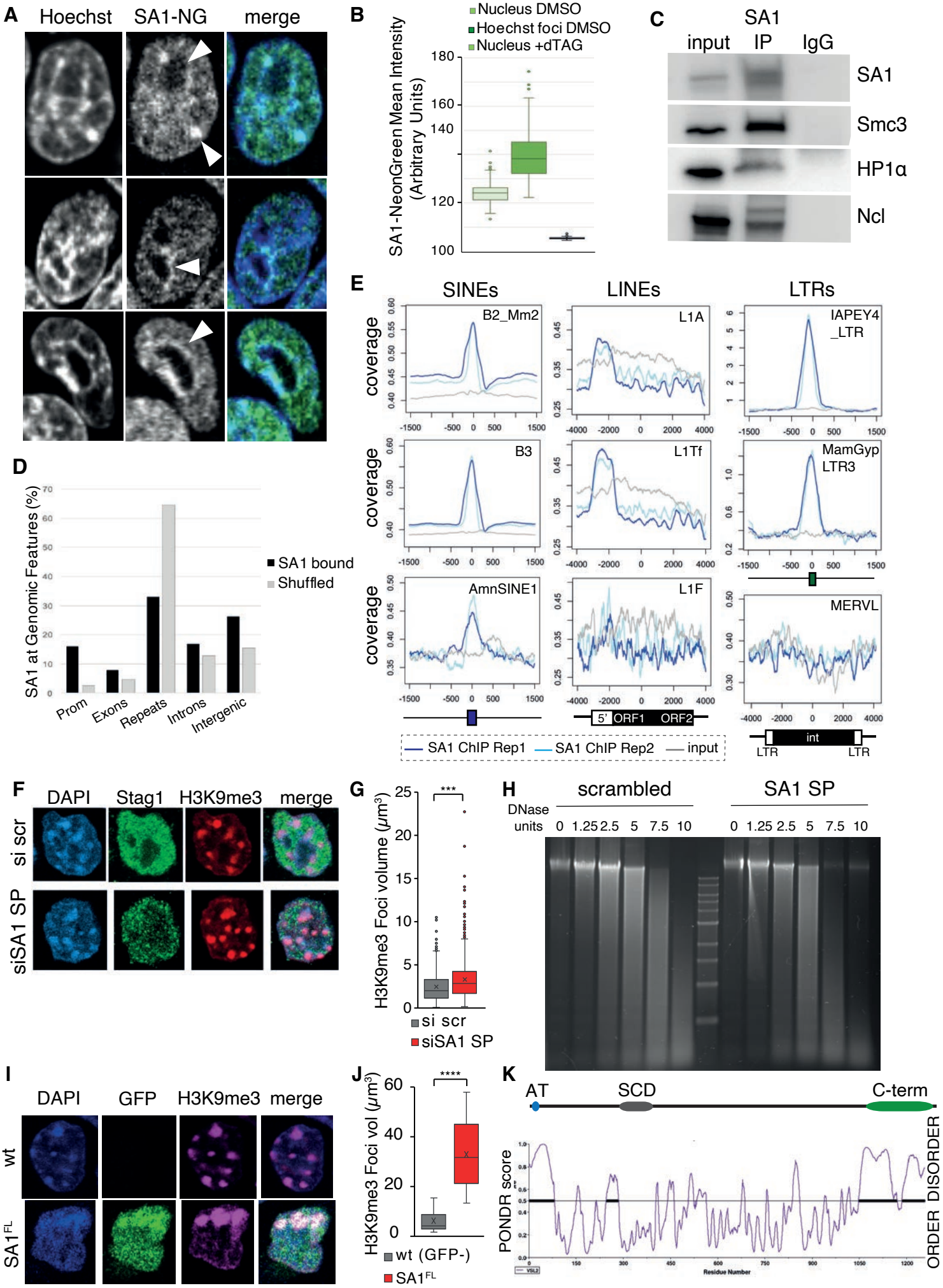


Figure 2. *Pezic et al.*



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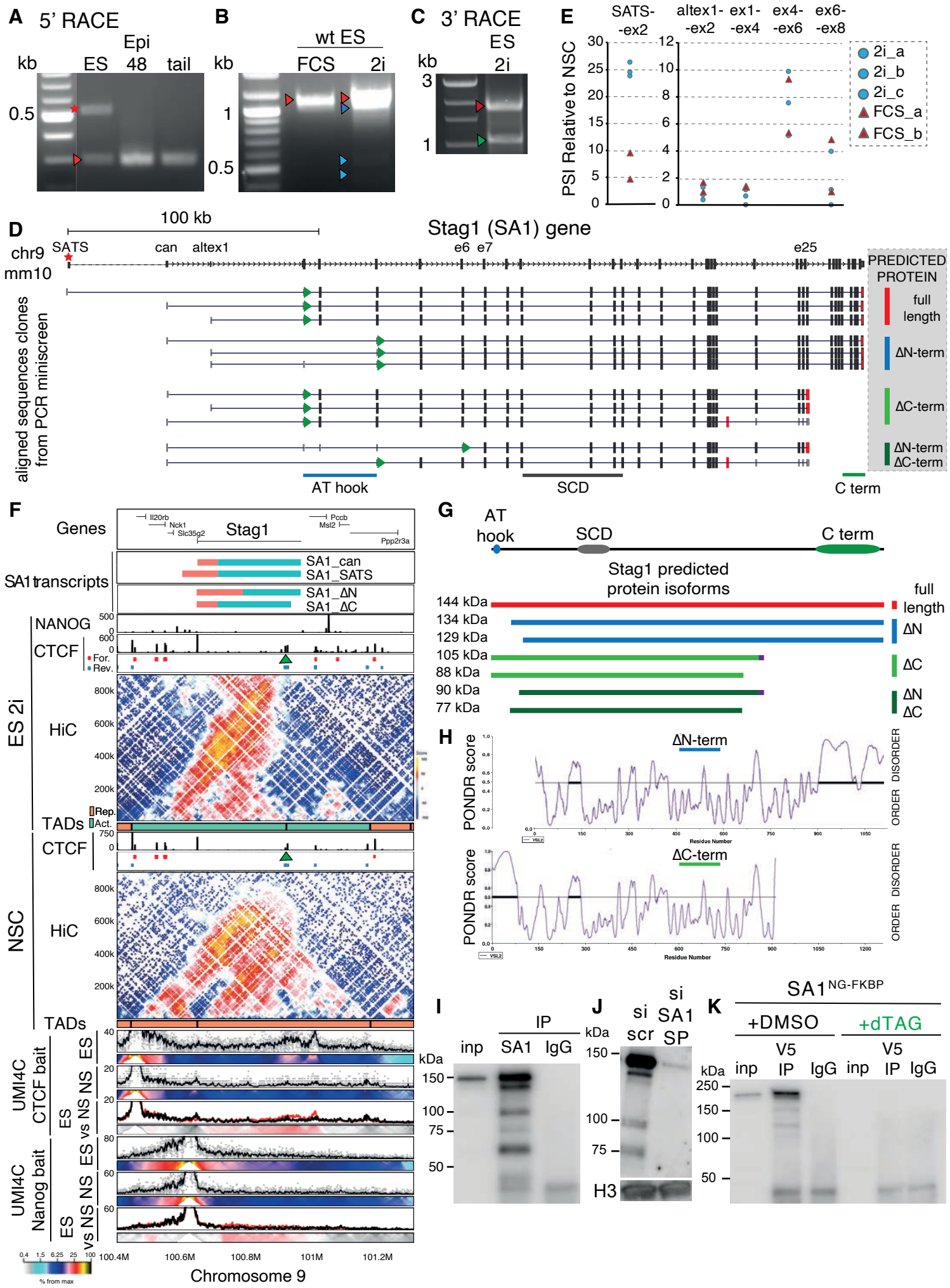

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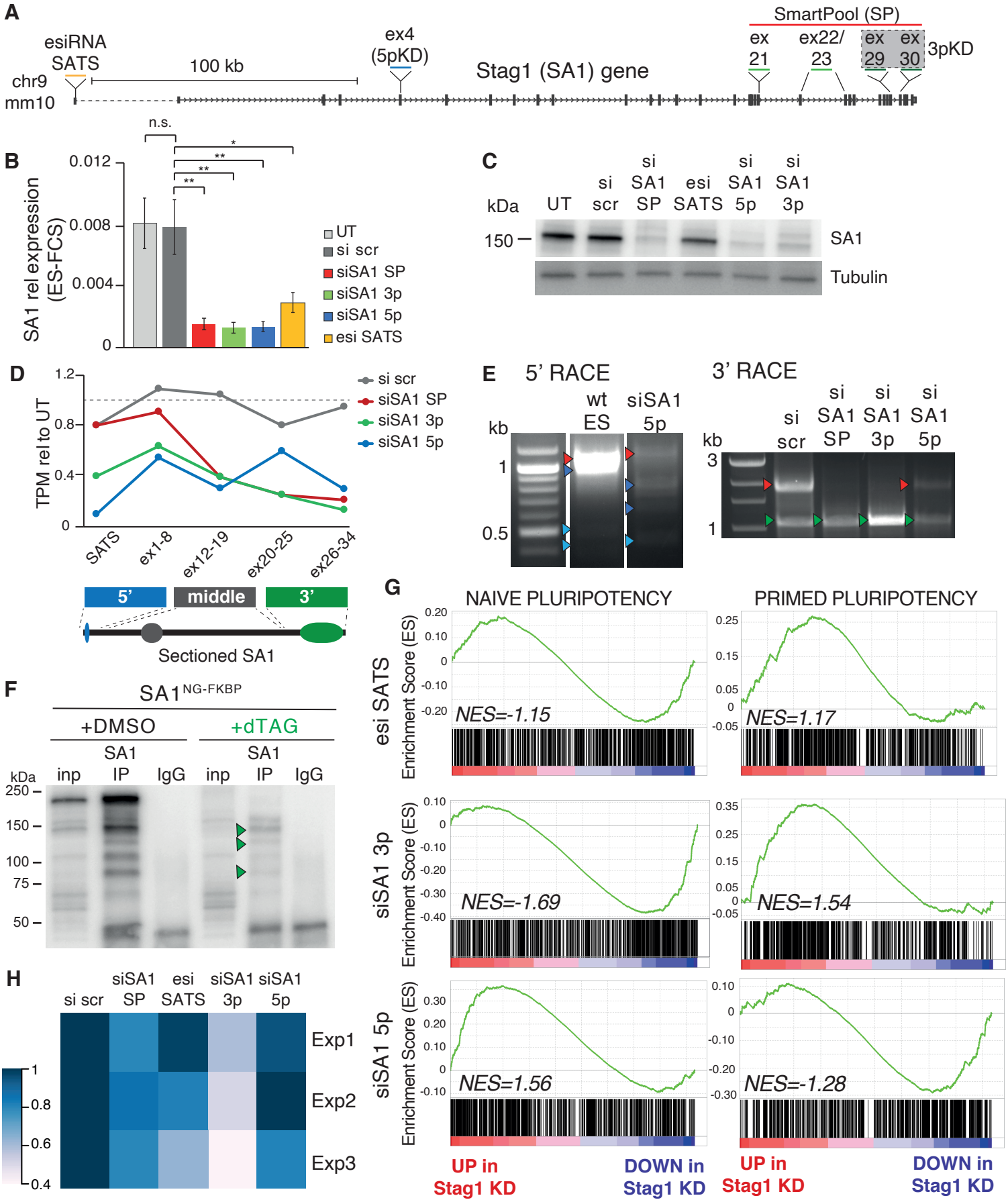


Figure 5.

Pezic et al.

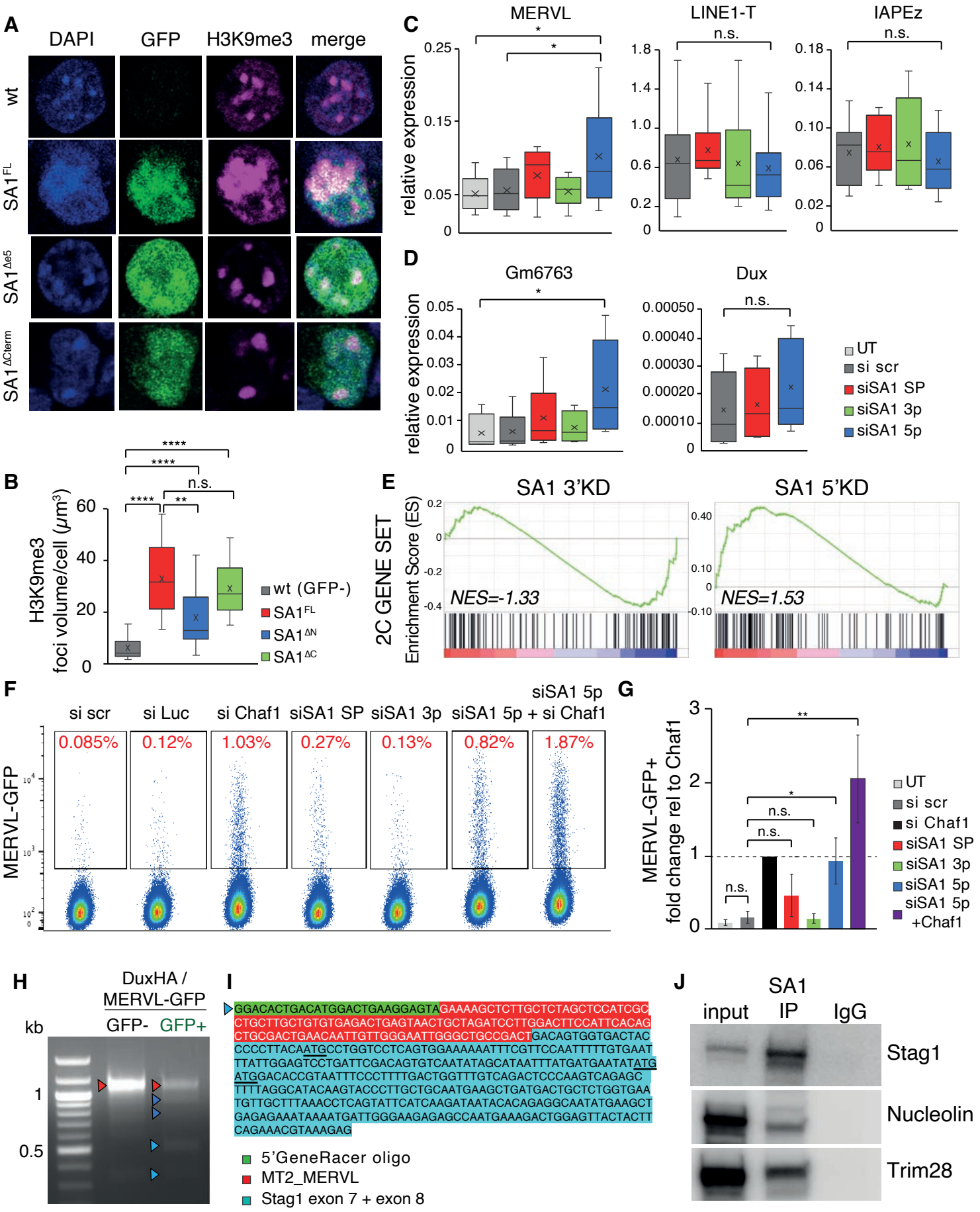
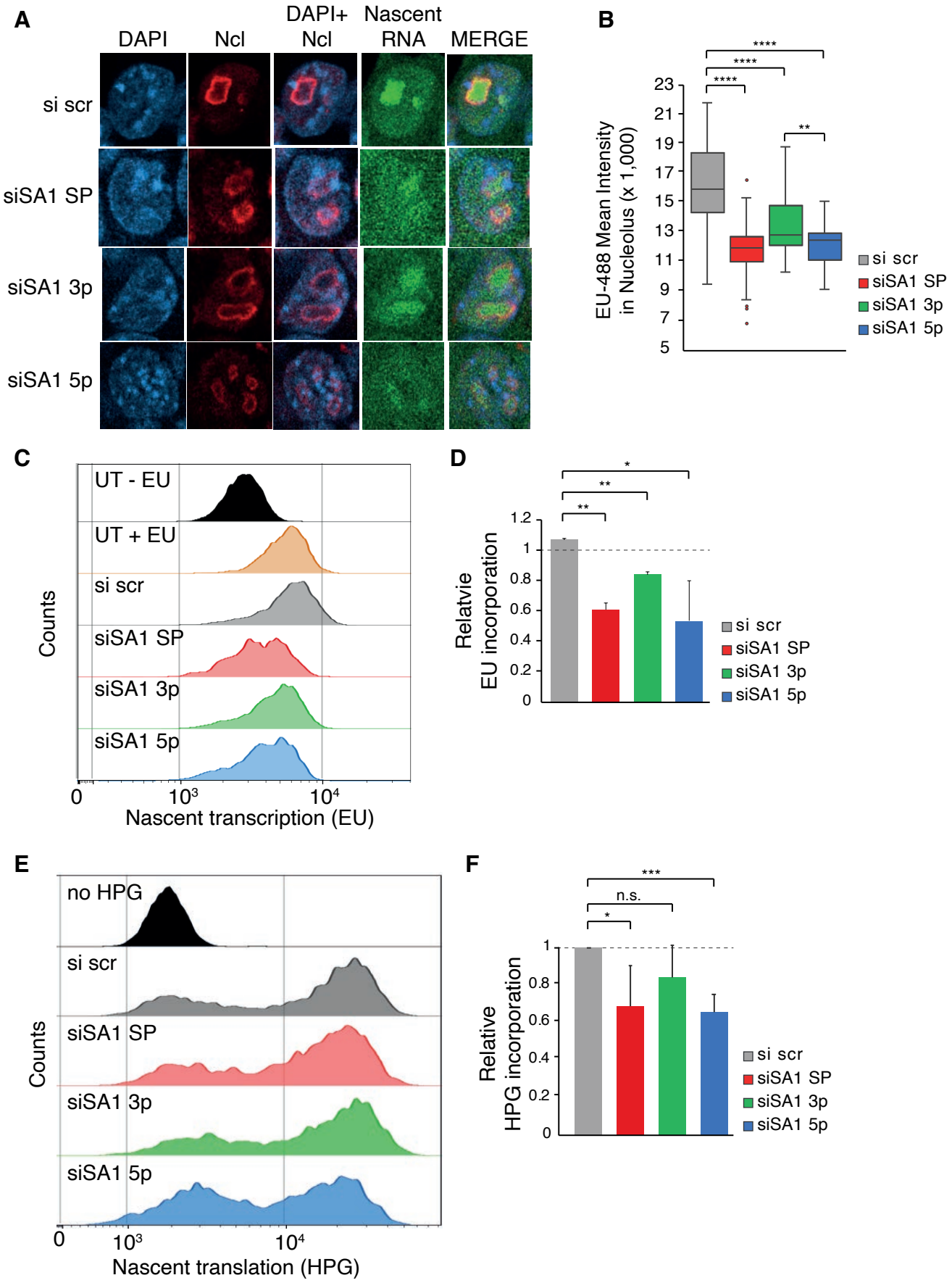


Figure 6.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siSA1 5p
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10³
10⁴
Nascent translation (HPG)
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Relative HPG incorporation
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0.8
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0.2
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si scr
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