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**Title:** A SUV39H1-low chromatin state characterises and promotes migratory properties of cervical cancer cells

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Running title: A SUV39H1-low state promotes CC migratory cells

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#### Abstract

Metastatic progression is a major cause of mortality in cervical cancers, but factors regulating migratory and pre-metastatic cell populations remain poorly understood. Here, we sought to assess whether a SUV39H1-low chromatin state promotes migratory cell populations in cervical cancers, using meta-analysis of data from The Cancer Genome Atlas (TCGA), immunohistochemistry, genomics and functional assays. Cervical cancer cells sorted based on migratory ability in vitro have low levels of SUV39H1 protein, and SUV39H1 knockdown in vitro enhanced cervical cancer cell migration. Further, TCGA SUV39H1-low tumours correlated with poor clinical outcomes and showed gene expression signatures of cell migration. SUV39H1 expression was examined within biopsies, and SUV39H1<sup>low</sup> cells within tumours also demonstrated migratory features. Next, to understand genome scale transcriptional and chromatin changes in migratory populations, cell populations sorted based on migration in vitro were examined using RNA-Seq, along with ChIP-Seq for H3K9me3, the histone mark associated with SUV39H1. Migrated populations showed SUV39H1-linked migratory gene expression signatures, along with broad depletion of H3K9me3 across gene promoters. We show for the first time that a SUV39H1-low chromatin state associates with, and promotes, migratory populations in cervical cancers. Our results posit SUV39H1-low cells as key populations for prognosis estimation and as targets for novel therapies.

#### Introduction

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Despite significant advances in vaccination and screening, cervical cancer represents one of the most common cancers in women in the developing world. While considerable insights have emerged on early stages of cervical cancer progression using *in vitro* and mouse models[1], advanced progression stages, including metastasis, are still poorly understood - in part due to due to extensive heterogeneity within and across human tumours. In particular, the emergence of migratory subpopulations within tumours represents a rate limiting step during multiphase process of metastasis. We have previously reported that one such population in cervical cancer progression, marked by CEACAM/CD66, shows enhanced tumorigenicity, migration, and associations with metastasis[2–4]. However, it is poorly understood what broad mechanisms

regulate such migratory populations during advanced progression, and how they would allow fate plasticity- the switch between proliferative and migratory states, allowing the formation of macroscopic distant metastases.

One possible mechanism that could regulate such plasticity during migration and metastasis is heterochromatin regulation, through metastable changes in chromatin conformation. We have previously reported that CD66-enriched spheroid cultures show differential expression of several chromatin regulators, including a depletion of heterochromatin regulator *SUV39H1*[2,3]. SUV39H1 effects transcriptional silencing through H3K9 trimethylation (H3K9me3), and SUV39H1-regulated heterochromatin has previously been demonstrated to play a barrier to cell fate transitions during IPSC reprogramming and T-cell lineage commitment[5–9]. In this study, we examined whether a SUV39H1-low chromatin state is associated with, and regulates, migratory populations in cervical carcinomas.

Metastatic transitions in cancers have frequently studied using treatment of cells with growth factors *in vitro* to induce an Epithelial to Mesenchymal Transition (EMT)[10]. While these approaches have been informative, alternative modalities of cell migration have also been reported in solid cancers[11], and in other contexts, EMT is not associated with migration, and is instead linked to functions such as chemoresistance[12,13]. To study chromatin changes directly associated with migratory traits, we analysed migrated and non migrated populations of cervical cancer cells, functionally segregated with the help of an *in vitro* transwell set up (Fig. 1a). As invasive and pre-metastatic traits prominently emerge during the carcinoma *in situ* stage, we used the SiHa cell line, which is derived from a primary cervical carcinoma. Combining this approach with an analysis of TCGA *SUV39H1*-low tumours, immunohistochemistry, genomics, imaging and RNAi, we demonstrate that a SUV39H1-low chromatin state associates with, and promotes, migratory cell populations in cervical cancers.

#### Results

#### Low SUV39H1 expression characterises cervical cancer migratory states in vitro

Initial observations showed a depletion of *SUV39H1* in CD66 enriched spheroid cultures[2], as well as in CD66+ve cells sorted from these cultures (Supplementary Fig. 1a). Given these findings, we sought to determine whether SUV39H1 depletion is directly linked to a migratory phenotype, and whether this depletion enhances cell migration. To examine cell populations

showing enhanced migration, we sorted migrated and non migrated populations from SiHa cells using a transwell set up (Fig. 1a). 1.9-fold lower SUV39H1 protein was noted in sorted migrated populations, relative to non-migrated populations (Fig. 1b). Next, we used endoribonuclease-prepared silencer RNAs (esiRNAs) to knock down SUV39H1 in SiHa cells, to avoid off-target effects using individual siRNAs. SUV39H1 knockdown led to a 3 and 2-fold enhanced transwell migration and Matrigel invasion, respectively, and 2-fold lower cleaved PARP under anoikis conditions (Fig. 1c-e). Under proliferation cues, these cells also show 2.7-fold enhanced clonogenic potential (Fig. 1f, Supplementary Fig. 1b). These phenotypes were accompanied by a 1.4-fold upregulation of CD66 at a protein level (Supplementary Fig. 1c). We also used a retroviral shRNA approach to knockdown SUV39H1. qPCR indicated that the SUV39H1 shRNA knockdown was successful (Supplementary Fig. 1d). Additionally, this approach further confirmed that a knockdown of SUV39H1 resulted in a small decrease in migratory ability (1.2-fold) (Supplementary Fig. 1f, g). Thus, SUV39H1 protein is depleted in migrated populations *in vitro*, and its knockdown results in enhanced migration and invasion.

#### Low SUV39H1 expression correlates with poor clinical outcomes in cervical cancers

Given that low SUV39H1 was noted in migrated populations, and SUV39H1 knockdown resulted in enhanced tumorigenic properties in vitro, we next asked if SUV39H1 abundance in tumours correlated with disease outcomes in human cervical cancers. We examined publicly available RNA-seq data from primary human cervical tumours (TCGA cervical squamous cell carcinoma and endocervical adenocarcinoma, CESC) [14,15]. Squamous Cell Carcinoma (SCC) cases were sorted into SUV39H1-low and SUV39H1-high groups, based on tumoural SUV39H1 mRNA expression. The SUV39H1-low group showed significantly lower overall survival over time, compared to the SUV39H1-high group (p= 0.012) (Fig. 2a, Supplementary Fig. 2a). We also examined whether SUV39H1 expression correlated with survival in other human cancers. Interestingly, among 21 TCGA cancers examined, cervical cancer was the only cancer in which low SUV39H1 correlated with poor survival (Supplementary Table 1, Fig. 2a-c, Supplementary Fig. 2b, c). 3 other cancers (Cutaneous melanoma, Renal clear cell carcinoma, and Renal papillary cell carcinoma) showed significant correlations (p<0.05) of SUV39H1 expression with survival, but here high SUV39H1 expression correlated with poor survival instead. Correlations with survival in SCC cases were not observed with SUV39H2, and HP1a homolog CBX5, which are known to synergise with SUV39H1 in some contexts to regulate

H3K9me3 (Supplementary Fig. 2d, e). Thus, low tumoural *SUV39H1* abundance correlates with poor clinical outcomes in cervical cancers, and this correlation may be unique to cervical cancers.

## Low SUV39H1 expression correlates with features of enhanced migration in cervical cancers

Poor clinical outcomes associated with SUV39H1-low cervical tumours could be due to various aspects of disease progression, such as metastasis, recurrence, and drug resistance. As CD66<sup>high</sup> populations also show SUV39H1 depletion, along with enhanced migration and associations with metastasis, we sought to examine whether SUV39H1-low tumours showed features of enhanced migration and invasion. We examined genes differentially expressed in cervical SUV39H1-low tumours. 1710 genes were enriched and 721 genes were depleted in SUV39H1-low tumours (Supplementary Table 2). These SUV39H1-low tumours showed an enrichment (>1.5 fold) of several migration and matrix remodelling associated genes, including TWIST1, ZEB1 and several MMPs (Fig. 2d). Gene Set Enrichment Analysis (GSEA) indicated an enrichment for a cell migration gene set in SUV39H1 in SUV39H1-low tumours (pvalue=0.0163) (Fig. 2e). Gene Ontology (GO) and KEGG pathway analysis revealed a significant (p<0.01) enrichment for terms including cell migration, vasculature development, TGF-β signalling, and cell adhesion in SUV39H1-low tumours (Fig. 2f, Supplementary Fig. 3a). The SUV39H1-low group also showed a significant (p<0.01) depletion of DNA replication and cell cycle signatures (Fig. 2d, Supplementary Fig. 3b). Finally, we noted that genes enriched in SUV39H1-low tumours show a statistically significant overlap with a CD66<sup>high</sup> cell profile[3] (Supplementary Fig. 3c). Thus, low SUV39H1 abundance in cervical tumours correlates with poor clinical outcomes, and SUV39H1-low tumours show gene expression signatures of cell migration.

## SUV39H1<sup>low</sup> cell populations within cervical tumours show features of enhanced migration and a bulk depletion of H3K9me3

We observed gene expression signatures of migration in *SUV39H1*-low tumours. The SUV39H1-low state of these tumours may be due to a global depletion of SUV39H1 in cells

within tumours, or due to varying proportions of cells with low and high SUV39H1 expression. To examine whether SUV39H1<sup>low</sup> cell populations are observed within tumours, and whether these putative SUV39H1<sup>low</sup> populations show features of migration, we examined individual tumour biopsies using Immunohistochemistry (IHC). We examined SUV39H1 staining intensity within sections, and broadly characterised cell populations as SUV39H1<sup>high</sup> and SUV39H1<sup>low</sup>. As a loss of cell-cell adhesion and proliferation programs and a gain of an elongated, "sarcomatoid-like" morphology are key facets of migration, we next examined for regions of serial sections showing depletion of CDH1 (ECAD) and P63. ECAD and P63 mark cell-cell adhesion and keratinocyte proliferation, respectively, and a depletion of these markers indicates a migratory state[16–18]. 5 out of 12 SCC biopsies showed SUV39H1<sup>-ve</sup>ECAD<sup>-ve</sup>P63<sup>-ve</sup> cell populations (Fig. 3, Supplementary Fig. 4, Supplementary Fig. 5), and cells in these populations also showed a sarcomatoid-like morphology. Other SCC sections showed less drastic depletion of SUV39H1, nonetheless, populations of cells showing lower SUV39H1 also showed lower ECAD and P63.

Since SUV39H1 is known to mediate transcriptional regulation through H3K9me3, we also assessed bulk H3K9me3 distribution in serial sections. We observed that SUV39H1<sup>low</sup> populations within tumours were also H3K9me3<sup>low</sup>, suggesting an altered chromatin state in these populations (Fig. 4a, Supplementary Fig. 5). Interestingly, in normal cervix sections, in sections showing the entire spectrum of differentiation, SUV39H1<sup>-ve</sup> and H3K9me3<sup>-ve</sup> cells were located in the basal stem layer, whereas the more differentiated, proliferative parabasal layer was SUV39H1<sup>high</sup> and H3K9me3<sup>high</sup> (Fig. 4b), suggesting a possible link between a SUV39H1<sup>low</sup> chromatin state and stemness. Thus, SUV39H1<sup>low</sup> cell populations are observed within tumours, and these populations show features of cell migration and a bulk loss of H3K9me3.

## Migrated populations of cervical cancer cells show SUV39H1-linked migratory gene expression signatures

We noted migratory gene expression signatures in *SUV39H1*-low tumours (Fig. 2d) and a bulk H3K9me3 depletion in SUV39H1<sup>low</sup> cells (Fig. 4a). This suggested that a SUV39H1-low, H3K9me3-low chromatin state may drive migratory populations. We sought to determine whether migrated populations showed 1. SUV39H1-linked transcriptional changes and 2. linked changes in H3K9me3 distribution, using migrated populations of SiHa (sorted using a transwell setup, Fig. 1a). First, we sought to determine whether migrated populations showed reflections of migration and EMT (Epithelial-Mesenchymal Transition) at a transcriptional level, and

whether these transcriptional changes showed parallels with transcription profiles of SUV39H1low tumours. The transcriptome of *in vitro* migrated cell populations was examined by RNA-Seq (Supplementary Fig. 6a, c). Migrated populations showed an upregulation of 944 genes and a downregulation of 531 genes, relative to non migrated populations. RNA-Seq analysis revealed the following findings: 1. Migrated populations showed enrichment of cell motion and cell migration Gene Ontology (GO) terms, along with DNA replication signatures (at  $p < 10^{-4}$ ) (Fig. 5a, b). Genes downregulated in migrated populations, on the other hand, showed enrichment for apoptosis and cell death GO processes (at  $p < 10^{-4}$ ). 2. An upregulation of known apex EMT transcription factors TWIST1, SNAIL2, and ZEB1 was observed in the migrated population (Fig. 5c). Differential expression of downstream effectors/ regulators of migration was also noted, including a downregulation of cell-cell adhesion marker CDH1/ECAD and an upregulation of FN1 (Fibronectin), CD44, MMP14 and MMP28 (Fig. 5d). 3. Transcription factors such as AP1, TGIF, STAT3, and SOX9 showed overrepresented target genes in migrated populations (Fig. 5e), indicating that these regulators may effect the transcriptional changes observed in migrated populations. 4. A significant overlap was noted between genes upregulated in migrated populations and genes upregulated in TCGA SUV39H1-low tumours (p=2.5x10<sup>-4</sup>) (Fig. 5f), highlighting a link with SUV39H1. Common genes upregulated in both these sets included migration-associated genes such as TWIST1, ZEB1, MMP2, and PKCA. Lastly, differential expression of SUV39H1 itself was not observed at the transcription level in migrated populations, indicating that altered levels of SUV39H1 protein observed in SiHa migrated populations are regulated post-transcriptionally. Thus, migrated populations of cervical cancer cells show reflections of migratory processes at a transcriptional level, and these signatures show a link with a SUV39H1 transcriptional signature.

Next, we knocked down SUV39H1 and assessed whether transcription factors *SNAIL1*, *SNAIL2*, *TWIST1* and *ZEB1* were transcriptionally upregulated, and also whether downstream migration regulators/indicators *ECAD*[18] and *MMP28*[19,20] were downregulated and upregulated, respectively. First, we performed an esiRNA knockdown of SUV39H1 in SiHa. qPCR showed a downregulation of *SNAIL1* and *SNAIL2*, while other transcription factors were not differentially regulated (Supplementary Fig. 7a). *ECAD* was also not differentially regulated, but we noted an upregulation of *MMP28*. Next, using qPCR in shRNA SUV39H1 knocked down SiHa cells, we observed that the *SNAIL2*, *TWIST1*, and *ZEB1* were downregulated (Supplementary Fig. 7b). *ECAD* was not differentially expressed, but *MMP28* was upregulated.

Lastly, we examined whether SUV39H1 knocked down cells would show different gene expression signatures under migratory conditions. We examined esiRNA SUV39H1 knocked down and control cells isolated during early migration using a transwell set up. Under these conditions, SUV39H1 knocked down cells showed a downregulation of *SNAIL1* and *SNAIL2*, and no differential expression of *TWIST1* and *ZEB1* was observed (Supplementary Fig. 7c). *ECAD* showed marginal downregulation, and *MMP28* was found to be upregulated. Thus, SUV39H1 knockdown leads to a downregulation of *SNAIL1* and *SNAIL2*, but an upregulation of *MMP28*.

#### Migrated populations of cervical cancer cells show a broad loss of H3K9me3

Given the transcriptional changes observed in migrated populations, we next sought to examine whether migrated populations show linked changes in distribution of H3K9me3, the histone mark associated with SUV39H1. We also sought to determine whether putative H3K9me3 alterations acted on a locus specific, gene specific manner[18], or whether broader chromatin changes operated across genomic classes[7,21]. First, bulk levels of H3K9me3 were assessed by western blotting and immunofluorescence. These approaches revealed 3 and 1.6-fold reductions in H3K9me3, respectively, in migrated populations (Fig. 6a, 6b-d left panels). The nuclei in migrated populations also appeared 1.2-fold larger (Fig. 6b-d right panels). The loss in H3K9me3 and increase in nuclear size together suggested a decondensed chromatin state in migrated populations. We also examined distribution of H3K4me3, a histone mark associated with transcriptional activation, and which is not associated with SUV39H1. We observe that unlike H3K9me3, H3K4me3 did not show detectable bulk differences in migrated populations. (Supplementary Fig. 8a, b).

Next, we assessed genomic locations at which differential H3K9me3 binding is observed, using H3K9me3 ChIP-Seq. H3K9me3 ChIP-seq profiles revealed broad regions of enrichment across the genome. Examining across genomic classes, an enrichment of H3K9me3 was observed over intergenic regions and repetitive elements, along with a depletion of H3K9me3 over more open chromatin- for example, exons and most promoters (Fig. 6e, Supplementary Fig. 9c). These trends were consistent with H3K9me3 binding patterns reported previously[22,23]. Further, migrated populations showed greater loss of H3K9me3 over open chromatin regions. Analysis of H3K9me3 distribution over gene bodies showed a depletion of over transcription

start sites (Supplementary Fig. 9a, b), and an increase in level along the gene body, which has been previously reported[24] and suggested to prevent aberrant transcription initiation within the gene body. Next, to understand links between changes in chromatin and the transcriptional state in migrated populations, results of the H3K9me3 ChIP-seq were compared against RNA-seq gene expression data. No direct correlation was observed between H3K9me3 occupancy and differential gene expression in migrated and non migrated populations (Supplementary Fig. 7d). However, a correlation was noted between changes in this methylation and the absolute level of mRNA expression. Specifically, while promoters of low expression genes (0-25 percentile expression) did not show significant changes in H3K9me3, promoters of high expression genes (75-100 percentile expression) showed low H3K9me3, and this mark is further depleted in migrated populations broadly across these promoters (p<10<sup>-4</sup>) (Fig. 6f, Supplementary Fig. 9e). Thus, migrated populations of cervical cancer cells show enlarged nuclei and a broad loss of H3K9me3, especially over promoter elements of highly expressed genes.

#### Discussion

We observed that a SUV39H1 knockdown enhances migration in cervical cancer cells (Fig. 1d, Supplementary Fig. 1e), *SUV39H1*-low cervical tumours and SUV39H1<sup>low</sup> cell populations within tumours show features of migration (Fig. 2e, Fig. 3), and that migrated populations show *SUV39H1*-linked transcriptional changes and a broad loss of H3K9me3 (Fig. 5a-d, f, Fig. 6a, b, f). Our observations indicate an anti-migratory role for SUV39H1 in cervical cancers, in contrast with pro-migratory roles reported in colon, breast and prostate cancer settings[25–27]. Reinforcing this complexity, across a panel of 21 cancers, *SUV39H1* expression showed a negative correlation with survival only in cervical cancer (Supplementary Table 1, Fig. 2a-c, Supplementary Fig. 2b, c). SUV39H1 has been suggested in oncogenic as well as tumour suppressor roles across cancers, depending on the context[25,26,28–30]. This is likely because SUV39H1, like most chromatin regulators, does not possess sequence specificity- the genomic loci to which it is recruited to depends on the transcription factor it associates with[18], and on the chromatin landscape, and these factors may be dependent on the tissue of origin, active oncogenes, and mutation spectrum across cancers.

*SUV39H1*-low tumours showed gene expression signatures of migration and enhanced TGF- $\beta$  signalling (Fig. 2d, f, Supp. Fig. 3a). We also note links between low SUV39H1 and CD66<sup>high</sup>

populations (Supplementary Figs. 1a, b, 3c); and low SUV39H1 in CD66 populations[2] may partly explain the migratory phenotypes observed in these cell populations. Further, sorted migrated populations of cervical cancer cells also reflect transcriptional signatures of migration and EMT, and these signatures show parallels with transcriptional signatures seen in *SUV39H1*-low tumours (Fig. 5a-d, f).

Low SUV39H1 abundance also correlates with low levels of its associated histone mark, H3K9me3. Interestingly, one deviation from the EMT model observed in migrated populations is the broad depletion of H3K9me3, especially over gene promoter elements (Fig. 6a, b, f), in contrast to the more specific loss of H3K9me3 at mesenchymal gene promoters observed in EMT models[18]. Along with nuclear enlargement observed in migrated populations (Fig. 6b, c, d, right panels), this may suggest a model of enhanced epigenome plasticity in migratory populations- that a depletion of SUV39H1 and H3K9me3 may render genes more susceptible to remodelling and changes in gene expression, in turn allowing cells to switch to a migratory state more efficiently in the presence of migration-inducing cues (Supplementary Fig. 10). SUV39H1 and H3K9me3 mediated heterochromatin have been demonstrated to play similar barriers to cell fate plasticity in IPSC reprogramming and T-cell lineage commitment[5–9]. A role in cell fate plasticity is further supported by a depletion of SUV39H1 and H3K9me3 in the basal, stem layer in the normal cervix (Fig. 4b). Chromatin-linked cell fate plasticity is increasingly emerging as a critical regulatory mechanism during cancer progression, including metastasis[21].

The consistent downregulation of *SNAIL1* and *SNAIL2* observed in SUV39H1 knocked down cells was non-intuitive, as our initial hypothesis predicted an upregulation of these transcription factors (Supplementary Figs. 7a-c). However, there was an upregulation of a downstream effector of migration, *MMP28*[19,20], consistent with the enhanced migratory phenotypes associated with SUV39H1. This data suggests a more complex regulatory mechanism, and it may be that SUV39H1 only effects a smaller subset of the broad transcriptional differences observed in migrated populations (e.g. MMP28 upregulation). Broad H3K9me3 depletion may maintain gene promoters in a poised state, however it is likely that additional regulators are required to shape the overall transcriptional changes observed in migrated populations. However, this subset of changes effected by SUV39H1 is sufficient to yield a phenotype of enhanced migration and invasion with SUV39H1 knockdown (Fig. 1d, Supplementary Fig. 1e). The data may also possibly suggest differences between migration and EMT, wherein migration associated-downstream effector genes are altered, but possibly via non-EMT-transcription factor regulated mechanisms.

Collectively, our findings highlight an association and role for a SUV39H1-low chromatin state in promoting migratory cell populations during advanced cervical carcinoma progression. In subsequent studies, it will be important to gain further insights into mechanisms by which SUV39H1 regulates migratory populations, the regulators of SUV39H1 itself, and the roles of other epigenetic regulators and networks in migratory and metastatic cell populations.

#### Materials and methods:

#### Cell culture

SiHa cells (purchased from ATCC) were cultured in Dulbecco's modified Eagle's Medium (DMEM) with 10% FCS. Cultures were used for assays within 27 passages of acquisition from ATCC. All cultures were routinely tested for mycoplasma using Mycoalert kit (Lonza) and were consistently mycoplasma negative, and examined for retention of described morphology and growth characteristics. For transient overexpression experiments, cells were transfected with an empty vector, pcDNA3, or pIX305 SUV39H1 LV V5 (DNASU plasmid repository) for SUV39H1 overexpression. Lipofectamine 2000 (Invitrogen) was used for transfection, and cells were used for molecular and phenotypic assays 48h post transfection. Transient knockdown experiments were performed using esiRNA, as the heterogeneous mixture of these endoribonuclease derived siRNAs provides greater target specificity than chemically synthesised siRNAs. For these experiments, mission esiRNAs (Sigma) targeting eGFP or SUV39H1 were transfected using lipofectamine RNAiMAX (Invitrogen). Cells were used for assays 48h post transfection. For shRNA knockdown of SUV39H1, shRNAs to SUV39H1 (pSMP-Suv39H1\_1) and Luciferase (pSMP-Luc as a control) were retrovirally transduced into SiHa cells. Cells were subjected to selection using puromycin, and cells were subsequently used for cell migration and qPCR assays. pSMP-Suv39H1 1 (Addgene plasmid # 36342) and pSMP-Luc (Addgene plasmid # 36394) were gifts from George Daley.

#### Transwell migration set up

0.5 X 10<sup>6</sup> cells were counted, resuspended in DMEM containing 1% FCS and seeded into the upper well of a 24 well matrigel coated (invasion) or uncoated (migration) transwell set up. DMEM containing 10% FCS was added to the lower well of the transwell set up as a chemoattractant. Cells were allowed to migrate 10 hours at 37C after seeding. Following this, cells at the bottom of the chamber were stained with Hoechst. Cells were imaged under a Nikon Inverted Microscope ECLIPSE TE2000-S, and counted using ImageJ. For extraction of migrated and non-migrated cell fractions for western blot, RNA seq and ChIP, 0.75 x 10<sup>6</sup> cells were seeded per well of a 6 well migration transwell set up. Cells were then scraped in PBS, pooled and used for further processing.

#### Western blots

Immunoblots were performed using antibodies against the following: SUV39H1 (Cell Signalling Technology, D11B6), H3K9me3 (Abcam, 8898), CD66 (Abcam, ab134074), Cleaved PARP (Cell signalling technology, 9541), GAPDH (Santa Cruz Biotechnology, sc-47724). Quantitation of western blots was performed using ImageJ, using GAPDH as a control for equal loading.

#### Immunohistochemistry

Formalin Fixed, Paraffin Embedded (FFPE) biopsy sections were obtained from Kidwai Cancer Institute, with appropriate institutional ethical approvals from the Kidwai Medical Ethics committee, including obtaining informed consent from each subject. Immunohistochemistry was performed on serial sections using Leica Novolink Polymer Detection system, according to manufacturer's instructions. The following modifications were made to the manufacturers protocol. A permeabilization step was added post antigen retrieval, using 0.1% Triton in TBS (TBS-T). All washes were performed in TBS-T. Additionally, primary antibody incubation was performed overnight at 4C, using antibodies to SUV39H1 (ab155164, abcam), H3K9me3 (ab8898, abcam), P63 (4A4) (sc-8431,Santa Cruz Biotech), CDH1/ECAD (H168) (cat no ab76055, abcam). For each biopsy, multiple fields (≥3) were imaged to verify co-expression in serial sections.

#### Analysis of TCGA data

Level 3 TCGA CESC RNA Seq data was obtained from the TCGA data portal (https://tcgadata.nci.nih.gov/). RNA seg RSEM gene counts as well as RSEM normalized gene counts, as well as corresponding clinical data, were downloaded. Clinical data from the samples was used to filter out adenocarcinomas, retaining only squamous cell carcinomas. Next, normalised gene count was used to divide patients based on SUV39H1 expression, using a median, tertile or guartile split. Overall patient survival corresponding to the two groups split by median was used to create Kaplan Meier plots depicted, using R packages survival (v2.41-3)[31,32] and ggplot2 (v2.2.1)[33]. Similar results were seen when different group splitting methods (median, tertile, quartile) were used. For survival data across TCGA cancers, web tool Oncolnc[34] was used, with a quartile split. For RNA seg differential expression, RSEM gene counts (non normalized) corresponding to the SUV39H1-low and SUV39H1-high tumours (quartile group split) were used. Normalisation and differential expression analysis was performed using EBseq (v1.10.0)[35,36]. Genes showing a >1.5-fold change and a FDR cutoff of 0.05 were considered to be significantly differentially expressed. Lists of differentially expressed genes were analysed using DAVID (v6.7) to obtain significantly enriched GO (GO BP-ALL) and KEGG terms. GSEA was performed using the GSEA software (v.2.0)[37], using normalised gene counts from SUV39H1-low and SUV39H1-high tumours, and using gene sets obtained from the MsigDB.

#### **Statistics**

Bar graphs indicate mean and standard error across biological replicates ( $n \ge 3$ ), unless otherwise indicated. n in all Figure legends corresponds to biological replicates. Significance was assessed using two-tailed t tests, and a p value of  $\le 0.05$  was considered as statistically significant. For immunofluorescence intensity and nuclear area quantitation, bars indicate median and interquartile range within a single experiment; two-tailed t-tests for these experiments were performed on medians from n $\ge 3$  biological replicates. For Kaplan Meier plots, statistical significance was assessed using a log rank test. Plots were generated using R through R Studio (v1.1.383), using R package ggplot2 (v2.2.1)[33].

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#### Data availability

The datasets generated and analysed in this study are included within the manuscript (and Supplementary) or have been deposited in the Gene Expression Omnibus (GEO, for RNA-Seq and ChIP-Seq data) under accession number GSE103794. All other data are available upon reasonable request.

Assays for phenotypes (clonogenic, proliferation, anoikis), Immunofluorescence, RNA-Seq, qPCR, ChIP-Seq are described in Supplementary Methods.

#### Author contributions:

**Conception and design:** C. Rodrigues, S. Krishna, P. Varga-Weisz, C. Pattabiraman, D. Notani

Development of methodology: C. Rodrigues, C. Pattabiraman

Acquisition of data: C. Rodrigues, C. Pattabiraman, A. Vijaykumar, R. Arora

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Analysis and interpretation of data: C. Rodrigues, S. Krishna, P. Varga-Weisz, D. Notani, R. Arora

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### Figure legends:

#### (All figures are 2-column fitting, colour images)

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**Figure 1. Low SUV39H1 expression characterises cervical cancer migratory states. a.** Schematic representation of an *in vitro* transwell set up, used to obtain populations of migrated and non migrated SiHa cells. **b.** Western blot showing abundance of SUV39H1 in migrated populations of cells (n=3). **c.** Quantitation of SUV39H1 knockdown efficiency, 48h post transfection, using western blotting (n= 3). **d.** Quantitation of migration and invasion in SUV39H1 kd cells using a transwell assay (n=3, \*, p<0.05). **e.** Cleaved PARP estimation by western blotting under anoikis conditions (n=4) **f.** Estimation of clonogenic potential using clonogenic assay (n=3, \*, p<0.05). Western blots depict mean fold intensity ± S.E.M.

**Figure 2.** Low SUV39H1 expression correlates with features of enhanced migration in human cervical cancers. **a.** Survival in cervical SCC patients with low v/s high tumoural SUV39H1 (median group split, n=94 for each group). Quartile split approach yielded similar results (Supplementary Fig. 5a). **b, c.** Survival in patients with low v/s high tumoural *SUV39H1* in Skin Cutaneous Melanoma (SKCM) and Kidney Renal Clear Cell Carcinoma (KIRC) datasets respectively (quartile split). **d.** Volcano plot depicting differential gene expression in *SUV39H1*-low tumours v/s *SUV39H1*-high tumours (N=47 for each group). Blue points indicate genes significant at cutoff fold change (1.5 fold) and FDR corrected q value (0.05). **e.** Gene set enrichment analysis (GSEA) plot examining enrichment for a cell migration gene set (M6281, from the Msigdb) in SUV39H1 low tumours (p-value=0.0163). **f.** Enriched KEGG pathway terms corresponding to genes upregulated in *SUV39H1*-low tumours. Arrows indicate adhesion and migration associated terms. Results shown in this figure are based on data obtained from The Cancer Genome Atlas Project (TCGA) Research Network (http://cancergenome.nih.gov/).

Figure 3. SUV39H1<sup>low</sup> cell populations within cervical tumours show features of enhanced migration. IHC staining of SUV39H1, CDH1(ECAD) and P63 in SCC serial biopsy sections (n=5 of 12, also ref. Supplementary Fig. 4). Arrows indicate populations with features of migration. For each biopsy, multiple fields ( $\geq$ 3) were imaged to verify co-expression in serial sections. Representative images from each biopsy are depicted. Scale bar, 50 µm.

**Figure 4. SUV39H1**<sup>low</sup> **cell populations show low bulk levels of H3K9me3.** IHC staining of SUV39H1 and H3K9me3 in **a**. serial SCC sections (n=3 of 9, arrows depict populations with sarcomatoid-like morphology) and **b**. serial normal cervix sections (n=2, arrows indicate basal and parabasal layers). Inset: representative enlarged regions from original images (dotted selection region). Scale bar, 50 µm.

**Figure 5. Migrated populations of cervical cancer cells show SUV39H1-linked migratory transcriptional signatures. a.** GO Biological Process terms corresponding to genes upregulated and downregulated in migrated populations (across replicates, n=3). **b.** Plots depicting mRNA abundance (y axis depicts normalised read count) of representative

differentially expressed genes *SNAIL2* and *CASP10* in migrated and non migrated populations, in a single replicate (n=1 of 3). **c**, **d**. Expression of core EMT transcription factors and downstream markers/effectors across replicates (n=3), FPKM values depicted as a heat map. **e**. Putative transcription factors active in migrated populations, inferred using overlap between genes upregulated in migrated populations and known UCSC ENCODE transcription factor binding sites (TFBS). **f**. Overlap of migrated population upregulated genes with set of genes upregulated in TCGA *SUV39H1*-low tumours.

**Figure 6. Migrated populations of cervical cancer cells show a global reduction of H3K9me3, the histone mark associated with SUV39H1. a.** H3K9me3 abundance in migrated populations of SiHa cells using western blotting (n=3). **b-d.** H3K9me3 abundance (left panels) and nuclear size (right panels) assessed by immunofluorescence. **b.** Representative confocal images. Scale bar, 35µm, **c.** Quantitation of H3K9me3 staining intensity in individual cells range from a single representative experiment (n=1 of 4 and 3, respectively). Bars depict median and interquartile range. **d.** Comparison of median H3K9me3 fluorescence intensity and nuclear area in migrated and non migrated populations across replicates (n=4 and 3, respectively). **e.** Enrichment of H3K9me3 ChIP reads against input across repetitive element (left) and gene element (right) classes (n=1 of 2 replicates). **f.** Distribution of H3K9me3 ChIP reads over promoters of low expressed (0-25 percentile) and high expressed (75-100 percentile) genes (n=1 of 2 replicates), \*\*\*, p<0.0001, Wilcoxon signed rank test.

#### **Supplementary Figures:**

**Supplementary figure 1. a.** qPCR assay showing relative mRNA expression of *SUV39H1* in CD66+ve cells, enriched from CaSki spheroids (n=4). **b.** Clonogenic assay images in control and SUV39H1 knocked down SiHa cells (n=1 of 3). **c.** Western blot showing abundance of CD66 in control and SUV39H1 kd cells (n=10). **d.** Quantitation of SUV39H1 knockdown efficiency in shRNA knocked down SiHa cells using qPCR (n= 3). **e.** Quantitation of migration in shSUV39H1 cells using a transwell assay (n=3, \*, p<0.05). **f.** Western blot showing SUV39H1 expression level after transfection of SUV39H1-V5 (n=4). Higher band size in SUV39H1 OE lane is due to the presence of V5 tag. **g.** Quantitation of migration of control and SUV39H1

overexpressing SiHa cells, using a transwell assay (n=7). Error bars denote S.E.M. Western blots indicate mean fold change  $\pm$  S.E.M.

**Supplementary figure 2. a-c.** Kaplan-Meier plots depicting survival in **a.** cervical SCC patients with low v/s high tumoural *SUV39H1*, using a quartile group split, (n=47 for each group). **b, c.** Glioblastoma multiforme (GBM) and Ovarian serous cystadenocarcinoma (OV) patients with low v/s high tumoural *SUV39H1* (quartile group split). **d, e.** cervical SCC patients with low v/s high tumoural *SUV39H2* and HP1a homolog *CBX5*, using a median group split (n=94 for each group). Similar results were obtained using quartile group split approach. The results shown in this figure are based upon data generated by the TCGA Research Network (http://cancergenome.nih.gov/.)

**Supplementary figure 3. a, b.** GO Biological process terms enriched in significantly differentially expressed genes in *SUV39H1*-low and *SUV39H1*-high tumours (n=47 for each group), using DAVID analysis. Arrows highlight migration associated processes. **c.** Overlap of TCGA *SUV39H1*-low upregulated genes with genes upregulated in CD66<sup>high</sup> populations (Pattabiraman et al., 2014), p-value computed using Fisher's exact test. Results shown in this figure are based on Level 3 RNA seq data obtained from The Cancer Genome Atlas Project (TCGA) Research Network (http://cancergenome.nih.gov/).

**Supplementary Figure 4.** IHC staining of SUV39H1, CDH1(ECAD) and P63 in SCC serial biopsy sections (n=7 of 12). Scale bar, 50 μm.

**Supplementary Figure 5. a.** Immunohistochemical staining of SUV39H1, H3K9me3, CDH1(ECAD) and P63 in a high-grade Squamous Intraepithelial Lesion (H-SIL) graded biopsy, showing features of projection into stroma (n=1). Arrows indicate cell populations with features of migration. Scale bar, 50 μm.

**Supplementary figure 6. a.** Heatmap showing correlation between RNA-Seq replicates derived from migrated and non migrated populations of SiHa cells. **b.** Differentially expressed long

noncoding RNAs in migrated cell populations (n=3), depicted as a heat map. Intensity denotes FPKM, rows transformed to have mean 0, variance 1. **c.** qPCR validation of RNA-Seq data (points represent values from biological replicates, n=3 or 4).

**Supplementary figure 7.** Expression of core EMT transcription factors (*SNAI1*, *SNAI2*, *TWIST1*, *ZEB1*) and downstream migration markers/effectors (*ECAD*, *MMP28*) across replicates, assessed by qCPR (n=3), in **a.** esiRNA SUV39H1 knocked down SiHa cells, relative to esi EGFP knocked down cells. **b.** shRNA SUV39H1 knocked down SiHa cells, relative to shLuc control cells. **c.** esiRNA SUV39H1 knocked down SiHa cells relative to esi eGFP knocked under transwell migratory conditions. Mean fold expression ± S.E.M.is indicated.

**Supplementary figure 8. a.** (Left) Representative image of H3K4me3 distribution assessed by immunofluorescence in migrated and non migrated populations (n=1 of 3). (Right) H3K4me3 Immunofluorescence quantitation, bars depict median and interquartile range from a single representative experiment (n=1 of 3). **b.** Comparison of median H3K4me3 fluorescence intensity in migrated and non migrated populations across replicates (n=3).

**Supplementary figure 9. a, b.** Average trend of H3K9me3 over all genes (gene ± 2kb) (c, d depict replicate sets). **c.** Enrichment of H3K9me3 ChIP reads against input across repetitive element (left) and gene element (right) classes in replicate ChIP-Seq set (n=1 of 2). **d.** Overlay of-RNA Seq differentially expressed genes on H3K9me3 ChIP-Seq data (n=1 of 2). Graph represents ChIP-Seq read density over all genes in migrated and non migrated populations. **e.** Distribution of H3K9me3 ChIP-Seq reads over promoters of low and high expressed genes seen in replicate ChIP-seq set (n=1 of 2). **f.** ChIP-qPCR validation of ChIP-Seq data. RPL30: Negative control, euchromatin region, SAT2: Positive control, heterochromatin locus.

**Supplementary figure 10.** Model of SUV39H1 and H3K9me3 based regulation of cervical cancer migratory populations.

#### Highlights:

- SUV39H1 is depleted in cervical cancer cell populations sorted based on migration
- SUV39H1 knockdown enhances cervical cancer cell migration and invasion
- SUV39H1-low tumours correlate with poor disease outcomes, signatures of migration
- SUV39H1<sup>low</sup> cells within cervical tumours show features of migration.
- ., bra Migrated populations show SUV39H1-linked migratory signatures, broad H3K9me3 •

Figure 1.



## Figure 2.



25

## Figure 3.



## Figure 4.



SUV39H1 H3K9me3 SUV39H1 H3K9me3

Case #17

b.

Case #18

## Figure 5.





## Figure 6.