Regulation of Heterochromatin Transcription by Snail1/LOXL2 during Epithelial-to-Mesenchymal Transition

Alba Millanes-Romero,1 Nicolás Herranz,2 Valentina Perrera,3 Ane Iturbide,1 Jordina Loubat-Casanovas,1 Jesús Gil,2 Thomas Jenuwein,3 Antonio García de Herreros,1,4 and Sandra Peiró1,*

1Programa de Recerca en Cancer, Institut Hospital del Mar d’Investigacions Mèdiques (IMIM), 08003 Barcelona, Spain
2Cell Proliferation Group, MRC Clinical Sciences Centre, Faculty of Medicine, Imperial College, W12 0NN London, UK
3Max Planck Institute of Immunology and Epigenetics, 79108 Freiburg, Germany
4Departament de Ciències Experimental i de la Salut, Universitat Pompeu Fabra, 08003 Barcelona, Spain
*Correspondence: speiro@imim.es
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SUMMARY

Although heterochromatin is enriched with repressive traits, it is also actively transcribed, giving rise to large amounts of noncoding RNAs. Although these RNAs are responsible for the formation and maintenance of heterochromatin, little is known about how their transcription is regulated. Here, we show that the Snail1 transcription factor represses mouse pericentromeric transcription, acting through the H3K4 deaminase LOXL2. Since Snail1 plays a key role in the epithelial-to-mesenchymal transition (EMT), we analyzed the regulation of heterochromatin transcription in this process. At the onset of EMT, one of the major structural heterochromatin proteins, HP1α, is transiently released from heterochromatin foci in a Snail1/LOXL2-dependent manner, concomitantly with a downregulation of major satellite transcription. Moreover, preventing the downregulation of major satellite transcripts compromised the migratory and invasive behavior of mesenchymal cells. We propose that Snail1 regulates heterochromatin transcription through LOXL2, thus creating the favorable transcriptional state necessary for completing EMT.

INTRODUCTION

Packaging genomic DNA into chromatin is critical for gene regulation in eukaryotic cells. Chromatin can be divided into euchromatin and heterochromatin, based on its differential compaction at interphase. Euchromatin is less condensed, more accessible for regulatory factors, and in general more easily transcribed. In contrast, heterochromatin is typically highly condensed and less accessible (Grewal and Jia, 2007). Histone-modifying enzymes, chromatin-remodeling complexes, and DNA methylation establish different ways of nucleosome packaging and chromatin organization. At the epigenetic level, heterochromatin is characterized by DNA methylation, histone hypoacetylation, histone 3 lysine 9 methylation (H3K9me), and histone 4 lysine 20 methylation (H4K20me) (Kouzarides, 2007). Heterochromatin is also enriched for the heterochromatin protein 1 (HP1α), which is essential for its formation and maintenance (Grewal and Jia, 2007). Several lines of evidence have suggested that heterochromatin formation and maintenance requires noncoding pericentromeric RNA transcription. In mice, noncoding pericentromeric transcripts, called major satellites, are tightly regulated during early mouse development and are essential for heterochromatin formation (Probst et al., 2010). Moreover, major satellite expression that takes place at different times during the cell cycle is linked with heterochromatin formation (Lu and Gilbert, 2007). Little is known about whether noncoding pericentromeric RNA is regulated through specific promoters and transcription factors. It has only recently been shown that the regulation of pericentromeric transcription by the transcription factor Pax3 is essential for maintaining heterochromatin integrity (Bulut-Karslioglu et al., 2012).

Transcriptional regulation is a highly complex event determined by the interplay of numerous factors, which converge in the transcriptional status of a gene. Thus, in addition to transcription factors, corepressors and coactivators are essential for determining the final output by modifying histones or mobilizing nucleosomes. We have recently characterized lysine oxidase-like 2 (LOXL2) as a histone-modifying enzyme (Herranz et al., 2012). LOXL2 is a member of the lysyl oxidase (LOX) family of proteins. These proteins are copper-dependent enzymes responsible for the oxidation of the amino group located in ε position in lysines (Lucero and Kagan, 2006). We have demonstrated that LOXL2 catalyzes an amino oxidase reaction to deaminate trimethylated lysine 4 on histone H3 (H3K4me3) and that it creates a type of H3K4 modification, the formation of a deaminated lysine (allysine) (Herranz et al., 2012). By removing an active mark from histone H3 (H3K4me3), LOXL2 inactivates its target promoters and can therefore be classified as a transcriptional repressor. We have demonstrated that deamination of histone tails is an additional mechanism to remove the important regulatory H3K4me3 histone mark.

The epithelial-to-mesenchymal transition (EMT) is critical during embryonic development, as it promotes the conversion of epithelial cells into mesenchymal cells, a process necessary...
for mesoderm formation, neural crest delamination, and secondary palate formation, among other things (Yang and Weinberg, 2008). The EMT process also takes place in pathological situations, such as cancer progression. During EMT, epithelial cells lose their epithelial characteristics, including adherent junctions and apical-basal polarity, and acquire a mesenchymal phenotype and the ability to migrate and invade. Recent evidence suggests that cells that have passed through the EMT program also acquire stem cell properties. Success in metastatic colonization is likely to be influenced by the cells’ capacity to self-renew and to avoid apoptosis, both consequences of EMT, which allow cells to spawn large malignant growths (De Craene and Berx, 2013). Among the several pathways and transcription factors involved in EMT, Snail1 transcription factor is known to play a critical role, being essential for triggering this process both during embryogenesis and in pathological situations (Lim and Thiery, 2012).

Chromatin reorganization is highly likely to occur during EMT because important changes in the cellular characteristics and gene expression occur during this process. However, only few pieces of evidence support this idea (McDonald et al., 2011). Because the transcription factor Snail1 plays an essential role in EMT through its interaction with LOXL2 (Peinado et al., 2005) and heterochromatin is also characterized by the absence of H3K4me3, we investigated the potential roles of Snail1 and the H3K4me3-remodeling enzyme LOXL2 in regulating heterochromatin reorganization during EMT.

RESULTS

Snail1 Is Essential for Maintaining the Pericentromeric Heterochromatin Organization in Mesenchymal Cells

In mice, reorganization of the genome during germ cell development and gametogenesis leads to changes that are clearly visible at the pericentromeric heterochromatin domains, because these domains are arranged in arrays up to several megabases in length (Probst et al., 2010). These heterochromatin domains, called chromocenters, are organized and maintained by structural proteins such as HP1α and can be easily visualized by staining for HP1α and H3K9me3 (Cheutin et al., 2003; Festenstein et al., 2003; Misteli et al., 2000; Schmiedeberg et al., 2004). We initially studied the contribution of Snail1 to the heterochromatin organization using primary mouse embryonic fibroblasts (pMEFs) derived from embryonic day 12.5 from Snail1-conditional mice (Snail1+/F/F/Cre+ and Snail1+/F/F/Cre−) (Batlle et al., 2013). Snail1 was efficiently ablated by Cre-mediated recombination after tamoxifen addition, as shown by western blot (Figure 1A). Heterochromatin was initially analyzed by 4’,6-diamidino-2-phenylindole (DAPI) staining. In order to quantify the Snail1-dependent heterochromatin organization, we counted the number of foci in Snail1+/F/F/Cre+ (CT) and Snail1+/F/F/Cre− (KO) pMEFs after tamoxifen treatment. This analysis revealed an important decrease in the number of heterochromatin foci per nucleus upon Snail1 depletion (Figure S1 available online). Figure 1B shows that the staining patterns for H3K9me3, a histone modification normally associated with heterochromatin, and for HP1α were similar to that observed for DAPI-stained nuclei. The graph shows that the average of foci number per nucleus was significantly lower in Snail1-depleted cells (Figure 1C). These observations suggest that pericentromeric heterochromatin loses its normal organization in pMEFs in the absence of Snail1.

Snail1 Regulates Pericentromeric Transcription and Is Enriched in Pericentromeric Regions

To determine whether the observed heterochromatin structural deficiencies are functionally important, we analyzed pericentromeric transcription by quantitative RT-PCR (qRT-PCR) in Snail1+/− knockout (KO) MEFs. We observed an increase in major satellite transcription of up to 50-fold in KO compared with control MEFs (CT) (Figure 2A). Other interspersed repetitive elements, such as minor satellites, L1 LINE, SINE1, and IAP1, were not affected (Figure 2A). Although the levels of major transcripts were elevated in the absence of Snail1, they were partially restored when Snail1 was reintroduced into KO MEFs (Figure 2A).

We also checked by chromatin immunoprecipitation (ChIP) whether Snail1 binds to pericentromeric regions. Indeed, endogenous Snail1 was enriched in major satellite regions in control MEFs. This enrichment was not observed when an irrelevant sequence (α-globin gene) was used as a negative control (Figure 2B). Due to the complexity of human pericentromeric sequences and the impossibility to predict in silico the Snail1 binding sites in human heterochromatin, we analyzed whether Snail1 also binds to pericentromeric regions in human cells by ChIP-seq analysis. We covered an average depth of 1 million reads that mapped to unique sites in the genome (Table S1). From the identified binding sites, more than 50% mapped to...
intergenic regions. Exhaustive analysis of intergenic sequences revealed putative binding sites of Snail1 to pericentromeric and centromeric regions of 14 human chromosomes (Figure 2C; Table S2). Using ChIP assays, we confirmed that Snail1 was bound to the pericentromeric regions of the three different human chromosomes analyzed (Figure 2D). ChiP of HP1α demonstrated that the binding sites corresponded to pericentromeric regions (Figure 2D).

**Pericentromeric Transcription Is Linked to H3 Oxidation**

Because the lack of H3K4 methylation in the pericentromeric regions is a feature of these regions and Snail1 represses transcription via the co-repressor LOXL2, which oxidizes histone H3 in lysine 4 (Herranz et al., 2012), we investigated whether LOXL2 is also involved in the regulation of pericentromeric transcription. We found that the observed upregulation of pericentromeric transcription in Snail1 KO MEFs shown in Figure 2A correlated with a decrease in the enrichment of oxidized H3 in major satellite regions (Figure 3A). To assess the contribution of LOXL2 in pericentromeric silencing, we infected Snail1 KO MEFs with a retrovirus vector expressing LOXL2 (Figure 3B, right panel). We observed that overexpression of LOXL2 led to a downregulation of major satellite transcription (Figure 3B, left panel).

LOXL2 knockdown (shLOXL2) in control MEFs induced an increase in major satellite transcript expression analyzed by qRT-PCR (Figure 3C). The RNA levels of other repetitive sequences, such as minor satellites, L1 LINE, SINE1, and IAP1, were not affected.
by the absence of LOXL2 (Figure 3C). Moreover, ChIP assays also showed that LOXL2 is bound to major satellites sequences (Figure 3D, left panel). In addition, loss of LOXL2 reduced H3 oxidation (Figure 3D, middle panel) and increased H3K4me3 levels (Figure 3D, right panel). These results suggested that LOXL2 is involved in the regulation of pericentromeric transcription.

**Pericentromeric Transcription Regulation Is Essential for a Complete EMT**

Because Snail1 is a transcription factor that plays a key role in EMT, we investigated whether it also regulates heterochromatin organization during this transition. For this, we used the well-established model of mouse mammary epithelial NMuMG cells treated with the transforming growth factor β (TGF-β) (Miettinen et al., 1994). HP1α foci were clearly visible in control cells and 24 hr after TGF-β treatment. However, after 8 hr, the number of HP1α foci per cell clearly decreased (Figures 4A and 4B).

Salt extraction of isolated nuclei released higher levels of endogenous HP1α 8 hr after TGF-β treatment as compared with the other time points, suggesting that a pool of HP1α was loosely bound to chromatin at that time point (Figure 4C, left panel). Total HP1α levels were constant in this fraction, as shown in the input by western blot (Figure 4C). Consistently, subcellular fractionation showed a decrease in HP1α levels in the chromatin fraction at 8 hr after TGF-β treatment (Figure 4C, right panel). These results suggested that TGF-β led to the transient loss of HP1α at pericentromeric heterochromatin during the first steps of the EMT process.

We next analyzed whether this heterochromatin reorganization was Snail1 dependent. NMuMG cells were transfected with a small interfering RNA (siRNA) control or siRNA against Snail1 in the presence of TGF-β. As expected, Snail1 protein levels were upregulated 8 hr after TGF-β treatment in cells with the siRNA control, but not in those transfected with Snail1 siRNA (Figures S2A and S2B). The numbers of HP1α foci were quantified in these conditions (Figure 4D). Strikingly, the number of HP1α foci was maintained 8 hr after TGF-β treatment in the absence of Snail1. As expected, the same results were obtained in LOXL2 knockdown conditions (Figure S3). These results suggested that heterochromatin reorganization, as observed by following HP1α localization during EMT, depends on the action of Snail1 transcription factor through LOXL2.

To investigate whether the heterochromatin reorganization that occurs during EMT is linked with pericentromeric transcription regulation, we analyzed major satellite transcription after...
TGF-β treatment in NMuMG cells. We observed an inverse correlation between Snail1 protein levels (Figure 5A, left panel) and the expression levels of major satellite transcripts (Figure 5A, right panel). ChiP experiments showed that Snail1 binds to major satellite regions (Figure 5B, left panel) in a TGF-β-dependent manner and that this binding associates with an increase in the levels of oxidized H3 in major satellite sequences (Figure 5B, right panel). In agreement, in Snail1 knockdown conditions, the repression of major satellite transcription was impaired (Figure 5C). Furthermore, interference RNA experiments demonstrated that LOXL2 participates in this pericentromeric transcription during the EMT, because it was deregulated in the absence of LOXL2 (Figure 5D, left panel). In agreement, the levels of oxidized H3 did not change after TGF-β treatment in LOXL2-depleted cells (Figure 5D, right panel). These results suggest that Snail1 works together with LOXL2 to actively regulate major satellite transcription upon EMT induction.

We next asked whether downregulation of this pericentromeric transcription is relevant for the EMT process. Major satellite transcripts were ectopically expressed in NMuMG cells (Figure S4A), and a TGF-β-dependent EMT was studied (Figure 6). We observed that ectopic expression of major satellite transcripts blocked the release of HP1α during EMT (Figures 6A and 6B). We next compared the global transcriptomes of NMuMG control cells to those of cells stably transfected with a major satellite construct and treated 8 hr with TGF-β. Gene ontology analysis revealed that the 1,269 genes were differentially regulated by TGF-β in major satellite stable cells with

Figure 4. HP1α Distribution Changes during EMT in a Snail1-Dependent Manner
(A) NMuMG cells were treated with TGF-β and immunolabeled with HP1α antibody or stained with DAPI. Scale bar, 10 µm.
(B) The graph depicts the average of foci number per nucleus of HP1α and DAPI-positive cells at different times of TGF-β treatment.
(C) Purified nuclei from NMuMG cells treated with TGF-β were extracted with NaCl (0.5 M), and the extracted fractions or the input were analyzed by western blotting (left panel). Quantification is shown at the bottom. Right panel: the release of HP1α from the chromatin fraction of NMuMG cells treated with TGF-β was monitored by western blot. HP1α levels were standardized using H3 levels. The quantification shows the increase or decrease in the amount of HP1α from treated as compared to untreated cells (time point 0), which was set as 1.
(D) NMuMG cells were transfected with an siRNA control and an siRNA against Snail1. After 24 hr, cells were treated with TGF-β. The average of HP1α-foci number per cell was quantified prior or 8 hr after TGF-β addition. Error bars indicate SD in at least three independent experiments. **p < 0.01.
respect to control cells. These genes corresponded mainly to those pathways associated with the cell cycle, cancer, cell death and survival, and cellular movement and assembly (Figure 6C; full list in Table S3). We observed that the classical mesenchymal genes activated during EMT, such as Fn1, Zeb1, Zeb2, Snai2 (Slug), and several Mmp, presented higher expression levels upon TGF-β treatment in control cells compared with major transfected cells (Figure 6C, right panel, negative log fold...
Figure 6. Ectopic Major Satellite Expression Alters the EMT

(A) Control or NMuMG cells ectopically expressing major satellites were treated with TGF-β and immunolabeled for HP1α. Scale bar, 10 μm. Inset shows magnified representative nucleus, scale bar 5 μm.

(B) The percentage of cells positive for HP1α-foci is shown in the graph.

(C) Gene ontology analysis of differentially expressed genes in major satellite-NMuMG cells compared with control cells 8 hr after TGF-β treatment. List of the selected mesenchymal and epithelial genes that are differentially expressed in major-NMuMG cells compared with control-NMuMG cells upon TGF-β treatment. LogFC indicates log2 fold changes.

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change). The observed changes for selected genes were validated by qRT-PCR (Figure 6D) as well as by western blot for FN1 and N-cadherin (Figure S4B). Although Coh1 RNA downregulation by TGF-β was not statistically different in the two cell populations, the loss of E-cadherin protein was not detected in major satellite cells upon 24 hr of TGF-β treatment (Figure S4C). In agreement, the scattered phenotype observed in control cells was less evident in overexpressing major satellite cells (Figure S4D). Thus, these results suggest that the conversion from epithelial to mesenchymal cells was affected when major satellite transcription was not regulated.

Migration and invasion assays were also performed to determine if these changes in gene expression affect the functional properties of mesenchymal cells. Using 10% fetal bovine serum (FBS) as a chemoattractant, we observed that the loss in migration and invasion capacity upon TGF-β treatment was impaired when the ectopic major satellites were expressed (Figure 6E).

Finally, we assessed the relevance of HP1α and major satellites in EMT by knocking down these two elements. The upregulation of mesenchymal markers observed 24 hr after TGF-β treatment in NMuMG cells was not significantly affected by depletion of HP1α (Figure 7A) or major satellites (Figure 7B).

DISCUSSION

Although it is known that heterochromatin is transcribed, many questions about this process remain unanswered, such as those regarding regulation of this process (which transcriptional factors are involved?) or whether the transcripts are transcribed from transposons or from specific promoters within the genomic DNA satellite region. Here, we show that heterochromatin organization in MEFs depends on the transcription factor Snail1. Few transcription factors have been implicated in genome organization so far (Bulut-Karslioglu et al., 2012; Hakim et al., 2013). In this article, we demonstrate that heterochromatin transcription is tightly regulated by the transcription factor Snail1, which acts through the coressor gene LOXL2.

It has been previously suggested that an epigenetic reprogramming occurs during EMT, because some histone modifications are globally modified (McDonald et al., 2011). We now propose that a general chromatin reorganization is required for a complete EMT transition. In this process, Snail1 plays a key role by repressing pericentromeric transcription (see Figure 7C). Snail1 is a master gene in triggering EMT because it is quickly activated during this process, induces the morphological and genetic changes characteristic of EMT, and is necessary for the completion of this process (Lim and Thiery, 2012). At the onset of EMT, coincident with Snail1 expression, we detected a repression in major satellite transcription leading to a transient release of HP1α from heterochromatin. This result is consistent with previous observations indicating that an RNA component is required to maintain HP1α bound to heterochromatin (Maison et al., 2011). We cannot discard that in the EMT context, Snail1-dependent HP1α posttranslational modifications, such as sumoylation (Maison et al., 2011), could also contribute to HP1α release from heterochromatin. In any case, this HP1α release is probably necessary to permit the heterochromatin reorganization that takes place during the EMT. In agreement, when major satellite transcription was impaired, by ectopically expressing these transcripts, HP1α release was blocked and many of the changes in gene expression characteristics of the EMT were prevented. Moreover, these cells presented lower invasion and migration capability than control cells upon TGF-β treatment, further indicating an incomplete EMT. Finally, we observed that HP1α or major satellite knockdown did not alter the TGF-β-induced EMT, suggesting that major satellite downregulation and HP1α release are important in the initial steps of the transition.

We have also partially elucidated the epigenetic mechanism by which Snail1 regulates the transcription of these heterochromatin transcripts. The histone-modifying enzyme LOXL2 removes the trimethylated amino group in lysine 4, after which the demethylated histone cannot be methylated. The presence of LOXL2 and oxidized H3 in heterochromatin would partially explain the absence of H3K4me3 in these regions. Importantly, oxidized H3 is not a static modification, because the levels of oxidized H3 change during EMT.

Our findings elucidate a mechanism guided by the transcription factor Snail1 and the histone deaminase enzyme LOXL2 that is critical for chromatin reorganization during the EMT process. These data provide evidence that transcription factors not only regulate specific gene transcription but also have a critical role in establishing a functional nuclear genome organization during the conversion of epithelial to mesenchymal cells. Active and inactive chromatin domains cluster in different spatial locations. Some genomic regions could move from one location to another during EMT, allowing or blocking gene transcription. Further work will be necessary to determine which these domains are and how they move during EMT. In any case, the heterochromatin regulation that takes place in a specific window of time during TGF-β-induced EMT is essential for the genome reorganization required to acquire mesenchymal traits.

EXPERIMENTAL PROCEDURES

Cell Lines, Transfection, Infection, and Cloning
SW620, NMuMG, HEK293T, HEK293 Phoenix cells, and MEFs were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) with 10% FBS (Invitrogen) at 37°C in 5% CO2. NMuMG cells were also supplemented with insulin (10 μg/ml). A final concentration of 1–5 ng/ml TGF-β was used to

(D) Validation by qRT-PCR of selected genes.
(E) Major satellite-NMuMG and control NMuMG cells were treated with TGF-β; after 24 hr, cells were reseeded on transwell chambers and incubated for 10 hr (left panel, migration) or placed in Matrigel-coated transwells and incubated for 24 hr (right panel, invasion). Nonmigrating and noninvasive cells were removed from the upper surface of the membrane, while cells present at the lower surface were fixed and stained with DAPI. The DAPI-stained nuclei were counted in four different fields per filter by ImageJ software.

Error bars indicate the SD for at least four independent experiments. *p < 0.05, **p < 0.01.
Figure 7. Depletion of HP1α or Major Satellites Does Not Affect EMT

(A and B) NMuMG cells were transfected with an siRNA control or an siRNA against HP1α (A) or with LNA-DNA control gapmers or with a mix of LNA-DNA gapmer1 and LNA-DNA gapmer2 to deplete major satellite expression (B). After transfection, cells were treated with TGF-β for 24 hr when indicated. Levels of FN1, Zeb1/Zeb2, Slug (Snail2), CD44, HP1α, and major satellites were determined by qRT-PCR. Error bars indicate SD in at least three independent experiments.

(C) Working model. Upon TGF-β induction of EMT, Snail1 is rapidly upregulated, binds to pericentromeric regions, and recruits LOXL2 to oxidize H3 and repress major satellite transcription. As a consequence, HP1α is released from heterochromatin, enabling chromatin reorganization and acquisition of mesenchymal traits. This Snail1 action is transient, and in 24 hr major satellite levels and HP1α binding to heterochromatin have been recovered. Therefore, for the conversion of an epithelial cell to a mesenchymal cell during TGF-β-induced EMT, there is a window of time during which major satellite transcription is downregulated due to the actions of Snail1 and LOXL2 and the oxidation of H3. HP1α is released from heterochromatin during this time frame. Altogether, this allows chromatin reorganization and mesenchymal traits to be acquired.
stimulate NMuMG cells and induce EMT, and cells were collected after 0, 2, 8, and 24 hr. pMEFs were obtained from conditional knockout mice (Snai1/Crof1 and Snai1/Crof1), and Snai1 deletion was induced by tamoxifen treatment (1 µM). Immortalized MEFs were obtained following the 3T3 protocol (Nilsauser and Green, 1965). MEFs were transfected with the pcDNA3 empty vector (MEFs CT) or pcDNA3-CreGFP to induce Snai1 deletion (MEFs KO). MEFs were transfected or infected with pBabe-mSnail1HA, PCMV-LXL2, or empty vectors and selected with puromycin (1 µg/ml). NMuMG cells were infected with retroviruses using pBabe-Maj-Sarat and pBabe-empty vectors and selected with puromycin (1 µg/ml). pBabe-Maj-Sarat plasmids were generated by subcloning PBR4MAj-9-2 plasmid (Lehnertz et al., 2003) into a pBabe-empty vector after EcoRI digestion. Two plasmids were generated: one with the insert oriented in the sense direction and the other in the antisense. In major satellite overexpression experiments, both vectors were coinfected. For retroviral infections, HEK293T Phoenix cells were transfected (day 0) using Lipofectamine 2000 reagent (Invitrogen) either with pCMV-LXL2 or pCMV-empty vectors to infect MEFs or with pBabe-Maj-Sarat and pBabe-empty vectors to infect NMuMG. The transfection medium was replaced with fresh medium after 24 hr (day 1), and the cell-conditioned medium at day 2 was filtered and used to infect target cells with 8 µg/ml polybrene. HEK293T Phoenix cells were incubated with fresh medium for a further 24 hr, and on day 3, a second infection with the conditioned medium and polybrene was performed. For lentiviral infection, HEK293T cells were used to produce viral particles. Cells were grown to 90% confluence and then transfected (day 0) with Lipofectamine 2000 reagent (Invitrogen) and Lipofectamine 2000 reagent (Invitrogen) either with pCMV-LOXL2 or pCMV-empty vectors to infect MEFs or with pBabe-Maj-Sarat and pBabe-empty vectors to infect NMuMG. The transfection medium was replaced with fresh medium after 24 hr (day 1), and the cell-conditioned medium at day 2 was filtered and used to infect target cells with 8 µg/ml polybrene. HEK293T cells were incubated with fresh medium for a further 24 hr, and a second infection with the conditioned medium and polybrene was performed on day 3. To downregulate Snail1 in NMuMG cells, mmSnail siRNA (QIAGEN; target sequence: 5′-CACCCTTGGATACAGCAAT-3′) was reverse transfected in NMuMG cells (96-well plates) at a final concentration of 30 nM siRNA using a 3.5% solution of HiPerFect transfection reagent (QIAGEN). The Cy3-labeled siGLO cyclin B siRNA (Dharmacon) was used to monitor transfection efficiency, and the AllStars scrambled siRNA and siRNA targeting GFP (QIAGEN) served as negative controls. Media was replaced 10 hr after transfection, and TGF-β (5 ng/ml) was added 24 hr later. Cells were fixed 8 hr after TGF-β addition with paraformaldehyde (PFA) 4% and then were stained by immunofluorescence.

To downregulate HP1α in NMuMG cells, mouse CBX5 siRNA (Dharmacon L-004799-01) was transfected in NMuMG cells at a final concentration of 5 nM siRNA with RNAiMax transfection reagent (Invitrogen) and Opti-Mem transfection media (Invitrogen) following manufacturers instructions. siGENOME nontargeting siRNA #3 (Dharmacon D-001210-03-20) was used as control. Media was replaced 24th hour and cells were treated with TGF-β overnight. To downregulate major satellite transcripts, NMuMG cells were transfected with LNA-DNA gapmers (sequences specified in Supplemental Information) with RNAiMax transfection reagent (Invitrogen) and Opti-Mem transfection media (Invitrogen) following manufacturers instructions. TGF-β was added at the time of transfection and RNA was collected after 24h.

Antibodies
The following antibodies were used: α-H3K9me3 (1:625, Millipore), α-H1p1α (h2164, Sigma), α-LXL2 (Abcam ab60753), α-LXL2 (Abcam ab55470), α-H3 (Abcam ab1791), α-Snail1 (Franci et al., 2006), α-tubulin (T9026, Sigma), α-pyruvate kinase (Chemicon ab1235).

Immunofluorescence
Cells were fixed with 4% PFA for 15 min at room temperature (RT) and blocked for 1 hr with 1% PBS-BSA. Cells were then incubated at room temperature for 2 hr with primary antibody, followed by 3 washes with PBS and a 1 hr incubation at RT with the secondary antibody. Cells were washed again three times with PBS and incubated for 5 min with DAPI (0.25 µg/ml) to stain cell nuclei before mounting them with fluoromount. Fluorescence images corresponding to DAPI, Snai1, (E-cadherin), HP1α, and H3K9me3 were acquired for each condition (ten fields per well) with the InCell 2000 automated epifluorescence microscope (GE) at 40× magnification. Images were analyzed using InCell investigator Software (GE) by first defining the cell nuclei with DAPI staining. The nuclei were then segmented using top-hat segmentation defining a minimum nucleus area of 100 µm². To define the expression of Snai1 protein by cell, the average intensity of pixels in the reference channel (Alexa 488) within the defined nuclear region was measured. HP1α foci within the nuclei (Alexa 594) were segmented using multiscale top-hat to define a granule size of 1–2 µm. Once each cell was assigned a nuclear intensity for the specific expression of Snai1 and a number for HP1α foci, a threshold filter defining positive and negative expressing cells was set. Threshold filter uses a histogram for data visualization. The cut-off of the filters was set as follows: Snai1 expression was measured in knockout MEFs or NMuMG untreated cells to define the negative population. Positive controls (e.g., TGF-β-treated cells) were analyzed to define the highly expressing population. The opposite criterion was followed to specify the filter settings for HP1α foci. Once the cut-off was set up, the analysis was carried out. The program thus assigns a definition of positive or negative to each cell and generates a percentage of both cell populations per well.

Subcellular Fractionation and Salt Extraction Experiments
For subcellular fractionation, cells were lysed in buffer A (10 mM HEPES [pH 7.8], 10 mM KCl, 1.5 mM MgCl₂, and 0.5 mM dithiothreitol [DTT]) supplemented with protease and phosphatase inhibitors for 10 min at 4°C. A one-third volume of 10% Triton X-100 was added, and samples were mixed for 30 s. Samples were then centrifuged at 15,000 x g at 4°C for 1 min. Supernatant was collected, and a 0.11 volume of buffer B (0.3 M HEKES [pH 7.8], 1.4 M KCl, and 30 mM MgCl₂) was added. After 30 min incubation at 4°C, samples were centrifuged for 15 min at 15,000 x g at 4°C, and supernatant was collected again (cytoplasmic fraction). Pelleted nuclei were resuspended in one-fifth the original volume in buffer C (20 mM HEPES [pH 7.8]), 25% glycerol, 0.42 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, and 0.5 mM DTT) and rotated for 30 min at 4°C. After 15 min centrifugation at 15,000 x g at 4°C, supernatant was collected (soluble nucleus) and separated from the pellet (chromatin fraction), which was resuspended in Laemmli buffer. Histone H3 and β-tubulin were used as chromatin and cytoplasmic markers, respectively. Salt extraction experiments were performed as described previously (Mesher et al., 2006).

Quantitative Reverse Transcription Coupled to PCR
After RNA extraction with TRIzol reagent (Invitrogen) and genomic DNA digestion with DNase turbo (Ambion) following the manufacturers’ instructions, real-time quantitative PCR experiments were done in a Light Cycler PCR machine (Roche). See Supplemental Experimental Procedures for primer sequences.

ChIP Experiments
ChIP experiments were performed as described previously (Herranz et al., 2008). Briefly, cells were crosslinked in 1% formaldehyde for 10 min at 37°C. Crosslinking was stopped by adding glycine to a final concentration of 0.125 M for 2 min at room temperature. Cell monolayers were scraped in cold lysis buffer (50 mM Tris [pH 8.0], 10 mM EDTA, 0.1% NP-40, and 1% glycerol) and incubated 20 min on ice. Nuclei pellets were lysed with SDS lysis buffer (1% SDS, 10 mM EDTA, and 50 mM Tris [pH 8.0]) and extracts were sonicated. Sonication was performed five times at 40% for 10 s (Branson) to generate 200 to 500 bp DNA fragments. Supernatants were diluted 1:10 with dilution buffer, and immunoprecipitation was done by rotation overnight at 4°C with primary antibody or irrelevant immunoglobulin Gs. DNA was purified with GFX kit from GE Healthcare and eluted in MilliQ water. To detect H3 oxidation, ChIP assays were performed as described previously (Herranz et al., 2012). Briefly, cells were incubated with activated biotin, crosslinked, and immunoprecipitated with anti-histone H3; immunocomplexes were re-extracted with SDS lysis buffer and reimmunoprecipitated with streptavidin-magnetic beads for 30 min at 4°C. Samples were then treated with elution buffer and incubated at 65°C to reverse formaldehyde crosslinking. Results were quantified by taking into account the total amount of H3 immunoprecipitated in each condition.
For ChIP sequencing (ChIP-seq) analysis, the Solexa library was prepared as recommended by the manufacturer (http://www.illumina.com). The size selection of this library was performed by gel electrophoresis, with subsequent excision and purification of DNA in the ~150–300 bp range. The positional resolution of ChIP-seq was improved by reducing the size, and narrowing the size range, of DNA collected from gel purification (Johnson et al., 2007). DNA sequencing of each sample was performed by the Solexa/Illumina protocol. Solexa ChIP and control reads were analyzed jointly to identify regions with an overrepresentation of reads in the ChIP sample versus the control sample. Reads obtained from the samples were aligned to the human reference genome using the GEM mapper, allowing up to three mismatches. Reads that were unambiguously mapped were selected, and site identification of short sequence reads (SISsRs), a peak-finding algorithm, was used to identify significantly enriched genomic locations (Jothi et al., 2008). Candidates for enriched regions were identified as aggregations of five or more ChIP reads that were neither separated by more than 100 bp nor present in the control sample. It was also determined whether flanking genes and promoter regions were associated with these peaks in the human genome.

**Gene Expression Analysis**

We measured gene-expression levels of control-NMuMG cells and major-satellite-NMuMG stable cells. For microarray analysis, amplification, labeling, and hybridizations were performed according to protocols from the Ambion WT Expression Kit (Ambion). Samples were labeled using the WT Terminal Labeling Kit (Affymetrix) and then hybridized to the GeneChip Mouse Gene 1.0 ST Array (Affymetrix) in a GeneChip Hybridization Oven 640. Washing and scanning were performed using the Hybridization Wash and Stain Kit and the GeneChip System of Affymetrix (GeneChip Fluidics Station 450 and GeneChip Scanner 3000 7G). After quality control of raw data, they were background corrected, quantile normalized, and summarized to a gene level using the robust multichip average, obtaining a total of 28,822 transcript clusters, excluding controls, which roughly correspond to genes. Linear Models for Microarray (LIMMA), a moderated t-statistics model, was used to detect differentially expressed genes between the conditions. Correction for multiple comparisons was performed using a false discovery rate. Genes with an adjusted p value less than 0.05 and with an absolute fold change value above 1.5 were selected as significant. Ingenuity Pathway Analysis v 9.0 (Ingenuity Systems; http://www.ingenuity.com) was used to functionally analyze the results.

**Migration and Invasion Assays**

For the migration experiments, major-NMuMG and control-NMuMG cells were treated with TGF-β; after 24 hr, 50,000 cells were resuspended in DMEM 0.2% FBS-0.1% BSA, reseeded on a transwell filter chamber (Costar 3422), and incubated for 6–8 hr. For invasion assays, cells were placed in Matrigel-coated transwell filter (BD356234) and incubated for 24 hr. In both cases, DMEM 10% FBS-0.1% BSA, reseeded on a transwell filter chamber (Costar 3422), and incubated for 24 hr. Migration and Invasion Assays

**ACCESSION NUMBERS**

The Gene Expression Omnibus accession number for the data referred to in this paper is GSE49073.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes four figures, three tables, and a primer list and can be found with this article online at http://dx.doi.org/10.1016/j.molcel.2013.10.015.

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