SUMMARY
Extensive changes in posttranslational histone modifications accompany the rewiring of the transcriptional program during stem cell differentiation. However, the mechanisms controlling the changes in specific chromatin modifications and their function during differentiation remain only poorly understood. We show that histone H2B monoubiquitination (H2Bub1) significantly increases during differentiation of human mesenchymal stem cells (hMSCs) and various lineage-committed precursor cells and in diverse organisms. Furthermore, the H2B ubiquitin ligase RNF40 is required for the induction of differentiation markers and transcriptional reprogramming of hMSCs. This function is dependent upon CDK9 and the WAC adaptor protein, which are required for H2B monoubiquitination. Finally, we show that RNF40 is required for the resolution of the H3K4me3/H3K27me3 bivalent poised state on lineage-specific genes during the transition from an inactive to an active chromatin conformation. Thus, these data indicate that H2Bub1 is required for maintaining multipotency of hMSCs and plays a central role in controlling stem cell differentiation.

INTRODUCTION
Both embryonic development and tissue homeostasis require the expansion and differentiation of multipotent self-renewing stem cells. Stem cells can differentiate into a variety of cell types depending upon the stimulus provided. A deeper understanding of the molecular mechanisms determining cell-type-specific differentiation may provide therapeutic opportunities for treating a variety of pathological conditions.

Stem cells exhibit specific gene expression signatures which dictate cell fate during differentiation (Fisher and Fisher, 2011). A unique pattern of posttranslational histone modifications in stem cells governs gene expression patterns and differentiation potential (Fisher and Fisher, 2011; Meissner, 2010). Genome-wide studies have revealed that many developmental genes exhibit “bivalent” chromatin domains displaying both “active” (H3K4me3) and “repressive” (H3K27me3) histone modifications (Azuara et al., 2006; Bernstein et al., 2006). This “poised” state is proposed to allow for correct temporal gene expression. The transcriptional apparatus and chromatin modifications exhibit a high degree of crosstalk. For example, phosphorylation of Ser2 (P-Ser2) within the heptapeptide repeat of the RNA polymerase II (RNAPII) C-terminal domain (CTD) provides a platform for recruiting the WW domain containing adaptor protein with coiled coil (WAC) (Zhang and Yu, 2011). In turn, WAC recruits the RNF20/RNF40 ubiquitin ligase complex which monoubiquitinates H2B (Kim et al., 2009; Zhu et al., 2005). Knockdown of either CDK9 (Pirngruber et al., 2009b) or WAC (Zhang and Yu, 2011) elicits an effect on H2Bub1 similar to that of RNF20 or RNF40 depletion.

Consistent with its dependence upon the elongating form of RNAPII, H2Bub1 is primarily associated with the transcribed regions of active genes (Minsky et al., 2008) where it facilitates transcriptional elongation (Pavi et al., 2006; Prenzel et al., 2011). However, despite its association with active genes, H2Bub1 is only required for the transcription of a subset of inducible genes (Prenzel et al., 2011; Shema et al., 2008). Consistent with a specific regulation of tumor-relevant transcriptional pathways, H2Bub1 is a proposed tumor suppressor whose levels decrease during tumor progression (Johnsen, 2012; Prenzel...
The inverse relationship between differentiation status and malignancy may indicate that decreased H2Bub1 in advanced tumors leads to a less differentiated phenotype. However, the role of H2Bub1, its modifying enzymes, and upstream regulators in controlling cell-fate determination in mammalian systems remains unexplored.

Here we demonstrate that H2Bub1 levels increase in diverse cell differentiation systems. Perturbation of H2B monoubiquitination via depletion of its ubiquitin ligases RNF40 and RNF20, or upstream regulators CDK9 and WAC impairs stem cell differentiation. RNF40 depletion affects multipotency by preventing the resolution of bivalent histone marks on lineage-specific genes. These results uncover a previously unknown function of H2Bub1 and its upstream regulatory pathway in stem cell differentiation and shed light into the mechanism by which its loss may increase malignant potential.

**RESULTS**

**H2Bub1 Increases during Cellular Differentiation**

We previously reported decreased H2Bub1 levels during malignant progression (Prenzel et al., 2011). Given the inverse correlation between tumor differentiation status and malignancy, we hypothesized that H2Bub1 may increase during normal cellular differentiation. We therefore examined H2Bub1 levels in various differentiation systems including telomerase-immortalized human mesenchymal stem cells (hMSCs) (Simonsen et al., 2002) differentiated to either the osteoblast (Figure 1A) or adipocyte lineages (Figure 1B), human fetal osteoblast (hFOB) cells (Figure 1C), mouse neurospheres (Figure 1D, see Figure S1A available online), and oligodendrocytes (Figure 1E). In each of the investigated mammalian model systems, H2Bub1 levels increased concomitantly with the induction of lineage specification which was accompanied by increased RNF40 protein levels in hMSC (Figure S1B). In contrast, the levels of H2A mono-ubiquitination remained constant during hMSC differentiation (Figure S1C).

In order to determine whether the increase in H2Bub1 levels during cellular differentiation is conserved across species, we performed immunofluorescence analyses of H2Bub1 in the Drosophila melanogaster ovary. As observed in undifferentiated mammalian cells, H2Bub1 was undetectable in Drosophila germline stem cells (characterized by the presence of phospho-Mad: Figure 1F). In contrast, differentiated cystoblasts displayed readily detectable H2Bub1 levels. Thus, increased H2Bub1 levels during cellular differentiation are common to multiple differentiation programs and across species.

**RNF40 Knockdown Impairs hMSC Differentiation**

To test whether H2Bub1 is required for differentiation, we examined the effects of RNF40 depletion on hMSC differentiation. As assessed by staining for differentiated osteoblasts (alkaline phosphatase) or adipocytes (oil red O), we observed a clear decrease in both differentiation models following RNF40 knockdown with independent RNF40 siRNAs (Figures 2A and 2B; Figures S2A–S2F). Consistently, no lipid droplet formation was observed by electron microscopy in RNF40-depleted cells grown under adipocyte differentiating conditions (Figure S2G).

**RNF40 Knockdown Impairs Differentiation-Regulated Transcription**

In order to determine the extent of the effects elicited by RNF40 knockdown on hMSC differentiation, we performed siRNA knockdown and transcriptome-wide gene expression microarray analyses of undifferentiated hMSCs and cells differentiated to the adipocyte or osteoblast lineages for 2 or 5 days. RNF40 knockdown had little or no effect on gene expression in undifferentiated hMSCs (Figure S2J). Strikingly, the differentiation-induced gene expression changes were significantly impaired following RNF40 knockdown (Figures 2C and 2D) without affecting the expression of the osteoblast and adipocyte-regulatory factors Runx2, Cebpβ, and Cebpβ (Figures S2H and S2I). The effects of RNF40 knockdown were evident for common (Figure S2K) as well as lineage-specific (Figures S2L and S2M) transcriptional programs. Pathways significantly enriched in gene sets differentially regulated in each differentiation program and by RNF40 depletion (Table S1) include focal adhesion (Figures S2N–S2Q), cell cycle (Figures S2R–S2U), adipokine signaling (Figures S2V and S2W), and pathways in cancer (Figures S2X–S2AA). Microarray data were verified on a number of osteoblast (Bglap, Alpl, and G6pd)- and adipocyte (Pdk4, Pparγ, and Rasd1)-specific genes (Figures 2E and 2F, respectively). Consistent with the obligate heterodimeric function of RNF20 and RNF40, RNF20 knockdown also decreased H2Bub1 levels and impaired the induction of osteoblast- and adipocyte-specific gene expression (Figures S2AB–S2AE). Importantly, RNF40 depletion did not affect mRNA levels of other pathway components (CDK9, WAC, RNF20; Figures S2AF and S2AG).

**CDK9 Controls hMSC Differentiation**

Since CDK9 controls the global and gene-specific levels of H2Bub1 (Pimruber et al., 2009b; Shchebet et al., 2012), we hypothesized that CDK9 may also be required for hMSC differentiation by directing RNF40-dependent H2B monoubiquitination through P-Ser2 RNAPII CTD. Consistent with this notion, the levels of P-Ser2 increased during hMSC differentiation (Figure S3A).

We next examined the effects of CDK9 knockdown on hMSC differentiation. Similar to the effects of RNF40 knockdown, adipocyte differentiation and gene expression (Figures 3C and 3D, Figure S3F) were impaired following CDK9 knockdown, as was the expression of the osteoblast marker genes Bglap and G6PD (Figure 3B) and global H2Bub1 levels (Figures S3B and S3C). Surprisingly, the induction of alkaline phosphatase activity (Figure 3A, Figure S3E) and Alpl gene expression (Figure 3B) was increased following CDK9 knockdown. Since alkaline phosphatase is also a marker of pluripotency, these results may indicate a reversion to an earlier stem cell phenotype. These effects were not due to impaired expression of H2Bub1 pathway regulators (Figures S3G and S3H). Thus, we conclude that CDK9 knockdown prevents hMSC differentiation irrespective of cell lineage.

**WAC Knockdown Phenocopies RNF40 Depletion**

WAC functionally links CDK9 and H2Bub1 during transcription by recruiting the RNF20/RNF40 complex to P-Ser2 RNAPII
Figure 1. H2Bub1 Levels Increase upon Differentiation

(A and B) Immortalized human mesenchymal stem cells (hMSCs) were induced to differentiate into the osteoblast (A) or adipocyte (B) lineages for 5 days. Protein extracts were analyzed by western blot using antibodies against H2Bub1 or H2B (left panel). The expression of the osteoblast (ALPL, BGLAP; A) or adipocyte (LPL, PPARG; B) marker genes was verified by qRT-PCR. Gene expression was normalized to a control gene (HNRNPK) and the undifferentiated condition and expressed as “relative mRNA levels.” Mean ± SD, n = 3. Statistical analysis, *p < 0.05; **p < 0.01; ***p < 0.001.

(C) Human fetal osteoblasts (hFOBs) were differentiated and analyzed by western blot for H2Bub1 and H2B as in (A). Induction of osteoblast marker genes (ALPL, BGLAP) was verified as in (A). Mean ± SD, n = 3.

(D) Mouse neurospheres were induced to differentiate and analyzed by western blot as in (A). Differentiation to glial cells was examined by immunofluorescence staining of GFAP (red). Nuclei were costained with DAPI (blue).

(E) Mouse oligodendrocytes were differentiated for 5 days and analyzed for H2Bub1 and H2B levels by western blot. Differentiation was verified by immunofluorescence staining against myelin basic protein (MBP, green) and actin (red).

(F) Immunofluorescence analysis of Drosophila melanogaster ovary stained for H2Bub1 and phosphorylated Mad (pMad; marker of proliferating stem cells). TF, terminal filament cells; CpC, cap cells; GSC, germline stem cells; Cyst, cystoblast; EC, escort cells.
Figure 2. RNF 40 Is Required for hMSC Differentiation

(A and B) hMSCs were transfected with control and RNF40 siRNAs and induced to differentiate into osteoblasts (A) or adipocytes (B) for 5 days. Differentiation was verified by alkaline phosphatase (A) or oil red O (B) staining (left). The stained area was quantified using ImageJ software and displayed as percentage of area (right). Mean ± SD, n = 3.
H2Bub1 Is Required for Stem Cell Differentiation

CTD (Zhang and Yu, 2011). Consistent with this report, WAC was required for the stability of the RNF20/40 complex (Figures S3I and S3J) and maintaining H2Bub1 levels (Figures S3K–S3N). Importantly, WAC knockdown impaired osteoblast (Figure 3E and Figure S3O) and adipocyte (Figure 3G and Figure S3P) differentiation and the induction of marker gene expression (Figures 3F and 3H). While RNF20, RNF40, and WAC protein levels were dependent upon one another (Figures S3I and S3J), their mRNA levels were unaffected by WAC knockdown (Figures S3Q and S3R). These results provide a mechanistic explanation for how CDK9 directs H2Bub1 during differentiation through RNAPII CTD phosphorylation and recruitment of the WAC/RNF20/RNF40 ubiquitin ligase complex. Perturbation of any component similarly impairs stem cell differentiation.

RNF40 Is Required for Resolution of Bivalency

In order to test whether the effects of RNF40, RNF20, CDK9, and WAC depletion on hMSC differentiation may be a direct effect of H2Bub1, we performed chromatin immunoprecipitation analyses on the adipocyte-specific genes PDK4, PPARG, and RASD1 as well as positive (GAPDH) and negative (TF1) control genes. Indeed, all three lineage-specific genes showed increased levels of H2Bub1 during differentiation (Figure 4A). Importantly, the levels of H2Bub1 were constantly present on the GAPDH gene and decreased following RNF40 knockdown but were constitutively absent from the silenced TFF1 gene.

Embryonic stem cells and some lineage-committed stem cells display a unique combination of both activating (H3K4me3) and repressing (H3K27me3) histone modifications on many lineage-specific genes (Collas, 2010; Fisher and Fisher, 2011). During differentiation, bivalency on adipocyte-specific genes is resolved in favor of an active state in which H3K4me3 is retained while H3K27me3 is lost (Collas, 2010; Noer et al., 2009). Perturbations in the resolution of bivalency impair the inducibility of lineage-specific genes during differentiation. The analysis of genome-wide chromatin immunoprecipitation-sequencing data (ChIP-Seq) from human embryonic stem cells (hESCs) and normal adipose nuclei confirmed that the investigated adipocyte-specific genes (PDK4, PPARG, RASD1) are all bivalent in hESC and lose their bivalency in favor of H3K4me3 during terminal adipocyte differentiation (Figure 4B).

Given the established dependence of H3K4me3 on H2Bub1 in some systems (Kim et al., 2009; Pirngruber et al., 2009a; Zhu et al., 2005), we hypothesized that the impaired differentiation observed following RNF40 knockdown (with no major effects on the global gene expression profile) may be due to changes in the bivalency status of differentiation-induced genes. Surprisingly, while adipocyte-specific genes (PDK4, PPARG, RASD1) exhibit significant levels of both H3K4me3 and H3K27me3 in the undifferentiated state (Figures 4C and 4D), neither the global (Figure S4) nor the gene-associated levels of H3K4me3 were reduced following RNF40 knockdown (Figure 4C). This supports the findings that H3K4me3 may be independent of H2Bub1 in some systems (Shema et al., 2008; Vethantham et al., 2012).

As previously reported (Noer et al., 2009), bivalency was resolved in favor of H3K4me3 on adipocyte-specific genes during differentiation (Figure 4D). In contrast, H3K27me3 was retained on bivalent adipocyte-specific genes following RNF40 knockdown. Thus RNF40-dependent H2B monoubiquitination appears to be dispensable for H3K4me3 but essential for the resolution of bivalency on lineage-specific genes.

DISCUSSION

In order to obtain a clearer understanding of the roles of specific chromatin modifications in the transition from stem cells into a differentiated phenotype, we investigated the effects of perturbing H2Bub1 levels during cellular differentiation. We show that both global and lineage-specific gene-associated H2Bub1 levels increase during hMSC differentiation. Although a recent study reported a decrease in H2Bub1 during myoblast differentiation (Vethantham et al., 2012), our data suggest that H2Bub1 levels increase in multiple diverse differentiation systems. Furthermore, this process is evolutionarily conserved. Notably, in addition to the investigated mouse and human cell culture differentiation models, we demonstrate that H2Bub1 is suppressed in stem cells in vivo. Although a suppression of H2Bub1 was previously suggested to be essential for stem cell maintenance in Drosophila (Buszczak et al., 2009), we now show that H2Bub1 levels indeed increase during differentiation and that its modifying enzymes and upstream regulators are essential for the differentiation process.

We have demonstrated that CDK9 activity is essential for the induction of lineage-specific gene expression. Data in Drosophila and C. elegans support this observation, since a suppression of CDK9 activity is required for stem cell maintenance (Batchelder et al., 1999; Hanyu-Nakamura et al., 2008; Zhang et al., 2003). Furthermore, it has been shown that CDK9 activity is required for differentiation in several mammalian cell systems, including adipocyte (Iankova et al., 2006), myoblast (Simone et al., 2002), monocyte (Yu et al., 2006), and cardiomyocyte differentiation (Kaichi et al., 2011). Given that a large fraction of unexpressed genes in ESCs exhibit paused RNAPII lacking P-Ser2 (Guenther et al., 2007), suppression of CDK9 activity may be a general mechanism employed for maintaining a stem cell state. Like bivalent chromatin domains, the presence of a paused RNAPII is expected to allow the timely induction of differentiation-related genes.

Specific chromatin states play an essential role in controlling pluripotency, and dynamic changes in these states are required for proper lineage-specific differentiation (Fisher and Fisher, 2011; Meissner, 2010). We have confirmed that adipocyte-specific genes exist in a bivalent chromatin state in multipotent hMSCs. In parallel with the global increase in H2Bub1 levels,
Figure 3. CDK9 and WAC Direct H2Bub1 during Differentiation

(A–H) hMSCs transfected with siRNAs against CDK9 (A–D) or WAC (E–H) were induced to differentiate into the osteoblast (A and B, E and F) or adipocyte (C and D, G and H) lineages. Cells were stained for alkaline phosphatase activity (A and E) or oil red O (C and G) for osteoblast and adipocyte lineages, respectively. Staining was quantified as in Figures 2A and 2B. Mean ± SD, n = 3. (B, D, F, and H) The knockdown efficiency and the effects on osteoblast- (ALPL, BGLAP, G6PD; B and F) and adipocyte-specific (PDK4, PPARG, RASD1; D and H) gene expression were verified by qRT-PCR and analyzed as in Figures 2E and 2F following 2 days of differentiation. Mean ± SD, n = 3.
H2Bub1 Is Required for Stem Cell Differentiation

A. 

**PDK4**

**PPARG**

**H2Bub1**

**RASD1**

**GAPDH**

**TFF1**

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**H3K4me3**

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D. 

**PDK4**

**PPARG**

**H3K27me3**

**RASD1**

**HIST1H2AC**

**TFF1**

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E. 

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this bivalency is resolved, and these genes become fully active and exhibit a decrease in H3K27me3 while retaining H3K4me3. In our model, tissue-specific transcription factors promote differentiation in a CDK9-dependent manner by directing H2Bub1 (Figure 4E). In turn, H2Bub1 (or possibly RNF40 directly) likely directs dynamic changes in chromatin structure which facilitate the demethylation of H3K27me3 during the transition from a repressive to an active chromatin state. Consistently, we and others have shown that RNF20 and RNF40 cooperate with the FACT histone chaperone complex to stimulate histone exchange and facilitate transcriptional elongation (Pavri et al., 2006; Prenzel et al., 2011). Consistent with a CDK9 → WAC → RNF20/ RNF40 → H2Bub1 → FACT regulatory circuit, the presence of both H2Bub1 and FACT on target genes depends upon CDK9 activity rather than transcriptional activity per se (Gomes et al., 2006; Pirngruber et al., 2009b). Interestingly, the chromatin remodeling factor CHD1 is required for pluripotency (Gaspar-Maia et al., 2009) and was independently identified in complexes containing CDK9 (Park et al., 2010) and FACT (Kelley et al., 1999). Furthermore, a recent study identified a functional interaction between H2Bub1 and Chd1 in yeast (Lee et al., 2012). Moreover, mammalian CHD1 interacts directly with H3K4me3 through its chromodomain (Simos et al., 2007) but is excluded from bivalent domains (Gaspar-Maia et al., 2009). Thus, the maintenance of H3K27me3 at bivalent genes following RNF40 knockdown may function in part by preventing CHD1 recruitment.

In conclusion, our data establish a role for CDK9, WAC, and RNF40 in directing H2B monoubiquitination and transcriptional reprogramming during stem cell differentiation. This effect appears to be due to dynamic changes in chromatin structure during differentiation, which facilitate a transition from an inactive to an active chromatin state. In keeping with this, we observed maintenance of H3K27me3 levels on bivalent adipocyte-specific genes following RNF40 knockdown. Thus these data place H2Bub1 and its regulatory pathway as central players in cell-fate determination and underscore the importance of dynamic changes in chromatin structure during cellular differentiation.

EXPERIMENTAL PROCEDURES

Cell Culture, Differentiation, and Knockdowns

hMSC and hFOB1.17 cells were cultured and differentiated as previously done (Simonsen et al., 2002; Harris et al., 1995) and are described together with staining procedures and the generation, culture, and differentiation of neuro-spheres and oligodendrocytes in the Supplemental Experimental Procedures.

Knockdown procedures and siRNAs utilized are listed in the Supplemental Information.

Western Blot Analysis, RNA Isolation, qRT-PCR, ChIP

Western blot analysis was performed with antibodies and dilutions listed in the Supplemental Information. RNA isolation, reverse transcription, ChIP, and quantitative RT-PCR were performed as described (Prenzel et al., 2011) with the modifications listed in the Supplemental Experimental Procedures. ChIP-Seq results were obtained from the NIH Roadmap Epigenomics Project (Bernstein et al., 2010), visualized using the UCSC Genome Browser, and used under permission from the NIH Epigenome Mapping Center at Broad Institute.

Microscopy

For analyzing H2Bub1 levels in vivo, ovaries from OregonR/Drosophila were fixed and stained as described in the Supplemental Experimental Procedures.

Microarray Studies

Whole-genome gene expression analysis was performed by the Vancouver Prostate Centre Laboratory for Advanced Genome Analysis (Vancouver, Canada) using the Illumina human HT-12 v4 beadchip. Gene expression data were analyzed as previously described (Prenzel et al., 2011). Gene set pathway analyses were performed as described in the Supplemental Experimental Procedures. All gene expression data will be made publicly available through the GEO repository.

ACCESSION NUMBERS

The Gene Expression Omnibus (GEO) accession numbers for the ES and adipose nuclei ChIP-Seq reported in this paper are GSM669889, GSM669897, GSM669925, and GSM669930. The GEO accession number for the gene expression microarray analyses reported in this paper is GSE38173.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures, one table, Supplemental Experimental Procedures, and Supplemental References and can be found with this article online at doi:10.1016/j.molcel.2012.05.022.

ACKNOWLEDGMENTS

The authors would like to thank S. Le Bihan and A. Haegeert for performing microarray experiments, and the NIH Epigenome Mapping Center at Broad Institute for allowing the use of the unpublished ChIP-Seq data. O.K. and Z.N. were supported by fellowships from the Dorothea-Schlo¨zer Program of the University of Göttingen and the German Academic Exchange Service (DAAD), respectively. This work was funded by an ERC Starting Grant to M.S.; the German Research Foundation (DFG) Research Group 942 to T.B.; and the State of Lower Saxony, Hannover, Germany (VWZN2562) and the DFG (JO 815/1) to S.A.J.

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Figure 4. RNF40 Expression Is Required for the Resolution of Bivalent Chromatin Marks

(A) ChIP analysis of H2Bub1 on adipocyte-regulated genes (PDK4, PPARG, and RASD1) upon RNF40 knockdown. hMSCs were differentiated to adipocytes for 2 days following RNF40 depletion for 24 hr. GAPDH serves as a positive control for an active gene displaying H2Bub1, and TFF1 serves as a control for an inactive gene with no detectable H2Bub1. Background (IgG) levels are indicated as dotted lines. Mean ± SD, n = 3.

(B) Human ESCs demonstrate bivalent domains containing both H3K4me3 and H3K27me3 on the adipocyte-regulated genes PDK4, PPARG, and RASD1 while this bivalency is resolved in favor of H3K4me3 in adipose nuclei. Red triangles indicate amplicons used for detection of bivalent domains; blue triangles indicate amplicons used for detection of H2Bub1 within the transcribed region.

(C and D) H3K4me3 (C) and H3K27me3 (D) occupancy on adipocyte-regulated genes as well as one active gene (HIST1H2AC) and one repressed (TFF1) control gene. ChIP analysis was performed in hMSCs as in (A) following RNF40 depletion. Mean ± SD, n = 3.

(E) Model of the role of H2Bub1 in differentiation. “4” in green circles, H3K4me3; “27” in red circles, H3K27me3; “120” in blue circles, H2Bub1; “P” in orange circles, Ser6 phosphorylation of CTD; “P” in green circles, Ser2 phosphorylation of CTD; TSS, transcription start site.
REFERENCES


