The Nucleosome Remodelling and Deacetylation complex suppresses transcriptional noise during lineage commitment

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Abstract

Multiprotein chromatin remodelling complexes show remarkable conservation of function amongst metazoans, even though components present in invertebrates are often found as multiple paralogous proteins in vertebrate complexes. In some cases, these paralogues specify distinct biochemical and/or functional activities in vertebrate cells. Here, we set out to define the biochemical and functional diversity encoded by one such group of proteins within the mammalian Nucleosome Remodelling and Deacetylation (NuRD) complex: Mta1, Mta2 and Mta3. We find that, in contrast to what has been described in somatic cells, MTA proteins are not mutually exclusive within embryonic stem (ES) cell NuRD and, despite subtle differences in chromatin binding and biochemical interactions, serve largely redundant functions. ES cells lacking all three MTA proteins exhibit complete NuRD loss of function and are viable, allowing us to identify a previously unreported function for NuRD in reducing transcriptional noise, which is essential for maintaining a proper differentiation trajectory during early stages of lineage commitment.

Keywords: chromatin; ES Cell; lineage commitment; NuRD; transcription

Introduction

Mammalian cells contain a number of proteins capable of using ATP hydrolysis to shift nucleosomes relative to the DNA sequence, thereby facilitating chromatin remodelling. In mammals, these ATP-dependent chromatin remodelling proteins usually exist within multiprotein complexes and play essential roles in the control of gene expression, DNA replication and repair (Hargreaves & Crabtree, 2011; Narlikar et al., 2013; Hota & Bruneau, 2016).

Nucleosome Remodelling and Deacetylation (NuRD) is one such multiprotein complex which is unique in that it contains both chromatin remodelling and protein deacetylase activity. NuRD is highly conserved amongst metazoans and has been shown to play important roles in cell fate decisions in a wide array of systems (Denslow & Wade, 2007; Signoret & Hendrich, 2015). For example, in embryonic stem (ES) cells, NuRD controls nucleosome positioning at regulatory sequences to finely tune gene expression (Reynolds et al., 2012; Bornelöv et al., 2018), and in somatic lineages, NuRD activity has been shown to prevent inappropriate expression of lineage-specific genes to ensure fidelity of somatic lineage decisions (Denner & Rauchman, 2013; Knock et al., 2015; Gomez-Del Arco et al., 2016; Loughran et al., 2017). It was recently demonstrated that this is achieved in both ES cells and B-cell progenitors by restricting access of transcription factors to regulatory sequences (Liang et al., 2017; Loughran et al., 2017; Bornelöv et al., 2018). Additionally, aberrations in expression levels of NuRD component proteins are increasingly being linked to cancer progression (Lai & Wade, 2011; Mohd-Sarip et al., 2017).

Nucleosome Remodelling and Deacetylation is comprised of two enzymatically and biochemically distinct subcomplexes: a chromatin remodelling and a deacetylase subcomplex. The chromatin remodelling subcomplex contains a nucleosome remodelling ATPase protein (Chd3/4/5) along with one of the zinc finger proteins Gatad2a/b and the Doc1/Cdk2ap1 protein, while the deacetylase subcomplex contains class I histone deacetylase proteins Hdac1/2, the histone chaperones Rbhp4/7, the Metastasis Tumour Antigen family of proteins, Mta1, Mta2 and Mta3 and, in pluripotent cells, the zinc finger proteins Sall1/4 (Lauberth & Rauchman, 2006; Allen et al., 2013; Kloet et al., 2015; Bode et al., 2016; Low et al., 2016; Miller et al., 2016; Spruijt et al., 2016; Zhang et al., 2016).

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While all three mutual exclusivity may be cell-type-specific (Hong Mta1 was shown to interact with Mta2 in MEL cells, indicating that MTA proteins are mutually exclusive within NuRD. In contrast, these studies did not detect biochemical interactions between previously been described. Another or with the various NuRD components in ES cells has not biochemical analysis of interactions of MTA proteins with one another or with the various NuRD components in ES cells has not previously been described.

Changes in subunit composition in large multiprotein, chromatin-modifying complexes such as PRC1 and BAF have been shown to correlate with distinct changes in function at sites of action in a cell-type-specific manner (Ho & Crabtree, 2010; Morey et al., 2012). The NuRD complex might therefore be expected to show similar diversity in both composition and function, and in fact, diversification of NuRD function has been described through differential incorporation of various isoforms of NuRD component proteins (Bowen et al., 2004). For example, Mbd2 and Mbd3 are mutually exclusive within NuRD (Le Guezennec et al., 2006). While Mbd2 is not required for mammalian development, Mbd3 is essential at early postimplantation stages in mice (Hendrich et al., 2001). Mbd2/NuRD is a methyl-CpG binding co-repressor complex which is dispensable for early development but Mbd3/NuRD, a transcriptional modulator found at sites of active transcription, has been shown to play important roles in regulation of cell fate decisions in multiple developmental systems (Feng & Zhang, 2001; Reynolds et al., 2012, 2013; Gunther et al., 2013; Shimbo et al., 2013; Menafra et al., 2014). NuRD complexes containing either CHD3, CHD4 or CHD5 play distinct roles during cortical development (Nitarska et al., 2016).

Further functional and biochemical diversification occurs through alternate use of the three MTA proteins within NuRD. MTA proteins function as a scaffold around which the deacetylase subcomplex is formed, comprising a 2:2:4 stoichiometry of MTAs: HDACs:RBBPs (Millard et al., 2013, 2016; Smits et al., 2013; Zhang et al., 2016). While invertebrates predominantly have a single MTA protein orthologue, most vertebrates have three. The three mammalian MTA proteins are highly conserved, differing from each other predominantly at their C-termini (Fig 1A). The MTA1 protein was originally identified because of its elevated expression in metastatic cell lines (Toh et al., 1994), and subsequently, all three MTA proteins have been shown to be upregulated in a range of different cancer types (Covington & Fuqua, 2014; Sen et al., 2014; Ma et al., 2016). The MTA1 and MTA3 proteins were shown to form distinct NuRD complexes in breast cancer cells and in B cells and were recruited by different transcription factors to regulate gene expression (Mazumdar et al., 2001; Fujita et al., 2003, 2004; Si et al., 2015). These studies did not detect biochemical interactions between MTA3 and the other MTA proteins, leading to the conclusion that MTA proteins are mutually exclusive within NuRD. In contrast, Mta1 was shown to interact with Mta2 in MEL cells, indicating that mutual exclusivity may be cell-type-specific (Hong et al., 2005). While all three Mta genes are expressed in ES cells, detailed biochemical analysis of interactions of MTA proteins with one another or with the various NuRD components in ES cells has not previously been described.

Functional evidence does not support a strict lack of redundancy amongst MTA proteins during mammalian development. While zygotic deletion of Chd4 or Mbd3 results in pre- or peri-implantation developmental failure, respectively (Kaji et al., 2007; O’Shaughnessy-Kirwan et al., 2015), mice deficient in any one of the three MTA proteins show minimal phenotypes. Mice lacking either Mta1 or Mta3 are viable and fertile (Manavathi et al., 2007; Mouse Genome Informatics), while mice lacking Mta2 show incompletely penetrant embryonic lethality and immune system defects (Lu et al., 2008). In the current study, we took a systematic approach to dissecting MTA protein biochemical and functional diversity. We find that, in contrast to what has been described in somatic cells, MTA proteins are not mutually exclusive within NuRD in ES cells and serve largely redundant functions. Furthermore, ES cells lacking all three MTA proteins are viable and represent a complete NuRD null, allowing us to identify a previously undetected function for NuRD in early stages of lineage commitment.

**Results**

MTA proteins are not mutually exclusive within the NuRD complex in ES cells

The absence of a detected interaction between the MTA2 and MTA3 proteins in human cells (Fujita et al., 2003; Si et al., 2015), and the observation that different MTA proteins can show different protein–protein interactions in B cells (Fujita et al., 2004) have led to the conclusion that the MTA proteins are mutually exclusive within NuRD and could hence confer functional diversity to the NuRD complex (Lai & Wade, 2011). To investigate the biochemical specificity of the MTA proteins in an unbiased manner, we used gene targeting of endogenous loci to produce three different mouse ES cell lines in which an epitope tag was fused to the C-terminus of each MTA protein (Fig 1A). Although MTA genes show different expression patterns in preimplantation mouse development, all three are expressed in peri-implantation and early postimplantation epiblast, the tissue most similar to the naïve ES cell state (Fig EV1). We therefore considered ES cells to be a good system in which to investigate the function of MTA proteins.

Each tagged protein was expressed at levels comparable to those of wild-type proteins and was found to interact with other NuRD component proteins by immunoprecipitation (Fig 1B). Each MTA protein was also able to immunoprecipitate the other MTA proteins in addition to unmodified forms of itself (Fig 1B). Mta3 was barely detectable in Mta1-3xFLAG immunoprecipitates by IP-western blot (though robustly found by mass spectrometry, see below), but Mta1 was robustly detected associating with Mta3-3xFLAG (Fig 1B). This could indicate that any Mta1-Mta3-containing NuRD complexes represent a relatively small proportion of nuclear Mta1, but a relatively large proportion of nuclear Mta3. Individual NuRD complexes contain two MTA proteins (Millard et al., 2013; Smits et al., 2013; Zhang et al., 2016), so the identification of an interaction between MTA proteins means that NuRD complexes in ES cells could contain either homodimers or heterodimers consisting of any combination of the three MTA proteins.

To investigate the potential biochemical diversification conferred by different MTA proteins in pluripotent cells, we used our tagged
Figure 1.
cell lines to identify proteins interacting with each of the MTA proteins by label-free quantitative mass spectrometry. Each protein robustly co-purified with all known NuRD component proteins, including each of the MTA proteins, confirming that MTA proteins are not mutually exclusive within NuRD in ES cells (Fig 1C). In addition to NuRD components, each of the MTA proteins co-purified with Wdr5 as well as a number of zinc finger proteins, most of which had previously been identified as NuRD-interacting proteins (Bode et al., 2016; Spruijt et al., 2016; Ee et al., 2017; Matsuura et al., 2017).

As NuRD is assembled from a deacetylase subcomplex and a remodeler subcomplex joined through the Mbd3 protein (Fig 1A), loss of Mbd3 is expected to result in dissociation of these two subcomplexes. Endogenous tagging for each MTA protein was performed in an ES cell line harbouring a floxed Mbd3 allele, and IP/Mass spectrometry was repeated in ES cells after Mbd3 deletion in order to enrich for interactions specific for the deacetylase subcomplex. The majority of interactions with non-NuRD components was lost after Mbd3 deletion, indicating that most of these proteins do not directly associate either with the MTA proteins or with the deacetylase subcomplex (Fig 1C). Exceptions to this were Zfp296, which was identified as interacting with all three MTA proteins in an Mbd3-independent manner, and Pwwp2a, which co-purified with Mta1 in both wild-type and Mbd3-null cells, consistent with recent reports (Link et al., 2016; Zhang et al., 2019). Notably, Zfp219 and Zfp512b both showed Mbd3-independent interactions specifically with Mta2 (Fig 1C).

By quantitating the abundance of peptides sequenced in each experiment, we found that the interactomes for both Mta1 and Mta2 showed a depletion of peptides associated with the remodeler subcomplex (i.e. Chd4, Gata2a/b and Cdk2ap1) in Mbd3-null cells, whereas proteins associated with the histone deacetylase subcomplex (MTA proteins, Hdac1/2, Sall proteins and Rbbp4/7) remained present at similar levels (Fig 1D). In contrast, the Mta3 interactome showed an increased interaction with Mbd2 and no relative loss of either subcomplex in the absence of Mbd3. Thus, both Mta1 and Mta2 can form part of a stable histone deacetylase-containing subcomplex in the absence of intact NuRD, but Mta3 is preferentially found in intact NuRD complexes. All three MTA proteins associated with Mbd2 in the absence of Mbd3, indicating that they all can contribute to Mbd2/NuRD. Together these data show that the MTA proteins are found exclusively within the NuRD complex in ES cells.

MTA proteins show subtle differences in genome-wide binding patterns

To test whether MTA proteins confer differential chromatin binding to NuRD complexes, we subjected our ES cell lines expressing epitope-tagged MTA proteins to chromatin immunoprecipitation followed by high throughput sequencing (ChiP-Seq). The binding profiles of the three proteins were largely, but not completely overlapping (Fig 2A and B). Mta3 binding was almost entirely associated with Mta1 and/or Mta2 binding, while 31% of Mta1 peaks and 22% of Mta2 peaks were not associated with any other MTA protein (Fig 2A and B). The overall efficiency of Mta3-3xFLAG ChIP-seq was lower than those for Mta1-3xFLAG or Mta2-GFP, possibly due to the lower levels of Mta3 present in ES cells, although replicates were highly correlated (Appendix Fig S1). While Mta3-bound sites can therefore be identified with high confidence, it remains formally possible that “Mta1-only” or “Mta2-only” sites also contain Mta3, albeit at levels beneath the detection threshold of our ChiP-seq. The vast majority of MTA peaks were also bound by Chd4, indicating that they represent NuRD-bound regions (Figs 2C and D, and EV2A and B).

Sites found associated with all three MTA proteins were highly enriched for Chd4 and Mbd3 binding, consistent with these being core NuRD binding regions (Fig 2D). These sites were also enriched for marks of active promoters (H3K4Me3, H3K27Ac) and active enhancers (H3K4Me1, H3K27Ac, P300; Fig 2D and E), both of which are hallmarks of NuRD-associated regions (Miller et al., 2016; Bornelöv et al., 2018). The same was true for sites bound by any two of the three MTA proteins (Fig EV2B). The majority of sites occupied by one MTA protein, but not the other two, were also occupied by Chd4 but to a lesser extent than is seen at core NuRD sites (Fig 2D and E). Sites associated with all three MTA proteins, presumably representing core NuRD-bound sites, were located both at transcription start sites and at distal locations, while sites bound by only one MTA protein were predominantly located distal to transcription start sites (Fig EV2C). Whereas Mta2-only and Mta3-only sites showed some enrichment for both H3K4Me1 and H3K4Me3, Mta1-only sites showed no enrichment for H3K4Me3 (Fig 2D and E). In all cases, MTA-only sites lacking Chd4, which could represent sites of binding by the histone deacetylase subcomplex only, showed characteristics of inactive enhancers, in that they were moderately enriched for H3K4Me1 and P300, but not for H3K4Me3, H3K27Ac or H3K36Me3 (Fig EV2D). None of the MTA-bound
Figure 2. MTA proteins show similar chromatin binding patterns.

A ChIP-seq peaks identified for Mta1 and Mta2 (left), Mta1 and Mta3 (middle) or Mta2 and Mta3 (right) are plotted by enrichment for each protein. Peaks called for both proteins are indicated in the overlap.

B Overlap of peaks identified by ChIP-seq for each MTA protein in wild-type ES cells. Total peak numbers are indicated below each protein name. Each ChIP-seq dataset was made from biological triplicates.

C Comparison of Mta1 and Mta2 peaks with Chd4 peaks, as in Panel (B).

D ChIP-seq enrichment for indicated proteins or histone modifications is plotted across different subsets of Mta-bound sites. Mta1/2/3 refers to peaks identified in all three ChIP-seq datasets, whereas “Mta1 Only”, “Mta2 Only” or “Mta3 Only” refer to peaks only called for that protein.

E Average enrichment of density plots in (D) is plotted for different subsets of Mta ChIP-seq peaks.
sequences showed any enrichment for methylated DNA (Fig EV2E). Genes associated with binding by only one or two MTA proteins, with or without Chd4, are associated with a similar distribution of GO terms (Fig EV2F) which is not consistent with the idea that the MTA proteins are directing NuRD activity to specific gene subsets.

**Mta1/Mta2/Mta3 triple knockout is a total NuRD null**

To investigate the degree of genetic redundancy amongst the different MTA genes, we obtained gene trap alleles for each gene from the European Conditional Mouse Mutagenesis Programme (Skarnes et al, 2011) as ES cell lines (Mta1 and Mta2) or as embryos (Mta3) (Fig EV3A-C). ES cell lines were used for morula aggregation to create chimaeric mice which were subsequently outcrossed to establish a mouse line. Conditional deletion alleles were generated for each line, which was subsequently bred with females expressing Sox2-Cre (Hayashi et al, 2002) resulting in deletion of floxed exons and the absence of any detectable protein production from each allele (Fig EV3D; see Materials and Methods for details). As has been reported previously, Mta1-/- and Mta3-/- mice were viable, and Mta2-/- mice showed incompletely penetrant embryonic lethality (Manavathi et al, 2007; Lu et al, 2008).

Mta1, Mta2 or Mta3-null ES cell lines derived from mice were morphologically indistinguishable from wild-type ES cells, as were ES cell lines deficient for combinations of pairs of MTA genes (Fig EV3E). While some differences in gene expression could be identified between single mutants and their respective parent lines (Fig EV3F and G), these changes were not sufficient to make them distinct from wild-type ES cells when compared to Mbd3-mutant ES cells, 3.5 dpc ICM and 4.5 dpc epiblast (Fig EV3H). We therefore concluded that any gene expression changes identified in single mutant ES cells were unlikely to be of consequence during early development, and that MTA proteins show functional redundancy during early mammalian development.

Mta1-/-/Mta2-/-/Mta3Flox/Flox ES cells (Mta12Δ) were subsequently created and expanded in culture. After transfection with a Cre expression construct to induce deletion of both Mta3 alleles, we recovered ES cells lacking all three MTA proteins (see Materials and Methods for details; Fig 3A). These Mta1-/-/Mta2-/-/Mta3-/- ES cells (subsequently referred to as Mta123Δ) appeared morphologically normal in standard, 2i + LIF culture (Fig 3B), but showed a considerable degree of spontaneous differentiation in serum/LIF culture. MTA proteins are therefore dispensable for ES cell viability.

Structurally, MTA proteins bridge an interaction between the deacetylase subcomplex with Mbd3 and the remodelling subcomplex (Fig 1A) so we predicted that loss of all three MTA proteins would prevent NuRD formation. Consistent with this prediction, we could detect no interactions between Chd4 and components of the deacetylase subcomplex (Hdac2, Rbpb4) in Mta123Δ ES cell nuclear extract by immunoprecipitation of endogenous proteins (Fig 3C). Surprisingly, despite being transcribed at normal levels, both Gatad2b and Mbd3 proteins were present at reduced levels in Mta12Δ cells and barely detectable in Mta123Δ cells (Figs 3C and EV4A), indicating that the MTAs are important for the stability of both of these proteins. To investigate this further, we monitored loss of protein stability in Mta1-/-/Mta2-/-/Mta3Flox/Flox ES cells after deletion using a tamoxifen-inducible Cre (Fig 3D). Loss of Mbd2, Mbd3, Gatad2a and Gatad2b protein stability was all coincident with loss of Mta3 protein, although Mbd3 and Gatad2b were already at reduced levels in the starting cell line (Mta12ΔMta3Flox/Flox) (Fig 3D). Gatad2b and Mta3 also show reduced protein stability in Mbd3Δ ES cells, indicating that a particular inter-dependency exists between these three proteins for stability (Fig EV4B). Furthermore, introduction of either Mta1, Mta2 or Mta3 into Mta123A ES cells at levels comparable to wild-type expression resulted in restoration of Mbd3 protein levels, demonstrating that contact with at least one of the MTA proteins is sufficient for Mbd3 protein stability (Fig 3E). In contrast to Mbd3-null ES cells which display a significant depletion, but not a complete loss of NuRD due to partial compensation by Mbd2 (Kaji et al, 2006), Mta123Δ ES cells were completely devoid of any detectable intact NuRD (Fig 3C and F). The Mta123A ES cells are thus a total NuRD-null ES cell line, which allowed us to examine, for the first time, the consequences of a complete loss of the NuRD complex in a viable mammalian cell system.

**NuRD suppresses transcriptional noise**

Twice as many genes showed an increase rather than a decrease in expression levels in Mta123A ES cells compared to control ES cells by RNA-seq (Fig 4A). The ratio of increased to decreased gene expression in Mta123Δ cells was very similar for genes bound by all three MTA proteins, Mta1+2, Mta1 only or Mta2 only (Fig 4B). NuRD’s global impact on transcription is therefore not grossly altered by the inclusion or absence of individual MTA subunits.

Comparing global gene expression profiles using principal component analysis (PCA) showed that Mta123Δ and Mbd3Δ ES cells were completely indistinguishable from wild-type ES cells, indicating that a particular inter-dependency exists between these three proteins for stability (Fig EV4B). Furthermore, introduction of either Mta1, Mta2 or Mta3 into Mta123Δ ES cells at levels comparable to wild-type expression resulted in restoration of Mbd3 protein levels, demonstrating that contact with at least one of the MTA proteins is sufficient for Mbd3 protein stability (Fig 3E). In contrast to Mbd3-null ES cells which display a significant depletion, but not a complete loss of NuRD due to partial compensation by Mbd2 (Kaji et al, 2006), Mta123Δ ES cells were completely devoid of any detectable intact NuRD (Fig 3C and F). The Mta123A ES cells are thus a total NuRD-null ES cell line, which allowed us to examine, for the first time, the consequences of a complete loss of the NuRD complex in a viable mammalian cell system.

**Figure 3.** Mta1/Mta2/Mta3 triple-null ES cells represent a complete NuRD KO.

A. Western blots of wild-type (Control), double or triple-null ES cell nuclear extract were probed with antibodies indicated at right. LaminB1 acts as a loading control.

Approximate sizes are indicated in kDa.

B. Phase-contrast images of wild-type or Mta123A ES cells in 2i+LIF conditions. Scale bars represent 100 μm.

C. Western blots of anti-Chd4 immunoprecipitation of nuclear extract from indicated cell lines (top) probed with antibodies indicated at right. For the Gatad2b and Mbd3 blots, arrowheads indicate target proteins, while an asterisk marks a non-specific IgG bands. 10% of the input was loaded into “Input” lanes. Approximate sizes are indicated in kDa.

D. Western blot of a time course of Mta3 deletion in Mta1ΔMta2ΔMta3Flox/Flox Cre-ER (Mta12Δ3Flox/Flox) or Control (Mta12Δ3Flox/Flox without Cre-ER) ES cells probed for indicated NuRD component proteins or RNA polymerase II as a loading control (rNPAP). The time course is indicated at the top as Days +/- tamoxifen.

E. Western blot showing rescue of Mta123A ES cells by ectopic expression of Mta1, Mta2 or Mta3 from a transgene (TG). Total RNA polymerase II acts as a loading control (rNPAP). For all western blots, molecular weight is indicated at left in kDa.

F. Model of NuRD complex structure. Upon loss of Mbd3, most of the NuRD complex falls apart into the Chd4-containing remodeller subcomplex and the MTA-containing deacetylation subcomplex, but some intact Mbd2-NuRD still remains. Upon loss of all three MTA proteins, no intact NuRD remains, both Mbd3 and Gatad2a/b become unstable and neither of the intact subcomplexes remain.
Figure 3.
Figure 4. The EMBO Journal 38:e100788 | 2019 © 2019 The Authors

Gene Expression Quartile:

| Category                                | Quartile I | Quartile II | Quartile III | Quartile IV |
|-----------------------------------------|------------|-------------|--------------|-------------|
| Genes near core NuRD peak (N=15210)     | 14.9%      | 38.8%       | 21.0%        | 7.4%        |
| Genes near an MTA-only peak (N=10280)   | 15.3%      | 39.0%       | 22.0%        | 7.6%        |
| Mbd2/NuRD (Chd4/MTA-Mbd3) genes (N=349) | 18.6%      | 44.4%       | 9.8%         | 3.8%        |
| MTA-only genes (N=151)                  | 17.6%      | 40.5%       | 16.7%        | 0%          |

Significantly DE
cells in self-renewal conditions were more similar to each other than either was to wild-type cells (Fig 4C). Mta123Δ ES cells were most distinct from wild-type cells, consistent with them representing a more complete NuRD knockout (Fig 4C). Consistent with this, genes driving the NuRD-specific separation (Principal Component 2; PC2) were generally misexpressed to a greater degree in Mta123Δ ES cells than in Mbd3Δ ES cells (Fig EV4C). Genes misexpressed in either Mbd3Δ or Mta123Δ ES cells were most significantly associated with the same top three GO terms, but the significance was greater in Mta123Δ ES cells as these cells misexpressed more genes in each category and generally to a greater extent than did the Mbd3Δ cells (Figs 4D and E, and EV4C).

Genes misexpressed in Mta123Δ ES cells were predominantly those normally expressed at lower levels (Fig EV4D). A very similar pattern was seen if we considered genes misexpressed in Mbd3Δ ES cells, with the major difference being that more genes were misexpressed in the Mta123Δ cells. The predominant biochemical difference between the Mbd3Δ cells and Mta123Δ cells was the lack of Mbd2/NuRD and of the MTA/HDAC subcomplex in the triple mutants, so we next asked whether we could detect evidence for specific functions for either of these complexes in ES cells. We identified genes near core NuRD peaks (N = 15,210), near Mta-only peaks (regardless of the presence of a NuRD peak; N = 10,280), genes associated with NuRD components Chd4 and MTA1s, but not Mbd3 (presumptive Mbd2/NuRD-only genes; N = 349), and genes containing MTA-only peaks, but no Chd4 peak (MTA-only genes; N = 151) (Fig 4F) and divided them into expression quartiles. While there are very few genes not associated with a NuRD peak, those few presumptive Mbd2/NuRD-only genes or MTA-only genes showed a similar misexpression pattern to those seen for core NuRD genes, in that the majority of misexpressed genes was in the second lowest expression quartile (Fig 4F). We therefore find no evidence for a specific gene regulatory function associated with an MTA/HDAC subcomplex. This is consistent with a recent report in which loss of Ppp5r2a/b resulted in only very slight alterations in nascent RNA production amongst a small subset of genes (Zhang et al., 2018). Nevertheless, this analysis further highlights the importance for NuRD in suppressing expression of lowly expressed genes. We therefore conclude that, in addition to fine-tuning gene expression as part of the Mbd3/NuRD complex, MTA proteins are also important for preventing inappropriate activation of a variety of different genes, or in preventing transcriptional noise in ES cells.

The NuRD complex safeguards cellular identity during differentiation

We next asked what impact complete loss NuRD activity had upon the differentiation capacity of Mta123Δ ES cells. Upon removal of the two inhibitors and LIF from the culture media, wild-type cells began to adopt the flatter morphology of neuroectoderm (Fig 5A; Ying et al., 2003). This was accompanied by downregulation of pluripotency-associated genes and the activation of a neural gene expression programme (Fig 5B). Mbd3Δ ES cells are able to respond to the absence of self-renewal factors but have a very low probability of adopting a differentiated fate when induced to differentiate in N2B27 conditions (Kaji et al., 2006; Reynolds et al., 2012). Consistent with these findings, after 5 days in differentiation conditions Mbd3Δ ES cells showed some signs of having responded to differentiation conditions, but still retained pockets of morphologically undifferentiated cells (Fig 5A, Middle). In contrast, the completely NuRD-null Mta123Δ ES cells appeared to have all exited the self-renewal programme and adopted a flat, monolayer morphology (Fig 5A, Bottom). The ability of Mta123Δ ES cells to undergo morphologically normal neuroectodermal differentiation was rescued upon re-expression of either Mta1, Mta2 or Mta3 (Fig EV5A).

Since the differentiation process may be considered as a combination of exit from self-renewal (downregulation of pluripotency-associated genes) and acquisition of lineage-specific gene expression programmes, we focussed on changes to these two classes of genes by RT-qPCR over a differentiation time course. Pluripotency-associated genes such as Esrrb, Nanog and Pou5f1 were downregulated during differentiation of wild-type, Mbd3Δ and Mta123Δ ES cells (Fig 5B). While this downregulation was not absolutely dependent on the presence of functional NuRD, the magnitude and kinetics of the response varied in a gene-dependent manner. Genes associated with acquisition of neural fate such as Nestin, Pax6, Ascl1 and Cdh2...
Figure 5.
were activated in the presence of wild-type NuRD, but this response was reduced or absent in the NuRD mutant lines (Fig 5B). In contrast, Mta123Δ ES cells aberrantly expressed markers of inappropriate lineages, such as Elf5 and Gata3 (trophectoderm—but not Cdx2 or Eomes), Ebf4, Nobox and Hcn4 (genes associated more generally with later stages of differentiation or transmembrane transport). Misexpression of these genes was also seen in Mbd3Δ cells, although not always to the same extent. Exogenous expression of either Mta1, Mta2 or Mta3 was able to rescue the ability of Mta123Δ ES cells to activate neural gene expression to different extents during differentiation (Fig EV5B), consistent with their ability to rescue morphological phenotypes.

The morphology of Mta123Δ cells induced to differentiate towards neuroectoderm was quite different from that of Mbd3Δ cells (Fig 5A), but there was no large-scale difference in gene categories changing between the two mutant lines after 48 h in differentiation conditions (Fig EV5C). In general, Mta123Δ cells misexpressed more genes from each category and to a greater extent than did Mbd3Δ cells (Fig EV5D), indicating that their abnormal morphology in differentiation conditions is most likely due to widespread gene misexpression generally, rather than activation of a specific differentiation pathway. Mta123Δ ES cells induced to differentiate towards a mesoderm fate similarly failed to appropriately activate differentiation markers, and exogenous expression of individual MTA proteins was again able to rescue the defect to varying degrees (Fig EV5E).

To assess the differentiation potential of Mta123Δ ES cells in an unbiased manner, we allowed control, Mbd3Δ and Mta123Δ ES cells to differentiate in embryoid bodies in basal media supplemented with serum (Doetschman et al, 1985). After 12 days, control cells had formed colonies containing numerous morphologically distinguishable cell types, silenced pluripotency-associated gene expression and activated expression of genes associated with all three germ layers (Fig 5C and D). In contrast, both Mbd3Δ and Mta123Δ cells showed less vigorous outgrowths consisting predominantly of a monolayer of flat cells (Fig 5C). While both mutant lines were able to silence expression of pluripotency-associated genes, both showed reduced activation of differentiation-associated genes, with Mta123Δ cells generally showing more profound expression defects than Mbd3Δ cells (Fig 5D). Together these data show that while NuRD is not strictly required for silencing of pluripotency genes, its activity is required for proper activation of lineage-appropriate genes and repression of lineage-inappropriate genes during differentiation.

To better understand how NuRD-null cells respond when induced to differentiate, we compared their gene expression profiles to a transcription landscape made from single cells taken from early mouse embryos (Figs 5E and EV5F and G) (Mohammed et al, 2017). In the self-renewing state, Mbd3Δ and Mta123Δ cells clustered near control (parental) lines near embryonic day 3.5 (E3.5) and E4.5 inner cell mass cells, as is expected for naïve mouse ES cells (Boroviak et al, 2014). After 48 hours of differentiation, control ES lines clustered with E5.5 epiblast cells, indicating that the ES cell differentiation process occurred analogously to development in vivo. While Mbd3Δ ES cells appear to have exited the self-renewing state and to have taken the same differentiation trajectory as wild-type cells (i.e. leftwards along PC1; Figs 5E and EV5G), they did not travel as far along this trajectory as wild-type cells, instead occupying a space between the E4.5 and E5.5 epiblast states. Mta123Δ cells travelled even less far along PC1 than Mbd3Δ ES cells, and rather than maintain the appropriate differentiation trajectory they also travelled along PC2, occupying a space between E4.5 epiblast and E4.5 primitive endoderm. This further demonstrates that not only is NuRD important for cells to be able to adopt the appropriate gene expression programme for a given differentiation event, but it is also important for cells to maintain an appropriate differentiation trajectory.

If Mta123Δ ES cells were undergoing a specific trans-differentiation event towards trophoderm during ES cell differentiation, then this could become more pronounced if exposed to normal differentiation conditions in a chimaeric embryo. If, in contrast, they are simply unable to differentiate properly, they would not be expected to contribute to early embryos. To distinguish between these possibilities, we assessed the ability of Mta123Δ ES cells to differentiate in chimaeric embryos. Equal numbers of control or Mta123Δ ES cells expressing a fluorescent marker were aggregated with wild-type morulae and allowed to develop for 48 hours. While wild-type cells contributed to the ICM of host embryos with 100% efficiency, Mta123Δ ES cells showed significantly reduced contribution and increased levels of apoptosis (Figs 6A and B, and EV5H). Those Mta123Δ cells that did survive in blastocysts were predominantly, but not always found in the inner cell mass. These cells expressed Sox2 but not Cdx2, indicating that they had not undergone inappropriate differentiation towards a trophoderm fate (Fig 6A and B). Nevertheless, no Mta123Δ cells could be found in E5.5 dpc embryos, indicating that they were unable to adapt to developmental cues and form epiblast. This is most consistent with Mta123Δ cells being unable to enter a normal differentiation path in an ICM environment. The ability to contribute to the ICMs of chimaeric embryos was rescued by constitutive expression of Mta2 in Mta123Δ cells (Fig 6B). We therefore propose that NuRD not only functions to establish the correct lineage identity of cells during the differentiation process, but also
Figure 6. NuRD-null ES cells cannot contribute to normal embryonic development.

A Composite images of representative chimaeric embryos made with control (Mta123Δ/Mta2+/-Mta3Flox/Flox) or Mta123Δ ES cells. ES-derived cells express the Kusabira Orange fluorescent marker. Sox2 indicates epiblast cells and Cdx2 is expressed in trophectoderm cells. Arrows indicate examples of K-Orange expressing cells in the mutant embryos. Scale bars = 20 μm.

B (Top) Number of K-Orange expressing cells observed in chimaeric embryos obtained using control ES cells, Mta123Δ ES cells or Mta123Δ ES cells in which Mta2 was reintroduced on a constitutively expressed transgene (Mta123Δ + Mta2TG). P-values calculated using a two-tailed t-test. (Middle) Mean number of K-Orange cells per embryo separated by Sox2 expression. P-values calculated using a two-tailed t-test. (Bottom) Number of K-Orange and Caspase-3-positive cells per embryo. P-values calculated using a one-tailed t-test: *P < 0.05, ****P < 0.0001, “ns” = not significant.

C Model of NuRD function during differentiation of pluripotent cells. NuRD facilitates lineage commitment of ES cells after exit from pluripotency (red arrows), allowing cells to form differentiated cell types (Top). In the absence of Mbd3, residual NuRD activity ensures cells retain the appropriate differentiation trajectory, but the cells are unable to reach a differentiated cell fate (dotted red arrows, Middle). In the absence of all three MTA proteins, there is no residual NuRD activity and ES cells are unable to either achieve appropriate lineage commitment, or to maintain the proper differentiation trajectory (dotted arrows, Bottom).
Different MTA proteins exhibit subtle differences in chromatin localisation or biochemical interaction partners, but we find no evidence for protein-specific functions in self-renewing or differentiate mouse ES cells. ES cells completely devoid of MTA proteins are complete NuRD nulls and are viable but show inappropriate expression of differentiation-associated genes, are unable to maintain an appropriate differentiation trajectory and do not contribute to embryogenesis in chimaeric embryos.

Protein subunit diversity is often found in chromatin remodelling complexes which specifies functional diversity (Hargreaves & Crabtree, 2011; Morey et al., 2012; Hota & Bruneau, 2016). This is also the case for the NuRD complex, where alternate usage of Mbd2/3, Chd3/4/5 and Mta1/2/3 has been found to result in alternate functions for NuRD complexes (Feng & Zhang, 2001; Fujita et al., 2003; Nitarska et al., 2016). Yet the findings by us and others (Manavathi et al., 2007 and Mouse Genome Informatics) that Mta1 and Mta3-null mice are viable and fertile, and that we detect no major differences in the abilities of different MTA proteins to rescue the Mta123A ES cell phenotypes indicate that MTA proteins exhibit considerable functional redundancy during development. The MTA protein family expanded from a single copy to three orthologues near the base of the vertebrate clade. Triplication of this protein family may have allowed early vertebrates to diversify NuRD function in specific somatic tissues, conferring a selective advantage. Our finding that MTA proteins are functionally redundant in early embryonic development indicates that the ancestral, essential function of the MTA proteins in early development has been retained by all three mammalian orthologues.

Different MTA proteins are capable of interacting with each other in ES cells, so how the mutual exclusivity reported in other cell types might be achieved is not clear. One possibility is that the variable inclusion in NuRD of zinc finger proteins, such as the Sall proteins in ES cells, could influence the MTA makeup of NuRD complexes. This class of variable NuRD interactors, which include Sall1/2/3/4, Zfp423 (Ebfaz), Zfp1/2 (Fog1/2) and Bcl11b, interact with RBBP and/or MTA proteins via a short N-terminal motif (Hong et al., 2005; Lauberth & Rauchman, 2006; Lejon et al., 2011). Of this class of proteins, Sall1 and Sall4 are the most highly expressed in ES cells, and Sall4 can associate with all three MTA proteins (Fig 1C; Miller et al., 2016). In contrast, the Zfp1/2 (Fog1) protein was shown to preferentially associate with Mta1 and Mta2, but not Mta3, in a somatic cell line (Hong et al., 2005). Hence, it is possible that different proteins using this N-terminal motif to interact with NuRD in different cell types could act to skew the proportion of different MTA proteins included in the NuRD complex.

This genetic dissection has revealed that NuRD component MTA proteins display a large degree of inter-dependence for protein stability, as has been reported for other multiprotein complexes (Roumeliotis et al., 2017). Mta1 and Mta2 both contain two distinct RBBP interaction domains, while Mta3 lacks the C-terminal most RBBP interaction domain (Millard et al., 2016). The Mta1 and Mta2 proteins show minimal loss of stability in the absence of Mbd3, but Mta3 requires an interaction with Mbd3 to be completely stable (Fig EV4B; Bornelov et al., 2018). It is possible that the additional interaction with an Rbbp protein confers stability to Mta1 and Mta2 in the absence of Mbd3. This would be consistent with our interpretation that Mta3 preferentially exists within an intact NuRD complex (Figs 1 and 2). Rbbp4/7 confers histone H3 binding to the NuRD complex, so Mta3-containing NuRD may bind chromatin less tightly than Mta1- and/or Mta2-containing NuRD.

We have used pluripotent cells lacking Mbd3 extensively to show that NuRD plays important roles in control of gene expression during early stages of exit from pluripotency in vivo and in culture (Kaji et al., 2006, 2007; Latos et al., 2012; Reynolds et al., 2012). Mbd3-null ES cells contain Mbd2/NuRD and thus represent a NuRD hypomorph, rather than a NuRD-null. ES cells lacking all three MTA proteins show no detectable NuRD formation (Fig 3), and therefore, we believe, represent a true NuRD null. Mta123A ES cells are similar to Mbd3Δ ES cells in that both misexpress a range of genes in 2iL conditions and both fail to properly differentiate. Yet the Mta123A ES cells misexpress more genes and generally to a greater extent than do Mbd3Δ cells in both self-renewing and differentiation conditions, and they show more pronounced differentiation defects. We propose that this phenotype is a direct consequence of NuRD’s function in suppressing transcriptional noise during cell fate decisions. Moderate levels of noise in the Mbd3Δ state allow cells to maintain a differentiation trajectory, but interfere with their ability to achieve the appropriate differentiation state (Fig 6C). In the higher levels of noise, characteristic of a complete absence of NuRD cells are unable to appropriately follow differentiation cues and maintain a coherent differentiation trajectory (Fig 6C). We therefore propose that NuRD encodes a highly conserved, redundant function to prevent transcriptional noise, as well as a further, Mbd3-dependent function to fine tune transcriptional outputs during differentiation.

Materials and Methods

Mouse embryonic stem cells

Mouse embryonic stem (ES) cells were grown on gelatin-coated plates in 2i/LIF conditions as described (Ying et al., 2008). All lines were routinely PCR genotyped and tested for mycoplasma. All ES cell lines used in this study are listed in Table EV1. The following “Knockout First” alleles were obtained from EUCOMM as heterozygous ES cell lines (Illustrated in Fig EV1A and B): Mta1tm1a(EUCOMM)Wtsi: https://www.mousephenotype.org/data/alleles/MGI:2150037/tm1a(EUCOMM)Wtsi Mta2tm1a(EUCOMM)Wtsi: https://www.mousephenotype.org/data/alleles/MGI:1346340/tm1a(EUCOMM)Wtsi

| © 2019 The Authors | The EMBO Journal | 38: e100788 | 2019 | 13 of 18 |
Embryonic stem cells were used to derive mouse lines by blastocyst injection using standard methods. ES cell derivation was performed by isolating ICMs and outgrowing in 2i/LIF media as described (Nichols et al., 2009).

The epitope-tagged ES cell lines Mta1-Avi-3×FLAG and Mta2-GFP were generated by traditional gene targeting, while the Mta3-Avi-3×FLAG line was generated using a CRISPR/Cas9 genome editing approach. Mta3 contains three alternate stop codons in exons 14, 16 and 17, with exon 15 accessed by alternate usage of a splice donor site in exon 14. Visual inspection of our RNA-seq data on a genome browser showed very little transcription beyond exon 14 in ES cells, indicating that the short isofrom was predominant (Appendix Fig S2). We therefore targeted the stop codon present in exon 14 and simultaneously destroyed the alternate splice donor site using a synonymous mutation (GAA TGT to GAA TGC) to prevent splicing of exons 2

Mesendoderm differentiation was performed as follows: ES cells were plated at 10⁴ cells/cm² in N2B27 on fibronectin-treated 6-well plates and cultured for 48 h. Medium was then replaced with medium conditioned with 10% foetal calf serum. After 3 days, embryoid bodies were allowed to form by allowing embryoid bodies to attach and outgrow on gelatin-coated plates in N2B27 + serum. Experiments were carried out in quadruplicate.

Mice

All animal experiments were approved by the Animal Welfare and Ethical Review Body of the University of Cambridge and carried out under appropriate UK Home Office licences. The Mta3 “Knockout First” allele was obtained from EUCOMM as a heterozygous mouse line (Illustrated in Fig EV1C):

Mta3tm3a(KOMP)Wtsi: (https://www.mousephenotype.org/data/allele/s/MGI:2151172/tm3a%2528KOMP%2529Wtsi/).

Heterozygous knockout first mouse lines were crossed to a Flp-deleter strain kindly provided by Andrew Smith (University of Edinburgh; Wallace et al., 2007) to generate conditional alleles. Mice harbouring conditional alleles were crossed to a Sox2-Cre transgenic line (Hayashi et al., 2002) to create null alleles. Mouse lines created in this study are listed in Table EV1.

Chimeraic embryos were made by morula aggregation with 8–10 ES cells per embryo as described (Hogan et al., 1994) and cultured for 24–48 h prior to fixation and immunostaining. ES cells used stably expressed a PiggyBac Kusabira Orange transgene.

Nuclear extracts and immunoprecipitation

For extraction of nuclear proteins, ES cells were lysed in ice cold Buffer A (10 mM HEPES, pH7.9, 1.5 mM MgCl₂, 10 mM KCl and 0.5 mM DTT) supplemented with protease inhibitors and containing 0.6% NP-40. Nuclear pellets were washed further with Buffer A to reduce cytoplasmic protein contamination before extraction by vigorous shaking at 4°C for 1 h in ice cold Buffer C (10 mM HEPES, pH7.9, 12.5% glycerol, 0.75 mM MgCl₂, 10 mM KCl, 0.5 mM DTT) with protease inhibitors followed by centrifugation to remove insoluble fraction.

For immunoprecipitation of FLAG-tagged proteins, anti-FLAG antibody was incubated with Protein G Sepharose beads (Sigma) for 1 h at room temperature. Nuclear extract (200 µg) was diluted in IP Buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 0.1% DOC) in the presence of protease inhibitors and benzamidine and incubated with antibody-bead conjugates at 4°C overnight. Beads were washed three times in ice cold IP Buffer and a further two times in IP Buffer containing 300 mM NaCl.

Chd4 immunoprecipitations were carried out at 4°C overnight using antibody to endogenous Chd4 with Protein G sepharose in 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 10% glycerol and washed as for the FLAG IPs before SDS–PAGE.

For immunoprecipitation of GFP-tagged proteins, Chromotek GFP-Trap agarose beads were used. Nuclear extract (200 µg) was incubated with 25 µl GFP-Trap beads in GFP IP buffer (10 mM Tris, pH 7.5, 150 mM NaCl, 0.5 mM EDTA) with Benzamidine and protease inhibitors for 1 h at 4°C. Beads were washed five times in IP buffer prior to western blotting.

IPs were carried out in bulk and loaded on multiple SDS gels for western blot analysis with various antibodies. Antibodies are listed in Table EV2.

Label-free pulldowns and label-free quantitation (LFQ) LC-MS/MS analysis

Label-free GFP pulldowns were performed in triplicate as previously described (Kloet et al., 2018). 2 mg of nuclear extract was incubated with 7.5 µl GFP-Trap beads (Chromotek) or 15 µl FLAG-sepharose beads (Sigma) and 50 µg/ml ethidium bromide in Buffer C (300 mM NaCl, 20 mM HEPES/KOH, pH 7.9, 20% v/v glycerol, 2 mM MgCl₂, 0.2 mM EDTA) with 0.1% NP-40, protease inhibitors and 0.5 mM DTT in a total volume of 400 µl. After incubation, 6 washes were performed: 2 with Buffer C and 0.5% NP-40, 2 with PBS and 0.5% NP-40, and 2 with PBS. Affinity purified proteins were subject to on-bead trypsin digestion as previously described (Smits et al., 2013). In short, beads were resuspended in 50 µl elution buffer (2 M urea, 50 mM Tris, pH 7.5, 10 mM DTT) and incubated for 20 min in a thermoshaker at room temperature. After addition of 50 mM iodoacetamide (IAA), beads were incubated for
Chromatin immunoprecipitation (ChIP), sequencing and analysis

Chromatin immunoprecipitations were carried out as previously outlined (Reynolds et al., 2012). For sequencing of ChIP DNA, samples from six (Mta1-FLAG and Mta2-GFP) and four (Mta3-FLAG) individual ChIP experiments were used. Antibodies used are listed in Table EV2. ChIP-seq libraries were prepared using the NEXTflex Rapid DNA-seq kit (Illumina) and sequenced at the CRUK Cambridge Institute Genomics Core facility (Cambridge, UK) on the Illumina platform. Low-quality reads and adaptor sequences were removed by Trim Galore! (version: 0.20.0; Krueger & Kelly, 2013) prior to the mouse reference genome (GRCm38/mm10) using Bismark (version: 0.20.0; Krueger & Andrews, 2011). Alignments were de-duplicated and further processed using deepTools computeMatrix and plotHeatmap (Ramirez et al., 2014). Venn diagrams were plotted using the R/Bioconductor Diffbind package. The trimmed ChIP-seq reads were aligned to the mouse reference genome (GRCm38/mm10) using bowtie (version: 0.12.8; options: -m 1 -v 1; Langmead, 2010). This was followed by peak calling using macs2 callpeaks against the corresponding inputs using default parameters and a Q-value of <0.05. The R/Bioconductor Diffbind package was used to compare each combination of bound MTA proteins against all others using cut-off values of RPKM > 1.5, logFC > 1 and FDR < 0.05. The peaks were processed and visualised by deepTools computeMatrix and plotHeatmap (Ramirez et al., 2014). Venn diagrams were plotted using the R/Bioconductor ChIPseeker package.

DNA Methylation data were obtained from Shirane et al (2016), low-quality reads and adaptor sequences were removed by Trim Galore! (version: 0.4.1), and reads were aligned to the mouse reference genome (GRCm38/mm10) using Bowtie (version: 0.12.8; options: -m 1 -v 1; Langmead, 2010). This was followed by peak calling using macs2 callpeaks against the corresponding inputs using default parameters and a Q-value of <0.05. The R/Bioconductor Diffbind package was used to compare each combination of bound MTA proteins against all others using cut-off values of RPKM > 1.5, logFC > 1 and FDR < 0.05. The peaks were processed and visualised by deepTools computeMatrix and plotHeatmap (Ramirez et al., 2014). Venn diagrams were plotted using the R/Bioconductor ChIPseeker package.

Gene expression analyses

Total RNA was purified using RNeasy Mini Kit (Qiagen) including on-column DNase treatment. First-strand cDNA was synthesised using SuperScript IV reverse transcriptase (Invitrogen) and random hexamers. Quantitative PCRs (qPCRs) were performed using TaqMan reagents (Applied Biosystems) on a QuantStudio Flex Real-Time PCR System (Applied Biosystems) or a StepOne Real-Time PCR System (Applied Biosystems). Gene expression was determined relative to housekeeping genes using the ΔCt method. TaqMan assays or PCR primers are listed in Table 1.

RNA-seq

Libraries for sequencing were prepared using the NEXTflex Rapid Directional RNA-seq kit (Illumina) or SMARTer® Stranded Total RNA-Seq Kit v2—Pico Input Mammalian (Takara Bio) and sequenced on the Illumina platform as for ChIP-seq libraries. Low-quality reads and adaptor sequences were removed by Trim Galore! (version: 0.4.1), and the trimmed reads were aligned to mouse reference genome (GRCm38/mm10) using TopHat (version v2.1.0; Kim et al, 2013). Read counts per gene were obtained using featureCounts (version 1.5.0; Liao et al, 2014) with annotations from Ensembl release 86 (Yates et al, 2016). Normalisation, with and without spike-ins, and differential expression analyses were performed using the R/Bioconductor Deseq2 package (version 1.14.1; Love et al, 2014). The R procmp function was used for principal component analysis (PCA). Gene Ontology (GO) analysis was performed using DAVID (da Huang et al, 2009b).

Statistical analyses

No power calculations were performed to predetermine sample sizes. Statistical analyses were performed using GraphPad Prism.
software. Images of cells or embryos are representative of at least three independent experiments. Statistical methods used to analyse high throughput sequencing data and proteomics data are described in the respective sections of the Materials and Methods.

Data availability

High throughput sequence datasets used in this manuscript are listed in Table EV3. ChIP-seq data are available with the GEO accession number GSE122833 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE122833). RNA-seq data are available with the GEO accession number GSE122696 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE122696). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD009855 (https://www.ebi.ac.uk/pride/archive/projects/PXD009855). All original western blot images are available at Mendeley Data: https://doi.org/10.17632/d25bgfn8hd.1.

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Author contributions

TB and BH devised the study; TB, SK, SG, RF, JC, NR and BH generated the data; MB, MR and SD analysed high throughput sequencing data; SK and MV generated high throughput sequencing data and proteomics data; TK and BH devised the study; TB, SK, SG, RF, JC, NR and BH generated the data; statistical methods used to analyse high throughput sequencing data and proteomics data are described in the respective sections of the Materials and Methods. Images of cells or embryos are representative of at least three independent experiments. Statistical methods used to analyse high throughput sequencing data and proteomics data are described in the respective sections of the Materials and Methods.

Conflict of Interest

The authors declare that they have no conflict of interest.

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