Loss of p300 and CBP disrupts histone acetylation at the mouse Sry promoter and causes XY gonadal sex reversal

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Abstract

CREB-binding protein (CBP, CREBBP, KAT3A) and its closely related parologue p300 (EP300, KAT3B), together termed p300/CBP, are histone/lysine acetyl-transferases that control gene expression by modifying chromatin-associated proteins. Here, we report roles for both of these chromatin-modifying enzymes in mouse sex determination, the process by which the embryonic gonad develops into a testis or an ovary. By targeting gene ablation to embryonic gonadal somatic cells using an inducible Cre line, we show that gonads lacking either gene exhibit major abnormalities of XY gonad development at 14.5 dpc, including partial sex reversal. Embryos lacking three out of four functional copies of p300/Cbp exhibit complete XY gonadal sex reversal and have greatly reduced expression of the key testis-determining genes Sry and Sox9. An analysis of histone acetylation at the Sry promoter in mutant gonads at 11.5 dpc shows a reduction in levels of the positive histone mark H3K27Ac. Our data suggest a role for CBP/p300 in testis determination mediated by control of histone acetylation at the Sry locus and reveal a novel element in the epigenetic control of Sry and mammalian sex determination. They also suggest possible novel causes of human disorders of sex development (DSD).

Introduction

In mammals, the development of the bipotential embryonic gonad as a testis or ovary is determined by the presence or absence of the Y chromosome. In the mouse, the gonads begin to form on the surface of the mesonephros by 9.5 days post coitum (dpc) with a thickening of the coelomic epithelium. In XY embryos, the transient expression of Sry (sex determination region on Y chromosome) in somatic cells of the undifferentiated gonad between 10.5 dpc and 12.0 dpc triggers testis differentiation (1). SRY, in conjunction with NR5A1 (SF1), directly upregulates expression of Sox9 (Sry-related HMG box 9) (2) and SOX9 activates a gene regulatory network that controls the differentiation of Sertoli cells and morphogenetic events required for testis formation, including formation of testis cords [reviewed in (3)]. In XX gonads, the absence of SRY results in the activation of ovary-promoting pathways involving RSPO1/WNT4/CTNNB1 and FOXL2 (4–9).

The timing of the expression of Sry is crucial for activation of the testis-determining pathway of gene expression; a
Results

Wt1ERT2Cre or tamoxifen treatment does not significantly disrupt testis determination

To study the role of CBP and p300 in sex determination, conditional deletion in embryonic gonadal somatic cells was performed by crossing Cbpflox/þ or p300flx/þ mice with mice carrying Cbpflx or p300O/E and tamoxifen-inducible Wt1CreERT2, in which Cre expression is driven by the endogenous Wt1 regulatory elements (41). Wt1 is first expressed in the coelomic epithelium of the urogenital ridge as early as 9.0 dpc (42) and expression continues in somatic cells of both the developing XY and XX gonad (43,44). Gene deletion was induced by tamoxifen administration at 9.5 dpc, prior to sex determination. To assess the efficiency of deletion, we analysed CBP and p300 protein levels in gonads at 14.5 and 11.5 dpc using immunohistochemistry (Supplementary Material, Fig. S1A and B). CBP and p300 were essentially ubiquitous in XY control (Wt1ERT2Cre/–) gonads and the adjacent mesonephros at both stages, while gonads predicted to lack CBP [Wt1ERT2Cre/–, Cbpflx/– (henceforth, Cbp cKO)] or p300 [Wt1ERT2Cre/–, p300flx/– (p300 cKO)] exhibited a strong reduction in CBP and p300 protein levels, respectively. These data suggest that one dose of tamoxifen at 9.5 dpc is sufficient to efficiently delete these two genes in the somatic cells as early as 11.5 dpc. However, as Wt1 is not expressed in germ cells, CBP and p300 were detected in these cells (Supplementary Material, Fig. S1A and B).

The Wt1ERT2Cre line is a knock-in and therefore embryos carrying this Cre are heterozygous for a Wt1 null allele. WT1 functions in the establishment of the early bipotential gonad and the up-regulation of the testis-determining gene Sry (15,45). In order to analyse the effect of Wt1 heterozygosity on sex determination, we performed wholemount in situ hybridization (WISHM) for Sox9, a major gene controlling testis differentiation and a Sertoli cell marker, on gonads from Wt1ERT2Cre/–, Sox9flx/– embryos, with and without tamoxifen dosing of the dam (Supplementary Material, Fig. S2A and B). Testis development and Sox9 expression at 14.5 dpc were not significantly affected by the presence of Wt1ERT2Cre or affected by tamoxifen treatment. However, a few cells positive for Stra8, a gene expressed in pre-meiotic germ cells, usually only detected in XX gonads at this stage, were observed at the poles of one XY gonad carrying Wt1ERT2Cre and treated with tamoxifen (Supplementary Material, Fig. S2B). This phenotype was also occasionally observed in XY gonads carrying Wt1ERT2Cre in the absence of tamoxifen treatment (data not shown). These results suggest that XY Wt1ERT2Cre/– gonads treated with tamoxifen can occasionally exhibit a short delay in testis determination, which explains the polar Stra8 expression; this is the case with other mutants that have a very minor effect on testis determination (46). No effect of Wt1ERT2Cre or tamoxifen was observed on XX gonad differentiation (data not shown). It is likely that these gonads are sensitized to any further disruptions to testis determination, rather like the situation in which B6 mice carry the domesticus Y chromosome from the AKR strain (B6.Ydom) (47,48). Therefore, for the rest of the study, we used gonads carrying Wt1ERT2Cre treated with tamoxifen as controls.
XY gonads following conditional deletion of Cbp or p300 exhibit partial sex reversal

To determine the effect of induced conditional deletion of Cbp and p300 on gonad differentiation, we analysed gonads at 14.5 dpc, a stage at which disruptions to sex determination are easily detectable. As expected, XY control gonads (Wt1ERT2Cre+/−) exhibited well differentiated testis cords expressing Sox9 (Fig. 1A and B, left), while in XX gonads typical ovarian morphology accompanied by strong expression of Stra8 was observed (Fig. 1A and B, right). XY gonads lacking Cbp (Cbp cKO) or p300 (p300 cKO) (Fig. 1A and B, respectively) exhibited a partial male-to-female gonadal sex reversal phenotype. The overall shape of the mutant gonads was curved, similar to XX control gonads. XY Cbp cKO gonads expressed Sox9 and AMH, markers of Sertoli cells, but testis cord organization was irregular (Fig. 1A). Furthermore, XY Cbp cKO gonads expressed Stra8 at the poles, a phenotype generally associated with a delay in the activation of the male pathway at the centre of the gonad (12,48,49). Immunohistochemical analyses of FOXL2, normally detected in granulosa cells of XX gonads at this stage, revealed some positive cells in the XY Cbp cKO gonads, indicating that some XY supporting cells had adopted an ovarian fate. A similar phenotype of partial gonadal sex reversal was observed in XY p300 cKO embryos at the same stage (Fig. 1B). However, there was more variability, as illustrated by Sox9 WIMSH; Sox9 was absent in some XY p300 cKO gonads, suggesting nearly complete sex reversal, while some expressed Sox9 and AMH in association with malformed testis cords. Moreover, Stra8 expression at the poles of the XY p300 cKO gonads extended further than in the XY Cbp cKO gonads, suggesting a more severe phenotype in the absence of p300 than in the absence of Cbp. FOXL2 was also detected in XY p300 cKO gonads (Fig. 1B).

XY gonads lacking two copies of p300 and one copy of Cbp exhibit complete sex reversal

In other contexts, it has been shown that p300 and Cbp can act redundantly (23). Therefore, in order to address whether Cbp can compensate for the loss of p300, we generated XY gonads conditionally lacking two copies of p300 and one copy of Cbp (Wt1ERT2Cre+/−, Cbp+/−, p300−/−, p300 cKO) (Fig. 1B) reveals partial gonadal sex reversal characterised by disruption to the expression of Sertoli cell markers Sox9, analysed by whole-mount in situ hybridisation (WIMSH) (top panel), and anti-Müllerian hormone (AMH), analysed by immunohistochemistry (bottom panel, AMH immunostaining in red). XY mutant gonads express the ovarian germ cell marker Stra8 (middle panels) at the poles as well as granulosa cell marker FOXL2 to variable degrees (bottom panels, green signal in immunostaining).

Reduced Sry and Sox9 expression in XY Cbp/p300 dcKO gonads at 11.5 dpc

In order to determine the molecular basis of the observed gonadal sex reversal at 14.5 dpc, we analysed the expression of Sry and its principal target gene, Sox9, at 11.5 dpc, at the time testis determination is initiated. Sry mRNA and SRY protein levels were drastically reduced in Cbp/p300 dcKO gonads compared with XY controls (Fig. 3A and B). Quantitative analysis of the level of Sry in sub-dissected gonads (lacking the mesonephros) by qRT-PCR revealed a reduction of approximately 6-fold in Cbp/p300 dcKO gonads (Fig. 3C). Analysis of Sox9 by WIMSH (Fig. 3D) and immunohistochemistry (Fig. 3E) revealed an absence of positive cells in Cbp/p300 dcKO gonads, consistent with the
Los cell marker FOXL2 (immunostaining, green signal). Wnt4 is a granulosa cell marker (immunostaining, red signal). Sex reversal is confirmed by ectopic expression of Sox9 in XY gonads. This phenotype observed at 11.5 dpc. The reduction in levels of Sox9 transcript, to approximately those found in XX controls, was confirmed by qRT-PCR (Fig. 3F). These data suggest that a failure to attain normal threshold levels of Sry at 11.5 dpc, with a consequent loss of Sox9, underlies gonadal sex reversal in Cbp/p300 dcKO gonads.

Disrupted histone acetylation at the Sry promoter in XY Cbp/p300 dcKO gonads

Our data reveal a role for Cbp/p300 in testis determination via a positive impact on Sry expression. Finally, we investigated a potential link between these two factors and activating histone marks at the Sry promoter using chromatin-immunoprecipitation (ChIP) from whole gonads (lacking the attached mesonephros) at 11.5 dpc (Fig. 4; see Materials and Methods for details). We determined the level of histone H3 acetylation of all lysine residues (pan-acetylated H3), lysine 9 (H3K9ac) and 27 (H3K27ac). K27 is a preferential target of Cbp/p300 acetylation, whilst K9 is acetylated by other histone acetyl-transferases, such as GCN5/PCAF (50). When compared with the mesonephrons, these three marks were enriched at the Sry promoter in XY B6 wild-type gonads, suggesting a correlation between histone H3 acetylation and Sry expression (Fig. 4A–C). Crucially, there was a reduction in the level of H3K27ac at the Sry promoter of Cbp/p300 dcKO gonads when compared with XY controls (tamoxifen-treated, Wt1ERT2Cre+/–, Cbpfl/fl, p300fl/fl) (Fig. 4F). In contrast, the levels of pan-acetylated H3 and H3K9ac remained unchanged in Cbp/p300 dcKO gonads compared with XY controls (Fig. 4D and E). These data suggest that Cbp/p300 activity is required for the establishment of a normal profile of positive histone acetylation marks at the Sry promoter.

Discussion

Here we demonstrate for the first time a role for Cbp/p300 in mammalian testis determination. Using an inducible Cre recombinase system to circumvent the embryonic lethality seen in Cbp and p300 knockout mice, we observed a partial male-to-female gonadal sex reversal in XY embryos with targeted ablation of either Cbp or p300 in somatic cells. Complete gonadal sex reversal occurred when only three out of four functional copies of Cbp/p300 were targeted for deletion. However, it should be remembered that the presence of Wt1ERT2Cre means that embryos are haploinsufficient for Wt1, a gene with a known role in testis determination. Although tamoxifen-treated Wt1ERT2Cre XY embryos develop testes as normal, it is likely that this haploinsufficiency makes some small contribution to the sex reversal phenotype. It is possible that in the presence of two wild-type copies of Wt1, it would be necessary to delete all four functional copies of Cbp/p300 in order to effect XY gonadal sex reversal.

Our observation that partial gonadal sex reversal is caused by deletion of either gene indicates that both Cbp and p300 play a role during testis determination. Although we observed a strong decrease of Cbp and p300 proteins in the somatic cells of the gonads at 11.5 dpc, we cannot formally exclude the possibility that deletion does not occur in all cells, or that this deletion is not early enough, and that, as a consequence, some residual p300 or CBP activity is responsible for the observed partial induction of testis differentiation in single gene mutants. However, previous studies have suggested that CBP and p300 can act redundantly. Both compound mutants exhibited complete gonadal sex reversal, with XY gonads developing as ovaries. These data suggest that both genes are involved in testis determination and can at least partially compensate for each other’s loss. Finally, it is noteworthy that sex-reversed mutant XY gonads robustly express Stra8 and FOXL2, suggesting that the ovarian program of differentiation, in contrast to the testicular, can occur when there is a major functional deficit in these two proteins.

We have demonstrated that, in the p300/Cbp dcKO, Sry expression is dramatically decreased at 11.5 dpc. This is sufficient to explain the loss of Sox9 expression at the same stage (2), and account for the sex reversal observed at 14.5 dpc. We sought a mechanistic explanation for this loss of Sry expression. Lysine 27 of histone H3 (H3K27), a known target of CBP/p300
Acetylation, shows higher levels of acetylation at the Sry promoter in XY gonads than in the mesonephros at the time of sex determination. Levels of pan-acetylated H3 and acetylated H3K9 are also higher in the gonad, suggesting a positive correlation between the level of H3 acetylation and Sry expression. These experiments were performed on whole gonads and, due to the heterogeneous collection of cell-types found in the gonad at this stage, may under-estimate the enrichment of acetylated H3 at the Sry promoter in gonadal somatic cells. In gonads depleted for both CBP and p300 using our conditional deletion strategy, the levels of H3K27 acetylation (H3K27ac) were significantly decreased, suggesting that the down-regulation of Sry in the absence of sufficient Cbp/p300 activity is caused by reduced histone acetylation at its promoter. Our data shed new light on a key developmental gene and its epigenetic control, which remains poorly understood (51). This is only the second report of an altered epigenetic mark at the Sry promoter in a sex-reversed mutant. Elevated levels of H3K9me2, an inhibitory mark, were observed in embryonic gonads lacking the H3K9 histone demethylase, JMJD1A (19). These data, taken together with those reported here, reveal a complexity of epigenetic regulation at the Sry locus required for its normal expression and, as a consequence, testis determination.

Levels of H3K9ac are higher in wild-type gonads than in mesonephros, but are unaffected by the loss of Cbp/p300, suggesting that some other unidentified histone acetyltransferases play a role in chromatin modification at the Sry locus. Notwithstanding the impact of CBP/p300 activity on
chromatin at the Sry promoter, which is required to initiate testis determination, we cannot exclude an indirect role i.e. functionally upstream of Sry, perhaps via the regulation of expression of some known regulators of Sry (Wt1, Cited2, Cbx2, Gat4 and others) revealed no differences between CBP/p300-deficient gonads and controls (Supplementary Material, Fig. S4). It should also be noted that in addition to their role as histone acetyl-transferases, CBP and p300 can also acetylate and regulate the activity of non-histone proteins, acting as gene regulatory hubs with at least 400 identified interacting partners, among them known regulators of Sry such as WT1, SF1, FOG2, GATA4 and (52).

Finally, a number of recent studies describe the use of exome sequencing of human patients with disorders of sex development (DSD), including partial and complete 46, XY gonadal dysgenesis (sex reversal), as a way of identifying the mutations causally responsible for the observed condition. It is noteworthy that two such studies report potentially disruptive mutations in human CBP and p300 (53,54). Whilst the conditional gene targeting strategy described here does not directly model the effects of constitutively acting human CBP/p300 gene mutations, it suggests that such variants may act by disrupting cell type-specific events, such as protein-protein interactions, required for human sex determination. The data reported here concerning the requirement for CBP and p300 in mouse testis determination suggest that such variants should be considered as potentially contributing to defects in human testis determination and warrant further investigation.

Materials and Methods
Mouse lines and generation of embryos
Mouse experimentation was approved by the Animal Welfare and Ethical Review Body at MRC Harwell. Breeding was performed by license under the Animals (Scientific Procedures) Act with approval from the U.K. Home Office (PPL 70/8898). Mice were housed in individually ventilated cages in a specific pathogen-free environment. Further details of micro- and macro-environmental conditions are available on request.

Adult mice were humanely euthanized by dislocation of the neck and embryos were decapitated in ice-cold, phosphate-buffered saline solution. The Cbpflox and p300flox lines have been previously described (55,56) and were maintained on the C57BL/6J (B6) background. In order to target gene deletion to somatic cells of the embryonic gonad, floxed mice were crossed with mice carrying the tamoxifen-inducible Wt1ERT2Cre allele (41), also maintained on B6. Different crosses were performed to generate mutant tissue for this study: i) Wt1ERT2Cre+/−, Cbpflox+/− × Cbpflox/fox; ii) Wt1ERT2Cre+/−, p300flox/fox × p300flox/fox; or iii) Wt1ERT2Cre+/−, p300flox/fox, Cbpflox/fox × p300flox/fox. Deletion was induced with a single dose of tamoxifen (Sigma T5648), dissolved in corn oil, at 200mg/kg per mouse.
administered by oral gavage at 9.5 days post coitum (dpc). Noon on the day of the copulatory plug was counted as 0.5 dpc.

Quantitative RT-PCR
Total RNA was extracted from one pair of gonads, dissected away from the mesonephroi, using the RNeasy plus microkit (Qiagen). 300 ng of RNA was reverse-transcribed using the high capacity RNA to cDNA kit (Applied Biosystem). qPCR of cDNA (qRT-PCR) was performed on a 7500 Real-Time PCR system (Applied Biosystems) using Fast SYBR Green Master Mix (Applied Biosystems) according to manufacturer’s instructions. At least 3 samples for each genotype were analysed. Data were normalized to Hprt1. Fold-change in expression was determined by the 2^ΔΔCT method. Statistical differences were determined by using a two-tailed Student’s t-test. Primers sequences are available on request.

Immunohistochemistry
Tissues were fixed overnight in 4% paraformaldehyde, embedded in paraffin and cut into 8μm sections. Analyses were performed on at least two independent samples per experiment. The following primary antibodies were used: AMH (SC28912, Santa-Cruz), SOX9 (AB5535, Millipore), FOXL2 (a kind gift from Dagmar Wilhelm), CBP (SC583, Santa-Cruz) and p300 (SC585, Santa-Cruz). Secondary antibodies were Alexafluor 594 and Alexafluor 488. Images were captured using a Zeiss 710 multiphoton microscope.

Wholemount in situ hybridization
Tissues were fixed overnight in 4% paraformaldehyde before storing at –20°C after serial methanol dehydration. The whole-mount in situ hybridization (WISH) protocol as well as the probes used have been previously described (Bogani et al., 2009). A minimum of three samples per genotype were analysed for each probe.

Chromatin immunoprecipitation
Chromatin immunoprecipitation (ChiP) was performed by modifying published methods of low cell number ChiP (57,58). Briefly, gonads or mesonephroi were fixed in 1% formaldehyde (room temperature, 8min), quenched with 1 volume of 250mM glycine and washed twice with chilled TBE (20 mM Tris-HCl, 150mM NaCl, 1mM EDTA) before freezing on dry ice and storing at –80°C. After thawing on ice, 3 pairs of gonads or mesonephroi were pooled and lysed with 100 l dilution buffer [50mM Tris-HCl pH 8.0, 10mM EDTA, 1% SDS, mini complete inhibitor (Roche)] on ice for 5 min. After centrifugation (500 g, 20 min, 4°C) samples were resuspended in 100 μl dilution buffer (16.7mM Tris-HCl pH 8.0, 167mM NaCl, 1.2mM EDTA, 1.1% Triton X100, 0.01% SDS) and sonicated for 7 cycles (30’ on/30’ off) using a Bioruptor water-bath sonicator (Diagenode, Belgium). After pre-clearing with a mix of protein G and A (Dynabeads, Invitrogen), sonicated chromatin was immunoprecipitated overnight with protein A- and G-coupled antibodies [H3K27Ac (Abcam, Ab4729), 0.5μg; pan-acetylated H3 (MD Millipore, 06–599), 1 μg; H3K9ac (Cell signalling, CB11), 0.5 μg]. 10% of each sample (input) was kept and used for calculation of enrichment. On the following day, beads were washed 3 times for 10 min at 4°C with low salt buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 2mM EDTA, 1% Triton X100, 0.1% SDS). After elution (50 mM Tris-HCl pH 8.0, 10mM EDTA, 1% SDS), samples were digested with proteinase K (2 μg/μl) and cross-linking for 6h at 68°C. Reverse cross-linked DNA was purified with Agencourt AMPure XP beads and diluted 3-fold before qPCR analysis. qPCR was performed on a 7500 Real-Time PCR system (Applied Biosystems) using Fast SYBR Green Master Mix (Applied Biosystems) according to manufacturer’s instructions. ChiP data were calculated as a ratio to input and were rescaled by normalizing to the control gene Gapdh. At least six measurements were made for each histone mark. Statistical differences were determined by using a two-tailed Student’s t-test. Primer sequences are available on request.

Supplementary Material
Supplementary Material is available at HMG online.

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Conflict of Interest statement. None declared.

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