Title: Histone H2A monoubiquitination in the thalamus regulates cocaine effects and addiction risk

Authors: Julian Cheron[1], Leonardo Beccari[2,3], Perrine Hagué[1], Romain Icick[4], Mathieu Defrance[5], Teresa Carusone[6], Chloé Despontin[1], Sagar Bhogaraju[6], Florence Vorspan[4], Jérôme Bonnefont[7], and Alban de Kerchove d'Exaerde[1,8*]

Affiliations :

[1]Université Libre de Bruxelles (ULB), ULB Neuroscience Institute, Neurophysiology Laboratory; Brussels, Belgium

[2]Centro de Biología Molecular Severo Ochoa, CSIC-UAM; Madrid, Spain

[3]Université Claude Bernard Lyon 1, Pathophysiology and Genetics of Neuron and Muscle, CNRS UMR 5261, INSERM U1315; Lyon, France

[4]INSERM UMRS_1144, Université Paris Cité; Paris, France

[5]Interuniversity Institute of Bioinformatics in Brussels, Université Libre de Bruxelles (ULB); Brussels, Belgium

[6]European Molecular Biology Laboratory; Grenoble, France

[7]Université Libre de Bruxelles (ULB), ULB Neuroscience Institute, Institut de Recherches en Biologie Humaine et Moléculaire (IRIBHM); Brussels, Belgium.

[8]WELBIO, Wavre, Belgium

*Corresponding author. Email: adekerch@ulb.ac.be

Abstract: The individual risk of developing drug addiction is highly determined by the epigenetic landscape[1,2]. Chromatin remodeling regulates drug-induced transcriptional and behavioral effects and the consequent development of addictive behaviors[2,3]. Several chromatin modifications in the ventral tegmental area and nucleus accumbens, including histone H3 methylation, H3 and H4 acetylation, have been implicated in drug addiction. Still, the contribution of other histones and their post-translational modifications (PTMs), such as monoubiquitination is unknown[4–8]. Here we found that H2A monoubiquitination in the paraventricular thalamus (PVT) plays a major role in cocaine-adaptive behaviors and local cocaine-evoked transcriptional repression. Mice undergoing chronic cocaine administration showed a specific increased monoubiquitination of H2A. Furthermore, we showed that this histone PTM is controlled, in the PVT, by an interaction between melanoma-associated antigen D1 (Maged1), a scaffold protein involved in drug addiction[9], and USP7, a deubiquitinase[10]. Accordingly, Maged1 specific inactivation in thalamic vGluT2 neurons, or USP7 inhibition, blocked cocaine-evoked H2A monoubiquitination and abolished cocaine locomotor sensitization. Finally, we identified genetic variations of *MAGED1* and *USP7* associated with modified transition to cocaine addiction and cocaine-induced aggressive behavior in human subjects. These findings identified a new epigenetic modification in a non-canonical reward pathway of the brain and a potent marker of epigenetic risk factor for drug addiction in human.

Main

The number of deaths associated with cocaine use disorder (CUD) is increasing, and in contrast to tobacco and opioid addiction, no medication has been approved for CUD[11]. Identifying risk factors and therapeutic targets is thus a pressing biomedical challenge. The neurobiological mechanisms that drive and sustain drug addiction involve the combination of neuronal and synaptic plasticity with modifications of gene expression, achieved, in part, through epigenetic mechanisms[1]. By regulating chromatin-related processes, drug-induced epigenetic alterations contribute to the aberrant gene expression and cellular function that underlie drug addiction pathogenesis[2]. Histone post-translational modifications (PTMs) that control chromatin structures regulate drug-adaptive behaviors and the subsequent transition to addiction[3,4]. The promoter of melanoma-associated antigen D1 (*Maged1*) was identified as one of the targets of histone 3 acetylation, a canonical PTM, induced in the (NAc) by chronic cocaine treatment[8]. Subsequently, we identified Maged1 as a master regulator of cocaine reward and reinforcement[9]. To date, however, the molecular mechanisms involving Maged1 in these cocaine-related phenotypes have not been characterize[9,12,13]. Despite its ubiquitous expression in the brain and its essential role in cocaine-related behaviors, *Maged1* is not required for cocaine-induced behaviors in dopaminergic or GABAergic cells[9], leaving excitatory glutamatergic cells as the main candidate.

Here, we specifically inactivated *Maged1* in glutamatergic vGlut1 or vGluT2 cells by crossing vGluT1- or vGluT2-cre-driver mice with *Maged1* floxed mice (*Maged1*^{loxP}). Notably, only vGluT2-mediated (but not vGluT1-mediated) *Maged1* inactivation (*Maged1* conditional knockout (cKO)) abolished cocaine-induced locomotor sensitization, a straightforward behavioral paradigm used to model drug-adaptive behavior[14–16] (Fig. 1a-f and Extended Data Fig. 1). In rodents, repeated cocaine injection induces gradually increased locomotor activity; classically after five days of consecutive injections, the locomotor response reaches a ceiling level. This state lasts for months after cocaine withdrawal[14]. Locomotor sensitization is thus thought to be linked with important aspects of vulnerability to drug addiction[17,18], relapse and drug craving[19,20]. We further excluded the contribution of glutamatergic telencephalic neurons by using an Emx1-cre driver line (Extended Data Fig. 2). To screen for potential vGluT2 nuclei involved in acquisition of this phenotype, we performed AAV retro mCherry injection in the NAc and revealed the amygdala, the ventral subiculum and the paraventricular thalamus (PVT) as the main vGluT2 nuclei targeting the NAc (Fig. 1g,h). Of these, *Maged1* mRNA in situ hybridization and immunohistochemistry (IHC) showed a singularly high level of Maged1 expression (Fig. 1i) in the PVT, which has recently been shown to be involved in drug addiction[21–24]. We therefore examined the impact of *Maged1* inactivation in the PVT via stereotactic injection of an adeno-associated virus (AAV) encoding a Cre recombinase in adult *Maged1*^{loxP} mice (Fig. 1j,k). Here again, we observed the abolition of cocaine-induced locomotor sensitization; thus, phenocopying *Maged1*-KO mice and excluding developmental effects (Fig. 1l). To test the sufficiency of Maged1 in the PVT to cocaine sensitization, we stereotactically injected an AAV expressing Maged1 into *Maged1*-KO mice and found that cocaine-evoked sensitization was restored (Fig. 1j,k,m). In contrast, stereotactic inactivation[9], (Extended Data Fig. 3) or re-expression (Extended Data Fig. 4) of *Maged1* in other vGluT2- or mixed vGluT1/vGLuT2 cell hubs of drug addiction, such as the prefrontal cortex (PFC), amygdala or ventral subiculum[25], did not lead to major significant changes in cocaine-induced locomotor sensitization. Taken together, these results point toward PVT as the key nucleus where Maged1 plays a role in drug addiction.

To identify the molecular mechanisms linking Maged1 in the PVT with cocaine-mediated behavior, we analyzed the transcriptome in the thalamus after chronic cocaine (or saline) administration in control and *Maged1*-cKO mice by RNA sequencing (RNA-seq) (Fig. 2a). In control mice, repeated cocaine injection induced upregulated expression of 252 genes and downregulated expression of 452 genes compared to the expression of these genes after saline treatment (Fig. 2b). The transcriptional response (and particularly the downregulation process, see supplementary text) to cocaine exposure by *Maged1*-cKO mice differed from that of control mice in terms of genes and their assigned GO terms (Fig. 2b and Extended Data Fig. 5 and 6a, and Table 1). Interestingly, a gene set enrichment analysis with curated gene sets revealed that cocaine-regulated genes in control but not in *Maged1*-cKO mice were targets of the Polycomb repressive complex (PRC) (Fig. 2c,d and Extended Data Table 1). Notably, the genes with expression similarly regulated by cocaine in the control and *Maged1*-cKO mice were not targeted by PRC action. For example, PRC2 mono-, di-, and tri-methylates lysine 27 of histone H3 (H3K27me1/me2/me3), which is catalyzed by EZH1/2[26,27], and PRC1 monoubiquitinates histone H2A on lysine 119 (H2AK119ub1), which is catalyzed by the E3 ligase RING1A/B[28] (Fig. 2d).

To understand how Maged1 mediates the profound transcriptional changes observed upon cocaine exposure (Fig. 2b-d), we aimed at identifying Maged1 protein partners by performing coimmunoprecipitation and mass spectrometry (co-IP-MS) experiments with an anti-Maged1 antibody that recognizes both the full length and truncated form (functional KO) in saline- and cocaine-treated mice (Fig 2e). We identified 404 potential Maged1-interacting proteins (see Supplementary Text and Table 2). Among them, histone H2A, in its abundantly expressed H2A.2 variant in the adult brain[29,30], and de-ubiquitinase USP7, a well-characterized PRC1 component[10,31,32], as well as several other proteins indirectly linked to PRC complexes, such as Praja1, hnRNPA2/B1, Myo5a, Dbn1 and Neb1/2, were identified (Fig. 2f)[33,34]. Within all the identified candidates, histone H2A was the only one whose i) interaction with Maged1 was disrupted in the *Maged1*-cKO samples and ii) displayed a differential association with Maged1 in cocaine and saline treated control mice (Fig 2f and Extended Data Table 2). USP7 behaved as a Maged1 partner under both saline and cocaine treatment conditions (Fig. 2f and Extended Data Table 2). To confirm these interactions, we performed Maged1 co-IP with mononucleosome samples extracted from the thalamus, followed by western blotting (WB) for protein identification. We confirmed that the specific link between Maged1 and H2A was evident only in the presence of cocaine, while USP7 coprecipitated with Maged1 under both cocaine and saline treatment conditions (Fig. 2g). In agreement with these IP data, we observed that Maged1 and USP7 protein levels were both higher in the PVT than in other parts of the thalamus (Extended Data Fig. 7).

Next, we postulated that the Maged1 interaction with H2A and USP7 contributes to the regulation of the monoubiquitination of H2A at lysine 119 (H2AK119ub1 (hereafter H2Aub)) as Mage proteins assemble with different E2/3 ubiquitin ligases[13,35]. This histone mark deposited by PRC1 and linked to gene repression[36,37] may explain how Maged1 represses gene expression in thalamic neurons upon chronic cocaine administration. To test this hypothesis, we performed co-IP experiments using an anti-H2Aub monoclonal antibody and observed that USP7 and Maged1 both linked with H2Aub under saline and cocaine treatment conditions (Fig. 2h and Extended Data Fig. 8). Notably, USP7 binding to H2Aub was significantly decreased in the *Maged1*-KO mice, indicating that Maged1 was necessary for this H2Aub-USP7 interaction (Fig. 2h and Extended Data Fig. 8). Furthermore, USP7-specific antibody coimmunoprecipitated H2Aub and H2A under saline and cocaine treatment conditions but not

in the Maged1-cKO thalamus samples (Fig. 2i). These data suggest that Maged1 plays a role in H2A monoubiquitination in thalamic neurons following cocaine intake, via Maged1 involvement with a PRC1 member. To test whether MAGED1 associates with USP7 in human cells, we performed co-IP-MS of MAGED1 in two different human cell lines: human embryonic kidney 293T (HEK293T) and neuroblastoma (SH-SY5Y) cells (Fig. j-m, Extended Data Table 2). The interaction with USP7 was thus confirmed in human cells (Fig. 2l,m). In neuronal cells, the known interaction between Maged1 and CREB[38] was also found (Fig. 2m). In addition, a novel interaction between MAGED1 and another H2A deubiquitinase, MYSM1, was also identified (Fig. 2m). These results indicated that the Maged1-USP7 axis is evolutionarily conserved and may regulate addiction behavior in humans through histone H2A PTM.

We therefore tested whether chronic administration of cocaine impacts H2A monoubiquitination in key brain regions involved in drug addiction and whether these changes depend on Maged1 function (Fig. 3a and Extended Data Fig. 9a). To this end, by WB using anti-H2Aub and anti-H2A specific antibodies, we analyzed H2A and H2Aub levels in the PFC, thalamus and NAc of control and *Maged1*-cKO animals after chronic cocaine or saline administration. The results showed a significant increase in H2Aub in all these nuclei (Fig. 3b-e) after cocaine administration. Remarkably, the cocaine-dependent increase in H2Aub was only abolished in the thalamus of the *Maged1*-cKO mice (Fig. 3e). These results are the first to show that cocaine treatment induces an increase in H2Aub in key structures involved in addiction and that Maged1 is crucial for cocaine-induced H2A monoubiquitination, specifically in the thalamus.

Because USP7 is a known PRC1 regulator with deubiquitinase activity[10,31,32], we hypothesized that USP7 plays a key role in the Maged1-dependent cocaine behavioral response. We therefore inhibited USP7 activity in vivo by administrating P5091, a selective USP7 inhibitor, in a two-injection cocaine sensitization protocol[15] (Fig. 3f). Interestingly, systemic injection of P5091 abolished locomotor sensitization, similar to the effect of *Maged1* inactivation (Fig. 3g,h and Extended Data Fig. 9a), with no effect on spontaneous locomotor activity (Extended Data Fig. 9b). To exclude a transient occlusion effect, we continued for an additional 3 days of daily cocaine injections and observed a sustained abolition of cocaine sensitization (Extended Data Fig. 9c). Similarly, an intrathalamic injection of P5091 significantly interfered with cocaine-induced locomotor sensitization (Fig. 3i,j). Similar to the observation with *Maged1*-cKO mice, P5091 treatment prevented the increase in H2A monoubiquitination induced by cocaine administration (Fig. 3k). These results showed that pharmacological abolition of cocaine-evoked H2Aub deposition in the thalamus impeded cocaine-evoked behavioral sensitization.

To further characterize the mechanisms by which Maged1 activity and H2A mono-ubiquitination contribute to cocaine sensitization, we compared H2Aub genomic coverage in the thalamus of control and *Maged1*-cKO mice after chronic cocaine or saline were administered by performing chromatin immunoprecipitation with sequencing (ChIPmentation)[39] (Fig. 3l). Analysis of the H2Aub coverage in these mice revealed a higher number of regions with this PTM in the cocaine- treated mice compared to saline-treated mice (13493 and 8174, respectively; +164.95% in the cocaine vs. the saline treated mice) (Fig. 3m). Supporting a regulatory function of this PTM, the same increase was observed when only gene promoter regions (defined as 5kb upstream and 2k downstream of the gene transcription start site (TSS)) were analyzed, and the largest fraction of H2Aub+ elements was located less than

5 kb upstream from the nearest TSS. Cocaine shifted this distribution significantly upward in the control but not in the *Maged1*-cKO mice (Fig 3n). Specifically, 1271 genes with H2Aub at their promoters were found in the control mice in the cocaine treatment condition, while only 143 loci showed H2Aub specific marks after saline administration (Fig. 3o). In contrast, the total number of H2Aub marks and promoters was comparable between the cocaine-injected (583 genes) and saline-injected (340 genes) *Maged1*-cKO animals (Fig. 3o). Furthermore, 84.03% (1068 genes) of genes displaying H2Aub enrichment upon cocaine administration in control mice did not show enrichment in *Maged1*-cKO animals, indicating that the loss of *Maged1* deeply impacted the H2Aub dynamics associated with the cocaine response (Fig. 3p,q). Cocaine-dependent H2Aub+ genes in control mice were associated with changes in biological process like ion transport, synaptic signaling, axonal guidance, neuron projection and cytoskeleton/microtubule organization (Extended Data Fig. 10 and Table 1) which were not found in *Maged1*-cKO mice (Extended Data Table 1). These results support the idea that Maged1 exerts its specific effects on the behavioral response to cocaine primarily through drug-induced PRC1 gene repression, as cocaine administration to control mice triggered coherent transcriptional and H2Aub dynamics (Extended Data Fig. 5d,6,9). Indeed, we found a significant overlap, between RNAseq and ChIP data, in the genes with transcriptional downregulation and those with promoters enriched in H2Aub marks after cocaine administration (Fig. 3r). Interestingly, we found that c1qL3, a signaling protein involved in synaptic projection and human CUD[40,41] was downregulated while displaying an increase in H2Aub on its promotor upon chronic cocaine administration, as well as HOPX and SCML4, important epigenetic regulators linked to PRC1.

To assess the potential contributions of *MAGED1* and *USP7* to CUD directly in humans, we conducted a within-case *MAGED1* and *USP7* gene analysis with 351 consecutively recruited outpatients with cocaine addiction and genetically verified Caucasian ancestry. After demonstrating the absence of structural variants in/near *MAGED1* or *USP7*, we focused on 175 nucleotide polymorphisms (SNPs). We investigated their potential associations with seven CUD-related phenotypes reflecting all stages of disease progression, correcting for multiple testing. The outpatient population comprised 77% men aged 38 +/- 9 years old. All the outpatients reported lifetime cocaine use and 72% of these men suffered from severe CUD (including to crack-cocaine). Patients carrying at least one alternate allele were compared to those carrying only reference allele(s). In the case of multiple statistical associations with a given phenotype, we retained lead SNPs most likely associated with the underlying biological signal by combining positional and functional data. First, the transition from 1st cocaine use to CUD was significantly associated with 17 SNPs in *MAGED1* (Fig. 4a,b), of which we identified two lead SNPs. These two SNPs showed opposite effects during the transition, as highlighted by Kaplan–Meier survival curves (Fig. 4c). An *in silico* analysis with human cells showed a significant association of these two SNPs with DNA methylation changes and thus altered gene expression. Second, we observed associations between 44 *USP7* SNPs and cocaine-induced aggression (Fig. 4a), of which we identified five lead SNPs. Four SNPs were in the alternate allele associated with lower aggression scores, and scores were higher for the alternate allele of the other SNP (Fig. 4c). Consistent with our preclinical data, these findings with human samples revealed that *MAGED1* and *USP7* polymorphisms are associated with CUD (Extended Data Table 3). These associations were independent of potential confounders, such as the self-reported amount of cocaine use, which is strongly related to cocaine-induced aggression and childhood hyperactivity and is associated with a shorter transition from first cocaine use to CUD (Extended Data Table 4 and 5). These data enabled us to designate

MAGED1 and *USP7* as contributors to the vulnerability for CUD and specific behavioral responses to cocaine.

Conclusion

Here we demonstrated that the major effect of Maged1 on drug-adaptive behavior is mediated through vGluT2 neurons in the PVT. Furthermore, we showed that the role played by Maged1 is bidirectional in the PVT of adult mice, with complete abolition and restoration of cocaine sensitization. Taking advantage of this phenotype, we found that *Maged1* inactivation significantly altered cocaine-evoked gene repression in the thalamus and further identified its involvement in PRC1 complex activity. The identification of USP7, a well-known PRC1 regulator, and H2Aub as Maged1-interacting partners, together with the observation that chronic cocaine exposure leads to an overall increase in H2Aub+ promoters, indicated that Maged1 mediates cocaine-dependent gene downregulation by controlling histone H2A monoubiquitination. Furthermore, inactivation of *Maged1* in vGluT2 neurons or inhibition of USP7 alters H2A monoubiquitination in the thalamus and cocaine-adaptive behavior. Considering that Maged1 enabled interactions between H2Aub and USP7, the Maged1-USP7 interaction likely constitutes a molecular switch linking PRC1-mediated gene repression to upstream signaling events[31].

Our proposed model (Extended Data Fig. 11) postulates that when cocaine is administered, Maged1-USP7 is recruited to H2A, activating or stabilizing PRC1 and leading to H2A monoubiquitination. Thus, in *Maged1*-cKO mice or after treatment with the USP7 inhibitor P5091, PRC1 activity is blocked, leaving it in an unresponsive state such that upstream events (such as cocaine exposure) do not induce its activity (Extended Data Fig. 11). This novel epigenetic mechanism highlighted in mice might be a first step toward developing CUD in human. Indeed, SNP mutations in *USP7* and *MAGED1* polydrug users led to aggressive behavior during cocaine use and a modified transition time to cocaine addiction, respectively. These findings establish a critical role for histone H2A monoubiquitination and its subsequent transcriptional changes, in regulating cocaine-adaptive behaviors, which may constitute an essential epigenetic risk factor for drug addiction in human. Although no medication has been developed to be an efficacious treatment for CUD, a chronic disease with harmful consequences worldwide[42,43], the regulation of this newly identified cocaine-induced epigenetic modification might be considered a new potential therapeutic target for prevention and harm reduction in active cocaine users.

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Author contributions:

Conceptualization: JC, AKE

Investigation: JC, LB, PH, RI, TC, CD, RI, FV, JB

Visualization: JC, LB, PH, RI, TC, FV, JB, AKE

Data Analysis: JC, LB, RI, MD, TC, SB, FV, AKE

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Project administration: JC, AKE

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Supplementary Materials

Supplementary Text

Extended Data Figures 1 to 11

Extended Data Tables 1 to 7

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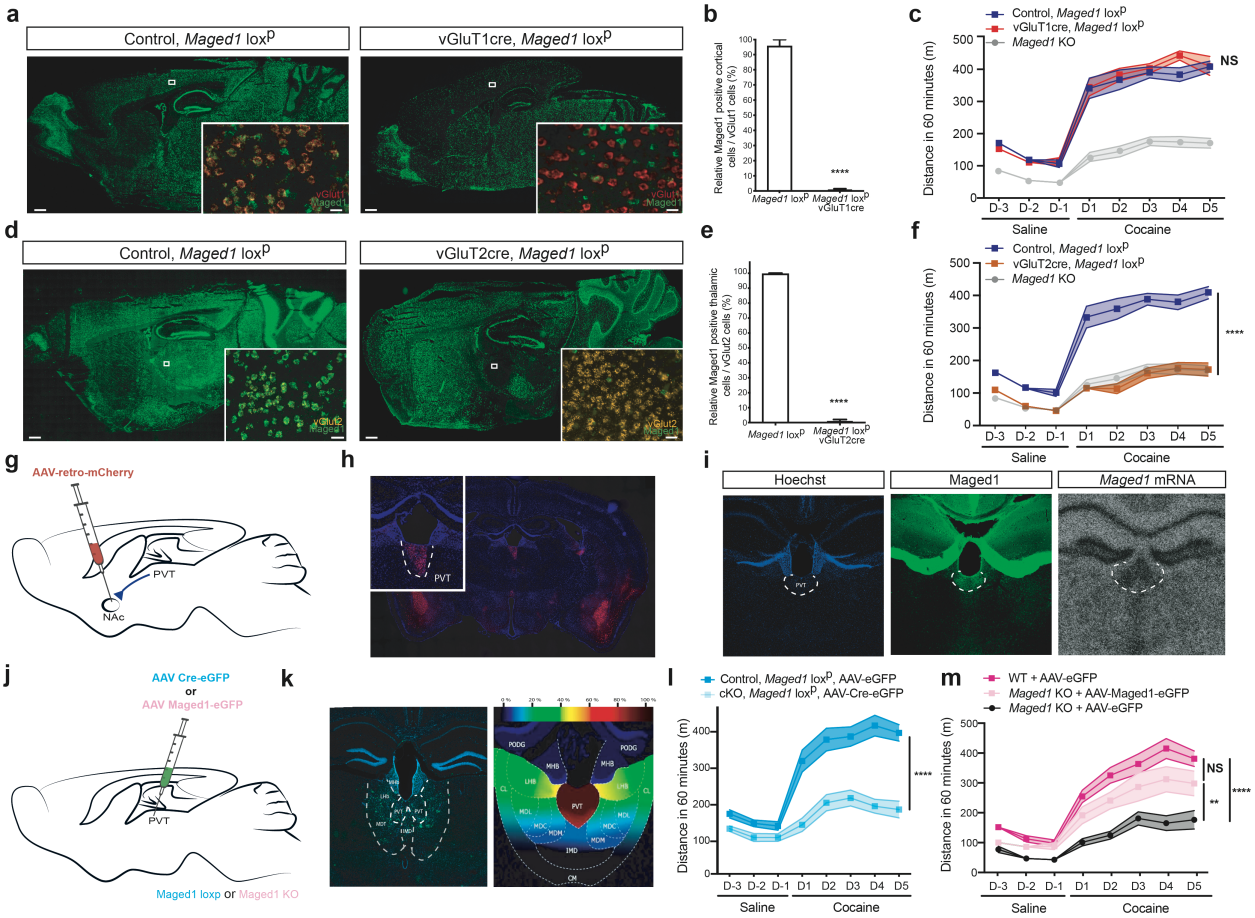

Fig. 1. *Maged1* inactivation in vGluT2 neurons completely recapitulates the effect of *Maged1* global inactivation and *Maged1* is 'necessary and sufficient' in the PVT. a,d, Fluorescent in situ hybridization (FISH) of *Maged1* mRNA (in green) (scale bar: 0.5 mm). Insets show multiplexed FISH with vGluT1 (in red) (**a**), vGluT2 (in orange) (**d**) and *Maged1* mRNA (x20 magnification) in the cortex (**a**) and in the thalamus (**d**) (scale bar: 20 µm (**a**), 30 µm (**d**). **b,e,** Relative percentage of *Maged1* mRNA-positive cells among vGluT1 mRNA-positive cells in the cortex (**b**), and vGluT2 mRNA-positive cells in the thalamus (**e**). n = 7 for each group (Mann–Whitney test, P < 0.0001). **c,f** Cocaine-induced locomotor sensitization, 20 mg/kg, intraperitoneal (ip) injection. In panel **c**: control, *Maged1* loxP, n = 13; vGluT1cre, *Maged1* loxP mice, n = 7, (two-way ANOVA, control versus vGluT1cre, *Maged1* loxP, P = 0.6651; days, P<0.0001; interaction factor (genotype X days) P = 0.5056). In panel **f**: control, *Maged1* loxP, n = 12; vGluT2cre, *Maged1* loxP mice, n = 9, (two-way ANOVA, control versus vGluT2cre, *Maged1* loxP, P < 0.0001; days, P<0.0001; interaction factor (genotype X days) P < 0.0001). **g,** Schematic of adeno-associated virus (AAV)-retro-mCherry injection in the nucleus accumbens (NAc). **h,** Wide-field image showing the mCherry signal specifically in the PVT and in other NAc-projecting regions, such as the amygdala. Inset shows a clear specific signal in the PVT with a relatively low signal in other thalamic nuclei, demonstrating the relative importance of PVT projecting to the NAc. **i,** Immunohistochemistry (IHC) staining of Maged1 (in green) and Hoechst staining (blue) and parallel in situ hybridization autoradiogram of *Maged1* mRNA. **j,** Schematic of inactivation (AAV-Cre-eGFP into *Maged1* loxP mice) and re-expression experiments in the PVT (AAV-*Maged1*-eGFP in the *Maged1*-KO). **k,** IHC staining showing the eGFP signal (in green) in the PVT (-2 mm from the bregma) (left), topographic representation of the targeted area; colors represent the percentage of the superimposed eGFP-targeted area (right). **l,m,** Inactivation

experiment (**l**) and re-expression experiment (**m**) constisting of a cocaine-induced locomotor sensitization, 20 mg/kg, ip injection. In panel **m**: control, *Maged1* loxP, AAV-eGFP mice, n = 10; cKO, *Maged1* loxP, AAV-Cre-eGFP mice, n = 11, (two-way ANOVA, control versus *Maged1*-cKO, $P < 0.0001$; days, $P<0.0001$; interaction factor (genotype X days) $P < 0.0001$). In panel **m**: control *Maged1*-KO, AAV-eGFP mice, n = 10; control *Maged1* WT, AAV-eGFP mice, n = 10; re-expressed in KO, AAV-*Maged1*-eGFP mice, n = 8, (two-way ANOVA, groups $P < 0.0001$; days, $P<0.0001$; interaction factor (genotype X days) $P < 0.0001$ and Sidak's post-test; re-expressed in KO versus control *Maged1* WT mice, $P = 0.0731$; re-expressed in KO versus control Maged1-KO mice, $P = 0.0072$; control *Maged1* WT versus control *Maged1*-KO, $P < 0.0001$). All error bars represent s.e.m.

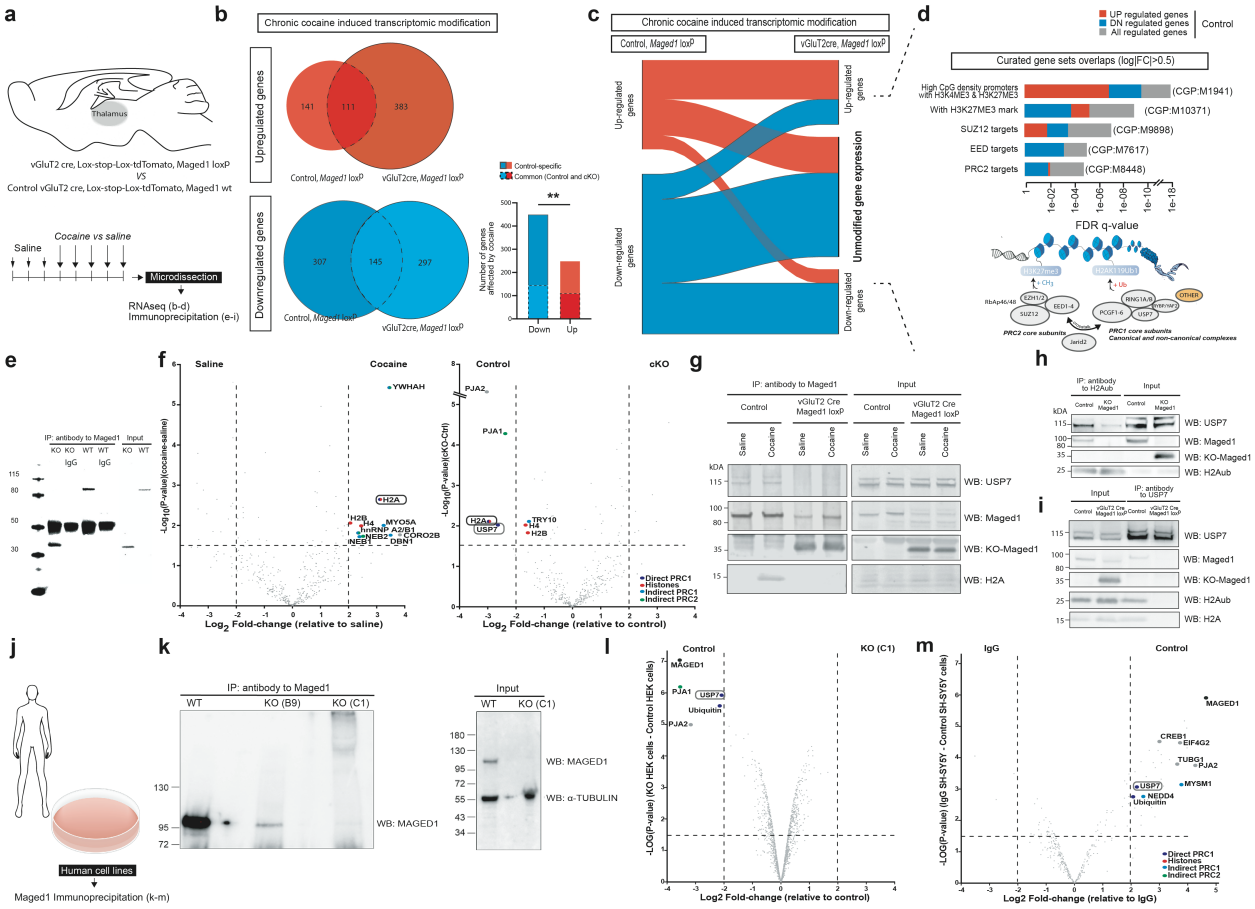

Fig. 2. *Maged1* inactivation modifies gene expression regulated by the Polycomb pathway through the USP7-H2A interaction. a, Method and time line for RNA sequencing and immunoprecipitation (IP) from microdissected mouse thalami. **b**, Venn diagram showing significantly up- and downregulated genes in control mice, *Maged1* loxP mice and *Maged1*-cKO, vGluT2 cre, *Maged1* loxP mice (log2|FC|≥0.5, FDR<0.05). The histogram on the bottom right shows that the proportions of overlapping up- and downregulated genes between control and *Maged1*-cKO group differ (chi-square, **P < 0.01). **c,** Alluvial plot showing the proportion of genes that are up- or downregulated in the control, *Maged1* loxP and are or are not modified in *Maged1*-cKO mice, vGluT2 cre, *Maged1* loxP mice (log2|FC|≥0.5, FDR<0.05). **d,** Curated gene set analysis showing statistically significant overlapping with literature-based gene categories and the unmodified genes in *Maged1*-cKO mice (n = 448 genes changed in the control) (top) and scheme of the Polycomb repressive complexes, PRC1 and PRC2, and their crosstalk (bottom). **e,** Control antibody (IgG) or anti-Maged1 antibody-based (IP) with in vivo thalamic tissue; anti-Maged1 antibody was used for western blotting (WB). **f,** Mass spectrometry (MS) results, for every identified protein, log2 FC values are plotted on the X-axis, and -log10 p values are plotted on the Y-axis of the volcano plot. On the right, relative abundance of proteins between Maged1 IP from control mice treated with saline (n = 5) and control mice treated with cocaine (n = 7), and on the left, between control mice (n = 7) and *Maged1*-cKO, vGluT2cre, *Maged1* loxp (n = 8) mice (both treated with cocaine) is shown. **g,** Mononucleosome (Mn) IP of Maged1 and its partners with USP7 and H2A identification by WB. **h,** MnIP of monoubiquitinated H2AK119 (H2Aub) and its partners with identification of Maged1 and USP7 by WB. **i,** MnIP of USP7 and its partners with

identification of Maged1 and H2Aub and H2A by WB. Panels **h** and **i** were performed under saline treatment conditions but are also representative of MnIP under cocaine treatment conditions. **j,** Method for IP with human cells (panels **k,l,m**). **k,** western blot (WB) analysis of wild type (WT) and C1 subclones of HEK293T lysate immunoblotted with anti-MAGED1 and anti-α-TUBULIN antibodies (left), WB showing IP of endogenous MAGED1 in WT, B9 and C1 subclones of HEK293T lysates with an anti-MAGED1 antibody (right). **l,m** MS results represented by volcano plots showing the relative abundance of proteins between MAGED1 IP from WT versus (C1)-KO HEK293T cells (**l**) and in the WT versus rabbit IgG (isotype control) in SH-SY5Y cells (**m**). PRAJA1/2, CREB1, NEDD4 (E3 ubiquitin ligase) and the PRC1 deubiquitinase USP7 are the main interactors of MAGED1. For MS results (**f,l,m**), proteins above the specified threshold (-log10 p value > 1.5 and log2|FC| > 2) are highlighted with their names and a color code (a direct PRC1 component is dark blue, histones are red, known PRC1 interactors are blue and known PRC2 interactors are green). Histone H4, H2B and Try10 are highlighted because of their proximity to the thresholds and their links with the H2A-PRC1 pathway.

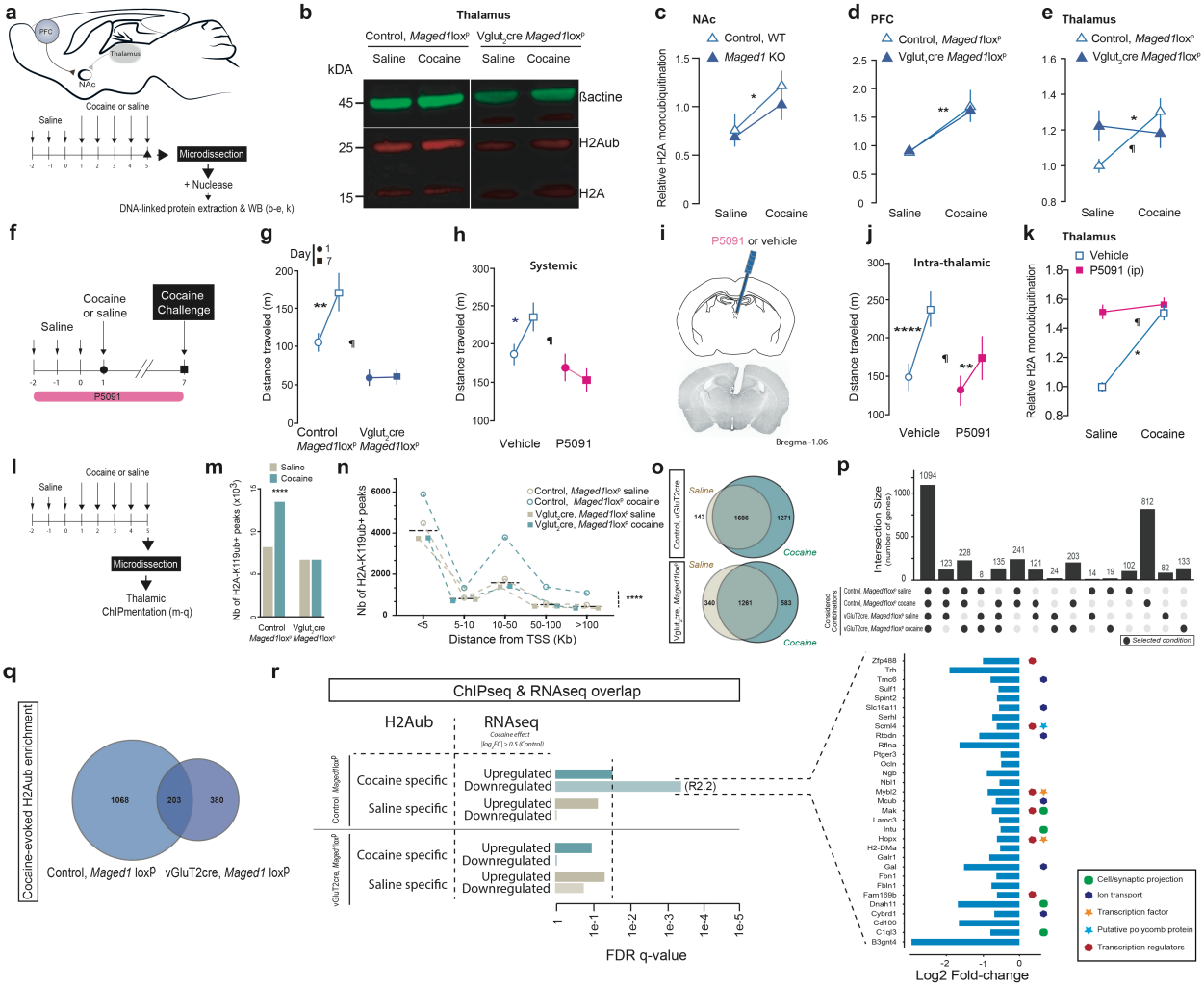

Fig. 3. Chronic cocaine administration increases H2A ubiquitination, which is impaired by *Maged1* inactivation or UPS7 inhibition. a, Method and time line of the experiment (cocaine, intraperitoneal (ip) injection, 15 mg/kg). Protein extraction was performed with nuclease to release protein linked to DNA. **b,** Representative fluorescence western blot (experiments performed with thalamus samples). **c,** Relative H2A monoubiquitination (H2AK119ub1, and 2Aub) in the NAc (**c**), in the PFC (**d**) and in the thalamus (**e**), quantified as the ratio between H2Aub and H2A signal intensities after saline or cocaine administration. In panel **c**: control saline, n = 6; control cocaine, n = 5; cKO saline, n = 5; cKO cocaine, n = 4, (two-way ANOVA, control versus *Maged1*-cKO, P = 0.3839; saline versus cocaine, *P = 0.0144; interaction factor, P = 0. 6853). In panel **d**: control saline, n = 9; control cocaine, n = 11; cKO saline, n = 10; cKO cocaine, n = 8, (two-way ANOVA, control versus *Maged1*-cKO, P = 0.8751, saline versus cocaine, **P = 0.0023; interaction factor, P = 0. 8428). In panel **e**: control saline, n = 14; control cocaine, n = 20; cKO saline, n = 13; cKO cocaine, n = 1, (two-way ANOVA, control versus *Maged1*-cKO, P = 0.0849; saline versus cocaine, P = 0.4907; interaction factor, ¶P = 0.0246, Sidak's post-test (saline versus cocaine), *P = 0.0281 for the control group). **f,** Scheme of cocaine sensitization (cocaine, ip injection, 15 mg/kg) during the USP7 inhibition experiment (P5091, ip injection, 10 mg/kg; intrathalamic injection, 0.1 mg/kg, and 400 nl). **g,** Cocaine-induced sensitization was abolished after inactivation of *Maged1* in vGluT2 neurons (*Maged1*-cKO, vGluT2cre *Maged1* loxp), control, n = 9; cKO, n = 8, (two-way

ANOVA, control versus cKO, P = 0.0007; days, P < 0.0216; interaction factor (genotype X days) ¶P = 0.0249; Sidak's post-test (D1 versus D7) **P = 0.0044). **h,** Cocaine-induced sensitization was abolished after systemic inhibition of USP7, control, n = 10; P5091, n = 8; (two-way ANOVA, vehicle versus P5091, P = 0.0224; days, P = 0.1790; interaction factor (P5091 X days) ¶P = 0.0125; Sidak's post-test (D1 versus D7) * P = 0.012). **i,** Diagram showing cannula implantation for injecting P5091 into the paraventricular thalamus (PVT) and immunohistochemical staining for Maged1. **j,** Cocaine-induced sensitization was significantly diminished after intra-PVT P5091 injections, control, n = 7; P5091, n = 8, (two-way ANOVA, vehicle versus P5091, P = 0.1987; days, P < 0.0001; interaction factor (P5091 X days) ¶P = 0.0265; Sidak's post-test (D1 versus D7), ****P < 0.0001, ** P = 0.011). **k,** Relative H2Aub in the thalamus, control saline, n = 10; control cocaine, n = 4; P5091 saline, n = 6; P5091 cocaine, n = 9 (two-way ANOVA, vehicle versus P5091, P = 0.0007; saline versus cocaine, P = 0.0171; interaction factor ¶P = 0.0414; Sidak's post-test (saline versus cocaine) *P = 0.0249 for control group). **h,j,k**, All control mice received vehicle injections. The distance traveled during cocaine-induced sensitization was recorded for 30 minutes. **l,** Time line of the experiment before microdissection of the thalamus used for ChIPmentation. **m,n,** Plots representing the number of H2Aub+ elements identified across different experimental conditions (**m**) and their distribution with respect to the nearest gene transcription start site (TSS) (**n**). The blue dashed line in panel **m** represents the median of 4 different experimental conditions. Repeated cocaine injection was associated with an increase in the total number of H2Aub+ elements compared to the saline condition; this increase was not observed in *Maged1*-cKO mice (chi-square, ****P<0.0001). **o-q**, Euler plots (**o,q**) and Upset (**p**) representing the number of H2Aub+ genes (displaying H2Aub+ elements at their promoters) across the different experimental conditions. Cocaine injection in control mice resulted in an increase in H2Aub+ genes (**o**, top). In contrast, in *Maged1*-cKO mice, the number of cocaine-specific H2Aub + genes was considerably lower than that in control mice (**o**, bottom), and only a minor fraction of cocaine-specific H2Aub+ genes were shared between control and *Maged1*-cKO mice (**q**). The Upset plot (**p**) represents the number of H2Aub+ genes in common between the different experimental groups; bars represent the number of H2Aub+ genes in common (Intersection size) between the groups indicated below each of them with a dark grey circle. **r,** Bar plot presenting the overlapping genes displaying H2Aub enrichment in saline or cocaine condition (in *Maged1* control and cKO mice) and up- or downregulated expression in *Maged1* control mice (log2|fold change, FC|≥0.5, false discovery rate (FDR)<0.05). A significant overlap was found between genes displaying H2Aub enrichment and that are downregulated after cocaine administration. These genes are highlighted on a bar plot with their associated log2FC in control mice on the right.

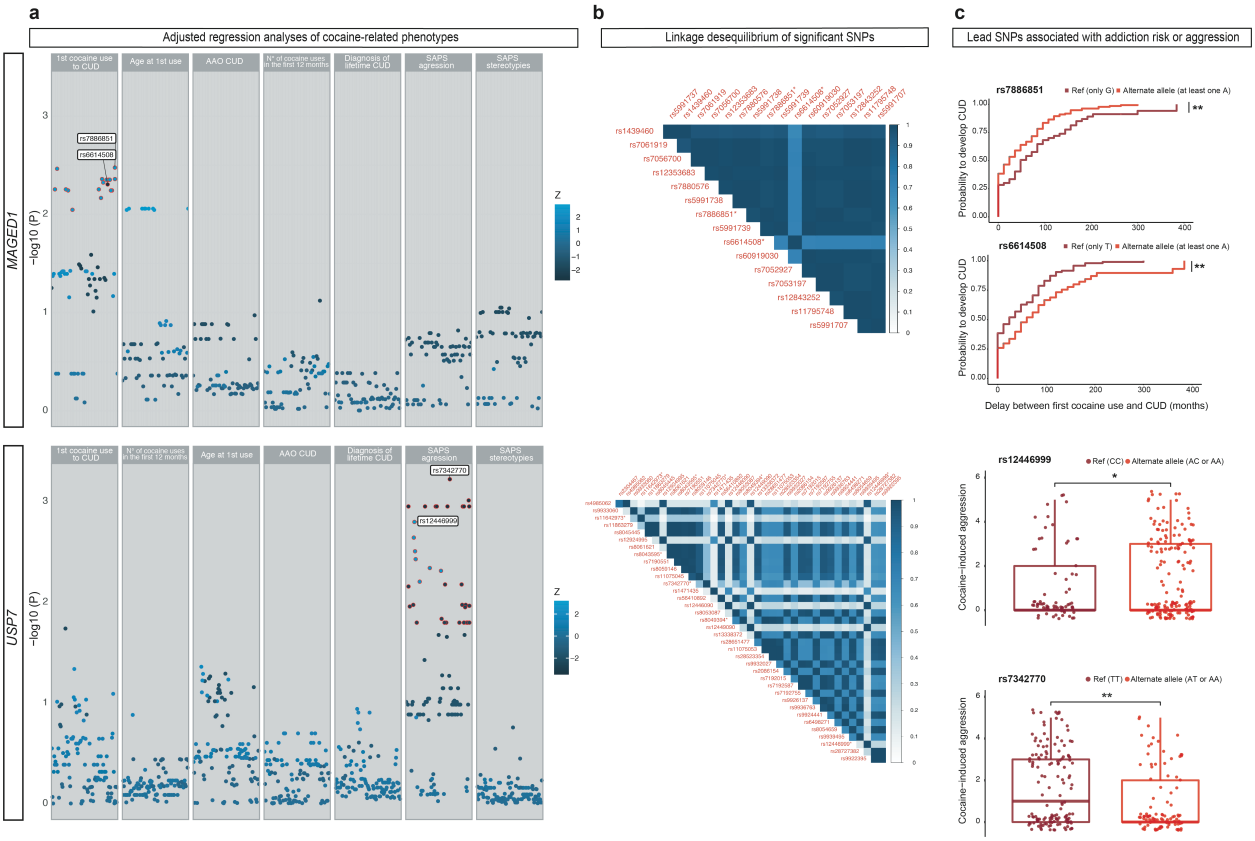

Fig. 4. Cocaine use disorder (CUD) is associated with small *MAGED1* and *USP7* polymorphisms in polydrug users. a, Scatterplots representing -log (p values) on the y-axis and effect sizes (Z) as the point color for 61 *MAGED1* single nucleotide polymorphisms (SNPs, upper panel) and 114 *USP7* SNPs (lower panel) tested using regression analyses with seven cocaine-related phenotypes (Cox regression for age at 1st cocaine use and transition from 1st cocaine use to cocaine user disorder (CUD), linear for the other), adjusted for sex and ancestry of 351 patients with strict Caucasian ancestry. Significant SNPs are circled in red, and the two most significant in each phenotype are labeled with their name. **b,** Linkage disequilibrium heatmaps showing increasing r^2 values, as indicated from white to dark blue with 16 *MAGED1* SNPs (data were not available for one SNP) significantly associated with the transition from 1st cocaine use to CUD (upper panel) and 37 *USP7* SNPs (data were not available for seven SNPs) significantly associated with cocaine-induced aggression (lower panel). SNPs are ordered according to their genomic position. Lead SNPs are marked with an asterisk*. **c,** First upper panel - Kaplan–Meier survival curves for *MAGED1* rs7886851-A, which is associated with the transition from first cocaine use to CUD. Log-rank test, **p value = 0.002. Second upper panel - Kaplan–Meier survival curves for *MAGED1* rs6614508-A, which is associated with the transition from first cocaine use to CUD. Log-rank test, **p value = 0.002. First lower panel – boxplot showing *USP7* rs12446999-A with aggression symptoms. N =237. Wilcoxon–Mann–Whitney *p value = 0.028. Second lower panel - boxplot showing *USP7* rs7342770 with aggression symptoms. N =237. Wilcoxon-Mann–Whitney **p value = 0.0014. Adenosine (A) is the alternate allele. AAO, age at onset; CUD, cocaine use disorder; SAPS, Scale for Assessment of Positive Symptoms – Cocaine-Induced Psychosis.

Methods

Animals

The Maged1-inactivated (Maged1 KO) and conditioned (Maged1 lox^p) mice used in the present study have been previously generated and described[44].
Maged1 KO do not show gross abnormalities and have normal general cognitive and sensori-motor abilities[9,44–46]. All our experiments were performed in 2- to 4-month-old mice. Our experiments were conducted on hemizygous Maged1 KO males and their wild-type littermates (Maged1 WT) generated by crossing heterozygous Maged1 KO/WT females with C57Bl6J males. Conditional knockout mice were generated by crossing heterozygous Maged1 lox^p/WT or homozygous Maged1 lox^p/lox^p females with different population selective Cre male mice. The Cre-mediated deletion of Maged1 was obtained using mice expressing Cre-recombinase under the control of the vGluT2 promoter, vGluT1 promoter and EMX1 promoter. vGluT1cre and vGluT2cre mice were obtained from the Jackson laboratory: Vglut1-IRES2-Cre-D, Stock No: 023527, Vglut2-ires-cre Stock No: 016963. EMX1cre mice[47]. All mice were backcrossed for at least eight generations in the C57Bl6J genetic background. Mice were housed by groups of 2–4 mice, in a 12 h light/dark cycle, with food and water available ad libitum. Experimental procedures were performed in accordance with the Animal Care Committee guidelines and approved by the approved by the ethical committee of the Faculty of Medicine of the Free University of Brussels.

Genotyping

We systematically check the genotype of each animal by PCR performed on DNA extracted from ear biopsies (1 2 mm). List of the primers used for the wild-type Maged1 and Maged1 KO and lox^p alleles are found in de Backer et al.[9]
For vGluT1cre allele, the primers vGluT1mutant CCC-TAG-GAA-TGC-TCG-TCA-AG, common ATG-AGC-GAG-GAG-AAG-TGT-GG, give an amplicon of 344 bp
For vGlut2cre allele, the primers vGluT2mutant (ATC-GAC-CGG-TAA-TGC-AGG-CAA) and common (CGG-TAC-CAC-CAA-ATC-TTA-CGG) give an amplicon of 600 bp
Emx1 : mutant (TCG-ATA-AGC-CAG-GGG-TTC) common (CAA-CGG-GGA-GGA-CAT-TGA) give an amplicon of 195 bp.

AAV stereotaxic injections

To inactivate Maged1 in a region-specific manner we used Maged1 lox^p mice in which we stereotaxically injected an AAV containing the Cre-recombinase in the ventral subiculum and in the PVT (from bregma, in mm): ventral subiculum, A-P -3,64, M-L +-3,15, D-V -4,72 (400 nl), PVT, A-P -1.46, M-L +-1.02, D-V -3.05, (500 nl) angle : 10°.
Behavior experiments after stereotaxic injection aiming at Maged1 inactivation were controlled by AAV-eGFP injection in littermate mice. In specified cases, control mice were Maged1 WT mice injected with AAV-eGFP-cre.
Mice were anaesthetized with avertin (2,2,2-tribromoethanol 1.25%; 2-methyl-2-butanol 0.78%, 20 ll/g, ip, Sigma-Aldrich) before bilateral injection of AAV on a stereotaxic frame (David Kopf Instruments). Injections were performed using a blunt needle connected to a 10µL syringe (Hamilton) and a microstereotaxic injection system (KD Scientific). The injection rate was fixed at 0.1 µL/min. The needle was left in place for 15 min after the injection before being slowly removed. To avoid bias, littermates were equally distributed between the two groups. After surgery, mice were allowed to recover for at least 3 weeks.
We used serotype 5 known for its neuronal tropism, and lowest abilities to migrate anterogradely[48].

Vectors used in this study were purchased from Penn Vector Core (University of Pennsylvania):

- AAV5.CMV.HI.eGFP-Cre.WPRE.SV40 (qPCR-based genome copy titer : 1.864e12)
- AAV5.CMV.PI.eGFP.WPRE.bGH (qPCR-based genome copy titer : 1.704e12)

To re-express Maged1 in a region-specific manner we used full Maged1 KO mice in which we stereotaxically injected an AAV containing Maged1 and an eGFP (linked with a P2A sequence, a 2A self-cleaving peptides inducing a ribosomal skipping during translation), either in the PFC, in the amygdala (PFC, A-P 1.9, M-L +-0.3, D-V -2.3 (600 nl) ; amygdala, A-P -1.0, M-L +-3.0, D-V -4.9 (600 nl)); or in the PVT. The vectors were generated by transient transfection (with X-tremeGENE) of HEK293 cells using three plasmids: our custom-made plasmid, cis ITR-containing CMV-eGFP-P2A-Maged1 (or just CMV-eGFP), the transplasmid encoding AAV replicase and capsid genes (pAAV.DJ/8) and the adenoviral helper plasmid followed by purification (with freezing and thawing and centrifugation) and qPCR-based genome copy titer quantification.

Cocaine-induced locomotor sensitization

Experiments were performed during the light period of the cycle. To evaluate spontaneous locomotor activity, mice were allowed to freely explore a wide open-field (OF; 40x40x40 cm), made of grey (wall) and white (flour) hard plastic, for 30 to 60 min (as specified). Locomotor activity was recorded in a low luminosity environment (18-22 lx) with a video camera placed on the ceiling above the arena and connected to an automated video tracking system (Ethovision XT 13-14, Noldus Information Technology). Classical cocaine-induced locomotor sensitization: the three-first days of experiment, mice were injected with saline and their locomotor activity was recorded, the next 5 days, mice were injected with cocaine instead of saline (20 mg/kg, intraperitoneal (i.p.)). When specified, we performed a two-injection protocol (15 mg/kg, i.p.) with 3 days of habituation and 7 days between the two cocaine injections, based on Valjent et al. work[15].

Immunohistochemistry

Mice were transcardially perfused with a 0.01 M PBS solution followed by paraformaldehyde 4% in 0.1 M phosphate buffer (pH 7.4). Brains were removed, fixed overnight in paraformaldehyde 4% and successively cryoprotected in 20 and 30% sucrose in 0.1 M phosphate buffer. 20-um-thick coronal sections were cut on a cryostat (-22°). Sections were incubated in 10% normal horse serum in 0.01 M PBS-0.1% Triton X-100 for 60 min at room temperature to block the nonspecific antibody binding. Sections were then incubated overnight at 4°C with the following required primary antibodies: chicken GFP (1:2000, Abcam, ab13970), rabbit mono-ubiquitylated histone H2A (1:1500, Cell Signaling, 8240S), goat USP7 (1/2000, Bethyl Laboratories, A303-943A), mouse mono-ubiquitylated histone H2A (1:75, Merck, 32160702), Maged1(1/300, Cosmo-Bio, BAM-74-112-EX), in 1% normal horse serum, 0.01 m PBS and 0.1% Triton X-100.

After three washes with PBS/0.1% Triton X-100, slices were incubated in PBS for 1 hour at room temperature and incubated 2 hours at room temperature with the appropriated Alexa488 (1/400, Invitrogen), Alexa594 (1/400, Invitrogen) Alexa647 (1/400, Invitrogen) secondary antibodies. Hoechst 33342 (1/5000, H3570, Invitrogen) was used (5 min and washed twice in PBS) for nuclear staining for 5 min.

The sections were next mounted on a Superfrost slide (Thermo Scientific) and dried using a brush before adding Glycergel mounting medium (Dako). Imaging was performed using a Zeiss LSM780 confocal microscope, an AxioImager Z1 (Zeiss) and a wide-field Fluorescent Microscope: Zeiss AxioZoom .v16 all controlled by the Zen Black software (Zeiss).

In situ hybridization autoradiography

Mice were deeply anesthetized using halothane and killed by dislocation and decapitation, and the whole brain was rapidly removed and frozen on dry ice. 18-um-thick coronal sections were prepared with a cryostat (CM 3050, Leica), mounted onto RNase-free glass-slides (SuperfrostPlus, Thermo Scientific) and stored at -20°C before experiment. In situ hybridization experiments were performed as previously described[9,49].

Fluorescent In situ hybridization with RNAscope

RNAscope ISH was used to detect cell type-specific expression of vGluT1 mRNA, vGluT2 mRNA, and Maged1 mRNA. Mice were deeply anesthetized, and the whole brain was removed and rapidly frozen on dry ice. Fresh-frozen tissue sections (16 µm thick) were mounted on positively charged microscopic glass slides (Fisher Scientific) and stored at −80 °C until RNAscope ISH assays were performed. Multiple target gene-specific RNAscope probes were used to observe the cellular distributions of vGluT1, vGluT2 and Maged1 in the whole brain by using a vGluT2 RNAscope probe (Mm-Slc17a6-C2-Vglut-2 probe, cat. no. 319171-C2, targeting 1986–2998 bp of the Mus musculus VgluT2 mRNA sequence, NM_080853.3), a custom-made Maged1 RNAscope probe (Mm-Maged1-O1, cat. no. 563041, targeting 901-1792 bp (targeting exon 4-12) of the Mus musculus Maged1 mRNA sequence, NM_019791.3), VgluT1 RNAscope probe (Mm-Slc17a7-C3-Vglut1 probe, cat. no. 416631-C3, targeting 464 - 1415 bp of the Mus musculus VgluT1 mRNA sequence, NM_182993.2). TSA fluorophores, cyanine3 (NEL744001KT), cyanine5 (NEL745001KT) and fluorosceine (NEL741001KT), were obtained separately at PerkinElmer. All these probes were designed and provided by Advanced Cell Diagnostics. The RNAscope mRNA assays were performed following the manufacturer's protocols. Stained slides were coverslipped with fluorescent mounting medium (ProLong Gold anti-fade reagent P36930; Life Technologies) and imaged with an AxioImager Z1 (Zeiss) microscope at 20-40x magnification with the use of Zen Black software (Zeiss). Cells expressing vGluT1 mRNA, vGluT2 mRNA, Maged1 mRNA in the cortex (PFC, motor (M1) and somato-sensorial cortex) and the PVT were counted in eight stereologically selected sections per brain under 40x magnification.

Flow cytometry and cell sorting

Animals were deeply anesthetized using halothane and killed by dislocation and decapitation. Brains were rapidly removed and immersed in ice-cold PBS where whole-thalamus were dissected[50,51]. We then performed a manual dissociation in iced-cold dissociation medium (98 mM Na_2SO_4, 30 mM K_2SO_4, 5.8 mM $MgCl_2$, 0.25nmM $CaCl_2$, 1 mM HEPES pH 7.4, 20 mM glucose, 125 uM NaOH)[52] and filtered the cells through a 40 um nylon mesh right before sorting. Cell sorting was performed using a FACS Aria III cell sorter supported by the FACSDiva software (BD Biosciences). Cells were selected by forward scatter, side scatter, Hoechst dye exclusion and red fluorescence. Cell sorting was carried out at the purity mode of sort precision using a low sheath pressure with a 70 µm nozzle and sorted cells were directly collected into ice-cold phenol and guanidine thiocyanate (QIAzol, QIAGEN) for subsequent RNA extraction. For the assessment of sort quality, cells were sorted in ice-cold PBS and re-analyzed. Total RNA extraction and DNase treatment were carried out using miRNeasy Micro Kit and RNase-free DNase set according to the manufacturer's instructions (QIAGEN).

Transcriptome analyses

Total mRNA from six sorted independent samples (3 vGluT2 Cre, loxp-stop-loxp-tdTomato, Maged1 lox^p and 3 vGluT2 Cre, loxp-stop-loxp-tdTomato, Maged1 WT) and from 15 unsorted independent samples (vGluT2 Cre::Maged1 loxp or WT, 8 with chronic cocaine, 7 with saline inejctions) was extracted using the QIAGEN miRNeasy micro kit according to the manufacturer's

recommendations. A high vGluT2 neurons proportion invited us to skip the FACS procedure for further transcriptomic experiment (Fig. 2) to avoid any transcriptomic modification relative to the sorting process, and we immediately mechanically dissociated the cells in phenol and guanidine thiocyanate (QIAzol, QIAGEN) which facilitate lysis of fatty tissues and inhibit RNases. Following RNA quality control assessed on a Bioanalyzer 2100 (Agilent technologies), the indexed cDNA libraries were prepared using the NEBNext rRNA Depletion Kit (New England BioLabs), followed by NEBNext Ultra II Directional RNA Library Prep, following manufacturer's recommendations. The multiplexed libraries (18 pM) were loaded on flow cells and sequenced with a 20 million reads depth, 2x100 bases paired-end (Novaseq 6000, Illumina).
Approximately 20 millions of paired-end reads per sample were mapped against the mouse reference genome (GRCm38.p4/mm10) using STAR 2.5.3a software[53] to generate read alignments for each sample. Annotations Mus_musculus.GRCm38.87.gtf were obtained from ftp.Ensembl.org. Read counts and transcript per million reads (TPMs) were generated using tximport R package version 1.10.0 and lengthScaledTPM method (Soneson et al., 2016) with inputs of transcript quantifications from tool salmon (Patro et al., 2017). Low expressed transcripts and genes were filtered based on analysing the data mean-variance trend. The expected decreasing trend between data mean and variance was observed when expressed transcripts were determined as which had ≥ 3 of the 40 samples with count per million reads (CPM) ≥ 1, which provided an optimal filter of low expression. A gene was expressed if any of its transcripts with the above criteria was expressed. The TMM method was used to normalise the gene and transcript read counts to log_2-CPM (Bullard et al., 2010). The principal component analysis (PCA) plot showed the RNA-seq data did not have distinct batch effects. Limma R package was used for 3D expression comparison (Ritchie et al., 2015; Law et al., 2014). To compare the expression changes between conditions of experimental design, the contrast groups were set as Ctrl.Saline-Ctrl.Cocaine, KO.Saline-KO.Cocaine, Ctrl.Saline-KO.Saline, Ctrl.Cocaine-KO.Cocaine, (Ctrl.Cocaine-Ctrl.Saline)-(KO.Cocaine-KO.Saline). For DE genes/transcripts, the log_2 fold change (L_2FC) of gene/transcript abundance were calculated based on contrast groups and significance of expression changes were determined using t-test. P-values of multiple testing were adjusted with BH to correct false discovery rate (FDR) (Benjamini and Yekutieli, 2001). A gene was significantly DE in a contrast group if it had adjusted p-value < 0.05.

Protein extraction

To understand by which means Maged1 modifies genes that are targeted by PRC, we tried to identify its protein partners in saline versus chronic cocaine conditions. On the fifth day of cocaine (or saline) injection, 5 minutes after injection, mice were deeply anesthetized using halothane and killed by dislocation and decapitation. Brains were rapidly removed and immersed in ice-cold PBS where PFC, NAc, and whole-thalamus were dissected. The tissue was mechanically dissociated using an electric rotating pestle (Ultra-Turrax, Merck) and cells were lysed in lysis buffer (before IP-MS and parallel WB: 50mM tris HCL pH 8, 150mM NaCl, 1mM EDTA, 0,5% NP-40, ; for subsequent IP-WB focusing on histones : 10mM tris HCl pH 8, 300mM sucrose, 300mM NaCl, 0,5% NP40) for 25 minutes on ice with protease inhibitor (Complete, Roche, diluted as recommended by the manufacturer) and phosphatase inhibitor (PhosSTOP, Roche, diluted as recommended by the manufacturer). For IP-MS, after a centrifugation at 4°C, 20 minutes, at 15000 g, only the supernatant was used. For histones related subsequent IP-WB, centrifugation at 4°C at 1200 g for 5 minutes separates soluble cytosolic and nuclear proteins (in the supernatant) from chromatin-linked protein (in the pellet). The pellet was treated with a nuclease (Benzonase, Merck,

cat. no. E1014) to release mononucleosomes [54] and related protein-interactions. Proteins were quantified using a Bradford assay (ThermoFisher, cat. no. 23246).

Immunoprecipitation with mouse lysates

30 ul of Dynabeads Protein A (Invitrogen, cat. no. 10001D) were washed 4 times in protein extraction lysis buffer (without protease or phosphatase inhibitors). The beads were then incubated with the IP antibody (2 ul of anti-Maged1, 7 ul of H2Aub and 7 ul of USP7 were used per sample) for 2 hours at room temperature. The beads were subsequently washed 4 times before proteins were added (500 ug of proteins was added for IP-MS, 1500 ug was used for IP-WB). Samples were incubated overnight with rotation at 4C.

For MS purpose: Beads were washed by the lysis buffer one time, and 3 times in trypsin digestion buffer (20 mM Tris.HCl pH 8.0, 2 mM CaCl2) and resuspended in 150 microliters trypsin digestion buffer.

For WB purposes: Beads were washed by the lysis buffer four times before being resuspended in 40 ul of 2x SDS buffer and thawed 15 minutes at 75°C. The beads were then removed using the magnetic rack.

Mass spectrometry from mouse lysates

Liquid chromatography–tandem mass spectrometry was done in Francis Impens Lab (VIB Center for Medical Biotechnology, UGent). After pull down, the washed beads were re-suspended in 150 µl trypsin digestion buffer and incubated for 4 hours with 1 µg trypsin (Promega) at 37 ˚C. Beads were removed, another 1 µg of trypsin was added and proteins were further digested overnight at 37 ˚C. Peptides were purified on SampliQ SPE C18 cartridges (Agilent), dried and re-dissolved in 20 µl loading solvent A (0.1% trifluoroacetic acid in water/acetonitrile (ACN) (98:2, v/v)) of which 2 µl was injected for LC-MS/MS analysis on an Ultimate 3000 RSLC nano LC (Thermo Fisher Scientific, Bremen, Germany) in-line connected to a Q Exactive mass spectrometer (Thermo Fisher Scientific). Peptides were first loaded on a trapping column made in-house, 100 µm internal diameter (I.D.) × 20 mm, 5 µm beads C18 Reprosil-HD, Dr. Maisch, Ammerbuch-Entringen, Germany) and after flushing from the trapping column the peptides were separated on a 50 cm µPAC™ column with C18-endcapped functionality (Pharmafluidics, Belgium) kept at a constant temperature of 35°C. Peptides were eluted by a stepped gradient from 98% solvent A' (0.1% formic acid in water) to 30% solvent B′ (0.1% formic acid in water/acetonitrile, 20/80 (v/v)) in 75 min up to 50% solvent B′ in 25 min at a flow rate of 300 nL/min, followed by a 5 min wash reaching 99% solvent B'. The mass spectrometer was operated in data-dependent, positive ionization mode, automatically switching between MS and MS/MS acquisition for the 5 most abundant peaks in a given MS spectrum. The source voltage was 3.0 kV, and the capillary temperature was 275°C. One MS1 scan (m/z 400−2,000, AGC target 3 ×E6 ions, maximum ion injection time 80 ms), acquired at a resolution of 70,000 (at 200 m/z), was followed by up to 5 tandem MS scans (resolution 17,500 at 200 m/z) of the most intense ions fulfilling predefined selection criteria (AGC target 50.000 ions, maximum ion injection time 80 ms, isolation window 2 Da, fixed first mass 140 m/z, spectrum data type: centroid, intensity threshold 1.3xE4, exclusion of unassigned, 1, 5-8, >8 positively charged precursors, peptide match preferred, exclude isotopes on, dynamic exclusion time 12 s). The HCD collision energy was set to 25% Normalized Collision Energy and the polydimethylcyclosiloxane background ion at 445.120025 Da was used for internal calibration (lock mass). QCloud was used to control instrument longitudinal performance during the project[55].

Analysis of the mass spectrometry data was performed in MaxQuant (version 1.6.11.0) with mainly default search settings including a FDR set at 1% on PSM, peptide and protein level.

Spectra were searched against the Mouse proteins in the Uniprot/Swiss-Prot reference database (release version of June 2019 containing 22,282 mouse protein sequences, UP000000589, downloaded from http://www.uniprot.org), supplemented with the protein A recombinant staphylococcus aureus protein sequence. The mass tolerance for precursor and fragment ions was set to 4.5 and 20 ppm, respectively, during the main search. Enzyme specificity was set as C-terminal to arginine and lysine, also allowing cleavage at proline bonds with a maximum of two missed cleavages. Variable modifications were set to oxidation of methionine residues, acetylation of protein N-termini. Matching between runs was enabled with a matching time window of 0.7 minutes and an alignment time window of 20 minutes. Only proteins with at least one unique or razor peptide were retained. Proteins were quantified by the MaxLFQ algorithm integrated in the MaxQuant software. A minimum ratio count of two unique and razor peptides was required for protein quantification. Further data analysis was performed with the Perseus software (version 1.6.2.1) after loading the proteingroups file from MaxQuant.

Western blot

Samples (input (eluted in 2x SDS buffer and thawed 15 minutes at 75°C) and IP proteins) were run in NuPAGE 4%–12% Bis-Tris Mini Protein Gel (10 wells) (ThermoFisher Scientific, NP0321BOX) at the voltage of 100V for 2.5 hours in a NuPAGE MOPS SDS Running buffer (ThermoFisher, cat. no. NP0001) and then transferred to Nitrocellulose Blotting Membrane (Bio-Rad, cat. no. 1704270, 0.2um) and placed in a Trans-Blot Turbo Transfer System (Bio-Rad) (1.3 A constant and up to 25 V) for 10 minutes. PageRuler Plus Prestained Protein Ladder, 10 to 250 kDa was systematically used (ThermoFisher, cat. no: 26619). The membrane was blocked in the intercept TBS blocking buffer (Li-Cor, cat. no. 927-60001) buffer for 1 hour at room temperature and subsequently incubated in the blocking buffer containing rabbit anti-Maged1, (1/3000, Cosmo-Bio, BAM-74-112-EX), goat anti-USP7 (1/700, Bethyl Laboratories, A303-943A), rabbit anti-H2A (1/1000, Merck, 07-146), and rabbit anti-mono-ubiquitylated H2A (1:1000, Cell Signaling, 8240S) antibodies overnight at 4C.
For qualitative analysis, this was followed by the incubation in the blocking solution containing secondary antibody anti-Rabbit or/and goat IgG antibody conjugated with HRP (GENA934, AP106P, Merck) at room temperature for 1 hour for enhanced chemiluminescence. Pierce ECL Western Blotting Substrate (Cat. #32106, ThermoFisher Scientific) was used for signal detection. For quantitative purposes, this was followed by the incubation in the blocking solution containing secondary antibody goat anti-Rabbit DyLight 680 (ThermoFisher, cat. no. 35568) or/and donkey anti-Goat DyLight 680 (ThermoFisher, cat. no. SA5-10090).

Human cell lines

Human embryonic kidney 293T (HEK293T) and human neuroblastoma cells (SH-SY5Y) were cultured in Dulbecco modified Eagle's medium containing 10% fetal bovine serum (FBS Qualified, Gibco) at 37°C in an atmosphere of 5% CO2.
The Maged1 KO in HEK cells was generated by using the RNA-guided CRISPR-Cas nuclease system[56]. For the design the Synthego and E-CRISP bioinformatic tools were used to minimize off-target effects. The input target genomic DNA sequence used was the human genome GRCh38.p13 from ENSEMBL where Maged1 gene locates on ChrX p11.22. As a result of the analysis six 20-nucleotide guide sequence were selected between exon 2 and exon 5 (gRNA1=GGCAGGGCGTTATACTACAG, gRNA2=CAAGGCGCTGTCTTCTACG, gRNA3=GCCTCAGCCTGCAAACACAG, gRNA4=AATGTTGAAGAGAACAGCAG, gRNA5=GTGGAATTGGCCAATCAGCG, gRNA6=GATGCTTAAGGACTACACAA). The 6

gRNAs were synthetized inside a U6RNA cassette between the U6 promoter and the gRNA scaffold by Twist Bioscience. Starting from the 6 cassettes nine different combinations of tandem gRNA were coupled in order to cut at the extremities of exon 2 and exon 5 (combo 1= gRNA1+4, combo 2= gRNA1+5, combo 3= gRNA1+6, combo 4= gRNA2+4, combo 5= gRNA2+5, combo 6= gRNA2+6, combo7= gRNA3+4, combo 8= gRNA3+5, combo 9= gRNA3+6) and directly transfected in HEK 293T cells with an empty Cas9 plasmid. HEK 293FT cells were maintained according to the manufacturer's recommendations. Cells were cultured in DMEM medium supplemented with 10% (vol/vol) FBS at 37 °C and 5% CO2. The cells were plated onto 6-well plates without antibiotics 16-24h before transfection and seeded at a density of 0.5 x 10^6 per well in a total volume of 2 ml. Cells were transfected with polyethylenimine (PEI) and Cas9 plasmid with the 2 gRNA mixed at equimolar ratios using 1µg of total DNA (0.7 µg of pX459-Cas9-Puro + 96ng for each gRNA), 2 µg/ml of puromycin was applied 24 hours after transfection for 48 hours. The clonal-density dilution procedure was then applied to isolate the clonal cell line and expanded for 2-3 weeks. Finally, the DNA was extracted by using Monarch® Genomic DNA Purification Kit (NEB, T3010S). For the analysis of microdeletions of the targeted exons an Out-Fwd (p1 GCACAGTCAGACCACAGTCACC) and Out-Rev primers (p2, GAGCCAACCATGGAAAGGAGGG) both of which were designed to anneal outside of the deleted region, were used to verify the successful deletion by product size. A parallel set of PCRs with In-Fwd (p1 same as above) and In-Rev primers (p3 CTGCGGTGGCCTGGTTAGTAG) was designed to screen for the presence of the WT allele. By analyzing the PCR result the combo 6 (gRNA 2+6) was chosen as the best combination to delete the region between exon 2 and 5, even if a wild type band is still present. This was expected since HEK293T cells have 3 X chromosomes containing the *MAGED1* gene, so the heterozygous clone selected (clone A1) was re-transfected with Cas9 plasmid + gRNA 2+6, two isolated sub-colonies were obtained and the abolished expression of MAGED1 at protein level was confirmed by WB (Fig. 2I). The sub-clone C1 was selected and used for subsequent experiments.

Immunoprecipitation from human cells

For MAGED1 co-IP in HEK cells a KO cell line for *MAGED1* was used as control, while in SH-SY5Y cells rabbit IgG-IP was used as isotype control respect to MAGED1 antibody (polyclonal IgG rabbit). Wild-type HEK293T, *MAGED1* KO HEK293T and SH-SY5Y cells were grown in triplicates in 150 mm dishes (20.0 x 106). Cells were lysed with lysis buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 1% Triton X-100) and supplemented with protease inhibitors (Roche, cat.no. 11836170001). Cell lysate was incubated for 30 min at 4°C, sonicated for 5 min at 0°C and then centrifuged at 15000 g for 15 minutes. The total protein concentration was determined by BCA assay and 500 µg of total proteins was incubated for 1 hour (4°C, rotary agitation) with 25 µl of Pierce™ Protein A Agarose beads (ThermoFisher, cat. no. 20333), centrifuged at 2500 g for 3 minutes at 4°C and the supernatant recovered as pre-cleared lysate. For the immune complex formation (total proteins + antibody) 500 µg of the pre-cleared lysate was incubated overnight (4°C, rotary agitation) with 2.5 µg of MAGED1 antibody (ThermoFisher, cat. no. PA5-99091) or 2.5µg of IgG rabbit (Biotechne, cat.no. NB810-56910) as isotype control. To reduce nonspecific binding and background, 25 µl of protein A Agarose beads were incubated for 1hr with 0.1% of BSA (4°C, rotary agitation) and washed two times with 500 µl of IP buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl). The pre-blocked protein A beads were added to the immune complex at 4°C for 4 hours under rotary agitation, centrifuged at 2500 g for 3 minutes at 4°C to discard the flowthrough and washed three times with 500 µl of IP buffer and one time with PBS only. The immune complex bound to the beads was eluted in 60µl of Laemmli buffer 2X (Biorad

cat.no. 1610747) incubated for 10 minutes at 95°C, centrifuged at 2500 g for 3 minutes and sent for MS analysis.

Mass spectrometry from human cells

Liquid chromatography–tandem mass spectrometry was here performed by the Proteomic Core Facility of EMBL (Heidelberg, Germany).

Sample preparation

Disulfide bridges were reduced in cysteine using dithiothreitol (56°C, 30 min, 10 mM in 50 mM HEPES, pH 8.5) and such reduced cysteines were then alkylated with 2-chloroacetamide (room temperature, in the dark, 30 min, 20 mM in 50 mM HEPES, pH 8.5). Established protocols [57,58] were followed and trypsin (Promega, sequencing grade,) was added (1/50 enzym/protein ratio) for overnight digestion at 37°C. Next day, the peptide recovery was performed by collecting supernatant on magnet and combining with second elution wash of beads with HEPES buffer. Peptides were labelled with TMT6plex[59] Isobaric Label Reagent (ThermoFisher) according the manufacturer's instructions. For the TMT6plex samples were combined and for the sample clean up an OASIS® HLB µElution Plate (Waters) was used. Offline high pH reverse phase fractionation was carried out on an Agilent 1200 Infinity high-performance liquid chromatography system, equipped with a Gemini C18 column (3 µm, 110 Å, 100 x 1.0 mm, Phenomenex)[60].

LC-MS\MS

An UltiMate 3000 RSLC nano LC system (Dionex) fitted with a trapping cartridge (µ-Precolumn C18 PepMap 100, 5µm, 300 µm i.d. x 5 mm, 100 Å) and an analytical column (nanoEase™ M/Z HSS T3 column 75 µm x 250 mm C18, 1.8 µm, 100 Å, Waters) were used. Trapping was carried out with a constant flow of trapping solution (0.05% trifluoroacetic acid in water) at 30 µL/min onto the trapping column for 6 minutes. Peptides were eluted by the analytical column with solvent A (0.1% formic acid in water, 3% DMSO) with a constant flow of 0.3 µL/min and increasing percentage of solvent B (0.1% formic acid in acetonitrile, 3% DMSO). The outlet of the analytical column was coupled directly to an Orbitrap Fusion™ Lumos™ Tribrid™ Mass Spectrometer (Thermo) using the Nanospray Flex™ ion source in positive ion mode. The peptides were loaded into the Fusion Lumos via a Pico-Tip Emitter 360 µm OD x 20 µm ID; 10 µm tip (New Objective) and a spray voltage of 2.4 kV was applied with capillary temperature of 275°C. Full mass scan (MS1) was acquired with mass range 375-1500 m/z in profile mode in the orbitrap with 60000 resolution. The maximum filling time was of 50 ms. Data dependent acquisition (DDA) was performed with the resolution of the Orbitrap set to 15000, with a fill time of 54 ms and a limitation of 1x105 ions. A normalized collision energy of 36 was applied. MS2 data was acquired in profile mode.

IsobarQuant[61] and Mascot (v2.2.07) were used to process the acquired data, which was searched against a Uniprot Homo sapiens proteome database (UP000005640) containing common contaminants and reversed sequences. The following modifications were included into the search parameters: Carbamidomethyl (C) and TMT6(K) (fixed modification), Acetyl (Protein N-term), Oxidation (M) and TMT6 (N-term) (variable modifications). For the full scan (MS1) a mass error tolerance of 10 ppm and for MS/MS (MS2) spectra of 0.02 Da was set. Other parameters chosen: Trypsin as protease with an allowance of maximum two missed cleavages: a minimum peptide length of seven amino acids; at least two unique peptides were required for a protein identification. The false discovery rate on peptide and protein level was set to 0.01. The raw output files of IsobarQuant (protein.txt - files) were processed using the R programming language (ISBN 3-900051-07-0). Only proteins that were quantified with at least two unique peptides were considered for the analysis. Raw signal-sums (signal_sum columns) were first cleaned for batch

effects using limma[62] and further normalized using variance stabilization normalization[63]. Proteins were tested for differential expression using the limma package. The replicate information was added as a factor in the design matrix given as an argument to the 'lmFit' function of limma. Also, imputed values were given a weight of 0.05 in the 'lmFit' function. A protein was annotated as a hit with a false discovery rate (fdr) smaller 5 % and a fold-change of at least 2 and as a candidate with a fdr below 20 % and a fold-change of at least 1.5.

USP7 inhibition and in vivo cannula infusion

USP7 inhibitor: P5091 (Selleck Chemicals, S7132) was prepared freshly before injection. P5091 was administered (i.p. or intrathalamic) every day during the two-injection cocaine sensitization protocol (just before saline or cocaine administration and at day 2-6) (Fig. 3).
For i.p. injection: the powder was first pre-dissolved in 5% v/v DMSO (vortex and sonication were used to aid dissolving and obtain a clear mix) then 95% v/v sesame oil (vortex was used to aid dissolving and obtain a clear mix) was added to the mix. Calculation was done to inject in i.p. a volume of 100 ul maximum and a dose of 10 mg/kg.
For cannula injections: the powder was first pre-dissolved in 54% v/v DMSO (vortex and sonication were used to aid dissolving and obtain a clear mix) then 26% v/v sesame oil (vortex and sonication were used to aid dissolving and obtain a clear mix) followed by 20% of artificial cerebrospinal fluid (NaCl 125 mM, KCl 2.5 mM, NaH2PO4 1.25 mM, NaHCO3 25 mM, MgCl2 1 mM, CaCl2 2 mM, D-glucose 25 mM) was added to the mix. Calculation was done to inject through cannula a volume of 400 nl and a concentration of 7.5 g/l.
We implanted 21-gauge guides that were cut 5.5 mm below a plastic pedestal (Plastics One Inc.) 0.2 mm above the PVT (from bregma, in mm): A-P -1.46, M-L +-1.02, D-V -2.8, angle: 10° and secured on the skull after H2O2 preparation using a dental cement (Metabond kit, Parkell Inc.). For daily injection, an internal cannula of 26-gauge projecting 0.2 mm below the guide was inserted into the guide and connected to a 10µL syringe (Hamilton). The injection rate was fixed at 0.1 µL/min. Contention was limited as the mice could freely move in their home cage during injection. No anaesthetics were administered for daily injections.

ChIPmentation

ChIPmentation experiments were performed based on the protocol of Schmidl C et al (doi: 10.1038/nmeth.3542) with minor modifications. The thalami of 2-4 WT or *Maged1* cKO mice injected either with cocaine or saline vehicle were microdissected and crosslinked in freshly prepared methanol free 1% formaldehyde solution (Thermofisher #28906) for 10 min at room temperature. The crosslinking reaction was stopped with0,125M Glycine solution and the samples were washed 3x with PBS and sored at -80C. Samples were then resuspended in cell lysis buffer (10 mM Tris pH 8.0, 10mM NaCl, 0,2% NP40, 1x EDTA free Proteinase inhibitor cocktail- PIC; Sigma # 11836170001) and nuclei were released by homogenization with a pestle B –douncer, centrifuged 8 min at 600g (4C) and resuspended in 130ul of sonication buffer (50 mM Tris pH 8.0, 10mM EDTA, 0,25% SDS, 1x PIC). After 15 min at 4C the resuspended nuclei were transferred to Covaris sonication microtubes (Covaris # 520045) and sonicated in a Covaris S220 (to fragment chromatin to an average size of 200-400bp. Sheared chromatin was centrifuged at >13.000g for 10 min to eliminate cell debris, and transferred to a new 1,5ml tubes and diluted 2,5x with ChIP dilution buffer (20mM HEPES pH 7.3, 1mM EDTA, 0,1% NP40, 150mM NaCl, 1x PIC). Anti-H2AK119ub antibody–Dynabeads protein G complexes were added to the diluted chromatin and incubated overnight at 4C with gentle rotation. For that, for each ChIP sample, 25ul of Dynabeads protein G magnetic beads (Thermofisher #) were transferred to 1,5 ml tubes and washed twice with 1 ml of blocking buffer (PBS1x, 5mgr/ml BSA, 0,5% Tween 20, 1XPIC), resuspended in

200ul of blocking buffer and conjugated with 4ugr of anti-H2AK119ub antibody (Cell Signaling, 8240S) for 3h at 4C in a rotating platform. Subsequently beads- antibody complexes were washed twice and resuspended in 15ul of blocking buffer and added to the diluted chromatin. For each wash, the beads were incubated 5 min at 4C on a rotating platform, collected for 2 min in a magnetic stand and resuspended in 1 ml of blocking buffer.

After overnight incubation chromatin –antibody- Dynabead complexes were washed twice with 1ml of RIPA/low salt buffer (10 mM Tris pH 8.0, 140mM NaCl, 0,1%, 1mM EDTA, Sodium Deoxicholate, 0,1% SDS, 0,1% Triton X100, 1XPIC), twice with 1ml of RIPA/high salt buffer (10 mM Tris pH 8.0, 500mM NaCl, 0,1%, 1mM EDTA, 0,1% Sodium Deoxicholate, 0,1% SDS, 0,1% Triton X100, 1XPIC), twice with 1ml of LiCl buffer (10 mM Tris pH 8.0, 250mM LiCl, 1mM EDTA, 0,5% Sodium Deoxicholate, 0,5% NP40, 0,1%, 1XPIC) and twice with 1ml 10mM Tris pH8.0. Washes were performed as described for the beads- antibody conjugation. After the last wash beads were resuspended in 200ul of 10mM Tris pH8.0 and transferred to a new tube, collected with the magnet and resuspended in 24ul of Tagmentation Mix (1:1 Illumina 2x Tagmentation buffer: 10mM Tris pH8.0). Resuspended chromatin- antibody complexes were incubated for 5 min at 37C and 1ul of Illumina TDE1 enzyme was added to each sample. After mixing each ChIP sample was tagmented for exactly 7 min at 37C and the reaction was immediately stopped by the addition of 1 ml of RIPA/low salt buffer. Samples were then washed once more with 1ml of RIPA/low salt buffer and once with 1ml TE buffer (10 mM Tris pH 8.0, 1mM EDTA). Finally, chromatin was eluted by resuspending beads in 100 ul of Elution buffer (10 mM Tris pH 8.0, 5mM EDTA, 0.1% SDS, 0.3M NaCl) supplemented with 50ugr of proteinase K and incubating at 65C overnight in an agitating thermoblock with heated lid. For Total Imput Chromatin (TIC) samples, 10 ul of sonicated chromatin were diluted 10x in elution buffer with 50ugr of proteinase K and decrosslinked at 65C overnight. In all cases, decrosslinked DNA was treated with 20ugr of RNAseA for 10 min at 37C, and purified with the Qiagen minielute PCR purification kit (Qiagen # 28004). Approx 10-20ng of TIC samples were then diluted in Illumina Tagmentation buffer and tagmented with the TDE1 enzyme as for ChIP samples. Finally, DNA was re-purified with the Qiagen minielute PCR purification kit and size-selected with the Pronex paramagnetic beads.

In all cases, 2 ul of each sample were used for library quantification using the KAPA Library Quantification Kit (Roche #KK4923). The rest of the DNA eluate (20ul) was used for library amplification using Nextera single-index primers as described in Schmidl C et al. Using the KAPA HiFi hotstart ready mix (Roche # KK2601). The number of cycles used to amplify each library was determined as the Ct value of the qPCR reaction +1. For library quantification and amplification the KAPA HiFi polymerase was preactivated by heating at 98C for 30 sec. In all cases, a nick repair step was performed before PCR amplification by incubating the PCR reactions at 72c for 6 min. PCR-amplified libraries were purified and size selected for fragments of 200-250bp using the Pronex paramegnetic beads (Promega # NG2001) according to manufacturer instructions. Finally, libraries were quantified using the Qubit High Sensitivity DNA quantification kit (ThermoFisher # Q32851) and analyzed by Fragment analyzer and sequenced pair paired-end sequencing 100bp in a MGISEQ-2000 platform (BGI sequencing services, Honk Kong).

H2Aub enrichment analysis

ChIPmentation sequencing data were analyzed using the public Galaxy server (https://usegalaxy.eu)[64] and personalized R scripts. First, adapter sequences were trimmed from both R1 and R2 read files using Cutadpat v3[65] and mapped against the mouse mm10 genome assembly using Bowtie2 (v 2.3.4.1)[66]. Significantly enriched regions were called using MACS2 (v

2.1.1.20160309.3) with broadpeak calling parameters[67]. The bam files from TIC samples were used as a control for MACS peak calling. Coverage files were normalized by the number of millions of valid paired reads of each sample. The R (v4.1.2) and Rstudio (1.4.1106) softwares were used for downstream analysis.

For the analysis of H2Aub+ elements, only MACS2 peaks that were in common in at least two biological replicates were selected for further analysis using the R GenomicRanges package (v1.44.0)[68]. The distance distribution of peaks from the nearest TSS was calculated using the chipenrich package[69]. The mouse GCR38 gene annotation was obtained using the R annotationHub package (v3.0.2) and used to select gene promoters (defined as the genomic region 5kb upstream and 2kb downstream of the gene TSS) overlapping with H2Aub peaks. Differentially enriched H2Aub+ genes were thus defined as those loci that displayed H2Aub+ promoters between two experimental conditions. We also identified differential H2A enriched genes by comparing H2Aub+ elements called in saline vs cocaine injected mice and attributing these peaks to genes using chipenrich package (polyenrich method). The conclusions of this analysis were overall similar to those reached based on the analysis of H2Aub promoters (data not shown). However, we preferred to base subsequent analyses on H2Aub+ promoter because of the limitations of long-range enhancer assignment to target genes.

Gene Ontology and curated gene set analysis

Gene Ontology analyses of biological processes of genes displaying either altered H2Aub binding in their promoters and/ or that were differentially expressed across the different experimental conditions were performed using the gprofiler2 (v0.2.1), ClusterProfiler (v4.0.5) and enrichplot (v1.12.3) packages[70,71]. For differentially expressed gene sets, gprofiler analysis was performed by ranking genes according to their FC value, while the unranked gprofiler test was used for gene sets displaying differential H2Aub binding in their promoters or for gene lists resulting from the crossing of RNAseq/ ChIPmentation data analysis. Finally, the GSEA software, a joint project of UC San Diego and Broad Institute[72], was used for curated gene set analysis of differentially expressed genes.

DATA deposition

All RNAseq and ChIPmentation data are deposited in GEO under accession number GSE208142. The R scripts used for this study are available in GitHub upon request.

Statistics

Statistical analyses were performed using GraphPad Prism 9 software (GraphPad Software Inc.) and SPSS statistics 27 (IBM).

Parametric data, provided by the Kolmogorov–Smirnov test, were analysed using a Student's t-test, one-way and two-way ANOVA tests (when a value was missing (because of a random reason, we performed a mixed-effects model instead of a two-way ANOVA) and Tukey's or Sidak's post hoc tests when appropriate, as indicated in figure legend. For nonparametric data, the Wilcoxon Mann-Whitney test for two-sample comparisons or the Kruskal–Wallis test for multiple comparisons were used. Differences were considered significant when $P < 0.05$. The results are expressed as mean ± standard error of mean (s.e.m.). Threshold for significance was set at $P < 0.05$. Sample sizes ("n") are given as the number of animals used for experiments.

For RNAseq statistical analysis we used the The 3D RNA-seq. To establish a significant differential list of genes, a FDR < 0.05 was chosen and a log2Fold-change threshold > 0.5 or <0.5 was applied when mentioned.

Human genotyping

Human genotyping was performed in collaboration with Romain Icick and Florence Vorspan (Université de Paris, Inserm UMR-S1144).

Sample selection and clinical assessment

Patients > 18 years seeking treatment for any SUD other than nicotine were consecutively recruited between April 2008 and July 2016 through two multicentric protocols. Both protocols and the current study were approved by the relevant Institutional Review Boards [CPP Ile-de-France IV and CEEI from the Institut de la Santé et de la Recherche Médicale (INSERM), IRB00003888 in July 2015, respectively]. All participants provided written informed consent for both the clinical and genetic assessments, and study records were continuously monitored by the hospital research administration. The research was conducted in accordance with the Helsinki Declaration as revised in 1989. Eligible participants were excluded if they had severe cognitive impairment or insufficient mastery of the French language preventing misunderstanding of the study purposes and assessments, if they had no social insurance, and if they were under compulsory treatment or mentorship.

Assessments included history of substance dependence using the E section of the Structured Clinical Interview for DSM-IV (SCID-IV)[73] and sociodemographic elements (currently married, yes vs. no; currently employed at least 20% time job, yes vs. no, ever been homeless – defined as having spent at least three months in the streets, yes vs. no). For the current study, we focused on cocaine-related variables that were modelled in the preclinical study, namely: lifetime cocaine dependence (presence/absence), age at onset (AAO) of first cocaine use and of cocaine dependence (transition from first to dependent cocaine use (hereafterm termed "TRANSCOC") - considered to be related to the rewarding properties of cocaine, which were modelled in the preclinical part - and the behavioral components of the Scale for Assessment of Positive Symptoms – Cocaine-Induced Psychosis (SAPS-CIP) considering total score, agression and stereotypies)[74].

Biological sampling and genetic analyses

DNA was extracted from whole blood using a Maxwell 16 PROMEGA® extractor (Promega France, Charbonnières-les-Bains, France). Purity assessment followed the procedures described by the Centre National de Recherche en Génomique Humaine. Participants were genotyped using the Infinium PsychArray (Illumina, San Diego, CA, USA) in two batches (2015 and 2017) by Integragen SA® (Evry, France) using the same pipeline. Genotype files were merged for the present study, keeping only bi-allelic variants common to both extractions. Then, PLINK 2.0[75] was used for quality control (QC), based on a consensus procedure for ancestry, relatedness and genetic discrepancies[76], followed by imputation on the Michigan Imputation Server v1.0.4 using the reference file hrc.r1.1.2016 through a SHAPEIT + MiniMac3 pipeline[77]. The number of post-QC variants increased from 500,000 to 7,833,448 SNPs. Chromosome X was imputed separately by Impute2 after pre-phasing by Shapeit4, following a consensus procedure[78], after exclusion of the pseudo-autosomal region. This increased the number of post-QC variants from 12,942 to 34,243.

Quality control and ancestry assessment were performed on a whole-genome level using a standard procedure[76] based on: an overall call rate >99%, missing genotypes per study and per individual <5%, Hardy-Weinberg equilibrium held at p >10-6 and relatedness inferior to the 2nd degree (in case two participants fell within such relatedness, the one with the highest overall call rate was kept for the study). From 592 genotyped patients, 522 were left for analysis, 351 of whom had strict Caucasian ancestry as compared to the distribution of variants in European from the 1000 genomes database.

The candidate gene study was performed using genomic positions (human genome version GChR37) of the genes prioritized by the preclinical study, keeping markers with minor allele frequency (MAF) >5%, namely: *MAGED1* (chromosome X, 72 markers), *USP7* (chromosome 16,

142 markers), and histone 2A genes (chromosome 1, HISTH2AC18 and HISTH2AC20, no marker). Variants showing significant associations with any of the phenotypes of interest were annotated for their biological function and plausible impact according to multiple knowledge-based repositories online, as previously suggested[79]: the Combined Annotation-Dependent Depletion (CADD) database[80], brain expression and methylation quantitative trait loci (eQTL through BRAINEAC, http://www.braineac.org/, and mQTL through the mQTLdb database, http://www.mqtldb.org), and their ability to bind DNA enzymes and/or modify DNA conformation (regulomeDB database http://www.regulomedb.org). We also considered linkage disequilibrium (LD, using the r2 measure), which indicates how minor alleles tend to be inherited altogether due to chromatin blocks. High LD (considered at r2 >0.9 in our study, based on European reference panels) indicate a similar genomic signal from possibly multiple SNPs, among which both the strongest statistical association and the highest functional impact can be used to identify the one(s) responsible for the biological signal behind the statistical association. Additionally, we identified CNVs following a pipeline[81] based on the Illumina® final report file generated by GenomeStudio v.2, which involves two programs, PennCNV[82] and QuantiSNP[83], in a combined algorithm. Both programs are based on the hidden Markov chain model but show different sensitivity and specificity. CNVs may be very rare, but represent the closest variation to gene deletion in rodent models.

Data analysis

Continuous variables were described by means (standard deviation, SD) or medians (interquartile range, IQR) depending on their distribution, and qualitative variables are described by absolute counts and frequencies. Nonparametric tests were used to identify the clinical and sociodemographic factors associated with cocaine-related variables.

SNP-based analyses were performed using PLINK linear regression adjusted for sex and the first ten principal components of ancestry, a stringent adjustment recommended to further minimize the risk of population stratification. Possibly censored variables related to time, namely: AAO of cocaine dependence and transition from 1st cocaine use to CUD, which were analyzed using Cox proportional regression models in R. All these analyses included age, biological sex, and the first ten ancestry components as covariates and cofactor. CNVs in the candidate genes -if any - were also tested for associations with cocaine-related variables based on frequency, size or type (duplication *vs.* deletion) in the subsample of Caucasian ancestry. Statistical significance was set at $p < 0.05$ after correction for multiple testing using a false discovery rate procedure (Benjamini-Hochberg, BH-FDR). Separately for chromosome X and 16, the *p*-values of all seven phenotypes and SNPs were listed together to yield the corresponding FDR. In the case of multiple statistical associations (which is expected when using imputation), we used linkage disequilibrium (LD, measured by r2) and *in silico* gene expression measures in the brain (http://www.braineac.org/, table S6) to identify lead SNPs as the most plausibly associated with the underlying biological signal. These lead SNPs (identified as having LD $r^2 < 0.9$, strong statistical association and reliable evidence for functional impact) were further characterized using graphical representations and adjustment for more sociodemographic and clinical confounders, depending on the phenotype they were associated with. Analyses were conducted with R 4.02 through R studio 1.4.1103 under Mac OS X.12.6. The current study follows the STREGA guidelines for the report of genetic studies[84]. The summary of the R session and the summary statistics (table S7) are available as Supplementary files. Of note, no QQ-plots were produced given the within-cases, candidate-gene design of the study, driven by preclinical experiments.

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