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**Author:** Helfricht, A.
**Title:** Chromatin modifiers in DNA repair and human disease
**Issue Date:** 2016-11-01
IDENTIFICATION OF EHMT1 AS A CHROMATIN FACTOR THAT NEGATIVELY REGULATES 53BP1 ACCRUAL DURING THE DNA DOUBLE-STRAND BREAK RESPONSE

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ABSTRACT

DNA double-strand breaks (DSB) are the most dangerous species of DNA damage and their repair is crucial to preserve genome stability. Upon DSB induction a highly advanced signaling cascade is activated that leads to several DNA damage-associated histone modifications and the recruitment of chromatin remodelers to make the chromatin more accessible for the accrual of DNA repair proteins. However, the immense crosstalk between these dynamic chromatin modifications is so far poorly understood. To identify novel chromatin regulators that are involved in the response to DSBs, we performed a siRNA screen monitoring the early and late response to DSBs by determining the formation of ionizing radiation (IR)-induced γH2AX and 53BP1 foci, respectively. Amongst others, we found the lysine methyltransferase EHMT1 to negatively regulate 53BP1 accrual to foci. We further show that EHMT1 itself is rapidly recruited to DSBs and promotes DSB repair via both major repair pathways, non-homologous end-joining and homologous recombination. EHMT1 targets H3K9 and other proteins for methylation and we propose that these modifications are likely important during the response to DSBs and for the preservation of genome stability. Future research will certainly demonstrate the exact role of EHMT1 in the DSB response.
INTRODUCTION

DNA double-strand breaks (DSBs) occur on a daily basis when both strands of the DNA duplex are broken. This type of lesions is highly toxic to cells and can be induced by various endogenous and exogenous sources. If not repaired accurately, DSBs can cause genome rearrangements or even cell death. Cells respond to DSBs by activating a complex signaling network that coordinates the recruitment of repair proteins, chromatin organization and cell cycle progression in order to provide time for DNA repair in a permissive chromatin environment.

Upon DSB induction, a series of chromatin modifications are initiated with the Ataxia telangiectasia mutated (ATM)-dependent phosphorylation of the histone H2A variant H2AX (termed γH2AX) being among the first. γH2AX in turn recruits Mediator of DNA damage checkpoint protein 1 (MDC1), which binds γH2AX directly through its BRCT (Lukas et al., 2011; Stucki et al., 2005). MDC1 further coordinates DNA damage-induced histone modifications by providing a binding platform for different chromatin modifying enzymes. First, MDC1 recruits the multisubunit chromatin remodeling NuA4 complex including the acetyltransferase TIP60 to sites of DSBs. Upon DSB induction, Histone protein 1 (HP1) is released from the damaged chromatin, ‘unmasking’ the abundant H3K9me3 mark to which TIP60 binds through its chromodomain. TIP60 then activates ATM and promotes the DSB response by acetylation of histone H4 at lysine (K) 16 (Kaidi and Jackson, 2013; Sun et al., 2009).

Second, the E3 ubiquitin-protein ligase RNF8 binds through its Forkhead-associated domain to phosphorylated MDC1 and initiates an ubiquitylation signaling cascade within the damaged chromatin (Huen et al., 2007; Kolas et al., 2007; Mailand et al., 2007). RNF8 ubiquitylates histone H2A, which recruits a second E3 ubiquitin-protein ligase RNF168 that amplifies the formed ubiquitin conjugates and also induces novel monoubiquitylation on H2AK15 and 16 (Doil et al., 2009; Gatti et al., 2012; Stewart et al., 2009).

Third, MDC1 attracts the histone lysine methyltransferase MMSET to which it binds in an ATM-dependent manner. MMSET, together with the H4K20 monomethyltransferase SETD8, locally increases de novo dimethylation of H4K20 (H4K20me2) at DSB sites (Oda et al., 2010; Pei et al., 2011). These events together contribute to the accumulation of further downstream signaling factors such as Tumor suppressor p53-binding protein 1 (53BP1), which directly binds as bivalent histone modification reader to ubiquitylated H2AK15 via its ubiquitylation-dependent recruitment motif (Doil et al., 2009; Fradet-Turcotte et al., 2013; Stewart et al., 2009) and to H4K20me2 via its Tudor domain (Botuyan et al., 2006; Zgheib et al., 2009). 53BP1 binding additionally requires the activity of the histone deacetylases HDAC1/2 to counteract TIP60-induced H4K16ac, since this enables local de novo H4K20me2 formation (Hsiao and Mizzen, 2013; Miller et al., 2010; Tang et al., 2013). Furthermore, the removal of the H4K20me2binders JMJD2A and L3MBTL1 is necessary to reveal this histone mark for 53BP1 binding (Acs et al., 2011; Lee et al., 2008; Mallette et al., 2012; Min et al., 2007). All these events are highly dynamic and scientists are only beginning to understand the immense crosstalk between these DNA damage-induced histone modifications.

Moreover, the structure and composition of chromatin can also be changed by ATP-dependent chromatin remodeling enzymes such as the ATPases Chromodomain-helicase-DNA-binding protein 4 (CHD4) and SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily A member 5 (SMARCA5/SNF2h). Both ATPases are recruited to DSBs and facilitate the efficient recruitment of RNF168, which leads to effective
ubiquitylation and BRCA1 accrual (Luijsterburg et al., 2012; Smeenk et al., 2013). Considering the incredible multitude of chromatin remodeling events during the DSB response, we expected novel chromatin regulating factors to participate in the signaling of DSBs and set out to identify those. To this end, we performed a high-throughput short interfering RNA (siRNA) screen for regulators of the DSB response by simultaneously monitoring the accrual of γH2AX, happening early during the DSB response, and the accumulation of downstream factor 53BP1 into ionizing radiation (IR)-induced foci, which occurs during the later steps of the response to DSBs. Genome-wide screens with a comparable read-out have been performed before (Doil et al., 2009; Paulsen et al., 2009), however so far did not lead to the identification of chromatin modifiers. Moreover, such screens often miss hits for instance due to less strong effects on the read-out. We therefore performed this dedicated high-content microscopy siRNA screen. Amongst others, we identified the histone Eu chromatric histone-lysine N-methyltransferase 1 (EHMT1), also named GLP, as a negative regulator of 53BP1 recruitment into IR-induced foci, while the formation of γH2AX was not affected in EHMT1 knockdown cells. Interestingly, we revealed that EHMT1 is rapidly recruited and promotes DSB repair via both major pathways, non-homologous end-joining (NHEJ) and homologous recombination (HR). Our results thus suggest a role for EHMT1 within the DSB response and EHMT1 is therefore an interesting and novel candidate for maintaining genome stability.

RESULTS

siRNA screen identifies novel chromatin regulators involved in the DSB response

In order to identify novel chromatin regulators involved in the response to DSBs, we carried out a siRNA screen using the Dhamacon Epigenetics SMARTpool library complemented with a custom made SMARTpool library comprising epigenetic modifiers containing a chromo-, bromo- or SANT domain, as well as SNF2-related genes (Table S1A). U2OS cells were reversely transfected with siRNA SMARTpools spotted in 96 well plates and after three days of cultivation, the cells were exposed to 2 Gy of IR. Subsequently, one hour later the cells were fixed and co-immunostained for γH2AX and 53BP1, which was followed by high-throughput confocal imaging. As a read-out the average number of γH2AX and 53BP1 foci/nucleus was determined in duplicate upon knockdown of all 227 targets. To control for siRNA transfection efficiency, we included a siRNA SMARTpool directed against the essential KIF11 gene in each plate, whose knockdown induces cell killing by generating mitotic spindle catastrophes (Weil et al., 2002). Indeed, the knockdown of KIF11 resulted in a ~ 90% reduction in cell viability (Fig. S1). Further controls per plate included siRNAs directed against Luciferase (Luc, negative control) and RNF8 (positive control). The latter is essential for 53BP1 accumulation, but not for γH2AX formation (Doil et al., 2009; Huen et al., 2007; Kolas et al., 2007; Mairland et al., 2007; Stewart et al., 2009). To provide an estimate of the variation within each 96-well plate, these control siRNAs were spotted three times on different locations on each plate. Next, the average numbers of 53BP1 foci of the negative and positive controls per location on the plate were used to calculate the Z-factor. This quality readout was performed for all plates and each time positively met the selection criteria [0.5 < Z-factor < 1] (data not shown). Hence, transfection variation within one 96-well plate did not vary strongly.

To exclude possible knockdown-induced cell growth defects a minimum of 100
Figure 1. RNAi screen identifies EHMT1 as a regulator of 53BP1 accumulation to DSBs. (A) Schematic of siRNA screen performed to identify novel chromatin regulators involved in the DDR. (B and C) Scatter plot of 124 Z-scores derived from the siRNA screen for γH2AX (B) and 53BP1 (C) foci formation using siRNA Smartpools. Luciferase and RNF8 are indicated as negative and positive control, respectively, for 53BP1 foci formation. The knockdown of targets depicted in red lead to an increase in foci formation, while the depletion of targets shown in blue was followed by a decrease in foci formation. (D and E) Results from secondary validation screen, where four individual siRNAs per target were used to validate the first 12 hits from the primary screen (as in B and C). Shown is the average number of γH2AX (D) and 53BP1 (E) foci/nucleus per siRNA per target from duplicate experiments. One and three times the standard deviation (s.d.) of the Luciferase control are indicated by dashed and continuous horizontal lines, respectively, in blue for an increase and in green for a decrease in average number of foci/nucleus. Confirmed hits are indicated in red where 3 out of 4 siRNAs caused a change in the average foci number/nucleus larger than three times the s.d. of Luciferase. Data of additional 36 hits is presented in Fig. S1.

cells per well were imaged and examined in each of two independent experiments. This criteria was not met for 106 siRNA SMARTpools and led to their exclusion from the dataset (Table S1A). Next, Z-scores were calculated from the average amount of foci per nucleus for each siRNA within one 96-well plate using the siLuc and siRNF8 controls as a reference. The average Z-score from the experimental duplicates provided a measure for the change
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in the amount of foci per nucleus upon siRNA treatment compared to control. As expected, depletion of RNF8 caused a dramatic drop in the number of 53BP1 IR-induced foci on each plate (Fig. 1C,E; Fig. S2B,D,F; Table S1A). The knockdown of 32 genes showed a significant effect on γH2AX foci formation, while the depletion of 70 genes by SMARTpools changed the average amount of 53BP1 foci per nucleus considerably, all meeting the selection criteria [Z-score < -1.5 or > 1.5 and p-value < 0.05] (Fig. 1B,C, Table S1A).

To validate the obtained hit list, we performed a deconvolution screen for which 48 targets were selected, that had been identified in other screens before, but had not yet been functionally characterized (Chou et al., 2010; Hurov et al., 2010; Matic et al., 2010; Matsuoka et al., 2007; Paulsen et al., 2009). For this deconvolution screen we employed four individual siRNAs per target within the same experimental set-up as described above (Fig. 1A,D,E; Table S1B). Here, the average number of foci per nucleus was determined directly from the obtained average foci numbers per nucleus after siRNA treatment from two individual experiments. A gene was considered a hit when at least three out of four siRNAs showed a difference in foci formation larger than three times the standard deviation (s.d.) of the siluc control. This approach provided more stringent selection criteria for the identification of hits than the thresholds applied in the initial siRNA screen, reducing the chance of obtaining false-positives. Summarizing our results, SDS3 knockdown lead to a decrease in γH2AX foci formation upon IR with all four siRNAs (Fig. S2E; Table S1B), while EHMT1, BRWD1 or MYST2 depletion caused an increase in 53BP1 foci formation after exposure to IR with three distinct siRNAs (Fig. 1D,E; Table S1B).

EHMT1 regulates 53BP1 recruitment into foci
To define whether the siRNA screen approach indeed identified novel factors involved in the DDR, we focused on the histone-lysine N-methyltransferase 1 (EHMT1, also named GLP). EHMT1 is a closely related paralog of EHMT2 (also G9a), both being mammalian lysine methyltransferases (KMTs) that mainly facilitate H3K9 mono- and dimethylation (H3K9me1/2) in euchromatin as well as the methylation of non-histone substrates. Although EHMT1 and EHMT2 can form homomeric complexes, they predominantly exist in a heteromeric complex formed via the interaction of their SET domains (Shinkai and Tachibana, 2012; Tachibana et al., 2005). Observed phenotypes were surprisingly identical in either EHMT1- or EHMT2-deficient mice with embryonic lethality around embryonic day 9.5. Moreover, both EHMT1 and EHMT2 knockout mouse ES cells show a clear reduction in global H3K9me1/2 levels (Tachibana et al., 2002; Tachibana et al., 2005). Importantly, no additive effect was measured in double knockout ES cells, indicating a cooperative rather than a redundant function of these enzymes, and thus an equally important role in the maintenance of H3K9me1/2 throughout chromatin (Tachibana et al., 2005; Tachibana et al., 2008). Interestingly, while mouse Ehmt2 has been shown to be unstable in Ehmt1-/- cells, Ehmt2-/- cells do not show a difference in Ehmt1 protein stability (Tachibana et al., 2005). And while EHMT2 has been shown to interact with a series of DNA-binding and transcriptional repressor proteins such as the DNA methylases DNMT1, DNMT3A and DNMT3B, as well as histone protein 1 (HP1) (Epsztejn-Litman et al., 2008; Shinkai and Tachibana, 2011), a subset of EHMT1 and EHMT2 was found in a multimeric complex together with other histone KMTs such as SUV39H and SETDB1, which can facilitate di- and trimethylation of H3K9 (Fritsch et al., 2010). Upon depositioning of H3K9me1/2 by the EHMT1/2 complex in euchromatin, a repressive chromatin state is induced that forms a substrate for trimethylation by SUV39H at heterochromatic regions as well as for HP1 binding (Bannister et al., 2001; Lachner et al.,
2001; Rice et al., 2003), which leads to heterochromatin formation. Furthermore, EHMT1 function has been suggested to play an important role during neuronal development since loss of function mutations in the EHMT1 gene or submicroscopic deletions of the distal long chromosome arm 9q lead to haploinsufficiency of EHMT1 causing Kleefstra syndrome (KS) (previously 9q subtelomeric deletion syndrome). KS-patients mainly display intellectual disability, childhood hypotonia and characteristic facial anomalies (Kleefstra et al., 1993; Kleefstra et al., 2012; Nillesen et al., 2011). Finally, EHMT1 as well as EHMT2 have been found to be overexpressed in various cancers (Guan et al., 2014; Huang et al., 2010). Concerning these phenotypes and the detected increase in 53BP1 foci formation upon IR exposure in our siRNA screen, we started a follow-up study addressing the role of EHMT1 during the response to DSBs. First, we used two siRNAs against EHMT1 which reduced 53BP1 focus formation in the deconvolution screen to forwardly transfect U2OS cells on 18

![Figure 2](image.png)

**Figure 2. Depletion of EHMT1 leads to an increase in 53BP1 foci formation upon ionizing radiation (IR).** (A) U2OS cells were treated with the indicated siRNAs. 48 hours later cells were either left untreated or were exposed to 2 Gy of IR. Cells were immunostained for γH2AX 1 h later. Representative images are shown of the 0,5 h time point. Quantification is depicted using the average number (nr) of γH2AX foci/nucleus obtained from 3 individual experiments where at least 75 cells were examined. Scale bar, 10 µm. (B) As in (A), but immunostained for 53BP1. (C) U2OS cells were transfected with indicated siRNAs and were stained with propidium iodide 48 h later. Cells were then subjected to flow cytometry analysis. Shown is the percentage of cells in G1 (black), S (dark gray) and G2/M phase (light gray). (D) Whole cell extracts from cells in (A) and (B) were subjected to western blot analysis.
mm coverslips and 48 h later, exposed cells to 2 Gy of IR. We determined γH2AX and 53BP1 foci formation after 0.5 and 1 h and again confirmed the increase in 53BP1 foci formation after IR, while depletion of RNF8 showed the expected decrease in 53BP1 recruitment (Fig. 2A,B) (Lukas et al., 2011). To exclude that this effect might indirectly be caused by cell cycle progression defects induced through EHMT1 depletion, we determined the percentage of U2OS cells present in G1, S and G2/M phase in control or EHMT1 knockdown cells. We did not detect a significant difference in cell cycle distribution after EHMT1 deletion, which was confirmed by western blot analysis (Fig. 2C,D). However, we did observe a partial decrease in H3K9me2 upon EHMT1 knockdown (Fig. 2D), which is in agreement with other reports (Chase and Sharma, 2013; Tachibana et al., 2005).

EHMT1 is rapidly recruited to DNA DSBs

Having identified EHMT1 as a novel factor that controls 53BP1 recruitment during the DSB response, we wondered whether EHMT1 itself is recruited to sites of DNA damage.

**Figure 3. EHMT1 is rapidly recruited to DNA double-strand breaks decorated with γH2AX.** (A) GFP-tagged mouse EHMT1 was expressed in U2OS cells which were subsequently subjected to laser micro-irradiation. After 10 min, cells were fixed and immunostained for γH2AX. EHMT1 co-localizes with γH2AX at DNA damage. (B) GFP-mEHMT1 recruitment to laser-induced DNA damage in cells from (A) was monitored in time. Representative images of EHMT1 recruitment of one cell at indicated time points are shown. (C) Immunostaining for γH2AX and EHMT1 at either no or FokI-induced DSBs, which was tagged with mCherry-LacR and re-located to a 200x integrated Lac operator genomic array in U2OS 263 ER-TA cells upon addition of Shield and 4-hydroxytamoxifen 6 h prior to fixation for translocation of FokI-fusion to the nucleus. Scale bars, 10 μm.
EHMT1 promotes DSB repair via Non-homologous end joining (NHEJ) and Homologous Recombination (HR)

In mammals, two major pathways have evolved to repair DSBs. The main pathway is called Non-homologous end-joining (NHEJ) and simply re-ligates the broken DNA ends back together throughout the whole cell-cycle, which can either happen in an error-free or error-prone fashion. The second repair pathway is termed homologous recombination (HR). The functioning of this pathway is restricted to S or G2-phase due to the requirement of a homologous or highly identical template, which is often provided by the sister chromatid (Chapman et al., 2012). To investigate whether EHMT1 contributes to DSB repair, we made use of two well-established reporter assays to monitor DSB repair efficiency in EHMT1-depleted Hek293T cells. The EJ5-GFP NHEJ reporter consists of a GFP gene, which is parted from its promoter due to an insertion of a Puromycine gene that is flanked by two I-SceI recognition sites. DSBs are induced upon transient expression of the rare-cutting I-SceI endonuclease and subsequent excision of the Puromycine gene. Repair of the broken DNA-ends via NHEJ fuses the promoter to the GFP gene and restores GFP expression, which can be measured by flow cytometry (Fig. 4A) (Bennardo et al., 2008). On the other hand, we employed the DR-GFP reporter to study HR, which consists of two differentially mutated GFP genes that are oriented as direct repeats. The upstream repeat carries an I-SceI restriction site, which inactivates gene function, whereas the downstream repeat is a 5’ and 3’ truncated version of the GFP gene. Transient expression of I-SceI leads to the induction of a DSB in the upstream GFP repeat, which can be repaired by HR using the downstream partial GFP sequence as a homologous template. This leads to the restoration of the GFP gene and consequently to GFP expression detectable by flow cytometry (Fig. 4C) (Weinstock et al., 2006). As expected, depletion of RNF8 and BRCA2 lead to a severe reduction in NHEJ and HR efficiency, respectively (Hu et al., 2014; Roy et al., 2012). Surprisingly, upon depletion of EHMT1 with three different siRNAs, the repair of DSBs via NHEJ as well as HR was considerably reduced (Fig. 4B,D). The knockdown of EHMT1 in Hek293T reporter cells (Fig. 4E) did not cause major changes in cell cycle distribution (Fig. 4F), suggesting that the observed effects were not indirect. The amount of EHMT1-depleted cells in G2/S-phase...
EHMT1 negatively regulates 53BP1 accrual during the DNA double-strand break response.

Figure 4. EHMT1 promotes the repair of DSBs via Non-homologous end joining (NHEJ) and Homologous Recombination (HR). (A) Schematic of the EJS-GFP reporter used to monitor NHEJ efficiency in Hek293T cells (see text for details). (B) EJS-GFP reporter cells were transfected with the indicated siRNAs. 48 hours later, cells were transfected with a control- or I-SceI expression vector (pCBASce). After additional 48 hours, cells were analysed for GFP expression by flow cytometry. The average of 2 experiments +/- s.e.m. is presented. (C) Schematic of the DR-GFP reporter exploited to investigate HR efficiency in Hek293T cells (see text for details). (D) DR-GFP reporter cells were treated the same way as described in (B). The average of 2 experiments +/- s.e.m. is shown. (E) Hek293T DR-GFP reporter cells were transfected with the indicated siRNAs, followed by transfection with the I-SceI expression vector 48 h later. Cells were stained with propidium iodide 24 h after that and subjected to flow cytometry analysis. The percentage of cells in G1 (black), S (dark gray) and G2/M (light gray) phase is shown. (F) Whole cell extracts from cells in (E) were subjected to western blot analysis.
might have been slightly less when compared to control cells, however it is unlikely that this small difference did cause the considerable drop in DSB repair efficiency upon EHMT1 knockdown. Therefore, these results suggest that EHMT1 promotes the effective repair of DSBs via NHEJ and HR.

**DISCUSSION**

Major cross-talk exists between histone modifications facilitating a permissive chromatin state in the vicinity of DSBs to promote their signaling and repair as part of the DSB response. In order to gain a better understanding of the spatio-temporal organisation of these chromatin modifications and to identify novel chromatin regulators with a role in the DSB response, we performed an siRNA-based high-throughput microscopy screen. With this approach we found the lysine methyltransferase (KTM) EHMT1 amongst several other hits to negatively regulate 53BP1 foci formation. Additionally, we showed that EHMT1 is rapidly recruitment to DSBs and that it promotes DSB repair via both major repair pathways, NHEJ and HR. EHMT1 thus is a novel candidate for the maintenance of genome stability.

**siRNA Screen for novel chromatin regulators**

By examining γH2AX or 53BP1 foci formation upon IR, we could monitor the early and late events during the response to DSBs. Hence, we not only gathered information about novel chromatin regulators and whether or not they have a role during the DDR, but could also define their moment of action more closely. 53BP1 thereby was a suitable read-out candidate to screen for, as several distinct chromatin modifications are required for and contribute to its accrual at DSBs.

We obtained a long list of possible chromatin regulators affecting either γH2AX and/or 53BP1 accrual to IR-induced foci from the primary screen. Among those hits, known regulators of γH2AX were found such as BAZ1B (WSTF), which is involved in the global phosphorylation of H2AX on Y142 (Xiao et al., 2009), a mark that needs to be removed upon damage induction for proper MDC1-binding to γH2AX at S139 (Cook et al., 2009; Krishnan et al., 2009). Moreover, depletion of the ATP-dependent chromatin remodeler SMARCA4 (BRG1) led to a decrease in γH2AX foci formation. This is in agreement with recent reports, which indicated that SMARCA4 is phosphorylated by ATM upon DSB induction and promotes γH2AX formation as well as DSB repair through the binding of acetylated histone H3 in γH2AX-containing nucleosomes (Kwon et al., 2015) (Table S1A). We further detected an increase in 53BP1 foci formation after IR in cells depleted from JMJD2A, which has been shown to mask H4K20me2, subsequently preventing 53BP1 binding at DSBs. For 53BP1 binding to occur, JMJD2A needs to be targeted for degradation through ubiquitylation by RNF168 upon DSB induction (Lee et al., 2008; Mallette et al., 2012). Likewise, the depletion of CBX5, better known as HP1α, was found to cause elevated levels of 53BP1 foci, which is in agreement with previously published results (Lee et al., 2013).

With a selection of 48 hits from this primary screen, a deconvolution screen was performed. We were able to confirmed 4 hits, of which we selected EHMT1 for a follow-up study. Its regulatory effect on 53BP1 accrual to DSBs was successfully validated during a second IR-induced foci experiment, where another format and different siRNA transfection method was used (Fig. 2A,B). This thus shows the ability of our screening approach to identify novel factors involved in the DSB response. However, potential hits might also
have been missed out on due to knockdown efficiency issues, since we could not control siRNA transfection efficiency per individual siRNA. Nonetheless, siKIF11 transfection led to 90% cell death and a strong decrease in 53BP1 foci formation was observed upon RNF8 depletion. Hence, the controls for siRNA transfection efficiency indicated the effectiveness of the applied transfection protocol. Additionally, the reproducibility of the generated data was confirmed by the calculation of the Z-factor for each plate, that all met the threshold criteria.

**Stringent selection during deconvolution screen**

Due to the biased target selection of epigenetic regulators and the high number of possible hits obtained from the primary screen, we stringently applied thresholds during the analysis of the deconvolution screen. Here, 3x the standard deviation of the siLuc control was used as selection criteria, which led to the confirmation of EHMT1 as a hit, but excluded its related heterodimer-partner EHMT2 from the hit list (Table 1B) (Tachibana et al., 2005). Interestingly, EHMT2 would have been a hit under the threshold of 1x the standard deviation (Fig. 1E; Table S1A,B). This less pronounced increase in 53BP1 foci formation in EHMT2-depleted cells could have been caused by insufficient siRNA transfection efficiency. On the other hand, this could also hint towards an independent function of EHMT1 in the response to DSBs. However, the H3K9 mono- and dimethylation activities were assigned to both KMTs and loss of one or the other leads do a clear decrease in global H3K9me1/2 levels (Tachibana et al., 2005; Tachibana et al., 2008). Subsequently, further verification of the role of EHMT2 in the DSB response either dependent or independent of EHMT1 is therefore required.

**EHMT1 recruitment to DSBs**

Although EHMT1 was identified as a negative regulator of 53BP1 accrual into IR-induced foci, we found that γH2AX formation remained unaffected in EHMT1-depleted cells (Fig. 1D,E; Table S1A,B). This suggests that the activity of EHMT1 is important for the more downstream steps of the DSB response. However, EHMT1 is recruited rather rapidly to DSBs (Fig. 3), which might hint towards a role in a process taking place immediately after DNA damage induction, yet one that controls 53BP1 recruitment. To further categorize EHMT1 into the numerous events of the DDR, the recruitment of other important DSB response factors such as MDC1, RNF8 or RNF168 to IR-induced foci or laser-induced DNA damage should be monitored in the absence of EHMT1. Moreover, the recruitment of several DSB response factors is highly dependent on the phosphorylation activity of ATM on serine (S) target sites. Both, EHMT1 and EHMT2, have shown to contain ATM-/ATR-target sites on Ser466 and Ser569, respectively (Matsuoka et al., 2007). It is therefore likely, that EHMT1 and EHMT2 are recruited in an ATM-dependent fashion, but this still requires experimental confirmation. Another way to rapidly recruit EHMT1 could be facilitated through the action of poly(ADP-ribose) polymerase 1 (PARP1), which attaches poly(ADP-ribose) chains onto itself and other target proteins upon DSB induction (Bekker-Jensen and Mailand, 2010; Smeenk et al., 2013). Since the recruitment of the histone tri-methylase SUV39H was found to be PARP-dependent (Ayrapetov et al., 2014), it would be interesting to investigate the contribution of PARP to EHMT1 recruitment in cells depleted from PARP or treated with an PARP inhibitor.

**Possible role of EHMT1 at DSBs**

Once EHMT1 is recruited to DSBs, it exerts a yet unknown function. However, it has been
generally described to mono- and dimethylate H3K9 within euchromatin, together with EHMT2 (Tachibana et al., 2005). Since di- and trimethylation of H3K9 was shown to locally increase upon DSB induction (Ayarpetov et al., 2014; Khurana et al., 2014), the question arises whether EHMT1/2 contribute to establish H3K9me2 at DSBs. For the binding of oligomerized 53BP1 at DSBs, RNF168-ubiquitylated H2AK15 (Fradet-Turcotte et al., 2013) and H4K20me2, established through the combined action of MMSET and SETD8, are required (Panier et al., 2012). But how could the H3K9 methyltransferase activity of EHMT1 affect 53BP1 accumulation? We hypothesize that it might perform the first two methylation steps on H3K9 upon DSB induction providing the substrate for SUV39H H3K9 trimethylation, which is an important mark for the recruitment and activation of TIP60 to DSBs (Sun et al., 2009). TIP60 binds H3K9me3 and acetylates H4K16 (Hsiao and Mizzen, 2013; Tang et al., 2013), which prevents de novo H4K20 mono- and dimethylation by SETD8 and MMSET (Huen et al., 2008; Pei et al., 2011). However, upon DSB induction the histone deacetylases HDAC1/2 are recruited and facilitate the deacetylation of H4K16 (Miller et al., 2010), paving the way for SETD8 and MMSET and promoting 53BP1 accrual. Other proteins bound to H4K20me2 such as L3MBTL1 and JMJD2A are then removed from chromatin in the vicinity to the DSB by eviction or proteasomal degradation (Acs et al., 2011; Mallette et al., 2012; Meerang et al., 2011). Hypothetically, when translating these events to the case of EHMT1-depletion, H3K9me3 would not be established for TIP60 binding, highly stimulating H4K20 methylation followed by an increase of 53BP1 assembly at DSBs, which describes the exact phenotype obtained during the siRNA screen and validation experiments (Fig. 1E,2B).

To investigate this hypothesis experimentally, one could use ChIP to examine whether a local decrease in H3K9 methylation levels at DSBs can be detected in EHMT1-depleted or -inhibitor treated cells compared to untreated cells. Additionally in a similar set-up, H4K16ac levels could be examined at DSBs looking for a decrease in H4K16ac in cells with no functional EHMT1 like it has been done for SUV39H-depleted cells showing a loss in H4K16-acetylation (Ayarpetov et al., 2014). This would indicate that EHMT1/2-mediated H3K9 methylation is required for TIP60 binding and activity. And since a portion of EHMT1 and EHMT2 was found to form a multimeric complex with SUV39H and the histone di-/trimethyltransferase SETD81 (Fritsch et al., 2010), the combined action of these histone mono-/di- and trimethylases seems plausible in order to facilitate DSB-dependent local H3K9me3 regulating 53BP1 accrual.

EHMT1 also would not be the first H3K9 dimethyltransferase implicated in the DSB response, since the PR domain zinc finger protein 2 (PRDM2), together with the repressive macrohistone variant macroH2A1, has been shown to promote the formation of condensed chromatin in a manner dependent on ATM and dimethylation of H3K9. These events ultimately facilitate DSB end resection, BRCA1 recruitment and DSB repair via HR (Khurana et al., 2014). Conversely, H3K9me3 has been suggested to only transiently increase following the rapid accumulation of the KAP1/HP1/SUV391H complex to DSBs. Once TIP60 is activated through the binding of the established H3K9me3 mark, it acetylates ATM and H4. This is immediately followed by ATM-dependent KAP1 phosphorylation, which leads to the release of the KAP1/HP1/SUV391H complex from chromatin (Ayarpetov et al., 2014). The authors reasoned that ATM activation functions as negative feedback loop through the removal of repressive SUV39H from DSBs, possibly limiting DSB repair. However, whether KAP1/HP1/ SUV39H only induces transient H3K9me3 is questionable, since SET just recently has been shown to be recruited to DSBs, where it interacts with KAP1 and induces the retention of KAP1 and HP1 at DSBs. When overexpressed, a compact chromatin state is established that
limits uncontrolled DSB signaling and inhibits DNA end resection as well as repair via HR during S/G2 phase of the cell cycle (Kalousi et al., 2015). Thus, that H3K9 methylation is strictly regulated during the DSB response to induce repressive chromatin formation either transiently or in general becomes increasingly clear. However, future research is required to define the persistence of H3K9me3 and the role of EHMT1/2 in H3K9me3 establishment at DSBs.

**Potential consequences of EHMT1 overexpression**
Where the depletion of EHMT1 leads to an increase in 53BP1 recruitment to DSBs, its overexpression might actively abrogate the response to DSBs by promoting H3K9 methylation and simultaneous HP1- or TIP60-binding that subsequently leads to H4K16-acetylation. This would result in a restrained availability of binding sites for 53BP1 at DSBs. When testing this hypothesis experimentally, we observed that transiently overexpressed Ehmt1 is rapidly recruited to DSB-containing laser tracks, where Ehmt1 remained present for at least 1 h at the site of DNA damage (Fig. 3A). Interestingly, upon a more closely investigation of those laser tracks, we could detect a decrease in the spreading of GFP-tagged Ehmt1 within the damaged chromatin compartment over time, which would support the hypothesis that Ehmt1 overexpression negatively regulates the DSB response. However, to map the consequences of EHMT1 overexpression, the track width, which is a measure reflecting the extent to which factors spread into the damaged chromatin compartment, should be determined in time after DNA damage induction by laser micro-irradiation for EHMT1 and 53BP1. If this theory holds, 53BP1 accrual would be clearly decreased and less expanded upon excessive EHMT1 expression. Additional research however needs to point out whether that is the case.

**EHMT1 also methylates non-histone targets**
EHMT1/2 can methylate itself, H3K9 and, several non-histone proteins. Methylation of the Widely-interspaced zinc finger-containing protein (WIZ) stabilizes EHMT1/EHMT2 complex formation through the binding of its sixth zinc-finger motif to the SET-domains of EHMT1/ EHMT2. WIZ thereby acts as an adaptor molecule that stabilizes EHMT2 and might drive the dominant heteromeric complex formation of EHMT1/2 in vivo (Tachibana et al., 2005; Ueda et al., 2006). Hence, WIZ might indirectly be involved in the regulation of 53BP1 levels during the DSB response via the action of the EHMT1/2-WIZ complex. Another established target of EHMT1/2 methylation is the tumor suppressor p53 which is primarily dimethylated on K737. This process in turn is regulated by the E3 ubiquitin ligase MDM2 (Chen et al., 2010; Huang et al., 2010). Upon DSB induction, MDM2 and p53 are phosphorylated by ATM leading to a de- or increase in their protein stability, respectively (Khosravi et al., 1999). However under these conditions, K737me2 levels of p53 remained the same, which indicates that this mark correlates with inactive p53. This is supported by the fact that upon EHMT1/EHMT2-depletion the levels of apoptotic cells increase due to p53 release from K373me2-mediated repression (Huang et al., 2010), something we did observe visually but did not measure in the performed cell-cycle experiments of EHMT1-depelted cells (Fig. 2C,4F). Whether and if so, how the methylation of these and possible unknown targets is related to the role of EHMT1 in regulating 53BP1 levels during the DSB response remains unclear and requires further investigation.

Additionally, EHMT1/EHMT2 targets have been identified by immunoprecipitating methylation target proteins with the GST-tagged methyl-binding domain of L3MBTL1 from
cells treated without or with an inhibitor for EHMT1/EHMT2 (UNC0638). Interestingly, amongst others the DNA repair factors DNA ligase 1 (LIG1), DNA-dependent protein kinase catalytic subunit (DNA-PKcs) and the chromatin remodeler SMARCA5 have been identified as methylation-candidate targets of EHMT1/EHMT2 (Moore et al., 2013). Future studies need to reveal the role of EHMT1/EHMT2-dependent methylation of these factors during the response to DSBs. However, there is also a possibility that EHMT1 might exert a yet unknown function, which is not connected to its described lysine methylation activity. In that case, recruitment studies of DSB response factors would provide insights on the spatio-temporal activity of EHMT1 during the DSB response and would lead to appropriate follow up studies.

**EHMT1 is involved in the efficient repair of DSBs via NHEJ and HR**
The well-established EJ5-GFP and DR-GFP reporters used to monitor DSB repair efficiency of NHEJ or HR, respectively, clearly suggest a role for EHMT1 during the repair of DSBs (Fig. 4A-D). As previously discussed, EHMT1 seems to regulate 53BP1 accrual, which has been identified as an important factor driving NHEJ by preventing resection at DSBs and the subsequent assembly of HR factors (Panier et al., 2012). However, EHMT1 depletion promotes both repair pathways in the employed reporter assays. To gain a better understanding of how EHMT1 can promote NHEJ as well as HR, a possible additive effect on DSB repair efficiency could be monitored by additional depletion of 53BP1 from siEHMT1 treated DR-GFP reporter cells. Moreover, besides the recruitment of 53BP1 and BRCA1 in siEHMT1 treated cells, the accumulation of DSB signalling factors like RNF8 and RNF168, DNA end resection factors like CtIP and RPA or DSB repair factors like XRCC4 and RAD51 could be monitored to locally laser micro-irradiated regions or IR-induced foci. This would more precisely define EHMT1’s mode of action during DSB signalling and repair. Finally, there is also a possibility that EHMT1 exerts diverse, yet unknown functions within the two different repair pathways. In any case, revealing the function of EHMT1 will instantly lead to a better understanding of how it can contribute to the repair of DSBs via both repair pathways.

**EHMT1 involved in intellectual disability syndrome and cancer**
Loss of function mutations in EHMT1 are one cause of the intellectual disability disorder Kleefstra syndrome in humans (Kleefstra et al., 1993; Kleefstra et al., 2012; Nillesen et al., 2011). This phenotype is also conserved in Drosophila where EHMT-deficiency apparently leads to defects in learning and memory (Kramer et al., 2011). Moreover, EHMT1 and EHMT2 knockout mice are embryonic lethal and global H3K9me1/2 levels are highly reduced in knockout ES cells (Tachibana et al., 2002; Tachibana et al., 2005), indicating an important role for EHMT1/2 activity in mammalian development. Furthermore, EHMT1 and EHMT2 have been reported to be overexpressed in various cancers (Guan et al., 2014; Huang et al., 2010), which suggests a role as putative oncogenes. Consequently, they may form promising anti-cancer drug targets for the development of chemical inhibitors. Encouragingly for such a purpose, EHMT2 knockdown appeared to inhibit tumor cell growth in vitro and induced extensive chromosome instability (Kondo et al., 2008). Consequently, EHMT1- and EHMT2-dependent maintenance of H3K9 methylation in euchromatin and/or methylation of other target proteins such as p53 and mentioned DNA repair factors seems highly important for the preservation of genome stability.
MATERIAL AND METHODS

Cell culture
U2OS cells, U2OS 263 cells containing a 200x integrated Lac operator genomic array and HEK293T cells were grown in DMEM (Gibco) containing 10% FCS (Bodinco BV) and 1% penicillin/streptomycin unless stated otherwise. U2OS 263 cells were a gift from Susan Janicki (Shanbhag et al., 2010) and were grown in the presence of G418 [400 µg/ml].

siRNA screen
siRNAs, from Dharmacon siGENOME® SMARTpool® Epigenetics siRNA library supplemented with 80 custom siGENOME® SMARTpool® siRNAs for the first screen and from a customized library containing sets of four single siRNA per target for the validation screen, were spotted into 96-well glass bottom plates. Additionally, the negative control Luciferase (Luc) and positive controls RNF8 and KIF11 were spotted 3 times at different locations per 96-well screening plate. Reverse siRNA transfection was performed by adding first HiPerFect transfection reagent (QIAGEN) to each well according to manufacturer instructions and secondly U2OS cells in DMEM (Gibco) containing 10% FCS (Bodinco BV). Cells were cultivated at 37°C and after 24 h, media was refreshed with DMEM containing 10% FCS and 1% penicillin/streptomycin. 48 hours later, cells were exposed to 2 Gy of ionizing radiation (IR) and fixed after 1 h at 37°C with 4% formaldehyde for 10 min. Cells were treated with 0.1% Triton X-100 in PBS for 5 min and rinsed with PBS, followed by equlibration of cells in PBS containing 5 g BSA/L and 1.5 g glycine/L prior to immunostaining for γH2AX (1:2000, #07-164, Millipore) and 53BP1 (1:1000, #NB100-304, Novus Biologicals). Detection of primary antibodies was accomplished using goat anti-mouse or goat anti-rabbit IgG coupled to Alexa 488 or 555 (Invitrogen Molecular probes). Cells were incubated with DAPI [0.1 µg/ml] and after several PBS washes kept in PBS at 4°C. High-throughput imaging was performed on a BD pathway equipped with a Nipkow spinning disc for confocal imaging and a 40x objective. Each screen was executed in duplicate and BD Image Data Explorer software version 2.3.1 was used from BD Biosciences for automated analysis to determine the average number of foci/nucleus. Z-scores were calculated from the duplicates per 96-well plate with following formula:

\[
Z-score = \frac{(x - \mu)}{\sigma}
\]

\(x -\) raw score,
\(\mu\) - mean of Luc per plate,
\(\sigma\) - std dev of Luc per plate (Doil et al., 2009).

Z-scores with a cut-off of 1.5 below or above the reference and a p-value lower than 0.05 were categorized as hit in the first screen using SMARTpool® siRNAs. During the validation screen the average amount of foci/nucleus was determined from duplicates employing the set of four single siRNAs per target of which at least three needed to cause a difference of more than 3 times the standard deviation from Luciferase to be assigned as hit.

Transfections and RNAi interference
siRNA and plasmid transfections were performed using Lipofectamine RNAiMAX (Invitrogen) or Lipofectamine 2000 (Invitrogen), respectively, according to the manufacturer’s instructions. During the follow-up study, the following siRNA sequences were used:
5’- CGUACGCAGAUACUGA -3’ (Luciferase, Dharmacon),
5’- GAGGGCCAAUGGACAAUUA -3’ (RNF8, Dharmacon),
5’- CAAACACGUGGUCAAGUA -3’ (EHMT1-1, Dharmacon),
5’- CAAGAAAGGCGCACUGAA -3’ (EHMT1-2, Dharmacon),
5’- GGAUUCUGUCUCAAGA -3’ (EHMT1-3, Dharmacon),
5’- AUAGUUGUGAACUGAGA -3’ (XRCC4, Dharmacon),
5’- GAAGAAUGCAGGUUUAAUA -3’ (BRCA2, Dharmacon).

Cells were transfected twice with siRNAs [40 nM] within 24 h and examined further 48 h after the second transfection unless stated otherwise.

Generation of DSBs
IR was delivered by a YXlon X-ray generator (YXlon International, 200 KV, 4 mA, dose rate 1.1 Gy/min). In U2OS 263 cells, DSBs were induced throughout the addition of Shield [1 µM] (Clontech) and 4-Hydroxytamoxifen [300 nM] to the growth media (Guan et al., 2014; Shanbhag et al., 2010) to induce nuclear expression of the mCherry-LacR-FokI fusion that localizes to the LacO array, where FokI induces DSBs (Shanbhag et al., 2010). Cells were subsequently fixed with 4% formaldehyde after 6 h followed by immunostaining.

Plasmid
GFP-mEHMT1 expression vectors were obtained from Yoichi Shinkai (Tachibana et al., 2005).

Laser micro-irradiation
Multiphoton laser micro-irradiation was carried out on a Leica SP5 confocal microscope equipped with an environmental chamber set to 37°C (Helfricht et al., 2013). Briefly, U2OS cells were grown on 18 mm glass coverslips and media was replaced with CO2-independent Leibovitz L15 medium, both supplemented with 10% FCS and 1% penicillin/streptomycin. Cells were placed in a Chamlide TC-A live-cell imaging chamber before imaging and were kept at 37°C. DSB-containing tracks (1.5 μm width) were generated with a Mira modelocked Ti:Sapphire laser (λ = 800 nm, pulselength = 200 fs, repetition rate = 76 MHz, output power = 80 mW). Typically, cells were micro-irradiated with 1 iteration per pixel using LAS-AF software, incubated for the indicated time-points at 37°C and subsequently fixed with 4% formaldehyde before immunostaining. For live cell imaging, confocal images were recorded before and after laser irradiation at different time intervals.

Immunofluorescent labelling
Immunofluorescent labeling of γH2AX and EHMT1 was performed as described previously (Helfricht et al., 2013). Briefly, cells were grown on glass coverslips and treated as indicated in the figure legends. Subsequently, cells were washed with PBS, fixed with 4% formaldehyde for 10 min and treated with 0.1% Triton X-100 in PBS for 5 min. Cells were rinsed with PBS and equilibrated in PBS containing BSA [5 g/l] and glycine [1.5 g/l] prior to immunostaining. Detection was done using goat anti-mouse or goat anti-rabbit IgG coupled to Alexa 488, 555 or 647 (Invitrogen Molecular probes). Samples were incubated with DAPI [0.1 µg/ml] and mounted in Polymount.

Microscopy analysis
Images of fixed samples were acquired on a Zeiss AxioImager M2 widefield fluorescence
microscope equipped with 40×, 63×, and 100× PLAN APO (1.4 NA) oil-immersion objectives (Zeiss) and an HXP 120 metal-halide lamp used for excitation, as well as ZEN software (2012). Fluorescent probes were detected using the following filters: DAPI (excitation filter: 350/50 nm, dichroic mirror: 400 nm, emission filter: 460/50 nm), GFP/Alexa 488 (excitation filter: 470/40 nm, dichroic mirror: 495 nm, emission filter: 525/50 nm), mCherry (excitation filter: 560/40 nm, dichroic mirror: 585 nm, emission filter: 630/75 nm), Alexa 555 (excitation filter: 545/25 nm, dichroic mirror: 565 nm, emission filter: 605/70 nm), Alexa 647 (excitation filter: 640/30 nm, dichroic mirror: 660 nm, emission filter: 690/50 nm). The average number of IR-induced foci per nucleus was determined using ImageJ and the IRIF analysis 3.2 Macro as previously described (Typas et al., 2015).

**Cell cycle profiling**

For cell cycle analysis cells were fixed in 70% ethanol, followed by DNA staining with 50 µg/ml propidium iodide in the presence of RNase A (0.1 mg/ml). Cell sorting was performed on a BD LSRII flow cytometer (BD Bioscience) using FACSDiva software version 5.0.3. Obtained data was quantified with Flowing software 2.5.1 (by Perttu Terho in collaboration with Turku Bioimaging).

**Western blot analysis**

Protein extracts were generated by direct lysis of cells in 2x Laemmli buffer and boiled for 10 min at 95°C. Proteins were size separated using Novex 4-12% Bis-Tris mini gels (Invitrogen) in 1x MOPS buffer (Invitrogen) and transferred to PVDF membranes, which were blocked in 4% milk for at least 30 minutes and incubated with the indicated antibodies overnight. Several wash steps before and after 1 h incubation with secondary antibodies rabbit-anti-700 and mouse-anti-800 (Sigma) were executed. Protein bands were visualized using the Odyssey infrared imaging system (Licor) according to manufacturer’s instructions.

**Antibodies**

Immunofluorescence and western blot analysis were performed using antibodies against γH2AX (1:1000-2000, #07-164, Millipore), 53BP1 (1:1000, #NB100-304, Novus Biologicals), EHMT1 (1:500, #B0422, R&D Systems), α-Tubulin (1:1000, #T6199 clone DM1A, Sigma), Histone H3K9me2 (1:500, #1220, Abcam) and Histone H3 (1:1000, #1791, Abcam).

Homologous recombination and Non-homologous end-joining repair assay

HEK293 cell lines containing a stably integrated copy of the DR-GFP or EJ5-GFP reporter, respectively, were used to measure the repair of I-SceI-induced DSBs via NHEJ or HR (Bennardo et al., 2008; Pierce and Jasin, 2014; Weinstock et al., 2006). Briefly, 48 h after siRNA transfection, cells were transfected with the I-SceI expression vector pCBASce and a mCherry expression vector (Pierce et al., 1999). 48 h later the fraction of GFP-positive cells among the mCherry-positive cells was determined by FACS on a BD LSRII flow cytometer (BD Bioscience) using FACSDiva softwared version 5.0.3. Quantifications were performed using Flowing software 2.5.1 (by Perttu Terho in collaboration with Turku Bioimaging).

**ACKNOWLEDGEMENTS**

We thank Louise von Stechow, Jordi Carreras Puigvert and Ram Sidappa for sharing experimental knowledge and assistance with spotting siRNAs.
REFERENCES


EHMT1 negatively regulates 53BP1 accrual during the DNA double-strand break response.


SUPPLEMENTAL INFORMATION

Figure S1. Knockdown efficiency confirmed with KIF11 knockdown. U2OS cells were reversely transfected with the indicated siRNAs and fixed after 3 days of cultivation. DNA was stained with DAPI to indicate cell nuclei, images were taken and the percentage of surviving cells in control and siKIF11 treated cells was estimated to 10%.

Figure S2. RNAi validation screen for novel regulators of γH2AX and 53BP1. Presented are the results from secondary validation screen, where four individual siRNAs per target were used to validate another 36 hits from primary screen (see first 12 hits in Fig. 1. D and E). Shown is the average number of γH2AX (A,C and E) and 53BP1 (B,D and F) foci/nucleus per siRNA per target from duplicate experiments. One and three times the standard deviation (s.d.) of the Luciferase control are indicated by dashed and continuous horizontal lines, respectively, in blue for an increase and in green for a decrease in average foci number/nucleus. Confirmed hits are indicated in red where at least 3 out of 4 siRNAs caused a change in average foci number/nucleus larger than three times the s.d. of Luciferase.
EHMT1 negatively regulates 53BP1 accrual during the DNA double-strand break response.

### EHMT1 DNA double-strand break response

**EHMT1_human**

```
MAAADAEAVPARGRPEPQDCCVKTTEYETPMAAEDGSAEQKAEGEANMAADGQPSQCE  59
```

**EHMT1_mouse**

```
MAAADAQAEVLQKTQEQYCCMKTELEDDQTTMAAEDGSTEQGGETPMAAEDGQPSQCE  60
```

**EHMT1_human**

```
N5DASHAAAKQTQDSARPVQDQGNTTLRIRAENUVQEDSMAAKQHVTADDQFTSTQV  119
```

**EHMT1_mouse**

```
KSGDPSHLNAPKHTQENTRASQEGTNRVSVRENGVSTETVQGHRHVTADDQFTSTQV  120
```

**EHMT1_human**

```
IGSNGYLNPALQLQPRFLRTSTLSPLLGHAAKTLPQAGKAGTRPSAFQPTQAPAPQRTL  179
```

**EHMT1_mouse**

```
IGSNGYFLNPALQLQPRFLRTSTLSPLLGHAAKTLPQGCACTLSALPQTPQAPAPQRTV  180
```

**EHMT1_human**

```
GEGSADTEDRKLAPGAGAVKVRHARKTMPSFKSVGVLHAASKDQPKEIVREARDKHPKKE  239
```

**EHMT1_mouse**

```
GEGSADTEDRKPASGAGAVKVRHARKTMPSFKSVLHLAAASKDHREVQDHKEEPKEDIR  237
```

**EHMT1_human**

```
NISDFGRQQLPFFSLHSLPQNCYMMATKQTCLPFLVAAYSAVRKRRMGSTYSVL  299
```

**EHMT1_mouse**

```
NISDCGRQQLPFFSLHSLPQNCYMMATKQTCLPFLVAAAYSAVRKRRMGSTYSVL  297
```

**EHMT1_human**

```
FJKKTKVLQRTVMFKSISHTVSTQGKEDLGASLSHLVQESMSDESEDSEELED  359
```

**EHMT1_mouse**

```
FJKKTKVLQRTVMFKSISHTVSTQGKEDLGASLSHLVQESMSDESEDSEELED  357
```

**EHMT1_human**

```
DGGHAEAAAPFEDTSRKSSEMSEADRAQKGMDEEEESEESVGTGEEBBGQDSDSSE  419
```

**EHMT1_mouse**

```
DGGHAEAAAPFEDTSRKSSEMSEADRAQKGMDEEEESEESVGTGEEBBBQDSDSSE  417
```

**EHMT1_human**

```
SIKKFFILKRGKGRKTSFWIPKARKRRRSRQKPSLALGSEYKSAGAEQTAPDGSSTGY  479
```

**EHMT1_mouse**

```
SIIKKFFILKRGKGRKTSFWIPKARKRRRSRQKPSLALGSEYKSAGAEQTAPDGSSTGY  477
```

**EHMT1_human**

```
MEVSLSDLRLVRKGILSSQ--EGLANPQVLGETDLQEVLCPSCRMETOPKRSREITL  537
```

**EHMT1_mouse**

```
MEVSLSDLRLVRKGILSSQ--EGLANPQVLGETDLQEVLCPSCRMETOPKRSREITL  537
```

**EHMT1_human**

```
NQCMATESVDHELGRCTSNVYKELMFNSKAPLLVLCEDHGRMVKQCCFCGESGYCTA  597
```

**EHMT1_mouse**

```
NQCMATESVDHELGRCTSNVYKELMFNSKAPLLVLCEDHGRMVKQCCFCGESGYCTA  597
```

**EHMT1_human**

```
QDHKEPKEDINR 237
```

**EHMT1_mouse**

```
QDHKEPKEDINR 237
```

**EHMT1_human**

```
ETLESALIDSEKPKKLRFYFHPQLYYSARQQELQKVLLMLVGGIDPKNFMEHQAQKRSPL  777
```

**EHMT1_mouse**

```
ETLESALIDSEKPKKLRFYFHPQLYYSARQQELQKVLLMLVGGIDPKNFMEHQAQKRSPL  775
```

**EHMT1_human**

```
HAEEAGHVDICMHLQAGANIDCSEDQDTRPLMEAANNHLEAVYKILKAGALVDPKOA  837
```

**EHMT1_mouse**

```
HAEEAGHVDICMHLQAGANIDCSEDQDTRPLMEAANNHLEAVYKILKAGALVDPKOA  835
```

**EHMT1_human**

```
EGSTCHLHAAKGYEVYVQLLSNGQMDVNCQDDGGTFPMIYAW抓捕EYVHDLVKLKLSSGK  897
```

**EHMT1_mouse**

```
EGSTCHLHAAKGYEVYVQLLSNGQMDVNCQDDGGTFPMIYAW抓捕EYVHDLVKLKLSSGK  895
```

**EHMT1_human**

```
DINRDNEEINCLHWAFAFSGCCVIAEILLAAKCDLHAVNIFHGSPLHLAAARENRYDCVL  957
```

**EHMT1_mouse**

```
DINRDNEEINCLHWAFAFSGCCVIAEILLAAKCDLHAVNIFHGSPLHLAAARENRYDCVL  955
```

**EHMT1_human**

```
FLSRDSVTLKNKEGETPQLCASLNSQVWSALQMSKALQDSAPDRSPFVERVSRDIARG  1017
```

**EHMT1_mouse**

```
FLSRDSVTLKNKEGETPQLCASLNSQVWSALQMSKALQDSAPDRSPFVERVSRDIARG  1015
```
**Figure S3.** EHMT1 protein sequence is quiet conserved between mouse and human.

Entries Q9H9B1 for human EHMT1 and Q5DW34 for mouse Ehmt1 were aligned using the Uniprot alignment tool available at www.uniprot.org. The conserved amino acids are indicated by a green asterisk.

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<th>Entries</th>
<th>Human Sequence</th>
<th>Mouse Sequence</th>
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<td>Q5DW34</td>
</tr>
<tr>
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<td><strong>LSMCWYDKDGRLLLPEFNMAEPP</strong>LIFECNHACSCWCRNCRNVRVQNLQRLQRRLQLYRTQDMG**</td>
<td>Q9H9B1</td>
</tr>
<tr>
<td>Q9H9B1</td>
<td><strong>WGVRSLQDIFPGTFVCEYV</strong>GELISDSEADVREEDSYLVQLDNKDGVEYCIDARFYGNVSR**</td>
<td>Q5DW34</td>
</tr>
<tr>
<td>Q5DW34</td>
<td><strong>FINHHCEPVNLVV</strong>RVFMHQLRFFPRIAFFSTRLIEAGEQGLGFDYGERFWDIKGLKL**</td>
<td>Q9H9B1</td>
</tr>
<tr>
<td>Q9H9B1</td>
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</tr>
<tr>
<td>Q5DW34</td>
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<td>Q9H9B1</td>
</tr>
</tbody>
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Table S1. List of siRNA screen gene targets and results. (A) List of 227 gene targets and positive (LUC) and negative (siRNAs) controls. Indicated are gene symbols, Gene ID, Accession numbers and the obtained -scores calculated from the average fold change and the standard deviation of the control Luciferase.

<table>
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<tr>
<th>Gene Symbol</th>
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<th>Accession Numbers</th>
<th>-Score</th>
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<td>NM_003655</td>
<td>-2.329</td>
<td>Increase in H2AX levels.</td>
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<tr>
<td>53BP1</td>
<td>NM_003883</td>
<td>NM_006044</td>
<td>1.320</td>
<td>Decrease in 53BP1 levels.</td>
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<tr>
<td>JMJD1A</td>
<td>NM_014952</td>
<td>NM_020920</td>
<td>0.099</td>
<td>No significant change.</td>
</tr>
<tr>
<td>JMJD2A</td>
<td>NM_032408</td>
<td>NM_01527</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(B) List of 48 gene targets and the average fold change of H2AX or 53BP1. Entry values are calculated as the average fold change of the four single siRNAs when compared to the deconvolution screen. Hit results are indicated in red when minimal 3 out of 4 siRNAs caused an increase (blue) or decrease (green) larger than three times the standard deviation of the control Luciferase.