Proteomic analysis of H3K36me3 and PSIP1/p75 (LEDGF) complexes reveal their wider role in DNA repair [version 2; referees: 1 approved, 1 approved with reservations]

Madapura M. Pradeepa 1,2, Gillian C.A. Taylor2, Alex von Kriegsheim2

1School of Biological Sciences, University of Essex, Colchester, CO4 3SQ, UK
2MRC Human Genetics Unit, MRC Institute of Genetics and Molecular Medicine, University of Edinburgh, Edinburgh, EH4 2XU, UK

Abstract

Background: Trimethylation at histone H3 at lysine 36 (H3K36me3) is associated with expressed gene bodies and recruit proteins implicated in transcription, splicing and DNA repair. PC4 and SF2 interacting protein (PSIP1/LEDGF) is a transcriptional coactivator, possesses a H3K36me3 reader PWWP domain. Alternatively spliced isoforms of PSIP1 binds to H3K36me3 and suggested to function as adaptor proteins to recruit transcriptional modulators, splicing factors and proteins that promote homology directed repair (HDR), to H3K36me3 chromatin.

Methods: We performed chromatin immunoprecipitation of H3K36me3 followed by quantitative mass spectrometry to identify proteins associated with H3K36 trimethylated chromatin in mouse embryonic stem cells (mESCs). Furthermore, we performed stable isotope labelling with amino acids in cell culture (SILAC) for a longer isoform of PSIP1 (p75) and MOF/KAT8 in mESCs and mouse embryonic fibroblasts (MEFS).

Results: Proteomic analysis of H3K36me3 chromatin show association of proteins involved in transcriptional elongation, RNA processing and DNA repair with H3K36me3 chromatin. Furthermore, we show DNA repair proteins like PARP1, gamma H2A.X, XRCC1, DNA ligase 3, SPT16, Topoisomerases and BAZ1B are predominant interacting partners of PSIP1/p75. We validated the association of PSIP1/p75 with gamma H2A.X, an early marker of DNA damage and also demonstrated accumulation of damaged DNA in PSIP1 knockout MEFs.

Conclusions: In contrast to the previously demonstrated role of H3K36me3 and PSIP1/p75 in promoting HDR in mammals, our data supports the wider role of H3K36me3 and PSIP1 in maintaining the genome integrity by recruiting several DNA repair proteins to transcribed gene bodies.
Corresponding author: Madapura M. Pradeepa (pmadap@essex.ac.uk)

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Both referees make very important suggestions to improve the manuscript, which we have addressed in our comments in response to the reviewers. We have made the following changes to this revised manuscript:

We have amended the name of the dataset files at the OSF site and have also included a new dataset which was missing in the original submission. We have accordingly changed the file names in the manuscript text.

We have discussed the possibility of PSIP1 binding to H3K36me2 at the promoter elements in the text, and cited new literature. We have also added a new reference to appropriate tables and figures for clarity.

As suggested by the reviewers, we agree that further experiments would increase the credibility of the conclusions drawn in this manuscript. However, we believe that the proteomic datasets published in this manuscript provide novel insights into the understanding of how histone modifications and chromatin proteins like PSIP1 might play major role in DNA repair pathways.

Introduction
PC4 and SF2 interacting protein (PSIP1) encode two splice variants – a shorter isoform called p52 and a longer isoform called p75. The N-terminal PWWP and adenine-thymine (AT) hook-like DNA binding domains are common to both isoforms (Figure 1A). The PWWP domain binds specifically to Histone H3 trimethylated at lysine 36 (H3K36me3); both PSIP1 isoforms and H3K36me3 co-occur at expressed gene bodies (Pradeepa et al., 2012; Pradeepa et al., 2014; van Nuland et al., 2013a). The C-terminal integrase binding domain is unique to p75, and is shown to interact with HIV integrase (Cherepanov et al., 2003; Ciuffi et al., 2005); the same domain binds to the mixed lineage leukaemia proteins (MLL1, MLL2 and Menin) (Pradeepa et al., 2014; van Nuland et al., 2013b; Yokoyama & Cleary, 2008). Psip1/p75 is a multifunctional chromatin protein that has been implicated in regulation of homeotic genes, cell survival, cancers and autoimmune diseases, and has also been shown to promote homology derived repair (HDR) by recruiting C-terminal binding protein interacting protein (CtIP) to double stranded breaks (DSB) (Basu et al., 2012; Ciuffi et al., 2005; Daniels et al., 2005; Desforges & Ciuffi, 2010; Pradeepa et al., 2014; Sutherland et al., 2006).

We have previously demonstrated the role of the p52 isoform in modulating alternative splicing by recruiting splicing factors to H3K36 trimethylated chromatin through its N terminal PWWP domain (Pradeepa et al., 2012). Work from other groups has shown that the PSIP1/p75 isoform interacts with: JPO2 (also known as R1, RAM2 and CDCA7L) (Bartholomeeusen et al., 2007), pogo transposable element with zinc finger domain (PogZ) (Bartholomeeusen et al., 2009), the activator of the S-phase kinase complex (CDC7-ASK) (Hughes et al., 2010), methyl CpG binding protein 2 (MeCP2) (Bartholomeeusen et al., 2009), and CtIP (Daugaard et al., 2012) and interacts-with-Spt6 (IWS1) (Tesina et al., 2015), in different genomic location or cellular contexts.

Figure 1. PSIP1/p75 domains and its functional interactors. (A) Cartoon of PSIP1 p52 and p75 isoforms showing methylated histone binding PWWP domain, DNA binding AT hook domain at the N-terminus and also the integrase binding domain (IBD) domain at the C-terminus of p75 that interacts with MLL1, Menin and JPO2. (B) Illustration showing crosslinked chromatin immunoprecipitation (xChIP) followed by mass spectrometry (MS) to identify proteins associated with histone modifications.
Several pieces of evidence support the role of H3K36me3 in the DNA damage response. In mammals, SETD2 mediated H3K36me3 is shown to recruit PSIP1/p75 to express genes, which upon DNA damage recruits the repair factors CIP and RAD51 to facilitate HDR (Aymard et al., 2014; Daugaard et al., 2012; Pfister et al., 2014). All this evidence supports a model in which PSIP1 is constitutively anchored to H3K36me3 chromatin at expressed gene bodies through its PWWP domain. Upon DNA damage, chromatin bound PSIP1/p75 recruits CIP and RAD51, which promotes HR repair by efficient resection, and protects these vulnerable regions of the genome from DNA damage. In the absence of SETD2 or H3K36me3, the chromatin association of PSIP1 is reduced, and DNA damage induced recruitment of repair proteins is impaired, leading to reduced resection and HDR. Another H3K36me3 reader – MRG15 – has been shown to recruit the partner and localiser of BRCA2 (PALB2) complex to undamaged chromatin (Bleuyard et al., 2017). Constitutive association of PALB2 to H3K36me3 chromatin at expressed gene bodies facilitates immediate availability of PALB2 upon DNA damage during active transcription and DNA replication. H3K36me3 is also shown to promote DNA mismatch repair by recruiting the mismatch recognition protein MutSβx through its PWWP domain (Li et al., 2013). In contrast to mammalian studies, in budding yeast, H3K36me3 promotes non homologous end joining (NHEJ) and inhibits HDR (reviewed in (Jha et al., 2014). Similar to yeast studies, H3K36me2, catalysed by SETMAR/Metnase also promotes NHEJ in human cells (Fnu et al., 2011). This suggests a complex role of H3K36 methylation in DNA repair choice and genome stability. Intriguingly, the PWWP domain of PSIP1/p75 is also shown to bind H3K36me2 in vitro and in vivo (Zhu et al., 2016), similarly, PSIP1/p75 is also detected near transcriptional start sites of Hox genes suggesting the possibility of binding of PSIP1/p75 to H3K36me2 at TSS and to H3K36me3 at the gene bodies (Pradeepa et al., 2014) SETD2, the only enzyme responsible for H3K36 trimethylation is mutated in cancers and is proposed to function as tumor suppressor (Li et al., 2016; Zhu et al., 2014). The methylated H3K36 reader – PSIP1 – is implicated in a variety of cancers (Basu et al., 2016; Daniels et al., 2005; French et al., 2016; Yokoyama & Cleary, 2008) and also implicated in resistance to chemotherapy induced cell death in prostate cancer (Mediavilla-Varela et al., 2009). This suggests that H3K36me3 and PSIP1 play an important role in DNA repair and that dysregulation of this pathway could cause human cancer.

We hypothesised that PSIP1 isoforms function as an adaptor protein to recruit various proteins involved in transcription, splicing and DNA repair to H3K36me3 sites via PWWP domain. We have previously shown that the p52 isoform binds to H3K36me3 and recruits splicing factors to exons. Here, we performed formaldehyde crosslinked chromatin immunoprecipitation (ChIP) followed by label-free quantitative mass spectrometry (xChIP-qMS) to identify proteins associated with H3K36me3 chromatin. We find several proteins implicated in transcriptional elongation, RNA processing and DNA repair associated with H3K36me3 in vivo. Furthermore, SILAC proteomics analysis of endogenous PSIP1/p75 complex shows that several DNA repair proteins interact with p75. We also detect a higher level of DNA damage in mouse embryonic fibroblasts (MEFs) derived from a Psip1 knockout mouse (Psip1<sup>−/−</sup>). We propose a wider role of H3K36me3 and PSIP1/p75 in maintaining genome integrity at the site of transcription.

**Results**

**xChIP-qMS identifies H3K36me3 associated proteins in vivo**

H3K36me3 is associated with actively transcribed gene bodies, preferentially exons of the expressed genes, suggesting its role in splicing and transcriptional elongation. H3K36me3 peptide pull-down followed by SILAC-MS identified that the PWWP domain has a putative H3K36me3 reader domain (Vermeulen et al., 2010). In order to capture the proteins that transiently and stably associate with H3K36me3, formalddehyde cross linked mouse embryonic stem cells (mESCs) were treated with hypotonic buffer to prepare nuclei and sonicated to obtain soluble chromatin, and were immuno-precipitated using H3K36me3 and pan H3 antibodies bound to magnetic beads (Figure 1B). This is a useful method to study the proteins that are associated with particular histone modifications in vivo. However, since the chromatin is crosslinked and fragmented by sonication to get 100–500bp DNA fragments, many proteins that do not directly bind to H3K36me3 but are bound directly to DNA or to other histone modifications, are also likely to be enriched. Hence, we performed ChIP with the same chromatin using H3 antibodies as control.

Label-free quantitative mass spectrometry analysis of two replicates ChIPs resulted in identification of several proteins, implicated in replication, transcription, RNA processing and DNA repair, which were quantitatively enriched in ChIP with anti-H3K36me3 normalised to anti-pan H3 antibodies (Figure 2 and Dataset 1). Association of RNA processing proteins with H3K36me3 is consistent with the role of PSIP1/p52 in binding to H3K36me3 and recruiting several RNA processing proteins to exons of expressed genes to modulate alternative splicing (Pradeepa et al., 2012). Similarly, MRG15 binds to H3K36me3 and recruits PTBP to modulate alternative splicing (Luco et al., 2010) and another PWWP domain protein B569 binds to H3K36me3 and recruits splicing factors to influence splicing pattern (Guo et al., 2014). Since H3K36me3 is located at expressed gene bodies, it is not surprising that we find several proteins implicated in transcription and transcriptional elongation (Dataset 1). Interestingly, we found 26 proteins that are implicated in DNA repair and are associated with H3K36me3 in vivo, with >1.5 ratio of H3K36me3 ChIP/ H3 ChIP (Figure 2 and Dataset 1). These include known interactors of H3K36me3 – PSIP1, SPT16, SSRP1 and MSH6 (Carvalho et al., 2013; Li et al., 2013; Pradeepa et al., 2012; Pradeepa et al., 2014). We detected many peptides mapping to the N-terminal domain that is common to both PSIP isoforms, however, we also found peptides mapping to the p75 specific C-terminal domain, suggesting the enrichment of both isoforms of PSIP1 in H3K36me3 chromatin.

**SILAC proteomics of PSIP1/p75 complex**

We and others have shown that PSIP1 is a H3K36me3 reader protein, binds to H3K36me3 both in vitro and in vivo and localises to expressed gene bodies, similar to H3K36me3. The p52 isoform of PSIP1 binds to H3K36me3 and recruits splicing factors to exons of expressed genes (Pradeepa et al., 2012). Similarly, the p75 isoform binds to H3K36me3 and recruits MLL proteins to expressed HOX genes (Pradeepa et al., 2014;
Figure 2. H3K36me3 associated proteins that are implicated in DNA repair. The label-free mass spectrometry quantitative output values assigned to each protein following immunoprecipitation from the mouse embryonic stem cells. The list of proteins associated with DNA repair function with the H3K36me3 vs H3 ratio of more than 1.5 (y-axis) are plotted (full list of proteins in Dataset 1). Horizontal scatter was added only to aid visibility of each protein and has no data correlate. The position of PSIP1 protein is highlighted in red.

van Nuland et al., 2013a; van Nuland et al., 2013b). p75 is also shown to promote HDR by recruiting CtIP and RAD51 to DSBs in a H3K36me3 dependent manner (Aymard et al., 2014; Daugaard et al., 2012; Pfister et al., 2014). In order to comprehensively identify both stable and transient interacting partners of p75, we performed immunoprecipitation (IP) of endogenous p75 protein in cells grown in SILAC media using previously characterised antibodies that specifically pull-down the p75 isoform of PSIP1 (Pradeepa et al., 2012; Pradeepa et al., 2014). IP with anti-MOF served as an irrelevant control and rabbit immunoglobulin (IgG) served as a negative control. mESCs and MEFs were first labelled for two weeks in light, medium and heavy SILAC cell culture media, followed by IP with negative control (rabbit IgG) anti-MOF (non-relevant chromatin protein) and anti-PSIP1/p75 antibodies, respectively (Figure 3A). The protein enrichment ratio was then calculated to identify proteins that are quantitatively enriched with p75 and MOF compared to negative control.

PSIP1/p75 interacts with DNA repair proteins
IP with PSIP1/p75 and MOF antibodies showed PSIP1 and MOF proteins with the highest SILAC ratio over negative control in respective IPs in both MEFs and mESCs (Table 1; Dataset 2 and Dataset 3). Proteins identified in PSIP1/p75 IPs are specific to this isoform of PSIP1, as the antibody used for IP is specific to the c-terminal domain of PSIP1/p75, which is absent in the p52 isoform (Figure 1A). Cell division cycle-associated 7 like (CDC7L), one of the known interacting partners of p75, had the second highest ratio in MEFs (Hughes et al., 2010). SILAC ratio for γH2A.X was similar to PSIP1 in mESCs, suggesting the co-occurrence of PSIP1 along with γH2A.X at the nucleosomal level. Interestingly, with the exception of XRCC1 all the other DNA repair proteins found in the p75 complex were also associated with H3K36me3 chromatin (Table 1 and Figure 2). Facilitates active transcription (FACT) heteromeric complex proteins – SSRP1 and SPT16 are associated with H3K36me3, whereas only SPT16 was detected in the PSIP1 complex in this study. However, SSRP1 has been recently shown to interact with PSIP1 (Lopez et al., 2016), which supports the functional interplay between PSIP1 and the FACT complex in transcriptional elongation and DNA repair. Immunoprecipitation done in mESCs detected fewer proteins and also lower SILAC ratio for p75 (Table 1; Dataset 3). Intriguingly, other PSIP1 interacting proteins – MLL1, MLL2, Menin, and/or CtIP – were not detected.
Figure 3. PSIP1 role in facilitating DNA repair. (A) Illustration showing schematics of SILAC immunoprecipitation for using rabbit IgG, anti-MOF and anti-PSIP1 antibodies in cells labelled with light (R0K0), medium (R6K4) and heavy (R10K8) SILAC media. (B) Western blotting with antibodies recognising MLL1, phosphorylated H2A.X (γH2A.X), H2A and H3K36me3, for HA-tag pulldowns from nuclear extracts of Psip1−/− MEFs rescued with HA-p52 and HA-p75. (C) Microscopic images of WT and Psip1−/− MEFs after Comet assay, a representative image from 16 microscopic fields are shown. (n=2 biological replicates). (D) Working model showing various DNA repair proteins that are recruited to H3K36me3 chromatin to modulate repair choice or enhance DNA repair at the site of transcription.

Table 1. List of proteins that associate with PSIP/p75 in mouse embryonic fibroblasts and stem cells. *proteins also found associated with H3K36me3 chromatin (Dataset 1); ND, proteins not detected.

<table>
<thead>
<tr>
<th>Proteins with higher SILAC ratio</th>
<th>Known function</th>
<th>P75/IgG (MEFs)</th>
<th>P75/IgG (mESCs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC4 and SFRS1-interacting protein*</td>
<td>Transcription, alternative splicing Promotes DNA repair</td>
<td>93.5</td>
<td>22</td>
</tr>
<tr>
<td>Cell division cycle-associated 7-like</td>
<td>Ser/Thr kinase protein</td>
<td>17.7</td>
<td>6</td>
</tr>
<tr>
<td>Histone H2A.X*</td>
<td>DNA damage response</td>
<td>ND</td>
<td>23</td>
</tr>
<tr>
<td>Isoform Alpha of DNA ligase 3</td>
<td>NHEJ, BER, SSBR</td>
<td>10.9</td>
<td>ND</td>
</tr>
<tr>
<td>PARP1*</td>
<td>DNA repair</td>
<td>8.7</td>
<td>4</td>
</tr>
<tr>
<td>XRCC1</td>
<td>single-strand DNA breaks repair</td>
<td>7.3</td>
<td>ND</td>
</tr>
<tr>
<td>FACT complex subunit SPT16*</td>
<td>Nucleosome exchange, DNA repair</td>
<td>5.9</td>
<td>6.3</td>
</tr>
<tr>
<td>DNA topoisomerase 1*</td>
<td>DNA replication &amp; repair</td>
<td>4.6</td>
<td>ND</td>
</tr>
<tr>
<td>Top2 alpha*</td>
<td>DNA replication &amp; transcription</td>
<td>9.2</td>
<td>ND</td>
</tr>
<tr>
<td>Top2 beta*</td>
<td>DNA replication &amp; transcription</td>
<td>ND</td>
<td>2.9</td>
</tr>
<tr>
<td>Tyrosine-protein kinase BAZ1B*</td>
<td>H2A.X kinase</td>
<td>3.6</td>
<td>ND</td>
</tr>
</tbody>
</table>
To validate the interaction of p75 with S139 phosphorylated histone H2AX (γH2A.X), ML1 and H3K36me3, we performed IP with α-HA-tag antibodies in Psip1 knockout MEFs (Psip1–/–), which are stably transduced with HA-Psip1/p75 and HA-Psip1/p52 (Figure 3B) (Pradeepa et al., 2014; Shun et al., 2007). Western blotting of HA IPs with anti-H3K36me3 confirmed the interaction of both p52 and p75 with H3K36me3, which is mediated by a PWWP domain common to both PSIP1 isoforms. Interestingly, we found specific association of a DNA damage marker γH2A.X, with PSIP/p75, but not the p52 isoform. This is consistent with the previously known function of p75 in DNA damage response (Aymard et al., 2014; Daugaard et al., 2012). PSIP1/p75 is known to interact with ML1, but it was not detected in our endogenous p75 IP-MS analysis. However, western blotting with anti-MLL1 shows specific interaction of HA-p75, but not HA-p52 with MLL1 (Figure 3B). These results confirm that although both PSIP1 isoforms are localised to H3K36me3 chromatin, only p75 associates with ML1, γH2A.X and other DNA repair proteins - consistent with previous reports showing both isoforms of PSIP1 have different protein partners and cellular function (Daugaard et al., 2012; Ge et al., 1998; Pradeepa et al., 2012; Pradeepa et al., 2014; Pradeepa et al., 2017).

Higher DNA damage in PSIP1 knockout cells

To examine whether PSIP1 has a direct role in the DNA-damage response, we performed comet assay with WT and Psip1–/– MEFs, which is a sensitive method to measure DNA damage in individual cells (Olive & Banáth, 2006). Visual scoring of comets showed a significantly higher number of comets with a higher concentration of DNA in Psip1–/– MEFs compared to WT (Figure 3C). This data supports our previous observation that human cells depleted of p75 show higher levels of unrepaired DNA compared to control, confirming the higher level of unrepaired DNA in cells lacking PSIP1. This strengthens the evidence for a role for PSIP1/p75 in maintaining genomic integrity.

Acetylation of histone H4 at lysine 16 (H4K16ac) and enzymes responsible for most of this modification TIP60 (KAT5) and MOF (KAT8) have been implicated with DNA repair (Kumar et al., 2013; Sharma et al., 2010; Tang et al., 2013). Recent work suggests a link between H4K16ac and H3K36me3 in DNA damage response (Li & Wang, 2017). We planned to perform SILAC IP for three different antibodies and were thus able to include anti-MOF IP along with p75 IP and IgG, allowing us to identify the protein partners of MOF along with PSIP1/p75 in mESCs and MEFs. MOF IP also acted as an unrelated chromatin protein control that is known to associate with active genes at regulatory elements (Li et al., 2012; Taylor et al., 2013). MOF protein has been shown to be associated with both male-specific lethal (MSL) and non-specific lethal (NSL) complexes (Cai et al., 2010; Li et al., 2009; Mendjan et al., 2006). Canonical MSL proteins were detected in MOF complex and there was no overlap between PSIP1 and MOF complex proteins, which shows the specificity of the IP. Intriguingly, we did not find association of MOF with NSL or DNA repair proteins (Dataset 2).

Although there have been efforts to study protein partners of PSIP1 and MOF, to our knowledge, this is the first study exploiting the utility of SILAC proteomics to investigate the cellular interactome of these two proteins without using overexpression or epitope tagging. The SILAC immunoprecipitation strategy used here is a very sensitive and powerful means of detecting transient and stable protein partners of chromatin associated proteins.

**Discussion**

Purified protein domains that recognise and bind to H3K36me3 can be studied using H3K36 trimethylated histone tail peptides (Pradeepa et al., 2012). In addition, immunoprecipitation of nuclear extracts with specific peptides followed by SILAC MS has been successfully used to identify methylated histone peptides, including H3K36me3 (Vermeulen et al., 2010). We now used x-ChIP-qMS, a useful method to identify proteins that interact transiently to histone modifications or chromatin proteins in vivo. A similar method was successfully used in *Drosophila* cells to identify MSL associated histone proteins and histone modifications (Wang et al., 2013). In this study, we identified proteins associated with H3K36me3 using x-ChIP-qMS. Further studies upon targeted mutations to histone modifying proteins (writers) or domains that recognises these histone modifications (readers) using gene editing methods will aid in validating the specific interaction of histone modifications and their reader proteins.

SETD2 is the only enzyme responsible for majority of H3K36 trimethylation in mammals, and its depletion reduces H3K36me3 levels, which results in a lower density of FACT subunits SPT16 and SSRP1 (Carvalho et al., 2013). Our data shows that FACT subunits are associated with both H3K36me3 and p75, suggesting the role of p75 in recruiting the FACT complex to transcribing gene bodies. Indeed, we have previously demonstrated the role of p75 in binding to H3K36me3 at expressed HOX genes and restraining their expression; we propose this regulation occurs at the stage of transcriptional elongation (Pradeepa et al., 2014). PSIP1/p75 and the SSRP1 complex were recently suggested to be important for the life cycle of HIV (Lopez et al., 2016). The FACT complex has been shown to facilitate the exchange between H2A.X and H2A (Heo et al., 2008).

Several pieces of evidence have emerged in recent years for the role of histone modifications in DNA repair. Mammalian Setd2 (homolog of Set2) catalyses H3K36me3 at expressed gene bodies in a transcription dependent manner. H3K36me3 in turn recruits PSIP1 (Daugaard et al., 2012; Pradeepa et al., 2012; Pradeepa et al., 2014), Mrg15 (Bleuyard et al., 2017; Luco et al., 2010), B56γ (Guo et al., 2014), Dnmt3a (Dhayalan et al., 2010) and Msh6 (Li et al., 2013), which mediate transcription, DNA methylation, alternative splicing, and DNA repair choice. A clear association of H3K36me3 with PSIP1 at expressed gene bodies and their association with several DNA repair proteins (this work) and HDR (Aymard et al., 2014; Daugaard et al., 2012), suggests H3K36me3 and PSIP1 pathway in genome stability. Although we have previously shown that both isoforms of PSIP1 bind to H3K36me3 through the common PWWP domain, it is only
the p75 isoform of PSIP1 that associates with γH2A.X (Figure 3B). Moreover, most of the previously known p75 interacting proteins are shown to bind to IBD in the C-terminus of p75 (Figure 1A). These data suggest the possibility of other known p75 interacting proteins like PogZ, JPO2, IWS1, MLL and ASK that binds to IBD (Tesina et al., 2015) in DNA repair pathways.

The constitutive chromatin association of repair factors with H3K36 trimethylated histones that are located at transcribed gene bodies suggests they have a key role in the maintenance of genome stability at the site of transcription. Identification of several DNA repair proteins that interact with PSIP1/p75 that are also associated with H3K36me3 suggests that H3K36me3 and PSIP1 have a wider role in DNA repair pathways. We propose a wide spectrum of roles for SETD2 dependent H3K36me3 and its readers in DNA repair and genome stability than previously suggested (Figure 3D). PSIP1/p75 is a stress survival protein, also implicated in various cancers including breast, ovarian, prostate and leukaemia, and has also been shown to promote resistance to chemotherapy induced cell death in prostate cancer. Further detailed studies are needed for better understanding of the importance of PSIP1 promoting DNA repair in stress response and in cancers.

**Methods**

**Cell lines**

Psip1−/− and its corresponding WT MEFs (Pradeepa et al., 2014; Shun et al., 2007) were a kind gift of Prof. Alan Engelman (Dana-Farber Cancer Institute, USA), and were cultured for two weeks in SILAC DMEM media (Dundee Cell Products), containing 10% fetal bovine serum (HyClone, GE Healthcare) and 1% Pen/Strep (Sigma Aldrich). mESCs (OS25, IGMM bios) were adapted to grow in DMEM media before they were cultured in SILAC DMEM media. MEFs and mESCs to be cultured in R10K8 media. R0K0 media; cells used for MOF IP were cultured in R6K4 media; and cells used for P75 IP were cultured in R10K8 media.

**ChIP mass-spectrometry**

mESCs were cultured in GMEM as described previously (Pradeepa et al., 2016). Cells were harvested by trypsinization and fixed immediately with 1% formaldehyde (Thermo Fisher, Cat. 28906) (25°C, 10 min) in PBS, and stopped with 0.125M Glycine. Cross linked cells were re-suspended in Farnham lysis buffer (5 mM PIPES pH 8.0, 85 mM KCl, 0.5% NP-40, Complete Mini EDTA-free protease inhibitor; Roche) for 30 minutes and centrifuged at 228 g for 5 minutes at 4°C. Nuclei were resuspended in RIPA buffer (1X PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS (filtered 0.2 -0.45 micron filter unit) + Complete Mini EDTA-free protease inhibitor; Roche) and sonicated using a Bioruptor® Plus sonication device (Diagenode) at full power for 50 minutes (30 seconds on, 30 seconds off) to produce fragments of 200–500 bp. 10 µg of each antibody was incubated with Protein A Dynabeads (ThermoFisher Scientific, 10001D) in 5 mg/ml bovine serum albumin (BSA) in PBS on a rotating platform at 4°C for two hours. An arbitrary concentration of 200 µg chromatin was incubated with antibody bound Dynabeads on a rotating platform at 4°C for 16 hours. Beads were washed 5 times (5 minutes each) on a rotating platform with cold LiCl wash buffer (100 mM Tris pH 7.5, 500 mM LiCl, 1% NP-40, 1% Sodium deoxycholate) and one time with RT TE buffer.

Antibodies used: 5 µg of rabbit IgG (Santa Cruz sc-2025), Histone H3 (rabbit polyclonal, Abcam, Ab 1791), H3K36me3 (rabbit polyclonal, Abcam, Ab 9050) were used per IP. For analysis by mass spectrometry, beads were washed 3 times with Tris-saline buffer, and excess buffer removed. ChiPed complexes were digested on beads, desalted and analysed on a Q-Exactive plus mass spectrometer, as previously described (Turrisi et al., 2014). Proteins were identified and quantified by MaxLFQ by searching with the MaxQuant version 1.5 against the mouse proteome database (Uniprot). Modifications included C Carbamyllation (fixed) and M oxidation (variable). Bioinformatic analysis was performed with the Perseus software suite.

**Immunoprecipitation**

10x14-cm dishes of cells were trypsinized and pelleted, resuspended in 5 ml of ice-cold swelling buffer (10 mM Hepes, pH 7.9, 1.5 mM MgCl2, 20 mM KCl, 0.5 mM DTT and protease inhibitors (Complete, Roche) for 5 min, and cells were broken open to release nuclei using a pre-chilled Dounce homogenizer (20 strokes with a tight pestle). Dounced cells were centrifuged at 228 g (2,000 rpm) for 5 min at 4°C to pellet nuclei and other fragments. The supernatant was discarded. The resulting nuclear pellet was resuspended in 5 ml of RIPA buffer containing 50 mM Tris, pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% Sodium deoxycholate, and protease inhibitors + Benzonase (Novagen, 10 µl/ml), incubated for 30 min on ice, and sonicated briefly on ice (10 x 30 s at full power in bioruptor). Extracts were cleared by centrifugation at 13000 RPM for 10 min at 4°C. Nuclear protein concentrations were measured using a Bradford assay.

Protein A Dynabeads (Life Technologies) were incubated with 5 µg of rabbit IgG (Santa Cruz sc-2025), anti-PSIP/p75 (rabbit polyclonal, Bethyl Laboratories, A300-848A) and anti-MOF (rabbit polyclonal, Bethyl Laboratories A300-992A) in 5% BSA in phosphate buffer saline for two hours, equivalent total protein amounts of extracts were incubated separately with antibodies bound to beads in a rotating platform at 4°C for 30 min. Beads were washed once with RIPA buffer and combined carefully after first wash step. After a further 4 washes, bound proteins were eluted in 4X SDS loading buffer (Life Technologies) with freshly added DTT at 95°C for 5 min. Samples were centrifuged at 11,000 RPM speed for 1 min and supernatant was collect in low binding tube. LC-MS/MS and quantification were carried out by Dundee Cell Products. Briefly, for SDS-PAGE, gel slices per fraction were cut and digested in-gel with trypsin. The purified peptides were then separated (Ultimate U3000, trap-enriched nanoflow LC-system, Dionex), and identified (LTQ Orbitrap XL, Thermo-scientific, via nano ESI ion source, Proxeon Biosystems). Quantification (MaxQuant, based on 2D centroid of isotope clusters within each SILAC pair) can distinguish between the samples, to give a ratio of protein of interest to IgG. Background proteins would be expected to have a ratio of 1:1 and are therefore disregarded.
HA-pulldown of p52 and p75
PSIP1 knockout MEFs stably rescued with HA-PSIP1/p52 and HA-PSIP1/p75 were immunoprecipitated with anti-HA antibodies. 5% input lysate and IPed proteins were separated on Novex 4–20% gels and transferred to PVDF membranes. Western blotting was performed by immunoblotting with MLL1 (Active Motif, 61295), gH2A.X (Millipore, 05-636), H2A and H3K36me3 (Abcam, ab9050) antibodies.

Comet assay
Comet assays for WT and Psip1 knockout MEFs were performed using Comet Assay kit (OxiSelect™), according to the manufacturer’s instructions.

Data availability
Raw data for this study are available from OSF http://doi.org/10.17605/OSF.IO/UAX7G (Praideepa, 2017). Dataset 1: ChIP MS data in mESCs, Dataset 2: PSIP1 p75 SILAC results in MEFs; Dataset 3: PSIP1 p75 SILAC results in mESCs, WT MEFs Comet assay data, PSIP1 KO Comet assay data, and uncropped blots for Figure 3B.

Competing interests
No competing interests were disclosed.

Grant information
This work was supported by the Wellcome Trust [085767]; and the Medical Research Council and centenary award to PM.

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Acknowledgements
We thank Prof Wendy Bickmore for support and discussions, Dr. Celine Courilleau for help with Comet assay and John Dawson for graphics. Current research in PM’s lab is supported by Wellcome Trust seed award [200598].

References

Kumar R, Honkoshi N, Singh M, et al.: Chromatin modifications and the DNA


Deepak Jha
Division of Pediatric Hematology and Oncology, Boston Children's Hospital and Dana-Farber Cancer Institute, Boston, MA, USA

I do not have any further comments.

Competing Interests: No competing interests were disclosed.

Referee Expertise: Epigenetics, Genome Stability, Cell Cycle, Cancer Epigenetics, Hematopoiesis

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

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Deepak Jha
Division of Pediatric Hematology and Oncology, Boston Children's Hospital and Dana-Farber Cancer Institute, Boston, MA, USA

The overarching goal of this manuscript by Pradeepa et al. is to identify if PSIP1 isoforms provide a binding platform for DNA repair proteins, which would explain how H3K36me3 functions in DNA repair. They use proteomics to identify, in a somewhat unbiased manner, the interacting partners of H3K36me3 chromatin, and PSIP1/p75. They found a substantial overlap between the binding partners of H3K36me3 and p75. Furthermore, they perform co-IP with Y-H2A.X to validate their Ip-mass spectrometry data. Finally, they show that Psip1/-/- cells have higher level of unrepaired DNA damage, thereby alluding to a role for Psip1 -/- in DNA repair.

Specific Comments:
1. As far as I can tell, all of the mass spectrometry experiments have been performed without the induction of any DNA damage. This implies that one is looking at some combination of intrinsic DNA damage associated binding partners, which would be heavily influenced by transcriptional...
biases (related to gene length, transcriptional frequency and exons), and cell cycle phases. To
directly make a conclusive statement regarding if \textit{PSIP1} acts as a scaffold for recruiting DNA repair
proteins, it would be beneficial to monitor the interaction(s) with and without DNA damage. Given
the extensive rewiring of cellular signaling after DNA damage, having a more direct readout of
differential interacting partners would be more beneficial to refine the various models presented in
figure 3d.

2. Figure 3 can be improved by testing some other key interacting partners from table 1. Specifically,
components of FACT complex and PARP1 should be tested in co-IP experiments since both of
them are key regulators of DNA repair as well.

3. Fig. 3: Additional evidence for unrepaired DNA damage/abrogated DNA damage signaling in
\textit{Psip1}\textsuperscript{-/-} should be provided. E.g. Y-H2A.X retention kinetics after DNA damage in \textit{Psip1}\textsuperscript{-/-} cells.

4. Datasets 1 and 2 should be labeled in the same manner as the OSF files.

5. The authors state “Interestingly, with the exception of…” and cite Fig.2 for this. They should also
refer to table1, alongside Fig.2 to clarify the overlaps.

6. Can the authors phenocopy fig.3c with a PWWP mutant of p75 in order to directly test if
H3K36me3- p75 are part of the same mechanism in DNA repair?

\textbf{Is the work clearly and accurately presented and does it cite the current literature?}
Partly

\textbf{Is the study design appropriate and is the work technically sound?}
Yes

\textbf{Are sufficient details of methods and analysis provided to allow replication by others?}
Yes

\textbf{If applicable, is the statistical analysis and its interpretation appropriate?}
Yes

\textbf{Are all the source data underlying the results available to ensure full reproducibility?}
Yes

\textbf{Are the conclusions drawn adequately supported by the results?}
Partly

\textbf{Competing Interests:} No competing interests were disclosed.

\textbf{Referee Expertise:} Epigenetics, Genome Stability, Cell Cycle, Cancer Epigenetics, Hematopoiesis

I have read this submission. I believe that I have an appropriate level of expertise to confirm that
it is of an acceptable scientific standard, however I have significant reservations, as outlined
above.
Jan De Rijck  
Department of Pharmaceutical and Pharmacological Sciences, KU Leuven, Leuven, Belgium

Several manuscripts have suggested a role for LEDGF/p75 in transcriptional elongation and DNA repair. Since LEDGF/p75 is known to read H3K36me3, a marker of active chromatin present in the body of genes, and since both H3K36me3 and LEDGF/p75 have been linked to DNA repair it is tempting to speculate that both are part of the same molecular mechanism. Pradeepa et al. tried to tackle this question by analysing and comparing the proteome of H3K36me3 and LEDGF/p75.

Comments:
- Recently, LEDGF/p75 has been suggested to interact with H3K36me2 next to H3K36me3 (Zhu et al. 2016 and Okuda, 2014). Some even claim that this interaction is preferred over H3K36me3. In this regard, LEDGF/p75 can also be found in promoter regions. Although this remains a matter of debate it should be mentioned in the introduction.
- The datasets in the supplemental material are not clear to me. Dataset 1 and 2 are mentioned but not clearly linked to the supplements. I could not find dataset 1.
- Although DNAse is used to overcome DNA bridging in the IP experiments, the xChip experiments are inevitably prone to DNA bridging. One can only claim that these proteins were in the neighbourhood of H3K36me3 marks. This should be clearly indicated in the discussion.
- In the text it is claimed that there is a strong overlay between both datasets in DNA repair proteins. The authors refer to figure 2 to support this statement. However, this figure only mentions H3K36me3 hits. The general overlay between both set could be presented in a better way.
- LEDGF is a major hit in the H3K36me3 xChIP. Looking at the sequenced peptides, can you speculate whether this was p52 or p75?
- Confirmation of some of the important hits by co-immunoprecipitation experiments as presented in figure 3b would strengthen the credibility of the manuscript.
- The fact that LEDGF/p75 has an effect on DNA damage response (figure 3c) is not new. However, neither the present manuscript nor previous manuscripts from other groups could provide a direct link. The authors claim that LEDGF/p75 and not p52 affects DNA repair. As such, there is a good chance that a protein binding to the IBD domain is responsible for this effect. Proteins binding the IBD are characterised by an IBM motif (Tesina et al., 2015).
  - Could the authors rescue the DNA repair defect in -/- cells by a mutant LEDGF/p75 protein not able to interact with IBM proteins?
  - CDC7/ASK is the only protein in table 1 with an IBM motif. Can HA-LEDGF/p75 rescue DNA repair upon knockdown of CDC7/ASK?

Is the work clearly and accurately presented and does it cite the current literature?  
Partly
Is the study design appropriate and is the work technically sound?  
Partly

Are sufficient details of methods and analysis provided to allow replication by others?  
Yes

If applicable, is the statistical analysis and its interpretation appropriate?  
Yes

Are all the source data underlying the results available to ensure full reproducibility?  
Partly

Are the conclusions drawn adequately supported by the results?  
Partly

**Competing Interests:** No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

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**Author Response 11 Oct 2017**

Pradeepa madapura, University of Essex, UK

We thank Dr. Jan De Rijck for critically reviewing this paper and also suggestions to improve the credibility of the manuscript. Please see our responses below for each of the specific points.

Recently, LEDGF/p75 has been suggested to interact with H3K36me2 next to H3K36me3 (Zhu et al. 2016 and Okuda, 2014). Some even claim that this interaction is preferred over H3K36me3. In this regard, LEDGF/p75 can also be found in promoter regions. Although this remains a matter of debate it should be mentioned in the introduction. *We have mentioned this in the introduction*

- The datasets in the supplemental material are not clear to me. Dataset 1 and 2 are mentioned but not clearly linked to the supplements. I could not find dataset 1. *We have added the datasets now clearly labeled*

- Although DNAse is used to overcome DNA bridging in the IP experiments, the xChip experiments are inevitably prone to DNA bridging. One can only claim that these proteins were in the neighborhood of H3K36me3 marks. This should be clearly indicated in the discussion. *We completely agree with the reviewer’s point, hence say these proteins are associated with H3K36me3*

- In the text, it is claimed that there is a strong overlay between both datasets in DNA repair proteins. The authors refer to figure 2 to support this statement. However, this figure only mentions H3K36me3 hits. The general overlay between both sets could be presented in a better way.
We have now referred to Table 1 and Figure 2 and dataset 1

- LEDGF is a major hit in the H3K36me3 xChIP. Looking at the sequenced peptides, can you speculate whether this was p52 or p75?

We have now looked into this and found p75 peptides in the mass spec data. So we cannot rule out the presence of shorter p52 isoform along with the p75 isoform. We didn’t find a short peptide that distinguishes between two isoforms. So our data shows both isoforms are likely to present in the H3K36me3 domain.

- Confirmation of some of the important hits by co-immunoprecipitation experiments as presented in figure 3b would strengthen the credibility of the manuscript.

We completely agree with the suggested confirmatory experiments by both reviewers will strengthen the credibility of the manuscript. We will be performing these experiments when we have resources, and we will update the manuscript.

- The fact that LEDGF/p75 has an effect on DNA damage response (figure 3c) is not new. However, neither the present manuscript nor previous manuscripts from other groups could provide a direct link. The authors claim that LEDGF/p75 and not p52 affects DNA repair. As such, there is a good chance that a protein binding to the IBD domain is responsible for this effect. Proteins binding the IBD are characterized by an IBM motif (Tesina et al, 2015).

It is an interesting point; we have now discussed this in the manuscript

- Could the authors rescue the DNA repair defect in -/- cells by a mutant LEDGF/p75 protein not able to interact with IBM proteins?

Thanks for suggesting this experiment, we would love to do this experiment in the future, but not for this manuscript

- CDC7/ASK is the only protein in table 1 with an IBM motif. Can HA-LEDGF/p75 rescue DNA repair upon knockdown ofCDC7/ASK?

These data could provide a direct link between LEDGF/p75 and DNA repair.

We appreciate the suggestions to improve the manuscript by both the reviewers. Due to limited resources, we cannot perform the suggested experiments at this time. However, we will perform the suggested experiments in future. We wished to share our data with this publication to wider readers at the earliest time point.

**Competing Interests:** No competing interests were disclosed.