PHD1 regulates p53-mediated colorectal cancer chemoresistance

Sofie Deschoemaeker1,2, Giusy Di Conza1,2, Sergio Lilla3, Rosa Martín-Pérez1,2, Daniela Mennerich4, Lise Boon1,2, Stefanie Hendriks1,2, Oliver DK Maddocks3, Christian Marx3,5, Praveen Radhakrishnan6, Hans Prenen7, Martin Schneider6, Johanna Myllyharju8, Thomas Kietzmann4, Karen H Vousden3, Sara Zanivan3 & Massimiliano Mazzone3,2,*

Abstract

Overcoming resistance to chemotherapy is a major challenge in colorectal cancer (CRC) treatment, especially since the underlying molecular mechanisms remain unclear. We show that silencing of the prolyl hydroxylase domain protein PHD1, but not PHD2 or PHD3, prevents p53 activation upon chemotherapy in different CRC cell lines, thereby inhibiting DNA repair and favoring cell death. Mechanistically, PHD1 activity reinforces p53 binding to p38α kinase in a hydroxylation-dependent manner. Following p53–p38α interaction and chemotherapeutic damage, p53 can be phosphorylated at serine 15 and thus activated. Active p53 allows nucleotide excision repair by interacting with the DNA helicase XPB, thereby protecting from chemotherapy-induced apoptosis. In accord with this observation, PHD1 knockdown greatly sensitizes CRC to S-FU in mice. We propose that PHD1 is part of the resistance machinery in CRC, supporting rational drug design of PHD1-specific inhibitors and their use in combination with chemotherapy.

Keywords chemotherapy resistance; colorectal cancer; DNA repair; prolyl hydroxylase domain proteins; tumor suppressor p53

Introduction

Resistance to chemotherapy remains a major clinical issue in the treatment of colorectal cancer (CRC). Response rates have already improved to about 30–40% over the past years with the introduction of the currently used FOLFOX and FOLFIRI regimens in patients with metastatic CRC, however still leaving room for further research on potential candidates causing chemorefractory disease (Prenen et al, 2013).

Prolyl hydroxylase domain proteins PHD1, PHD2, and PHD3 (codified by EGLN2, EGLN1, and EGLN3, respectively) are oxygen-sensitive enzymes initially known for their ability to target the hypoxia-inducible transcriptional factors HIF-1α and HIF-2α for proteasomal degradation (Epstein et al, 2001). Besides controlling cellular adaptation to hypoxic conditions, it is now clear that PHDs are also involved during cell damage and metabolic stress (Aragones et al, 2008; Schneider et al, 2010; Leite de Oliveira et al, 2012). Recently, we have shown that inhibition of PHD2 mounts an adaptive response in mice treated with chemotherapeutic drugs, resulting in the protection against their toxic side effects (Leite de Oliveira et al, 2012). Additionally, PHD1 or PHD2 inhibition confers organ protection against ischemic damage (Aragones et al, 2008; Schneider et al, 2010; Takeda et al, 2011). However, proteins other than HIF-1α and HIF-2α (including PKM2, FOXO3a, ATF4, RPB1, HCLK2, and β2-adrenergic receptor) have been proven to be alternative targets of PHD1–3 (Epstein et al, 2001; Mikhaylova et al, 2008; Chan et al, 2009; Xie et al, 2009, 2012; Xue et al, 2010; Ameln et al, 2011; Hiwatashi et al, 2011; Takeda et al, 2011; Scholz et al, 2013; Wong et al, 2013; Zheng et al, 2014). In some cases, substrate hydroxylation by PHD1–3 does not initiate a consensus for proteasomal degradation. For example, RPB1 is positively regulated by PHD1-mediated hydroxylation (Mikhaylova et al, 2008). This suggests that many PHD targets and functions are still undiscovered. This is also reflected by the lack of a conclusive role for PHDs in the context of cancer biology in general. More specifically in CRC, PHD2 and PHD3 have been suggested to act as tumor suppressors because of their decreased expression in the cancer compared to the normal tissue (Chan et al, 2009; Xue et al, 2010). However, the expression data on PHD1 in CRC are not unison with some studies reporting...
decreased expression of PHD1 and others showing no alterations (Jubb et al, 2009; Rawluszko et al, 2013). Importantly, none of these studies correlate the activity (rather than the expression) of these enzymes to disease onset and outcome or even, more specifically, to CRC response to chemotherapeutic regimens.

The transcription factor p53 is undoubtedly the most characterized cell stress sensor and tumor suppressor. p53 is usually phosphorylated and activated upon oncogene activation and DNA damage resulting in growth arrest and DNA repair or cell death induction, depending on the extent of the damage (Vousden & Lane, 2007). In CRC, p53 is mutated in about 50% of patients. However, p53 is never the primary hit and a clear correlation between p53 induction, depending on the extent of the damage (Vousden & Lane, 2009). Indeed, upon 5-FU treatment, p53 phosphorylation, which was largely prevented upon silencing of PHD1 (Fig 1F). To extend our findings to different CRC cell lines other than HCT116, we used LIM1215 carrying wild-type p53 (Chen et al, 2014). PHD1 mRNA levels were 82.1% reduced in PHD1-silenced cells compared to their scrambled control (Fig 1G). Treatment with 5-FU resulted in increased p53 levels and increased p53 phosphorylation at Ser15 compared to untreated cells; PHD1 silencing strongly prevented this induction (Fig 1H).

Altogether, these data provide evidence that, in the context of colorectal cancer, a drop in PHD1 levels reduces p53 phosphorylation following the administration of three different chemotherapeutics commonly used in the clinical treatment of CRC.

**Results**

**PHD1 silencing hinders p53 activation upon chemotherapy treatment**

To evaluate the possible effect of PHD1–3 on p53 activation upon chemotherapy treatment, we silenced EGLN2, EGLN1, or EGLN3 (coding for PHD1, PHD2, and PHD3, respectively) in p53^{wt/wt} HCT116 cells (also denoted HCT116) (Sur et al, 2009) and treated them with 5-FU, PHD1, PHD2, and PHD3 RNA transcripts after knockdown were reduced, respectively, by 86.4, 91.1 and 84.7% compared to the scrambled control (Fig 1B). Evaluation of p53 activation was done by Western blotting for p53 phosphorylation at Ser15 (p53 pS15), frequently associated with the initial steps of p53 activation (Meek & Anderson, 2009). Upon 5-FU treatment, HCT116 showed an increased p53 accumulation and phosphorylation at Ser15 in the scrambled control cells (Fig 1A). Silencing of PHD2 or PHD3 did not affect either p53 levels or phosphorylation both at baseline and after 5-FU treatment. However, PHD1 knockdown significantly reduced p53 phosphorylation at Ser15 upon 5-FU treatment compared to the scrambled control (Fig 1A and C). Similar results were obtained by using a different siRNA against PHD1 (Fig 1D and E).

To address whether the reduction in p53 phosphorylation upon PHD1 silencing also holds true upon different chemotherapeutics clinically used in CRC treatment, we exposed HCT116 to either SN-38 or oxaliplatin. In scrambled control cells, both drugs induced p53 phosphorylation, which was largely prevented upon silencing of PHD1 (Fig 1F). To extend our findings to different CRC cell lines other than HCT116, we used LIM1215 carrying wild-type p53 (Chen et al, 2014). PHD1 mRNA levels were 82.1% reduced in PHD1-silenced cells compared to their scrambled control (Fig 1G). Treatment with 5-FU resulted in increased p53 levels and increased p53 phosphorylation at Ser15 compared to untreated cells; PHD1 silencing strongly prevented this induction (Fig 1H).

Altogether, these data provide evidence that, in the context of colorectal cancer, a drop in PHD1 levels reduces p53 phosphorylation following the administration of three different chemotherapeutics commonly used in the clinical treatment of CRC.

**PHD1 silencing sensitizes colorectal cancer cells to chemotherapy**

In order to find out whether the reduction in p53 phosphorylation after chemotherapy following PHD1 knockdown could affect cell death in a p53-dependent manner, we treated PHD1-silenced p53^{wt/wt} HCT116 and the previously described p53^{-/-} HCT116 lacking full-length p53 (Sur et al, 2009) with 5-FU for 24 h (Fig EV1A). As expected, the treatment caused the cleavage of caspase-3 and parp in p53^{wt/wt} HCT116; however, this induction was further enhanced upon silencing of PHD1 (Fig 2A). Though caspase-3 cleavage was also induced in p53^{-/-} HCT116 cells (albeit to a lower extent), it was not enhanced upon silencing of PHD1, suggesting that PHD1 might underlie resistance to chemotherapy by modulating p53 phosphorylation (Fig 2A). The same results were confirmed by FACS analysis on HCT116 cells stained with propidium iodide, by ELISA nucleosome detection and by TUNEL immunofluorescence staining of fixed cells (Figs 2B and EV1B and C). These findings were recapitulated by using a second siRNA against PHD1 (Fig 2C). Similar to what was observed with 5-FU, treatments with either SN-38 or oxaliplatin were also able to induce apoptosis of p53^{wt/wt} HCT116 cells and this response was further enhanced by PHD1 silencing (Fig 2D and E). To validate our observations in a different cell type, we showed that PHD1 silencing was also able to sensitize p53^{wt/wt} LIM1215 cells to 5-FU (Fig 2F).

To link the effect of PHD1 silencing on chemoresistance to the negative regulation of p53 phosphorylation at Ser15, we made use of p53^{-/-} HCT116 cells and reconstituted them with either wild-type

**Figure 1. Silencing of PHD1 decreases p53 phosphorylation in response to chemotherapy in CRC cells.**

A Western blot for p53, phosphorylated p53 at Ser15 (p53 pS15), and vinculin (vinc) in HCT116 treated with 300 μM 5-FU for 8 h and silenced for PHD1, PHD2, or PHD3.

B qRT-PCR for PHD1, PHD2, and PHD3 in HCT116 silenced for PHD1 (*p = 0.0006 toward Scr), PHD2 (*p = 0.0004 toward Scr), or PHD3 (*p = 0.0075 toward Scr).

C Western blot of p53, p53, PHD1, and vinculin in HCT116 treated with 300 μM 5-FU for 8 h and silenced for PHD1 or a scrambled (Scr) control.

D qRT-PCR for PHD1 showing silencing efficacy with both siRNAs (1 (% = 0.0017 toward Scr) and 2 (% = 0.0023 toward Scr) compared to the scrambled control in HCT116. A two-tailed unpaired t-test was performed with n = 3/group.

E Western blot for p53, p53, and vinculin in HCT116 silenced for PHD1 with two different constructs (constructs 1 and 2) upon exposure to 300 μM 5-FU for 8 h.

F Western blot for p53, p53, and pS15 in HCT116 silenced for PHD1 and treated with either 200 nM SN-38 or 20 μM oxaliplatin for 8 h.

G RNA levels of PHD1 in LIM1215 silenced for PHD1. (*p = 0.0099 toward Scr in a two-tailed unpaired t-test with n = 3/group).

H Western blot for p53, p53, and vinculin in LIM1215 upon silencing of PHD1 and treatment for 8 h with 200 μM 5-FU.

Data information: Vinculin (vinc) was used as a loading control in (A, C, E, F, H). Source data are available online for this figure.
Figure 1.

A  5-FU 300 µM (8 h)

B  PHD1 transcription in HCT116

C  5-FU 300 µM (8 h)

D  PHD1 transcription in HCT116

E  5-FU 300 µM (8 h)

F  SN-38 200 nM (8 h)  Oxaliplatin 20 µM (8 h)

G  PHD1 transcription in LIM1215

H  LIM1215  5-FU 200 µM (8 h)

Figure 1.
HCT116 responded to 5-FU similar to their p53<sup>wt/wt</sup> HCT116 counterpart, and correspondingly, p53 phosphorylation at Ser15 was not detected in p53<sup>wt/wt</sup> or p53<sup>−/−</sup> HCT116 cells, hindering p53 phosphorylation at Ser15.

**PHD1 silencing improves the response of CRC to 5-FU treatment**

To evaluate whether the aforementioned findings are also relevant in more complex systems, we initially performed a colony formation assay in p53<sup>wt/wt</sup> HCT116 carrying a doxycycline-inducible shScr or shPHD1 construct. After treatment for 24 h with 1 µg/ml of doxycycline, cells were exposed to 5-FU in combination with doxycycline and then assessed for the ability to form foci *in vitro*. In the scrambled control, 5-FU treatment decreased colony formation compared
to the untreated cells, but this difference was even further decreased upon silencing of PHD1 (Figs 3A and EV2A). In contrast, no differences in colony formation capacity were detected between PHD1-silenced and control p53−/− HCT116 cells upon their treatment with 5-FU (Fig EV2A and B), highlighting the dependency of p53 in the PHD1-mediated resistance against cytostatic drugs.

Following these results, we investigated the preclinical relevance of these findings in vivo. To this end, nude mice were subcutaneously injected with p53wt/wt or p53−/− HCT116, where conditional silencing of PHD1 was achieved by doxycycline administration when tumors reached 250 mm³. Forty-eight hours after doxycycline administration, mice received a weekly treatment with the maximum tolerated dose of 100 mg/kg 5-FU. While tumor growth was not altered in untreated mice carrying p53wt/wt HCT116 shScr or shPHD1 tumors, 5-FU treatment reduced tumor volume by 39.5% in p53wt/wt HCT116 shScr, but by 70% in mice carrying a tumor silenced for PHD1 (Fig 3B). In contrast, 5-FU-treated mice carrying p53−/− HCT116 shScr or shPHD1 tumors did not show any differences in tumor growth, providing evidence for the p53 dependency of these findings (Fig 3C). These results show that PHD1 silencing can increase the sensitivity of CRC to chemotherapeutic drugs both in vitro and in vivo in a p53-dependent manner.

**PHD1 hydroxylase promotes p53 phosphorylation upon chemotherapy**

Mechanistically, PHDs have been shown to affect other proteins in both hydroxylation-dependent and hydroxylation-independent manners (Mikhaylova et al, 2008; Chan et al, 2009; Xue et al, 2010; Hiwatashi et al, 2011; Takeda et al, 2011; Zheng et al, 2014). To evaluate whether the enzymatic function of PHD1 was required for p53 regulation, scrambled and PHD1-silenced HCT116 cells were treated with the non-specific prolyl hydroxylase inhibitor DMOG in the presence or absence of 5-FU. DMOG treatment alone did not significantly affect p53 levels or phosphorylation at Ser15; however, it did decrease p53 phosphorylation upon 5-FU treatment in control cells, thus mimicking the effect of PHD1 knockdown. PHD1 silencing did not further reduce the phosphorylation of p53 (Fig 4A), providing evidence that PHD1 promotes p53 phosphorylation through its hydroxylase function. To exclude that HIFs could play a role in this process as they have been shown to influence p53 levels and activity (Sermeus & Michiels, 2011), we silenced HIF-1α or HIF-2α in combination with PHD1 in HCT116. Upon treatment with 5-FU, silencing of PHD1 in combination with HIF-1α or HIF-2α knockdown still impaired p53 Ser15 phosphorylation compared to...
The scrambled control (Fig EV3A–C), indicating that PHD1-mediated p53 regulation is HIF independent.

To investigate whether PHD1 could interact with p53, Flag-tagged PHD1 was overexpressed in HEK293T cells and immunoprecipitated using anti-Flag antibodies. From this analysis, we observed that endogenous p53 was able to bind PHD1 (Fig 4B). Furthermore, to evaluate whether this interaction was direct or indirect, we performed an IP with an unrelated IgG control antibody. The data obtained show that PHD1 could interact with p53 also at the endogenous level in HCT116 cells (Fig EV4A).

In summary, we can conclude that PHD1 through its hydroxylase function and binding with p53 allows p53 phosphorylation at Ser15 upon chemotherapy treatment.

Reduced p53 hydroxylation impairs p38α-mediated p53 phosphorylation

To study how PHD1 silencing could affect p53 phosphorylation at Ser15, we first assessed which of the known p53-kinases were involved in p53 phosphorylation at Ser15. Therefore, we silenced Chk1, Chk2, ATR, ATM, p38α, and DNA-PK in HCT116 cells upon treatment with 5-FU (Bode & Dong, 2004; Meek & Anderson, 2009) (Fig EV4A and B). Unexpectedly, at least under the experimental conditions tested, only p38α silencing could completely reduce p53 phosphorylation at Ser15 to a similar extent as silencing of PHD1 (Fig EV4A). To confirm whether PHD1 could indeed affect p53 phosphorylation through p38α, we silenced PHD1 and p38α, alone or in combination (Figs 5A and EV4C). After treatment with 5-FU, silencing of both p38α and PHD1 did not show an additive reduction in p53 phosphorylation at Ser15, therefore suggesting that PHD1 hydroxylase function allows proper p53 phosphorylation by p38α (Fig 5A). To further confirm the specificity of these findings for p38α, we performed an in vitro kinase assay with p38α, p38β, and p38γ on p53 isolated from cells expressing either a control (mirSIMA) or an artificial miRNA against PHD1 (mirPHD1). This revealed that only phosphorylation of p53 by p38α, but not by p38β or p38γ, was prevented when PHD1 was lacking (Fig 5B). Finally, we proved that, upon 5-FU treatment, PHD1 silencing significantly reduced p53 binding to p38, confirming that hydroxylation of p53 is required for p38 interaction to p53 (Fig 5C). These data show that lack of PHD1 prevents proper p53 phosphorylation through a reduced binding of p53 to p38α upon chemotherapy treatment.

**PHD1 silencing reduces p53-mediated DNA repair following chemotherapy**

To investigate how the silencing of PHD1 could result in an increased apoptotic response, we initially focused our attention on...
the transcription of several important target genes of p53 such as CDKN1A, GADD45, MDM2, BAX, and PUMA (Menendez et al., 2009). Although all these genes were induced by 5-FU treatment in the scrambled control condition, PHD1 silencing did not affect their transcription compared to the control both at baseline and upon 5-FU treatment (Fig 6A). Therefore, we evaluated whether transcriptional activity was at all required for this specific process by treating HCT116 cells with 5-FU together with the transcription inhibitor α-amanitin. The proper function of the compound was confirmed by quantification of PUMA transcription, a downstream target of p53 (Fig EV5A). Silencing of PHD1 was still able to increase parp cleavage upon combined treatment with 5-FU and α-amanitin. Thus suggesting that p53-mediated transcription is not the cause for the increased cell death observed after chemotherapy upon PHD1 silencing (Fig 6B). In line with this, we also evaluated apoptosis after 5-FU treatment in p53<sup>+/+;</sup> HCT116, carrying a p53 DNA contact mutant, incapable of DNA binding and transcription despite a well-preserved tridimensional structure (Muller & Vousden, 2013). As already suggested by the use of α-amanitin, also in these cells we noted that silencing of PHD1 upon 5-FU treatment could still reduce Ser15 phosphorylation (Fig 6C and D) and results in increased apoptosis as detected by Western blot for cleaved caspase-3 (Fig 6E). These experiments confirm the importance of a transcription-independent function of p53.

As p53 has been shown to influence DNA repair in a transcription-independent manner, and as DNA repair can increase the resistance of cancer cells toward chemotherapy (Bouwman & Jonkers, 2012; Gordon & Nelson, 2012), we evaluated the DNA damage present in the cells. Indeed upon 5-FU treatment, DNA damage, detected by phosphorylation of histone H2AX (pH2AX) as a marker, was induced in the scrambled control and this was further increased in PHD1-silenced cells (Fig 7A). The increased DNA damage consequent to PHD1 silencing after chemotherapy treatment was p53 dependent as pH2AX accumulation in p53<sup>−/−</sup> HCT116 upon 5-FU treatment was equal in either the presence or absence of PHD1 (Fig 7A). More specifically, the reduced phosphorylation of p53 at Ser15 is required for the increased DNA damage, as there are no longer differences between the Scr control and siPHD1 condition in

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**Figure 5.** PHD1 hydroxylase favors p38α-mediated p53 phosphorylation.

A Western blot for p53 pS15, p53, and vinculin (vinc) in HCT116 treated with 300 μM 5-FU upon silencing of PHD1 and p38α, alone or in combination. Vinculin (vinc) is used as a loading control.

B Detection of p53 pS15 and p53 after *in vitro* phosphorylation by p38α, p38β, or p38γ of p53 immunoprecipitated from cells silenced for a control (SIMA) or PHD1.

C Detection by Western blot of p53 and p38 from whole cell extracts (WCE) or after immunoprecipitation of p53 from cell silenced for a Scr control or siPHD1 and treated for 1 h with 300 μM 5-FU.

Source data are available online for this figure.
pH2AX accumulation upon 5-FU treatment in the p53<sup>S15A→p53</sup>−/− HCT116 cells (Figs 7B and EV1D). These results were also confirmed with a second siRNA against PHD1 (Fig 7C) as well as upon SN-38 or oxaliplatin treatment (Fig 7D) and in the p53<sup>R248/-</sup> HCT116 cells (Fig 7E).

Previous studies have shown that p53 can stimulate the nucleotide excision repair (NER) pathway (engaged after 5-FU-, SN-38-, and oxaliplatin-induced DNA damage) in a transcription-independent manner through its direct interaction with XBP and XPD, two components of transcription factor II human (TFIIH).
**PHD1 causes CRC resistance to chemotherapy**

Sofie Deschoemaeker et al

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**Figure 7.**

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**H**

**CHEMORESISTANCE**

- PHD1
- DNA damage
- DNA repair response
- activation p53

**CHEMOSENSITIVITY**

- PHD1
- DNA damage
- DNA repair response
- activation p53

**Survival**

- PHD1
- DNA damage
- DNA repair response
- activation p53

**Apoptosis**
PHD1, when overexpressed in colorectal cancer cells, can decrease the role of PHD1 in cancer. Previous reports have shown that murine panel). This leads to increased DNA damage and cell chemotherapy treatment is prevented. It follows that p53 is not any longer able to bind XPB, thereby impairing the DNA repair capacity and thus enhanced apoptotic response to chemotherapy (right panel).

Data information: Vinc is used as a loading control in (A–E, G).
Source data are available online for this figure.

(Sengupta & Harris, 2005). When assessing the physical interaction of p53 with XBP or XPD, we noticed that PHD1 knockdown strongly reduced the binding of p53 and XBP upon treatment with 5-FU (Fig 7F), whereas no interaction between XPD and p53 was detected (Fig EV5B). To confirm that indeed, these findings are dependent on XBP, we silenced PHD1 either alone or in combination with XBP in HCT116 treated with 5-FU (Fig EV5C). The silencing of XBP caused a strong induction of DNA damage as detected by phH2AX upon 5-FU treatment and this was not further enhanced upon additional silencing of PHD1 (Fig 7G). These data show that impaired binding of p53 to XBP is indeed the underlying cause of the reduced DNA repair capacity of the cells after PHD1 blockade. In essence, the reduced p53 phosphorylation at Ser15 upon PHD1 silencing reduces p53 binding to XBP in the NER complex upon chemotherapeutic challenge, thereby increasing DNA damage and consequently cell death upon cytostatic insult (Fig 7H).

Discussion
Chemotherapy remains the most widely used cancer treatment. In the past, much attention has been paid on the mechanisms underlying chemotherapy resistance. In our study, we aimed to investigate the interplay between PHDs and p53 and their potential role in the response of CRC to chemotherapy. The model we propose relies on PHD1 hydroxylase function allowing p38α-dependent phosphorylation of p53 in response to cytostatic damage in colorectal cancer cell lines. As a consequence, p53 can interact with the NER machinery and more specifically with XBP. This promotes DNA repair and results in the resistance toward the cytostatic effects of chemotherapy (left panel). On the other hand, when PHD1 is lacking, the p53-kinase p38α can no longer bind, and therefore, p53 phosphorylation upon chemotherapy treatment is prevented. It follows that p53 is not any longer able to bind XBP, thereby impairing the DNA repair capacity linked to p53 activity. This leads to increased DNA damage and cell death in response to the chemotherapeutic treatment (Fig 7H, right panel).

With these findings, we are adding another layer of complexity to the role of PHD1 in cancer. Previous reports have shown that murine PHD1, when overexpressed in colorectal cancer cells, can decrease tumor growth through the reduction of HIF-1α and VEGF (Erez et al, 2003). On the other hand, a reduction in PHD1 levels in breast cancer can hinder tumor growth due to the accumulation of FOXO3a and consequent suppression of cyclin D1 eventually leading to a decreased proliferation (Zhang et al, 2009; Zheng et al, 2014). In non-cancerous cells, the situation is even more complex with increased HIF-2α and MYC-dependent proliferation in liver tissue of Phd1 KO mice after liver resection (Mollenhauer et al, 2012), but decreased proliferation upon PHD1 silencing in HeLa ovarian cells because of reduced hydroxylation of the centrosome component Cep192 (Moser et al, 2013) or eventually decreased enterocyte apoptosis in Phd1 knockout mice affected by colitis (Tambuwala et al, 2010). In our current study, we uncover that PHD1 silencing does not affect the colony formation capacity and tumor growth under basal conditions, but it increases sensitivity of CRC cells toward the cytostatic effects of chemotherapeutic drugs such as 5-FU, SN-38, or oxaliplatin in vitro and in vivo. These effects are independent of HIF signaling as proven by: (i) direct interaction of PHD1 and p53, (ii) PHD1-mediated p53 regulation, (iii) the absence of detectable HIF-2α levels, (iv) the lack of modulation of HIF-1α by PHD1 silencing, and (v) reduced phosphorylation at Ser15 of p53 upon 5-FU treatment after silencing of PHD1 even in the absence of HIF-1α or HIF-2α. Together with our experiments in p53−/− HCT116 cells supporting the p53 dependency of the effects on DNA damage, apoptosis, and colony formation upon PHD1 regulation, these findings exclude the possibility that the previously shown interplay between HIFs and p53 (Sermeus & Michiels, 2011) can take part in the mechanism here described.

With our work, we show that PHD1 binds to p53 and that its hydroxylase function is required for the effects observed on p53 phosphorylation. This would suggest that indeed, p53 could be hydroxylated. The direct binding of p53 to PHD1 observed in vitro supports this hypothesis, as no other intermediate proteins are required for the interaction. Hereby, prolyl hydroxylation would represent a new posttranslational modification in the p53 field that adds to the long list of already known posttranslational p53 modifications (Bode & Dong, 2004). So far it was believed that the phosphorylation of p53 at Ser15 was one of the initial steps of p53 activation (Bode & Dong, 2004; Meek & Anderson, 2009). Our data would however suggest that binding of PHD1 to p53 and potential hydroxylation of p53 precede its phosphorylation adding specificity to the phosphorylation process of p53 and directing p53 activity toward DNA repair and cytostatic signaling.
When looking further downstream in the p53 pathway, our results suggest that a reduction in p53 phosphorylation at Ser15 without altering the total p53 levels affects specifically the transcription-independent DNA repair pathway of p53. Indeed, transcription of some important p53 target genes such as CDKN1A, MDM2, and PUMA is not altered upon PHD1 silencing after 5-FU treatment. This is in line with some previous evidence showing that p53 phosphorylation is not the main determinant of p53 transcriptional activity as p53 transcription can also be triggered by mere p53 stabilization without phosphorylation (Stommel & Wahl, 2004; Kruse & Gu, 2009). This could explain the lack in transcriptional differences observed in our model, but also uncovers a new role for p53 phosphorylation at Ser15 in NER. Previous findings have shown that p53 can positively influence NER and that p53 can do this independently of transcription through the binding with XPB and XPD (Sengupta & Harris, 2005; Chang et al., 2008). In our results, we did not observe binding between p53 and XPD, but observed a robust binding between XPB and p53 after 5-FU treatment, which was strongly reduced upon PHD1 silencing and thus upon reduced p53 phosphorylation at Ser15, which is followed by impaired DNA repair and resultant apoptosis. Despite that NER has already been involved in resistance to chemotherapy (Dabholkar et al., 2000; Reed et al., 2003; Bohanes et al., 2011), to our knowledge, this is the first report showing that p53 phosphorylation can affect the DNA repair machinery, though further investigations will be required to explain how posttranslational modifications in p53 can elicit this effect.

Here, we also show that mutation of p53 in position R248, a hot spot mutation in CRC, recapitulates the effects seen with wild-type p53. So far, mutations in p53 have been considered to be inactivating and rendering p53 incapable to perform any of its regular functions (Muller & Vousden, 2013). However, our data clearly demonstrate that a p53 DNA contact mutant is still regulated by PHD1 and retains at least a partial DNA-repair-promoting activity. Therefore, potential blockage of PHD1 could also be clinically relevant in patients carrying DNA contact mutations in p53—which are frequently reported in CRC.

The clinical usage of PHD1-specific inhibitors has been already proposed in the context of breast cancer, ischemic liver disease, and colitis (Zhang et al., 2009; Schneider et al., 2010; Tambuwala et al., 2010). Particularly in CRC, specificity of PHD inhibitors is warranted because PHD2 inhibition has been shown to increase tumor growth and PHD3 blockage has been associated with a reduced apoptotic response to irradiation (Chan et al., 2009; Xie et al., 2012). With our data, we pave the opportunity to design and validate PHD1-specific inhibitors in colorectal cancer patients carrying wild-type or DNA contact mutant p53 aiming to increase their sensitivity to currently used chemotherapeutic treatments.

Materials and Methods

Cell culture

HEK293T human embryonic kidney cells, HCT116 human colon carcinoma cells (containing wild-type p53: p53wt/wt HCT116, further noted as HCT116; lacking p53: p53−/− HCT116 and p53R248/− HCT116, a kind gift from Prof. Karen Vousden, Glasgow), and the human colon cancer cell line LiM1215 (a kind gift from Prof. Sabine Tejpar, Leuven) were maintained at 37°C at 95% air and 5% CO2 in DMEM (Gibco, life technologies) supplemented with 10% fetal bovine serum (FBS, Gibco), 2 mM L-glutamine (Gibco), and 1% penicillin/streptomycin (PenStrep, Gibco). All cancer cell lines underwent mycoplasma testing before their use. Negative mycoplasma contamination status was verified using LookOut Mycoplasma PCR Kit (Sigma) and MycoAlert Mycoplasma Detection Kit plus Assay Control (Lonza). All cell lines were not maintained longer than 10 passages in culture to perform experimental procedures. Transfection of siRNA was performed with lipofectamine RNAiMAX (Life Technologies) according to the supplier’s protocol. Catalog numbers for the different siRNAs used can be found in Table 1.

Cell treatments

The chemotherapeutics 5-fluourouracil (5-FU, Sigma-Aldrich), 7-ethyl-10-hydroxycamptothecin (SN-38, Sigma-Aldrich), oxaliplatin (Selleck Chemicals), MG-132 (Calbiochem) as well as the PHD inhibitor dimethyloxaloylglycine (DMOG, Frontier Scientific) were prepared in DMSO stock solutions and diluted to their final concentration in DMEM (10% FBS, 2 mM L-glutamine, and 1% PenStrep). Treatment times and doses were used as indicated in the results.

Western blot

Protein extraction was performed using RIPA buffer [50 mM Tris pH 8, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate (SDS)] containing phosphatase (PhosSTOP Roche) and protease (Complete Roche) inhibitors. When the detection of apoptosis was required, the supernatants of the cells were also collected for Western blot analysis. The following antibodies were used for the detection of the proteins by immunoblotting: rabbit anti-PHD1 (Novus, NB100-310, 1/250 in 5% milk), mouse anti-p53 (Santa Cruz Biotechnology, DO1, 1/4,000 in 5% milk), rabbit anti-phospho-serine 15 p53 (Cell Signaling, #9284, 1/1,000 in 5% milk), mouse anti- vinculin (Sigma-Aldrich, V9131, 1/5,000 in 5% milk), mouse anti-caspase-3 (Cell Signaling, #9665, 1/500 in 5% milk), rabbit anti-cleaved parp (Cell Signaling, #5625, 1/1,000 in 5% milk), rabbit anti-PHD1 (Novus, NB100-310, 1/250 in 5% milk), mouse anti-p53 (Santa Cruz Biotechnology, DO1, 1/4,000 in 5% milk), rabbit anti-p53 (Santa Cruz Biotechnology, FL-393, 1/1,000 in 5% milk), mouse anti- vinculin (Sigma-Aldrich, V9131, 1/5,000 in 5% milk), rabbit anti-caspase-3 (Cell Signaling, #9665, 1/500 in 5% milk), rabbit anti-phospho-serine 15 p53 (Cell Signaling, #9284, 1/1,000 in 5% BSA), rabbit anti-ph2AX (Cell Signaling, #2577, 1/1,000 in 5% BSA), rabbit anti-cleaved parp (Cell Signaling, #5625, 1/1,000 in 5% BSA), rabbit anti-p38 (Cell Signaling, #9212, 1/1,000 in 5% BSA), and rabbit anti-XPB (Santa Cruz Biotechnology, sc-293, 1/500 in 5% milk). Secondary goat anti-mouse and goat anti-rabbit antibodies directly conjugated to horseradish peroxidase were used (Santa Cruz Biotechnology, 1/4,000 in 5% milk), and blots were developed using ECL (Life Technologies) or Super Signal West Femto (Thermo Scientific) with a CCD camera (ImageQuantTM LAS 4000). All Western blot experiments were repeated at least three times in independent experiments, and uncropped figures can be found in the Source Data.

Co-immunoprecipitation

Co-immunoprecipitation of PHD-Flagged proteins in HEK293T cells was performed by lysing the cells in RIPA buffer. After
pre-clearing of 1 mg of protein lysate with 30 μl of non-conjugated Sepharose beads (GE Healthcare life sciences), 30 μl of ANTI-Flag<sup>®</sup> M2 Affinity Gel (Sigma-Aldrich, pre-blocked with 0.05% BSA) was added to the pre-cleared lysates. After a 2-h incubation at 4°C, the bead–sample complexes were washed three times with RIPA buffer. Afterward, 20 μg of Flag peptide (Sigma-Aldrich) diluted in 80 μl of TBS buffer [50 mM Tris–HCl pH 7.4, 150 mM NaCl, containing protease inhibitors (Complete Roche)] was added to the pre-cleared lysates. After a 2-h incubation on a rotating wheel at 4°C, the eluates were analyzed by immunoblot as described above. Finally, the eluted proteins were analyzed by immunoblot as described above with the following antibodies: 1/3,000 mouse anti-p53 (DOI Santa Cruz) in 5% milk.
and 1/200 sheep anti-PHD1 (200 µg/ml R&D Systems AF6394) in 5% BSA.

**Quantitative real-time PCR (qRT–PCR)**

RNA from cells was extracted using the Qiagen Mini kit following the manufacturer’s protocol. One microgram of RNA was reverse-transcribed to cDNA by using the Qiagen QuantiTect Reverse Transcription kit, according to the manufacturer’s protocol. Afterwards, the cDNA was diluted 10 times before further use. qRT–PCR was performed using commercially available primers for the studied genes (Table 2) and TaqMan Fast Universal PCR Master Mix (Applied Biosystems). In a total volume of 10, 2 µl of the diluted cDNA was added to 500 nM primers and Fast Master Mix and was pipetted in a 96-well MicroAmp plate (Applied Biosystems). This plate was analyzed on the 7500 Fast Real-time PCR system (Applied Biosystems). Gene transcription was presented as the number of mRNA copies of the gene of interest with respect to the β-actin copies in each sample.

**Immunocytochemistry**

HCT116 cells seeded on coverslips in 12-well format were stained according to the manual of the ApopTag® Fluorescein In Situ Apoptosis Detection Kit (Merck Millipore), and the apoptotic area was quantified by microscopic analysis with an Olympus BX41 microscope and CellSense imaging software. ApopTag analysis showing the average ± s.e.m. from three biological replicates wherefrom each time three fields were analyzed.

**Cell cycle analysis**

Supernatants derived from HCT116 cells were collected, and adherent cells were subsequently trypsinized, added to the supernatant previously collected, and centrifuged at 300 g for 5 min. After one wash with PBS, cells were fixed with 1 ml of 70% ethanol. Cells were incubated 2 h or overnight at 4°C, prior to another centrifugation at 300 g for 5 min. Supernatant was removed and the pellet was resuspended in 200 µl of PBS, containing 500 µg of RNase (10 mg/ml). About 200 µl of propidium iodide (0.1 mg/ml) was added to a final volume of 400 µl. Samples were incubated for 1–2 h at 37°C and subsequently analyzed by fluorescence-activated cell sorting by using FACS Canto II (BD Bioscience). FACS analysis was performed on three independent experiments wherefrom a representative experiment is shown.

**ELISA nucleosome detection**

The nucleosome detection was performed by using the Roche Cell Death Detection ELISA Plus according to the manufacturer’s protocol. ELISA was performed in two independent experiments with three biological replicates each, wherefrom one representative experiment is shown.

**Stable cell line generation**

Full-length Flag-tagged wild-type and different proline mutant forms of p53 were generated by Genscript in a lentiviral vector where p53 is under the RSV promoter as described before (Kaeser et al, 2004). A small amount of lentivirus was used to transduce the p53−/− HCT116 cells in order to obtain one copy of the lentiviral vector/cell. Selection with puromycin (Sigma-Aldrich) was then performed to achieve a homogeneous population.

**In vitro kinase assay**

The Flag-tagged p53 proteins were immunoprecipitated from whole cell extracts dissolved in lysis buffer [50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 2 mM EDTA, 2 mM EGTA, 10 mM Na4P2O7, 1 mM PMSF, and complete protease inhibitor cocktail tablet (Roche)]. To recover Flag p53 immunoprecipitates, 500 µg of total protein was incubated with 2 µg Flag antibody for 1 h at 4°C before Sepharose beads (30 µl per reaction mixture) were added for 12 h. After washing with lysis buffer, the immunocomplexes were washed twice with kinase assay buffer (25 mM MOPS pH 7.2, 25 mM MgCl2, 5 mM EGTA, 2 mM EDTA, 250 µM DTT, 6 mM β-glycerophosphate). Then, the samples were incubated in 10 µl kinase assay buffer containing 20 ng of active, recombinant full-length p38 (SignalChem), and 250 µM ATP at 30°C for 25 min. Thereafter, proteins were denatured by incubating with 4× SDS sample buffer at 95°C for 10 min. Following separation of the proteins by 10% SDS–PAGE, the gel was blotted onto a nitrocellulose membrane, and proteins were analyzed as described before. Experiment was performed twice with the image depicted being a representative of both independent experiments.

**Colony formation assay**

p53wt/wt and p53−/− HCT116 transduced with a doxycycline-inducible shScr or shPHD1 construct were treated for 24 h with 1 µg/ml doxycycline (Sigma-Aldrich). Following treatment for 8 h with 300 µM 5-FU or control, the cells were detached and seeded at a cell density of 2,000 cells/well in culture medium containing 1 µg/ml doxycycline. Afterward, colony formation capacity was followed over time and medium was changed every 2–3 days to maintain doxycycline treatment. Colonies were stained with crystal violet and analyzed with ImageJ. Experiments are performed three times, and analysis is a representative experiment.
The paper explained

Problem
Resistance to chemotherapy remains a major clinical issue in the treatment of colorectal cancer (CRC) and other cancers. Response rates in patients with metastatic CRC have already improved to about 30–40% over the past years with the introduction of the FOLFOX and FOLFIRI treatment regimens. However, there is still much room for improvement and thus further research on potential candidates causing chemoresistant disease.

Results
We demonstrate that PHD1 mediates CRC resistance against 5-FU, irinotican, and oxaliplatin by favoring p53 phosphorylation. This post-translational modification of p53 allows the interaction between p53 and XBP, a component of the nucleotide excision repair machinery, ultimately leading to the resolution of DNA damage caused by these genotoxic drugs which enables cancer cells to survive and proliferate. By inhibiting PHD1 in CRC cells carrying wild-type or DNA contact mutant p53, we show increased cell death in response to chemotherapy and improved disease outcome in mouse models of CRC.

Impact
Our data support the design, validation, and use of PHD1-specific inhibitors in colorectal cancer patients carrying wild-type or DNA contact mutant p53, with the aim to increase sensitivity to currently used chemotherapeutic treatments.

Tumor experiments
NMRI nude (Harlan) mice, 6-8-week-old males with a weight between 30 and 40 g, were maintained under SPF conditions with free access to water and food pellets in cages with 4–6 mice/cage. Mice were injected subcutaneously in the flank with 3 free access to water and food pellets in cages with 4–6 mice/cage. Mice were injected subcutaneously in the flank with 3 × 10⁶ cells of the stably transduced p53wt/wt HCT116 p53wt−/− HCT116 shScr or shPHD1 cell lines. Tumors were measured in a blinded manner with a caliper, and tumor volume was calculated using the formula: \[ V = \pi \times (d^2 \times D)/6, \]
where \( d \) is the minor tumor axis and \( D \) is the major tumor axis. When tumors reached a volume of 200 mm³, mice were given access to drinking water containing 1 mg/ml doxycycline in H₂O containing 5% sucrose until the end of the experiment. Two days later, when tumors reached approximately 250 mm³, the mice were randomly assigned to receive either treatment with 100 mg/kg (i.p.) or a control solution was started. Afterwards, mice were treated twice more at days 7 and 14 after treatment start. Eighteen hours after the last dose of 5-FU, mice were euthanized. Housing and all experimental animal procedures were approved by the Institutional Animal Care and Research Advisory Committee of the KU Leuven (P096-2012) and reported according to the ARRIVE guidelines. The experiments were performed two times, with the experiment shown being a representative experiment.

Statistics
Data entry and all analyses were performed in a blinded fashion. All statistical analyses were performed using GraphPad Prism software. Statistical significance was calculated by two-tailed unpaired t-test on two experimental conditions or two-way ANOVA when repeated measures were compared, with \( P < 0.05 \) considered statistically significant. Exact \( P \)-values are indicated in the figure legends, except when \( P < 0.0001 \) as prism does not provide an exact \( P \)-value below this point. Data were tested for normality using the D’Agostino–Pearson omnibus test (for \( n > 8 \)) or the Kolmogorov–Smirnov test (for \( n \leq 8 \)) and variation within each experimental group was assessed. Detection of mathematical outliers was performed using the Grubbs’ t-test in GraphPad. Sample sizes for all experiments were chosen based on previous experiences, and \( n \)-numbers given in the figure legends always represent biological replicates. All graphs show mean values ± s.e.m.

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Author contributions
SD performed experimental design, all experiments, acquisition of data and analysis, interpretation of all data and wrote the manuscript. SL and SZ performed all MS design and analysis. GDC, RM-P, SH, LB, PR, HP and MS performed and analyzed experiments. DM and TK performed and analyzed kinase assays. ODKM, CM and KHV performed optimization for immunoprecipitation experiments. JM provided purified recombinant PHD1. MM performed experimental design, analysis of data, conducted scientific direction, and wrote the manuscript.

Conflict of interest
The authors declare that they have no conflict of interest.

References
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Dabholkar M, Thornton K, Vionnet J, Bostick-Brunton F, Yu JJ, Reed E (2000) Increased mRNA levels of xeroderma pigmentosum complementation group B (XPD) and Cockayne’s syndrome complementation group B (CSB) without increased mRNA levels of multidrug-resistance gene (MDR1) or metallothionein-II (MT-II) in platinum-resistant human ovarian cancer tissues. *Biochem Pharmacol* 60: 1611 – 1619


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