Identification of novel DNA-damage tolerance genes reveals regulation of translesion DNA synthesis by nucleophosmin

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Cells cope with replication-blocking lesions via translesion DNA synthesis (TLS). TLS is carried out by low-fidelity DNA polymerases that replicate across lesions, thereby preventing genome instability at the cost of increased point mutations. Here we perform a two-stage siRNA-based functional screen for mammalian TLS genes and identify 17 validated TLS genes. One of the genes, NPM1, is frequently mutated in acute myeloid leukaemia (AML). We show that NPM1 (nucleophosmin) regulates TLS via interaction with the catalytic core of DNA polymerase-η (polη), and that NPM1 deficiency causes a TLS defect due to proteasomal degradation of polη. Moreover, the prevalent NPM1c+ mutation that causes NPM1 mislocalization in ~30% of AML patients results in excessive degradation of polη. These results establish the role of NPM1 as a key TLS regulator, and suggest a mechanism for the better prognosis of AML patients carrying mutations in NPM1.
The high abundance of DNA damage has led to the evolution of a variety of DNA-damage tolerance and repair pathways that minimize potential deleterious effects on DNA replication and gene expression, thereby preventing genome instability and a plethora of pathological conditions. Most of the damage is repaired by accurate DNA repair mechanisms, primarily during the G1 phase of the cell cycle. However, during S phase, DNA replication encounters DNA lesions that have escaped repair, or that were newly formed, leading to the arrest of replication forks and/or the formation of single-stranded gaps, which may further lead to the formation of double-stranded breaks (DSB) and genome instability. These replication obstacles are dealt with by DNA-damage tolerance, of which two main strategies are known: (1) translesion DNA synthesis (TLS), whereby specialized low-fidelity DNA polymerases replicate across the damaged DNA region in a process that is inherently error prone; and (2) homology-dependent repair in which the gap opposite the DNA lesion is filled-in by either physical transfer of the complementary strand or by using the latter as a template for gap-filling repair or template switch repair.

The importance of TLS is revealed by investigating one of them, nucleophosmin, encoded by the NPM1 gene. We show that NPM1 regulates TLS by protecting polζ from proteasomal degradation, and that a mutation results in elevated ultraviolet sensitivity, and 12 known TLS genes (FDR < 10%; Supplementary Table 1). Importantly, known TLS genes, but not other DNA repair pathways, were highly represented among the hits (Fig. 1d), suggesting that the XPA deficiency indeed enriched for genes involved in TLS.

The second screening stage was performed using a newly developed high-throughput TLS assay. It is a modification of an assay based on plasmids containing a site-specific DNA lesion opposite a gap, previously successfully used to study TLS. The high-throughput TLS system was utilized to screen 237 siRNA hits from the primary screen for involvement in TLS across each of two major ultraviolet irradiation-induced DNA lesions, namely thymine–thymine 6-4 photoproduct (TT 6-4 PP) and thymine–thymine cyclobutane pyrimidine dimer (TT CPD; Fig. 2c–e). Screening was performed in four independent biological replicas, exhibiting good reproducibility (Fig. 1b). Of note, the product of a successful TLS event in the Fluc system still carries the lesion on the non-transcribed strand, which might interfere with Fluc expression. To test this possibility, we constructed the expected TLS product, namely a fully double-stranded plasmid with the lesion on the non-transcribed strand, and found that its Fluc expression in XPA cells was essentially identical to that obtained with a control plasmid without the lesion (Supplementary Fig. 2a).

The high-throughput TLS system was utilized to screen 237 siRNA hits from the primary screen for involvement in TLS across each of two major ultraviolet irradiation-induced DNA lesions, namely thymine–thymine 6-4 photoprotein (TT 6-4 PP) and thymine–thymine cyclobutane pyrimidine dimer (TT CPD; Fig. 2c–e). Screening was performed in four independent biological replicas performed on different days and exhibited high reproducibility (Fig. 2f). To normalize for differential damage-independent knockdown effects on Fluc and Rluc expression, each siRNA was also tested using Fluc and Rluc plasmids both carrying gaps but no lesions (NLs), and the results served to correct the data (Supplementary Fig. 2b,c). TLS elevation or inhibition scores were calculated with respect to control siRNAs, and were subjected to statistical analysis (Supplementary Fig. 2d–f). This screen resulted in 47 novel hits and 12 known TLS genes (FDR < 10%; Supplementary Data 1 and Supplementary Table 1). Importantly, known TLS genes, but not other DNA repair pathways, were further enriched among the hits (Fig. 2h and Supplementary Table 2), indicating high specificity of the screening method towards the TLS pathway. The ATR signalling pathway was also enriched to some extent, in which two main strategies are known: (1) translesion DNA synthesis (TLS), whereby specialized low-fidelity DNA polymerases replicate across the damaged DNA region in a process that is inherently error prone; and (2) homology-dependent repair in which the gap opposite the DNA lesion is filled-in by either physical transfer of the complementary strand or by using the latter as a template for gap-filling repair or template switch repair.

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Figure 1 | Primary screen for ultraviolet sensitivity in NER-deficient human cells. (a) An outline of the primary screen. In brief, NER-deficient XPA cells were transplanted with the siRNA libraries on day 0, exposed to ultraviolet C (UV) irradiation (1 J m⁻²) on day 2 and analysed for cell viability on day 4. (b) Data reproducibility. Luminescence values of three biological replicas that were preformed on different days were plotted against each other. (c) Histogram describing ultraviolet-sensitivity fold change (FC) caused by the siRNAs (median values over the three replicas). Dashed red lines denote the distribution borders of negative control samples. (d) Enrichment of TLS, DNA repair and DNA-damage response pathways within the screen hits. Dashed red line corresponds to a hypergeometric test P value 0.05. NER, nucleotide excision repair; BER, base excision repair; MMR, mismatch repair; FA, the Fanconi anemia pathway; NHEJ, non-homologous end joining; HRR, homologous recombination repair; ATM and ATR, the two main DNA-damage response pathways. See also Supplementary Fig. 1, Supplementary Data 1 and Supplementary Table 1.

Functional validation of candidate TLS genes. To further validate the hits, a second version of the TLS assay was developed based on quantitative PCR (qPCR) readouts rather than gene expression (Fig. 3a). In brief, following TLS in mammalian cells, the plasmids were extracted under alkaline conditions, which denatured gapped plasmids but not the covalently closed products of TLS or the control gap-filling reactions. Remnants of gapped plasmids were digested with S1 single-stranded endonuclease. TLS efficiency was calculated as the ratio between the products of qPCRs that targeted the filled-in gap-lesion plasmid and the filled-in control gapped plasmid, and was normalized to control siRNA-treated samples (Fig. 3a). The qPCR-based TLS assay was utilized to examine the involvement of 10 selected hits across the major tobacco smoke-induced DNA adduct benzo[a]pyrene-guanine (BP-G), a non-ultraviolet lesion. Six hits, namely Papd7, Ruvbl2, Trip11, Npm1, Abh2 and Ube2e1, significantly affected TLS across both DNA lesions, although the effects obtained for Abh2 and Ube2e1 were rather small (Fig. 3b, t-test, P values < 0.05; knockdown efficiencies are shown in Fig. 3c). Cyld, Mmc3, Delre1a and Ercc1 affected TLS across TT CPD and two adducts formed by the sunlight-induced TT CPD and two adducts formed by the chemotherapy drug cisplatin, namely cisPt-GG and cisPt-GTG. As can be seen in Fig. 4a, in MEF cells in which Npm1 was knocked down, TLS across each of the three lesions was significantly reduced (two-tailed t-test, P values < 0.02), with the TT CPD being most affected. In contrast, knocking down Npm1 on polη-deficient PolH⁻/⁻ MEF cells did not significantly affect TLS across the TT CPD lesion (Supplementary Fig. 3a,b), further suggesting that the effect of NPM1 on TLS across TT CPD is mediated via polη. This effect was not indirectly mediated via APE1, the base excision repair endonuclease that is regulated by NPM1, since knocking down the expression of Ape1 in the MEF cells had no effect on TLS across TT CPD (Supplementary Fig. 3c,d). Since NPM1 was reported to control the levels of POLH, we tested TLS across three lesions that are bypassed by polη: the sunlit...
NPM1 interacts with DNA pol\(\eta\) in the nucleus. We next examined the interaction between pol\(\eta\) and NPM1 by co-immunoprecipitation (co-IP) of the endogenous NPM1 with endogenous pol\(\eta\). As can be seen in Fig. 4b, immunoprecipitation (IP) of pol\(\eta\) with a specific antibody caused co-precipitation of NPM1. No NPM1 was observed when a control IgG was used (Fig. 4b, lane 2). To probe the subcellular compartment in which NPM1 and pol\(\eta\) interact, we resorted to the proximity ligation assay (PLA). As can be seen in Fig. 4c, interaction foci between pol\(\eta\) and NPM1 were clearly observed in human MRC5sv cells and localized mainly in the nucleus (the green spots in Fig. 4c upper panel, and quantified in Fig. 4d). These interaction foci were not observed in control XPV cells that lack pol\(\eta\) (Fig. 4c middle panel, and Fig. 4d), but appeared in XPV cells complemented with the POLH gene, encoding pol\(\eta\) (Fig. 4c lower panel, and Fig. 4d). The interaction foci appear to be distributed throughout the nucleus but were excluded from the nucleoli (Supplementary Fig. 5a). Similarly, in MRC5sv cells co-expressing green fluorescent protein (GFP)-tagged pol\(\eta\) and mCherry-tagged NPM1, GFP-pol\(\eta\) was distributed throughout the nucleus and in reduced amounts in the nucleoli (Supplementary Fig. 5b). Taken together, these results indicate that NPM1 interact with pol\(\eta\) in the nucleoplasm.

We next examined whether DNA-damage induction affects the interaction between NPM1 and pol\(\eta\). Exposure of the MRC5sv cells to ultraviolet irradiation resulted in a decrease in the amount of pol\(\eta\) while the total levels of NPM1 were not significantly altered (Fig. 4e, input blot). Interestingly, following ultraviolet irradiation the interaction between pol\(\eta\) and NPM1 was transiently lost, and reappeared at later time points, as detected by co-IP (Fig. 4e). Similarly, analysis by the PLA showed a transient loss of interaction between pol\(\eta\) and NPM1 following ultraviolet irradiation (Fig. 4f, quantified in Fig. 4g). Ultraviolet irradiation of MRC5sv cells co-expressing GFP-tagged pol\(\eta\) and mCherry-tagged NPM1 resulted in recruitment of pol\(\eta\) but not of NPM1 into replication foci (Supplementary Fig. 5b), further supporting the observation that NPM1 releases pol\(\eta\) following ultraviolet irradiation. To identify the region of pol\(\eta\) that interacts with NPM1, a series of FLAG-pol\(\eta\) deletion mutants was constructed and expressed in HEK293 cells. While IP of

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**Figure 2 | Secondary functional TLS screen.** (a,b) The plasmids used for the secondary TLS screen: A Flu- expressing gapped plasmid carrying a site-specific DNA lesion on the non-transcribed strand and a gap on the transcribed strand (a) and a control RLuc-expressing gapped plasmid without a lesion (b). (c) An outline of the secondary TLS screen. In brief, XPA cells were transfected with the siRNAs on day 0 and with the reporter plasmids on day 2. Fluc and RLuc signals were measured on day 3. (d,e) Histograms describing TLS FC effects caused by the siRNAs (median values over four replicas that were performed on different days). Dashed red lines denote the distribution borders of negative control samples. Data from the TT 6-4 PP TLS screen is presented in (e), and that from the TT CPD TLS screen in (f). (g) Luminescence values of the four biological replicas plotted against each other from the TT 6-4 PP screen (a). (h) Enrichment of TLS, DNA repair and DNA-damage response pathways within the screen hits. The dashed red line corresponds to a hypergeometric test \(P\) value 0.05. See also Supplementary Fig. 2, Supplementary Data 1 and Supplementary Tables 1 and 2.
NPM1 resulted in co-precipitation of FLAG-pol\(\eta\) mutants lacking its regulatory carboxy terminus (Fig. 4h lanes 8–9, and Fig. 4i), it failed to co-precipitate the FLAG-pol\(\eta\) mutants lacking the catalytic core of the polymerase (Fig. 4h lanes 2–7, and Fig. 4i). This suggests that NPM1 interacts with the catalytic core of pol\(\eta\).

**NPM1 protects DNA pol\(\eta\) against proteasomal degradation.**

To examine whether NPM1 affects pol\(\eta\) stability, we measured by flow cytometry the level of ectopically expressed GFP-POLH in MRC5sv cells that stably express this construct. Knocking down NPM1 in these cells resulted in a threefold decrease in the expression of the GFP-pol\(\eta\) construct (Fig. 5a, median values). When cells expressing GFP-RAD18, a gene fusion of the main E3 ligase responsible for monoubiquitination of PCNA were analysed, no reduction in RAD18 expression was obtained following knockdown of NPM1 (Supplementary Fig. 6), which is in agreement with the functional PCNA ubiquitination in these cells. Next, we tested whether NPM1 regulates the protein level of endogenous pol\(\eta\). Knocking down the expression of NPM1 in XPA cells caused a significant fourfold decrease in the amount of the NPM1 protein (Fig. 5b, lanes 1 and 2). A similar result was obtained with the MRC5sv cells (3.4-fold decrease; Fig. 5c, lanes 1 and 2). Under these conditions, the amount of pol\(\eta\) was significantly reduced both in the XPA cells (3.8-fold decrease; Fig. 5b, lanes 1 and 2) and the repair-proficient MRC5sv cells (2.9-fold decrease; Fig. 5c, lanes 1 and 2), without a significant change in POLH mRNA level, as measured by qPCR (Fig. 5d). A similar effect was observed in ultraviolet-irradiated cells (Fig. 5b lanes 3 and 4, and Fig. 5c lanes 3 and 4, corresponding to XPA and MRC5sv cells, respectively). Importantly, ectopic expression of an siRNA-resistant NPM1 construct rescued the reduction in pol\(\eta\) caused by the silencing of endogenous NPM1 (Fig. 5e). Thus, a reduction in the amount of NPM1 causes a decrease in the amount of endogenous pol\(\eta\) at the post-transcriptional level. A similar reduction was also observed with pol\(\eta\), which is another TLS DNA polymerase, but not with pol\(\theta\), the closest homologue of pol\(\eta\) in mammalian cells (Fig. 5f). Inhibiting the proteasome using MG132 caused an increase in pol\(\eta\) levels in cells pre-treated with siRNA against NPM1, but not in those treated with control siRNA (Fig. 5g). This indicates that NPM1 has an important function in maintaining the stability of pol\(\eta\), and protecting it from proteasomal degradation. The E3 ubiquitin ligases PIRH2 and MDM2 were implicated in the proteasomal degradation of pol\(\eta\). Nevertheless, knocking down each of these enzymes or both, in addition to knocking down NPM1, did not rescue the pol\(\eta\) level (Supplementary Fig. 7a,b), suggesting that the degradation of pol\(\eta\) upon NPM1 deficiency is not mediated solely by PIRH2 or by MDM2.

**NPM1c+ mutations phenocopy the deficiency in DNA pol\(\eta\).**

The C terminus of NPM1 is mutated in \(~30%\) of all AML patients, resulting in loss of the nucleolar localization signal and the generation of a de novo nuclear export signal. This leads to mislocalization of mutant NPM1 (designated NPM1c+), and partially of wild-type (wt) NPM1 interacting with it, in the cytoplasm.\(^{40,43}\) These mutations strongly correlate with better response of patients to chemotherapy and better clinical outcome.\(^{40,44}\) Because of the role of NPM1 in stabilizing pol\(\eta\) in the nucleus, we hypothesized that mislocalization of NPM1 to the cytoplasm might cause destabilization of pol\(\eta\), similar to the effect that we have observed in cells in which NPM1 was knocked down. To this end, we examined pol\(\eta\) levels in OCI/AML2 and OCI/AML3 cell lines derived from AML patients, either without or with the NPM1c+ mutation, respectively. As can be seen in Fig. 6a, the amount of pol\(\eta\) was much lower in the NPM1c+ AML cell line compared with the NPM1wt AML cell line, whereas the POLH mRNA levels were similar (Fig. 6b). Consistently with the lower pol\(\eta\) amount, TLS across a TT CPD was lower (two-tailed \(t\)-test, \(P\) value <0.003), and more mutagenic (\(\chi^2\)-test, \(P\) value <0.01) in the NPM1c+ AML cells compared with the NPM1wt AML cells (Fig. 6c). Proteasome inhibition using

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**Table 1 | Summary of the novel TLS genes identified by the 2-stage screen.**

| Gene symbol | Ultraviolet-sensitivity screen | 6-4 PP TLS screen | CPD TLS screen | Examined in MEFs | Function category |
|-------------|-------------------------------|------------------|---------------|----------------|------------------|
|             | Fold change | \(P\) value | Fold change | \(P\) value | Fold change | \(P\) value |             |               |                 |
| ALKBH2      | 2.2          | \(\times 10^{-5}\) | 1.5          | \(\times 10^{-4}\) | 0.9          | \(\times 10^{-1}\) | ✓             | Replication/repair |
| DCLRE1A     | 1.6          | 2.3\(\times 10^{-3}\) | 1.7          | 1.2\(\times 10^{-2}\) | 1.1          | 4.0\(\times 10^{-1}\) | ✓             |                 |
| ERCC4       | 3.2          | \(<1\times 10^{-5}\) | 2.8          | 1.3\(\times 10^{-5}\) | 1.2          | 1.8\(\times 10^{-1}\) | ✓             |                 |
| MCM3        | 2.2          | \(<1\times 10^{-7}\) | 1.6          | 6.0\(\times 10^{-3}\) | 1.5          | 7.0\(\times 10^{-3}\) | ✓             |                 |
| RUVBL2      | 2.7          | 8.0\(\times 10^{-7}\) | 1.7          | 1.1\(\times 10^{-3}\) | 1.3          | 7.5\(\times 10^{-2}\) | ✓             |                 |
| RPN1        | 2.4          | \(<1\times 10^{-7}\) | 1.5          | 3.4\(\times 10^{-3}\) | 1.1          | 3.2\(\times 10^{-1}\) | ✓             | Proteasomal degradation |
| SMURF2      | 1.6          | 2.1\(\times 10^{-4}\) | 1.8          | 8.6\(\times 10^{-6}\) | 1.5          | 2.8\(\times 10^{-3}\) | ✓             |                 |
| UBE2E1      | 1.5          | 1.1\(\times 10^{-3}\) | 3.0          | 5.3\(\times 10^{-7}\) | 2.5          | 1.2\(\times 10^{-6}\) | ✓             |                 |
| UBE2G2      | 0.7          | 1.3\(\times 10^{-3}\) | 1.7          | 3.4\(\times 10^{-4}\) | 1.1          | 2.1\(\times 10^{-1}\) | ✓             |                 |
| NPM1        | 1.7          | 3.8\(\times 10^{-6}\) | 1.8          | 4.4\(\times 10^{-3}\) | 1.6          | 2.9\(\times 10^{-3}\) | ✓             | Chaperon        |
| DNAJC6      | 1.5          | 2.4\(\times 10^{-3}\) | 1.7          | 3.2\(\times 10^{-4}\) | 1.1          | 2.5\(\times 10^{-1}\) | ✓             |                 |
| TRIP11      | 1.6          | 7.5\(\times 10^{-6}\) | 2.5          | \(<1\times 10^{-7}\) | 2.2          | \(<1\times 10^{-7}\) | ✓             | Structural Golgi |
| VCP/IP1     | 0.7          | 1.6\(\times 10^{-3}\) | 1.8          | 3.3\(\times 10^{-5}\) | 1.3          | 2.3\(\times 10^{-2}\) | ✓             | Other           |
| CYLD        | 1.6          | 8.7\(\times 10^{-5}\) | 2.3          | 1.5\(\times 10^{-4}\) | 1.3          | 8.3\(\times 10^{-2}\) | ✓             |                 |
| OTUB2       | 2.0          | 1.0\(\times 10^{-7}\) | 1.8          | 5.6\(\times 10^{-4}\) | 1.4          | 1.6\(\times 10^{-2}\) | ✓             |                 |
| PAPD7       | 1.4          | 2.0\(\times 10^{-2}\) | 1.5          | 1.0\(\times 10^{-3}\) | 1.1          | 1.9\(\times 10^{-1}\) | ✓             |                 |
| SENP2       | 1.7          | 1.3\(\times 10^{-6}\) | 1.1          | 2.5\(\times 10^{-1}\) | 1.6          | 2.7\(\times 10^{-3}\) |               |                 |

The table lists the 17 novel TLS genes identified by the two-stage screen, divided into functional categories.
MG132 rescued the level of pol\(Z\) in the NPM1c\(+\) AML cell line (Fig. 6d), indicating that pol\(Z\) is subjected to excessive proteasomal degradation, as was the case in cells in which NPM1 was knocked down. To further support the notion that it is the mislocalization of NPM1 in the AML cells that causes the pol\(Z\) deficiency, we treated the cells with Leptomycin B, an inhibitor of nuclear export via exportin 1, previously shown to prevent the exclusion of NPM1 from the nucleus\(^{46}\). As can be seen in Supplementary Fig. 8, NPM1 was present mainly in the cytoplasm in NPM1c\(+\) AML cells, but not in the NPM1\(wt\) AML cells, as expected. Upon addition of Leptomycin B, The NPM1c\(+\) AML cells exhibited nuclear localization of NPM1, similarly to the NPM1\(wt\) cells. Examining the level of pol\(Z\) under these conditions revealed that Leptomycin B treatment did not change the amount of pol\(Z\) in NPM1\(wt\) AML cells, but caused a significant increase in pol\(Z\) in the NPM1c\(+\) AML cells (Fig. 6e), consistent with a role of NPM1 in stabilizing pol\(Z\) in the nucleus.

Furthermore, ectopic expression of mCherry-NPM1c\(+\) in HEK293 cells that stably express GFP-pol\(Z\) caused a significant twofold decrease in the amount of GFP-pol\(Z\) as indicated by flow cytometry analysis (Fig. 6f,g). This effect was not observed when mCherry-NPM1\(wt\) or mCherry alone were expressed (Fig. 6h–k).
Taken together, these results establish a role for NPM1 in the regulation of TLS, and suggest that the prevalent NPM1c+ mutation results in reduced levels of pol\(\eta\), thereby leading to defective TLS.

**Discussion**

The multiplicity of TLS DNA polymerases and their low fidelity suggest the existence of a complex regulation to ensure their action at the right place and the right time. The molecular basis of

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**Figure 4 | NPM1 interacts with DNA pol\(\eta\) and regulates pol\(\eta\)-promoted TLS.** (a) NPM1 is required for effective TLS across site-specific TT CPD, or the cisplatin-induced site-specific lesions cisPt-GG, and cisPt-GTG in MEFs. Results of gapped plasmid-based, colony-forming TLS assays are presented as the mean \(\pm\) s.e.m. of three biological replicas. Two-tailed t-test, \(P\) values <0.02 for all tested lesions. (b) Co-IP of pol\(\eta\) and NPM1 in extracts from MRC5sv cells. (c) PLA of pol\(\eta\) and NPM1 in MRC5sv cells (upper panel), pol\(\eta\)-deficient XPV cells (middle panel) and XPV cells complemented with ectopic pol\(\eta\) expression (bottom panel). Blue, DAPI (4,6-diamidino-2-phenylindole) staining of the DNA in the nucleus; green, pol\(\eta\)-NPM1 interaction detected by the assay. White scale bars correspond to 10 \(\mu\)m. (d) Quantification of the interacting foci shown in (c), based on at least 140 cells of each type. Mean values \(\pm\) s.e.m. are presented. (e–g) Pol\(\eta\)-NPM1 interaction is transiently lost following ultraviolet irradiation. (e) Co-IP of pol\(\eta\) and NPM1 extracted from MRC5sv cells at different time points after ultraviolet irradiation with 10 J m\(^{-2}\). (f) PLA of pol\(\eta\) and NPM1 in unirradiated and ultraviolet irradiated cells. Blue, DAPI staining of the DNA in the nucleus; Green, pol\(\eta\)-NPM1 interaction. White scale bars correspond to 10 \(\mu\)m. (g) Quantification of the interacting foci shown in (f), based on at least 100 cells of each type. Mean values \(\pm\) s.e.m. are presented. (h) NPM1 interacts with the catalytic core of pol\(\eta\). NPM1 was precipitated from HEK293FT cells expressing a series of FLAG-POLH deletion mutants. Co-precipitation of the FLAG-POLH mutants is shown in the upper panel, whereas the expression of POLH mutants before precipitation is shown in the lower panel. (i) The functional domains of pol\(\eta\). PAD, polymerase-associated domain; PIP1 and PIP2, PCNA-interacting protein domains 1 and 2, respectively; UBZ, ubiquitin-binding zinc finger; NLS, nuclear localization signal. All blots are representative of three independent experiments. IB, immunoblotting; siCont, control siRNA. POLH FL, POLH full length.
Figure 5 | NPM1 protects DNA pol δ from proteasomal degradation. (a) MRC5sv cells stably expressing GFP-POLH were treated with siRNA against NPM1 or non-targeting control siRNA (siCont). GFP-polδ levels were quantified by flow cytometry. (b) Immunoblot of polδ extracted from human XPA cells (b) and MRC5sv cells (c) pretreated with siRNA against NPM1 or non-targeting siCont. When indicated, cells were ultraviolet-irradiated 15 h before extraction with 2 J m⁻² (b) or 10 J m⁻² (c). Upper and lower blots were done with the same extracts. (d) Polδ downregulation is not at the transcription level. qPCR of POLH and NPM1 mRNA levels from cells pretreated with siRNA against NPM1, normalized to non-targeting siRNA-treated cells. Mean values ± s.e.m. of three replicas are presented. (e) Immunoblot of polδ extracted from MRC5sv cells pretreated with siRNA against NPM1 and complemented by ectopic expression of an siRNA-resistant NPM1 construct. (f) Polδ, but not polη, is also affected by NPM1 knockdown. Immunoblots of polη and polδ extracted from MRC5sv cells pretreated with siRNA against NPM1, or non-targeting siCont. (g) Accumulation of polη following proteasomal inhibition. MRC5sv cells were treated with non-targeting siRNA or siRNA against NPM1 and then subjected to MG132 for the indicated time periods in order to inhibit proteasomal activity. Tubulin served as a loading control. All blots are representative of three independent experiments. Irad., irradiated.

the regulation that enables a low and tolerable mutation burden is only partially understood. We reasoned that given its complexity, TLS in mammalian cells is regulated by additional as yet unidentified genes, and searched for those using a two-stage functional siRNA screen. Having two functional assays, one of which was specific for TLS, and a subsequent stringent validation protocol that included analysis by additional TLS assays, was anticipated to reduce the number of false hits. The high selectivity of the screens for TLS genes is demonstrated by the high enrichment of known TLS genes within the hits, in contrast to other DNA repair pathways. The switch to mouse cells for the subsequent qPCR validation enabled us to gain more validation power by using different siRNA oligos to target the mouse genes, and by demonstrating that the hits are not cell type- or human-specific. It should be noted that the secondary gapped plasmid-based screen used in this study measures TLS outside the chromosomal context, and hence TLS genes that are associated with chromatin structure or replication fork progression are unlikely to be scored. Such TLS genes might have been detected in the primary ultraviolet-sensitivity screen. The E3 ligase RAD18 that mediates PCNA monoubiquitination was not scored in our screen, although we have previously shown that it is required for effective TLS in the gap-lesion plasmid assay. This might have been caused by inefficient knockdown of the gene. Our screening strategy had led to the identification of 17 new TLS genes, six of which, namely NPM1, SMURF2, UBE2E1, CYLD, OTUB2 and VCPPIP1, do not have obvious homologues in E. coli or S. cerevisiae. Of the 17 TLS genes, 10 were further examined by a transcription-/translation-independent qPCR version of the TLS assay in mouse cells, and at least four, namely CYLD, NPM1, PAPD7 and RUVBL2, also affected mutation rates across the BP-G lesion, further supporting their role in TLS. Most of our hits were detected when screening for TLS across the TT 6-4 PP lesion, with only few exceptions detected in the TT CPD screen, perhaps owing to the robustness of CPD bypass by the highly specialized polη. Virtually, all of our hits seem to have a positive role in TLS, with the exception of USP1, which is a known negative TLS regulator responsible for de-ubiquitination of monoubiquitinated PCNA.

Most of the new TLS hits can be classified into four functional categories, including DNA replication and repair genes, genes affecting proteasomal degradation, chaperons and Golgi-related genes (Table 1). The replication/repair category contains five genes, including the helicase subunit MCM3, which is involved in the formation of replication forks; ALKBH2, which is involved in a direct reversal repair pathway; DCLRE1A, which functions in the repair of DNA crosslinks; ERCC4, which function in HRR as well as in NER; and RUVBL2. Scoring those genes as regulators of
TLS suggests cross talk between DNA replication, repair and damage tolerance. Interestingly, the recruitment of DCLRE1A to sites of damage requires the ubiquitination of PCNA by RAD18 in a similar manner to TLS polymerases. Since TLS polymerases at sites of damage and was recently suggested to regulate DNA-damage tolerance.

The next two categories include four genes involved in protein degradation by the proteasome and two genes encoding chaperons, consistent with the importance of regulating the stability of TLS components. UBE2E1 and UBE2G2 are E2 ubiquitin ligases; SMURF2 is an E3 ubiquitin ligase; and RPN1 forms part of the regulatory subunit of the 26S proteasome. The chaperons group includes NPM1, which is discussed below, and DNAJC6, which belongs to the DNAJ/HSP40 family of chaperons, and promotes uncoating of clathrin-coated vesicles.

Figure 6 | The NPM1c+ mutation causes DNA polγ degradation and TLS deficiency. (a) Immunoblot of polγ extracted from OCI/AML2 and OCI/AML3 cells expressing wt NPM1 and NPM1c+ alleles, respectively. (b) qPCR of POLH and NPM1 mRNA levels from OCI/AML2 and OCI/AML3 cells. Mean values ± s.e.m. of three replicas are presented, two-tailed t-test, P value <0.003. Mutagenicity was addressed by sequencing 96 TLS events per cell line, χ²-test, P value <0.01. (d) Proteasomal inhibition rescued Polγ deficiency in NPM1c+ AML cells. Polγ levels in OCI/AML2 and OCI/AML3 cells pretreated with MG132 for the indicated time to inhibit proteasomal activity. Tubulin served as a loading control. (e) NPM1 nuclear retention rescued Polγ deficiency in NPM1c+ AML cells. Polγ levels in OCI/AML2 and OCI/AML3 cells pretreated with Leptomycin B (LMB) for the indicated time or its solvent as a control. Tubulin served as a loading control. (f-k) Density plots and histograms representing fluorescence-activated cell sorting analysis of HEK293 cells stably expressing GFP-polγ, and partially transfected with mCherry-tagged NPMc+ (f-g), wt NPM1 (h-i) and control mCherry (j-k). Data from mCherry-negative (untransfected) cells in (g-k) are in red and correspond to the left y axis, while that from mCherry-positive (transfected) cells are in blue and correspond to the right y axis. All blots are representative of three independent experiments. siCont, control siRNA.
Of note, the *Caenorhabditis elegans* homologue of DNAJC6 (w07a8.3) was previously scored as a hit in a mutagenesis screen, which further supports its involvement in TLS. The category of Golgi-related genes includes TRIP11 and VCP/P1. VCP/P1 is a deubiquitinating enzyme that mediates the reassembly of Golgi stacks after mitosis. Importantly, it also interacts with VCP/p97, which was recently found to regulate TLS. There are four additional TLS hits including two de-ubiquitination enzymes, namely OTUB2 and CYLD, the latter being a negative regulator of the NF-kB pathway; SENP2 that participates in SUMO signalling; and PAPD7 that is an RNA-specific ribonucleotidyl transferase that plays a role in the turnover of aberrant mRNAs. The new TLS genes, which belong to diverse pathways and functions, are likely to be useful for further analysis of TLS in mammalian cells and its interaction with other cellular pathways.

The finding that NPM1 is a regulator of TLS adds an unexpected dimension to the role of this multi-functional protein in preventing genome instability and uncovering a new key player of TLS regulation via pro-tosomal degradation. By protecting pol from degradation, NPM1 enables TLS to fulfill its function of overcoming replication blockades, thereby preventing DSB and genome instability. It was previously shown that two E3 ubiquitin ligases promote polyubiquitination of pol and target it for proteasomal degradation: MDM2 (ref. 59) and CRL4(CDT2) (ref. 60). In addition, PIRH2 targets pol to proteasomal degradation via a ubiquitination-independent pathway. Thus, the cellular levels of pol are tightly regulated. Human HSP90 was reported to facilitate the corrected folding of pol into its active form, and GEI-17 was shown to prevent the polyubiquitination and subsequent degradation of pol by CRL4(CDT2) following genotoxic stress in *C. elegans*. While GEI-17 is dispensable for pol stability at steady state, NPM1 is critical for maintaining a functional pool of pol in the absence of external DNA damaging agents. Thus, even if GEI-17 activity is conserved in mammals, it does not seem to overlap with the role of NPM1 in protecting pol stability, but rather work subsequently. The transient release of pol from NPM1 following ultraviolet irradiation might enable pol to reach damaged sites in the DNA through its affinity to monoubiquitinated PCNA and preform TLS, however, this dynamics of pol needs further investigation.

It was recently reported that pol is involved in the resistance to aRAC, a drug routinely used to treat AML, which exerts its therapeutic activity via incorporation into DNA and inhibiting replication. Thus, our results may explain, at least in part, the better prognosis and response to chemotherapy treatment of AML patients carrying the NPM1c+ mutation, which causes exclusion of NPM1 from the nucleus, leading to a pol deficiency, and therefore more effective killing of the leukaemic cells by drugs such as aRAC. Pol was recently implicated in the cellular response to doxorubicin treatment. Although we demonstrated that the NPM1c+ OCI/AML3 AML cells carry low levels of pol, they were reported to be more resistant to doxorubicin than OCI/AML2 cells. The sensitivity of the AML cells to doxorubicin is most likely governed by processes other than TLS such as DSB repair and apoptosis, which might also be regulated by NPM1. Such processes can mask the effect of pol, which might be secondary for this drug.

The results presented here underscore the critical role of NPM1 in maintaining genome stability, and highlight pol and NPM1, and in particular their interaction, as potential targets for development of drugs for AML therapy.

**Methods**

**Plasmids construction.** pGL4.13 and pGL4.73 reporter plasmids (Promega) served as backbones for the reporter gapped plasmids used in the screen. Restriction-free cloning was performed using PfuUltra II Fusion High-Fidelity DNA Polymerase (Stratagen). The following cloning steps were performed: (i) BsAI and BstXI restriction sites were mutated; (ii) the SV40 promoter was replaced with a CMV promoter obtained from pRL-CMV (Promega); (iii) the CMV promoter was relocated to the region between the CMV promoter and the reporter gene; and (iv) the AMP resistance cassette was replaced with a chloramphenicol resistance one, and was relocated in the fire cistron construct to the region between the CMV promoter and the reporter gene. The multiple-tagged NPM1 and NPM1c constructs were produced by fusing the mCherry coding region (Clontech) to the carboxyl terminus of the human NPM1 or NPM1c coding region. Flag-tagged pol truncations were produced by introducing the FLAG epitope in the amino terminus of the human POLH coding region, and deleting the indicated fragments by restriction-free cloning. siRNA-resistant human NPM1 construct was designed and introducing silence mutations in the sequence corresponding to the siRNA oligo.

**Ultraviolet-sensitivity screen.** Human XPA cells were reverse-transfected in 96-well plates with 25 nM siGenome SMARTpool siRNA libraries (Dharmacon), using Hiperfect transfection reagent (Qiagen). Following incubation of 2 days, cells were washed twice with Hanks’ buffer (Sigma) and irradiated in Hanks’ buffer at a dose of 1 J m⁻² ultraviolet C using a low-pressure mercury lamp (TUV 15W G15T8, Philips). Following irradiation, the cells were maintained under growth conditions for 48 h. Cell viability was determined using CellTiter-Glo (Promega). In order to reduce misinterpretation of toxic effects of certain siRNAs, each sample was normalized using its corresponding non-irradiated sample transfected with the same siRNA. Ultraviolet sensitivity was calculated with respect to control wells.

**TLS screen.** Human XPA cells were transfected with the siRNA hits from the ultraviolet-sensitivity screen at a concentration of 10 nM. The cells were incubated for 2 days, after which they were co-transfected with a mixture of 2.5 ng Fluc gap-lesion plasmid and 2.5 ng Rluc control gapped plasmid. Following incubation of 24 h, Fluc and Rluc signals were sequentially measured using the dual-luciferase reporter assay (Promega). TLS extent values were calculated as the ratio between Fluc and Rluc signals in respect to samples transfected with control siRNA, and were corrected for having two different reporters as described in the statistical analysis section. Exclusion of siRNAs-positive hits due to off-target effects of the siRNA was achieved by de-convolution of selected siRNA pools and testing each of the four siRNA oligos separately. Gap-lesion plasmids were constructed as described.

**Statistical analysis of the primary TLS-specific screen.** Viability was measured using luminescence readouts for a set of 1,100 siRNA pools with or without ultraviolet irradiation in three independent biological replicates. This can be represented by a non-parametric test (Fisher’s exact test). A fold change of 2 was used to select siRNAs.

\[
\text{Fold change} = \frac{\log_{2}(\text{no UV} / \text{UV})}{\log_{2}(\text{cont} / \text{UV})}
\]

\[
D_{\text{cont}} = I_{\text{cont}} - I_{\text{UV}}
\]

This represents the ultraviolet effect associated with a specific siRNA in a specific repeat with respect to the control ultraviolet effect. According to the null hypothesis, this statistic is distributed with zero mean and an intensity-dependent
under growth conditions for 3 days, after which they were lysed using RIPA buffer supplemented with protease inhibitor cocktail and phenylmethylsulfonyl fluoride (PMSF) (Sigma). For FCNA ubiquitination measurements, Triton-soluble fractions were extracted by incubating the cells for 5 min at 4°C in buffer A (100 mM NaCl, 300 mM sucrose, 3 mM MgCl₂, 10 mM PIPES (pH 6.8), 1 mM EGTA and 0.2% Triton X-100). Following removal of the buffer (Triton-soluble fraction), cells were rinsed once with cold PBS, and the adhering cellular material (Triton-insoluble fraction) was collected by scraping and then extracted by incubating at 37°C for 45 min in buffer B (same as buffer A but containing 50 mM NaCl and no Triton X-100) supplemented with 60 units DNase (Sigma). Protein concentration was determined using the BCA protein assay (Thermo Scientific). Protein extracts were fractionated by SDS–polyacrylamide gel electrophoresis, after which they were transferred to a polyvinylidene difluoride (PVDF) membrane and probed with antibodies specific for pol(8-7, Santa Cruz Biotechnology, diluted 1:500), pol(60,668, Novus, diluted 1:1,000), polkb (ab57070, Abcam, diluted 1:1,000), Npm1 (FC82291, Sigma, diluted 1:2,000), FLAG (M2, Sigma, diluted 1:1,000), APE1 (AB194, Abcam, diluted 1:2,000), and tubulin (DM1A, Sigma, diluted 1:20,000). Proteins were visualized using SuperSignal West Pico Chemiluminescent substrate (Pierce). Quantification was done by ImageJ software. Uncropped blots are presented in Supplementary Fig. 9.

### Co-immunoprecipitation.
Cells were rinsed twice in cold PBS and lysed in Triton lysis buffer (20 mM Tris-Cl, pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 10% glycerol and 1% Triton X-100) supplemented with protease and phosphatase inhibitors (Sigma) for 1 h at 4°C. Samples were pre-treated with protein Ag/PLUS agarose beads (Santa Cruz Biotechnology), and then incubated with 2 µg rabbit anti polk (H1930, Santa Cruz Biotechnology), 2 µg rabbit IgG (Santa Cruz Biotechnology) or 2 µg mouse anti FLAG (M2, Sigma) for 2 h, followed by adding protein Ag/PLUS agarose beads or Protein A/G MagBeads (Genscript) for additional 1 h. Beads were washed three times with lysis buffer, and two additional times with lysis buffer that contained also 250 mM NaCl. Beads were boiled in SDS buffer, and were taken for immunoblot analysis.

### Immunostaining and PLA.
The PLA assay was performed according to the manufacturer's instructions (OLINK Bioscience). In brief, cells were plated on 12 mm coverslips pre-coated with 10 µg/ml fibronectin (Sigma) and fixed with 3% paraformaldehyde. When indicated, cells were ultraviolet irradiated with 10 mJ·cm⁻² at time points before fixation. Permeabilization was done with 0.1% Triton X-100. Cells were blocked and then probed with rabbit anti polk (AB1728, Abcam, diluted 1:500) and mouse anti-NPM1 (FC82291, Sigma, diluted 1:500) Mouse antibody anti-FLAG (M2, Sigma) for 2 h, followed by adding protein Ag/PLUS agarose beads or Protein A/G MagBeads (Genscript) for additional 1 h. Beads were washed three times with lysis buffer, and two additional times with lysis buffer that contained also 250 mM NaCl. Beads were boiled in SDS buffer, and were taken for immunoblot analysis.

### Proteasome or nuclear export inhibition.
Proteasomal activity was inhibited by treating MRC5 or AML cells with 10 µM MG132 (Sigma) for 1–4 h. NPM1c + nuclear export was inhibited by treating AML cells with 20 nM Leptomycin B (Santa Cruz Biotechnology) for 3–7 h.

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Author contributions
O.Z., A.Z. and Z.L. designed the research; O.Z. and N.M.-N. performed the research; A.Z., O.Z., E.D., R.N., N.B.-C. and Z.L. analysed the data; U.S., N.E.G., T.C., S.S., C.E.C., M.P.M., R.R., and B.F. contributed new reagents/analytic tools; O.Z., A.Z., R.N., N.E.G., T.C., S.S., C.E.C., M.P.M., B.F., E.D. and Z.L. wrote the manuscript.

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