Regulation of the error-prone DNA polymerase Polκ by oncogenic signaling and its contribution to drug resistance

Kelsey Temprine1,2,4, Nathaniel R. Campbell1,2, Richard Huang1, Erin M. Langdon4,5, Theresa Simon-Vermot1, Krisha Mehta5, Averill Clapp6, Mollie Chipman1,2, Richard M White16

The DNA polymerase Polκ plays a key role in translesion synthesis, an error-prone replication mechanism. Polκ is overexpressed in various tumor types. Here, we found that melanoma and lung and breast cancer cells experiencing stress from oncogene inhibition up-regulated the expression of Polκ and shifted its localization from the cytoplasm to the nucleus. This effect was phenocopied by inhibition of the kinase mTOR, by induction of ER stress, or by glucose deprivation. In unstressed cells, Polκ is continually transported out of the nucleus by exportin-1. Inhibiting exportin-1 or overexpressing Polκ increased the abundance of nuclear-localized Polκ, particularly in response to the BRAFV600E–targeted inhibitor vemurafenib, which decreased the cytotoxicity of the drug in BRAFV600E melanoma cells. These observations were analogous to how *Escherichia coli* encountering cell stress and nutrient deprivation can up-regulate and activate DinB/pol IV, the bacterial ortholog of Polκ, to induce mutagenesis that enables stress tolerance or escape. However, we found that the increased expression of Polκ was not exclusively mutagenic, indicating that noncatalytic or other functions of Polκ could mediate its role in stress responses in mammalian cells. Repressing the expression or nuclear localization of Polκ might prevent drug resistance in some cancer cells.

INTRODUCTION

Errors in DNA replication can lead to increased mutation rates, thereby contributing to cancer pathogenesis. For example, somatic or germline mutations in the proofreading domain of DNA polymerase delta (Polδ) or epsilon (Pole) can lead to tumors with markedly increased numbers of point mutations (1–3). Aside from these two main replicative polymerases, a number of other DNA polymerases have been identified that may contribute to cancer initiation or progression (4). For example, inactivation of DNA polymerase eta (Ponh) is associated with xeroderma pigmentosum variant (XP-V), which predisposes patients to ultraviolet (UV)–induced skin cancers (5). In addition, DNA polymerase iota (Polι) is up-regulated in esophageal squamous cell cancer, and its expression levels positively correlate with lymph node metastasis/clinical stage (6). During the revision of this manuscript, a study identifying a role for multiple error-prone polymerases in resistance to targeted therapies, such as cetuximab, in colorectal cancer was published (7).

The roles of other DNA polymerases in this process are less well understood but likely could contribute to tumor progression. One such polymerase is DNA polymerase kappa (Polκ), which is a member of the Y-family of DNA polymerases that plays an essential role in the DNA damage tolerance process of translesion synthesis (8, 9). Several previous studies have shown that overexpression of Polκ can contribute to tumorigenesis and drug resistance in cancer (10–13). For example, overexpression of Polκ in glioblastoma cells increases resistance to the DNA-damaging agent temozolomide (13), and it has also been found to be substantially overexpressed in lung cancer (10).

Polκ can replicate DNA in both an error-free and error-prone manner during translesion synthesis (14). It can bypass thymine glycols in a relatively error-free manner (15), whereas it bypasses N2-acetylaminofluorene adducts in a more error-prone manner (16). When replicating on undamaged DNA, Polκ has a markedly high error rate due to a relatively large active site and lack of a proofreading domain (17). Using in vitro assays, it has been shown to have error rates as high as 1 error per 200 bp when replicating on undamaged DNA (18). For this reason, it is considered an “error-prone” polymerase that can induce untargeted mutations while acting either directly at the replication fork or by filling in postreplication gaps (19). The range of errors introduced by Polκ span virtually all substitutions, although to differing degrees (with a high rate of T → G substitutions), as well as a preponderance of deletions (17). These error rates are substantially higher than those found for the replicative polymerases Polδ and Polε.

In addition to these roles in DNA repair, recent data have also demonstrated that Polκ may have a noncatalytic function (20). Human lymphoblastic Nalm6 cells, which have intact p53 signaling and MSH2 activity, were engineered to express a catalytically dead (CD) D198A/E199A Polκ mutant, which completely lost all polymerase activity yet maintained normal protein expression. The CD mutant was then compared to complete knockouts (KO) or wild-type (WT) cells for their ability to protect against a panel of genotoxic stressors. Whereas the KO cells were highly sensitive to oxidizing agents such as hydrogen peroxide and menadione, the CD cells showed no such defects and were able to protect against these agents similar to WT cells. The mechanisms by which catalytically deficient Polκ may protect against such damage remain unclear, but it is posited to...
be due to its interactions with other proteins such as the DNA repair protein REV1 and/or play a role in the repair of oxidized deoxy-nucleotide triphosphates (dNTPs).

Given the diverse role of Polk, it is important that cells regulate both its expression and access to DNA. How Polk is regulated can be informed by several decades of work studying DinB, the *Escherichia coli* ortholog of Polk, which is regulated by the “SOS”/DNA damage response, along with the RpoS/starvation stress response (21, 22). Under stress conditions, *E. coli* can temporarily increase their mutation rate using a process known as stress-induced mutagenesis (22–25). This hypermutation is enacted as part of double-strand DNA break repair, which becomes mutagenic due, in part, to the activity of DinB (26). This mutagenic process is regulated at three levels, as recently reviewed (24): (i) a double-strand break (27, 28), (ii) activation of the SOS/DNA damage response (23), and (iii) activation of the generalized sigma S (RpoS) stress response (22). The SOS response, when coupled with the RpoS stress response, allows first for up-regulation of DinB (29) and then subsequent usage of this error-prone polymerase for mutagenic repair, which results in the base substitutions and indels that are commonly observed (22). It is likely that deficiencies in mismatch repair (MMR) contribute to this process since overexpression of MutL inhibits mutation in stationary phase but not during growth (30), whereas both MutS and MutH can be down-regulated, in part, by the RpoS stress response pathway (31).

In contrast to those in *E. coli*, the mechanisms regulating the expression and localization of Polk in mammalian cells are less well understood. In normal human tissues, Polk is widely expressed at the mRNA level (32), whereas in the mouse, it is highly enriched in the adrenal cortex and testis (33). In the mouse, protein expression using a peptide-generated antibody was noted in adrenal cortex, pachytcne cells in meiosis I, postmeiotic spermatids, and some epithelial cells in the lung and stomach (33). At the cellular level, numerous studies using overexpression of enhanced green fluorescent protein (EGFP)–Polk fusion proteins have demonstrated that Polk is strongly enriched in the nucleus (34, 35). However, antibody staining of endogenous Polk protein using antibodies generated with either peptide fragments or full-length proteins has shown variable expression in both the cytoplasm and the nucleus (33). Analysis of the Polk promoter has shown consensus binding sites for Sp1 and cyclic adenosine 3′,5′-monophosphate response element–binding protein (CREB), both of which have been reported to transcriptionally activate its expression (36, 37).

Although recent observations demonstrate that Polk can promote tumorigenesis and drug resistance (10–13), it is unknown what regulates its expression in cancer and to what extent this depends on its mutagenic properties. Ectopic expression of Polk allows it to become part of the replication machinery, even in the absence of external stress, indicating that high levels of it could be sufficient to induce new mutations, likely in concert with other alterations in DNA repair machinery (34). This has important clinical implications since dysregulation of Polk expression could therefore contribute to tumorigenic phenotypes by affecting its normal subcellular localization. In this study, we demonstrate that deprivation of oncogenic signaling in melanoma, lung cancer, and breast cancer cell lines up-regulates Polk and confines it to the nucleus. These pathways converge on phosphatidylinositol 3-kinase (PI3K)/mammalian target of rapamycin (mTOR) signaling, a central regulator of nutrient status in the cell (38) that may be analogous to the generalized stress factor RpoS in *E. coli* (24). When cells are nutrient replete and have intact mTOR signaling, Polk is primarily present in the cytoplasm; when cells are starved or mTOR is inhibited, Polk shifts primarily to the nucleus, suggesting that the cell can dynamically regulate Polk in response to cell stress. In line with this, we find that Polk can be rapidly exported back out of the nucleus by the nuclear export machinery, implying that cells may normally use nuclear export to prevent excess Polk in the nucleus. When this dynamic regulation is subverted by forced nuclear over-expression of Polk, we find that this increases resistance to the BRAFV600E inhibitor vemurafenib in melanoma cells. Unexpectedly, we find little evidence that Polk is highly mutagenic in these melanoma cells, indicating that it may play other currently unknown roles in inducing drug resistance. Our data suggest a mechanism by which mammalian cancer cells regulate the levels and localization of Polk, dysregulation of which can contribute to drug resistance.

### RESULTS

**MAP kinase inhibition induces POLK mRNA up-regulation and changes the subcellular localization of its protein**

Given the role of stress in up-regulating DinB/pol IV in *E. coli*, we first asked whether cell stress regulated Polk expression in cancer. We reasoned that drugs blocking oncogenic drivers would induce cell cycle arrest and, ultimately, apoptosis and would represent an extreme form of cell stress. In human melanoma, the most common activating mutation occurs at BRAFV600E, which activates downstream mitogen-activated protein kinase (MAPK) kinase (MEK) and extracellular signal–regulated kinase (ERK) signaling. This provides the melanoma cells with a substantial growth advantage. Small molecules targeting the BRAF/MEK/ERK pathway have been developed, some of which are being clinically used to treat melanoma patients (39–41). BRAF inhibitors such as vemurafenib can induce cell stress before overt death of the cancer cell, which can occur via the endoplasmic reticulum (ER) stress pathway (42) and is associated with stress-induced senescence (43). On the basis of this finding, we first asked whether inhibition of the MAP kinase pathway could induce Polk expression in melanoma.

To assess this, we treated the BRAFV600E mutant melanoma A375 cell line with the BRAFV600E kinase inhibitor PLX4032 (vemurafenib) (39) and measured the abundance of transcripts encoding Polk (*POLK* mRNA) by quantitative reverse transcription polymerase chain reaction (qRT-PCR) from 2 to 72 hours after exposure. This revealed an up-regulation in expression of Polk at the transcript level that peaked at 24 hours and was sustained thereafter (Fig. 1A). We next examined expression of Polk at the protein level under similar conditions. We used an antibody raised against full-length human Polk protein and verified its specificity using short hairpin RNA (shRNA) knockdown and overexpression of endogenous Polk (fig. S1, A to C). Although Polk has been largely reported to be nuclear localized when overexpressed as an EGFP-tagged fusion protein (34, 35), we unexpectedly found that the Western blot using an antibody raised against the full-length protein showed that endogenous Polk was expressed in both cytoplasmic and nuclear fractions (Fig. 1B). This expression pattern is similar to that of the related family member Polh, where both cytoplasmic and nuclear expression is seen [Human Protein Atlas (44)]. Whereas treatment with vemurafenib did not change overall protein levels of Polk, it instead induced a specific increase in nuclear Polk and corresponding
Actin or lamin-B1 served as the loading control. Mean of the Western blot data relative to the DMSO control.

**Different BRAFV600E mutant melanoma cell lines (A375 and SK-MEL28), whether downstream inhibitors would elicit similar effects. In two

| Fig. 1. Treatment of melanoma cells with BRAF or MAP kinase inhibitors modulates Polk expression and localization. (A) qRT-PCR to detect the mRNA expression of Polk relative to the DMSO control was performed on A375 cells treated with DMSO or 5 μM PLX4032 for 2, 8, 24, 48, or 72 hours. Mean ± SEM, n = 3 experiments; *P < 0.05, paired two-tailed t test. (B) Western blot analysis for Polk was performed on A375 cells treated with DMSO (−) or 5 μM PLX4032 (+) for 48 hours. (C) Quantification of the Western blot data relative to the DMSO control. β-Actin or lamin-B1 served as the loading control. Mean ± SEM from five to eight independent experiments; ns = nonsignificant, **P < 0.01, paired two-tailed t test. (D) Immunofluorescence staining of A375 cells treated with DMSO or 5 μM PLX4032 for 24 hours. Scale bars, 10 μm. (E) Quantification of the percentage of cells with nuclear enrichment of Polk. Mean ± SEM; at least 2500 cells were counted for each sample across 22 fields of view, ***P < 0.001, paired two-tailed t test. (F) Immunofluorescence staining of A375 cells treated with DMSO or ERK inhibitors (10 μM CI-1040 and 10 μM U0126) for 24 hours. Scale bars, 10 μm. (G) Quantification of the percentage of cells with nuclear enrichment of Polk. Mean ± SEM; at least 1000 cells were counted for each sample across 18 fields of view. ***P < 0.001, paired two-tailed t test. (H) Immunofluorescence staining of A375 cells treated with DMSO or ERK inhibitors (1 μM ulixertinib and 1 μM SCH772984) for 24 hours. Scale bars, 10 μm. (I) Quantification of the percentage of cells with nuclear enrichment of Polk. Mean ± SEM; at least 250 cells were counted for each sample across six fields of view. ***P < 0.001, paired two-tailed t test.

We confirmed this redistribution using immunofluorescence, wherein acute exposure to PLX4032 induced a shift of Polk from the cytoplasm to the nucleus (Fig. 1, D and E). Cytoplasmic localization of a DNA polymerase that can shift from the cytoplasm to the nucleus has been reported previously (45–47), suggesting that this mode of regulation might be relevant for Polk under physiologic conditions. Because BRAF activates downstream MEK/ERK pathway signaling, we also examined whether downstream inhibitors would elicit similar effects. In two different BRAFV600E mutant melanoma cell lines (A375 and SK-MEL28), we found that both MEK and ERK inhibitors (40, 41) produced an up-regulation of POLK mRNA and a nuclear shift of Polk that are similar to that seen after BRAF inhibition (Fig. 1, F to I, and fig. S2, A to D).

**DNA damage or cell cycle arrest is not sufficient for Polk localization**

The induction of DinB in bacteria relies upon two related systems: the SOS/DNA damage response and the RpoS-controlled general/starvation stress response. We reasoned that the nuclear localization
of Polk in response to MAP kinase inhibition might act through one of these two mechanisms. To test this, we treated A375 melanoma cells with vemurafenib and then checked for markers of DNA damage (48), including gamma H2AX (γH2AX), 53BP1, phosphorylated Chk1 (p-Chk1), and phosphorylated Chk2 (p-Chk2) but saw no induction of any of these markers (fig. S3A). We also tested whether knockdown of p53, which is a regulator of the DNA damage response pathway (49), would prevent the observed subcellular shift but failed to see any change in the nuclear localization of Polk after treatment with PLX4032 (fig. S3, B to D). Another possibility was that cell cycle arrest was the signal, as would be expected during a starvation response or during MAP kinase inhibition. To test this, we treated A375 melanoma cells with either the CDK4/6 inhibitor PD0332991 (50) or the BRAFV600E inhibitor vemurafenib and then assessed the cell cycle using flow cytometry and Pol differentiations led to a near-complete G1 arrest (fig. S4A); however, treatment using immunofluorescence. As expected, both of these interventions were specific to melanoma/the BRAF pathway or whether they could be activated by RAF/MEK/ERK signaling either directly or by cross-pathway activation (51–54), would prevent the observed subcellular shift but failed to see any change in the nuclear localization of Polk after treatment with PLX4032 (fig. S3, B to D). Another possibility was that cell cycle arrest was the signal, as would be expected during a starvation response or during MAP kinase inhibition. To test this, we treated A375 melanoma cells with either the CDK4/6 inhibitor PD0332991 (50) or the BRAFV600E inhibitor vemurafenib and then assessed the cell cycle using flow cytometry and Polk localization using immunofluorescence. As expected, both of these interventions led to a near-complete G1 arrest (fig. S4A); however, treatment with the CDK4/6 inhibitor resulted in little to no nuclear Polk (fig. S4, B and C). Together, these data suggested to us that other mechanisms might be responsible for the induction of Polk.

mTOR inhibition rapidly induces Polk nuclear accumulation

As we saw no effect of cell cycle inhibition or DNA damage, we next turned to mTOR signaling, a central sensor and effector of growth factor signaling, nutrient status, and stress (38). The mTOR pathway can be activated by RAF/MEK/ERK signaling either directly or by cross-pathway activation (51–54). In addition, BRAF inhibition with vemurafenib has recently been shown to induce the ER stress response (42, 55), which is associated with suppression of mTOR signaling (56). On the basis of this finding, we reasoned that MAP kinase inhibition might be acting to dampen downstream mTOR signaling to mediate the effect on Polk. To test this, we treated A375 melanoma cells with inhibitors of BRAF/MEK/ERK signaling for 24 hours and then measured mTOR pathway activation using levels of phosphorylated S6 (p-S6), a target of the kinase S6K (38, 57). We found that these MAP kinase inhibitors potently decreased levels of p-S6 (Fig. 2, A and B, and fig. S5, A and B) and that this inversely correlated with the shift of Polk to the nucleus: Cells with the lowest level of p-S6 had the highest levels of nuclear Polk (Fig. 2C). These observations prompted us to then test whether inhibitors of the mTOR pathway itself would lead to an effect on Polk. We treated A375 melanoma cells with several inhibitors of the PI3K/mTOR pathway, including mTOR inhibitors (rapamycin or PP242) or a PI3K inhibitor (LY29002). We found that these inhibitors potently induced nuclear Polk to a level comparable to that seen with the BRAF/MEK/ERK inhibitors, but it occurred much more rapidly (Fig. 2, D and E). Whereas the MAP kinase inhibitors took ~24 hours for full induction of nuclear Polk, the mTOR pathway inhibitors could do this in as little as ~6 hours. In addition, drugs that activate the ER stress pathway, which can be activated by BRAF inhibitors (fig. S6, A and B) as well as dampen mTOR signaling, produced a similar effect (fig. S6, C and D).

Polk dysregulation occurs in other cancer types

We next wished to determine whether the observed effects on Polk were specific to melanoma/the BRAF pathway or whether they could be activated by RAF/MEK/ERK signaling either directly or by cross-pathway activation (51–54). In addition, BRAF inhibition with vemurafenib has recently been shown to induce the ER stress response (42, 55), which is associated with suppression of mTOR signaling (56). On the basis of this finding, we reasoned that MAP kinase inhibition might be acting to dampen downstream mTOR signaling to mediate the effect on Polk. To test this, we treated A375 melanoma cells with inhibitors of BRAF/MEK/ERK signaling for 24 hours and then measured mTOR pathway activation using levels of phosphorylated S6 (p-S6), a target of the kinase S6K (38, 57). We found that these MAP kinase inhibitors potently decreased levels of p-S6 (Fig. 2, A and B, and fig. S5, A and B) and that this inversely correlated with the shift of Polk to the nucleus: Cells with the lowest level of p-S6 had the highest levels of nuclear Polk (Fig. 2C). These observations prompted us to then test whether inhibitors of the mTOR pathway itself would lead to an effect on Polk. We treated A375 melanoma cells with several inhibitors of the PI3K/mTOR pathway, including mTOR inhibitors (rapamycin or PP242) or a PI3K inhibitor (LY29002). We found that these inhibitors potently induced nuclear Polk to a level comparable to that seen with the BRAF/MEK/ERK inhibitors, but it occurred much more rapidly (Fig. 2, D and E). Whereas the MAP kinase inhibitors took ~24 hours for full induction of nuclear Polk, the mTOR pathway inhibitors could do this in as little as ~6 hours. In addition, drugs that activate the ER stress pathway, which can be activated by BRAF inhibitors (fig. S6, A and B) as well as dampen mTOR signaling, produced a similar effect (fig. S6, C and D).

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We next wished to determine whether the observed effects on Polk were specific to melanoma/the BRAF pathway or whether they could...
be applied more broadly. To test this, we examined POLK mRNA levels and subcellular localization in breast and lung cancer cell lines, which harbor unique oncogenic dependencies that differ from melanoma. PC-9 lung cancer cells harbor activating mutations in EGFR and are critically dependent on that growth factor signaling pathway. We treated PC-9 cells with either the EGFR inhibitor erlotinib (58) or the BRAFV600E inhibitor PLX4032 (which we would expect to have no effect as these cells contain WT BRAF) and then measured the expression of Polk and determined its localization. Both high and low doses of erlotinib caused induction of Polk mRNA and a shift of Polk to the nucleus in the PC-9 cells, but as expected, this effect was not seen with PLX4032 (Fig. 3, A and B, and fig. S7A). Analogous to what we observed for melanoma, this shift of Polk to the nucleus was accompanied by a loss of p-S6 in the lung cancer cells (Fig. 3, A and C). A similar effect was seen in a breast cancer cell line. SK-BR3 cells, which overexpress the HER2 oncogene, were treated with the HER2 inhibitor lapatinib (59) or the BRAFV600E inhibitor PLX4032. Lapatinib caused a marked induction of Polk expression, nuclear accumulation of Polk, and suppression of p-S6 (Fig. 3, D to F, and fig. S7B), an effect that was not seen with PLX4032 in this cell line as expected. These results demonstrate that the observed effects on Polk are specific to the driver oncogene and that they can be generalizable to multiple cancer types.

Glucose starvation phenocopies the effects on subcellular localization of Polk

mTOR could elicit the observed effect on Polk through multiple downstream mechanisms, but because E. coli use the starvation response as part of DinB up-regulation/activation, we wondered whether, by inhibiting mTOR, we were mimicking a starvation

![Image](http://stke.sciencemag.org/)

<ref>Fig. 3. The effects on Polk and p-S6 are driver gene specific across tumor types. (A) Immunofluorescence staining of PC-9 cells treated with DMSO, 5 μM PLX4032, or erlotinib (0.1 or 1 μM) for 24 hours. Scale bars, 10 μm. (B) Quantification of the percentage of cells with nuclear enrichment of Polk. Mean ± SEM; at least 100 cells were counted for each sample across four fields of view. ns = nonsignificant, ***P < 0.001, paired two-tailed t test. (C) Quantification of the percentage of cells with p-S6 (S240/244). Mean ± SEM; at least 100 cells were counted for each sample across four fields of view. ns = nonsignificant, ***P < 0.001, paired two-tailed t test. (D) Immunofluorescence staining of SK-BR3 cells treated with DMSO, 5 μM PLX4032, or lapatinib (0.1 or 1 μM) for 24 hours. Scale bars, 10 μm. (E) Quantification of the percentage of cells with nuclear enrichment of Polk. Mean ± SEM; at least 200 cells were counted for each sample across seven fields of view. *P < 0.05, ***P < 0.001, paired two-tailed t test. (F) Quantification of the percentage of cells with p-S6 (S240/244). Mean ± SEM; at least 200 cells were counted for each sample across seven fields of view. *P < 0.05, ***P < 0.001, paired two-tailed t test.</ref>
response. To test this idea, we examined the effect of nutrient deprivation on Polk localization. We grew A375 melanoma cells in a variety of media conditions in which we selectively removed serum, glucose, or glutamine and measured Polk localization by immunofluorescence. Whereas we found that both glutamine and serum deprivation had small effects on nuclear Polk localization, glucose starvation resulted in a highly significant induction of nuclear Polk (Fig. 5A). Because glucose is a major carbon source for rapidly growing cancer cells, this may be analogous to the starvation response induced in E. coli by deprivation of lactose, which is an important carbon source for bacteria.

**Exportin-1 plays a role in regulating the subcellular localization of Polk**

These data led us to ask what mechanisms the cells may use to control cytoplasmic versus nuclear localization of Polk. Two possibilities for the effects we observed were that they were mediated by either degradation of Polk or relocalization of Polk from the cytoplasm to the nucleus. A previous analysis of the protein structure of Polk demonstrated that the full-length protein contains a bipartite nuclear localization signal (NLS) towards the 3’ end of the gene, and computational analysis also indicated a likely nuclear export signal. Deletion of the NLS region results in the protein completely localizing to the cytoplasm, which suggested to us that the localization results we saw above might be controlled by the nuclear import and/or export machinery. To test this, we used inhibitors of importin-β or exportin-1 (CRM1) and tested whether these affected the shift of Polk in response to BRAF inhibition in melanoma cells. We found that importazole (61), which inhibits importin-β, had little effect on Polk localization (fig. S9, A and B). In contrast, cotreatment with leptomycin B (62), which inhibits exportin-1, accelerated the rate at which Polk localized to the nucleus: Whereas 3 hours of PLX4032 typically only leads to ~30% induction of nuclear Polk, in the presence of leptomycin B, this increased to 60% (Fig. 4, A and B). In addition, we noted that leptomycin B alone (even in the absence of PLX4032) induced a small but significant increase in nuclear Polk, indicating that Polk normally cycles between the cytoplasm and nucleus. To further test the contribution of the nuclear export machinery, we performed washout experiments. A375 cells were treated with PLX4032 to induce nuclear Polk and then the drug was washed away (Fig. 4C). After washout, Polk relocates to the cytoplasm within 24 hours. However, if leptomycin B is added after PLX4032 washout, Polk remains present in the nucleus (Fig. 4, D and E). In contrast, we found little evidence that PLX4032 directly affected Polk degradation. Treatment of A375 cells with the proteasome inhibitor MG132 (63) increased Polk protein levels to a similar extent in dimethyl sulfoxide (DMSO) or PLX4032-treated cells (fig. S10). Collectively, these data indicate that oncogenic signaling regulates the nuclear localization of Polk at least, in part, through the export machinery.

**Polk overexpression can lead to increased drug resistance but is minimally mutagenic on its own**

These data suggested a model in which rapidly growing cancer cells generally keep Polk at relatively low levels and sequestered in the cytoplasm due to active nuclear export. Previous work has shown that forced overexpression of Polk, which is predominantly nuclear, can contribute to temozolomide resistance in glioblastoma, which occurs by activation of ATM-CHK1 signaling, which promotes homologous recombination-dependent DNA repair (13). This led us to ask whether nuclear Polk might contribute to drug resistance in melanoma as well either through its mutagenic activity or through nonmutagenic capabilities (20). To test this, we generated a doxycycline-inducible Polk overexpression construct for use in A375 melanoma cells (Fig. 5A). We then isolated multiple single-cell clones from this population and expanded them in culture in the presence or absence of doxycycline for 3 months. As expected, these cells showed strong induction with increased nuclear localization of Polk after addition of doxycycline (fig. S1, B and C). We then tested both populations for sensitivity to either the BRAFV600E inhibitor PLX4032 or the CDK4/6 inhibitor PD0332991 (Fig. 5A). For both clones, the population that had experienced long-term Polk overexpression showed a modest increase in growth in PLX4032 across multiple different doses compared to its respective negative control cells (Fig. 5B), consistent with a mild increase in resistance to the drug. In contrast, there was no significant difference between the two populations with regard to their responses to PD0332991 (Fig. 5C).

To test whether this effect on drug resistance was likely mediated by new mutations, we used a mutation reporter assay (Fig. 6A), similar to (64). We stably overexpressed GFP in the doxycycline-inducible Polk melanoma cells and FACs (fluorescence-activated cell sorting)–sorted them to yield a population that is 100% GFP+. In the presence of a factor that increases mutation rate, stochastic mutation of the GFP transgene will lead to a loss of GFP expression due to random frameshifts/indels/single-nucleotide variants (SNVs). As a positive control, we used CRISPR to knock out the canonical MMR proteins MLH1, MSH2, MSH6, and PMS2. As expected, loss of the MMR proteins led to a loss of GFP-positive cells as measured by FACs (Fig. 6, B and C). In contrast, we found that overexpression of Polk failed to increase the mutation rate in this assay, even when combined with the defects in MMR (Fig. 6, B and C). To confirm this, we isolated DNA from the A375 cells and performed MiSeq analysis of this transgenic insertion to a depth of ~10,000×. This analysis failed to demonstrate any new mutations in the Polk-overexpressing clones, supporting the observation of the FACs readout. It is possible that Polk can only be fully mutagenic in concert with complete suppression of MMR. Our studies with individual MMR CRISPRs did not show synergy with Polk, but we did not attempt to knock out all MMR genes at the same time. Thus, while we cannot fully exclude other low-level mutagenic activity induced by Polk in these cells, these data suggest that Polk can contribute to BRAF inhibitor resistance in A375 melanoma cells but that mechanisms outside of its canonical ability to induce large numbers of new mutations may be contributory.

**Polk regulates genes associated with drug resistance and immune surveillance**

To gain further insight into the mechanisms by which Polk might regulate sensitivity to BRAF inhibitors, we performed CRISPR inactivation of Polk in A375 melanoma cells (fig. S1D) and measured their sensitivity to vemurafenib. This showed that at low doses of drug, knockdown of Polk enhanced sensitivity to vemurafenib, essentially the converse of what we observed with the overexpression of Polk (Fig. 7A). We then performed RNA sequencing (RNA-seq) of the Polk CRISPR cells and compared their gene expression profile to cells that had been treated with a nontargeting control single guide RNA (sgRNA). Overall, we found a total of 591 dysregulated genes (310 up-regulated and 281 down-regulated at a log2-fold change = 2.0 and Padj < 0.05; table S3). We then performed gene set
enrichment analysis (GSEA) on these genes to identify the most altered pathways (Fig. 7, B and C, and table S3). Among the most highly enriched pathways, we found that the Polk knockdown cells shared several gene signatures identified by Rambow et al. (65), as those associated with minimal residual disease after treatment with BRAF/MEK inhibitors, a state strongly associated with resistance to these therapies. For example, Rambow et al. identified both a “neuro” and an “immune” subtype of cell associated with resistance, and we found that the Polk CRISPR cells strongly dysregulated several key genes expressed in those cells, such as ANGPTL4, LOXL2, ADAMTS4, (70, 71), MSH2 (72), MSH6 (73), or PMS2 (74) are associated with Lynch syndrome, which predisposes patients to a wide variety of tumors including colon and gynecologic cancers (75, 76). Large-scale genome sequencing efforts have identified point mutations in the replicative polymerases Polδ or Polε, which inactivate the proofreading capacity of these proteins and induce a large number of mutations in the tumors that emerge (1–3). Several studies have now identified overexpression of Polk in human tumors such as lung cancer or glioblastoma (10, 11). Because Polk lacks a proofreading domain, its overexpression can be associated with resistance, and LICAM. We also found that the Polk knockdown cells dysregulated pathways associated with the response to oxidative stress (TRPM2, SNCA, and MMP3), which is consistent with the reports described above positing that the CD Polk may regulate drug resistance via repair of oxidized dNTPs (20). Further supporting this idea, we also identified numerous genes associated with the nucleotide patch pathway (SMUG1, TDG, and POLD4). In addition to these pathways, we also unexpectedly found a strong enrichment for immune-related genes in the Polk knockdown cells. For example, we found an up-regulation of genes associated with an immune signature in the The Cancer Genome Atlas (TCGA) cohort (FDSCP, MAGEA1, MAGEC1, and CXCL11) and, more specifically, an increase in those associated with signaling mediated by interferon-α/β (IFN-α/β), such as OASL and OASL, which are involved in the degradation of viral or cellular RNAs. This suggests a plausible idea that dysregulation of Polk (either by BRAF inhibition, starvation, or cellular stress more generally as we have shown) may influence the ability of tumor cells to respond to the immune system in vivo. Previous studies have suggested that error-prone polymerases such as Polλ or Polδ play a role in immune responses, but this is thought to be primarily via their effects on somatic hypermutation (66–69). It is also possible that some of the effects we see are due to the CRISPR itself, but whether there is an additional effect of Polk on regulation of immune surveillance is an intriguing idea that will need further experimental validation in future studies.

**DISCUSSION**

There have been multiple reports of how alterations to DNA replication and repair processes can contribute to tumor initiation and progression. For example, defects in MMR proteins such as MLH1 and MSH2 are associated with Lynch syndrome, which predisposes patients to a wide variety of tumors including colon and gynecologic cancers (75, 76). Large-scale genome sequencing efforts have identified point mutations in the replicative polymerases Polδ or Polε, which inactivate the proofreading capacity of these proteins and induce a large number of mutations in the tumors that emerge (1–3).

Several studies have now identified overexpression of Polk in human tumors such as lung cancer or glioblastoma (10, 11). Because Polk lacks a proofreading domain, its overexpression can be associated with resistance, and LICAM. We also found that the Polk knockdown cells dysregulated pathways associated with the response to oxidative stress (TRPM2, SNCA, and MMP3), which is consistent with the reports described above positing that the CD Polk may regulate drug resistance via repair of oxidized dNTPs (20). Further supporting this idea, we also identified numerous genes associated with the nucleotide patch pathway (SMUG1, TDG, and POLD4). In addition to these pathways, we also unexpectedly found a strong enrichment for immune-related genes in the Polk knockdown cells. For example, we found an up-regulation of genes associated with an immune signature in the The Cancer Genome Atlas (TCGA) cohort (FDSCP, MAGEA1, MAGEC1, and CXCL11) and, more specifically, an increase in those associated with signaling mediated by interferon-α/β (IFN-α/β), such as OASL and OASL, which are involved in the degradation of viral or cellular RNAs. This suggests a plausible idea that dysregulation of Polk (either by BRAF inhibition, starvation, or cellular stress more generally as we have shown) may influence the ability of tumor cells to respond to the immune system in vivo. Previous studies have suggested that error-prone polymerases such as Polλ or Polδ play a role in immune responses, but this is thought to be primarily via their effects on somatic hypermutation (66–69). It is also possible that some of the effects we see are due to the CRISPR itself, but whether there is an additional effect of Polk on regulation of immune surveillance is an intriguing idea that will need further experimental validation in future studies.

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with increased mutation rates and drug resistance (12, 13). However, despite the potential importance of Polk in promoting tumor progression, little is known about the mechanisms by which it is regulated. In this study, we identified oncogenic signaling and nutrient status as one potential regulator of Polk expression and localization in cancer.

Polk belongs to a family of related Y-family DNA polymerases, which all function in translesion synthesis, a major DNA damage tolerance pathway (8, 9). In normal physiology, these polymerases play an important role in bypassing stalled replication forks that are induced by DNA damaging agents. Because they all lack proofreading domains, they have a propensity to introduce errors during replication, although depending on the specific lesion, they can also act in an error-free manner. For this reason, these Y-family polymerases can be a double-edged sword since they allow for replication past damaged regions of DNA and cell survival but may do so at a cost of new mutations (77). In vitro studies have demonstrated that these polymerases can act with extraordinarily low fidelity, with error rates ranging from $10^{-1}$ to $10^{-4}$, compared to $~10^{-6}$ for the normal replicative polymerases Polδ or Polε (17, 78, 79). For this reason, it is important that cells regulate the expression and localization of these polymerases such that they only act when the cell is under genomic stress.

Although DNA damage is likely a major endogenous inducer of Polk, our data would suggest that mammalian cells harbor the capacity...
to induce the expression of Polk under other forms of cell stress, namely, loss of oncogenic signaling and/or nutrient starvation. This may be analogous to E. coli, whereby cells under starvation stress can up-regulate DinB/pol IV (the bacterial ortholog of Polk) (21, 22). However, in E. coli, in addition to activating DinB, the RpoS and SOS pathways have other effects on the cells, including suppression of MMR (31), and all of these processes are necessary to induce new mutations (24). In addition, previous work has demonstrated that cancer cells harboring mutations in the proofreading domain of Polk also experience loss of MMR enzymes (80), and more recently, it has been shown that suppression of MMR is necessary for these Polk mutants to markedly increase their mutation rates (81). Notably, in mouse cells, overexpression of DNB1 (the mouse ortholog of Polk) has been shown to be highly mutagenic and causes a 10-fold increase in point mutation rate (82), although the status of MMR was not directly assessed. In contrast, in our cultured A375 melanoma cells, we found little evidence for Polk being nearly this mutagenic and no evidence that it cooperates with MMR deficiency in generation of such mutations. In part, this could relate to the particularities of melanoma cell lines, which already have very high mutation burdens due to UV-induced DNA damage that occurs in the patients from whom these cell lines are derived (83). In future studies, it will be of great importance to elucidate the situation or cell types in which Polk may be more or less mutagenic.

Despite the lack of compelling evidence of mutagenesis in these melanoma cells, our studies do suggest that prolonged expression of Polk can be associated with modest resistance to BRAF inhibitors such as vemurafenib. Both genetic and nongenetic mechanisms of BRAF inhibitor resistance have been described (84–93), and many of the mutations that lead to drug resistance are pre-existing in the population when examined by deep sequencing. It is possible that Polk overexpression is mutagenic in our studies (and that those
mutations contribute to the observed drug resistance) but that our technologies were not sufficient to quantify this. However, it is important to note that we cannot exclude other nonmutagenic functions of Polk that could account for the increased drug resistance. For example, noncatalytic functions of Polk (devoid of polymerase activity) are associated with the ability of cells to resist oxidative damage (20). In line with this, BRAF inhibitors such as vemurafenib are known to be potent inducers of ROS in melanoma cells (94), which leads to the activation of pyruvate dehydrogenase kinase. Our CRISPR and RNA-seq studies are consistent with these nonmutagenic functions of Polk, as we noted a statistically significant enrichment for genes associated with oxidative stress response and nucleotide repair. A few other studies have also observed scant cytoplasmic shuttling of Polk in HeLa cells, including data from the Human TCGA somatic mutations dataset (67). In HeLa cells, including data from the Human TCGA somatic mutations dataset (67), we identified a large number of genes associated with oxidative stress response and nucleotide repair. We also unexpectedly noted that loss of Polk dysregulates a large number of genes associated with immune response, especially those in the IFN pathway. Whether Polk could have an unexpected interaction with the efficacy of immunotherapies is unexplored but will be important to functionally test in future studies. Moreover, given the availability of small-molecule inhibitors of Polk (95, 96), it will be of future interest to determine whether the administration of such inhibitors could forestall the development of resistance to either targeted or immune-based therapies.

One unexpected finding in our study was the observation that Polk can exist in a cytoplasmic form, at least under certain conditions. Numerous previous studies have shown that Polk is primarily a nuclear protein, but these largely relied upon overexpression of an EGFP-Polk fusion protein (34, 35). In our hands, overexpression of Polk also strongly up-regulated nuclear expression, which we believe likely reflects saturation of the export machinery. This would be consistent with our data using leptomycin B, which suggested that one important mechanism of regulation for Polk is nuclear-cytoplasmic shuttling. A few other studies have also observed scant cytoplasmic Polk in HeLa cells, including data from the Human TCGA somatic mutations dataset (67).

Fig. 7. CRISPR of Polk demonstrates pathways involved in drug resistance and immune response. (A) Schema detailing how A375 cells were treated with control or Polk sgRNA and Cas9, single-cell clones were created, and the resulting cells were used to measure resistance to PLX4032. The graph shows the resistance of control and Polk KO cells to PLX4032 as determined by the CyQUANT Direct assay. For each population, the graph shows the resistance of control and Polk KO cells as determined by the CyQUANT Direct assay. (B) RNA-seq was performed on the control and Polk KO cells. Dual waterfall plot of top/bottom 50 gene sets from GSAA comparison POLK sgRNA versus control sgRNA ranked by normalized association score (NAS). (C) Heatmap of top leading edge genes from selected gene sets (up to 10 genes per gene set).

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Control sgRNA</th>
<th>POLK sgRNA</th>
<th>POLK sgRNA #1</th>
<th>POLK sgRNA #2</th>
</tr>
</thead>
<tbody>
<tr>
<td>OAS1</td>
<td>1.5</td>
<td>0.9</td>
<td>0.8</td>
<td>0.7</td>
</tr>
<tr>
<td>OAS2</td>
<td>2.0</td>
<td>1.8</td>
<td>1.7</td>
<td>1.6</td>
</tr>
<tr>
<td>OAS3</td>
<td>2.5</td>
<td>2.3</td>
<td>2.2</td>
<td>2.1</td>
</tr>
</tbody>
</table>

**Legend:**
- **CTRL sgRNA**
- **POLK sgRNA #1**
- **POLK sgRNA #2**

**Significance:**
- **P < 0.01**
- **P < 0.001**
Protein Atlas (44), which used antibodies raised against peptide antigens. One important difference in our studies is that we used a monoclonal antibody raised against the full-length protein. Examination of Polk transcript variants in Ensembl (97) reveals that humans transcribe at least two versions of Polk (Polk-201 and Polk-216), such that antibodies raised against short peptides versus full-length protein may recognize different transcripts with different localizations. The exact reasons for this discrepancy will await further studies of Polk structure regarding posttranslational modifications of nuclear localization or export signals or variations in transcript abundance in different cell types. Notably, another Y-family polymerase Polη has also been reported to have nuclear localization when overexpressed as an EGFP fusion protein (98), yet data from the Human Protein Atlas (44) show a predominantly cytoplasmic localization with nuclear enrichment in some cells, similar to what we saw with Polk.

Our data indicate that mTOR and nutrient sensing regulate the localization of Polk, and this pathway is more broadly known to affect nuclear-cytoplasmic shuttling of multiple proteins (99–106). For example, PI3K-AKT-mTOR pathway signaling has been shown to prevent nuclear accumulation of glycogen synthase kinase 3β (GSK3β) such that inhibition of this pathway leads to a robust increase in nuclear GSK3β (104, 105). Furthermore, mTOR complex 1 (mTORC1)–dependent phosphorylation of the transcription factor TFEB (transcription factor EB) promotes its association with members of the 14-3-3 family of proteins, thereby forcing its retention in the cytosol (103). However, the network connecting the mTOR pathway to the DNA damage response continues to be elucidated (107–110). One study (110) showed that mTOR-S6K signaling leads to phosphorylation and subsequent degradation of E3 ubiquitin ligase RNF168; hyperactivation of mTOR by the kinase LKB1 leads to decreased levels of RNF168 and increases DNA damage. One possible explanation for our observed results is that mTOR (or one of its downstream targets) could directly phosphorylate Polk and thereby affect its localization as has been shown for other proteins that undergo nuclear-cytoplasmic shuttling (111–114). An important area for future exploration is understanding the ways in which mTOR may interact with the maintenance of genome stability.

MATERIALS AND METHODS

Cell culture

Cell culture was performed at 37°C in a humidified atmosphere containing 5% CO₂. A375 and SK-MEL28 cells were obtained from the American Type Culture Collection. PC-9 cells were a gift from C. Rudin [Memorial Sloan Kettering Cancer Center (MSKCC), New York, USA]. SK-BR3 cells were a gift from S. Chandarlapaty (MSKCC, New York, USA). Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; A375 and SK-MEL28), RPMI1640 (PC-9), or DMEM/F12 (SK-BR3) supplemented with 2 mM glutamine, penicillin (100 IU/ml), streptomycin (100 μg/ml), and 10% heat-inactivated fetal bovine serum. Cell lines were regularly tested and verified to be mycoplasma negative by the MycoAlert Mycoplasma Detection Kit (Lonza).

Cell treatments

Inhibitors were maintained until collection, unless otherwise noted: PLX4032 (Selleck), CI-1040 (Selleck), U0126 (Sigma-Aldrich), Ulixertinib (Selleck), SCH772984 (Selleck), erlotinib (Selleck), rapamycin (Selleck), PFP242 (Abcam), LY294002 (Sigma-Aldrich), bleomycin (Sigma-Aldrich), brefeldin A (Sigma-Aldrich), thapsigargin (Sigma-Aldrich), tunicamycin (Sigma-Aldrich), importazole (Sigma-Aldrich), leptomycin B (Sigma-Aldrich), and MG132 (Sigma-Aldrich). Lapatinib and PD0332991 were gifts from S. Chandarlapaty. Complete media consisted of DMEM (without glucose or glutamine) supplemented with 25 mM glucose, 6 mM glutamine, and 10% heat-inactivated fetal bovine serum. Media without glucose consisted of DMEM (without glucose or glutamine) supplemented with 6 mM glutamine and 10% heat-inactivated fetal bovine serum. Media without glutamine consisted of DMEM (without glucose or glutamine) supplemented with 25 mM glucose, 6 mM glutamine, and 1% heat-inactivated fetal bovine serum.

Generation of inducible Polk overexpression cells

For inducible overexpression of Polk, the human Polk open reading frame was amplified from a constitutive Polk overexpression plasmid, which was a gift from J. S. Hoffmann (Toulouse, France), and cloned using the In-Fusion Cloning kit (Clontech) into the pSIN-TREtight-MCS-IRECherry-PGK-Hygro vector, which has a doxycycline-inducible promoter and adds an IRES-mCherry to the C terminus of Polk, to generate TetRE-POLK-IRES-mCherry. The pSIN-TREtight-MCS-IRECherry-PGK-Hygro vector and its corresponding tet-activator (rtTA3) RIEP vector were gifts from S. Lowe (MSKCC, New York, USA). The following primers were used for amplification: TRE-hpolk F1 forward, CGGTACCCGGGATCCACCATGATGACAAAAAG; TRE-hpolk F1 reverse, TTAGTCTTCGCGGCCGCTTACTTAAAAATATATCAAGGG. Lentivirus was produced by transfection of human embryonic kidney (HEK)–293T cells with TetRE-POLK-IRES-mCherry and the packaging plasmid psiAmpho (which was a gift from R. Levine, MSKCC, New York, USA) at a 1:1 ratio. Transfection was performed using Fugene (Promega) reagent. The viral supernatant was collected 48 and 72 hours after transfection, filtered through a 0.45-μm filter (Thermo Fisher Scientific), and added to A375 cells with polybrene (8 μg/ml; Thermo Fisher Scientific). Infected cells were selected by treatment with hygromycin (1 mg/ml; Thermo Fisher Scientific). Lentivirus for RIEP (TetA) was produced the same way and then used to infect A375 cells already containing TetRE-POLK-IRES-mCherry. Doubly infected cells (TetRE-POLK-IRES-mCherry/TetA) were selected by treatment with puromycin (10 μg/ml; Thermo Fisher Scientific) and hygromycin (1 mg/ml). Single-cell clones were then isolated, and their induction efficiency was tested using flow cytometry. Clones #1 and #2 showed the best induction after addition of doxycycline (1 μg/ml; Sigma-Aldrich) compared to uninduced controls, so they were selected for future experiments.

To add constitutive GFP expression to these cells, we used pBABE-GFP, which was a gift from W. Hahn (Addgene plasmid #10668). pBABE-GFP (SV40-p-GFP) expresses humanized Renilla GFP (hrGFP) under the SV40 promoter in the pBABE backbone. Lentivirus for pBABE-GFP was produced in HEK-293T cells using the same protocol detailed above and then used to infect A375 cells already containing TetRE-POLK-IRES-mCherry/TetA (doxycycline-inducible Polk overexpression cells). Infected cells were selected using flow cytometry for GFP-positive cells.

Inducible shRNA knockdown of Polk and p53

For inducible knockdown of Polk, two different shRNAs (115) targeting Polk were generated using PCR. The two Polk shRNAs...
Recombinant plasmids targeting p53 (sh-p53-1 and sh-p53-2) were gifts from S. Lothe. Different hairpins targeting p53 were designed using restriction digestion and cloned into the pEGFP-C1 vector (Clontech). The following oligos were used as the templates for amplification: sh-Pol-1, CCTCGGA. Plasmids containing either a control hairpin (sh-Ctrl) or two different hairpins targeting p53 (sh-p53-1 and sh-p53-2) were gifts from S. Lothe. For all hairpins, lentivirus was produced by transfection of HEK-293T cells with each hairpin plasmid with the packaging plasmids psPAX2 and pMD2G at a 4:3:1 ratio. Transfection was performed using Lipofectamine 2000 (Life Technologies) reagent. The viral supernatant was collected at 48 and 72 hours after transfection of HEK-293T cells with each hairpin plasmid with the packaging plasmids psPAX2 and pMD2G at a 4:3:1 ratio. Transfection was performed using Lipofectamine 2000 (Life Technologies) reagent. The viral supernatant was collected at 48 and 72 hours after transfection and frozen at −80°C. Virus was later thawed and added to A375 cells, and infected cells were selected by treatment with puromycin (1 μg/ml). Induction after treatment with doxycycline (1 μg/ml) was verified using flow cytometry.

**Total RNA extraction, cDNA isolation, and qRT-PCR analysis**
Total RNA from treated cells was extracted using the Quick-RNA Mini-Prep kit (Zymo), and RNA concentration was determined using a Nanodrop spectrophotometer. cDNA isolation was performed using reverse transcription (RT) of total RNA using SuperScript III First-Strand Synthesis SuperMix (Thermo Fisher Scientific) according to the manufacturer's guidelines. qRT-PCR reactions were performed using a CFX384 Touch machine (Bio-Rad) using iQ SYBR Green Supermix (Bio-Rad). RNA expression levels were calculated using the comparative C_{T} method (2^{-ΔΔC_{T}}) normalized to β-actin. The qRT-PCR primer sets used in this study are listed in table S1.

**Western blot analysis**
For whole-cell lysates, cells were washed 1× with phosphate-buffered saline (PBS; Invitrogen) and then lysed in RIPA buffer (EMD Millipore) containing 1× HALT Combined Protease and Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific) for 20 min at 4°C. Cell lysates were clarified by centrifugation at 10,000 rpm for 10 min at 4°C. For cytoplasmic or nuclear fractions, the Subcellular Protein Fractionation Kit for Cultured Cells (Thermo Fisher Scientific) was used according to the manufacturer's protocol. Protein concentration was measured with Bradford reagent (Sigma-Aldrich), and samples were resolved by 4 to 15% or 12% SDS–polyacrylamide gel electrophoresis gels (Bio-Rad) for Polk or p53, respectively. Proteins were transferred onto nitrocellulose membranes and subjected to standard immunoblotting. ECL Prime (Amersham) was used as the developing reagent.

The following antibodies for Western blotting were used: Polk (ab57070, Abcam, 1:10,000), p53 (sc-126, Santa Cruz Biotechnology, 1:10,000), mCherry (ab167453, Abcam, 1:1000), lamin-B1 (ab133741, Abcam, 1:10,000), β-actin (A5441, Sigma-Aldrich, 1:20,000), antimouse (ab97046, Abcam, 1:10,000), and anti-rabbit (ab97051, Abcam, 1:10,000).

**Immunofluorescence**
Cells were cultured on Millicell EZ SLIDE four- or eight-well glass slides (EMD Millipore). Cells were fixed with 4% paraformaldehyde (Santa Cruz Biotechnology) at 37°C for 15 min with washed PBS, and then blocked with 5% goat serum (Thermo Fisher Scientific) and 0.2% Triton X-100 (Thermo Fisher Scientific) in PBS for 1 hour. The cells were incubated with primary antibody in antibody dilution buffer [PBS with 1% bovine serum albumin (Sigma-Aldrich) and 0.2% Triton X-100] at 4°C overnight, then washed three times with PBS, and incubated with Alexa-conjugated secondary antibody for 2 hours at room temperature. After washing three more times with PBS, cells were stained with 4′,6-diamidino-2-phenylindole (DAPI; 0.1 μg/ml; Thermo Fisher Scientific), mounted with Dako fluorescence mounting media (Agilent), and imaged on a Zeiss Axio Imager A2.

The following immunofluorescence antibodies were used: Polk (ab57070, Abcam, 1:500), p-S6 (5364, Cell Signaling, 1:1000), γH2AX (2577, Cell Signaling, 1:200), 53BP1 (ab175933, Abcam, 1:200), p-Chk1 (2348, Cell Signaling, 1:100), p-Chk2 (2661, Cell Signaling, 1:500), anti-mouse Alexa-488 (4408, Cell Signaling, 1:1000), anti-rabbit Alexa-488 (4412, Cell Signaling, 1:1000), and anti-rabbit Alexa-594 (8889, Cell Signaling, 1:1000).

**Quantification of nuclear versus cytoplasmic enrichment of Polk**
To quantify nuclear versus cytoplasmic enrichment, we created a nuclear mask from the DAPI image of each cell, which allowed us to quantify the number of cells per field. This mask was then overlaid onto the staining with Polk, and each cell was manually scored as to whether the staining was exclusively nuclear (i.e., perfectly overlapped with the DAPI mask) or not. The calculation of “% of cells with nuclear enrichment of Polk” was then calculated as the number of cells with strictly nuclear staining divided by the total number of cells. Because we found that this method correlated with our cell fractionation Western blots but was more scalable across many conditions and also allowed for additional stainings (i.e., phospho-S6), this method was applied to each figure.

**Cell cycle analysis**
Cells were treated with the indicated inhibitors for 24 hours and fixed in 70% ethanol (Thermo Fisher Scientific) at 4°C for at least 4 hours. Later, the cells were washed with PBS and stained with 20 μg of propidium iodide (Thermo Fisher Scientific), 200 μg of RNase A (Thermo Fisher Scientific), and 0.1% Triton X-100 in PBS for at least 30 min. The labeled cells were analyzed using a Fortessa flow cytometer, and the Dean-Jett Fox model in FlowJo was used to determine which cells were in G1, S, and G2-M.

**KO of MMR genes and Polk using the Alt-R CRISPR-Cas9 system**
Two sgRNAs for each MMR gene or Polk were designed using the CHOPCHOP online tool (117). KO was performed in A375 cells using the Alt-R CRISPR-Cas9 system [Integrated DNA Technologies (IDT)], which uses sgRNAs against the gene of interest and labeled tracrRNAs to guide Cas9 protein to a specific genomic location. Proper cutting by Cas9 was verified using the Surveyor Mutation Detection Kit (IDT) after amplification of the targeted area using the associated forward and reverse primers. The sgRNA and validation primer sequences used in this study are listed in table S2.
Drug resistance assays

Two different clones of doxycycline-inducible Polk overexpression cells (clone #1 and clone #2) were generated. We then divided each population equally and treated them with or without doxycycline (1 µg/ml) for ~3 months to generate Polk-overexpressing cells and their corresponding negative control population. All populations were then switched to media without doxycycline, plated in 96-well plates, and exposed to various doses of PLX4032 or PD0323991. After 4 days (PLX4032) or 7 days (PD0323991), cell number was determined using the CyQUANT Direct Cell Proliferation Assay kit (Thermo Fisher Scientific). The technical replicates for each dose were averaged and then normalized against the 0 µM dose condition to determine the growth relative to DMSO. The PLX4032 resistance assay detailed above was also repeated for control and Polk KO (two different sgRNAs) cells.

Mutagenesis assays

Doxycycline-inducible Polk overexpression A375 cells (clone #1 and clone #2) constitutively expressing GFP under the SV40 promoter with control or MMR KO were generated. We then divided each population equally and treated them with or without doxycycline (1 µg/ml) to generate Polk-overexpressing cells and their corresponding negative control population. After 14 days of treatment, both populations were trypsinized, stained with DAPI (1 µg/ml), and analyzed using a Fortessa flow cytometer. FlowJo was used to determine the percentage of GFP-negative and GFP-positive cells in each population.

RNA-seq and analysis

This was extracted as described above. Purified RNA was delivered to GENEWIZ (South Plainfield, NJ) for mRNA preparation with the TruSeq RNA V2 kit (Illumina) and 150- bp paired-end sequencing on the Illumina HiSeq2500. After quality control with FASTQC (Babraham Bioinformatics) and trimming with TRIMMOMATIC (118), reads were aligned to GRCh38 (Ensembl version 90) using STAR (119), with quality control via SeQC (120). Differential expression was calculated with DESeq2 (121) using the output of the --quantMode GeneCounts feature of STAR. Z scores of log2-transformed normalized counts from the rlog function were used for heatmaps. Pathway analysis was performed with Gene Set Association Analysis (GSEA) (122, 123) using the following parameters: gseamatic Weighted_KS, demetric Sig2Noise, permute gene_set, rnd_type no_balance, scoring_scheme weighted, and norm MeanDiv.

Statistical tests

The following statistical tests were used: paired two-tailed t-test (all qRT-PCR, Western blot analysis, and immunofluorescence), χ² tests (cell cycle analysis), Pearson correlation coefficient (comparison of rates of p-S6 loss versus nuclear Polk enrichment), two-way analysis of variance (ANOVA), and Fisher’s least significant difference (LSD) test (drug resistance assays).

SUPPLEMENTARY MATERIALS

stke.sciencemag.org/cgi/content/full/13/629/eaau1453/DC1

Fig. S1. Validation of the Polk antibody.

Fig. S2. MAPK inhibition in melanoma cell lines increases POLK mRNA levels and changes the subcellular localization of Polk.

Fig. S3. DNA damage is not associated with the subcellular shift of Polk.

Fig. S4. Cell cycle inhibition is not responsible for the shift in the subcellular localization of Polk.

Fig. S5. MAPK inhibition decreases the abundance of p-S6.

Fig. S6. BRAF inhibition decreases the abundance of p-S6.

Fig. S7. The effects on subcellular localization of Polk.

Fig. S8. Glucose starvation also modulates the subcellular localization of Polk.

Fig. S9. Inhibiting importin β decreases the abundance of p-S6.

Fig. S10. Inhibition of the proteasome does not have a differential effect on the protein level of Polk after treatment with DMSO or PLX4032.

Table S1. qRT-PCR primers used in the study.

Table S2. sgRNAs and validation primers used for MMR and Polk KO.

Table S3. RNA-seq and GSEA analysis on Polk-CRISPR A375 cells.

Data file S1. Differentially expressed genes between A375 cells with POLK sgRNA versus control or MMR KO.

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Regulation of the error-prone DNA polymerase Polκ by oncogenic signaling and its contribution to drug resistance


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Polκ manages stress

DNA polymerase κ (Polκ) is a traditionally error-prone polymerase that is overexpressed in some tumors. Temprine et al. found that Polκ facilitates tumor cell survival in response to oncogenic mutations (such as those in the kinases BRAF or EGFR), targeted kinase inhibition, oxidative stress, or starvation. These cellular stresses shifted Polκ from its largely cytoplasmic distribution to a nuclear localization in various tumor cell types, but without increasing mutagenesis. Knocking down Polκ decreased, whereas blocking its nuclear exit increased, the resistance of melanoma cells to the BRAF-targeted inhibitor vemurafenib, suggesting that targeting Polκ may reduce drug resistance in cancer patients.