SHORT COMMUNICATION
Crosstalk between NRF2 and HIPK2 shapes cytoprotective responses

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Homeodomain interacting protein kinase-2 (HIPK2) is a member of the HIPK family of stress-responsive kinases that modulates cell growth, apoptosis, proliferation and development. HIPK2 has several well-characterised tumour suppressor roles, but recent studies suggest it can also contribute to tumour progression, although the underlying mechanisms are unknown. Herein, we have identified novel crosstalk between HIPK2 and the cytoprotective transcription factor NRF2. We show that HIPK2 is a direct transcriptional target of NRF2, identifying a functional NRF2 binding site in the HIPK2 gene locus and demonstrating for the first time a transcriptional mode of regulation for this kinase. In addition, HIPK2 is required for robust NRF2 responsiveness in cells and in vivo. By using both gain-of-function and loss-of-function approaches, we demonstrate that HIPK2 can elicit a cytoprotective response in cancer cells via NRF2. Our results have uncovered a new downstream effector of HIPK2, NRF2, which is frequently activated in human tumours correlating with chemoresistance and poor prognosis. Furthermore, our results suggest that modulation of either HIPK2 levels or activity could be exploited to impair NRF2-mediated signalling in cancer cells, and thus sensitise them to chemotherapeutic drugs.

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INTRODUCTION
Homeodomain interacting protein kinase-2 (HIPK2) is a member of the HIPK family of stress-responsive kinases, and it modulates cell proliferation, differentiation, apoptosis and development. HIPK2 responds to a variety of physiological stresses, transforming these cues into changes in transcriptional programs, which in turn enables cells to adapt to and survive the original insult. Although HIPK2 is highly regulated at the posttranslational level, essentially no information exists about its transcriptional regulation.

HIPK2 is considered to be a potential haplo-insufficient tumour suppressor as it can promote apoptosis in response to chemotherapy drugs and radiation, mainly by phosphorylating p53 at S46, which then induces expression of pro-apoptotic genes. Additionally, HIPK2 can also protect cells against genome instability induced by genotoxic agents by promoting DNA damage repair pathways. Interestingly, accumulating evidence suggests that HIPK2 may also support tumour progression; the expression of HIPK2 is significantly higher in cervical cancer than in healthy tissue and in aggressive meningiomas (when compared with benign meningiomas), where it positively associates with tumour progression. HIPK2 is also amplified in pilocytic astrocytoma and in ovarian and prostate tumours (The Cancer Genome Atlas), and positively associates with cell growth in various cancer cell lines. These results imply that HIPK2 might play a dual role in cancer depending on context, either acting as a tumour suppressor or facilitating tumour progression. While the pathways involved in the tumour suppressor role of HIPK2 are relatively well understood, the underlying mechanisms mediating its cytoprotective function(s) remain unclear.

NRF2 (nuclear factor erythroid 2 (NF-E2) p45-related factor 2, encoded by NFE2L2) is the master regulator of oxidative stress responses, which allows adaptation and survival during stress conditions. NRF2 controls the expression of a battery of genes, which encode antioxidant and drug-metabolising enzymes, as well as drug transporters (for example, HO1, GSTs, NQO1 and MRPs), all of which contain antioxidant response elements (AREs) within their promoter/enhancer regions. In normal cells, NRF2 activity is kept low under non-stress conditions by its rapid proteasomal degradation, which is principally mediated by KEAP1 activation of NRF2 is especially relevant in lung tumours, where sustained activation protects tumour cells against chemo- and radiotherapy and can promote metabolic activities that support cell proliferation and tumour growth. Not surprisingly, therefore, NRF2 is often constitutively activated in human tumours, where it is associated with poor prognosis. While NRF2 activity is kept low under non-stress conditions by its rapid proteasomal degradation, which is principally mediated by KEAP1, it has become apparent that its sustained activation protects tumour cells against chemo- and radiotherapy and can promote metabolic activities that support cell proliferation and tumour growth. Not surprisingly, therefore, NRF2 is often constitutively activated in human tumours, where it is associated with poor prognosis.

Although NRF2 is cytoprotective and its transient activation is linked with chemoprevention, it has become apparent that its sustained activation protects tumour cells against chemo- and radiotherapy and can promote metabolic activities that support cell proliferation and tumour growth. Not surprisingly, therefore, NRF2 is often constitutively activated in human tumours, where it is associated with poor prognosis.
by loss of PTEN, suggesting that aberrant activation of NRF2 is a common event in many cancer types. In this study, we describe for the first time the existence of crosstalk between HIPK2 and NRF2 in which NRF2 regulates HIPK2 expression, and in turn, HIPK2 positively shapes the NRF2 response.

RESULTS AND DISCUSSION
NRF2 transcriptionally regulates basal and inducible levels of HIPK2
Previously, we and others have shown that HIPK2 can affect redox balance and might regulate oxidative stress responses. As NRF2 is arguably the main transcription factor associated with oxidative stress responses, we wanted to test whether there was a link between NRF2 and HIPK2. To do so, we first used A549 cells (lung cancer cells that possess constitutively active NRF2 due to inactivating mutations on KEAP1). We found that NRF2 knockout cells (NRF2-KO) have reduced protein and messenger RNA (mRNA) basal levels of HIPK2; conversely, NRF2 reconstitution restored both the protein and the mRNA levels of HIPK2 (Figure 1a). To monitor NRF2 activity, we used the prototypic NRF2 target gene NQO1 (Figure 1a; Supplementary Figure S1A). To our knowledge, there are no studies to date addressing the transcriptional regulation of HIPK2. We therefore confirmed the effect of NRF2 on HIPK2 mRNA levels, first by using different NRF2-KO cell lines (Supplementary Figures S1B and S1C) and second, by using various short hairpin RNA (shRNAs) against NRF2 (Supplementary Figure S1D). These results demonstrate that NRF2 regulates the steady-state levels of HIPK2.

Figure 1. For caption see page 6206.
In order to answer whether NRF2 activation is also able to induce HIPK2, we used two different approaches. In a pharmacological approach, we used two classical NRF2 activators, hydrogen peroxide (H₂O₂) and sulforaphane (SFN), which disrupt the KEAP1-dependent NRF2 degradation. We found that NRF2 activators increase both mRNA and protein levels of HIPK2 in H1299 cells (lung cancer cells with functional KEAP1), and that this induction requires NRF2 (Figure 1b). Of note, other KEAP1-proficient cell lines showed similar behaviour (Supplementary Figure S1E), and an overexpressed flag-tagged HIPK2 construct does not get stabilised by neither of NRF2 activators (Supplementary Figure S1F).

Additionally, we used a genetic model: by deleting the KEAP1-binding motif within the endogenous NRF2, we produced cells that harbour a constitutively active NRF2 gain-of-function (GOF) mutant. Such GOF mutations are often found in tumours, and have been associated with malignancy.⁴³⁻⁴⁵ Thus, NRF2-GOF cells provide a physiologically relevant model of sustained NRF2 activation in malignant cells. We found that H1299 NRF2-GOF cells have elevated protein and mRNA levels of HIPK2 and NQO1 when compared with their WT counterparts (Figure 1c; Supplementary Figure S1G). The upregulation of HIPK2 observed in NRF2-GOF cells is not cancer cell type-specific (Supplementary Figure 1H). This model demonstrates that sustained NRF2 activation increases HIPK2 expression.

To identify the region(s) in the HIPK2 gene that is responsible for its regulation by NRF2, we performed an in silico analysis of the HIPK2 promoter to identify potential AREs that might be bound by NRF2. The minimal proposed consensus ARE sequence is TGASmNGC (where S = C or G).⁵² As active AREs are often located close to the transcription start site, we focused on two ARE sequences we identified between −1 and −2000 bp within the HIPK2 promoter, referred as ARE1 (at −246 bp: 5′-TGAGAGGG-3′) and ARE2 (at −1794 bp: 5′-GACTTGAG-3′). Additionally, Malhotra et al.⁵² using ChIP-seq in mouse cells, detected 1256 peaks as potential NRF2 binding sites. Among these, we identified a peak within a HIPK2 intronic region; as this ARE sequence is conserved in humans (named intronic ARE: 5′-gTGACTCAGC-3′), we analysed all three potential sites by studying NRF2 occupancy via ChIP-qPCR analyses. We used DLD1 cells to immunoprecipitate endogenous NRF2 and to interrogate its ability to bind to the three potential AREs. The analysis was performed by comparing the amount of material immunoprecipitated with anti-NRF2

**Figure 1.** HIPK2 is a novel NRF2 target gene. (a) All cell lines used in the study have been validated by STR profiling and were routinely tested for mycoplasma. When applicable, the differences between groups were determined by unpaired Student’s t-test. Analyses were performed using GraphPad Prism (GraphPad Software Inc., La Jolla, CA, USA); a P-value of < 0.05 was considered significant. **P** ≤ 0.05, **PP** ≤ 0.01, **PPP** ≤ 0.001. The endogenous NFE2L2 gene, which encodes NRF2, was edited by transfecting cells with plentiCRISPR-v2 (a gift from Dr Feng Zhang, Addgene plasmid #52961) containing a guide RNA (gRNA) directed against the KEAP1-binding domain within the NFE2L2 locus (5′-TGAGAGGCCAGATATAGATCT-3′). CRISPR-mediated gene editing with this gRNA produced NRF2 knockout clones (NRF2-KO), and NRF2 gain-of-function clones (NRF2-GOF). NRF2-GOF clones were those that the Cas9-mediated cleavage was repaired in frame but introducing mutations (deletions or insertions) within the KEAP1-binding domain. After 2 days of puromycin selection, cells were cloned selectively by serial dilution, and positive clones were identified as previously described.⁶⁶ Control cells (referred as wild-type) comprises the pooled population of surviving cells transfected with plentiCRISPRv2 vector (empty backbone) treated with puromycin. Mutational gene changes in NFE2L2 were validated by sequencing of their genomic DNA. All our results were validated using at least two different clones from each gRNA. Lentiviral infection was performed as previously described.¹⁰ Left panel: A549 cells were grown in DMEM (Thermo Fisher Scientific, Waltham, MA, USA) containing 10% fetal bovine serum (FBS). A549 control cells (WT) infected with empty vector were compared with CRISPR-mediated NRF2 knockout cells (NRF2-KO) infected with empty vector or with lentivirus encoding NRF2 (NRF2 Rec). Protein levels of human NRF2 (ab62352, Abcam, Cambridge, UK), HIPK2 and NQO1 were analysed by western blotting. Tubulin was used as a loading control (TU-02, Santa Cruz Biotechnology, Dallas, TX, USA). Note that antibodies against human NRF2 recognise a non-specific protein as a faster migrating band; such band has been previously validated.⁶⁶ The right panel: TaqMan analyses of HIPK2 mRNA levels in A549 control cell lines (WT) compared with NRF2-KO cells or NRF2-KO cells reconstituted with NRF2 (NRF2 Rec). The HIPK2 mRNA levels in A549 control cell lines (WT) compared with NRF2-KO cells or NRF2-KO cells reconstituted with NRF2 (NRF2 Rec). The HIPK2 mRNA levels in WT cells were set as 1. Values are means plus s.d. from three independent experiments. RNA was extracted using RNeasy kit (Qiagen, Hilden, Germany), and reverse-transcribed to complimentary DNA (cDNA) using Omniscript RT kit (Qiagen) according to the manufacturer’s instructions. Resulting cDNA was analysed using TaqMan Universal Master Mix II (Life Technologies, Carlsbad, CA, USA). Gene expression was determined using an Applied Biosystems (Foster City, CA, USA) 7300 Real-Time PCR system by the comparative ΔΔCt method. The following primers and probes were used in the study: HIPK2-F 5′-CATGAAGCAGACAGCCG-3′, HIPK2-R 5′-CATCAATGTCGACCTTC-3′, HIPK2-Probe 5′-GATGATATGGCCCAGGTGA-3′, hActin-F 5′-GGCCGGCTACAGCTTCA-3′, hActin-R 5′-TCTTCTTAATGTCGACCGAT-3′, hActin-Probe 5′-CACACCAGCCAGAGGGAGA-3′. (b) WT or NRF2-KO H1299 were treated with vehicle, 100 μM hydrogen peroxide (H₂O₂) or with 3 μM Sulforaphane (SFN). After 3 h, cells were lysed and subjected to western blotting or mRNA analyses (RT-qPCR). Upper panel: levels of HIPK2 mRNA were analysed. The mRNA levels in vehicle-treated WT cells were set as 1. Values are means plus s.d. from five independent experiments. Sulforaphane (1-isothiocyanato-4(R,S)-methylsulfanyl) butane) was obtained from LKT Labs (St. Paul, MN, USA). Lower panel: the levels of the indicated proteins were analysed. HO1 antibody was purchased from Biovision (Biovision Inc., Milpitas CA, USA). (c) H1299 control cells (WT) were compared with CRISPR-mediated NRF2 gain-of-function (NRF2-GOF) H1299 cells. Results obtained using five independent NRF2-GOF clones are shown. Left panel: the levels of the indicated proteins were analysed. Right panel: Tagman analysis of HIPK2 mRNA levels. (d) ChIP analysis of NRF2 occupancy within the HIPK2 proximal promoter (ARE1 and ARE2), HIPK2 intronic region (Intron) and the previously characterised ARE within the NQO1 promoter.⁷¹ Chip analyses were performed as previously described⁶⁸ using the following primers: NQO1-ARE-F 5′-CCCTTTTAGAGCTGGACGAGGA-3′, NQO1-ARE-R 5′-TGACCGAGGAGAACTTGTTAT-3′, HIPK2-ARE-Intron-F 5′-GCTCCATTATACCTTGCAG-3′, HIPK2-ARE-Intron-R 5′-AGCATGATCCACACGACATG-3′, Hipk2-ARE-1 5′-GGAAGCCGACACCCAGAGG-3′, Hipk2-ARE-2 5′-GAGTGGTGAAG-3′, Hipk2-ARE-3 5′-GGCTGTCGCTTTTTTCATCAGAGG-3′. Left panel: the amount of material immunoprecipitated with anti-NRF2 (ab62352, Abcam) in DLD1 (WT) versus NRF2-KO cells was compared. Right panel: we compared the amount of material immunoprecipitated with anti-NRF2 in DLD1 (WT) versus DLD1 NRF2-GOF cells. All data were normalised against the input lysates before enrichment by immunoprecipitation. Values are means plus s.d. from three independent experiments. (e) Luciferase gene reporter assay to identify functional AREs within the HIPK2 locus. The proximal HIPK2 promoter (−1 to −2000) and the HIPK2 intronic region (a 700 bp region spanning the identified ARE) were cloned into the basic-pGL3-luc vector, upstream and downstream of the luciferase gene, respectively. We used Nqo1-luc and mutARE-Nqo1-Luc as positive and negative controls, respectively.⁶⁶ The indicated constructs were transfected into RL-34 cells (grown in DMEM containing 10% FBS) using Lipofectamine 2000. About 24 h after transfection, cells were treated either with vehicle or with 50 μM of TBE-31.¹⁵ Sixteen hours later, cells were lysed and their luciferase expression analysed using the Luciferase Assay System from Promega (Madison, WI, USA). The luciferase levels in vehicle-treated cells were set as 1. Values are means plus s.d. from five independent experiments.

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antibodies in WT and NRF2-KO DLD1 cells (Figure 1d, left panel). Additionally, we compared the amount of material immunoprecipitated with anti-NRF2 or anti-IgG in DLD1 cells (Supplementary Figure S1I). Our results showed that at basal conditions NRF2 binds to the ARE1 and to the intronic ARE, but not to the more distal ARE2 sequence. Furthermore, we tested whether activation of NRF2 leads to an increase in its binding to the HIPK2 locus. To do so, we used NRF2-GOF DLD1 cells as a model for NRF2-sustained activation and we compared them with DLD1 WT cells, and found that NRF2 activation leads to an enrichment of NRF2 bound to the HIPK2 locus (Figure 1d, right panel). Finally, to address the potential functional relevance of the two identified sites, we used a luciferase-based genetic reporter assay. We cloned the proximal promoter of HIPK2 and the ARE-containing intronic region of HIPK2 upstream and downstream of the luciferase gene, respectively, and individually mutated the promoter ARE1 sequence and the intronic ARE sequence. We transfected these constructs into RL-34 cells (which are highly responsive to NRF2 inducers53) and tested their response to the NRF2 inducer TBE-3154,55 (Figure 1e). We used plasmids containing either the promoter of NQO1 fused to luciferase (WT) or the promoter of NQO1 with a mutated ARE sequence fused to luciferase (MUT)56 as a positive and a negative control, respectively. Our results showed that while the ARE1 sequence situated within the HIPK2 promoter does not control luciferase expression, the ARE sequence within the intronic region of HIPK2 is responsible for the TBE-31-mediated induction of luciferase, highlighting the functional relevance of this ARE sequence. Together, these results show that NRF2 regulates both basal and inducible levels of HIPK2 at the transcriptional level via an intronic ARE sequence. The identification of a functional intronic ARE, although rare, has been recently reported for another NRF2 target gene.57 To our knowledge, this is the first demonstration of a transcriptional mode of regulation for HIPK2.

HIPK2 supports NRF2 antioxidant response

Having demonstrated that HIPK2 is regulated by NRF2, we then studied whether HIPK2 affects NRF2-dependent responses. First, we found that HIPK2 overexpression promoted the accumulation of a nuclear, lambda phosphatase-sensitive form of NRF2 (in both endogenous and overexpressed NRF2) (Figure 2a; Supplementary Figure S2A). These results suggest that HIPK2 can activate NRF2 by promoting its nuclear accumulation, in a similar way as exposure to the oxidant H2O2 does (Supplementary Figure S2B). To test this possibility, we compared wild-type (WT) and HIPK2 knockout MEF cells and found that HIPK2-deficient cells had lower basal protein levels of NRF2 and the NRF2 target NQO1 and GSTM1 (Figure 2b), as well as an impaired induction of NQO1 after SFN treatment, as measured by enzyme activity (Figure 2c). Furthermore, HIPK2 reconstitution rescued (i) the basal mRNA levels of NRF2 target genes (Figure 2d) and their induction (in response to oxidants) (Supplementary Figure 2C) without affecting the mRNA levels of HIPK2.
NRF2 itself, and (ii) the basal NRF2 protein levels, and its response to oxidants (measured by induction of NQO1 and HO1 levels upon exposure to H$_2$O$_2$) (Figure 2e). In these experiments, we used HIPK1/2-double knockout MEF cells to avoid potential compensation from HIPK1; $^2$$^3$$^5$$^8$ similar results were obtained in single HIPK2 knockout cells (Supplementary Figure S2D). Interestingly, the effect of HIPK2 on NRF2 is kinase dependent, as a HIPK2 kinase-deficient mutant form (KD) did not rescue the basal NRF2 levels or the NRF2-mediated response (Figure 2e). However, based on our results we cannot distinguish between the effect of HIPK2 on NRF2 being direct or indirect.

To test the relevance of HIPK2 in the regulation of NRF2 in human cancer cells, we produced CRISPR-mediated HIPK2 knockout cells. We found that HIPK2 knockout decreased NRF2 levels in both H1299 and A549 lung cancer cells (Figure 2f). These results were confirmed in various cell lines using shRNAs against HIPK2 (Supplementary Figures S2E and S2F).

To test whether HIPK2 regulates NRF2 response in vivo, we analysed Hipk2, Nqo1 and Nrf2 mRNA levels from livers of wild-type and HIPK2 knockout mice treated with a single dose of the NRF2 inducer TBE-31. Compared to wild-type mice, HIPK2-deficient mice exhibited impaired induction of Nqo1 by TBE-31 (Figure 2g), with significantly affecting Nrf2 mRNA levels (Supplementary Figure S2G).

These results establish that HIPK2 contributes substantially to the NRF2-mediated responses both in cells and in vivo. Moreover, they highlight the potential role HIPK2 may play in ensuring a robust adaptive response against oxidative stress and xenobiotics. Of note, this new link between HIPK2 and oxidative stress responses is conserved throughout evolution, as a recent study demonstrated that in Caenorhabditis elegans, HIPK-1 (the single homologue of HIPKs), confers resistance to oxidative stress. $^9$

Physiological relevance of the crosstalk between HIPK2 and NRF2 in rodent liver. NRF2 is well-characterised as being cytoprotective in healthy tissue. In clear contrast, NRF2 increases resistance against a wide variety of chemotherapeutic drugs in malignant tissue. The fact that HIPK2 positively regulates NRF2 suggests that this HIPK2/NRF2 axis could represent a new pathway by which HIPK2 prevents tumour initiation. However, the existence of such axis provides a means by which HIPK2 might, via NRF2, play a hitherto unrecognised role in mediating survival of malignant cells upon challenge with chemotherapeutic drugs.

To address whether HIPK2 affects cell responses to chemotherapeutic drugs via NRF2, we used two different approaches. First, we reconstituted HIPK1/2-KO cells with HIPK2 (or with an empty vector) and exposed both isogenic cell lines to increasing concentrations of the commonly used chemotherapeutic drug doxorubicin. We found that, compared to HIPK1/2-KO MEFs, cells reconstituted with HIPK2 exhibited higher cell viability (Figure 3a) and a striking reduction of apoptosis (measured by PARP cleavage) in response to doxorubicin, correlating with higher levels of NRF2 and NQO1 (Figure 3b). In full agreement with the apoptosis data, HIPK2-reconstituted cells were more resistant to doxorubicin (measured by cell viability), and this resistance was significantly reduced by NRF2 knockdown (Figure 3c). Second, we...
knocked out HIPK2, NRF2 or both genes in H1299 lung cancer cells, and measured their sensitivity to cisplatin compared with wild-type cells. HIPK2 knockout (or knockdown) increased the sensitivity to cisplatin as seen by a colony-formation assay (Figure 3d). Furthermore, whereas knockout of NRF2 sensitised cells to cisplatin, a double knockout of both NRF2 and HIPK2 did not increase drug sensitivity further as shown by a cell viability assay (Figure 3e).
These results confirm that HIPK2 can promote cell survival upon challenge with chemotherapeutic drugs; this, together with the involvement of the well-established pro-survival factor NRF2 makes a strong case for the idea that, by activating NRF2, HIPK2 could support cancer cell survival.

CONCLUSIONS

Herein, we demonstrate for the first time that HIPK2 is regulated at the transcriptional level and that HIPK2 is an NRF2 target gene. This is a notable finding because most HIPK2 regulatory mechanisms described to date rely on posttranslational modifications and thus, our results add an extra regulatory layer to the control of HIPK2 activity. Additionally, our data place HIPK2 as a critical kinase, shaping the NRF2 response. This is important in the cancer biology field for two reasons: First, our discovery reveals HIPK2 to be a common apical regulator of two major stress regulated pathways, NRF2 and p53, and thus a decisive factor controlling cancer cell fate by being coupled to both cell death and cell survival. It is important to highlight that although HIPK2 can protect healthy tissue against tumour initiation by promoting both DNA repair and cytoprotection (shown in this study), activation of the same pro-survival pathways in malignant tissue could lead to aberrant cell survival and enhanced chemoresistance (see model in Figure 4), particularly when apoptotic pathways are impaired (that is, in the absence of a functional p53). In this context, it will be important in the future to address whether HIPK2 plays opposing roles depending on the stages of tumour development (for example, preventing initiation but accelerating progression) as has also been proposed for NRF2.60 Interestingly, HIPK2 also controls the levels of Notch1, a well-known factor with a dual role in cancer, which in common with NRF2, can act as both tumour suppressor and oncogene, depending on the context, adding strength to the idea of a context-dependent role for HIPK2 in cancer. Second, our data suggest that inhibition of HIPK2 could be a plausible mechanism by which the NRF2 pathway could be suppressed, thereby providing a new strategy to overcome NRF2-associated resistance to therapies in malignant cells. It is recognised that aberrant-sustained activation of NRF2 can promote chemoresistance and radioresistance, and therefore, inhibition of NRF2 in these settings should increase the efficacy of anticancer therapies.

In summary, our results provide new information supporting the already well-established tumour suppressor role of HIPK2, and also could explain how under certain conditions (for example, cancer cell chemoresistance due to upregulated NRF2) HIPK2 might provide cancer cells with a survival advantage.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Fig. 4. Schematic representation of the proposed differential role of HIPK2 in healthy and malignant tissue. HIPK2 pro-apoptotic role can promote clearance of damaged cells in healthy tissue and also the elimination of malignant cells in response to cancer therapeutic. In contrast, HIPK2 pro-survival role might help cancer avoidance by preventing DNA mutations in healthy tissue, but it might also protect malignant cells against genotoxic insults.
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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)