miR-625-3p regulates oxaliplatin resistance by targeting MAP2K6-p38 signalling in human colorectal adenocarcinoma cells

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Oxaliplatin resistance in colorectal cancers (CRC) is a major medical problem, and predictive markers are urgently needed. Recently, miR-625-3p was reported as a promising predictive marker. Herein, we show that miR-625-3p functionally induces oxaliplatin resistance in CRC cells, and identify the signalling networks affected by miR-625-3p. We show that the p38 MAPK activator MAP2K6 is a direct target of miR-625-3p, and, accordingly, is downregulated in non-responder patients of oxaliplatin therapy. miR-625-3p-mediated resistance is reversed by anti-miR-625-3p treatment and ectopic expression of a miR-625-3p insensitive MAP2K6 variant. In addition, reduction of p38 signalling by using siRNAs, chemical inhibitors or expression of a dominant-negative MAP2K6 protein induces resistance to oxaliplatin. Transcriptome, proteome and phosphoproteome profiles confirm inactivation of MAP2K6-p38 signalling as one likely mechanism of oxaliplatin resistance. Our study shows that miR-625-3p induces oxaliplatin resistance by abrogating MAP2K6-p38-regulated apoptosis and cell cycle control networks, and corroborates the predictive power of miR-625-3p.

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Colorectal cancer (CRC) is the third most commonly diagnosed malignant disease in the western world\(^1\). Approximately 25% of the patients present with a disseminated, stage IV disease and in further 10–15% of patients with initially localized disease, metastases will develop within 5 years. However, no predictive biomarker for standard chemotherapeutic treatment is available and as many as 30% of the patients do not obtain an objective response to first-line treatment\(^2\). Thus, the identification of predictive biomarkers for response is of great importance.

MicroRNAs (miRNAs) are endogenous, small non-coding RNAs that play essential roles in the regulation of gene expression\(^3\), and which have been linked to chemotherapy resistance\(^4\). Recently, miR-625-3p was reported to be positively associated with lack of response to first-line oxaliplatin (oxPt)-based treatment in two independent cohorts of patients with metastatic CRC (mCRC)\(^5\). While that study suggested high expression of miR-625-3p to be a novel predictive marker for oxPt-resistance in a subset of mCRC patients, a possible functional relationship between miR-625-3p and cellular drug sensitivity was not examined.

Here, we have constructed a transposon-based doxycycline (DOX) inducible vector to investigate the role of miR-625-3p in modulating oxPt sensitivity in CRC cells in vitro. Our results show that on exposure to oxPt ectopic expression of miR-625-3p increases cell viability by decreasing apoptosis. Furthermore, we have identified direct and indirect targets of miR-625-3p dysregulation in these cells and in mCRC patients treated with first-line oxPt. We show that miR-625-3p directly targets and inhibits the mitogen activated protein kinase (MAPK) kinase MAP2K6 (also known as MKK6). As a consequence, we find that miR-625-3p-induced resistance is associated with reduced MAP kinase signal transduction after genotoxic stress leading to a reduction of p53-mediated apoptosis and an increase in cell cycle progression signals.

**Results**

**Ectopic expression of miR-625-3p promotes oxPt resistance.** We constructed a Sleeping Beauty (SB) transposon vector (pSBInducer), which allows for stable expression of small interfering RNAs (siRNAs) and miRNAs in a DOX-inducible manner (Supplementary Fig. 1), and consequently, robust downregulation of targeted genes in mammalian cells (Supplementary Fig. 2).

We used pSBInducer to introduce miR-625-3p expression (or control shRNA designed not to target any human transcripts) in the microsatellite stable and microsatellite unstable CRC cell lines SW620 and HCT116, respectively (Supplementary Fig. 1). Forty-eight hours of DOX induction led to a decrease of miR-625-3p expression in SW620 and HCT116, respectively (Supplementary Fig. 1). Forty-eight hours of DOX induction led to a decrease of miR-625-3p expression in SW620 and HCT116, respectively (Supplementary Fig. 1). Forty-eight hours of DOX induction led to a decrease of miR-625-3p expression in SW620 and HCT116, respectively (Supplementary Fig. 1).

We used pSBInducer to introduce miR-625-3p expression (or control shRNA designed not to target any human transcripts) in the microsatellite stable and microsatellite unstable CRC cell lines SW620 and HCT116, respectively (Supplementary Fig. 1). Forty-eight hours of DOX induced the expression of miR-625-3p approximately three-fold in HCT116 cells, which is comparable to the previously reported difference in miR-625-3p expression in SW620 and HCT116 cells (Supplementary Fig. 1). Forty-eight hours of DOX induction induced miR-625-3p expression by more than 400 fold (Supplementary Fig. 3). Ectopic expression of miR-625-3p has no significant effect on cell growth in SW620 cells, whereas in HCT116 cells, a slight (28%) increased viability was observed (Fig. 1a).

DOX-induced SW620 and HCT116 cells were next treated with increasing concentrations of oxPt for 48 h and cell viability assessed. In both cell lines miR-625-3p induction increased oxPt resistance over a range of concentrations (Fig. 1b), which translated into an increase in the half maximum inhibitory concentration IC\(_{50}\) (causing 50% inhibition of viability) from 1.6 µM in HCT116 to 28.8 µM in HCT116 and from 1.3 µM in SW620 to 6.1 µM in SW620 cells (Fig. 1c). There was no difference in IC\(_{50}\) between vector control cells and their parental wild-type counterparts (Fig. 1c). This indicates that miR-625-3p functionally is associated with increased resistance to oxPt in CRC cells.

**Increased miR-625-3p expression reduces oxPt-induced cell death.** To determine whether inhibition of cell death was a contributing factor to the observed oxPt resistance in HCT116 and SW620 cells, we performed a lactate dehydrogenase activity (LDH) assay. Induction of miR-625-3p in HCT116 cells inhibited drug-induced cell death when exposed to oxPt (Fig. 2a). A small decrease in cell death was also observed for 2 and 8 µM oxPt in miR-625-3p overexpressing SW620 cells although this was only borderline significant (Fig. 2a).

To confirm that the oxPt resistance phenotype was a general consequence of miR-625-3p induction, we used a flow cytometry-based Annexin-V/propidium iodide (PI) cell death assay on three randomly selected, independent HCT116 and SW620 single cell clones (these are biological replicates since Sleeping Beauty mediated transposition is near-random and individual low-passage cell clones harbour unique pSBInducer integrations\(^6\)). In agreement
with the LDH assay, the Annexin-V/PI assay demonstrated that miR-625-3p indeed reduced oxPt-induced cell death (Fig. 2b). The percentage of apoptotic cells in non-treated cells was similar in control and miR-625-3p cell clones, while the death rate upon exposure to oxPt was reduced from 81% in control cells to below 50% in the HCT116.625 cell clones. The same experiment was also performed with a single cell-derived SW620 clone, which revealed a similar effect (reduction in death rate from 51% in SW620.ctrl to 33% in SW620.625 cells; Supplementary Table 1).

To investigate whether sensitivity towards oxPt could be restored by reducing miR-625-3p levels, the most oxPt-resistant HCT116.625 clone (clone #1) was transfected with an inhibitor of miR-625-3p (an anti-miR). The anti-miR significantly increased oxPt sensitivity towards 64 μM oxPt as assessed by LDH assay. Data were presented as mean increase in 64 μM oxPt-induced cell death ± s.e.m. (n = 5). *P ≤ 0.05 (t-test).

In conclusion, our data demonstrate that ectopic expression of miR-625-3p promotes resistance towards oxPt in CRC cells, and that this resistance is caused, at least in part, by inhibition of oxPt-induced cell death.

miR-625-3p transcripts are associated with oxPt response.

To identify genes associated with the oxPt-resistant phenotype, transcriptional profiles of DOX-induced SW620.625 and SW620.ctrl cells were generated (Fig. 3a). We reasoned that a stronger impact on target miRNAs would be seen in SW620.625 cells as compared with HCT116.625 cells owing to the higher miR-625-3p levels in the former (Supplementary Fig. 3).

In total, 216 and 163 genes were up- and downregulated, respectively, in miR-625-3p expressing SW620.625 cells (absolute fold change > 1.5; Supplementary Data 1). We noted upregulation of several genes encoding ATP-binding cassette (ABC) transporter proteins (for example, ABCA6, FC = 17.4; and ABCA9, FC = 2.8, see Supplementary Data 1), however, the particular ABC proteins previously implicated in multi-drug resistance (for example, MDR1/ABCB1 and MRPI/ABCC1) were not dysregulated. Since no obvious pathways or single genes
with strong connection to drug resistance were identified, we addressed whether the dysregulated genes might be relevant in a clinical setting. To this end, we profiled 26 microsatellite stable primary tumours from mCRC patients receiving oxPt-based therapy as first-line treatment. Objective best response to treatment was then used as phenotype labels (Non-responder and Responder) in a gene set enrichment analysis. Interestingly, we found enrichment for SW620.625 upregulated genes among the non-responding patients (Fig. 3b). These data indicate a clinical relevance for the oxPt-resistant phenotype induced by ectopic miR-625-3p overexpression.

The MAPK kinase MAP2K6 is a direct target of miR-625-3p.

To identify miR-625-3p target genes, we searched the transcriptional profile for mRNAs with miR-625-3p target sequences that were downregulated in the SW620.625 cells. Overall, we found enrichment for mRNAs containing the miR-625-3p 7-mer target sequence (CTATAGT) in their 3’UTR among downregulated genes (Fig. 4a). To select putative target genes for experimental validation, we used the mirmap tool, which applies multiple predictors to generate a combined score of miRNA–mRNA repression strength (from 0 to 100; ref. 8). We selected the eight most downregulated genes with a miRmap score of 85.49 (Fig. 4f). To experimentally confirm this, an ~175 base fragment of the MAP2K6 3’UTR centred around this putative seed site was cloned into the 3’UTR of a Renilla Luciferase reporter gene construct (3’UTR WT Luc reporter). When transfected into HEK293T cells together with pre-miR-625-3p, Luc expression was reduced with 75% as compared with mock transfected cells (that is, Luc reporter with no MAP2K6 3’UTR) (Fig. 4g). The reduction was specifically related to miR-625-3p since co-transfection with a control pre-miR (Scr) had no effect on Luc (Fig. 4g). Furthermore, specific mutation of the miR-625-3p seed sequence (3’UTR mut1 and mut2) completely abolished miR-625-3p-mediated reduction of Luc (Fig. 4g).

Altogether, the data strongly support that MAP2K6 is a direct and functional target of miR-625-3p.

MAP2K6–MAPK14 signalling mediates oxPt response.

MAP2K6 is a dual specificity protein kinase, which transduces cellular and environmental stress signalling to its substrates, the p38 MAP kinases (MAPK11–14; ref. 10). In support of miR-625-3p regulating MAP2K6 signalling, we observed reduced phosphorylation of MAPK14 Tyr180/Y182 upon miR-625-3p-induction (Fig. 5a). To appreciate the resulting change in MAPK14 activity, we quantified the MAPK14 substrates HSPB1Ser82 (ref. 11), 4E-BP1Ser65 (ref. 12) and CDC25cSer216 (ref. 13) from multiple western blots; this showed 1.7–2.5- and 1.8–6.6-fold reduction of substrate phosphorylation in HCT116.625 and SW620.625, respectively (Fig. 5a).

To mechanistically investigate the role of MAP2K6 in oxPt response in CRC cells, we stably expressed MAP2K6 lacking the miR-625-3p binding site in HCT116.625 cells. Western blotting confirmed high ectopic MAP2K6 levels after DOX induction compared with endogenous MAP2K6 (Fig. 5b), which is relatively lowly expressed in HCT116 cells compared with other CRC cells (Fig. 4d and Supplementary Fig. 7). First, we addressed the immediate changes in MAPK14 activity upon 30 min oxPt treatment. OxPt exposure in HCT116.ctrl.mock control cells led
to increased MAPK14\textsuperscript{Tyr180/Y182} phosphorylation and a concurrent increase in MAPK14 activity (3.0-, 4.6- and 2.7-fold increased phosphorylation of HSPB1\textsuperscript{Ser82}, 4EBP1\textsuperscript{Ser65} and CDC25c\textsuperscript{Ser216} phosphorylation) was less than in HSPB1\textsuperscript{Ser82}, 4EBP1\textsuperscript{Ser65} and CDC25c\textsuperscript{Ser216} phosphorylation before treating them with oxPt for 30 min (Fig. 5c). No hyperactivation was observed, in fact the induced increase in HSPB1\textsuperscript{Ser82}, 4EBP1\textsuperscript{Ser65} and CDC25c\textsuperscript{Ser216} phosphorylation (2.2-, 1.5- and 1.6-fold increased HSPB1\textsuperscript{Ser82}, 4EBP1\textsuperscript{Ser65} and CDC25c\textsuperscript{Ser216} phosphorylation) was less than in HCT116.ctrl.mock cells and comparable to HCT116.625.map2k6 cells (Fig. 5c). This suggests the presence of feedback mechanisms such as the dual-specificity protein phosphatases\textsuperscript{14} or that other signalling components become limiting\textsuperscript{15}.

We next investigated how ectopic expression of the miR-625-3p insensitive MAP2K6 variant affected the ability of miR-625-3p to inhibit oxPt-induced cell death (Fig. 5d). As expected, after 48 h of oxPt treatment cell death was reduced in HCT116.625.mock compared to HCT116.ctrl.mock cells. The introduction of ectopic MAP2K6, however, resensitized HCT116.625.map2k6 cells to oxPt, reaching the same level of cell death as HCT116.ctrl.mock cells. In agreement with the changes in MAPK14 activity assessed after 30 min oxPt treatment (Fig. 5b,c), the control experiment with HCT116.ctrl.map2k6 cells confirmed that expression of MAP2K6 did not lead to oxPt hypersensitivity (Fig. 5d). Taken together, these findings indicate that increased oxPt resistance mediated by miR-625-3p is conveyed through its target MAP2K6.

To further corroborate the importance of MAP2K6 for mediating the effect of miR-625-3p in CRC cells, we generated stable HCT116 cells lines expressing a dominant-negative version of MAP2K6 harbouring a K82A mutation, which abolishes kinase activity\textsuperscript{16}. Western blotting showed the dominant-negative MAP2K6 to be expressed at many times higher level than the endogenous MAP2K6 (Fig. 5e). Dominant-negative MAP2K6 expressing cells showed a ~40\% reduction in 64 \&mgr;M oxPt-induced cell death compared with control HCT116 cells (Fig. 5e), and hence, mimics the phenotype of miR-625-3p overexpressing cells.

We finally asked whether MAP2K6 might be correlated with miR-625-3p and chemotherapy response in patients? Indeed, although not reaching significance, we found that MAP2K6 was
miR-625-3p negatively correlates with miR-625-3p expression in 26 mCRC tumours (Pearson’s $r = -0.22$; Fig. 5f). In addition, we also found MAPK26 to be slightly downregulated in non-responder patients compared with responder patients (Fig. 5g). Altogether, these data suggest that the oxPt-resistant phenotype induced by miR-625-3p in CRC cells operates through the direct target MAPK26.

**miR-625-3p dysregulates MAPK signalling pathways.** The results presented above indicates that the p38 MAPK subfamily (MAPK11–14) could be implicated as a mediator of platinum drug-induced stress signalling including apoptosis, a concept that has been exploited by others. We therefore profiled the phosphoproteome of both untreated and oxPt-treated HCT116 and HCT116.625 cells using stable isotope labelling by amino acid (SILAC)-based mass spectrometry of TiO$_2$-enriched phosphopeptides (Fig. 6a). We detected 9,423 distinct phosphopeptides on 3,217 different proteins including 177 kinases and 50 phosphatases (Supplementary Fig. 8a). We found between one and three phosphosites per protein on >75% of all detected proteins (Supplementary Fig. 8b), and...
detected predominantly serine phosphorylations (8,582 versus 816 and 25 threonine and tyrosine phosphopeptides, respectively; Supplementary Fig. 8c).

We first looked at the overall effect on the phosphoproteome after 48 h of increased miR-625-3p levels. Proteins with altered phosphorylations were mostly associated with GO terms involved with increased GTPase activity in nucleus, cytoplasm and adherence junction components, and with the mTOR, ErbB, insulin signalling and MAPK pathways (Fig. 6b). To look for changes in the activities of individual kinases, we did kinase substrate enrichment analysis (KSEA) using a merged collection of specific kinase phosphorylation site mappings obtained from the Human Protein Reference Database20, PhosphoSitePlus21 and PhosphoELM22 (see ‘Methods’ section). KSEA indicated decreased activity of MAPK8, MAPK14, MAPK1 and mTOR kinases, and increased activity of the CDK7, PRKACA and CSNK2A1 kinases, respectively, after miR-625-3p induction (Fig. 6c). In agreement, the mean log2 ratios of MAPK14, mTOR and MAPK1 substrate groups were significantly lower than the experimental mean, and for the PRKACA substrate group it was significantly higher (Fig. 6d). Collectively, this indicates that miR-625-3p overexpression leads to decreased activity of mTOR, MAPK1 and the MAPK14 kinases. The latter in agreement with the notion that miR-625-3p targets MAP2K6.

To more specifically investigate the role of MAPK14 in oxPt resistance, we first inactivated MAPK14 signalling (indicated by reduced HSPB1Ser82 phosphorylation) in HCT116 and SW620 cells through siRNA mediated depletion of MAPK14 (Supplementary Fig. 9). MAPK14 depletion was associated with increased resistance to oxPt-induced cell death. In HCT116 cells the induced death was reduced to 50% of control cells, and in SW620 cells to 85% of controls (Fig. 7a). Second, we used the small molecule inhibitor SB203580 to obstruct oxPt-induced MAPK14 activation in HCT116 cells as indicated by reduction of HSPB1Ser82 phosphorylation (Fig. 7b). SB203580 treatment reduced oxPt-induced cell death to less than 75% of control cells (Fig. 7c). A similar reduction in oxPt-induced cell death was also observed in SW620 cells (Fig. 7c). We also tested a second MAPK14 inhibitor, SB202190, which reduced the oxPt sensitivity in HCT116 cells, but not SW620 cells (Fig. 7c). Taken together, our observations in HCT116 and SW620 support that aberration of MAPK14 signalling plays a role in oxPt resistance.

Speculating whether the observations could be generalized and extended to additional CRC cell lines we generated stable, inducible miR-625-3p expression in the HCC2998 CRC cell line (Fig. 7d). This line represents a tumour etiology distinct from HCT116 and SW620 by being microsatellite stable, expressing a truncated TP53 variant, and displaying a hypermutator phenotype as a consequence of a POLE missense mutation23. miR-625-3p levels in HCC2998.625 cells after DOX induction was increased >20-fold (Supplementary Fig. 10) and associated with decreased MAP2K6 levels as well as with decreased MAPK14 activity (Fig. 7d). Similar to HCT116 and SW620 cells, ectopic
miR-625-3p expression reduced the 64 µM oxPt-induced cell death to ~75% of control cells (Fig. 7e). Using the same conditions as above (Fig. 7c), chemical inhibition of MAPK14 signalling in HCC2998 cells SB202190 also reduced oxPt induced cell death to ~70%, while SB203580 had no effect (Fig. 7f).

To further generalize the involvement of MAPK14 signalling in oxPt response, the two MAPK14 inhibitors were applied to four additional CRC cell lines. In all four cell lines, MAPK14 inhibition reduced the sensitivity to oxPt (Fig. 7g). Taken together, these data show that inhibition of MAPK14 phenocopies the effect of miR-625-3p overexpression and supports the notion that the MAP2K6–MAPK14 signalling network plays a central functional role in miR-625-3p-induced oxPt resistance (Fig. 7h).

The phosphoproteomic response to oxPt in CRC cells. To further characterize the role of miR-625-3p during oxPt treatment in CRC cells, we delineated phosphorylation changes associated with the immediate (30 min) response to oxPt in control CRC cells. Totally, we detected 205 phosphopeptides with phosphoserines/threonines preceding a glutamine, which are potential substrates of ATM and ATR DNA damage response kinases (Fig. 8a)24. The pS/pTQ motif was enriched among peptides that had increased phosphorylation after oxPt treatment (Fig. 8b), indicating that the DNA damage response signalling was induced after 30 min of oxPt exposure. Although phosphorylation of pS/pTQ motifs increased upon oxPt treatment, the general trend was the opposite. Indeed, we found more than three times as many phosphopeptides with decreased phosphorylation (n = 993) compared with phosphopeptides with increased phosphorylation (n = 313) after oxPt treatment (Fig. 8c), suggesting global dephosphorylation in CRC cells immediately after oxPt exposure similar to what has been observed after cisplatin treatment25. Dysregulated phosphoproteins were associated with processes involved in chromatin remodelling, mitotic cell cycle, microtubule organisation and pathways such as mTOR, cell cycle, ErbB and MAPK signalling (Supplementary Fig. 11). KSEA analysis suggested increased activities of ribosomal protein S6 kinases beta-1 and alpha-1 (RPS6KB1 and RPS6KA1), and various protein kinases known to be implicated in genotoxic stress signalling (PKRAC, PKRCD and PKRD1)26–29 as well as AKT1 (Fig. 8d). Reduced activities were found for cyclin-dependent kinases (Fig. 8e).
kinase 1 and 2 (CDK1 and CDK2) and polo-like kinase 1 (PLK1; Fig. 8d), in agreement with all three being positively involved in cell cycle progression and inhibition of DNA damage response.30,31 The importance of these kinases in the immediate cellular response to oxPt was also supported by increased mean log2 phosphorylation ratios for the RPS6KB1, RPS6KA1, PRKD1, AKT1 and PRKACA substrate groups, and by decreased ratios for the CDK1 and CDK2 substrate groups (Fig. 8e).

miR-625-3p blocks the normal cellular response to oxPt. We next investigated whether miR-625-3p expression affected the predicted activities of the oxPt-regulated kinases identified by KSEA (see Fig. 8d,e). In the 625 + OX/ctrl + OX experiment, a mean log2 ratio different from zero is expected for kinases whose activities after oxPt treatment are altered by increased miR-625-3p levels, while unaffected kinases will have a mean log2 ratio around zero. The mean phosphorylation ratios for the oxPt-induced PRKD1 and AKT1 substrate groups were decreased, while CDK1 and CDK2 substrates on average showed increased phosphorylation levels (Fig. 9a). Strikingly, the strongest change in mean log2 phosphorylation ratios were found for the MAPKAPK2 substrate group (MAPK14 substrate and binding partner) whose log2 ratio was decreased after oxPt.
Phosphorylation motif blockage of oxPt-induced signalling also was evident on a damage. blocks signalling cascades central in the normal response to DNA resistance phenotype (Fig. 2a,b), this suggested that induced nuclear lamina breakdown, increased phosphorylation was observed on multiple residues on LMNA in expressing cells, whereas unphosphorylated FZR1 in control cells suggested decreased CDK signalling at G0 or early G1 (ref. 67). In support of mitotic-G1/S checkpoint or in early M phase was also indicated by S138/S151 phosphorylations on inactivated FZR1 (also known as CDH1) in miR-625-3p expressing cells, whereas unphosphorylated FZR1 in control cells suggested decreased CDK signalling at G0 or early G1 (ref. 67). In support of mitotic-induced nuclear lamina breakdown, increased phosphorylation was observed on multiple residues on LMNA in miR-625-3p cells; On the contrary, these became dephosphorylated after oxPt treatment in control cells suggesting decreased cell cycle progression (also see Supplementary Fig. 14). (displayed as fcounts, groups involved in oxPt response were calculated for the 625 experiment. In agreement with the significantly altered substrate phosphorylation motifs identified for the ctrl −2 increase/no change in intensity, respectively, in HCT116.625 as compared with HCT116.ctrl cells. lysates from oxPt-treated HCT116.ctrl and HCT116.625 cells. Individual substrates are indicated with a dot with red and black indicating increase or representing Lamin A and C isoforms are indicated (normalized to

Figure 9 | Critical components of the cellular response to oxPt are blocked in cells with increased miR-625-3p levels. (a) Mean log2 ratios of substrates groups involved in oxPt response were calculated for the 625 + OX/ctrl + OX data. (b) Mean log2 for the MAPKAPK2 substrate group. (c) The most significantly altered substrate phosphorylation motifs identified for the ctrl + OX/ctrl and 625 + OX/ctrl + OX experiments identified using KSEA (displayed as fcounts, P ≤ 0.05 indicated with **`). Mean log2 ratios for substrates with these motifs were calculated. Coloured boxes in the boxplots of a, b and c indicate P ≤ 0.05, z-test; and NS, not significant. On the basis of similarity, the 16 individual motifs were grouped into the four motif groups indicated above. Note that the experimental mean log2 ratios for clarity have been omitted in b and c. (d) oxPt treatment in HCT116.ctrl cells led to dephosphorylation of Serine 130 (S130) of Cyclin-Dependent Kinase Inhibitor 1 (CDKN1A, also known as p21CIP1), which has been linked to increased stability of CDKN1A and inhibition of CDK/cyclin-mediated cell cycle progression65. In contrast, increased S130 phosphorylation was seen in cells with ectopic miR-625-3p expression. As indicated, this phosphorylation may itself be mediated by elevated CDK activity66. Increased CDK activity at the G1/S checkpoint or in early M phase was also indicated by S138/S151 phosphorylations on inactivated FZR1 (also known as CDH1) in miR-625-3p expressing cells, whereas unphosphorylated FZR1 in control cells suggested decreased CDK signalling at G0 or early G1 (ref. 67). In support of mitotic-induced nuclear lamina breakdown, increased phosphorylation was observed on multiple residues on LMNA in miR-625-3p cells; On the contrary, these became dephosphorylated after oxPt treatment in control cells suggesting decreased cell cycle progression (also see Supplementary Fig. 14). (e) Western blotting against the CDK1 substrate phospho-LAMIN A/C522 on lysates from oxPt-treated HCT116.ctrl and HCT116.625 cells. Quantification of bands representing Lamin A and C isoforms are indicated (normalized to β-actin signal). (f) Western blotting against the phosphorylated CDK motif p-TPXK on lysates from oxPt-treated HCT116.ctrl and HCT116.625 cells. Individual substrates are indicated with a dot with red and black indicating increase or decrease/no change in intensity, respectively, in HCT116.625 as compared with HCT116.ctrl cells.

treatment in HCT116.625 cells (Fig. 9b). The mean log2 ratios for all the five substrate groups were in the opposite direction in the 625 + OX/ctrl + OX as compared with the OX + ctrl/ctrl experiment. In agreement with the miR-625-3p-induced oxPt resistance phenotype (Fig. 2a,b), this suggested that miR-625-3p blocks signalling cascades central in the normal response to DNA damage.

Further, we investigated whether miR-625-3p-mediated blockage of oxPt-induced signalling also was evident on a phosphorylation motif level. KSEA analysis and mean log2 phosphorylation ratios on motif groups (that is, phosphopeptides with a similar 15 amino acid-motif centred on the phosphorylated residue) suggested that oxPt treatment of control cells led to increased kinase activities directed towards serines that are preceded by one or two basic arginine residues (R-pS motifs), or followed by an acidic aspartate (pS-D motifs) (Fig. 9c). Dephosphorylation after oxPt treatment was seen on proline directed motifs with or without a single trailing basic residue (pS/pTP-R/K and pS/pTP motifs; Fig. 9c), which are typically associated with the CDK, MAPK and GSK families32. In contrast, the oxPt response in the context of miR-625-3p led to increased pS/pTP-R/K-associated kinase activity, and generally, decreased R-pS-directed activity, while phosphorylations on pS/pTP motifs, in general, were similar in ctrl and 625 cells (Fig. 9c).

We used the network-based NetworKIN data set33 to identify kinases most likely associated with the differentially phosphorylated R-pS, pS-D and pS/pTP-R/K motifs (Supplementary Fig. 12). A significant association was found between the oxPt-induced motifs (R-pS and pS-D) and multiple kinase families including AKT1 and AKT2 kinases, protein kinase A, Calcium/Calmodulin-Dependent Protein Kinase II kinases (CAMKII), as well as HIPK2 and PAK kinases. The miR-625-3p specific pS/pTP-R/K motif was most strongly associated with cyclin-dependent kinases (CDK1, CDK2 and CDK5), and to a lesser extent with MAP kinases and TTK kinase. As expected, many of these kinases are involved in DNA damage response...
(for example, AKT, CAMKII, HIPK2 and PAK) and cell cycle regulation (for example, CDK, MAPK and TTK). Furthermore, several of them overlap with the kinases identified in the substrate group analysis.

To identify individual phosphoproteins associated with the observed progressive cell cycle signalling, we first defined regulatory classes based on those phosphopeptides with phosphorylation changes in opposite direction in the ctrl+/Ox/ctrl and 625+OX/ctrl+OX experiments (Supplementary Fig. 13). Among these, we identified several cell cycle-associated proteins, including CDKN1A, FXR1 and LAMIN A/C (Fig. 9d), with differential phosphorylation patterns that supported increased cell cycle progression in oxPt-treated HCT116.625 cells compared with control cells (Supplementary Fig. 14).

Phosphospecific western blotting against LAMIN A/C Ser22 (Fig. 9e) — a known CDK1 target at the onset of mitosis — confirmed the observed increase in lamin phosphorylation (Supplementary Fig. 14), which is a marker of nuclear envelope disassembly during mitosis. Interestingly, increased LAMIN A/C Ser22 phosphorylation in oxPt-treated HCT116.625 cells appeared to be a consequence of an increase in the LAMIN C over the LAMIN A isoform (Fig. 9e).

To confirm increased CDK activity after oxPt treatment in HCT116.625 cells, we did phosphospecific western blotting against the most differentially phosphorylated CDK motif pTPXK (Fig. 9c, a target for CDKs 1 and 2, among others). This revealed increased phosphorylation in oxPt-treated HCT116.625 cells at the majority of CDK substrates consistent with increased activity (Fig. 9f).

Finally, we found that phosphorylation of ATM/ATR pT/pSQ motifs in the oxPt-treated HCT116.625 cells was significantly increased (P<0.05, Fisher’s exact test), indicating that alteration of cell cycle signalling in these cells was not related to lack of DNA damage sensing per se (Supplementary Fig. 15a,b). This suggests that mir-625-3p acts after, or independently of, the immediate ATM/ATR-mediated DNA damage response (Supplementary Fig. 15c).

Altogether, these analyses are in support of the hypothesis that mir-625-3p induces blockage of signalling pathways involved in normal oxPt response, which, among other things, culminates in increased cell cycle progression signals relative to control cells.

Discussion

Previously, we reported that high expression of mir-625-3p in primary tumours of mCRC patients was associated with an odds ratio above 6 for a poor response to first-line oxPt-based therapy. In the present work, we have shown that mir-625-3p functionally leads to oxPt resistance by preventing the DNA damage response system to induce cell cycle arrest and apoptosis. Furthermore, we have identified MAP2K6 as a functional target for mir-625-3p, and as a mediator of mir-625-3p-induced oxPt resistance. To the best of our knowledge, MAP2K6 is the first functionally documented target of mir-625-3p, and conversely, mir-625-3p is the first described microRNA targeting MAP2K6. MAP2K6 (together with MAP2K3) catalyses dual phosphorylation of the TGY motif in the activation loop of the four p38 MAPK isoforms (MAPK11–14; refs 35–37), and as such conveys p38-mediated cellular stress signalling. The presented results are consistent with a model where mir-625-3p through downregulation of MAP2K6 impairs p38-MAPK stress signalling (Fig. 7h and Supplementary Fig. 15c). It is important to emphasize, however, that our model only addresses mir-625-3p signalling through MAP2K6. It is likely that mir-625-3p additionally could mediate resistance by regulating other unknown target proteins.

On the basis of our results using chemical inhibitors and MAPK14 knockdown, and in agreement with other studies, we are inclined to believe that the MAPK14 isoform of p38 is a mediator of mir-625-3p-induced oxPt resistance. We are aware of the discrepancy in the effect on oxPt sensitivity after chemical inhibition in two (SW620 and HCC2998) out of seven cell lines tested, which we attribute to the cell-specific off-targeting effects known to exist for SB203580 and SB202190 (refs 40,41). Our phosphoproteome data in exponentially growing unstressed CRC cells also revealed that MAPK14 was the kinase whose activity (on a substrate level) was mostly affected by mir-625-3p induction. Finally, oxPt treatment showed increased activity of the MAPKAPK2 kinase, which is a canonical MAPK14 substrate and binding partner responsible for nuclear translocation of MAPK14 after stress. This suggests that MAPK14–MAPKAPK2 activation plays a role during oxPt response in cancer cells. Such notion is further supported by our observation of reduced activity of MAPKAPK2 in oxPt-resistant HCT116.625 cells.

We observed resistance to oxPt after mir-625-3p induction in all three cell models — with the strongest phenotype obtained in HCT116 cells — despite different levels of induction (3× in HCT116, 25× in HCC2998 and >400× in SW620) and different degrees of MAP2K6 reduction (0.8× in HCT116, 0.4× in HCC2998 and 0.2× in SW620). This indicates that the resulting level of MAP2K6 protein — rather than changes in mir-625-3p and MAP2K6 per se — determines response to oxPt. Alternative explanations include cell-specific wiring and dependencies of the MAP2K6–MAPK14 signalling pathway, and diversity in a stress mediator downstream of MAPK14. An interesting candidate is TP53, which is mutated in SW620 and HCC2998 cells but wild type in HCT116. These hypotheses will have to be addressed in future studies.

Induction of p38 signalling by platinum-based drugs has been ascribed a pro-apoptotic role in multiple types of cancer cells. On the other hand, p38 may also induce survival signals after cytotoxic stress. In fact, MAP2K3/6-p38-MAPKAPK2/3 activation has recently emerged as a third signalling axis during DNA damage response, alongside ATM-CHEK2 and ATR-CHEK1 (refs 48,49). In this setting, p38 signalling functions as a cell cycle checkpoint by deactivating CDC25s, cyclinE and CDK1 to prevent premature mitotic entry. Thus, the outcome from dysregulated p38 signalling in drug-treated cancer cells appears to be a function of several factors including the extent and nature of the cellular insult. In that respect, we note that increased sensitivity to the topoisomerase I inhibitor irinotecan (another drug used to treat CRC patients) has been shown to correlate with decreased p38 phosphorylation in CRC patients. Following this, CRC patients with high mir-625-3p levels and reduced MAP2K6–MAPK14 signalling, and therefore resistance to oxPt, may instead benefit from irinotecan treatment as first-line therapy.

The findings reported suggest that the expression level of mir-625-3p, possibly in combination with the expression level and activity of MAP2K6 and MAPK14, has the potential to serve as a biomarker for predicting response to oxPt. Since up to 20% of mCRC patients show high mir-625-3p expression, the number of patients that potentially could benefit from quantification of the mir-625-3p biomarker is substantial. In addition, the observation that anti-mir-625-3p treatment makes cells with high mir-625-3p level responsive to oxPt, indicates that it may be possible to sensitize patients with high mir-625-3p expressing cancers to oxPt by mir-625-3p antagonist treatment before, or simultaneously with, oxPt treatment.

In conclusion, we have shown that overexpression of mir-625-3p in CRC cells can induce resistance to oxPt by directly targeting MAP2K6 and consequently inactivating genotoxic stress signalling conveyed by the MAP2K6–MAPK14 pathway.
Methods

Patients. Fresh frozen primary tumour biopsies originated from 26 patients who were treated with oxP and 5-FU as first-line therapy for mCRC in the Departments of Odense University Hospital and Aarhus University Hospital, Denmark, as described in ref. 52. Informed consent was obtained from all the patients. The study was approved by the national ethics committees and governmental authorities in Denmark and was conducted in accordance with the Declaration of Helsinki. The patients were grouped according to objective therapy response into nine poor responders (best response being either ‘Progressive disease’ or ‘Stable disease’) and 17 good responders (‘Partial response’ or ‘Complete response’).

Cell lines. HEK293 Flp FRT/eGFP was a gift from Jacob Giehm Mikkelsen, Aarhus University, while CRC cells originated from the ATCC and NCI-60 were authenticated by our in-house STR analysis (http://identicell.dk), and were tested negative for mycoplasma using MycoSensor PCR Assay Kit (Stratagene). The cells were propagated and frozen. We generated MAP2K6 (or Mock) expressing cells by transposing HCT116.625 (and HCT116.ctrl) cells with pSBInducer vector according to the strategy outlined above. From this, we removed the tRFP-miR30-shRNA-miR30 element (see Supplementary Fig. 1) expressing pSBInducer version according to the strategy outlined above. In all the steps, plasmid DNA was purified with GeneElute Plasmid Miniprep Kit (Sigma-Aldrich). Correct insertion was confirmed by sequencing and with appropriate restriction digestions.

Generation of pSBInducer cells. To generate pSBInducer cells, approximately 10 mio. cells were transfected with 1,500 ng pSBInducer.shRNA DNA (siEGFP, mir-625-3p or scramble) and 1,500 ng pCMV-SB100XCO helper plasmid (or, as a negative control, 1,500 ng pUC19 DNA) using 15 μl Lipofectamine 2000 (Invitrogen) in 500 μl Opti-Mem 1 Medium (Gibco, Invitrogen-Life Technologies). After transfection, cells were incubated for 24 h before refreshing the media. The cells were treated with a puromycin concentration of 1 μg·mL⁻¹ (HCT116 and HCC2998) or 2 μg·mL⁻¹ puromycin (SW620 and HEK293 Flp FRT/eGFP) for 5 days to eliminate control transfected cells. We used the iRFP fluorescence marker to sort for cell populations expressing the siRNA after induction; these cells were frozen and used for subsequent experiments. All the experiments were conducted with low-passage (<10 passages after sorting) cell populations. Single cell clones were generated from single RFP-positive cells sorted directly into 96 wells from where they were propagated and frozen. We generated MAP2K6 (or Mock) expressing pSBInducer cells with low-passage (8,000 cells were visualized although at least 50,000 cells were counted) and treated with 64 μM oxP for 48 h. Adherent and non-adherent cells were collected, pooled and stained using the Annexin V–FITC Apoptosis Detection Kit (Roche Applied Science). Cellular death (LDH release) was measured using the CytoTox96 Non-Radioactive Cytotoxicity Assay (Promega Corporation;#G3220). Fluorescence signal was measured using a multi-well ELISA reader (Synergy HT-reader, BioTek).

HCT116 625 (and HCT116.ctrl) cells were transfected with pINDUCER11 vector (in which the puromycin resistance gene is replaced by FLAG-MAP2K6 and FLAG-MAP2K6DN, we first used the pINDUCER vector to sort for cell populations expressing the shRNA after induction; these cells were frozen and used for subsequent experiments. All the experiments were conducted with low-passage (<10 passages after sorting) cell populations. Single cell clones were generated from single RFP-positive cells sorted directly into 96 wells from where they were propagated and frozen. We generated MAP2K6 (or Mock) expressing pSBInducer cells with low-passage (8,000 cells were visualized although at least 50,000 cells were counted) and treated with 64 μM oxP for 48 h. Adherent and non-adherent cells were collected, pooled and stained using the Annexin V–FITC Apoptosis Detection Kit (Roche Applied Science). Cellular death (LDH release) was measured using the CytoTox96 Non-Radioactive Cytotoxicity Assay (Promega Corporation;#G3220). Fluorescence signal was measured using a multi-well ELISA reader (Synergy HT-reader, BioTek).

Anti-miR and siRNA experiments. For anti-miR experiments, cells were DOX-induced and treated with 64 μM oxP for 48 h. Adherent and non-adherent cells were collected, pooled and stained using the Annexin V–FITC Apoptosis Detection Kit (Sigma-Aldrich) according to the manufacturer’s instructions. For miRNA experiment, single-strand cDNA was synthesized using the Superscript Reverse Transcriptase Kit (Life Technologies) and qRT–PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems) as described in the protocol. Small RNA and mRNA expression was normalized with 5S and GAPDH, respectively. Samples with a mean Ct > 40 were assigned ‘Undetermined’. All qRT–PCR measurements were done on a 7900 HT instrument (Applied Biosystems). MAPIK4 miRNA was detected using TaqMan Assay Hs01051512_m1 (Cat# 4331182 Applied Biosystems) and normalized to UBC.

RNA extraction, reverse transcription and qRT–PCR. Total RNA from cell lines was purified using QIAzol Lysis Reagent (Quagen) according to the manufacturer’s guidelines. RNA quality and integrity was ensured according to Agilent 2100 Bioanalyzer runs (RIN score > 9.5 for all samples; Agilent Technologies). Small RNA expression levels were quantified with qRT–PCR according to the protocol of the Universal CDNA synthesis kit (Exonix) using miRCURY LNA Universal RT microRNA PCR assays (Exonix) and SYBR Green master mix (Exonix) according to the manufacturer’s instructions. For mRNA detection, single-strand DNA was synthesized using the Superscript Reverse Transcriptase Kit (Life Technologies) and qRT–PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems) as described in the protocol. Small RNA and mRNA expression was normalized with 5S and GAPDH, respectively. Samples with a mean Ct > 40 were assigned ‘Undetermined’. All qRT–PCR measurements were done on a 7900 HT instrument (Applied Biosystems). MAPIK4 miRNA was detected using TaqMan Assay Hs01051512_m1 (Cat# 4331182 Applied Biosystems) and normalized to UBC.

Cell viability and death assays. Cell viability was measured using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay (Roche Applied Science). Cellular death (LDH release) was measured using the CytoTox96 Non-Radioactive Cytotoxicity Assay (Promega Corporation). Fluorescence signal was measured using a multi-well ELISA reader (Synergy HT-reader, BioTek).

Annexin V–PI apoptosis assay. For the apoptosis assay, cells were DOX-induced and treated with 64 μM oxP for 48 h. Adherent and non-adherent cells were collected, pooled and stained using the Annexin V–FITC Apoptosis Detection Kit (Sigma-Aldrich) according to the manufacturer’s protocol. Flow cytometry was performed at the FACs Core Facility, The Faculty of Health Sciences, Aarhus University, Denmark on a FACSaria III (BD Biosciences). Flowjo software version 8.8.3 (Tree Star Inc.) was used for data analysis. Initially, cells were gated with forward scatter-area (FSC-A) versus side scatter-area (SSC-A) followed by SSC-A versus forward scatter-height (FSC-H) to obtain cell singlets after which the percentage of cells in each quadrant of the fluorescein isothiocyanate (FITC) versus PI plot were obtained. For clarity only n = 8,000 cells were visualized although typically at least 50,000 cells were counted.

Western blotting. Protein extraction and western blotting analysis were performed according to standard procedures. Antibodies were GFP (1:1,000, Abcam, ab1218), β-actin (1:25,000, Abcam, ab9900), tubulin (1:5,000, Abcam, ab2791), p38/2/MAPK14 (1:500, Santa Cruz Biotehologies, SC-81621), MAP2K6/MKK7 (1:500–1:1,000), Cell Signaling (1:8550), MAP2K6/MKK7 (1:1,000, Santa Cruz Biotehologies, SC-1042), IRAK2 (1:1,000, Cell Signaling, #3467), phospho-Thr180/Tyr182-p38a/p46/MAPK14 (1:750, Cell Signaling, #9211), phospho-Ser82-HSPB1 (1:2,000, Cell Signaling, #2406), phospho-Ser261-CDC25c (1:750, Cell Signaling, #4901), phospho-Serb5-6EBP1 (1:750–1:1,000, Cell Signaling, #9456), phospho-52-Lamin A/C (1:1,000, Cell Signaling, #2026) and phospho-CDK Substrate (1:1,000, Cell Signaling, #3437). Densitometrical quantification of MAP2K6 protein and MAPK14 phospho-substrates was done in ImageJ using β-actin and tubulin as loading controls (Supplementary Fig. 16).

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Silac labelling and phosphoprotein enrichment. The HCT116.625 and HCT116.ctrl cells were grown in SILAC RPMI 1640 medium (PAA, Cat. #EI15-007) supplemented with heavy isotopes of arg10.13C6.15N4 (1.14 mM) and lys13C6.15N2 (0.22 mM); Cambridge Isotope Laboratories, Cat. #CNLM-539-H and #CNLM-291-H), medium isotopes Arg6-13C and Lys4-D4 (Cat. #CCLM-2265-H and #DLM-2640-O) or light isotopes Arg0 and Lys0 (Sigma). After >6 cell passages, we ensured that the incorporation rate was >95%. We also ensured that the pH 8.0 at 80% of cell density was within the recommended range for stable isotope incorporation. Two triplicate miRmap tool (http://mirmap.ezlab.org/app) was used with standard parameters using the options ‘Species’ = Human and ‘miRNA’ = hsa-miR-625-3p; Candidate target genes with a miRmap score > 75 were extracted.

Proteome and phosphoprotein data analyses. From the two (HCT116.625 and HCT116.ctrl) triplicate proteome intensity data, we made log2(625/ctrl) ratios, and used only distinct proteins that were detected in all three ratios (n = 2,410). All proteins with an absolute log2(625/ctrl) > 0.58 are listed in Supplementary Data 2. SW620 phosphopeptides were generated by separating protein lysates on a denaturing gel (10% acrylamide gel, 20% and 45% acrylamide) and was exposed to 16°C for 15 min. For phosphopeptide-enriched samples, the column oven (PRSO-V1, Sonation GmbH, Biberach, Germany) and the LC system was interfaced with the Q Exactive mass spectrometer (Thermo Scientific). Formic acid 0.1% was used to buffer the pH in the two running buffers used. The total gradient was 250 min followed by a 15 min washout and re-equilibration. In addition, the flow rate started at 250 nl min⁻¹ and 5% ACN with a linear increase to 25% ACN over 220 min followed by 30 min linear increase to 60% ACN. The washout followed with 60% ACN for 5 min followed by re-equilibration with a 5 min linear gradient back down to 5% ACN, which were maintained for the last 5 min. For phosphopeptide-enriched samples, the Q Exactive was operated with a Top12 method using a full scan range resolution 17,500 and 44 ms fill time. Isoelectric focusing was used followed by a Top10 method and fragment scan 17,500 and 44 ms fill time. Mass spectrometry was performed with a Top10 method and fragment scan resolution 17,500 and 44 ms fill time. The empirical distribution of the ranks for genes with and without the target motif complementary to the seed sequence of SW620 was compared using a Kolmogorov–Smirnov test. The web-based tool TargetScan (6.2) for all human annotated transcripts, and the most conserved target motif was compared using an in-gel digestion followed by nLC-MS60. In HCT116 cells, MAP2K6 were quantified against the human Uniprot database (April 2012 release). Carbamidomethylation of cysteine was specified as fixed modification for both groups. For the proteome data, variable modifications were considered for oxidation (M), N-terminal acetylation (N), and thiocyanate formation (S). The mass between run option was enabled, and the minimum score for both modified and unmodified peptides were set to 25, we used false discovery rate cut-off of 1% on peptide level.

Pompe disease and phosphoprotein treatment. Pompe disease and phosphoprotein treatment. We merged and manually curated three collections of known or believed to catalyse the phosphorylation. The phosphorylation positions acted as unique identifiers. Log2 ratios of normalized phosphopeptide intensities were generated for each (control + ox/ctrl, 625/ctrl, 625 + ox/ctrl and 625 + ox/ ctrl + OX experiment, and the mean log2 ratio calculated for phosphopeptides detected in at least two out of three experimental conditions, while singly detected phosphopeptides were discarded. We used a t-test to test the null hypothesis of no difference, that is, H0: log2 ratio = 0, and estimated the local false discovery rate to obtain the confidence of individual log2 ratios being false positives. The Enrichr tool was used to obtain enrichment scores (P-values) to KEGG pathways and GO terms. The most significant pathways were selected with a Bonferroni-corrected p value of 5 (lowest P values) for each category were reported. To detect changes in kinase activities, we applied the KSEA framework developed by Cassado et al. This is based on assigning individual phosphopeptides to one or more substrate groups according to the kinase(s) known or believed to catalyse the phosphorylation. To obtain such information, we merged and manually curated three collections of kinase phosphorylation mappings obtained from the Human Protein Reference Database29. PhosphoSitePlus and PhosphoELM. From this collection, we extracted those phosphorylated substrates detected in our experiments, and used this to make 25 substrate groups with at least 10 substrate members. The fraction delta-count (FDC) was then calculated as the number of substrates with increased (log2 ratio > 0, Np) minus the number of substrates with decreased (log2 ratio < 0, Nn) phosphorylation divided by the total number of substrates in the group, that is: FDC = (Np − Nn)/(Np + Nn). A Benjamin–Hochberg corrected P-value from a hypergeometric test was calculated to indicate the statistical significance of enrichment. We performed this analysis with the “enrichr” R package with the gene set collection as the “KSEA” collection.
using the z statistics (z = (x − μ)/σ/N0.5), where s is the population standard deviation and N the number of substrates in the group). The Z values were converted into two-sided p values via the standard normal distribution (P = 2P(−abs(z))). We corrected for multiple testing with the Benjamini–Hochberg procedure. To find altered phosphorylation motifs by KSEA for the ctrl + OX/ctrl and 625 + OX/ctrl + OX data, we first extracted 15 amino acids–windows (7 + 1 + 7) around the central phosphorylated amino acid for all serine and threonine phosphopeptides (detected in two out of three experimental triplicates). Proteins with phosphorylations within seven amino acids from the N or carboxy (C) termini were discarded. Phosphopeptides were then subjected to the motif-x algorithm64 using the following parameters: ‘occurrences’ = 5, ‘significant’ = 0.000001 and ‘background’ = IP1 Human Proteome. This resulted in 84 different motifs based on 7,850 phosphopeptides detected in the experiment. We further restricted KSEA to 51 motifs that were detected at least 50 times. To find kinases whose activities were associated with individual substrates and motifs, we obtained the netWoKIN data set of kinase substrate mappings33, but restricted us to entries with a high confidence (arbitrarily chosen as a netwoKIN score > 3), corresponding to 36,972 out of the total 304,338 distinct kinase substrates in netwoKIN. To predict kinase activities associated with the differentially phosphorylated motifs (P-R, P-S and P-T) we simplified the 15 amino acid motifs to 11 amino acids (5 + 1 + 5) motifs to directly use the 11 amino acids–phosphorylation windows provided for substrates in the netwoKIN data. Note that this resulted in the ‘…SP..’ motif. A motif was removed from the analysis since it is minimally described by a 6 + 1 + 6 format. We counted the enrichment score (ES) for each motif being associated with a given kinase as: ESm motif,k = (n(k)(m,N)/N0.5)/N0.5, where n(k) is the number of times a kinase k is mapped to a motif m, Nm is the total number of motifs m, Nk is the total number kinases k and N is the total number of kinase motif mappings. We used a BH-adjusted P value from a two-sided Fisher’s exact test as a significance value for the association, and for each motif selected the top-10 kinases with the smallest P value and with at least 100 observations for a motif (n(k) ≥ 100). Due to overlap between motifs, this resulted in 39 distinct kinases.

Statistical analysis. If not mentioned otherwise, a two-sided Student’s t-test was performed to evaluate statistical significance of differences in means, and the Fisher’s exact test used to test independence of count data. Experiments were performed at least three times and P values ≤0.05 were considered significant (indicated with “*” in figures).

Data availability. Proteome data are available via ProteomeXchange with identifier PXD002172. Clinical and cell line expression data can be obtained via GEO with identifiers GSE83129 and GSE83131, respectively. The authors declare that all other data supporting the findings of this study are available within the article and its Supplementary Information Files or from the corresponding author upon reasonable request.

References
1. Jemal, A. et al. Global cancer statistics. CA Cancer J. Clin. 61, 69–90 (2011).
2. Schmitt, H. J. et al. ESMO Consensus Guidelines for management of patients with colon and rectal cancer: a personalized approach to clinical decision making. Ann. Oncol. 23, 2479–2516 (2012).
3. Bartel, D. P. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 116, 281–297 (2004).
4. Blower, P. E. et al. MicroRNAs modulate the chemosensitivity of tumor cells. Mol. Cancer Ther. 7, 1–9 (2008).
5. Rasmussen, M. H. et al. High expression of microRNA-625-3p is associated with poor response to first-line oxaliplatin based treatment of metastatic colorectal cancer. Mol. Oncol. 7, 637–646 (2013).
6. Hackett, C. S., Geurts, A. M. & Hackett, P. B. Predicting preferential DNA acids–phosphorylation windows provided for substrates in the netwoKIN data. Statistical analysis and docking interactions in serine/threonine protein kinases. Mol. Cell. 43, 788–797 (2012).
7. Horn, B. et al. Differential activation of MAPKAP kinases 2. J. Biol. Chem. 287, 5065–5081 (2012).
8. Yoshida, K., Wang, H. G., Miki, Y. & Kufe, D. Protein kinase Cdelta is responsible for constitutive and DNA damage-induced phosphorylation of Rad9. EMBO J. 22, 1431–1441 (2003).
9. Archambault, V. & Glover, D. M. Polo-like kinases: conservation and divergence in their functions and regulation. Nature Rev. Mol. Cell. Biol. 10, 269–275 (2009).
10. Satyanarayana, A. & Kaldis, P. Mammalian cell-cycle regulation: several CDks, numerous cyclins and diverse compensatory mechanisms. Oncogene 28, 2925–2939 (2009).
11. Goldsmith, E. J., Akella, R., Min, X., Zhou, T. & Humphreys, J. M. Substrate and docking interactions in serine/threonine protein kinases. Chem. Rev. 107, 4946–4977 (2011).
12. Horn, H. et al. KinomeXplorer: an integrated platform for kinome biology studies. Nat. Methods 11, 603–604 (2014).
13. Heald, R. & McKeon, F. Mutations of phosphorylation sites in lamin A that prevent nuclear lamina disassembly in mitosis. Cell 61, 579–589 (1990).
14. Goedert, M., Cuenda, A., Craxton, M., Jakes, R. & Cohen, P. Activation of the type I protein kinase A is mediated by RI subunit interprotein disulfide bond formation. J. Biol. Chem. 281, 21827–21836 (2006).
15. Eisenberg-Lerner, A. & Kimchi, A. PKD is a kinase of Vps34 that mediates Oxidant-induced activation of type I protein kinase A is mediated by RI subunit interprotein disulfide bond formation. J. Biol. Chem. 281, 21807–21816 (2006).
41. Elkins, J. M. et al. Comprehensive characterization of the Published Kinase Inhibitor Set. Nat. Biotechnol. 34, 95–103 (2016).

42. Ben-Levy, L., Casadio, R., Paterson, H. & Marshall, C. J. Nuclear export of the stress-activated protein kinase p38 mediated by its substrate MAPKAP kinase-2. Curr. Biol. 8, 1049–1057 (1998).

43. Deacon, K., Mistry, P., Chernoff, J., Blank, J. L. & Patel, R. p38 Mitogen-activated protein kinase mediates cell death and p21-activated kinase mediates cell survival during chemotherapeutic drug-induced mitotic arrest. Mol. Biol. Cell. 20, 3011–3021 (2009).

44. Zhu, Y., Regunath, K., Jacq, X. & Prives, C. Cisplatin causes cell death via TAB1 regulation of p53/MDM2/MDM2/MDM3. Gesh Dev. 27, 1739–1751 (2013).

45. Germani, A. et al. Targeted therapy against chemoresistant colorectal cancers: Inhibition of p38alpha modulates the effect of cisplatin in vitro and in vivo through the tumor suppressor FoxO3A. Cancer Lett. 344, 110–118 (2014).

46. Pereira, L., Igea, A., Canovas, B., Dolado, I. & Nebreda, A. R. Inhibition of p38 MAPK sensitizes tumour cells to cisplatin-induced apoptosis mediated by reactive oxygen species and JNK. EMBO Mol. Med. 5, 1759–1774 (2013).

47. Phong, M. S. et al. p38 mitogen-activated protein kinase promotes cell survival in response to DNA damage but is not required for the G2(0)/DNA damage checkpoint in human cancer cells. Mol. Cell. Biol. 30, 3816–3826 (2010).

48. Llopis, A. et al. The stress-activated protein kinases p38alpha/beta and JNK1/2 cooperate with Chk1 to inhibit mitotic entry upon DNA replication arrest. Cell Cycle 11, 3627–3637 (2012).

49. Reinhardt, H. C., Aslanian, A. S., Lees, J. A. & Yaffe, M. B. p53-deficient cells rely on ATM- and ATR-mediated checkpoint signaling through the p38MAPK/ MK2 pathway for survival after DNA damage. Cancer Cell 11, 175–189 (2007).

50. Reinhardt, H. C. et al. DNA damage activates a spatially distinct late cytoplasmic cell-cycle checkpoint network controlled by MK2-mediated RNA stabilization. Mol. Cell 40, 34–49 (2010).

51. Paillas, S. et al. Targeting the p38 MAPK pathway inhibits irinotecan resistance in colon adenocarcinoma. Cancer Res. 71, 1041–1049 (2011).

52. Rasmussen, M. H. et al. High expression of microRNA-625-3p is associated with poor response to first-line oxaliplatin based treatment of metastatic colorectal cancer. Mol. Oncol. 7, 637–646 (2013).

53. Meereby, K. L. et al. The pINDUCER lentiviral toolkit for inducible RNA interference in vitro and in vivo. Proc. Natl Acad. Sci. USA 108, 3665–3670 (2011).

54. Derbyshire, Z. E., Hafter, U. M., Heimark, R. L., Sy, T. H. & Vaillancourt, R. R. Angiotensin II stimulated transcription of cyclooxygenase II is regulated by a novel kinase cascade involving Pyk2, MEKK4 and annexin II. Mol. Cell. Biochem. 271, 77–90 (2005).

55. Zanie, B. W. et al. Mammalian mitogen-activated protein kinase pathways are regulated through formation of specific kinase-activator complexes. J. Biol. Chem. 271, 29876–29881 (1996).

56. de Hoon, M. J., Imoto, S., Nolan, J. & Miyano, S. Open source clustering software. Bioinformatics 20, 1453–1454 (2004).

57. Saldanha, A. J. Java Treeview—extensible visualization of microarray data. Bioinformatics 20, 3246–3248 (2004).

58. Kelstrup, C. D. et al. Rapid and deep proteomes by faster sequencing on a benchtop quadrupole ultra-high-field Orbitrap mass spectrometer. J. Proteome Res. 13, 6187–6195 (2014).

59. Cox, J. & Mann, M. MaxQuant enables high peptide identification rates, individualized p.p.-range mass accuracies and proteome-wide protein quantification. Nat. Biotechnol. 26, 1367–1372 (2008).

60. Shavchenko, A., Tomas, H., Havlis, J., Olsen, J. V. & Mann, M. In-gel digestion for mass spectrometric characterization of proteins and proteomes. Nat. Protoc. 1, 295–296 (2006).

61. UniProt, C. UniProt: a hub for protein information. Nucleic Acids Res. 43, D204–D212 (2015).

62. Strimmer, K. A unified approach to false discovery rate estimation. BMC Bioinformatics 9, 303 (2008).

63. Chen, Y. Y. et al. Enrichr: interactive and collaborative HTML5 gene list enrichment analysis tool. BMC Bioinformatics 14, 128 (2013).

64. Schwartz, D. & Gygi, S. P. An iterative statistical approach to the identification of protein phosphorylation motifs from large-scale data sets. Nat. Biotechnol. 23, 1391–1398 (2005).

65. Bornstein, G. et al. Role of the SCFSkp2 ubiquitin ligase in the degradation of p21Cip1 in S phase. J. Biol. Chem. 278, 25752–25757 (2003).

66. Jarvilaatu, A. et al. Phosphorylation of the cyclin-dependent kinase inhibitor p21Cip1 on serine 130 is essential for viral cyclin-mediated bypass of a p21Cip1-imposed G1 arrest. Mol. Cell. Biol. 26, 2430–2440 (2006).

67. Qiao, X., Zhang, L., Gamper, A. M., Fujita, T. & Wan, Y. APC/C-Cdh1: from cell cycle to cellular differentiation and genomic integrity. Cell Cycle 9, 3904–3912 (2010).

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Author contributions

M.H.R. and I.L. designed and performed the experiments, as well as analysed most of the data. M.H.R., I.L., R.R.-C. and J.V.O. designed, performed and analysed the proteome part. L.S.T., T.P.H., F.H. and P.P. collected patient material and evaluated responses to therapy. M.H.R., I.L. and B.P.-B. designed the vectors. M.H.R., M.M.N. and J.S.P. performed the bioinformatics. M.H.R., I.L., T.F.O. and C.I.A. conceived the overall study design and wrote the paper.

Additional information

Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

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