The FOXM1-PLK1 axis is commonly upregulated in oesophageal adenocarcinoma

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BACKGROUND: The transcription factor FOXM1 is an important regulator of the cell cycle through controlling periodic gene expression during the G2 and M phases. One key target for FOXM1 is the gene encoding the protein kinase PLK1 and PLK1 itself acts in a positive feedback loop to phosphorylate and activate FOXM1. Both FOXM1 and PLK1 have been shown to be overexpressed in a variety of different tumour types.

METHODS: We have used a combination of RT–PCR, western blotting, tissue microarrays and metadata analysis of microarray data to study whether the FOXM1-PLK1 regulatory axis is upregulated and operational in oesophageal adenocarcinoma.

RESULTS: FOXM1 and PLK1 are expressed in oesophageal adenocarcinoma-derived cell lines and demonstrate cross-regulatory interactions. Importantly, we also demonstrate the concomitant overexpression of FOXM1 and PLK1 in a large proportion of oesophageal adenocarcinoma samples. This co-association was extended to the additional FOXM1 target genes CCNB1, AURKB and CKS1. In a cohort of patients who subsequently underwent surgery, the expression of several FOXM1 target genes was prognostic for overall survival.

CONCLUSIONS: FOXM1 and its target gene PLK1 are commonly overexpressed in oesophageal adenocarcinomas and this association can be extended to other FOXM1 target genes, providing potentially important biomarkers for predicting post-surgery disease survival.

Oesophageal cancer is the sixth most common cause of cancer death in the United Kingdom and has a poor prognosis with 5-year survival rates of <10% (Coupland *et al*, 2012). The majority of oesophageal cancers are of the adenocarcinoma histological sub-type, the incidence of which was rapidly increasing but has now stabilised (Coupland *et al*, 2012). Aetiological factors associated with the development of oesophageal adenocarcinoma include the presence of Barrett's metaplasia, obesity, widespread eradication of *Helicobacter pylori* infection and an ageing population (Koike *et al*, 2001; Solaymani-Dodaran *et al*, 2004; Hampel *et al*, 2005; Adams *et al*, 2007). The majority of patients present with late-stage disease that is not amenable to endoscopic or surgical resection and have a poor response to conventional systemic chemotherapy (Adams *et al*, 2007). Due to the late presentation of the disease, new screening methods that detect oesophageal malignancy at an earlier clinical stage and allow curative therapy are urgently required (Lao-Sirieix *et al*, 2009). Recent attempts at identifying new prognostic markers for oesophageal adenocarcinomas have focussed on using microarray analysis of mRNA expression patterns, and have led to the identification of four (Peters *et al*, 2010) and two (Kim *et al*, 2010) gene signatures which are of prognostic value. Thus, further analysis of gene expression signatures, or the coordinated upregulation of components of molecular pathways might provide further advances in this area.

FOXM1 is a member of the forkhead transcription factor family, which has been shown to have an important role in controlling the cell cycle (Laoukili *et al*, 2007; Koo *et al*, 2012). In particular, FOXM1 controls mitotic entry through the periodic upregulation of a group of genes that are maximally expressed as cells progress through late G2 and into M phase (Laoukili *et al*, 2005). Two of its target genes are *CCNB1* and *PLK1*, and these form part of a kinase-driven positive feedback loop that leads to the phosphorylation of FOXM1 and potentiation of its activity (Major *et al*, 2004; Fu *et al*, 2008). Thus, there is an intricate inter-regulatory relationship between FOXM1 and PLK1 that creates a cell-cycle control switch. The link between FOXM1 and cell-cycle control suggests that it is likely to contribute to the aberrant cell proliferation associated with malignancy. Indeed, FOXM1 has been shown to be upregulated in a range of different tumour types (Koo *et al*, 2012). More recently, FOXM1 was shown to be overexpressed and of potential prognostic significance in oesophageal squamous cell carcinomas (Hui *et al*, 2012) but before this study, its status in oesophageal adenocarcinomas was unknown. Similarly, the FOXM1 target gene *PLK1* has also been shown to be overexpressed

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in a wide range of tumours (Strebhardt and Ullrich, 2006), including oesophageal squamous carcinomas (Feng *et al*, 2009; Zhao *et al*, 2010). Molecularly, novel functions for FOXM1 have been identified in cancer cells beyond simply the acceleration of G2–M phase progression (Raychaudhuri and Park, 2011; Koo *et al*, 2012). This is exemplified by its ability to promote the nuclear translocation of β-catenin in gliomas, and hence activate a programme of Wnt target genes (Zhang *et al*, 2011). Together, these observations indicate that FOXM1 and PLK1 are likely central regulators in carcinogenesis and are potential therapeutic targets.

In this study, we investigated the expression of FOXM1 and PLK1 in oesophageal adenocarcinomas with particular emphasis on examining whether there was evidence for co-expression, and hence upregulation of the FOXM1-PLK1 regulatory axis. We show that there is widespread coordinate overexpression of FOXM1 and PLK1 in oesophageal adenocarcinomas, thereby providing the potential for feedback potentiation of FOXM1 activity. Indeed, we extend these studies to demonstrate the coordinate upregulation of a group of additional FOXM1 target genes across a high proportion of oesophageal adenocarcinomas. Some of these target genes appear to be useful prognostic indicators of survival following surgery. PLK1 inhibitors are currently being developed for cancer therapy (Strebhardt and Ullrich, 2006; Lens *et al*, 2010) and it is likely that cells demonstrating an upregulated FOXM1-PLK1 axis will be particularly susceptible to such treatment.

MATERIALS AND METHODS

Tissue collection

Ethical approval was granted by Wrightington Wigan and Leigh Ethics Committee, UK in 2004. Tissue was collected from 70 patients with oesophageal adenocarcinomas, 28 with Barrett's metaplasia and 55 healthy controls. Adenocarcinomas at the gastro-oesophageal junction were classified as oesophageal adenocarcinomas. Age and date at diagnosis, gender, co-morbidity, smoking status and survival were recorded. Details of the histological grade of tumour and stage, using the TNM and AJCC criteria were collected. Information on treatments including surgery, chemotherapy, radiotherapy and palliation were also recorded. Biopsy samples, ~ 4 mm in size, were taken at the time of endoscopic examination. Biopsy and surgical samples were rapidly frozen in liquid nitrogen and stored at $-80\,^{\circ}$C until needed. Paraffin blocks were used to construct tissue microarrays (TMA) for immunohistochemistry (IHC). Haematoxylin and eosin stained sections from the TMA's were characterised by two expert histopathologists and in the case of adenocarcinoma samples, were determined to contain a large proportion of tumour cells. Frozen biopsy and surgical samples were used for RNA extraction.

Cell lines, cell culture and western analysis

OE33, Flo1 (oesophageal adenocarcinomas), OE21 (oesophageal squamous cell carcinoma) and Het1A (normal oesophageal) cell lines were grown as described previously (Keld *et al*, 2010). For nocadazole block experiments, cells were grown for 24 h and then arrested at G2/M with 100 ng ml^{-1} nocadazole for 16 h followed by mRNA or protein extraction. Double thymidine block was performed as described previously (Whitfield *et al*, 2000). For experiments requiring PLK1 inhibition, 100 nM R-BI2536 (Activate Scientific GmbH, Prien, Germany) was added 7 h before harvesting. Cell lysis and western analysis were carried out essentially as described previously (Keld *et al*, 2010). Following transfer onto a nitrocellulose membrane, proteins were detected with ERK2 (New England Biolabs, Ipswich, ME, USA), FOXM1 (SC-502; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or PLK1 (F-8; Santa Cruz Biotechnology) antibodies.

RNA isolation and RT–PCR analysis

RNA was extracted, and analysed as described previously (Keld *et al*, 2010). Real-time RT–PCR was carried out using the primer pairs; FOXM1 (total), ADS2552 (5′-CCTCAAACCCAAACCAGC TA-3′) and ADS2553 (5′-GAAGCCACTGGATGTTGGAT-3′); PLK1, ADS2550 (5′-AAGAGGAGGAAAGCCCTGAC-3′) and ADS2551 (5′-TTCTTCCTCTCCCCGTCATA-3′); FOXM1a, ADS2558 (5′-CCA TAGCAAGCGAGTCCGCA-3′), and ADS2559 (5′-CAGCTAGCAGC ACTGATAAAC-3′); FOXM1b, ADS2562 (5′-CAGCACTGAGAGGA AGCGCA-3′) and ADS 2563 (5′-TCGTTTCTGCTGCTTAAAC-3′); FOXM1c, ADS2560 (5′-CGAGCACTTGGAATCACAGCAGA-3′) and ADS2561 (5′-TCCTCAGCTAGCAGCACCTTGG-3′); CCBN1, ADS1728 (5′-GGCCAAAATGCCTATGAAGA-3′) and ADS1729 (5′-AGATGTTTCCATTGGGCTTG-3′); AURKB, ADS2669 (5′-TGG GACACCCGACATCTTAACGC-3′) and ADS2670 (5′-ACCTTGAGC GCCACGATGAAATGG-3′); 18S, ADS4005 (5′-CGGCTACCACATC CAAGGAA-3′) and ADS 4006 (5′-GCTGGAATTACCGCGGCT-3′); GAPDH, ADS2184 (5′-ACAGTCAGCCGCATCTTCTT-3′) and ADS2185 (5′-TTGATTTTGGAGGGATCTCG-3′). Real-time PCRs were run on a Rotor Gene RG-3000 (Qiagen Ltd, Crawley, UK) and analysed with Rotor-Gene 6 software (Qiagen Ltd). Data are presented relative to 18S RNA levels in the same samples. For relative comparison of mRNA levels from tissue specimens, data were further normalised to the level of each gene in a standard concentration of RNA isolated from Het1a cells.

Immunohistochemistry

Tissue microarrays blocks were constructed as described previously (Keld *et al*, 2010). Arrays were stained with FOXM1 (SC-500; Santa Cruz Biotechnology) at a 1 : 40 dilution. A negative control slide was created by combining antibody with five-fold excess of blocking peptide (SC-500P) in Tris-buffered saline (TBS) and incubated overnight before adding to sections. Multiple cores for each specimen were constructed and initially scored by MD blinded to the clinical details. Cells were scored on the basis of intensity (score of 0–3) and the percentage of cells stained. The highest score in the triplet of cores was recorded. These two scores were then multiplied to make an overall IHC score. Two histopathologists (JC and SJH), both with a gastrointestinal interest, reviewed a selection of IHC sections, including comparison with positive and negative control sections, in order to confirm accuracy. Tissue microarrays spots which have both normal and tumour cells present only had the tumour-specific part scored. Negative expression was considered to be an IHC score of <45, low expression between 46 and 90, moderate expression 91–150 and high expression was between 151 and 300.

siRNA and plasmid transfection

Short interfering (si) RNAs directed against human FOXM1, PLK1 and GAPDH (SMARTpools; Dharmacon, Chicago, IL, USA), and a non-targeting scrambled sequence (Santa Cruz Biotechnology) were used. Cells were transfected as described previously (Keld *et al*, 2010). siRNA treatment was performed for 48 h before protein or mRNA expression analysis in an effort to minimise the potentially toxic effects caused by the loss of PLK1.

Microarray analysis

The recently published microarray data sets profiling gene expression of 28 normal and 64 oesophageal adenocarcinoma samples were downloaded from Gene Expression Omnibus (accession number: GSE13898) (Kim *et al*, 2010). Pearson's correlation coefficients (PCCs) were computed to identify a set of genes that have similar expression pattern with FOXM1, and

stringent threshold was applied on the PCC results (0.8 < r-value). Hierarchal clustering and visualisation of the expression levels of the identified genes were performed by MultiExperiment Viewer, a part of TM4 microarray software suite (Saeed et al, 2006)

RESULTS

The expression of FOXM1 and PLK1 in oesophageal-derived cell lines

FOXM1 is generally present in many cell types but its expression is thought to be limited to proliferating cells (Laoukili et al, 2007; Koo et al, 2012). Similarly, its target gene *PLK1* is also expressed under these conditions across many cell types (reviewed in Lens et al, 2010). To establish whether FOXM1 and PLK1 are expressed in oesophageal cells, we first determined whether protein could be detected in a range of oesophageal-derived cell lines, including non-tumourigenic Het1a cells, and cells originating from squamous carcinomas (OE21) or adenocarcinomas (OE33 and Flo1). For each cell line, asynchronously growing cells, or cells treated with nocodazole to enrich for the G2–M phase population, were analysed by western blotting. All of the cell lines express FOXM1 to similar levels (Figure 1A, upper panel). FOXM1 levels were unaffected by nocodazole treatment, but with the exception of OE33 cells, a lower mobility band was induced by this treatment which is indicative of the expected phosphorylation of FOXM1 that occurs in the G2–M phase (Figure 1A, upper panel, lanes 4–6) (Major et al, 2004). PLK1 was also detected at similar levels across all cell lines, and as expected, its expression was increased upon nocodazole treatment, with the exception of OE33 cells (Figure 1A, lower panel). However, OE33 cells responded poorly to nocodazole treatment, and do not readily accumulate in G2–M (data not shown).

Next, we focussed on oesophageal adenocarcinoma cells and analysed the expression of *FOXM1* and *PLK1* at the mRNA level. Cells were arrested in early S phase by using a double thymidine block, and then released into fresh media to allow synchronous progression through the cell cycle. *FOXM1* expression showed a modest oscillatory pattern during the cell cycle, peaking just before cells accumulated maximally in the G2–M phase of the cell cycle (Figure 1B, top panels). *PLK1* expression showed a similar oscillatory pattern, although higher amplitude changes are observed. The kinetics of induction vary between the two cell lines but this reflects the more rapid transition of OE33 cells into the G2–M phase of the cell cycle (Figure 1B, bottom panels).

Together, these results demonstrate that FOXM1 and PLK1 are expressed at both the mRNA and protein levels in oesophageal-derived cells, including oesophageal adenocarcinoma cell lines. Generally, there is little difference in their expression among these proliferating cells, although differences in cell-cycle progression are detected among oesophageal adenocarcinoma cell lines.

The FOXM1-PLK1 regulatory circuit is operative in oesophageal adenocarcinoma cells

FOXM1 controls *PLK1* expression and PLK1 forms part of a positive feedback loop to phosphorylate and hyperactivate FOXM1 (Figure 2A; Laoukili et al, 2005; Fu et al, 2008). We therefore examined whether this regulatory circuit is operational in oesophageal adenocarcinoma cells. First, we depleted FOXM1 or PLK1 using siRNA, and examined the expression of each protein. OE33 cells were treated with siRNA constructs for 48 h, at which point, although cell-cycle arrest is apparent, extensive cell death was not observed (data not shown). As expected, FOXM1 siRNA resulted in efficient FOXM1 depletion and PLK1 siRNA caused a loss of PLK1 (Figure 2B). However, FOXKM1 depletion also led to reductions in PLK1 levels, whereas PLK1 depletion also reduced FOXM1 protein expression to some degree. These observations are consistent with the known regulatory properties of FOXM1 as an activator of *PLK1* transcription. To confirm that FOXM1 was regulating PLK1 expression at the transcriptional level, we examined *PLK1* expression by real-time RT–PCR. Depletion of FOXM1 led to a significant decrease in *PLK1* levels (Figure 2C,
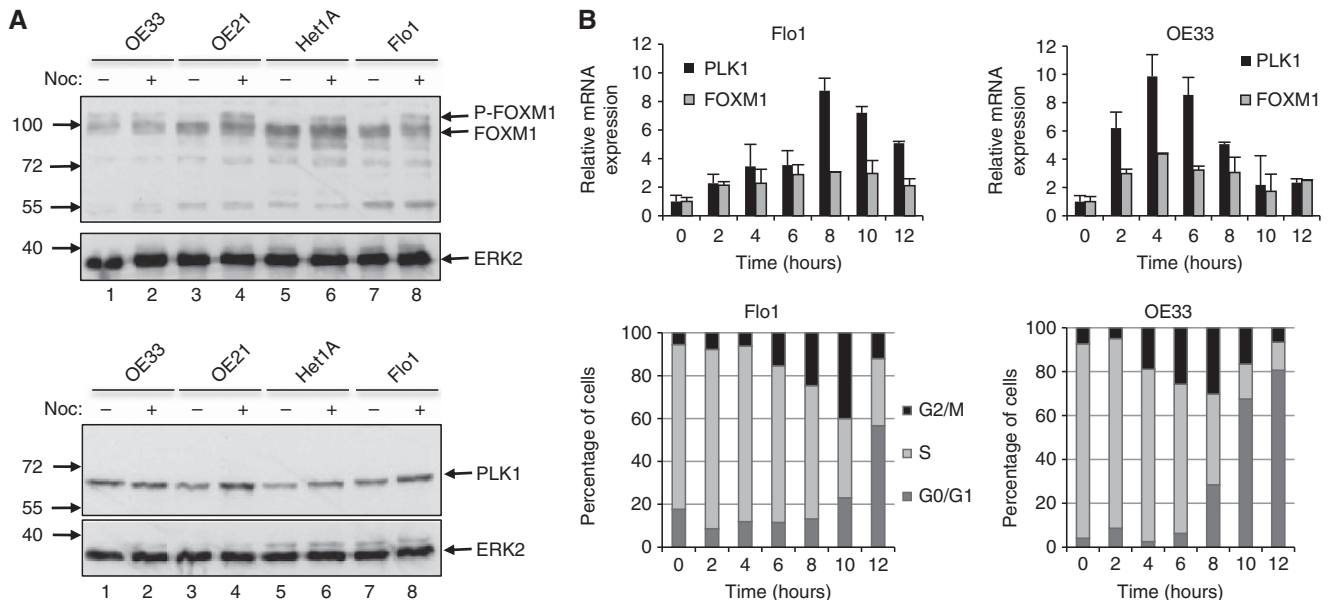

Figure 1 The expression of FOXM1 and PLK1 in oesophageal-derived cell lines. (**A**) Western blot analysis of FOXM1 (top panel) and PLK1 (bottom panel) protein expression in OE33, OE21, Het1a and Flo1 oesophageal cell lines treated with DMSO or with nocodazole (noc) for 16 h. The locations of the molecular weight markers are shown on the left (kDa). The positions of the bands corresponding to FOXM1 and PLK1 are shown and lower mobility bands consistent with phosphorylation of FOXM1 (P-FOXM1) are indicated. ERK2 was used as a loading control. (**B**) Real-time RT–PCR analysis of *FOXM1* (grey bars) and *PLK1* (black bars) expression in Flo1 (left) and OE33 (right) cells following release for the indicated time points from a double thymidine block (top graphs). The mean expression from two independent experiments relative to time zero (taken as 1) is shown. Error bars indicate the standard deviation. The corresponding cell cycle profiles are shown beneath each graph.

bottom panel). A reciprocal effect of PLK1 depletion was seen on *FOXM1* levels (Figure 2C, top panel). Due to the potential indirect effects of PLK1 depletion on cell-cycle properties, we also used a PLK1 inhibitor, BI2536, to cause immediate PLK1 inhibition in cells released from a double thymidine-induced cell-cycle arrest. Long-term treatment with the PLK1 inhibitor leads to the death of many cell types (reviewed in Strebhardt and Ullrich, 2006), including oesophageal adenocarcinoma cells (data not shown), therefore, we limited our gene expression analysis to 7 h after release from the arrest, as cells progress from late G2 into M phase. We used this approach to analyse the expression of *PLK1* and two other FOXM1 target genes *CCNB1* and *AURKB*, at this point of the cell cycle. All three genes showed reduced expression levels, although the changes in *AURKB* expression did not reach statistical significance (Figure 2D). There was also a reduction in *FOXM1* mRNA levels. Thus, active PLK1 is required for maximal activation of FOXM1 target genes.

Together, these results indicate that the FOXM1-PLK1 regulatory circuit is intact in oesophageal adenocarcinoma cells, with FOXM1 controlling the expression of *PLK1* and other cell-cycle target genes, and PLK1 feeding back to accentuate this control.

FOXM1 and PLK1 are overexpressed in oesophageal adenocarcinomas

Having established the expression of FOXM1 and PLK1 in oesophageal adenocarcinoma-derived cell lines, we next wanted to determine whether their expression differed between normal oesophageal tissue, and samples derived from patients with oesophageal adenocarcinomas. We collected biopsy samples from 19 patients with normal oesophageal tissue, and 69 oesophageal adenocarcinoma samples with 34 matched samples from adjacent normal tissue. An additional 12 samples were obtained from tissues diagnosed histologically to demonstrate features of Barrett's oesophageal tissues and 9 matched samples from adjacent normal tissue. The samples from normal oesophageal tissue provide control reference points for examining the changing expression levels of *FOXM1* and *PLK1* expression in the adenocarcinoma samples.

First, we analysed the expression of *FOXM1* and *PLK1* by real-time RT–PCR. Both genes are expressed at low levels in normal oesophageal tissue, but they are both overexpressed in a large proportion of the oesophageal adenocarcinoma samples (Figure 3A). The average expression levels were three-fold and four-fold over those found in normal tissue for *FOXM1* and *PLK1*, respectively (P-values <0.001), and 40% and 53% of samples express significantly increased levels of *FOXM1* and *PLK1*, respectively. The median level and the overall distribution of expression levels for both genes are significantly higher in the adenocarcinoma samples than in normal tissue or in tissues with Barrett's metaplasia (Figure 3B). There are three different *FOXM1* isoforms generated by alternative splicing (Ye *et al*, 1997). We therefore designed primer pairs to specifically detect different *FOXM1* splice forms and analysed whether any particular splice form was overexpressed among the adenocarcinoma samples. In general, elevated levels of *FOXM1a* and *FOXM1b* were seen in all cancer samples, whereas there was more heterogeneity with *FOXM1c* (Figure 3C). However, as expected, very few of the splice forms were overexpressed in samples from normal tissue. Thus,
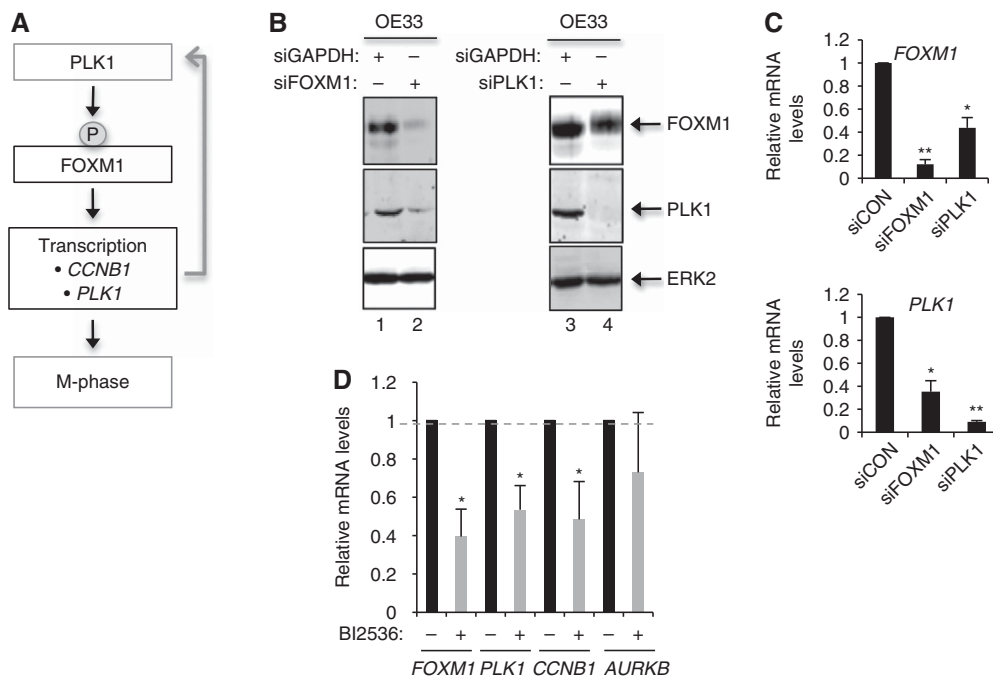

Figure 2 Regulatory inter-relationships between FOXM1 and PLK1 in oesophageal adenocarcinoma cells. (**A**) Model of the regulatory inter-relationships between FOXM1 and PLK1. FOXM1 activates PLK1 expression that feeds back to phosphorylate (P) to further enhance FOXM1 activity. (**B**) Western analysis of FOXM1 and PLK1 protein expression following siRNA-mediated knockdown of *FOXM1* (left) and *PLK1* (right) in OE33 cells. Control cells were treated with siRNAs targeting GAPDH. ERK2 is a loading control. The locations of the bands corresponding to each of the proteins are shown on the right. (**C**) RT–PCR analysis of *FOXM1* (top) and *PLK1* (bottom) mRNA expression following siRNA-mediated knockdown of *FOXM1* and *PLK1* in OE33 cells. As a control, cells were treated with non-targeting siRNAs (siCON). Data are the average of two independent experiments and presented as the mean and standard deviations of gene expression relative to cells treated with the non-targeting control (taken as '1'). (**D**) RT–PCR analysis of the expression of *FOXM1* mRNA, and its target genes *PLK1*, *CCNB1* and *AURKB* following cell synchronisation with double thymidine treatment and subsequent release into media containing BI2536 or DMSO (negative control). Cells were harvested 7 h after release. The experiments were repeated three times and data are presented as the mean and standard deviation of the three experiments relative to DMSO-treated cells (taken as 1). Statistical significance is indicated on the graphs (*P-value <0.05; **P-value <0.01).

although there is a clear pattern of *FOXM1* overexpression in oesophageal adenocarcinomas, there is no particular preference for a particular splice form.

Having established that FOXM1 and PLK1 are overexpressed in oesophageal adenocarcinomas at the mRNA level, we next analysed their expression at the protein level. First, we used TMAs
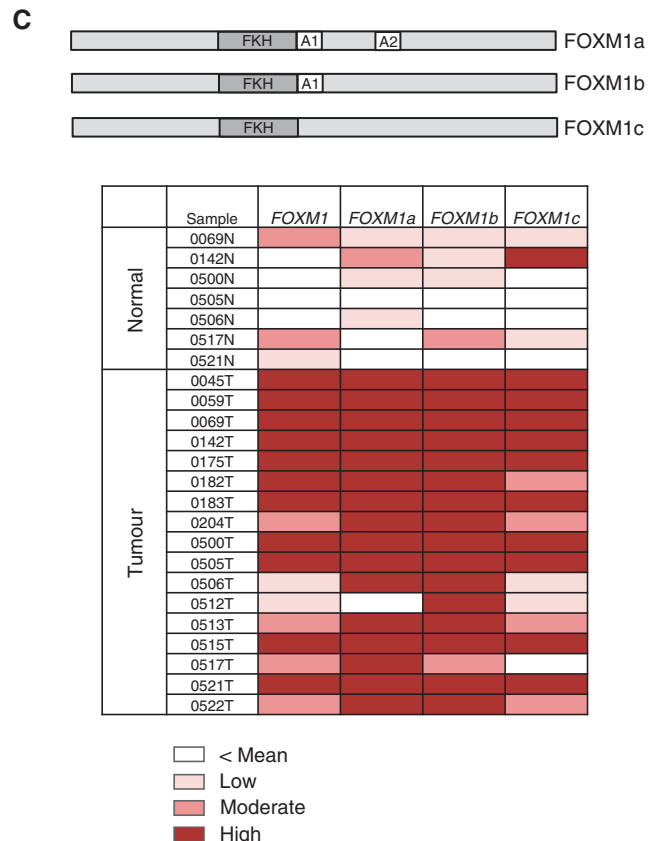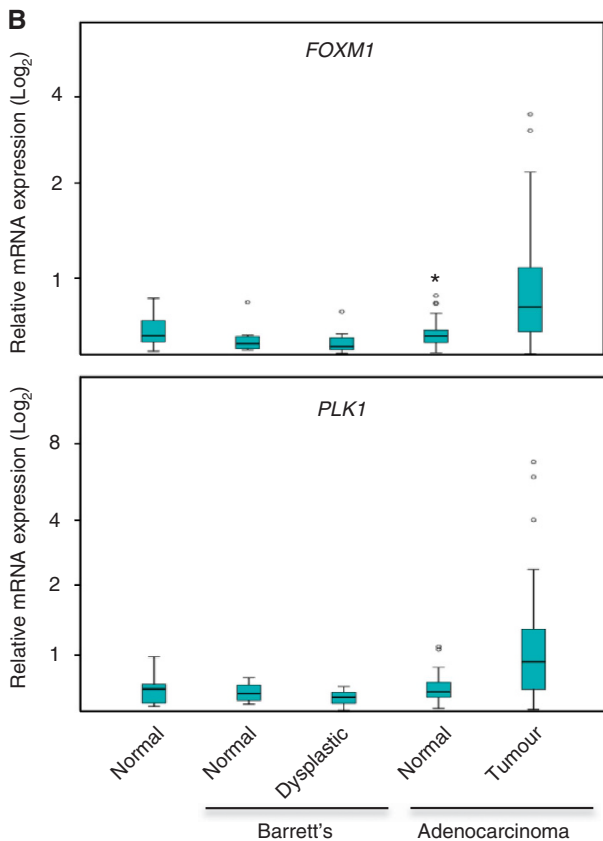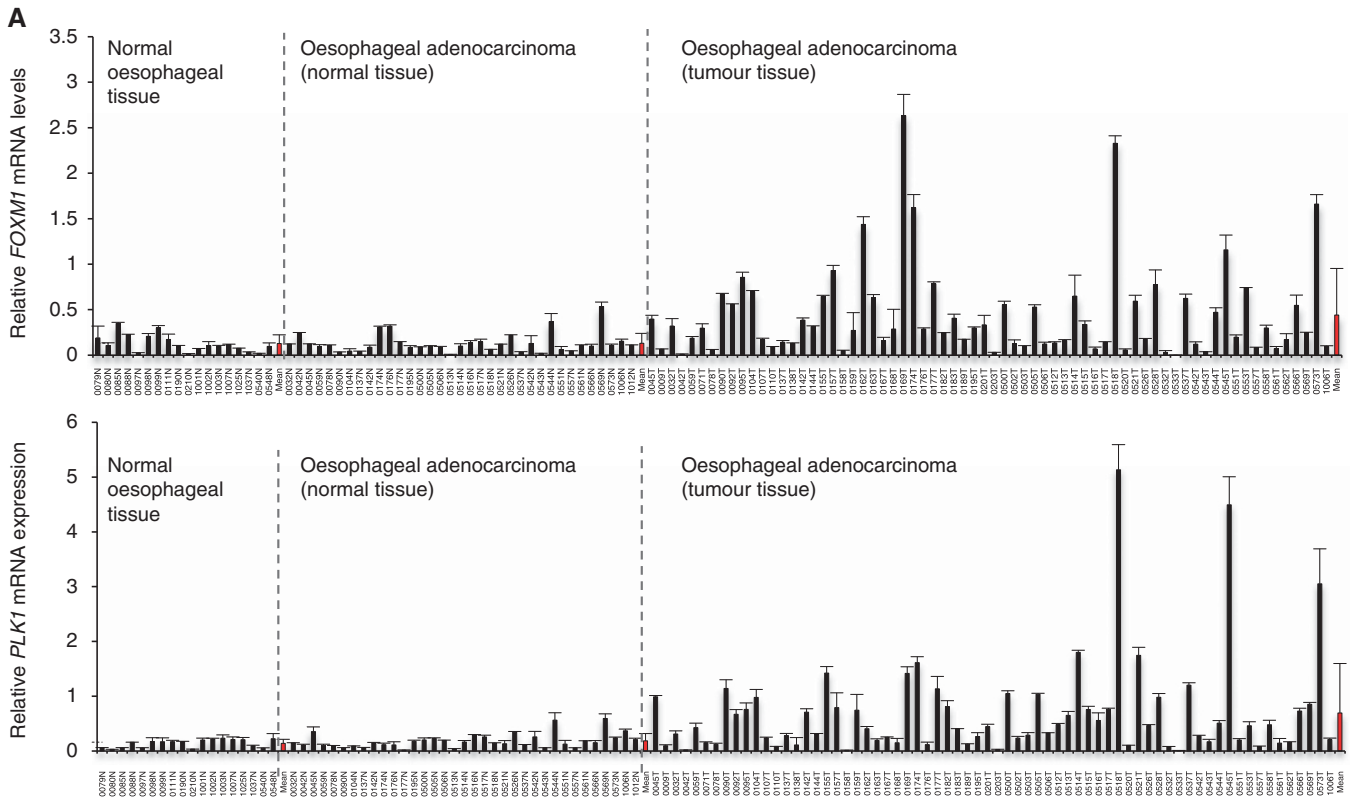
to probe for expression of FOXM1 across a collection of 15 samples from normal tissue and 117 samples from oesophageal adenocarcinomas. A range of different levels of FOXM1 expression were seen among tumour samples (Figure 4A). A significantly higher proportion of oesophageal adenocarcinoma samples expressed moderate or high levels of FOXM1 compared with normal tissue (Figure 4B; P-value 0.004), and this was reflected in the higher median and overall distribution of expression scores across the TMA in oesophageal adenocarcinoma samples (Figure 4C). We also attempted to analyse PLK1 expression using TMAs but were

unable to find a suitable antibody (data not shown). As an alternative method for determining protein levels, we used western analysis to compare FOXM1 expression across normal and tumour samples. Among seven matched samples, four showed elevated FOXM1 expression in the cancer-derived tissue (Figure 4D, upper panel, lanes 4 and 8; lower panel, lanes 5 and 9); high levels of FOXM1 were also observed in one of two additional tumour samples analysed (Figure 4D, upper panel, lanes 1 and 2). These results are broadly consistent with what is seen in the TMAs and RT–PCR analyses, where 40–60% of tumour samples express high
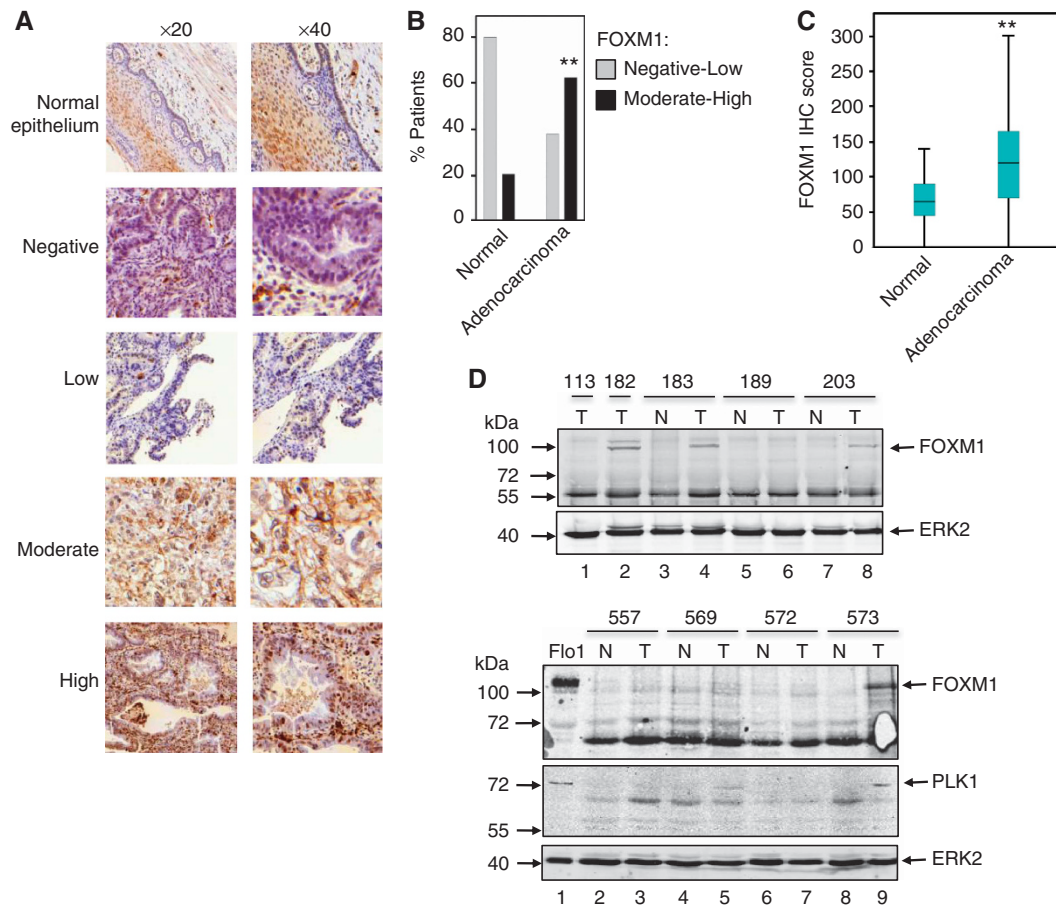

Figure 4 FOXM1 and PLK1 protein expression in oesophageal adenocarcinomas. (**A**) Examples of TMA staining for FOXM1 protein (brown stain) in normal epithelium and adenocarcinomas. Examples of negative, low, moderate and high level staining are shown at × 20 (left) and × 40 (right) magnification. (**B**) The proportion of patients in each class with negative-low FOXM1 staining (hatched bars) and moderate-high FOXM1 staining (black bars) in the TMAs is shown. **Represents P-value = 0.04 (χ^2). (**C**) Box plots of FOXM1 protein expression in oesophageal tissue samples from healthy controls, or oesophageal adenocarcinoma patients. Median values of FOXM1 are indicated for each tissue type. The box plot demonstrates the interquartile range. **Represents P-value = 0.003 (T-test). (**D**) Western blots of FOXM1 and PLK1 expression in oesophageal adenocarcinoma specimens. The positions of the bands corresponding to FOXM1 and PLK1 and the locations of the molecular weight markers are shown (kDa). Results of paired normal oesophageal mucosa (N) and tumour (T) specimens or unpaired tumour samples from oesophago-gastrectomy samples are shown. Flo1 cell lysate is shown in the left lane (bottom panel). ERK2 was used as a loading control.

Figure 3 FOXM1 and PLK1 mRNA expression in oesophageal adenocarcinomas. (**A**) RT–PCR analysis of *FOXM1* (top) and *PLK1* (bottom) mRNA expression in oesophageal adenocarcinoma samples (right), normal oesophagus from healthy patients (left) and paired samples of normal tissue from patients with oesophageal adenocarcinoma (centre). Data are shown relative to 18S mRNA in oesophageal tissue specimens and are standardised to the expression of the corresponding gene in Het1a cells. The average relative mRNA level and standard deviations derived from two readings from one sample are shown. The individual tissue specimens are numbered and tissue is grouped by tissue subtypes. The mean gene expression for each category of samples is shown in red. (**B**) Box plots of *FOXM1* (top) and *PLK1* (bottom) mRNA expression in oesophageal tissue samples from healthy controls, or oesophageal adenocarcinoma patients. Median values of *FOXM1* expression are indicated for each tissue type. The box plot demonstrates the interquartile range and the high outliers are indicated. (**C**) Heatmap showing the relative mRNA expression of total *FOXM1* or the isoforms *FOXM1a*, *FOXM1b* and *FOXM1c* (the presence of alternatively spliced exons A1 and A2 is shown schematically above) in samples from normal oesophageal tissue and oesophageal adenocarcinoma. mRNA expression is calculated relative to 18S ribosomal RNA. Each horizontal bar represents a single sample. The heatmap was composed based on the mean mRNA expression of the normal oesophageal samples. Low expression is defined as above this mean. Moderate and high expressions are > 1s.d. and > 2s.d. above this mean.

levels of FOXM1. Moreover, the high levels of FOXM1 protein seen in 182T, 183T and 573T samples correlate with the high mRNA levels observed by RT–PCR. Conversely, 557T and 189T samples were negative for both protein and mRNA. We also looked at PLK1 protein levels in a subset of these samples, and found elevated PLK1 levels in two out of four tumour samples (Figure 4D, bottom panel; lanes 5 and 9). Importantly, both of these samples also contained elevated FOXM1 levels, which is consistent with the regulatory inter-relationship between these two proteins.

Taken together, these results demonstrate that FOXM1 is upregulated at both the mRNA and protein levels in a large proportion of oesophageal adenocarcinomas. PLK1 is also commonly upregulated at the mRNA level in oesophageal adenocarcinoma and more limited analysis suggests that this is reflected at the protein level.

The FOXM1 target gene network is upregulated across oesophageal adenocarcinomas

Our results demonstrate that FOXM1 and its target gene *PLK1* are upregulated in a large proportion of oesophageal adenocarcinoma samples. To establish whether there is a correlation in their expression across tumour samples, we compared their relative expression at the mRNA level. High level *FOXM1* expression is often correlated with high *PLK1* expression (Pearson correlation = 0.730; Figure 5A and B). Next, we investigated whether the correlation between *FOXM1* and *PLK1* expression extended to other FOXM1 target genes; we selected a number of tumour samples with high levels of *PLK1* expression and 12 normal samples, and tested for the mRNA expression levels of *CCNB1*, *AURKB* and *CKS1* (Figure 5B). In general, high *FOXM1* levels were associated with a high level expression of all four of its target genes (in 12 out of 14 cases). Little expression of *FOXM1* was observed in normal samples, and likewise, minimal or no expression of its target genes was generally observed. To extend this analysis, we took advantage of a recently published study in which the authors used expression microarray analysis to determine the gene expression profiles of 28 normal and 64 oesophageal adenocarcinoma samples from patients who subsequently had their tumours surgically removed (Kim *et al*, 2010). Preliminary analysis of the expression patterns of *FOXM1* and its target genes *PLK1*, *CCNB1*, *AURKB* and *CKS1* demonstrated a close correlation of their expression profiles across tumour samples (PCCs all > 0.8 for pairwise comparisons with *FOXM1*). We therefore examined the expression profiles of these five genes compared with 100 randomly selected genes. Hierarchal clustering revealed that *FOXM1* and its four target genes formed a distinct cluster of genes, thereby confirming their close association of expression pattern among these tumour samples (Figure 6A). Next, we analysed this subgroup of genes in more detail, and this enabled us to segregate the tumour samples into three broad clusters, where all five genes are highly expressed (cluster a), all five genes show low expression (cluster b) and where there is variable expression of these genes (cluster c) (Figure 6B). This spread of expression values is consistent with that demonstrated within our own patient cohort, where some tumour samples show high level coordinate upregulation of FOXM1 and its targets whereas there are other tumours where the expression is generally lower. To establish whether there is any prognostic significance to the expression of *FOXM1* and/or its target genes, we monitored survival rates of patients with tumours from clusters a to c (Figure 6C). A Kaplan–Meier plot showed a worsening trend for better survival as the expression of *FOXM1* and its target genes increased, that is, cluster A patients fared worse, whereas cluster B patients fared the best. However, the differences failed to reach statistical significance. Interestingly, when the expression of FOXM1 and several of its target genes were analysed individually, high expression levels always produced a trend for shorter survival
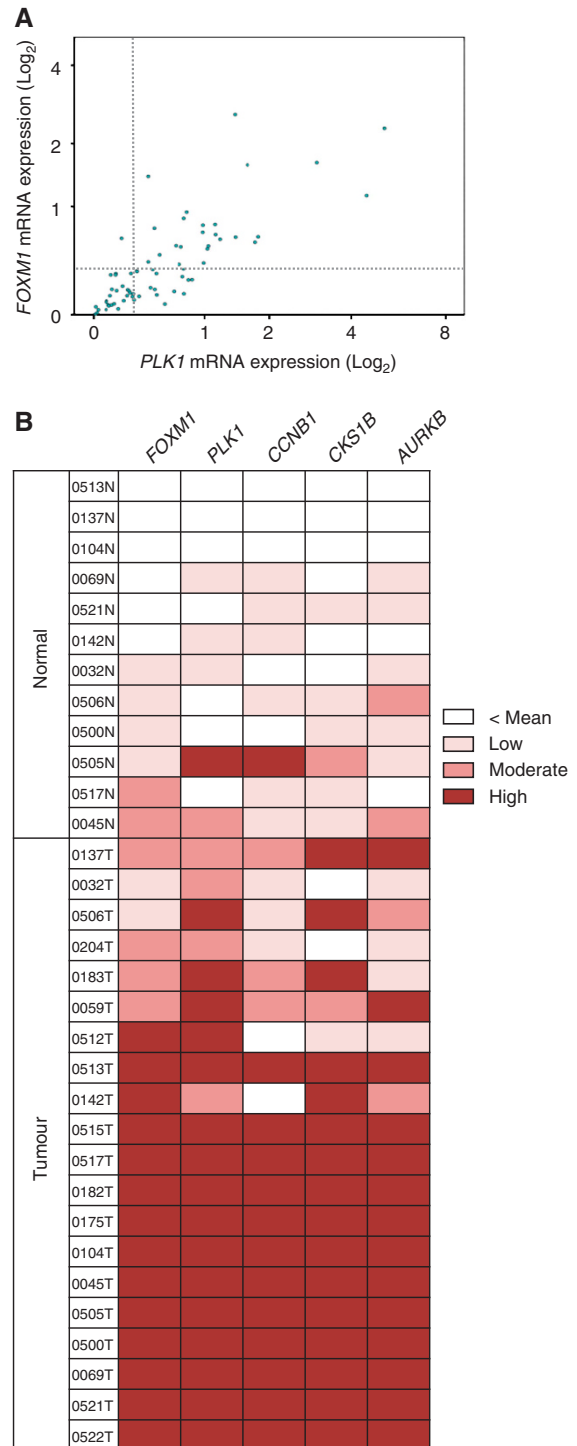

Figure 5 The inter-relationship between FOXM1 and its targets in oesophageal adenocarcinomas. (**A**) Scatter plot of *FOXM1 vs PLK1* mRNA expression levels among samples from oesophageal adenocarcinomas. Data are from Figure 3A. Dotted lines represent the gene expression value corresponding to high level expression (i.e., > 2s.d. above the mean of the normal samples). (**B**) Heatmap showing relative mRNA expression of *FOXM1*, *PLK1*, *CCNB1*, *AURKB* and *CKS1B* in samples from normal oesophageal tissue and oesophageal adenocarcinoma. mRNA expression is calculated relative to 18S ribosomal RNA. Each horizontal bar represents a single sample. The heatmap was composed based on the mean mRNA expression of the normal oesophageal samples. Low expression is defined as above this mean. Moderate and high expressions are > 1s.d. and > 2s.d. above this mean.
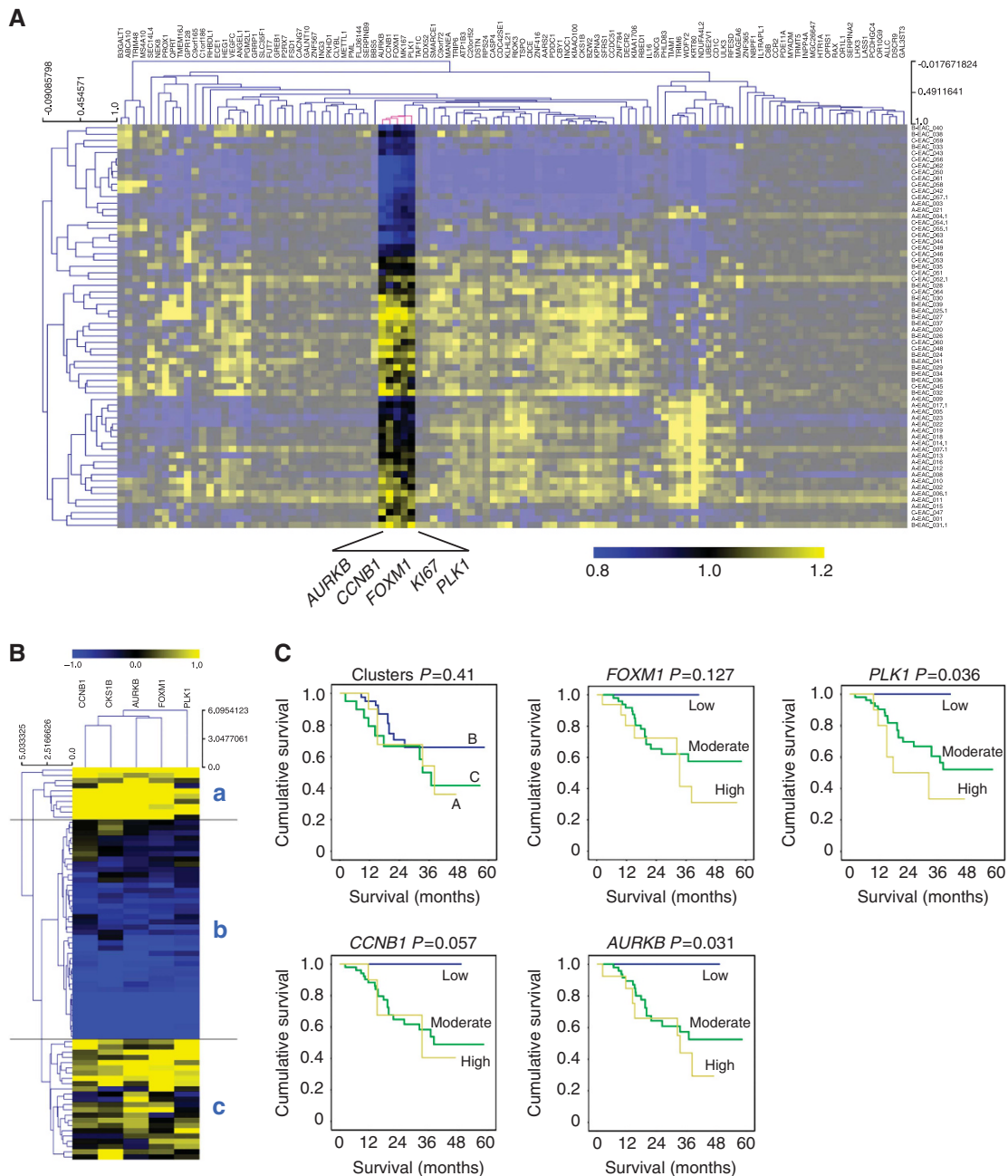
Figure 6 The FOXM1 target gene network expression in oesophageal adenocarcinomas. (**A**) Hierarchal k-means clustering of gene expression levels in oesophageal adenocarcinoma samples. *FOXM1, PLK1, CCNB1, AURKB* and *CKS1B* and 100 additional genes taken at random were clustered according to their expression across 78 adenocarcinoma samples (Kim *et al*, 2010). A tightly associated subcluster containing *FOXM1* and several target genes is highlighted (PCCs >0.8). (**B**) Unsupervised k-means clustering of the expression of *FOXM1* and the target genes *PLK1, CCNB1, CKS1* and *AURKB* in oesophageal adenocarcinoma samples. Three major clusters (a–c) are indicated. (**C**) Kaplan–Meier curves of overall survival in patients with oesophageal adenocarcinoma, grouped according to the clusters in (**B**), or due to individual mRNA expression levels for *FOXM1, PLK1, CCNB1* and *AURKB*. Expression is defined as low (<1s.d. above mean of normal samples), high (>1s.d. above mean of normal samples) and moderate (between these values). Log rank probabilities between low and high expression are shown.

times, and in the case of *PLK1* and *AURKB*, the differences in survival rates between patients with high *vs* low level expression reached statistical significance (*P*-values 0.036 and 0.031, respectively).

In summary, these data clearly demonstrate the coordinate upregulation of *FOXM1* and its target genes in a subset of oesophageal adenocarcinomas. Furthermore, the analysis of the expression of this cohort of genes has the potential to provide prognostic indicators of survival of patients following surgery.

DISCUSSION

FOXM1 and PLK1 overexpression has been observed in a variety of different tumour types (reviewed in Strebhardt and Ullrich, 2006 and Koo *et al*, 2012). However, before this study, their interrelationship within tumours had not been investigated. Here, we have demonstrated that both FOXM1 and PLK1 are commonly overexpressed in oesophageal adenocarcinomas. Thus, FOXM1 and PLK1 overexpression appears to be a frequent event in

Genetics and Genomics

oesophageal tumourigenesis, as previous studies demonstrated that they are overexpressed in oesophageal squamous cell carcinomas (Feng *et al*, 2009; Zhao *et al*, 2010; Hui *et al*, 2012). Importantly, in our study we also found that FOXM1 and PLK1 are generally co-expressed, as might be predicted from their regulatory relationship where FOXM1 controls *PLK1* transcription (Laoukili *et al*, 2005). Indeed, we have also shown that there is coordinate upregulation of other FOXM1 target genes including *CCNB1*, *AURKB* and *CKS1*, in many oesophageal adenocarcinomas. As both PLK1 and CCNB1 form part of a positive feedback loop that potentiates FOXM1 activity (Major *et al*, 2004; Fu *et al*, 2008), this regulatory loop will be potentiated in oesophageal adenocarcinomas. Functionally, all of the FOXM1 target genes that we have analysed are involved in cell-cycle control, chiefly in the late G2 and M phases of the cell cycle, and thus will contribute to the increased proliferative rates seen in the cancer.

Cell line models demonstrated that the FOXM1-PLK1 regulatory axis is operational in oesophageal adenocarcinoma cells. However, little difference was seen in the expression of FOXM1 and PLK1 between adenocarcinoma-derived cell lines, and the non-tumourigenic Het1a cells. However, this was not particularly surprising as it is well established that FOXM1 and its targets are expressed in proliferating cells, as part of the normal physiological control of the cell cycle (reviewed in Laoukili *et al*, 2007). Subtle differences were however seen in cell-cycle control in adenocarcinoma-derived cell lines, as OE33 and Flo1 cells progressed at different rates through the G2 and M phases following release from a double thymidine block. Furthermore, OE33 did not respond well to synchronisation with nocadazole, which probably accounts for the lack of induction of a lower mobility phosphorylated form under these conditions. Overall, the cell line models were not particularly informative about FOXM1 function in cancer cells compared with what is already known about its role in controlling cell-cycle progression.

The *FOXM1* transcript undergoes alternative splicing to produce three alternative isoforms (a–c) (Ye *et al*, 1997). FOXM1b and c are transcriptionally active whereas FOXM1a is transcriptionally inert. All three isoforms are expressed in adenocarcinoma samples but no clear pattern of alternative expression was observed, indicating that no particular isoform is dominant. Thus, transcriptional control of *FOXM1* expression rather than alternative splicing is defective in these cancer cells.

Previous studies based on microarray analysis of samples from oesophageal adenocarcinomas have revealed two and four gene signatures that have prognostic value (Kim *et al*, 2010; Peters *et al*, 2010). Our studies investigating FOXM1 and its target genes suggest that these genes may also represent useful prognostic indicators. Although analysis of our own samples failed to provide significant correlations with individual clinical parameters, metadata analysis of microarray data derived from patients with adenocarcinomas (Kim *et al*, 2010) showed that a five gene signature consisting of FOXM1 and four of its target genes was indicative of survival rates in this group of patients. However, although the overall signature failed to reach statistical significance, the analysis of two FOXM1 target genes, *PLK1* and *AURKB*, did indicate that their expression was predictive for long-term survival. Interestingly, a previous study also identified *PLK1* mRNA expression as a potential prognostic marker, but this

finding was not further validated (Peters *et al*, 2010). These findings raise the possibility that further analysis of FOXM1 and its targets might reveal more robust prognostic indicators of cancer status and progression.

PLK1 inhibitors are currently being developed as cancer therapeutics (reviewed in Lens *et al*, 2010). As PLK1 has a pivotal role in amplifying the output of the FOXM1 regulatory module, and is itself part of this module that is upregulated in oesophageal adenocarcinomas, this suggests that such inhibitors should be of clinical use in treating patients with oesophageal adenocarcinoma. In particular, the subset of patients that overexpress FOXM1 and its target genes might be particularly susceptible to these inhibitors. Indeed, we have shown that treatment of adenocarcinoma cells with the PLK1 inhibitor BI2536 leads to a reduction in the expression of FOXM1 and its target genes (Figure 2D) and ultimately a block in cell proliferation (data not shown).

CONCLUSIONS

In summary, this study shows that the FOXM1-PLK1 axis is commonly upregulated in samples from patients with oesophageal adenocarcinoma. Further analysis demonstrates that additional FOXM1 targets are upregulated in these cancer cells. Our results indicate that FOXM1 and its target genes represent molecular markers for this malignancy and candidate novel prognostic indicators of survival.

Conflict of interest

The authors declare no conflict of interest.

Author contributions

MD contributed to the study design, conducted the majority of the experiments and helped with manuscript preparation. JC and SH assisted with tissue sampling and IHC analysis. HV and CW assisted with the TMA experiments. NH performed the bioinformatics analysis. AY provided clinical training, contributed to study design and coordination and data interpretation. ADS contributed to experimental design and coordination and wrote the manuscript. All authors read and approved the final manuscript.

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Genetics and Genomics