Supplemental Information

Materials and Methods

Site-directed mutagenesis

The following primers were used: 5'-GAGCAGTGGGCCAGCTAAAGGCAGAG GCCGCC-3' and 5'-GGCGGCCTCTGCCTTTAGCTGGCCCACTGCTC-3' were used for making mutant E2F-1 R109K. 5'-AGCTCGGGGCAAAGGCCGCCATC-3' was used for making mutant E2F-1 R111K. 5'-GGGCAGAGGCAAACATCCAGGAA-3' was used for making mutant E2F-1 R113K. 5'-AGTGGGCCAGCTCGGGGCAAAGGCAAACATCCAGGAAAAGGT GTG-3' was used for making mutant E2F-1 KK. 5'-AGAGCAGTGGG CCAGCTAAGGGCAAGGGCAAGCATCCAGGAAAAGGTGTG-3' was used for making mutant E2F-1 KKK. Quick Change Mutagenesis Kit (Stratagene) was used.

Purification of GST-E2F-1 fusion proteins

Wild-type GST-E2F-1 and the GST-E2F-1 mutants were expressed in *E.coli* BL21-DE3 (Stratagene) cells by induction with isopropyl β-D-1-thiogalactopyranoside (IPTG) (0.1mM). Cell pellets resuspended in lysis buffer consisting of phosphate buffered saline containing protease inhibitor cocktail (1mM PMSF, leupeptin [1μg/ml], aprotinin [1μg/ml], and pepstatin A [1μg/ml]) were sonicated. 1% Triton-X100 was then added and the cells lysed at 4°C for 30min. Following centrifugation at 13,000rpm for 30 min the supernatants were incubated with glutathione affinity resin (Pharmacia) for 2h at 4ºC. The beads were then washed three times with lysis buffer containing 0.1% Triton-X100, once with lysis buffer without Triton-X100 and then once with elution buffer (50mM Tris-HCl pH8, 50mM NaCl).

GST fusion proteins were eluted from the beads by using 20mM glutathione dissolved in elution buffer.

***In vitro* methyltransferase assays**

Flag-PRMT5 plasmid was transfected into U2OS cells for 48h. Cells were lysed and Flag-PRMT5 was immunoprecipitated with Flag-agarose beads (Sigma) and then eluted. The elute was mixed with substrates (GST fusion proteins or E2F-1 peptide residue 100 to 120) in methylation reaction buffer (50mM Tris, 0.1mM EDTA, 50mM NaCl) with ^{3}H labelled SAM (as $-CH_3$ group donor) to a volume of 40µl and incubated at 30°C for 90 min. Half of the reactions were then spotted onto p81 membrane circles (Whatman) and air dried. The membranes were then washed three times, 5min each in 50ml of wash buffer (46mM $NaHCO_3$, 4mM Na_2CO_3, pH 9.2). After washing briefly with acetone, membranes were air dried, placed in scintillation vials, immersed in scintillation fluid (Beckman Coulter) and disintegrations per minute (DPM) measured in a scintillation counter. The other half of the reaction was run on SDS-PAGE gel and used to detect ^{3}H auto-radioisotope/ methylation signal.

GST binding assay

GST or GST tagged proteins (10µg each) were incubated with glutathione Sepharose beads and U2OS cell lysates for 3h. Beads were then washed with TNN buffer 3 times. SDS sample buffer was added to beads and samples were analysed by immunoblotting.

Preparation and purification of anti-MeR E2F-1 antibody

The E2F-1 peptides, either unmethylated or with R111 and R113 (bold) symmetrically methylated (CESSGPARG**R**G**R**HPGKG), were injected into rabbits for antibody preparation

(Alta Bioscience). Rabbit serum diluted in PBS was purified by peptide affinity chromatography as described (Jansson et al, 2008).

Analysis by tandem mass spectrometry

MCF-7 cells were transfected with Flag-E2F-1 and 20 hours later harvested in TNN buffer and immunoprecipitated as described with anti-Flag peptide antibody. Proteins were eluted with Flag peptide and after addition of NuPage LDS sample buffer (Invitrogen), eluted proteins were separated by SDS-PAGE on NuPAGE Bis-Tris precast gels (Invitrogen) using NuPAGE MPS SDS running buffer (Invitrogen). Gels were stained using the SilverQuest^{TM} Silver Staining Kit (Invitrogen). The Flag-E2F-1 protein band was excised and applied for tryptic in-gel digest as described previously (Jansson et al, 2008). GST-E2F-1 was used in an *in vitro* methylation reaction using unlabelled SAM (Sigma). The reaction was halted by addition of NuPage LDS sample buffer (Invitrogen) and separated by SDS-PAGE on NuPage 10% Bis-Tris pre-cast gels (Invitrogen) using NuPage MOPS SDS running buffer (Invitrogen). Gels were stained with GelCode Blue (Thermo) and the GST-E2F-1 protein band was excised for in-gel tryptic digestion as described previously (Jansson *et al*, 2008). The individual tryptic peptides were analysed by LC-MS/MS using a quadrupole time-of-flight tandem mass spectrometer (QTof Premier, Waters) coupled to a nano-UPLC system (NanoAcquity, Waters) using a reversed phase 75µm x 250mm column, as described (Batycka et al, 2006). MS/MS spectra were processed using ProteinLynx Global Software (PLGS, version 2.3) and analysed using MassLynx software (version 4.1). In addition, MS/MS spectra were searched against the Swissprot database using Mascot (version 2.3.01, Matrixscience) using the following parameters: peptide tolerance=0.2 Da; fragment ion tolerance: 0.1Da; fixed modification: carbamidomethylation; variable modifcations: oxidation (M), deamidation (NQ), mono-/di-/tri-methylation (R), following the guidelines reported previously (Taylor & Goodlett, 2005). The analysis of immunoprecipitated E2F-1 digested

material was performed by LC-MS/MS using an orbitrap Velos (Thermo) coupled to a nano-UPLC system (NanoAcquity, Waters) using a reversed phase 75μm x 250mm column as described (Fischer et al, 2011). MS/MS spectra were searched against the IPI human database (v.3.80, 86719 entries) in Mascot v2.3.01, allowing one missed cleavage and 20ppm / 0.5 Da mass deviations in MS / MSMS, respectively. Carbamidomethylation of cysteine was a fixed modification. Oxidation of methionine, mono-/di-trimethylation of arginine and deamidation of asparagine and glutamine were used as variable modifications.

Histopathology

Paraffin-embedded formalin-fixed tissue from different tumour types were cleared of paraffin in Citroclear, and rehydrated through graded alcohol baths. After a rinse in water, sections were heated at 95°C for 20min in 10mM sodium citrate, pH 6 for antigen retrieval. Slides were incubated in 0.5% hydrogen peroxide for 15min to inactivate endogenous peroxidases. Slides were washed in phosphate buffered saline/0.1% Tween 20 (PBST) for 5min before being blocked in serum for 20 min. Slides were incubated in primary antibodies; anti-PRMT5 or anti-E2F-1 overnight at 4°C. This was followed by corresponding anti-HRP secondary antibodies, ABC reagent (Vectorlabs) and then substrate (DAB; Vectorlabs). Slides were placed in hematoxylin (Sigma) for 5s for nuclear counterstaining and then mounted with coverslips using AquaTex (Merck). Sections were examined under a light microscope.

Tissue source and histopathology examination

For the lymphoma tissue, a tissue microarray of 22 patients with mantle cell lymphoma and 66 patients with follicular lymphoma were analysed. Medical reports of clinical information were not available. The study was approved by Oxfordshire Research Ethics Committee (ref. CO2.216). For colorectal tissue, the study group included patients who had undergone surgical resection with primary colorectal cancer (Stage II or III) between 2001 and 2004 (SI

Table 1). All tumour biopsies were analysed in triplicate, and each patient had a matched normal tissue sample. Medical reports of clinical information regarding demographics, treatment details and patient outcome were available for all patients with a follow-up of nearly 5 years. This study was covered by ethics approval reference MREC/00/7/412 VICTOR trial.

Evaluation

PRMT5 and E2F-1 expression were scored blind independently by two assessors. Scoring criteria were as follows; frequency was scored as the percentage of cells per tissue section staining positive (either overall or nuclear staining) with antibodies for either PRMT5 or E2F-1. A scoring scale was given as follows; PRMT5; 0 = 0-19%, 1 = 20-39%, 2 = 40-59%, 3 = 60-79%, 4 = 80-100% and E2F-1; 0 = 0-9%, 1 = 10-19%, 2 = 20-29%, 3 = 30-39%, 4 = 40-50%. In addition to frequency, intensity of staining was scored subjectively as the degree of staining for PRMT5 and E2F-1. For both proteins a score was given as follows, 0 = negative, 1 = weak, 2 = moderate and 3 = strong staining. Finally an overall staining score (OSS) was obtained by adding both the scores for frequency and intensity to give a mark of 0 to 7. In some of the figures to simplify the analysis, an OSS of 0-2 was deemed low (i.e. low frequency and weak intensity), 3-5, moderate (i.e. low frequency and high intensity, or high frequency and low intensity) and 6-7 high (i.e. high frequency and high intensity). Chi squared tests were determined using MedCalc software.

Supplemental information Figure 1

Arginine methylation of E2F-1

a) Wild-type E2F-1, KK or KKK mutants were transfected into U2OS cells and after 48h immunostained with the anti-HA antibody.

b) E2F transcription upon PRMT5 depletion. PRMT5 (P) or control (C) non-targeting siRNA was transfected into U2OS cells together with p73-luciferase, cyclin E-luciferase or Cdc6-luciferase for 48h, together with pCMV-βgal to monitor transfection efficiency. Relative luciferase activity is shown (i) together with expression level of endogenous proteins (ii); n=2.

c) ChIP analysis in U2OS cells transfected with PRMT5 (P) or control (C) siRNA followed by immunoprecipitation with anti-acetylated (Ac) histone (H)3 lysine (K)4 antibodies on the indicated E2F target genes quantitated by real-time PCR**.**

d) Representative images of PRMT5 and E2F-1 immunohistochemistry (IHC) in an MDA-MB-231 breast cancer cell xenograft showing examples of E2F-1 and PRMT5 staining cells; note that both positive and negative E2F-1 and PRMT5 stained tumour cells are evident (indicated by arrows; 10x and 40x magnification, as indicated).

e) U2OS cells were transfected with expression vectors encoding the indicated E2F-1 derivative (adjusted for equal protein expression), together with p73-luciferase (for 48h) and pCMV-βgal to monitor transfection efficiency. Relative luciferase activity is shown (luciferase/βgal) together with the expression level of ectopic proteins underneath; n = 3.

f) Apoptosis upon PRMT5 and p73 depletion. PRMT5 (P), p73 (73) or control (C) non-targeting siRNA was transfected into U2OS cells and harvested at 72h post-transfection and analysed by FACS. The graph represents the relative change in the sub-G1 population compared to the control treatment, and the level of PRMT5, E2F-1

and p73 is shown underneath; n=3. p73 levels were 2.2 fold higher in the P treatment relative to C treatment, and 0.8 fold in the P/73 treatment relative to C treatment.

g) Mass spectrometric profile of human E2F-1 (Sprot Acc Nr: Q01094) covering residues 91-113 (i) after *in vitro* methylation by PRMT5. Analysis by LC-MS/MS revealed the presence of four methyl groups present in the peptide fragment ARGRGR (108-113) in E2F-1. The MS/MS spectrum of the modified tryptic peptide RLDLETDHQYLAESSGPARGRGR + 4 x CH_2- $[M+5H]^{5+}$ 528.9Da (MW 2639.4 Da) is shown. Fragment ions are indicated as b and y ions. *represents loss of NH_3. Analysis by tandem mass spectrometry (ii) revealed the E2F-1 derived tryptic peptides 92-109 (unmodified), 91-111 (+ di-Met(R)) and 91-113 (+ 2 di-Met(R)), consistent with arginine methylation (di-methyl groups) at R_{111} and R_{113}.

Supplemental information Figure 2

a) Representative images of PRMT5 IHC in follicular B cell lymphoma. The follicles containing malignant cells which stain intensely with anti-PRMT5 are indicated (i), and at higher magnification (40x) in (ii).

b) Regulation of PRMT5 and E2F-1 under DNA damage. U2OS cells were treated with UV light (50 J m^{-2}) and at the indicated time (h) after treatment, harvested and immunoblotted with anti-PRMT5 and anti-E2F-1. Actin served as the loading control.

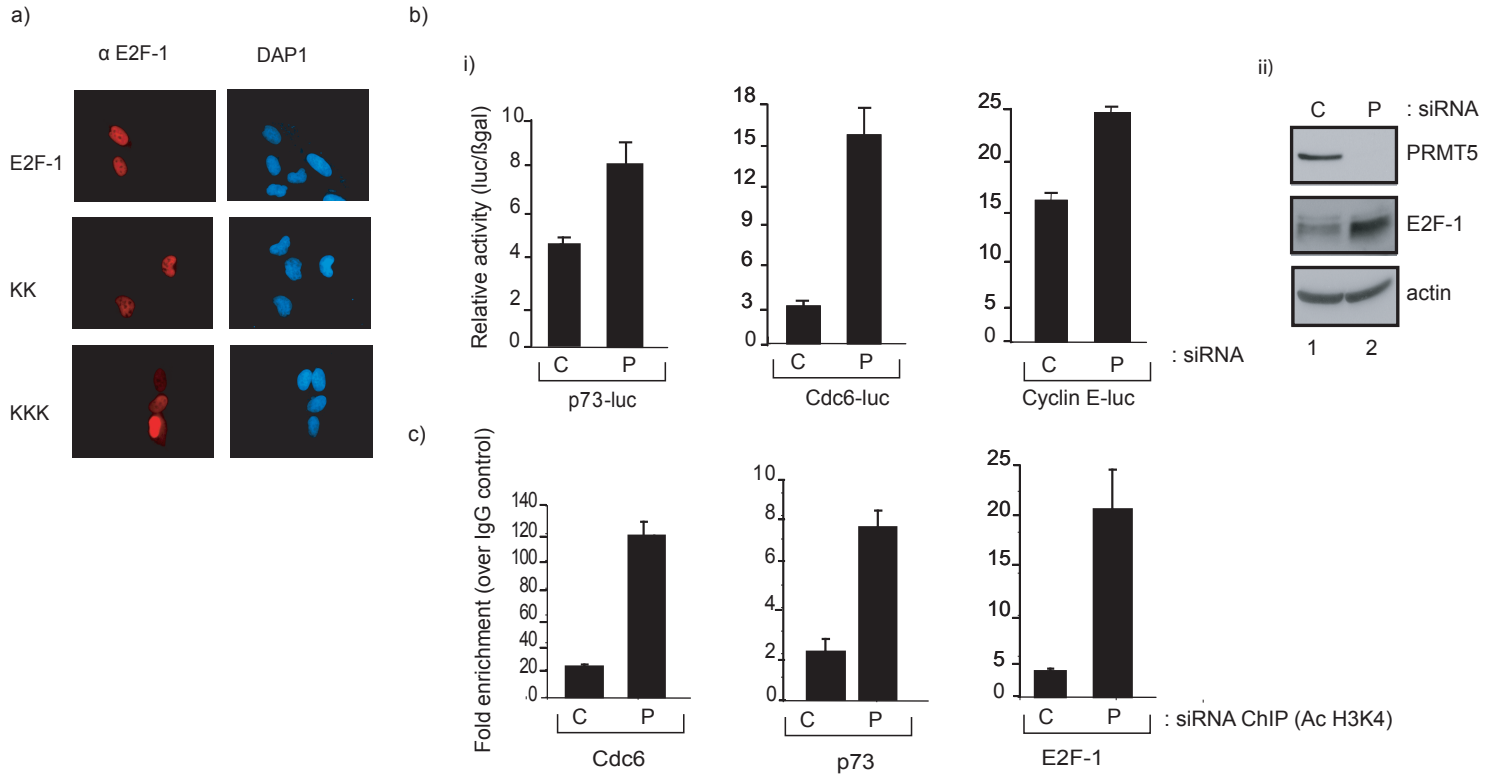
c) Expression vectors (1µg) encoding the indicated E2F-1 derivatives were transfected into U2OS cells and treated as described with cyclohexamide. Cells were harvested at 30, 60 and 120 min, followed by immunoblotting with anti-HA (i), and further quantitated in (iii). Actin served as the loading control (ii); n = 2.

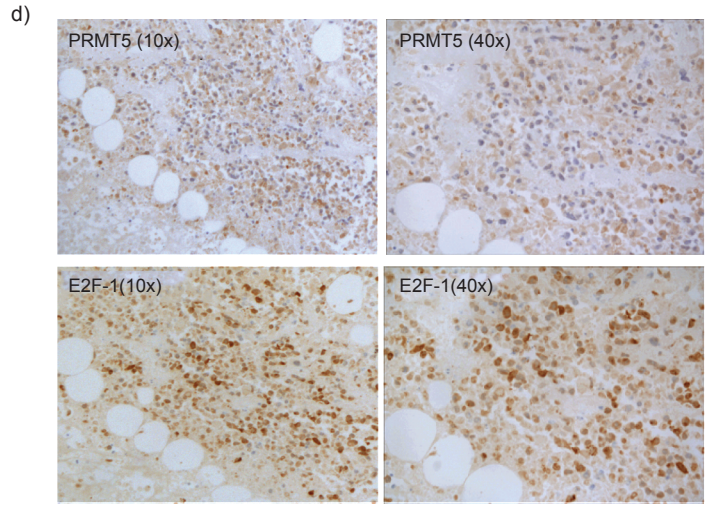
Supplemental information Table 1

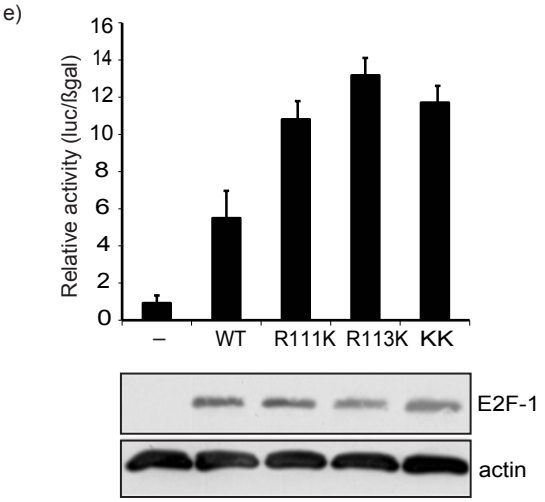
Demographic and clinical details of patient population

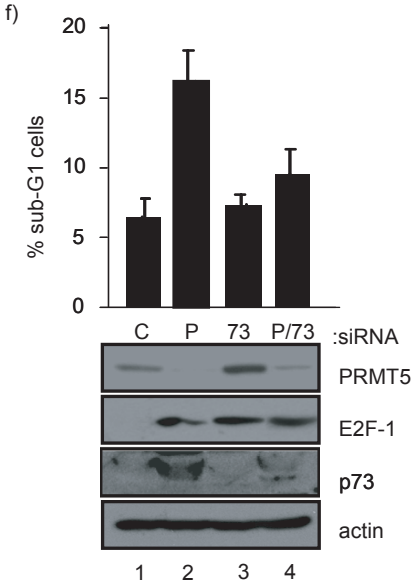
		VICTOR patients (N=2434) N (%)	Patients without Samples (N=1428) N (%)	Patients with samples (N=1006) N (%)	Patients analysed (N=108) N (%)
Age	Age < 50	181 (7.44)	109 (7.63)	72 (7.16)	8 (7.41)
	Age 50-59	590 (24.24)	349 (24.44)	241 (23.96)	26 (24.07)
	Age 60-69	940 (38.62)	557 (39.01)	383 (38.07)	34 (31.48)
	Age 70 +	723 (29.7)	413 (28.92)	310 (30.82)	40 (37.04)
Cancer Site	Colon	1592 (65.41)	914 (64.01)	678 (67.40)	73 (67.59)
	Junction	181 (7.44)	102 (7.14)	79 (7.85)	8 (7.41)
	Rectum	661 (27.16)	412 (28.85)	249 (24.75)	27 (25.00)
Stage	Stage II	1159 (47.62)	667 (46.71)	492 (48.91)	34 (31.48)
	Stage III	1275 (52.38)	761 (53.29)	514 (51.09)	74 (68.52)
Chemotherapy	None	1580 (64.9)	943 (66.04)	369 (36.68)	31 (28.70)
	Yes	854 (35.1)	485 (33.96)	637 (63.32)	77 (71.30)
Radiotherapy	Yes	299 (12.3)	191 (13.38)	108 (10.74)	10 (9.26)
	None	2135 (87.7)	1237 (86.62)	898 (89.26)	98 (90.74)
Gender	Male	1560 (64.09)	650 (64.61)	910 (63.73)	68 (62.96)
	Female	874 (35.91)	656 (35.39)	518 (36.27)	40 (37.04)
Age	Age (mean (SD))	63.78 (9.68)	63.52 (9.54)	64.15 (9.87)	64.91 (10.14)
Treatment	Placebo	1217 (50.0)	722 (50.6)	706 (49.4)	51 (47.22)
	Vioxx	1217 (50.0)	495 (49.2)	511 (50.8)	57 (52.78)

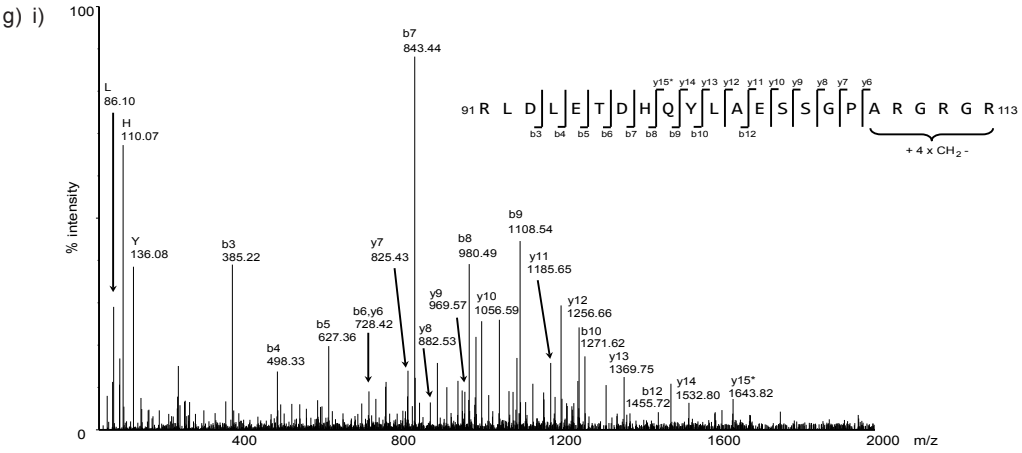
SI Fig 1







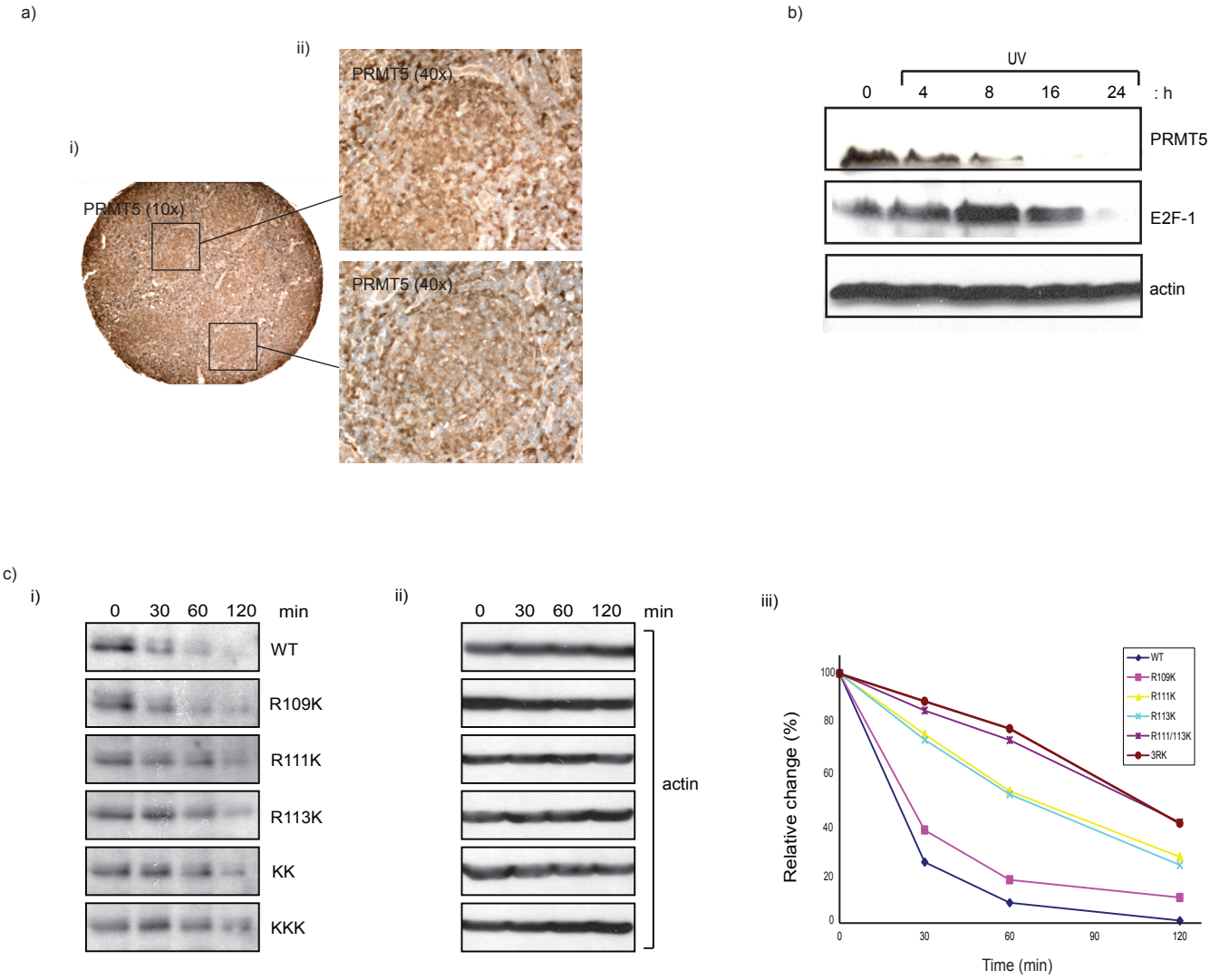




ii)

Peptide	Observed mass (m/z)	Expected mass	Calc. mass	Delta mass	Mascot Score
$_{92}$LDLETDHQYLAESSGPAR$_{109}$	667.99	2000.94	2000.94	-0.00	37
$_{91}$RLDLETDHQYLAESSGPARGR$_{111}$+ di-Met(R)	600.55	2398.17	2398.20	-0.03	42
$_{91}$RLDLETDHQYLAESSGPARGRGR$_{113}$ + 2 di-Met(R)	528.86	2639.34	2639.35	-0.01	60

SI Fig 2


a)
i)
PRMT5 (10x)
ii)
PRMT5 (40x)
PRMT5 (40x)
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UV
0
4
8
16
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PRMT5
E2F-1
actin
c)
i)
0 30 60 120 min
WT
R109K
R111K
R113K
KK
KKK
ii)
0 30 60 120 min
actin
iii)
Relative change (%)
Time (min)
WT
R109K
R111K
R113K
R111/113K
3RK