Truncating mutation in the autophagy gene UVRAG confers oncogenic properties and chemosensitivity in colorectal cancers

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Autophagy-related factors are implicated in metabolic adaptation and cancer metastasis. However, the role of autophagy factors in cancer progression and their effect in treatment response remain largely elusive. Recent studies have shown that UVRAG, a key autophagic tumour suppressor, is mutated in common human cancers. Here we demonstrate that the cancer-related UVRAG frameshift (FS), which does not result in a null mutation, is expressed as a truncated UVRAG⁵⁵ in colorectal cancer (CRC) with microsatellite instability (MSI), and promotes tumorigenesis. UVRAG⁵⁵ abrogates the normal functions of UVRAG, in a dominant-negative manner. Furthermore, expression of UVRAG⁵⁵ can trigger CRC metastatic spread through Rac1 activation and epithelial-to-mesenchymal transition, independently of autophagy. Interestingly, UVRAG⁵⁵ expression renders cells more sensitive to standard chemotherapy regimen due to a DNA repair defect. These results identify UVRAG as a new MSI target gene and provide a mechanism for UVRAG participation in CRC pathogenesis and treatment response.
Colorectal cancer (CRC) remains one of the most widespread malignancies worldwide. Approximately 15% of sporadic CRC and 90% of Lynch syndrome (hereditary nonpolyposis colorectal cancer) exhibit a microsatellite instability (MSI) phenotype, caused by a deficiency in DNA mismatch repair (MMR) that progresses with a high rate of insertions/deletions to repetitive DNA sequences, termed microsatellites. Increasing evidence suggests that MMR deficiency per se is not sufficient to drive cell transformation and tumorigenesis, but that microsatellite mutations in a limited number of target genes might be positively selected during tumour development and underlie MSI-associated pathogenesis and treatment response.

Frameshift (FS) mutations of several autophagy-related genes, including Atg2b, Atg5, Atg9b, Atg12 and UVRAG (ultraviolet radiation–associated gene), were recently reported in gastric cancer and CRC with MSI. Nevertheless, the functional consequences and key molecular events downstream of these mutations have not been extensively investigated.

Our previous studies have established UVRAG as a critical regulator of intracellular membrane trafficking, including autophagy and chromosomal stability. UVRAG contains four functional domains, that is, a proline-rich domain, a lipid-binding C2 domain, a Beclin1-binding coiled-coil domain (CCD) and a C-terminal domain presumed to be unstructured and involved in centrosome integrity and DNA damage repair. Importantly, all the different activities of UVRAG are functionally independent, suggesting biological interaction and coordinated regulation of the different processes under diverse environmental cues. Although most cellular studies to date have considered UVRAG as a tumour suppressor in human cancers, the genetic linkage of UVRAG mutations in major tumour types and the significance of these mutations in tumour pathogenesis remains less understood.

Here we show that MSI CRCs with the FS mutation in UVRAG express a truncated UVRAG protein, referred to here as UVRAGFS. In addition to losing the wild-type (WT) UVRAG functions, this nonsense mutant acts as a dominant-negative mutant and contributes to the oncogenesis and tumour metastasis of CRC, likely by antagonizing the activity of UVRAGWT as a tumour suppressor. UVRAGFS expression also increases the sensitivity to anticancer agents such as 5-fluorouracil (5-FU), oxaliplatin and irinotecan, routinely prescribed as adjuvant therapies for CRC patients. Our data thus identified the underlying pathogenic mechanisms beyond autophagy that are associated with UVRAGFS-positive cancers and suggest that expression of UVRAGFS might also be a predictive factor for chemotherapy response.

Oncogenic property of the UVRAGFS mutation. To probe whether the UVRAGFS mutant represents a mere loss of WT function or imparts oncogenic properties, we established MSS SW480 and MSI HCT116 cell lines stably expressing Flag-tagged UVRAGWT and UVRAGFS at equivalent levels. UVRAGFS-transduced cells showed increased proliferation and enhanced anchorage-independent growth in soft agar and tumour formation with accelerated kinetics. To further test whether expression of UVRAGFS is sufficient to transform noncancerous cells, we used NIH3T3 mouse embryonic fibroblasts stably expressing UVRAGWT or UVRAGFS. Compared with control (3T3.Vec), UVRAGFS–3T3 cells had elevated growth rate, formed larger colonies when plated at low density and induced anchorage-independence growth, whereas UVRAGWT exerted the opposite effects.

Dominant-negative effect of UVRAGFS on autophagy activation. UVRAGFS retains the N-terminal proline-rich and C2 domains, and the partial CCD required for Beclin1-mediated autophagy. To assess whether this mutation is indeed expressed in MSI cells, we generated an antibody specifically recognizing UVRAGFS, but not UVRAGWT, using the FS-derived neopeptide (31KKKVNACS37) as antigen. UVRAGFS expression was detected in all MSI cell lines carrying the FS mutation, but not in MSI or MSS cells that are WT for UVRAG (Fig. 1b). Notably, the overall expression of UVRAGWT was diminished in MSI cells with the FS mutation (Fig. 1b), and the levels of UVRAGFS were inversely correlated with the expression of UVRAGWT in all tested cell lines (Fig. 1c). This was consistent with the UVRAG expression profile from the CRC cell lines of the NCI-60 panel. Therein, a significant reduction of UVRAGWT expression was detected in UVRAGFS-positive KM12 and HCT116 CRC cells compared with other CRC cells without UVRAGFS (Supplementary Fig. 1d). In addition, the UVRAG FS mutation was present in one of the four analysed cases of human primary CRC with MSI (fourth column in Fig. 1d), but not in primary MSS CRC or in normal colorectal mucosa (Fig. 1d, Supplementary Table 1). This is in line with a previous report that evaluated the mutation frequencies in 137 genes in MSI cancers, revealing the high frequency of the A10 UVRAG FS mutation that was found in 33% CRC, 8% endometrial and 7.8% gastric cancers with MSI (Supplementary Fig. 1e). Whole-genome sequencing analysis of a large cohort of gastric cancers (Pfizer and UHK; n = 100) also confirmed the presence of the UVRAG FS mutation in MSI gastric cancer (40%) by collecting neopeptide (234KKKVNACS241) as antigen.

Material and methods. Here we show that MSI CRCs with the FS mutation in UVRAG express a truncated UVRAG protein, referred to here as UVRAGFS. In addition to losing the wild-type (WT) UVRAG functions, this nonsense mutant acts as a dominant-negative mutant and contributes to the oncogenesis and tumour metastasis of CRC, likely by antagonizing the activity of UVRAGWT as a tumour suppressor. UVRAGFS expression also increases the sensitivity to anticancer agents such as 5-fluorouracil (5-FU), oxaliplatin and irinotecan, routinely prescribed as adjuvant therapies for CRC patients. Our data thus identified the underlying pathogenic mechanisms beyond autophagy that are associated with UVRAGFS-positive cancers and suggest that expression of UVRAGFS might also be a predictive factor for chemotherapy response.
autophagosome-associated lipidated LC3 (LC3-II) in noncancerous NIH3T3 cells. As shown previously, UVRAGWT or rapamycin markedly promoted autophagy, as evidenced by increased GFP–LC3 puncta per cell, increased LC3-II conversion and increased response to the late-stage autophagy inhibitor Bafilomycin A1 (Fig. 3a,b). In sharp contrast, UVRAGFS did not demonstrate any proautophagic activity. Furthermore, UVRAGWT autophagy-promoting activity was

Figure 1 | Identification of UVRAG FS mutation in CRC cell lines and primary tumours. (a) Sequencing analysis of UVRAG at the location of the A10 repeat in MSS (HCC2998, COLO205, SW620, SW480 and HT29) and MSI (HCT15, SW48, HCT116, RKO, LIM2405, LS180 and KMT2) CRC cell lines. Arrows indicate the heterozygous deletion of one A in UVRAG A10 in MSI cell lines. (b,c) Wild-type (WT) and FS mutant UVRAG protein expression in MSS and MSI CRC cell lines. Whole-cell lysates (WCL) of MSS and MSI CRC cell lines were immunoprecipitated with anti-UVRAGFS followed by immunoblotting with anti-UVRAGFS, or they were directly probed with antibodies targeting UVRAGWT or γ-H2AX. Actin served as a loading control. Densitometric quantification of protein expression is shown in (c). Dash lines indicate average band intensities of all the tested cell lines. Note reduced UVRAGWT expression in MSI CRC cells expressing UVRAGFS. (d) H&E (first row) and immunohistochemical analysis of UVRAG (second row), Ki67 (fourth row), and γ-H2AX (5th row) in paired human primary CRC specimen obtained from three separate patients with their corresponding status of UVRAG FS mutation (third row) provided. The bar plots (right) are the quantification of the levels of Ki67 and γ-H2AX (denoted by arrows) in the paired tissues with WT or mutant UVRAG. HPF, high-power field. ***P<0.001 (Mann–Whitney test); Scale bar, 50 μm.
abrogated when UVRAGFS was added to the cells dose dependently (Supplementary Fig. 3a). UVRAG interacts with Beclin1 through their respective CCD, resulting in activation of Beclin1-associated Vps34 kinase. On UVRAGFS expression, the endogenous association between UVRAGWT and Beclin1 was diminished, and UVRAGFS was able to sequester the Beclin1 and UVRAG proteins in vivo, in line with its dominant-negative effect (Supplementary Fig. 3b, Fig. 3c). Accordingly, Vps34 enzymatic activity was significantly reduced in UVRAGFS cells (Fig. 3d), as illustrated by decreased punctate staining of the Vps34 kinase product, phosphatidylinositol 3-phosphate. Impaired autophagy was also observed in vivo in NIH3T3 tumour xenografts expressing UVRAGFS (Fig. 2e), showing increased levels of p62, an autophagic substrate. To explore whether autophagy inhibition underlies UVRAGFS-mediated oncogenesis, we examined the transforming effect of UVRAGFS in autophagy-null Atg5-deficient MEFs.
UVRAG<sup>FS</sup> promoted cell proliferation (Fig. 3e) and colony growth in soft agar (Fig. 3f–h), irrespective of the autophagy status. These data support a direct role of UVRAG<sup>FS</sup> in promoting tumorigenesis independently of autophagy.

**Figure 3 | UVRAG<sup>FS</sup> inhibits cellular autophagy in a dominant-negative manner.** (a) NIH3T3 cells stably expressing vector, UVRAG<sup>WT</sup>, and UVRAG<sup>FS</sup> were transfected with GFP–LC3 and treated with rapamycin (100 nM). GFP–LC3 puncta per cell were quantified as shown in representative images shown. Data represent the means ± s.d. (n = 6). *P < 0.05. Scale bar, 10 µm. (b) Western blot analysis and densitometric quantification (underneath the blot) of the LC3-II/LC3-I ratios in NIH3T3 cells treated with rapamycin in the presence or absence of Bafilomycin A1 (100 nM). N, normal condition; R, rapamycin; R + B, rapamycin + Bafilomycin A1. (c) Schematic depiction of the dominant-negative action of UVRAG<sup>FS</sup> on the UVRAG-Beclin1 interaction by sequestering both. (d) UVRAG<sup>FS</sup> inhibits Beclin1-associated VPS34 kinase activity. NIH3T3 cells from (a) were transfected with p40(phox)-PX-GFP (to monitor phosphatidylinositol 3-phosphate formation). At 16 h post-transfection, cells were subjected to confocal microscopy and p40(phox)-PX-GFP puncta per cell were quantified. Data represent the means ± s.d. (n = 3). **P < 0.01. Scale bar, 10 µm. (e) UVRAG<sup>FS</sup> promotes cell proliferation in Atg5-knockout iMEFs. Atg5<sup>+/+</sup> and Atg5<sup>−/−</sup> iMEF cells stably expressing vector and UVRAG<sup>FS</sup> were seeded and counted in triplicate on day 8. Values are mean ± s.d. (n = 3). UVRAG and Atg5 expression was assessed by western blot with actin serving as a loading control. *P < 0.05; **P < 0.01. (f–h) Anchorage-independent growth of Atg5-knockout iMEFs expressing UVRAG<sup>FS</sup>. Note the larger and greater number of colonies in UVRAG<sup>FS</sup>-expressing cells. Colony diameters (g) and numbers (h) were quantified from 20 random HPFs. Data are the means ± s.d. (n = 3). *P < 0.05; ***P < 0.001. Scale bar, 50 µm.

**UVRAG<sup>FS</sup> induces chromosomal instability and centrosome amplification.** Because the role of UVRAG in cancer has been linked to its ability to maintain chromosomal stability<sup>17</sup>, we investigated the effect of UVRAG<sup>FS</sup> on overall chromosomal
stability in genetically stable mouse embryonic stem cells. Spectral karyotyping analysis showed that, unlike control cells that were mostly diploid, UVRAGFS-embryonic stem cells were highly heterogeneous with respect to both structural and numerical aberrations as compared with the vector control (Fig. 4a, Supplementary Fig. 4a) with a greater than sevenfold increase in aneuploidy in UVRAGFS cells (Supplementary Fig. 4b). These results indicate that UVRAGFS elicits severe chromosomal instability and aneuploidy. To validate this, we analysed the Pfizer and UHK cohort of gastric cancers, and observed significantly enhanced chromosomal rearrangement in UVRAGFS MSI gastric cancers as compared with UVRAGWT MSI gastric cancers (Fig. 4b). In fact, UVRAGFS gastric cancers had substantially more protein-altering mutations and single-nucleotide variants than UVRAGWT MSI and MSS gastric cancers (Supplementary Fig. 4c). Moreover, the FS mutation appeared to be more frequent in gastric cases with advanced tumour, node, metastasis stage (Supplementary Fig. 4d). Thus, UVRAGFS may predispose MSI cancers to increased genetic instability and cancer progression.

UVRAGWT has been shown to associate with the centrosome protein CEP63 (ref. 17), contributing to chromosomal stability by preventing centrosome overduplication17. UVRAGFS expression in SW480 cells was sufficient to induce a marked increase in the incidence and degree of centrosome amplification compared with control (Fig. 4c). Consistent with the consensus that centrosome amplification causes erroneous chromosomal segregation30, we detected spindle malformation, chromosomal miss segregation and prolonged mitosis in UVRAGFS clones, whereas UVRAGWT clones behaved in the opposite manner (Fig. 4d, Supplementary Fig. 5a). Unlike WT, UVRAGFS was unable to associate with CEP63 (Fig. 4e), failing to colocalize with CEP63 and the centrosome marker, γ-Tubulin (Fig. 4f). UVRAGFS disrupted UVRAGWT-CEP63 interaction (Fig. 4g) and displaced UVRAG from the centrosome in a dominant-negative manner (Supplementary Fig. 5b). These results indicate that centrosome amplification induced by UVRAGFS might play a role in UVRAGFS-associated chromosomal aneuploidies.

UVRAGFS promotes cell invasion and metastasis outgrowth. Centrosome amplification per se has been shown to promote cell invasion through inappropriate microtubule nucleation and Rac-1 activation31, a small GTPase important for the control of cell invasiveness and metastasis32,33. Indeed, pull-down assay in UVRAGFS SW480 cells detected a more than twofold Rac1 activation, which could be blocked by Taxol, but not by the autophagy inhibitor chloroquine or the anticancer reagent 5-FU (Fig. 5a), indicating a requirement for dynamic microtubules. Consistent with increased Rac1 activation, UVRAGFS enhanced the cell motility of SW480 cells in a wound-healing assay, which was inhibited by Taxol (Fig. 5b). It also enhanced HCT116 cell migration through a collagen matrix, whereas UVRAGWT exerted an inhibitory effect (Supplementary Fig. 6a,b). Spleen injection of non-metastatic SW480 cells expressing UVRAGFS into nude mice resulted in a higher incidence of liver metastasis and a greater number of colonization in the lungs, kidney and peritoneum, whereas no colonization was found in the control group (Fig. 5c,d, Supplementary Fig. 6c). UVRAGFS-induced tumour metastases were confirmed in an independent mouse metastasis model with SW480 cells expressing GFP–UVRAGFS, as determined by bioluminescence imaging of metastatic lesions (Supplementary Fig. 6d). These results indicate that UVRAGFS enhances the metastatic capacity of CRC cells.

Autophagy has been postulated to be exploited by metastatic tumours to survive unfavourable conditions34. Nevertheless, UVRAGFS-metastatic tumours displayed higher levels of p62 than primary tumours, indicative of suppressed autophagy (Fig. 5e). Moreover, UVRAGFS metastatic tumours exhibited decreased apoptosis, as shown by decreased caspase 3 activation (Fig. 5f). Hence, in this context, autophagy is not the driving mechanism for metastatic colonization in CRC. Nevertheless, we observed other pathological differences that may account for increased metastasis on UVRAGFS expression. The colonized CRC tumours had reduced levels of the epithelial cell marker E-cadherin but increased levels of the mesenchymal markers, N-cadherin and vimentin (Fig. 5e), suggesting an induction of epithelial-mesenchymal transition (EMT) in the process of colonization. Indeed, expression of UVRAGFS in SW480 cells downregulated E-cadherin and upregulated N-cadherin and vimentin, whereas expression of UVRAGWT had the opposite effect (Fig. 5f). Importantly, UVRAGFS-associated EMT was efficiently reverted by Taxol without affecting Taxol-induced cell death (Fig. 5f, Supplementary Fig. 6f). Consistent with our in vitro observations, the primary MSI colon tumour with UVRAGFS exhibited elevated expression of N-cadherin and vimentin, along with significant reduction in E-cadherin levels, which were not detected in tumours with UVRAGWT (Supplementary Fig. 6e). These results indicate that UVRAGFS expression, which triggers centrosome amplification and Rac1 activation, can activate the EMT program and promote cell invasion and tumour metastasis.

UVRAGFS affects CRC response to chemotherapy. We next investigated the possible clinical relevance of UVRAGFS by testing the response of CRC to 5-FU chemotherapy, the first-line treatment for CRC patients, using a tumour xenograft model. Surprisingly, UVRAGFS expression significantly increased tumour sensitivity to 5-FU treatment with an approximate 10-fold reduction in tumour volumes after a 4-week administration of 5-FU (Fig. 6a), compared with a less than twofold reduction in the control group (Fig. 6a–c). Histological analyses revealed a significant reduction in cell proliferation and an increase in the number of cells undergoing apoptosis in 5-FU-treated UVRAGFS tumours, in concordance with induced tumour shrinkage (Fig. 6d). In addition, UVRAGFS expression in CRC cells markedly increased their sensitivity to other DNA-based cytotoxic anticancer agents, including oxaliplatin and irinotecan, as shown by reduced rates of clonogenic survival, whereas UVRAGWT cells were resistant to the drugs (Fig. 6e). To examine the unexpected role of UVRAGFS in tumour chemosensitivity, we measured the levels of γ-H2AX, a sensitive marker of double strand breaks (DSBs)35, and observed that UVRAGFS SW480-tumours accumulated higher levels of γ-H2AX than the controls, which further increased with 5-FU that produces DNA strand breaks (Fig. 6d). Consistent with our observation in xenograft tumours, UVRAGFS expression resulted in a significant increase of γ-H2AX foci and levels in SW480 CRC cells (Supplementary Fig. 7a,b). Furthermore, the overall levels of γ-H2AX were higher in MSI CRC cell lines expressing UVRAGFS compared with the WT counterparts, and likewise, were significantly different between UVRAGFS-positive and -negative primary tumours (Fig. 1b,d). Adding UVRAGWT to UVRAGFS, positive HCT116 and RKO cells at different doses clearly suppressed the levels of DSBs (Supplementary Fig. 7c), highlighting a direct involvement of UVRAGFS in genetic stability. To determine whether the observed accumulation of DSB in UVRAGFS cells reflects impaired DNA repair, we measured unrepaired DSBs after ionizing radiation (IR) using the comet assay. We found that IR induced comparable levels of DNA damage in vector, UVRAGWT and UVRAGFS cells (10 min
Figure 4 | UVRAGFS promotes chromosomal instability and centrosome amplification. (a) Representative SKY analysis of mouse embryonic stem (ES) cells expressing vector or Flag-UVRAGFS. Average chromosomal aberrations per cell were quantified. *P < 0.05, Wilcoxon Signed-rank Test. (b) Chromosomal rearrangement and single-nucleotide variants (SNVs) in UVRAGFS gastric cancer. Representative Circos plots of the UVRAGWT and UVRAGFS subtypes of MSI gastric cancer in the Pfizer and UHK cohorts20. The inner circle denotes chromosomal structural variants: ITX (intrachromosomal translocation), blue; DEL (deletion), red; CTX (interchromosomal translocation), green; INV (inversions), pink; Tandem duplication, black. In the second circle, each dot denotes one somatic SNV, coloured according to six mutation types: T > G, pink; T > C, green; T > A, gray; C > G, black; C > A, blue; C > T, red. The outer circle denotes 23 chromosomes. Boxplot represents the total chromosomal structure variants (SV) count in the UVRAGWT and UVRAGFS MSI gastric cancers (Mann–Whitney test). (c) UVRAGFS induces centrosome amplification. SW480.Vector and SW480.UVRAGFS cells with different centrosome numbers were immunostained for γ-Tubulin and DAPI and quantified (Data are the means ± s.d., n = 200 cells obtained by gathering data from three independent experiments). (d) centrosome. Scale bar, 10 μm. (e) Representative confocal images of spindle malformation in mitotic Vector, UVRAGWT, and UVRAGFS SW480 cells co-stained with anti-γ-Tubulin (red) and anti-α-Tubulin (green) for the mitotic asters. The percentages of cells with disorganized spindle were quantified. Data are the means ± s.d. (n = 200 cells obtained by pooling data from three independent experiments). Scale bar, 10 μm. (f) UVRAGFS is defective in CEP63 binding. Whole-cell lysates (WCL) of 293T transfected with Flag-UVRAGWT or Flag-UVRAGFS were immunoprecipitated with anti-Flag followed by IB with anti-CEP63. (g) Representative image showing dissociation of UVRAGFS from the centrosome. HeLa cells expressing Flag-UVRAGWT or Flag-UVRAGFS were stained with anti-Flag (green), anti-γ-Tubulin (red), and anti-CEP63 (blue). Scale bar, 10 μm. (g) UVRAGFS inhibits UVRAG-CEP63 interaction. The 293T cells were transfected with increasing amounts of Flag-UVRAGFS. WCL were immunoprecipitated with anti-CEP63 or anti-Flag, followed by immunoblotting with anti-UVRAG or anti-CEP63 as indicated.
Figure 5 | UVRAG<sup>FS</sup> activates Rac-1 and promotes tumour metastasis in vitro and vivo. (a) Rac-1 activation by UVRAG<sup>FS</sup>. Western blot shows a pull-down experiment to detect GTP-bound Rac1 in SW480.UVRAG<sup>FS</sup> cells and on drug treatment. Histogram shows quantification from three independent experiments. (b) Representative images of scratch-wound healing exhibit the motility of SW480.UVRAG<sup>FS</sup> cells. Cell motility into the wound area was taken at 0 and 16 h as marked by red lines. Wound-healing index was quantified (right). Data are the means ± s.d. (n = 3). **P < 0.01; ***P < 0.001; NS, not significant. Histogram shows quantification from three independent experiments. (c,d) UVRAG<sup>FS</sup> enhances tumour metastasis in mice inoculated by intrasplenic injection of SW480.Vector and SW480.UVRAG<sup>FS</sup> cells. Schematic depiction of the procedure (c, left) and representative images (c, right) of upper abdominal organs at 8-week post-injection are shown (c). The number of metastatic nodules (liver and lung) was quantified (d). H&E staining was performed on serial sections of metastatic tumours (M) and normal (N) liver and lung are shown below. Scale bar, 1 mm. Arrows in c represent the metastasis foci. Results are representative of 10 mice per group. Fisher’s exact test was used. (e) Immunohistochemistry analysis of autophagy, apoptosis and EMT status of primary tumours (left panel) and metastasis nodules (right panel). The bar graphs represent the quantification of the indicated protein markers. Data are the means ± s.d. (n = 3). *P < 0.05. Scale bar, 50 μm. (f) Western blot of the EMT-related protein expression in SW480 cells expressing UVRAG<sup>FS</sup> and on Taxol treatment.
post-IR in Fig. 7a). However, a high persistence of comet tails was observed 24 h post-irradiation in UVRAGFS cells, whereas UVRAGWT cells have repaired most of the damaged DNA. These data indicate that UVRAGFS disrupts the rapid repair process of DSBs. The inhibitory effect of UVRAGFS on DSB repair was also detected in the autophagy-competent Atg3+/+ and the autophagy-null Atg3−/− cells (Supplementary Fig. 7d), suggesting minimal participation of autophagy in the elevated DNA damage induced by UVRAGFS expression.

UVRAGFS is defective in the repair of DNA damage. We then asked whether UVRAGFS-associated DNA damage results from suppression of UVRAGWT function, which is known to promote DSB repair by NHEJ (non-homologous end joining) through interaction with the Ku70/Ku80/DNA–PKcs complex. Unlike with UVRAGWT, no physical interactions between UVRAGFS and DNA–PK proteins could be detected (Supplementary Fig. 7e). Moreover, UVRAGFS failed to translocate to sites of laser-induced DNA damage stripes containing γ-H2AX, whereas

Figure 6 | UVRAGFS sensitizes CRC to DNA damage-inducing chemotherapy. (a) Mice bearing SW480.Vec and SW480.UVRAGFS tumours were treated with saline or 5-FU over day 1–30 when the tumour volume reached 200 mm³. Mean relative tumour volume (n = 10), is expressed compared with tumour volumes on day 1. *P < 0.05; **P < 0.01; ***P < 0.001. (b) Relative tumour volume for individual mice treated in a at day 25. (c) Representative images of mice bearing SW480-xenografts on the day 0, 21–28 of chemotherapy (left). Western blots showed UVRAGFS expression in representative tumours. Scale bar, 5 mm. (d) Immunohistochemical analysis of SW480-xenografts harvested from mice treated for 28 continuous days with 5-FU. Representative sections were stained (left) as indicated and staining-positive cells were quantified (right) as means ± s.d. Scale bar, 10 μm. *P < 0.05; **P < 0.01; ***P < 0.001. (e) SW480.Vector, SW480.UVRAGWT and SW480.UVRAGFS were treated with the indicated doses of 5-FU, Oxaliplatin and Irinotecan, followed by colony survival assay. Data are the means ± s.d. (n = 3). *P < 0.05; **P < 0.01.
UVRAG<sup>WT</sup> was enriched at the damaged sites of DSBs (Supplementary Fig. 7f). As expected, ectopic expression of UVRAG<sup>FS</sup> blocked UVRAG-Ku70/Ku80 interaction, and disturbed Ku/DNA–PKcs complex formation after IR, concomitant with increased sequestration of UVRAG<sup>WT</sup>, again highlighting the dominant-negative effect of the FS mutation (Fig. 7b). To further establish a link between UVRAG<sup>FS</sup> and the DNA-damaging phenotype observed, we evaluated the DNA repair capacity in UVRAG<sup>FS</sup> cells, using a NHEJ repair reporter, the EJ5-GFP system. Expression of UVRAG<sup>FS</sup> alone markedly reduced the rate of NHEJ repair by over 50%, whereas it had no discernible effect on DNA homologous recombination repair (Fig. 7c). Treating cells with Nu7441, a specific inhibitor of DNA–PKcs, followed by immunoblotting with the indicated antibodies. (Supplementary Fig. 7f). As expected, ectopic expression of UVRAG<sup>FS</sup>, and that it influences the expression and function of UVRAG<sup>WT</sup> in a dominant-negative manner. Furthermore, mutated UVRAG alleles sensitize CRC to DNA damage-inducing treatment, making the UVRAG<sup>FS</sup> genotype a possible predictive factor for the response to chemotherapy treatment.

In this study, we found that the heterozygous deletion of the UVRAG A<sub>10</sub> exonic DNA repeat resulted in the expression of a truncated protein using an antibody specifically recognizing UVRAG<sup>FS</sup>, and that it influences the expression and function of UVRAG<sup>WT</sup> in a series of CRC cell lines and primary CRCs. Contrary to our findings, a previous study showed by immunoblotting that the levels of UVRAG<sup>WT</sup> appeared to be unaffected by the occurrence of the UVRAG FS mutation in three MSI CRC cell lines carrying the FS mutation (HCT116, LoVo and RKO), two of which having also been used in our study (Fig. 1b). While it is difficult to explain the discrepancy between this published work and ours, it might be due to differences in experimental design and/or to different sources or passage numbers of CRC cell lines used in both studies. Nonetheless, our results are consistent with the gene expression data retrieved from a GeneChip analysis of NCI-60 cancer cell lines from TSRI (The Scripps Research Institute; data are accessible at BioGPS: http://biogps.org), correlating reduced UVRAG<sup>WT</sup> expression in

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**Figure 7 | UVRAG<sup>FS</sup> inhibits NHEJ repair.** (a) Neutral comet assay shows a delay of DNA DSBs repair in UVRAG<sup>FS</sup> cells. SW480 cells stably expressing empty vector (first row), UVRAG<sup>WT</sup> (second row) or UVRAG<sup>FS</sup> (third row) were treated with 1 Gy IR. The DNA damage levels of the cells before IR, 10 min post IR and 24 h post IR were assessed. Representative comet images are shown in the left panel and quantifications are shown on the right. (b) UVRAG<sup>FS</sup> inhibits UVRAG interaction with Ku70 and Ku80 and the interaction of Ku70/80 with DNA–PKcs. The 293T cells transfected with increasing amounts of Flag-UVRAG<sup>FS</sup> were treated with IR (5 Gy). WCL were immunoprecipitated with anti-DNA–PKcs or anti-UVRAG, followed by immunoblotting with the indicated antibodies. (c) HEK293 cells stably expressing the EJ5-GFP reporter for NHEJ and the DR-GFP reporter for homologous recombination (HR) were transfected with an empty vector or Flag-UVRAG<sup>FS</sup> before the induction of DSBs by SceI transfection. The DNA repair activities as assessed by the reconstituted GFP signals were quantified by fluorescence-activated cell sorting. Data shown represent mean ± s.d. (n = 3). **P < 0.01.

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**Discussion**

Microsatellite instability as a result of MMR deficiency has been widely observed in human CRC. However, little is known of the biological consequences and pathogenic mechanisms associated with the selective gene targeting by MSI. Herein, we demonstrate that the autophagic tumour suppressor UVRAG represents a new *bona fide* MSI target gene in CRC and, likely, other MSI-related tumours, and that the truncating mutation in UVRAG enhances cellular transformation and penetrance of CRC tumour by interfering with the tumour-suppressing functions of UVRAG<sup>WT</sup> in a dominant-negative manner. Furthermore, mutated UVRAG alleles sensitize CRC to DNA damage-inducing treatment, making the UVRAG<sup>FS</sup> genotype a possible predictive factor for the response to chemotherapy treatment.

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**Discussion**

Microsatellite instability as a result of MMR deficiency has been widely observed in human CRC. However, little is known of the biological consequences and pathogenic mechanisms associated with the selective gene targeting by MSI. Herein, we demonstrate that the autophagic tumour suppressor UVRAG represents a new *bona fide* MSI target gene in CRC and, likely, other MSI-related tumours, and that the truncating mutation in UVRAG enhances cellular transformation and penetrance of CRC tumour by interfering with the tumour-suppressing functions of UVRAG<sup>WT</sup> in a dominant-negative manner. Furthermore, mutated UVRAG alleles sensitize CRC to DNA damage-inducing treatment, making the UVRAG<sup>FS</sup> genotype a possible predictive factor for the response to chemotherapy treatment.

In this study, we found that the heterozygous deletion of the UVRAG A<sub>10</sub> exonic DNA repeat resulted in the expression of a truncated protein using an antibody specifically recognizing UVRAG<sup>FS</sup>, and that it influences the expression and function of UVRAG<sup>WT</sup> in a series of CRC cell lines and primary CRCs. Contrary to our findings, a previous study showed by immunoblotting that the levels of UVRAG<sup>WT</sup> appeared to be unaffected by the occurrence of the UVRAG FS mutation in three MSI CRC cell lines carrying the FS mutation (HCT116, LoVo and RKO), two of which having also been used in our study (Fig. 1b). While it is difficult to explain the discrepancy between this published work and ours, it might be due to differences in experimental design and/or to different sources or passage numbers of CRC cell lines used in both studies. Nonetheless, our results are consistent with the gene expression data retrieved from a GeneChip analysis of NCI-60 cancer cell lines from TSRI (The Scripps Research Institute; data are accessible at BioGPS: http://biogps.org), correlating reduced UVRAG<sup>WT</sup> expression in...
a subset of CRC cell lines with the UVRAG FS mutation. Taken as a whole, our findings and those of others suggest that inactivation of UVRAG is selected for during the progression of colorectal tumours, and that UVRAG WT plays a suppressor role in colorectal tumorigenesis.

Previous studies have indicated that autophagy protects genome integrity presumably by removing aged or damaged proteins and organelles. We observed a significant reduction of autophagy by UVRAG FS in CRC cells and primary tumours, which was even greater in the metastases. Of note, a previous study suggested that UVRAG WT lost Beclin1-binding activity due to the frameshift truncation. However, we found that even though UVRAG FS lost more than 50% of CCD of UVRAG WT, it still retains a small alpha-helix structure in the CCD and remains competent for UVRAG and Beclin1 interaction, thereby neutralizing their proautophagic effect in a dose-dependent manner. However, autophagy loss could not prevent the transformed phenotype induced by UVRAG FS, indicative of an autophagy-independent oncogenic mechanism associated with UVRAG WT as previously suggested.

We found that ectopic expression of UVRAG FS per se in both embryonic stem cells and cancer cells results in extensive centrosome amplification and concomitant aneuploidy. Indeed, this cancer-associated mutated UVRAG, which lacks CEP63-binding ability, is more than just a relic of UVRAG inactivation, it this cancer-associated mutated UVRAG, which lacks CEP63-binding ability, is more than just a relic of UVRAG inactivation, it

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For multichannel imaging, fluorescent staining was imaged sequentially in line-interface modes to eliminate crosstalk between the channels. The step size in the z axis varied from 0.2–0.5 mm to obtain 16 slices per imaged file. All experiments were independently repeated several times. The investigators conducted blind counting for quantification. Values indicate the mean ± s.d. of at least three independent experiments.

**Histopathology and immunohistochemistry.** Tissue sections from the indicated mouse models were fixed in 10% buffered formalin and embedded in paraffin. Tissue sections were routinely stained with haematoxylin and eosin. For immunohistochemistry staining, tissue slides were deparaffinized in xylene and rehydrated in alcohol. Endogenous peroxidase was blocked with 3% hydrogen peroxide. Antigen retrieval was achieved using a microwave and 10 mM citric acid buffer (pH 6.0). Sections were then incubated overnight at 4 °C with the primary antibody. Antibody binding was detected with Envision Dual Link System-HRP DAB kit (K4065, Dako). Sections were then counterstained with haematoxylin. For negative control, the primary antibody was replaced with the buffer. The mitotic index was quantified by viewing and photographing 10 random high-power-field of each tissue section on a Nikon microscope, using a 40 × objective. For evaluation and scoring of immunohistochemical data, we randomly selected 10 fields within the tumour area under high-power magnification (× 400) for evaluation. The investigators conducted blind counting for each quantification-related study.

**Immunoblotting and immunoprecipitation.** For immunoblotting, polyribodies were resolved by SDS–PAGE and transferred to a PVDF membrane (Bio-Rad). Membranes were blocked with 5% non-fat dry milk, and probed with the indicated antibodies. HRP-conjugated goat secondary antibodies were used (1:10,000, Invitrogen). Immunodetection was achieved with the Hyglo chemiluminescence reagent (Denville Scientific), and detected by a Fuji ECL machine (LAS-3000). For co-immunoprecipitation, cells were lysed in 1% NP40 lysing buffer (25 mM Tris pH 7.5, 300 mM NaCl, 1 mM EDTA, 1% NP40), supplemented with a complete protease inhibitor cocktail (Roche). After preclearing with protein A/G agarose beads for 1 h at 4 °C, whole-cell lysates were used for immunoprecipitation with the indicated antibodies. Generally, 1–4 μg commercial antibody was added to cell lysate, which was incubated at 4 °C for 8–12 h. After addition of protein A/G agarose beads, incubation was continued for another 2 h. Immunoprecipitates were extensively washed with NP40 lysis buffer and eluted with SDS–PAGE loading buffer by boiling for 5 min before resolution by SDS–PAGE.

**Soft agar anchorage-independent growth assay.** To evaluate anchorage-independent colony formation, engineered cells (10⁴) were suspended in complete medium containing 0.3% Noble agar (Difco) supplemented with 2 μg ml⁻¹ puromycin and plated in 6-well plates over a basal layer of 0.5% agar in complete medium. Colonies were scored 21 days after plating and were photographed by viewing and photographing 10 random fields within the tumour area under high-power magnification (× 400) for evaluation. The investigators conducted blind counting for each quantification-related study.

**Wound healing assay.** The cell invasive activity was determined using the wound healing assay. Briefly, cells (2.5 × 10⁵) plated in 12-well chamber were grown into a 100% confluent monolayer culture. The confluent monolayer was scratched with a pipette tip, followed by media replacement. After 24 h, the width of the mean wound distance was calculated using software connected to Nikon Eclipse digital inverted microscope. To evaluate the 'wound closure', 10 randomly selected points along each wound were marked, and the horizontal distance the migrating cells travelled into the wound was measured.

**Cell migration assays.** A Transwell system (Corning, NY, USA) was used to evaluate cell migration. The upper and lower chambers were separated by a polycarbonate membrane with pores of 8-μm coated with fibronectin (BD Biosciences, CA, USA) on the lower surface. Cells (2 × 10⁴) suspended in serum-free medium were seeded onto the upper chamber, and 800 μl of medium with 10% FBS was added to the lower chamber. Two days later, a 0.5% agarose was incubated with 5% CO₂. The medium was removed from the upper chamber. The non-invading cells on the upper side of the chamber were scraped off with a cotton swab. Cells on the bottom side of the membrane were fixed, stained with crystal violet and mounted. The migration activity of cancer cells was determined by counting cells in 10 different viewing fields using a microscope at × 200 magnification. Each assay was repeated three times.

**Clonogenic cell survival assay.** The log-phased cells were plated in six-well plates overnight, allowing cells to attach to the plates. After chemotherapy drug treatment (24 h exposure), cells were trypsinized, counted and replated at appropriate dilutions for colony formation. After 10–14 days of incubation, colonies were fixed with methanol/acetic acid (3:1), stained with crystal violet and counted. Plating efficiency was determined for each individual cell line and the surviving fraction (SF) was calculated based on the number of colonies that arose after treatment, expressed in terms of plating efficiency. Each experiment was repeated three times.

**In vivo tumorigenesis assay.** To measure in vivo tumorigenicity, engineered NIH3T3 and SW480 cells expressing WT or the mutant form of UVRAG (5 × 10⁶) were transplanted into the flanks of six-week-old female ncr nude mice (Charles River). Ten mice per cell line were used. Mice were monitored triweekly for the development of tumours, and necropsied after a 3-week observation period. The tumour growth was monitored by measurements of tumour length (L) and width (W) and tumour volume was calculated using the following formula: Volume = (L × W²)/2. All animal studies were performed in compliance with the University of Southern California Institutional Animal Care and Use Committee guidelines.

**In vivo metastasis assay.** A midline incision was made on the left flank, and the spleen was exteriorized. SW480 cells (10⁵) were injected into the spleen, after which the wound was closed with surgical metal clips. The mice were sacrificed after 8 weeks, and their spleen, liver, lungs and lymph nodes were removed and examined for tumour metastases. The organs specimens were formalin-fixed and paraformaldehyde-insulted for histology analysis. Alternatively, GFP-labelled cells can be tracked using bioluminescence imaging at the end of experiment. Briefly, mice were placed in the induction chamber with 2% isoflurane in oxygen. GFP activity was quantified using an IVIS 200 image system. Images were taken with an emission wavelength of 465 and emission wavelength ranging from 500 to 540. Imaging processing and analysis, including flat fielding, adaptive background subtraction and spectral unmixing were performed with Living Image 3.0 software.

**Autophagy analyses.** Quantitative GFP–LC3 light microscopy assay was performed in NIH3T3 cells expressing the WT or FS mutant of UVRAG, then transfected with a GFP–LC3-expressing plasmid. Autophagy was then induced by puromycin and plated in 6-well plates over a basal layer of 0.5% agar in complete medium containing 100 μM Bafomycin A₁ for 2 h. LC3 mobility shift and levels were detected by immunoblotting.

**Neutral comet assay.** Neutral comet assay was performed using the CometAssay kit ( Trevigen) following the manufacturer's instruction. Briefly, 10 μl of cell suspension (10⁵ cells/ml) was carefully mixed with 90 μl of molten LMAgarose. After solidification, slides were immersed in Lysis Solution at 4 °C for 1 h, and equilibrated in chilled neutral electrophoresis buffer for 30 min. Electrophoresis was performed in neutral electrophoresis buffer for 1 h with an electric field of 1 volt cm⁻¹. Slides were further treated with DNA Precipitation Solution, followed by 70% ethanol for 30 min each at room temperature. After air drying, cells were stained with SYBR Green (1 μg ml⁻¹) or Propidium Iodide (1 μg ml⁻¹). Comet images were captured using an epifluorescence microscope (Nikon Eclipse C1). To analyse the images, cells were scored into three categories based on tail length (no tails, tail length shorter than 20 μm, tail length longer than 20 μm), and quantified.

**Laser microirradiation.** Laser microirradiation was done essentially as described before19. Cells grown on coverslips were incubated for 24 h in medium containing 10 μM BrdU (Sigma-Aldrich). Laser microirradiation was carried out with a Nikon C1 confocal microscope (Nikon) equipped with a 37 °C CO₂ chamber and a diode laser (Melles Gritol). DSBs restricted to the laser path were generated across the nuclei in 50 cells per coverslip, using the 100 × oil objective and 30% of laser power (λ = 405 nm) for 100 scans. Cells were then returned to tissue culture incubator at 37 °C, fixed 1 h later and analysed by flow cytometry. UVRAG expression was verified by western blotting. The repair activity of DSB generated by I-Cre recombinase was measured by GFP–LacI reporter (pCRTAsce), together with pmCherry as a transfection indicator. Cells were collected after another 48 h, and analysed by standard flow cytometry. UVRAG expression was verified by western blotting. The repair activity of DSB generated by I-Cre was calculated by the percentage of GFP-positive (repaired) cells in the mCherry-positive cells (transfected).

**Chromosomal analysis by SKY.** SKY analysis of embryonic stem cells was performed. Briefly, metaphase chromosome was prepared from exponentially growing cells after treatment with colcemid (KaryoMAX, GIBCO) at 0.1 μg ml⁻¹ for 1 h (ref. 31). Cells were swollen in prewarmed 0.56% KCl for 10 min at 37 °C, then carefully fixed in methanol/acetic acid (3:1) overnight and kept at −20 °C. Metaphase spreads were prepared by dropping cells in the fixative onto chilled slides (Fisher Scientific) at 25°C and 60% of humidity. After air drying and pepsin digestion, slides were denatured at 80°C for 5 min, hybridization was performed using SKY probe (Applied Spectral Imaging, San Diego) and
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Author contributions

S.H. performed most experiments of this study, analysed the data, and conducted bioinformatics analysis. Z.Z. conducted DNA damage repair analyses. X.Z., S.O., B.M. and J.L. contributed to the oncogenesis assays. B.V. performed live animal imaging acquisition, J.Y., Y.Y., D.C., S.P., T.Z., S.P., M.L., Y.Z., G.L. and S.M. participated in the data and sample collection. K.M. helped with the tumour metastasis study. C.L. designed and analysed the experiments, and wrote the manuscript.

Additional information

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

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