Investigating the Impacts Reduced *USP4* has on Chromosome Instability and its Implications in Colorectal Cancer

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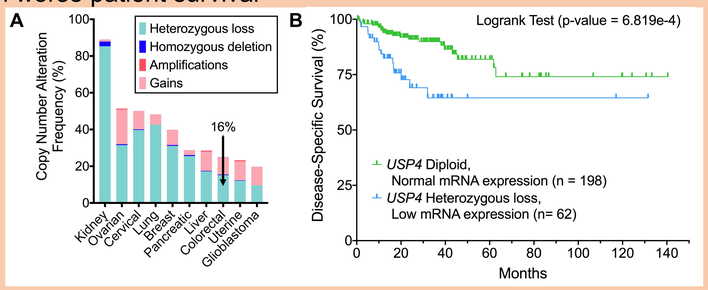
Introduction and Rationale

Colorectal cancer is one of the leading cancers diagnosed and one of the deadliest cancers in Canada – accounting for ~12% of new cancer cases and causing 9,700 deaths in 2020[1]. With these devastating statistics, it is imperative to further our understanding of the underlying genes, proteins and pathways driving cancer development and progression. Chromosome instability (CIN) is a form of genome instability, defined as an increase in the rate at which whole chromosomes or chromosome fragments are gained or lost[(2)]. CIN is associated with aggressive tumours, metastasis and poor patient prognoses, driving cancer development and progression in ~85% of colorectal cancers[(2,3)], yet the molecular alterations driving CIN remain largely unknown.

Recent findings from the McManus laboratory suggest reduced expression of genes regulating ubiquitin dynamics may be key drivers of CIN. Preliminary data from an siRNA-based screen of ~700 ubiquitination/deubiquitination genes identified *USP4* (Ubiquitin-specific peptidase 4) as a putative driver of CIN. *USP4* encodes a deubiquitinating enzyme involved in various cellular functions, including Wnt/***β***-catenin signalling, DNA damage repair, chromosome segregation and mRNA splicing[4,5]**.** Clinical patient data highlight the clinical relevance of *USP4* in colorectal cancer, as it is heterozygously lost in ~16% of colorectal cancers and is associated with worse patient outcomes (**Figure 1**)[6]. Here, we investigated the impact reduced *USP4* expression has on the development of CIN and colorectal cancer through assessment of CIN phenotypes in two non-malignant colonic epithelial cell lines, 1CT and 1CT-derived, A1309.

Figure 1. *USP4* is frequently lost in colorectal cancer and associated with worse patient survival



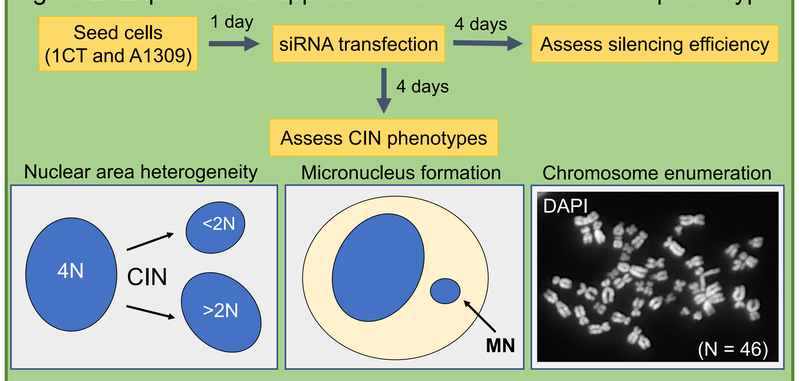
A. Bar graph showing *USP4* gene copy number alteration frequencies in 10 common cancer types, from TCGA patient data (PanCancer Atlas)[6]**.** *USP4* is heterozygously lost in 16% of colorectal cancers (shown with arrow) **B.** Kaplan-Meier curve of disease-specific survival in colorectal cancer shows significantly worse disease-specific survival for patients with *USP4* heterozygous loss, relative to diploid cases.

Hypothesis

We hypothesize that diminished *USP4* expression will induce chromosome instability that promotes colorectal cancer development and pathogenesis.

Materials and Methods

Figure 2. Experimental approaches used to evaluate CIN phenotypes



General workflow of experiments employed to investigate the role of reduced *USP4* expression in the development of CIN in two non-malignant, karyotypically stable human colonic epithelial cell lines 1CT and A1309 (1CT-derived). A1309 harbors protein alterations in KRAS (p.G12V), TP53 (wild-type, 50% knockdown), APC (truncation at codon 1309, 70% knockdown). Once effective silencing is established, nuclear areas, micronucleus (MN) formation, and chromosome spread assays were conducted.

Results

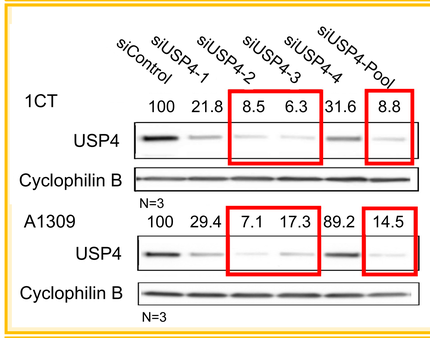
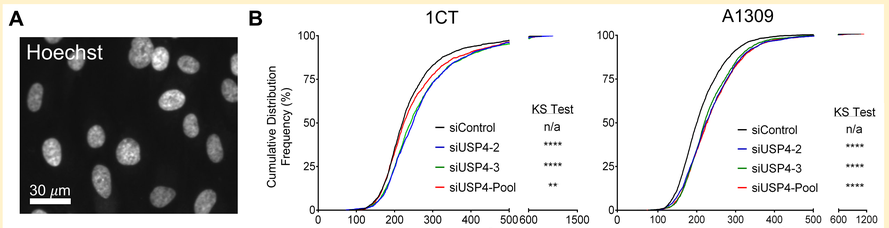


Figure 3. USP4 protein expression is effectively reduced following siRNA-based silencing

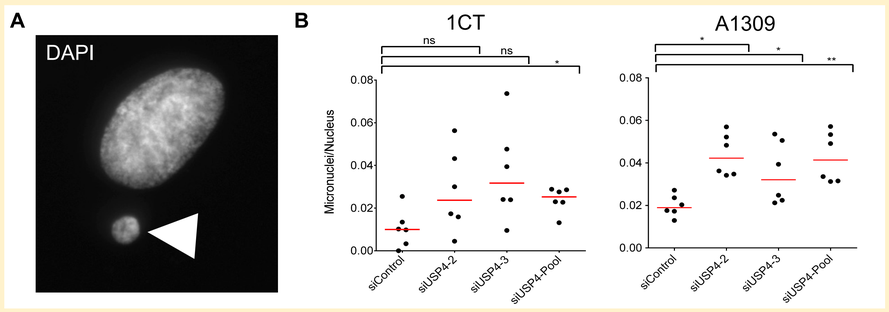
Western blots showing USP4 protein expression levels 4-days post-transfection of four individual siRNAs (Dharmacon, siON-TARGET*plus*) along with the pool (all four individual siRNAs) in 1CT (top) and A1309 (bottom), relative to non-targeting control (siControl), (N=3). The top two most effective individual siRNAs (siUSP4-2 and -3) along with the pool were employed in subsequent experiments.

Figure 4. Diminished *USP4* expression induces changes in nuclear areas



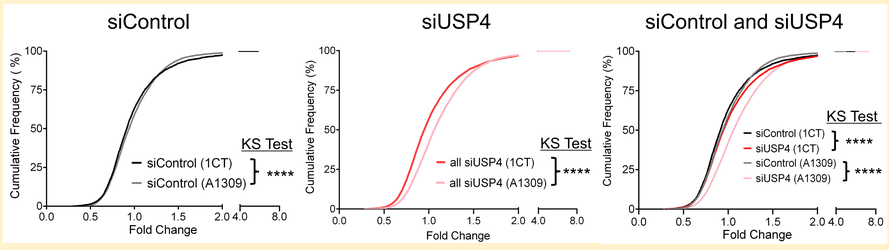
A Micrograph of Hoechst-counterstained nuclei (1CT). **B** Cumulative distribution frequency plots identify significant increases in nuclear area heterogeneity following *USP4*-silencing relative to control. Two-sample Kolmogorov-Smirnov (KS) tests for significance: p-value <0.01(**), <0.0001(****), n/a (not applicable); [N=3, n>100].

Figure 5. Micronucleus formation is increased following *USP4* silencing



A Micrograph of DAPI-counterstained nucleus (1CT) with adjacent micronucleus (shown with white arrowhead). **B** Dot plots show increases in micronucleus formation following *USP4* silencing in 1CT (left) and A1309 (right), compared to control (red bar = medians). Mann-Whitney tests for significance: p-value >0.05 (ns), <0.05 (*), <0.01(**), <0.0001(****), n/a (not applicable); [N=3, n >100].

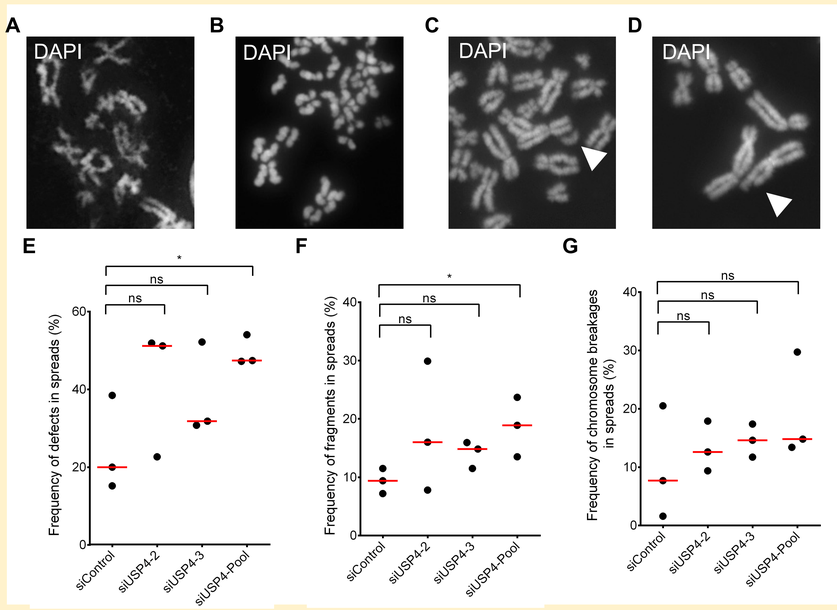
Figure 6. *USP4* silencing synergizes with genetic defects in A1309 to exacerbate increases in nuclear area distributions.



USP4-silenced conditions demonstrate a significant rightward shift in nuclear area distributions compared to control, with exacerbated increases in nuclear areas in A1309 than in 1CT. Cumulative distribution frequency graphs comparing fold change in nuclear areas for siControl (left) – black (1CT) and gray (A1309); siUSP4-2, -3 and -Pool combined – red (1CT) and pink (A1309); and all together. Nuclear areas were normalized to the mean its respective replicate control and statistically compared using two-sample KS tests for significance: p-value <0.0001(****); [N=3].

Results

Figure 7. USP4 silencing induces aberrant chromosome phenotypes



USP4 silencing is associated with increases in aberrant chromosome mitotic spreads in 1CT, compared to control. Example images of monitored aberrant chromosome phenotypes: **(A)** compaction defect, (**B**) cohesion defect, **(C)** fragments (white arrowhead), and **(D)** breakages (white arrowhead). Dot plots showing percentage of chromosome spreads with defects: **(E)** overall –fragments, breakages, and compaction and cohesion defects; (**F**) fragments**;** (**G**) breakages, (N=3, n>60). Experimental conditions were statistically compared to control using Mann-Whitney tests for significance: p-value >0.05 (ns), <0.05 (*); [N=3, n>69]

Conclusions and Significance

- Diminished *USP4* expression induces CIN in 1CT and A1309, with increases in nuclear area heterogeneity, increases in micronucleus formation, and trending increases in aberrant chromosome phenotypes.
- CIN phenotypes in A1309 compared to 1CT, namely more extreme shifts in nuclear area distributions (increases in nuclear area heterogeneity suggest large-scale chromosome gains/losses) highlight potential synergy between diminished *USP4* and alterations associated with colorectal cancer development (KRAS, TP53, and APC).
- These findings identify *USP4* as a driver of CIN in a colonic epithelial context, which may have implications in colorectal cancer development and pathogenesis

Future Directions

- Generation and validation of *USP4* gene knockouts in 1CT and A1309
- Assess impacts on chromosome numbers, dynamics of CIN over time
- Roles in development of cancer-associated characteristics (cellular transformation)

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