**INTRODUCTION**

Anaplastic lymphoma kinase-positive anaplastic large cell lymphoma (ALK+ALCL) is a specific type of T/null-cell non-Hodgkin's lymphoma recognized by the World Health Organization Classification Scheme for hematologic malignancies. Most of these tumors are characterized by the expression of the oncogenic fusion protein NPM-ALK, which mediates tumorigenesis by exerting its constitutive tyrosine kinase activity on various substrates. We recently identified MSH2, a protein central to DNA mismatch repair (MMR), as a novel binding partner and phosphorylation substrate of NPM-ALK. Here, using liquid chromatography–mass spectrometry, we report for the first time that MSH2 is phosphorylated by NPM-ALK at a specific residue, tyrosine 238. Using GP293 cells transfected with NPM-ALK, we confirmed that the MSH2 Y238F mutant is not tyrosine phosphorylated. Furthermore, transfection of MSH2 Y238F into these cells substantially decreased the tyrosine phosphorylation of endogenous MSH2. Importantly, gene transfection of MSH2 Y238F abrogated the binding of NPM-ALK with endogenous MSH2, re-established the dimerization of MSH2:MSH6 and restored the sensitivity to DNA mismatch-inducing drugs, indicative of MMR return. Parallel findings were observed in two ALK+ALCL cell lines, Karpas 299 and SUP-M2. In addition, we found that enforced expression of MSH2 Y238F into ALK+ALCL cells alone was sufficient to induce spontaneous apoptosis. In conclusion, our findings have identified NPM-ALK-induced phosphorylation of MSH2 at Y238 as a crucial event in suppressing MMR. Our studies have provided novel insights into the mechanism by which oncogenic tyrosine kinases disrupt MMR.

**MATERIALS AND METHODS**

Cell lines, cell culture and gene transfection

GP293 cells were maintained as previously described. Tet-on HEK293/NPM-ALK cells and ALK+ALCL cell Karpas 299 and SUP-M2 were cultured as previously described. Tet-on HEK293 cells were
purchased from Clontech Laboratories (Mountainview, CA, USA) and Karpas 299 and SUP-M2 cells were purchased from the ATCC (Manassas, VA, USA) and were recently tested for mycoplasma infection. GP293 cells were transiently transfected with expression vectors using Lipofectamine 2000 (Invitrogen, Life Technologies, Grand Island, NY, USA) according to the manufacturer’s protocol.

Gene expression vectors and site-directed mutagenesis
His-biotin (HB)-tagged MSH2 was constructed by cloning MSH2 from pcDNA3-MSH217 (a gift from Dr Meuth) into the HB-tagged vector described previously. Site-directed mutagenesis of tyrosine (Y) 238 of MSH2 to phenylalanine (F; MSH2 Y238F) was performed using the QuikChange Site Directed Mutagenesis Kit (Agilent Technologies, Mississauga, ON, Canada). The NPM-ALK expression vector was a gift from Dr Morris.16

Tandem affinity purification under denaturing conditions, on-bead protein digestion and liquid chromatography–mass spectrometry
Tandem affinity purification and liquid chromatography–mass spectrometry under denaturing conditions using GP293 cells transfected with HB-MSH2 and NPM-ALK were performed as previously described. Mass spectrometry results were confirmed using a biological replicate.18,19

Generation of Tet-on ALK+ALCL MSH2/MSH2Y238F cell lines
Tet-on ALK+ALCL MSH2Y238F cell lines were generated as outlined in the Supplementary Methods.

Immunoprecipitation, His-based protein purification and western blotting
All communoprecipitation and immunoprecipitation experiments were performed as previously described using 1000 μg of lysate in Cell Lytic M (Sigma Aldrich, Oakville, Ontario, Canada). No-antibody controls were included, although not always shown. Biotin-based protein purification was performed as previously described using 500 μg of lysate in RIPA buffer.18 These experiments were repeated at least three times. The following antibodies were used: anti-MSH2 (EMD, Billerica, MA, USA, IP/co-IP, catalog number NA27), anti-MSH6 (BD Biosciences, San Jose, CA, USA, catalog number 610919), anti-MSH2, anti-phospho-tyrosine, anti-ALK, anti-total NPM-ALK, anti-His, anti-β-actin (Santa Cruz Biotechnology, Dallas, TX, USA; sc-47778).

Functional assay for MMR: β-galactosidase reporter plasmid
Tet-on HEK293/NPM-ALK cells were transfected with the pcAR-OF β-galactosidase reporter plasmid,20 treated with doxycycline (DOX, Sigma Aldrich) and analyzed for β-galactosidase production, as previously described.4 Samples were read in sextuplicate, and the experiment was repeated three times.

Functional assay for MMR: sensitivity to DNA-damage-inducing drugs
GP293 cells were transfected with HB-EV, or HB-MSH2Y238F and NPM-ALK, for 24 h, and then they were plated into 48-well plates. Twenty-four hours later, cells were treated with 0, 0.5, 1, 2, 3 or 4 μM N-methyl-N-nitrosourea (MNNU) for 48 h, and cell viability was assessed by MTS (Promega, Madison, WI, USA). Tet-on SUP-M2 MSH2Y238F cells were treated with 0 or 500 ng/ml DOX for 48 h, followed by the addition of 0 or 100 μM MNNU or 5 μg/ml 5-fluorouracil (5-FU). After 24 h, cell viability was assessed, and then re-measured every 2 days. Samples were analyzed in triplicate and the experiment was repeated three times.

Apoptosis analysis
Apoptosis analysis was performed on Tet-on SUP-M2 and Karpas 299 MSH2Y238F cells treated with 0, 100 and 500 ng/ml DOX, and stained after 48 h with Annexin-V-FITC/PI (BD Biosciences) according to the suggested protocol. Analysis was performed on a BD FACs Calibur (Flow Cytometry Laboratory, Department of Experimental Oncology, Cross Cancer Institute).

Cell cycle analysis was performed using propidium iodide staining followed by flow cytometry, as previously described21 on Tet-on SUP-M2 MSH2Y238F cells treated with 0 and 500 ng/ml DOX for 48 and 120 h, respectively. Samples were analyzed in triplicate, and the experiment was repeated three times.

Statistical analysis
Data are expressed as mean ± s.e.m., and significance was determined from three independent experiments by Student’s t-test (GraphPad Prism, La Jolla, CA, USA). Sample size was chosen to give adequate power to the statistical calculations. No sample results were excluded from calculations.

RESULTS
Identification of Y238 of MSH2 as the crucial site for NPM-ALK-induced p-Y
Using NetPhos 2.0,22 we analyzed the MSH2 protein sequence for potential p-Y sites that may be targeted by NPM-ALK. We identified 13 putative sites in the MSH2 protein (Supplementary Figure 1 and Supplementary Table 1). The tyrosine (Y) residue with the highest score was Y238 (0.981, * in Supplementary Figure 1), located in the connector domain required for the formation of the MMR ternary complex.

To identify the specific residue(s) phosphorylated in the presence of NPM-ALK, tandem-affinity purification was performed. To achieve this, we used GP293 cells transiently transfected with both HB-tagged MSH2 and NPM-ALK. Using these cell lysates, we performed liquid chromatography coupled with tandem-affinity purification mass spectrometry. With the total peptide sequence coverage of MSH2 being 78%, the only detectable phosphorylated residue in the presence of NPM-ALK was Y238 (Supplementary Figure 2). The same experiment was done on GP293 lysate expressing HB-MSH2 and pcDNA3, and no evidence of phosphorylation was detected.

NPM-ALK mediates p-Y of MSH2 at tyrosine 238
To examine the functional significance of phosphorylation of MSH2Y238F, site-directed mutagenesis was performed on the HB-MSH2 plasmid to change the residue to F, a residue biochemically similar to Y that cannot be phosphorylated. HB-empty vector (HB-EV), HB-MSH2 or HB-MSH2Y238F was transfected into GP293 cells with or without NPM-ALK expression (Figure 1a). Immunoprecipitation using an anti-MSH2 monoclonal antibody was then performed on lysate depicted in Figure 1a. In the absence of NPM-ALK (Figure 1b, lanes 1–3), there was no convincing evidence of tyrosine phosphorylation of endogenous MSH2, HB-MSH2 or HB-MSH2Y238F, compared with the EV control. In the presence of NPM-ALK (Figure 1b, lanes 4–7), phosphorylation of endogenous MSH2 was readily detectable. Importantly, the phosphorylation of endogenous MSH2 in the presence of HB-MSH2Y238F was substantially (>60%) reduced (lane 7), suggesting that the MSH2Y238F mutant exerts a dominant negative effect on endogenous MSH2 p-Y.

Rather surprisingly, phosphorylation of the HB-tagged MSH2 proteins was not detectable in this experiment. However, as shown in Figure 1c, when streptavidin agarose resin was used to pull down the exogenous HB-MSH2 or HB-MSH2Y238F, NPM-ALK mediated p-Y of HB-MSH2 (lane 5), whereas there was only a barely detectable signal for HB-MSH2Y238F (lane 6). The discrepancy between Figures 1b and 1c regarding the phosphorylation of HB-MSH2 is likely owing to the fact that the anti-phospho-tyrosine antibody does not recognize its epitope on HB-MSH2 when it was pulled down by an anti-MSH2 antibody.

Taken together, these results suggest that NPM-ALK phosphorylated at MSH2Y238F. Furthermore, as site-directed mutagenesis of Y238 almost completely abrogated the p-Y signal induced by NPM-ALK, Y238 is the predominant p-Y site on MSH2 in this context. In conclusion, MSH2Y238F appears to have a dominant
previously published findings that overexpression of MMR proteins can lead to deregulation of MMR, mimicking what is seen in cells lacking certain MMR genes.\textsuperscript{20,23,24}

With the induction of NPM-ALK (that is, 400 ng \textmu g of DOX), we identified a significant increase in \(\beta\)-galactosidase, which is in concordance with our previous findings.\textsuperscript{4} Enforced expression of HB-MSH2 resulted in a further increase in \(\beta\)-galactosidase activity over cells expressing HB-EV (\(P < 0.01\)) (Figure 2a, 400 DOX). In contrast, enforced expression of HB-MSH2\textsuperscript{238F} did not significantly alter the \(\beta\)-galactosidase activity compared with cells expressing HB-EV (Figure 2b, 400 DOX). These findings strongly suggest that MSH2\textsuperscript{238F} has a substantial biological difference from MSH2 in the presence of NPM-ALK.

To further assess whether enforced expression of HB-MSH2\textsuperscript{238F} has the ability to neutralize the MMR-deregulating effect of NPM-ALK, we transfected HB-EV or HB-MSH2\textsuperscript{238F} into GP293 cells with or without the co-transfection of NPM-ALK. These cells were subsequently treated with MNU to assess cell viability. Of note, MNU, a mono-functional methylating agent that induces aberrant bases, is widely used to assess MMR in vitro.\textsuperscript{25} MNU generates DNA-damage-induced apoptosis that is MMR-dependent. Thus, in the absence of MMR, cells are resistant to MNU treatment.\textsuperscript{26} As shown in Figure 2c, in GP293 cells transfected with HB-EV, cells co-transfected with NPM-ALK showed a significantly higher cell viability than those co-transfected with EV in the presence of 1–3 m\(\textmu\) MNU (\(P < 0.05, 1–2 \text{ m}\mu\text{M}; P < 0.01, 3 \text{ m}\mu\text{M})). In contrast, in cells transfected with HB-MSH2\textsuperscript{238F}, the co-transfection of NPM-ALK did not result in any significant difference in cell viability, as compared with those co-transfected with the EV (Figure 2d). Thus, the HB-MSH2\textsuperscript{238F} mutant appeared to have neutralized the MMR-deregulating effect of NPM-ALK.

Enforced expression of HB-MSH2\textsuperscript{238F} alters MSH2:MSH6 (MutS\(\alpha\)) binding

Previously published data from our group demonstrated that NPM-ALK expression blocked the interaction of MSH6 and MSH2,\textsuperscript{4} which is critical for MMR. We hypothesized that p-Y of MSH2\textsuperscript{238} is involved in this process. Thus, we performed coimmunoprecipitation experiments using GP293 cells transiently transfected with HB-EV or HB-MSH2\textsuperscript{238F}. The cell lysate used for this experiment was initially subjected to western blotting, and the expression of endogenous and HB-tagged exogenous MSH2 proteins was confirmed (Figure 3a, lanes 1 and 2). Another set of cell lysates was similarly generated, except with the co-transfection of NPM-ALK (Figure 3a, lanes 3 and 4).

Using these two sets of cell lysates, the physical binding between MSH2 and MSH6 was assessed using coimmunoprecipitation with an anti-MSH6 antibody. In the context of MMR, MSH2 has multiple binding partners,\textsuperscript{27–29} whereas MSH6 only interacts with MSH2.\textsuperscript{27} Thus, using an anti-MSH6 antibody (rather than anti-MSH2) for the pull-down is more informative. As shown in Figure 3b, we only detected endogenous MSH2 bound to HB-MSH2\textsuperscript{238F}. The cell lysate used for this experiment was initially subjected to western blotting, and the expression of endogenous and HB-tagged exogenous MSH2 proteins was confirmed (Figure 3a, lanes 1 and 2). Another set of cell lysates was similarly generated, except with the co-transfection of NPM-ALK (Figure 3a, lanes 3 and 4).

Enforced expression of HB-MSH2\textsuperscript{238F} restores MMR in vitro

To determine whether Y238 is biologically important in the context of MMR, we expressed HB-EV, HB-MSH2 and HB-MSH2\textsuperscript{238F} in HEK293 cells in which the expression of NPM-ALK is DOX-inducible.\textsuperscript{4} To assess MMR, cells were transiently transfected with the MSH2 gene in frame, resulting in measurable enzymatic activity.\textsuperscript{25} β-actin was used as a control for equal protein loading. As shown in Figure 4a, enforced expression of HB-MSH2 or HB-MSH2\textsuperscript{238F} led to a statistically significant increase (\(P < 0.001\) and \(P < 0.01\)) in \(\beta\)-galactosidase activity over cells transfected with HB-EV (Figures 2a and b). This suggests that overexpression of these MSH2 proteins alone can impair MMR, which substantiates
Enforced expression of HB-MSH2\textsuperscript{Y238F} alters MSH2-NPM-ALK binding

Using the cell lysate depicted in Figure 3a, we assessed how MSH2\textsuperscript{Y238F} may affect the interaction of endogenous MSH2 with NPM-ALK. As shown in Figure 3c, when MSH2 was pulled down with an anti-MSH2 antibody, the interaction between NPM-ALK and endogenous MSH2 was readily detectable, as previously described.\textsuperscript{4,18} In contrast, the MSH2-NPM-ALK interaction was not detectable by immunoprecipitation when HB-MSH2\textsuperscript{Y238F} was expressed (lane 4). When pull-down of HB-MSH2\textsuperscript{Y238F} was done by biotin pull-down assay, we readily identified an interaction between NPM-ALK and HB-MSH2\textsuperscript{Y238F} (Figure 3d, lane 4). Taken together, these findings suggest that HB-MSH2\textsuperscript{Y238F} blocks the interaction between NPM-ALK and endogenous MSH2, possibly by binding to NPM-ALK and sequestering it away from endogenous MSH2.

Enforced expression of MSH2\textsuperscript{Y238F} decreases MSH2 p-Y and restores MSH2:MSH6 (MutS\textalpha) formation in ALK+ALCL cells

The biological importance of MSH2\textsuperscript{Y238F} phosphorylation in the context of NPM-ALK pathobiology was next validated in ALK+ALCL cell lines, which express NPM-ALK at the steady state. Using retroviral vectors, Karpas 299 and SUP-M2 cells stably transduced with an inducible MSH2\textsuperscript{Y238F} vector were generated, and were termed Tet-on MSH2\textsuperscript{Y238F} cells. The Tet-on SUP-M2 and Karpas 299 MSH2\textsuperscript{Y238F} cells showed a dose-dependent increase in the expression of total MSH2 protein upon DOX treatment (Figures 4a and b). Of note, both Karpas 299 and SUP-M2 have endogenous MSH2 expression, as evidenced at 0ng/ml DOX.

Using these Tet-on MSH2\textsuperscript{Y238F} cells, MSH2\textsuperscript{Y238F} expression was turned on at a low level (resulting in ~40% increase in the total MSH2 protein level) and MSH2 phosphorylation was assessed by immunoprecipitation. The expression of MSH2\textsuperscript{Y238F} in SUP-M2 cells resulted in an ~70% reduction in detectable p-Y (Figure 4c). Similar results were achieved in Karpas 299 cells, in which MSH2\textsuperscript{Y238F} decreased MSH2 phosphorylation by 50% (Figure 4d).

As the expression of MSH2\textsuperscript{Y238F} resulted in a decrease in detectable MSH2 p-Y, we then asked whether MSH2\textsuperscript{Y238F} was capable of restoring the interaction of MSH2:MSH6, which would indicate a partial restoration of MMR in these cells. As measured by co-IP using an anti-MSH2 antibody, expression of MSH2\textsuperscript{Y238F} in the Tet-on SUP-M2 cells (Figure 4e) and Karpas 299 cells (Figure 4f) resulted in a dose-dependent increase in the amount of MSH6 bound to MSH2.

ALK+ALCL cells are more sensitive to DNA-damage-inducing drugs upon enforced expression of MSH2\textsuperscript{Y238F}

As our previous results have shown that MSH2\textsuperscript{Y238F} is capable of restoring MSH2:MSH6 formation in ALK+ALCL, we then asked whether cells expressing MSH2\textsuperscript{Y238F} also have restored MMR function. Owing to technical reasons, we were unable to use the pCAR-OF plasmid described above, as it would have involved multiple gene transfection into ALK+ALCL cells, which significantly decreased cell viability (not shown). Instead, we assessed MMR indirectly by treating these cells with MNU. In addition, we also assessed MMR by treating these cells with the thymidylate synthase inhibitor 5-FU, as resistance to 5-FU has been associated with loss of MMR.\textsuperscript{35} As shown in Figures 5a and b, experiments using both MNU and 5-FU showed similar results. The expression...
of MSH2Y238F (induced by the addition of 500 ng/ml of DOX) in the presence of the DNA-damaging drugs resulted in a significant increase in loss of viability from day 3 onward. These findings support the concept that MSH2Y238F restores MMR in ALK+ALCL cells. Interestingly, expression of MSH2Y238F alone, without the addition of MN1 or 5-FU, resulted in a significant loss of viability (Figures 5c, P < 0.001 until day 7). The decrease in statistical significance on day 9 can be attributed to the fact that only one dose of DOX was given at the beginning of the experiment, and the expression of MSH2Y238F slowly faded away.

Enforced expression of MSH2Y238F induces spontaneous apoptosis in ALK+ALCL cells

As our results depicted in Figure 5 suggest that expression of MSH2Y238F alone can decrease cell viability of ALK+ALCL cells, we performed studies to analyze changes in the cell cycle and apoptosis induced by MSH2Y238F. By propidium iodide staining, at 48 and 120 h after MSH2Y238F expression, there was a significant increase in the fraction of apoptotic cells in the Tet-on SUP-M2 MSH2Y238F cell line (Supplementary Figure 3). The average percentage of cells in each stage of cell cycle is shown in Supplementary Table 2 (n = 3). At 48 and 120 h post MSH2Y238F expression, the increase in the percentage of cells in the apoptotic/SubG1 phase was statistically significant (P < 0.01).

Tet-on SUP-M2 and Karpas 299 MSH2Y238F cells were subjected to Annexin V/propidium iodide staining to determine whether the expression of MSH2Y238F resulted in a change in cells undergoing early- or late-stage apoptosis. As shown in Figure 6a, increasing expression of MSH2Y238F resulted in a dose-dependent increase in the fraction of cells undergoing both early (lower right quadrant) and late (upper right quadrant) apoptosis.

A graphical representation of the total apoptotic Tet-on SUP-M2 MSH2Y238F cells is shown in Figure 6b (n = 3); when the percentages of early, late and dead cells were added together (that is, total apoptotic cells), a significant increase in apoptosis was measured in cells treated with 100 ng/ml DOX (P < 0.05). In the Tet-on Karpas 299 MSH2Y238F cells, a significant dose-dependent increase in the percentage of total apoptotic cells was detectable in cells treated with both 100 and 500 ng/ml DOX (P < 0.05) (Figure 6c). Apoptosis in the Tet-on SUP-M2 MSH2Y238F cells was confirmed by western blotting for cleaved caspase 3 expression (Figure 6d).

**DISCUSSION**

The finding that oncogenic tyrosine kinases can impair MMR is relatively recent, and has not been extensively studied. In 2008, it was described that BCR-ABL, an oncogenic fusion tyrosine kinase, can induce MSI, but the underlying mechanism was not investigated. In a previous publication from our laboratory, we found that the oncogenic tyrosine kinase NPM-ALK induced the phosphorylation of MSH2 at unknown tyrosine residues, leading to MMR dysfunction. We also reported that ALK+ALCL cell lines and patient samples exhibited MSI and abnormal MSH2 cytoplasmic localization, which are both evidence of MMR deficiency. In this study, we tested the hypothesis that p-Y of MSH2 is a critical step in the deregulation of MMR by NPM-ALK. First, using liquid chromatography–mass spectrometry, we report for the first time that Y238 is a site that can be tyrosine phosphorylated by

![Figure 3](image-url) Enforced expression of HB-MSH2Y238F alters MutSα (MSH2:MSH6) formation and the MSH2-NPM-ALK interaction. (a) Western blot analysis of GP293 cell lysate transfected with HB-EV or HB-MSH2Y238F and pcDNA3 (lanes 1–2) or NPM-ALK (lanes 3–4). The western blot was probed with anti-MSH6 and anti-ALK antibodies. Anti-MSH2 antibody was used to detect endogenous MSH2 (lower band) and HB-MSH2/MSH2Y238F expression levels (upper band, HB). Anti-β-actin was used as a loading control. All panels are from the same western blot. (b) MSH6 was precipitated from cell lysate shown in a under co-IP conditions using an anti-MSH6 antibody. The resulting immunoblot was probed with MSH2 and MSH6 antibodies to measure the MSH2/MSH6 (MutSα) interaction. Densitometry shows the intensity of endogenous MSH2 normalized to MSH6, relative to lane 1. No exogenous MSH2 protein was detected by co-IP. All panels are from the same western blot. (c) MSH2 was precipitated from cell lysate a under co-IP conditions using an anti-MSH2 antibody, and the resulting immunoblot was probed with anti-ALK and anti-MSH2 to detect the interaction of MSH2 (endogenous and HB-tagged):NPM-ALK. All panels are from the same western blot. (d) Biotin purification of HB-MSH2Y238F from a, probed with anti-ALK and anti-MSH2 to measure the HB-MSH2Y238F-NPM-ALK interaction. All panels are from the same western blot, and a representative result (from the same experiment) of three independent experiments is shown.
this defect was restored by MSH2 Y238F. Second, in the presence of NPM-ALK, binding was disrupted in the presence of NPM-ALK, 4 and needs further delineation, we believe that results from this study support the concept that p-Y of MSH2 Y238 is a critical step by which NPM-ALK regulates MMR. Taken together, reduced the binding between NPM-ALK and endogenous MSH2, although the mutant itself can bind to NPM-ALK. Taken together, the most likely scenario we have considered is that MSH2 Y238F restores MMR by sequestering NPM-ALK away from the endogenous MSH2 protein, leading to a substantial increase in the availability of endogenous MSH2:MSH6 heterodimers. This is demonstrated in a model shown in Figure 7, consistent with the observation that MSH2 Y238F exerts a dominant negative effect on NPM-ALK-mediated p-Y of endogenous MSH2.

The finding of phosphorylation of MSH2 at tyrosine 238 directly affecting its MMR function is highly novel. Y238 is located in the connector domain of the MSH2 protein, which allows for the intramolecular interactions of MutSα with downstream proteins. 3,4 In the context of MMR function, a number of clinically relevant missense mutations have been identified in the connector domain of the MSH2 protein in Lynch Syndrome patients, leading to loss of MMR and MSH2 protein expression through a decrease in MSH2 protein stability. 3,5,6 Thus, phosphorylation of the connector domain of the MSH2 protein could significantly affect its ability to bind downstream proteins required to initiate the MMR signaling cascade.

To further understand the significance of p-Y of MSH2, we assessed the effect of the MSH2 Y238F mutant on the sensitivity to DNA-damaging agents in ALK+ALCL cells, specifically MNU and 5-FU, which induce DNA damage processed directly and indirectly to bind downstream proteins required to initiate the MMR signaling cascade. In the context of MMR, MNU treatment creates O6-methylguanine lesions in the DNA that are mispaired with thymine (T), and the resulting O6-methylguanine:T mismatch is detected by MMR and MutSα. Through the consequent repair process, a T is reinserted opposite the O6-methylguanine, reinitiating a futile MMR cycle resulting in double-stranded DNA breaks, G2 arrest and cell death. 26 MMR processing after 5-FU treatment is similar to that of 5-FU:Guanine lesions detected by MutSα.

Figure 4. Enforced expression of MSH2Y238F in ALK+ALCL cell lines alters MSH2 phosphorylation and MutSα formation. (a) Tet-on SUP-M2 MSH2Y238F cell lysate, in which the expression of MSH2 Y238F was correlated with the dose-dependent increase of DOX. (b) Tet-on Karps 299 MSH2 Y238F cell lysate depicting the dose-dependent increase in MSH2 Y238F expression with DOX treatment. In a and b, a β-actin antibody was used as a loading control, and densitometry of MSH2 expression relative to β-actin is shown. (c) Tet-on SUP-M2 MSH2 Y238F and (d) Tet-on Karps 299 MSH2 Y238F cells were treated with 0 and 50 ng/ml DOX, and the resulting cell lysate was subjected to IP using an anti-MSH2 antibody (+). p-Y of MSH2 was measured using an anti-p-Y antibody. No antibody controls (−) were included to account for nonspecific binding to the beads. Densitometry for phosphorylated MSH2 relative to total MSH2 is shown. (e) MSH2 was precipitated from Tet-on SUP-M2 MSH2 Y238F lysate treated with 0, 50 and 500 ng/ml DOX under co-IP conditions. The resulting immunoblots were probed with an anti-MSH6 and an anti-MSH2 antibody to assess the amount of MSH6 precipitated relative to MSH2. Densitometry for MSH6:MSH2 is shown. All panels in f are from the same western blot. Results are representative of at least three independent experiments.
We provide evidence that the expression of the MSH2 Y238F mutant significantly increased the sensitivity to MNU and 5-FU, suggesting a restoration of MMR in ALK+ALCL cells. Our data also suggest that enforced expression of MSH2 Y238F alone can induce apoptosis in two ALK+ALCL cell lines. Exactly how this mutant mediates this biological effect is unknown. Nevertheless, apart from its role in MMR, MSH2 is known to carry important roles in the regulation of cell cycle checkpoint and apoptosis in both MMR-proficient and -deficient cells. It has been reported that overexpression of MSH2 induced cell death in MMR-functional GP293 and MMR-deficient SK-UT-1 cells. Loss of MMR has been associated with a decrease in apoptosis and an increase in UVB-associated DNA-damage adducts in the epidermis of MSH2-null mice, accompanied by an increase in UVB-associated tumors and loss of phosphorylated p53. The resistance of MMR-deficient cells to certain DNA-damaging agents is partially through a loss of DNA-damage-induced cell cycle arrest; MSH2 associates with the checkpoint proteins ATR, CHK1 and CHK2, both in vitro and in vivo, mediating the initiation of G2/M cell cycle arrest. Although we have not yet understood the exact mechanisms underlying the induction of apoptosis following overexpression of MSH2 Y238F, we can speculate that ALK+ALCL cell lines carry a large number of DNA-damage adducts that, upon restoration of MMR, lead to an MMR-driven apoptotic signaling cascade.

There is accumulating evidence that cancers with oncogenic tyrosine kinases have dysfunctional DNA-damage response directly related to kinase-driven aberrant downstream signaling processes. As another oncogenic tyrosine kinase, BCR-ABL, has been shown to inhibit MMR, and receptor tyrosine kinases have similar features in regard to activity and signal transduction, MMR dysfunction through MSH2 phosphorylation may be shared across multiple oncogenic tyrosine kinases. Furthermore, with the emergence of ALK as a molecular driver of a number of other cancer types, including non-small-cell lung cancer, diffuse large B-cell lymphoma, breast cancer, retinoblastoma, colon carcinoma and esophageal squamous cell carcinoma, we believe that detection of MSH2 phosphorylation at Y238 may be of great clinical and diagnostic significance. We believe that p-Y of MSH2 at Y238 and the subsequent inhibition of DNA MMR may be a universal mechanism by which oncogenic tyrosine kinases potentiate tumorigenesis. We are currently in the process of characterizing a phospho-MSH2 Y238F-specific antibody in our laboratory, and we are assessing its utility for clinical use (manuscript in preparation).

In conclusion, we would like to reiterate our findings that overexpression of wild-type MSH2 (in the form of HB-MSH2) consistently resulted in suppression, rather than improvement, of the MMR function. Although we were initially perplexed with these seemingly paradoxical results, our literature search has revealed that multiple overexpression of key DNA MMR genes (for example, MSH2) suppresses MMR. Although the mechanism underlying this paradoxical effect is not completely understood, we speculate that this may be related to the fact that DNA MMR proteins (for example, MSH2) often require heterodimerization with another partner, such as MSH6. Thus, it is perceivable that gene transfection of wild-type MSH2 may substantially decrease the formation of the MSH2:MSH6 heterodimer relative to other MSH2 heterodimers (with partners other than MSH6), which is suboptimal for MMR. The observation that overexpression of the MSH2 Y238F mutant, but not wild-type MSH2, can result in improvement of MMR also highlights the concept that the biological effects associated with this MSH2 mutant are not simply due to the expression of an excessive amount of MSH2 protein.
CONCLUSION

In summary, we have presented evidence that NPM-ALK phosphorylates MSH2 at a specific site, Y238, creating a functional loss of MMR. Our data suggest that enforced expression of the MSH2Y238F phosphorylation-less mutant is able to restore MMR through a dominant-negative effect, whereby its expression blocks NPM-ALK interaction with endogenous MSH2, allowing for the formation of the endogenous MutSα heterodimer.
The importance of detecting MSH2 p-Y in oncogenic tyrosine kinase-expressing cancers clinically needs to be further defined.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

ACKNOWLEDGEMENTS
This work was supported by an operating research grant from the Canadian Institute of Health Research (RL and SEA), and by contributions by JTB from the University Health Foundation – Medical Research competition (2010). KMB is a graduate student supported by doctoral studentships from the Canadian Institute of Health Research, Alberta Innovates Health Solutions and the University of Alberta (President’s Doctoral Award of Distinction), and is a Killam Scholar (Izaak Walton Killam Memorial Scholarship). FW was supported by a post-doctoral fellowship from the Alberta Innovates Health Solutions and the University of Alberta (President’s Doctoral Award of Distinction), and is a Killam Scholar (Izaak Walton Killam Memorial Scholarship). The authors thank Dr P Kaiser for the gift of the HB expression vector, Dr M Meuth for the gift of the MSH2 expression vector and Dr S Morris for the gift of the MSH2 and NPM-ALK expression vectors. The authors also thank K Jung and Dr David Murray for proofreading the manuscript.

AUTHOR CONTRIBUTIONS
KMB, SEA and RL designed the experiments. KMB performed the majority of the experiments. KMB, SEA and RL participated in data interpretation. KMB and RL wrote the manuscript. PW and FW performed mass spectrometry and the associated data analysis. LL provided supervision of the mass spectrometry experiments. CW and JTB provided experimental assistance.

REFERENCES
1 Campo E, Swerdlow SH, Harris NL, Pileri S, Stein H, Jaffe ES. The 2008 WHO classification of lymphoid neoplasms and beyond: evolving concepts and practical applications. Blood 2011; 117: 5019–5032.  
2 Morris SW, Kirstein MN, Valentine MB, Dittmer KG, Shapiro DN, Saltman DL et al. Fusion of a kinase gene, ALK, to a nucleolar protein gene, NPM, in non-Hodgkin’s lymphoma. Science 1994; 263: 1281–1284.  
3 Pearson JD, Lee JK, Bacani JT, Lai R, Ingham RJ. NPM-ALK: the prototypic member of a family of oncogenic fusion tyrosine kinases. J Signal Transduct 2012; 2012: 123253.  
4 Young LC, Bone KM, Wang P, Wu F, Adam BA, Hegazy S et al. Fusion tyrosine kinase NPM-ALK deregulates MSH2 and suppresses DNA mismatch repair function: novel insights into a potent oncoprotein. Am J Pathol 2011; 179: 411–421.  
5 Li GM. Mechanisms and functions of DNA mismatch repair. Cell Res 2008; 18: 85–98.  
6 Kunkel TA, Eri DA. DNA mismatch repair. Annu Rev Biochem 2005; 74: 681–710.  
7 Sehgal R, Sheahan K, O’Connell PR, Hanly AM, Martin ST, Winter DC. Lynx syn-drome: an updated review. Genes (Basel) 2014; 5: 497–507.  
8 Krskova-Honzatka L, Cermak J, Sajdova J, Stary J, Sedlacek P, Sieglova Z. Microsatellite instability in hematological malignancies. Leuk Lymphoma 2002; 43: 1979–1986.  
9 Nkomdedeu JF, Perea G, Estivill C, Lasa A, Carnicer MJ, Brunet S et al. Microsatellite instability is not an uncommon finding in adult de novo acute myeloid leukemia. Ann Hematol 2005; 84: 368–375.  
10 Degroote A, Knippenberg L, Vander Borght S, Spaepen M, Matthys G, Schaeffer DF et al. Analysis of microsatellite instability in gastric mucosa-associated lymphoid tissue lymphoma. Leuk Lymphoma 2013; 54: 812–818.  
11 Miyashita K, Fuji K, Yamada Y, Hattori H, Taguchi K, Yamanaka T et al. Frequent microsatellite instability in non-Hodgkin lymphomas irresponsive to chemotherapy. Leuk Res 2008; 32: 1183–1195.  
12 Kanan P. Mechanisms of tolerance to DNA damaging therapeutic drugs. Carcinogenesis 2001; 22: 1931–1937.  
13 Christmann M, Tomicic MT, Kaina B. Phosphorylation of mismatch repair proteins MSH2 and MSH6 affecting MutS alpha mismatch-binding activity. Nucleic Acids Res 2002; 30: 1959–1966.  
14 Hernandez-Pigeon H, Quillet-Mary A, Louat T, Schambourg A, Humbert O, Selves J et al. hMutS alpha is protected from ubiquitin-proteasome-dependent degrada-tion by atypical protein kinase C zeta phosphorylation. J Mol Biol 2005; 348: 63–74.  
15 Hernandez-Pigeon H, Laurent G, Humbert O, Salles B, Lautier D. Degradation of mismatch repair hMutSalpha heterodimer by the ubiquitin-proteasome pathway. FEBS Lett 2004; 562: 40–44.  
16 Hegazy SA, Wang P, Anand M, Ingham RJ, Gelebart P, Lai R. The tyrosine 343 residue of nucleophosmin (NPM)-anaplastic lymphoma kinase (ALK) is important for its interaction with SHP1, a cytoplasmic tyrosine phosphatase with tumor suppressor functions. J Biol Chem 2010; 285: 19813–19820.  

Figure 7. Schematic model of how MSH2*Y238F is postulated to restore MMR. Our working model based on data published in this manuscript, as well as our previously reported findings, is that NPM-ALK, through an interaction with MSH2 that leads to MSH2 p-Y at residue 238, sequesters MSH2 from MSH6, blocking MutSα formation and its translocation to the nucleus in the presence of DNA damage. Our data from this manuscript, using both GP293 and ALK+ALCL cells, suggest that when its expression is enforced MSH2 Y238F blocks the phosphorylation of endogenous MSH2, by preferentially binding to NPM-ALK. Endogenous MSH2 is then capable of binding to MSH6, forming the MutS heterodimer, and restoring MMR, even in the presence of NPM-ALK.
17 Zhang H, Richards B, Wilson T, Lloyd M, Cranston A, Thorburn A et al. Apoptosis induced by overexpression of hMSH2 or hMLH1. Cancer Res 1999; 59: 3021–3027.
18 Wu F, Wang P, Young LC, Li R, Li L. Proteome-wide identification of novel binding partners to the oncogenic fusion gene protein, NPM-ALK, using tandem affinity purification and mass spectrometry. Am J Pathol 2009; 174: 361–370.
19 Wang P, Wu F, Zhang J, McMullen T, Young LC, Ingham RJ et al. Serine phosphorylation of NPM-ALK, which is dependent on the auto-activation of the kinase activation loop, contributes to its oncogenic potential. Carcinogenesis 2011; 32: 146–153.
20 Nicolaides NC, Littman SJ, Modrich P, Kinzler KW, Vogelstein B. A naturally occurring hPM2 mutation can confer a dominant negative mutator phenotype. Mol Cell Biol 1998; 18: 1635–1641.
21 Armanious H, Gelebart P, Anand M, Belch A, Lai R. Constitutive activation of the human MutSalpha DNA lesion recognition complex. Blood 2011; 117: 6237–6246.
22 Blom N, Gammeltoft S, Brunak S. Sequence and structure-based prediction of eukaryotic protein phosphorylation sites. J Mol Biol 1999; 294: 1351–1362.
23 Marra G, Iaccarino I, Lettieri T, Roscilli G, Delmastro P, Jiricny J. Mismatch repair deficiency associated with overexpression of the MSH3 gene. Proc Natl Acad Sci USA 1995; 92: 8568–8573.
24 Gibson SL, Narayan J, Hegan DC, Buermeyer AB, Liskay RM, Glazer PM. Overexpression of the DNA mismatch repair factor, PMS2, confers hypermutability and DNA damage tolerance. Cancer Lett 2006; 244: 195–202.
25 Jiricny J. The multifaceted mismatch-repair system. Nat Rev Mol Cell Biol 2006; 7: 335–346.
26 Hickman MJ, Samson LD. Apoptotic signaling in response to a single type of DNA lesion, O(6)-methylguanine. Mol Cell Biol 2004; 14: 105–116.
27 Acharya S, Wilson T, Gradia S, Kane MF, Guerrette S, Marsischky GT et al. hMSH2 forms specific mispair-binding complexes with hMSH3 and hMSH6. Proc Natl Acad Sci USA 1996; 93: 13629–13634.
28 Plotz G, Radel J, Brieger A, Trojan J, Zeuzem S. N-terminus of hMLH1 confers interaction of hMutLalp and hMutLbeta with hMutSalpha. Nucleic Acids Res 2003; 31: 3217–3226.
29 Rasmussen LJ, Rasmussen M, Lee B, Rasmussen AK, Wilson DM 3rd, Nielsen FC et al. Identification of factors interacting with hMSH2 in the fetal liver utilizing the yeast two-hybrid system. In vivo interaction through the C-terminal domains of hexo1 and hms2 and comparative expression analysis. Mutat Res Rep 2000; 460: 41–52.
30 Li LS, Morales JC, Vegil M, Sedwick D, Greer S, Meyers M et al. DNA mismatch repair (MMR)-dependent 5-fluorouracil cytotoxicity and the potential for new therapeutic targets. Br J Pharmacol 2009; 158: 679–692.
31 Stoklosa T, Poplawski T, Kopytna M, Nieborowska-Skorska M, Basak G, Slupianek A et al. BCR/ABL inhibits mismatch repair to protect from apoptosis and induce point mutations. Cancer Res 2008; 68: 2576–2580.
32 Bellacosa A, Cicchillitti L, Schepis F, Riccio A, Yeung AT, Matsumoto Y et al. MEDI1, a novel human methyl-Cpg-binding endonuclease, interacts with DNA mismatch repair protein MLH1. Proc Natl Acad Sci U S A 1999; 96: 3969–3974.
33 Mihaylova VT, Bindra RS, Yuan J, Campisi D, Narayanan L, Jensen R et al. Decreased expression of the DNA mismatch repair gene Mlh1 under hypoxic stress in mammalian cells. Mol Cell Biol 2003; 23: 3265–3273.
34 Warren JJ, Pohlahaus TJ, Changela A, Iyer RR, Modrich PL, Beese LS. Structure of the human MutSalpha DNA lesion recognition complex. Mol Cell 2007; 26: 579–592.
35 Ollila S, Sarantaus L, Karioila R, Chan P, Hampel H, Holinski-Feder E et al. Pathogenicity of MSH2 missense mutations is typically associated with impaired repair capability of the mutated protein. Gastroenterology 2006; 131: 1408–1417.
36 Ollila S, Dermadi Bебеb, Dјiricz J, Nijstom M. Mechanisms of pathogenicity in human MSH2 missense mutants. Hum Mutat 2008; 29: 1355–1363.
37 Ademson BL, Kravik KL, De Angelis PM. DNA damage signaling in response to 5-fluorouracil in three colorectal cancer cell lines with different mismatch repair and TP53 status. Int J Oncol 2011; 39: 673–682.
38 Aebi S, Fink D, Gordon R, Kim HK, Zheng H, Fink JL et al. Resistance to cytotoxic drugs in DNA mismatch repair-deficient cells. Clin Cancer Res 1997; 3: 1763–1767.
39 Kinsella TJ. Coordination of DNA mismatch repair and base excision repair processing of chemotherapy and radiation damage for targeting resistant cancers. Clin Cancer Res 2009; 15: 1853–1859.
40 O'Brien V, Brown R. Signalling cell cycle arrest and cell death through the MMR System. Carcinogenesis 2006; 27: 682–692.
41 Zeng X, Kinsella TJ. A novel role for DNA mismatch repair and the autophagic processing of chemotherapeutic drugs in human tumor cells. Autophagy 2007; 3: 368–370.
42 Stoic J, Cеjca P, Jiricny J. High doses of SN1 type methylating agents activate DNA damage signaling cascades that are largely independent of mismatch repair. Cell Cycle 2003; 4: 473–477.
43 de las Alas MM, Aebi S, Fink D, Howell SB, Los G. Loss of DNA mismatch repair: effects on the rate of mutation to drug resistance. J Natl Cancer Inst 1997; 89: 1537–1541.
44 Seifert M, Reichrath J. The role of the human DNA mismatch repair gene MSH2 in DNA repair, cell cycle control and apoptosis: implications for pathogenesis, progression and therapy of cancer. J Mol Histol 2006; 37: 301–307.
45 Peters AC, Young LC, Maeda T, Tron VA, Andrew SE. Mammalian DNA mismatch repair protects cells from UVB-induced DNA damage by facilitating apoptosis and p53 activation. DNA Repair (Amst) 2003; 2: 427–435.
46 Young LC, Peters AC, Maeda T, Edelmann W, Kucherlapati R, Andrew SE et al. DNA mismatch repair protein Msh6 is required for optimal levels of ultraviolet-B-induced apoptosis in primary mouse fibroblasts. J Invest Dermatol 2003; 121: 876–880.
47 Young LC, Thuilen KJ, Campbell MR, Tron VA, Andrew SE. DNA mismatch repair proteins promote apoptosis and suppress tumorigenesis in response to UVB irradiation: an in vivo study. Carcinogenesis 2004; 25: 1821–1827.
48 Adamson AW, Beardsley DI, Kim WJ, Gao Y, Baskaran R, Brown KD. Methylator-induced, mismatch repair-dependent G2 arrest is activated through Chk1 and Chk2. Mol Cell Biol 2005; 16: 1513–1526.
49 Wang Y, Qin J, MSH2 and ATR form a signaling module and regulate two branches of the damage response to DNA methylation. Proc Natl Acad Sci USA 2003; 100: 15387–15392.
50 Dierov J, Sanchez PV, Burke BA, Padilla-Nash H, Putt ME, Ried T et al. BCR/ABL induces chromosomal instability after genotoxic stress and alters the cell death threshold. Leukemia 2009; 23: 279–286.
51 Zhao R, Yang FT, Alexander DR. An oncogenic tyrosine kinase inhibits DNA repair and DNA-damage-induced Bcl-xl deamidation in T cell transformation. Cancer Cell 2004; 5: 37–49.
52 Skorski T. Oncogenic tyrosine kinases and the DNA-damage response. Nat Rev Cancer 2002; 2: 351–360.
53 Hallberg B, Palmer RH. Mechanistic insight into ALK receptor tyrosine kinase in human cancer biology. Nat Rev Cancer 2013; 13: 685–700.
54 Drummond JT, Genschel J, Wolf E, Modrich P. DHFR/MSH3 amplification in methotrexate-resistant cells alters the hMUTSalpha/hMUTSbeta ratio and reduces the efficiency of base-base mismatch repair. Proc Natl Acad Sci USA 1997; 94: 10144–10149.
55 Scherbakovova PV, Kuniel TA. Mutator phenotypes conferred by MLH1 overexpression and by heterozygosity for mlh1 mutations. Mol Cell Biol 1999; 19: 3177–3183.

This work is licensed under a Creative Commons Attribution 4.0 International License. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder to reproduce the material. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/