

THE EFFECTS OF THE LINE-1 PROTEIN ORF1P
ON MAPK P38 PATHWAYS

by

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ABSTRACT

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By BRIAN EZELL

Under the direction of PAMELA COOK, Ph.D.

Transposable elements make up approximately 50% of the human genome and include endogenous retroviruses, retrotransposons, and remnants of inactive transposons. LINE-1 is the only currently active autonomous retrotransposon in humans and replicates via a “copy-and-paste” mechanism termed retrotransposition. This process is highly mutagenic to genomic DNA. In addition, LINE-1 encodes two proteins, ORF1p and ORF2p, that can also perturb cellular function. LINE-1 is repressed in most somatic cells, but approximately 80 – 100 of the > 500,000 genomic copies of LINE-1 are capable of re-activation in epithelial precancerous and malignant cells and by exposures to environmental toxicants. Previous studies have shown that LINE-1 retrotransposition depends on activation of the mitogen activated protein kinase (MAPK) p38. Prior data in our lab showed that p38 phosphorylates the LINE-1 protein ORF1p on motifs whose phosphorylation is required for LINE-1 function, supporting a possible mechanistic role for p38 in LINE-1 activation. The experiments outlined in this thesis test our hypothesis that ORF1 exerts reciprocal effects on p38 by measuring p38 mRNA and protein expression in the presence or absence of transfected ORF1. In addition, we determined

the effect of ORF1p on the phosphorylation of p38 on Thr180/Tyr182, which is required for p38 kinase activity. Phosphorylation of p38 induces biological responses such as inflammation, apoptosis, growth, or cell differentiation via activation of multiple, complex downstream pathways. Deregulation of p38 is implicated in the pathologies of many diseases, including cancer. Identification of crosstalk between LINE-1 and p38 will therefore inform our understanding of how LINE-1 contributes to tumorigenesis, tumor progression, and cellular responses during environmental exposures.

INTRODUCTION

Transposable Elements

Transposable elements are stretches of DNA embedded in our genome that collectively constitute approximately half the human genome (Anwar, Wulaningsih, & Lehmann, 2017). Transposable elements are either class 1 (retrotransposons) or class 2 (DNA transposons). Retrotransposons are then further subdivided into long terminal repeat (LTR) retrotransposons and non-LTR retrotransposons. Non-LTR retrotransposons consist of long interspersed nuclear elements (LINEs) and short interspersed nuclear elements (SINEs). LINE-1 is referred to as an autonomous non-LTR retrotransposon because it encodes proteins required for its own retrotransposition. SINEs are referred to as non-autonomous because they do not encode proteins required for their mobilization (Hancks & Kazazian, 2012). Thus, SINE retrotransposition cannot occur without LINE-1 activation. LINEs and SINEs are the only active transposable elements in the human genome, but they are repressed in most healthy somatic cells. Exceptions include embryonic stem cells and neuronal precursor cells (Garcia-Perez et al., 2007) (Macia et al., 2016). Transposable elements can cause genomic damage via insertions, deletions, inversions, non-allelic homologous recombination, and pseudogene insertions (Konkel & Batzer, 2010). Though previously considered junk DNA, transposable elements have a powerful impact on genome stability and cause somatic mutations, which can lead to cancer and other diseases (Collier & Largaespada, 2007).

Although normally repressed in somatic cells, non-LTR retrotransposons have

been found to be reactivated by certain environmental toxicants. Heavy metals such as mercury, cadmium, arsenic, nickel, and cobalt increase the number of LINE-1 inserts and reduce LINE-1 RNA silencing (Morales, Servant, Ade, & Roy-Engel, 2016). Ionizing radiation and UV radiation was also shown to increase LINE-1 RNA copies (Morales et al., 2016). Importantly, LINE-1 activation by certain environmental toxicants has been shown to depend on activation of MAPKs p38 and ERK1/2 (Okudaira et al., 2010).

LINE-1 elements are abundant in the human genome. There are about 500,000 copies. A full-length human LINE-1 element is approximately 6 kb and contains two open reading frames (ORFs), designated ORF1 and ORF2, which express the proteins ORF1p and ORF2p, respectively. Both ORFs are essential to LINE-1 retrotransposition. About 100 of these LINE-1 elements, termed “hot L1s”, account for the majority of the retrotransposition events (Brouha et al., 2003). This is in part because many are truncated at the 5' end and missing part or entire ORFs (Ardeljan, Taylor, Ting, & Burns, 2017).

LINE-1 activity has been found in many different types of epithelial tumors and may play a significant role in tumorigenesis and the evolution and progression of tumors. LINE-1 ORF1p has been found in breast, lung, liver, esophageal, colon, pancreatic, ovarian, and prostate tumors (Ardeljan et al., 2017). Matched normal tissues in these various cancers do not typically express ORF1p. Expression of ORF1p is a hallmark of epithelial cancers and may be a biomarker of neoplasia (Ardeljan et al., 2017). LINE-1 activity is increased in patient tissues extracted from tumors, many cancer cell lines, and is

correlated with metastasis (Kemp & Longworth, 2015).

ORF1p

ORF1p is the most highly expressed protein encoded by LINE-1 (Dai, LaCava, Taylor, & Boeke, 2014). ORF1p has reported tumorigenic properties (Li et al., 2014). Reported tumorigenic characteristics of ORF1p include increased cell proliferation, increase in chemoresistance, and inhibition of apoptosis (Li, 2014. Lu, 2013). Still, little is known about how ORF1p affects cellular pathways such as MAPKs. An ORF1p monomer is 40 kDa, forms trimers, and has an RNA and single-stranded DNA binding region (Naufer et al., 2016) (Callahan, Hickman, Jones, Ghirlando, & Furano, 2012). ORF1p is heavily phosphorylated and requires phosphorylation of S18, S27, T203, and possibly T213 for LINE-1 retrotransposition (Cook, Jones, & Furano, 2015). These four amino acids are target sites for proline-directed protein kinase (PDPK), part of canonical MAPK motifs known as S/T-P motifs. MAPKs phosphorylate serine or threonine adjacent to a proline in the +1 position, known as S/T-P motifs. P38-delta phosphorylates ORF1p on S18 and S27, which are 2 of the 3 S/T-P motifs whose phosphorylation is required for LINE-1 retrotransposition (Cook & Tabor, 2016). In addition, our lab has data showing that the other isoforms of p38 can also target S18 and S27 (*Figure 1*).

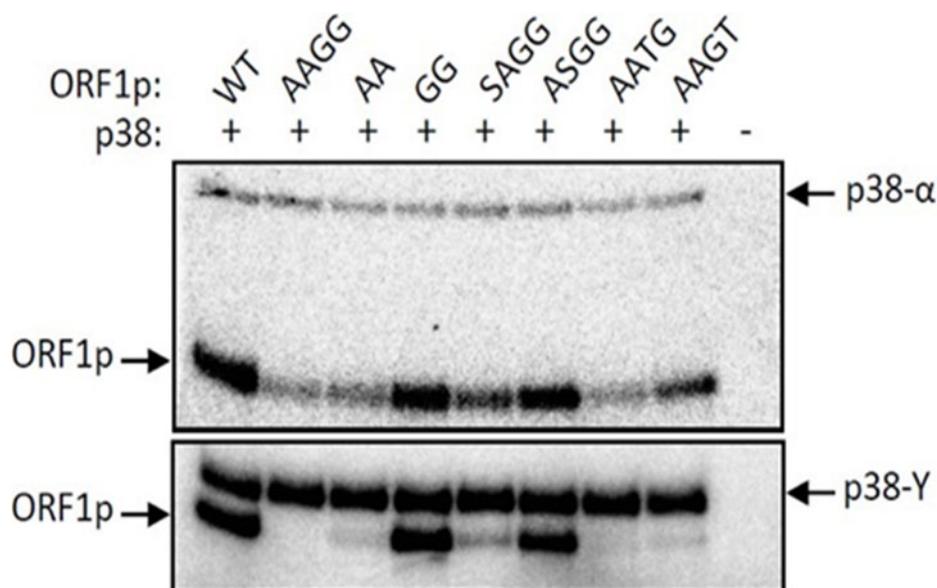


Figure 1. p38 kinases phosphorylate ORF1p on amino acids whose phosphorylation is required for LINE-1 activity. p38 isoforms (85 nM) were incubated with ORF1p (200 μM) WT or S/T-P mutants. Autoradiograms show [γ - 32 P]-ATP incorporation into ORF1p (and p38, which autophosphorylates). ORF1p mutants: AAGG (S18A/S27A/T203G/T213G), AA (S18A/S27A), GG (T203G/T213G), SAGG (S27A/T203G/T213G), ASGG (S18A/T203G/T213G), AATG (S18A/S27A/T213G), and AAGT (S18A/S27A/T203G). Data for p38- β were identical to p38- α ; p38- δ results were published in Cook & Tabor, 2016, Mobile DNA. Data for other p38 isoforms are unpublished.

The serine PDPK sites interact with the proline isomerase Pin1, which is a key regulator of S/T-P PDPK sites (Cook et al., 2015). This finding further suggests that ORF1p may have a regulatory role in MAPK and other PDPK pathways. Another finding relevant to our hypothesis that a feedback loop may exist between ORF1p and p38 was a report that ORF1p appears to mediate the induction of p38- δ in rheumatoid arthritis synovial fibroblasts (Kuchen et al., 2004). Moreover, it was also reported that ORF1p recruitment to chromatin relied on p38 activation (Ishizaka, Okudaira, & Okamura, 2012).

ORF2p

ORF2p is approximately 150 kDa and has endonuclease and reverse transcriptase activity essential to LINE-1 retrotransposition (Ostertag et al., 2001). ORF2p endonuclease activity, as well as mutagenic changes that occur during retrotransposition, are a significant source of DNA damage and could be a potential early diagnostic marker for colon or prostate cancer (De Luca et al., 2016). One study showed that expression of ORF2p increased in transgenic mice at early onset of breast cancer (Gualtieri et al., 2013). ORF2p endonuclease and reverse transcriptase activity can be greatly reduced by the mutations D205A and D702A respectively (Kines et al., 2016).

LINE-1 Replication Cycle

Once LINE-1 repression mechanisms have been overcome, LINE-1 can begin its replication cycle. The two main proteins encoded by LINE-1 are open reading frame 1 protein "ORF1p" and open reading frame 2 protein "ORF2p" (*Figure 2*). These two proteins will associate with LINE-1 mRNA to form an L1-ribonucleoprotein particle (L1-RNP). The L1-RNP then gains access to genomic DNA by an unknown mechanism. LINE-1 RNA is reverse transcribed by ORF2p to synthesize cDNA. LINE-1 cDNA mutagenically integrates into the genome with the help of ORF1p, ORF2p, and cellular factors. During integration, the 5' end is often truncated, losing its promoter and sometimes ORFs as well

(Ardeljan et al., 2017).



Figure 2. LINE-1 is a bicistronic gene containing a 5' UTR with an internal promoter and exon regions for two open reading frames (ORF1 and ORF2) that are divided by an intergenic region. *Images in this document are presented in color.*

Mitogen Activated Protein Kinases (MAPKs)

MAPKs are expressed in all human cells. The canonical MAPK pathway is a phosphorylation cascade triggered by an extracellular stimulus. A ligand binds to the extracellular portion of a membrane-bound receptor leading to the activation of a G protein, which in turn activates a MAPK kinase kinase (MAPKKK) that phosphorylates a MAPK kinase (MAPKK) that phosphorylates a MAPK that activates downstream kinases and transcription factors (Cargnello & Roux, 2011). MAPKs regulate many cellular processes, including cell differentiation, migration, inflammation, apoptosis, cell cycle progression, motility, and cell survival (Cargnello & Roux, 2011). MAPKs are activated by, among other things, growth factors, stress stimuli, and cytokines. Activation of MAPKs is initiated by dual phosphorylation of specific threonine and tyrosine amino acids in a Thr-Xaa-Tyr sequence (Xaa is any amino acid) (Cuenda & Rousseau, 2007). MAPKs are PDPKs,

meaning they phosphorylate proteins on a S/T-P motif. The most well-characterized MAPKs include: extracellular signal-regulated kinases ERK1/2, p38 isoforms (α , β , γ , δ), and the c-Jun amino N-terminal kinases JNK1/2/3.

MAPKs p38(α , β , γ , and δ)

P38- α and - β have been found ubiquitously expressed in human cells, however p38- γ and - δ are more restricted in their expression (Jiang et al., 1996). Phosphorylated p38 is the active form of p38 and it is most often phosphorylated by active MAPKs 3/6 that are highly selective for p38 but not JNKs or ERK1/2 (Cuenda & Rousseau, 2007). P38 regulates important cellular functions such as proliferation, apoptosis, cell survival, and inflammation by phosphorylating downstream protein kinases and transcription factors. P38 is activated in response to UV light, environmental stress, inflammatory cytokines, as well as by some of the same environmental toxicants that activate LINE-1. For example, LINE-1 activation was reported to depend on activation of p38 when cells were exposed to 6-formylindolo[3,2-b]carbazole (FICZ), benzo[*a*]pyrene (B[*a*]P), or 3-methylchroranthrene (3-MC) (Okudaira et al., 2010).

MAPKs ERK1/2

ERK1/2 is traditionally activated through a phosphorylation cascade initiated by a

mitogen binding to the extracellular growth factor receptor (EGFR). This leads to the activation of RAS, which phosphorylates RAF, which phosphorylates MAPKs 1/2 that will phosphorylate MAPKs ERK1/2.

Significance of MAPK Deregulation

Deregulation of MAPKs has been implicated in many diseases including cancer, autoimmune, and inflammatory diseases. MAPK p38 inhibitors are being investigated in approximately 30 clinical trials to determine their effects, alone or in addition to chemotherapy drugs, on different types of cancers (Zou, 2017). However, if ORF1p deregulates p38, targeting ORF1p may be an attractive strategy, since ORF1p is expressed in cancer cells but not somatic cells, whereas MAPKs like p38 are expressed in healthy cells also. This would therefore limit off-target effects of p38 inhibitors in healthy cells. In addition, inhibiting the function of ORF1p could simultaneously inhibit mutagenesis due to LINE-1 retrotransposition.

Hypotheses

Specifically, I hypothesize that ORF1-mediated deregulation of p38 in cancer cells occurs both at the transcriptional and post-translational levels. We therefore tested the effects of ORF1p on p38 mRNA levels and p38 phosphorylation on residues T180 and Y182.

To test these hypotheses, HeLa cells were transfected with ORF1 using an expression plasmid also carrying the puromycin-resistance gene, *PAC*. After transfection, cells were then selected with puromycin for 120 hours, based on the protocol by Kutchen et al., where it was reported that ORF1 upregulated the expression of p38-delta after being expressed for approximately 5 days. RNA was harvested, cDNA synthesized, and RTqPCR performed using TaqMan probes specific for each p38 isoform. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), beta-actin, and 18s ribosomal RNA were used as housekeeping genes to standardize data.

To test the effect of ORF1p on p38 phosphorylation, HeLa cells were transfected with ORF1 as above, and whole cell lysates were harvested and used for Western blot analysis of p38 phosphorylation.

Summary of Conclusions

RTqPCR and Western blots showed no significant difference in p38 expression in HeLa cells transiently transfected with LINE-1 ORF1. Western blot analyses indicated ORF1p-mediated changes in the levels of activating phosphorylation on p38 and ERK1/2s; however, the direction of change depended on the time of harvest, and some experiments showed no difference in phosphorylation in response to ORF1p expression.

MATERIALS AND METHODS

Plasmids

pcDNA3.1(+)-puro: (EV) Is based on the commercially available pcDNA3.1(+)-Neo construct (Invitrogen). The *neo* gene was removed and replaced with the PAC gene, which confers resistance to puromycin.

pcDNA3.1(+)-puro-ORF1: Contains the coding sequence for the LINE-1 protein ORF1p with a Flag tag at the C-terminus. This plasmid was previously described in (Cook et al., 2015).

Cell Culture

HeLa-JVM Cervical Cancer cells, generously supplied by the John Moran, PhD at University of Michigan Medical School, and 2102EP embryonal carcinoma cells were cultured in DMEM (Dulbecco's Modified Eagle Medium) with stable L-Glutamine. HCT-116 Colorectal Cancer Cells from the American Type Culture Collection (ATCC) were cultured in McCoy's 5A medium with L-Glutamine.

All cell lines were supplemented with 10% fetal bovine serum (FBS), 25 µg/mL of Amphotericin B, 10,000 µg/mL of streptomycin, and 10,000 units/mL of penicillin (Gibco). However, no antibiotic/antimycotic supplementation was given during experiments. All these cells are cultured in a humidified tissue culture incubator at 37C with 5% CO₂.

Transfections

Fugene-6 transfections were performed according to product literature using a DNA:reagent ratio of 1:3. Mirus (LT1, X2, and HeLa Monster) transfections were performed according to product literature using a DNA:reagent ratio of 1:3.

Puromycin Selection

For HeLa cells, 24 hours post transfection media was replaced and supplemented with puromycin at a concentration of 10ug/mL. 48 hours post transfection media was replaced and supplemented with puromycin at a concentration of 2.5ug/mL.

RNA Isolation

RNA was isolated from cells using the PureLink RNA Mini Kit (Ambion) along with the addition of On-Column PureLink DNase treatment. RNA purity and concentration was determined using spectrometry with the NanoDrop 2000. Samples were stored at -80C.

RNA-cDNA synthesis

High-Capacity RNA-to-cDNA kit (Applied Biosystems) was used for RNA-cDNA reverse transcription. Thermocycling conditions consisted of heating to 37C for 1 hour, heating to

95C for 5 minutes, and finally holding temperature at 4C.

Real-Time Quantitative Polymerase Chain Reaction

RT-qPCR was performed using TaqMan qPCR probes and TaqMan 2X Fast Universal PCR Master Mix. A master mix was first made consisting of cDNA, TaqMan Fast Universal PCR Master Mix, and nuclease-free H₂O. ThermoFisher TaqMan probes (p38 α , p38 β , p38 γ , p38-delta, GAPDH, 18s, beta-actin, and a custom ORF1 probe) with respective assay ID codes (Hs01051152_m1, Hs01558722_m1, Hs00268060_m1, Hs00559623_m1, Hs02786624_g1, Hs99999901_s1, Hs99999903_m1, APHGATC) were used for RTqPCR. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), 18s rRNA, and beta-actin were used as housekeeping genes. In each 10uL qPCR reaction there was 2uL of cDNA, 5uL of TaqMan 2X Fast Universal PCR Master Mix, 2uL nuclease-free H₂O, and 1uL of TaqMan probe specific to the target. Technical replicates of qPCR reactions were done in triplicate. Reactions and data collection were performed in the StepOne Real-Time PCR System by Applied Biosystems. Data was analyzed via the delta-delta Ct method to show relative gene expression. Ct (cycle threshold) is defined as the number of PCR cycles it takes for the specific fluorescent signal to cross a defined threshold. $\Delta\text{Ct} = (\text{Ct of the gene of interest}) - (\text{Ct of the housekeeping gene})$. $\Delta\Delta\text{Ct} = \Delta\text{Ct} (\text{treated sample}) - \Delta\text{Ct} (\text{untreated sample})$. By plugging in the $\Delta\Delta\text{Ct}$ in this equation $2^{-\Delta\Delta\text{Ct}}$, we calculate the fold difference in cDNA of the gene of interest in a treated sample verse an untreated sample. This

reflects the gene of interest's RNA expression in the cell.

Harvesting Cell lysates for Western blot

Cells were washed twice with HBSS then lysed in buffer containing 1% SDS, 10 mM Tris pH 7.4, 1 mM EDTA, and 3X HALT protease and phosphatase inhibitors. Cells were then scraped and collected into prechilled QIAshredders and centrifuged at 20,000 x g for 30 seconds. Lysates were transferred to a chilled microcentrifuge tube and centrifuged at 4C, 20,000 RCF for 10 minutes. While avoiding the pellet of cell debris, lysates were transferred to a clean chilled microcentrifuge tube. Lysates were frozen at -80C unless running on a gel that day.

Protein Quantification

To quantify protein lysate, the DC Protein Assay (Bio-Rad) was performed according to product literature. The NanoDrop 2000 was used to obtain 750nm readings.

Tris Glycine SDS PAGE

1X NuPage LDS sample buffer and 2.5% 2-Mercaptoethanol were mixed with cell lysates. Samples were then heated to 90C for 5 minutes before loading into Novex WedgeWell gels. Precision Plus Protein Dual Color Standard was used as a protein ladder. Electrophoresing was performed in the Thermo Fisher Mini Gel Tank.

Western Blotting

Cell lysates were fractionated by SDS-PAGE and subsequently transferred to a nitrocellulose membrane using the XCell SureLock Mini-Cell chamber according to the manufacturer's protocols. After incubation with blocking buffer (5% Dry non-fat milk, 0.1% Tween-20, TBS 1X, and H₂O) for 60 min, the membrane was incubated with antibodies against p38 (1:1000), p-p38 (1:1000), p-ERK1/2 (1:2000), beta-tubulin (1:5000), Flag (1:1000), LINE-1-ORF1p (1:1000) in binding/wash buffer (2.5% dry non-fat milk, .1% Tween-20, TBS 1X, and Q H₂O) at 4 °C for approximately 12 hours. Membranes were washed three times for 15 min and then incubated in TBS with a 1:10,000 dilution of horseradish peroxidase-conjugated secondary antibody (anti-rabbit or anti-mouse) for 1 hour. Blots were washed with TBS 5 times for 15 minutes and developed with chemiluminescent substrate (SuperSignal West Pico PLUS) according to the manufacturer's protocol. Blots were imaged using a BioRad ChemiDoc or X-ray film autoradiography.

Primary Antibodies

Anti-p38 rabbit polyclonal antibody #9212s from Cell Signaling Technology recognizes total p38 α , - β and - γ MAPK protein. Anti-p-p38 rabbit monoclonal antibody #4511s from Cell Signaling Technology detects p38 MAPK when phosphorylated at

Thr180/Tyr182. Anti-p-ERK1/2 rabbit monoclonal antibody #4370 from Cell Signaling Technology detects p-ERK1/2 when phosphorylated at Thr202/Tyr204. Anti-beta-tubulin mouse monoclonal antibody, catalog # MA5-16308, from Invitrogen detects beta-tubulin. This is a loading control antibody. Anti-flag M2 mouse monoclonal antibody, lot# SLBT6752, from Sigma Aldrich, detects the flag peptide sequence DYKDDDDK.

Secondary Antibodies

Goat-anti-rabbit polyclonal antibody supplier no. 474 1506 detects rabbit antibodies.

Anti-mouse polyclonal antibody from Sigma Aldrich, detects mouse antibodies.

RESULTS

Characterizing relative LINE-1 ORF1 RNA expression among cell lines.

Since our goal was to determine the effect of ORF1p expression on p38 RNA and phosphorylation, we first needed to determine whether the cell lines we intended to use expressed significant levels of endogenous ORF1p. Our initial plan was to transfect ORF1p into cell lines that did not express ORF1p, and to use siRNA to knock down ORF1p expression in cell lines expressing high levels of ORF1p. RNA harvested from untreated HeLa, HCT-116, 2102EP, and SW620 was analyzed via RTqPCR for relative ORF1 expression.

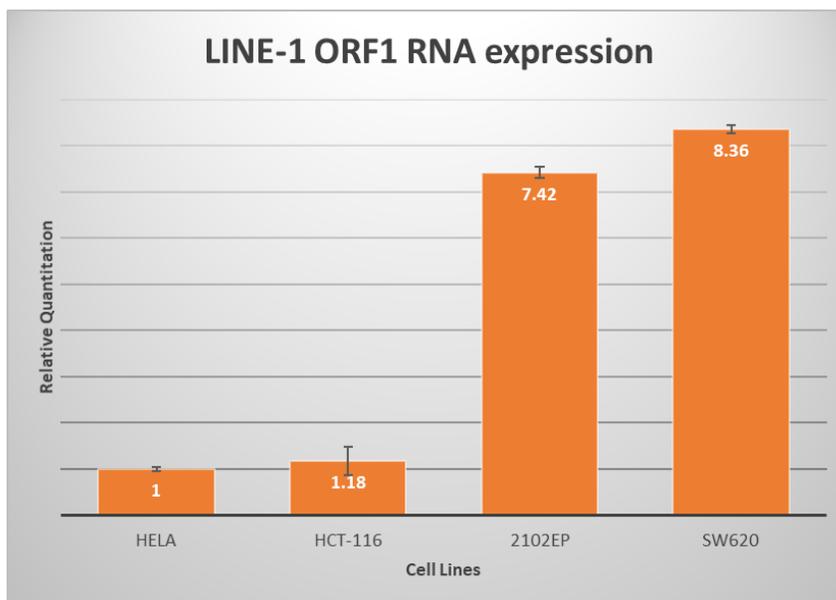


Figure 3. ORF1 RNA expression among different cell lines. HCT-116 and SW620 are both colorectal cancer cell lines, yet SW620 cells have nearly 8-fold more ORF1 RNA expression than HCT-116 cells. 2102EP cells have previously been shown to express higher levels of endogenous ORF1p compared to HeLa cells (Metzner, Jäck, & Wabl, 2012). Error bars reflect the standard error of the mean (SEM) of triplicate technical replicates. *Images in this document are presented in color.*

The effects of transiently transfected ORF1 on p38 RNA expression in HeLa cells.

Because HeLa cells express relatively low levels of ORF1 RNA, we chose this line for our gain-of-function studies in which we transfected ORF1 to determine its effects on p38.

Figure 3 indicates that exogenous expression of transfected ORF1 did not significantly affect the expression levels of any p38 isoform in HeLa cells. Significance is defined as a change greater than two-fold (Karlen et al., 2007). These results show ORF1p effect on p38-delta in HeLa cells was insignificant, in contrast to another study (Kuchen et al., 2004), which showed ORF1p effects on p38-delta in a different cell type.

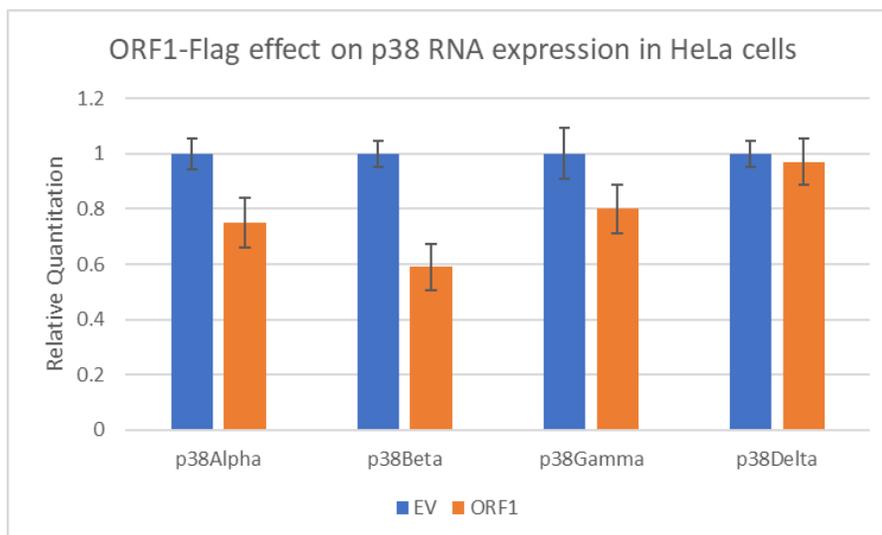


Figure 4. ORF1 induces no significant difference in p38 RNA expression in HeLa cells. HeLa cells were plated in 12-well plates and transfected with 1 ug ORF1p using Fugene-6 transfection reagent. Puromycin was applied at 10 ug/ml 24 hours after transfection, then lowered to 2.5 ug/ml at 48 hours post transfection and maintained until RNA harvest at 120 hours post transfection. RTqPCR reactions were performed as described in Materials and Methods using TaqMan probes for each p38 isoform. Relative expression levels are indicated. Error bars reflect the standard error of the mean (SEM) of triplicate technical replicates. *Images in this document are presented in color.*

The effects of transiently transfected ORF1 on the phosphorylation of p38 and ERK1/2 in HeLa cells

In order to determine the effects of ORF1p on the activating phosphorylation of p38 and ERK1/2, we transfected ORF1 into HeLa cells, then monitored p38 and ERK1/2 phosphorylation using antibodies. P-p38 antibodies are specific for phosphorylated T180 and Y182 phosphorylation, and p-ERK1/2 antibodies are specific for T202 and Y204. Phosphorylation of these residues indicates activation. Flag antibodies were used to

confirm that the plasmid had indeed expressed ORF1 and that samples had not been loaded in incorrect lanes. Beta tubulin antibodies were used to confirm equal loading and representation of cell lysates.

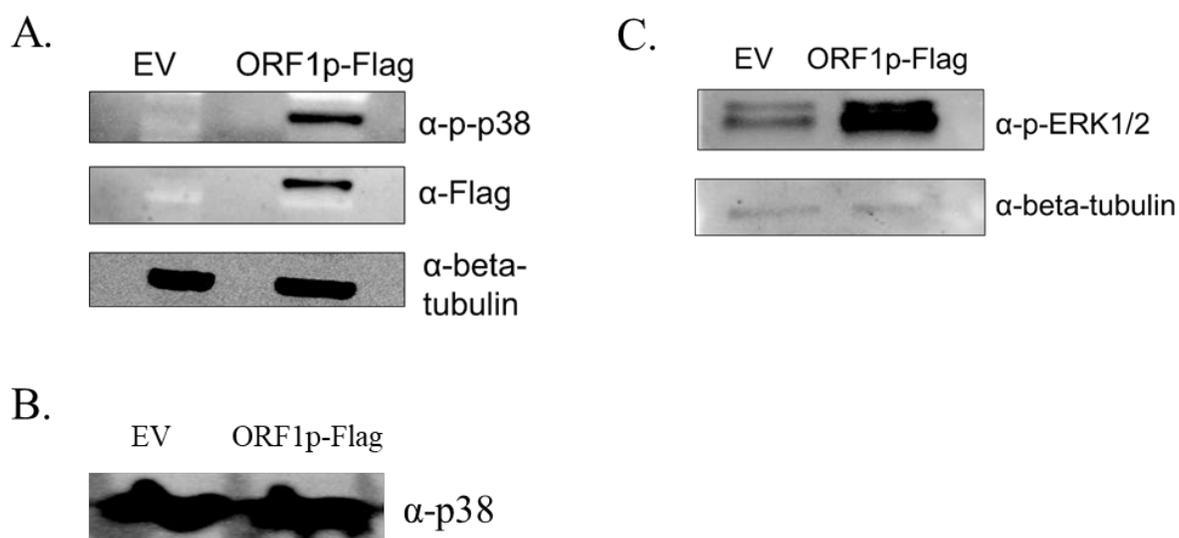


Figure 5. ORF1 fosters p38 and ERK1/2 phosphorylation in HeLa cells. HeLa cells were plated in T75 flasks and transfected with 20 ug ORF1 using Fugene-6 transfection reagent. Puromycin was applied at 10 ug/ml 24 hours after transfection, then lowered to 2.5 ug/ml at 48 hours post transfection and maintained until cell lysate harvest at 120 hours post transfection.

Although the results shown in *Figure 5* indicate that ORF1p induces p38 and ERK1/2 phosphorylation, subsequent experiments did not produce consistent results. For example, *Figure 6* shows equivalent p38 phosphorylation. We also investigated the effects of certain ORF1p mutations on its ability to induce p38 phosphorylation. As shown in Fig. 11, we used the ORF1 mutants C111F and R261A/R262A. C111F is an ORF1 mutant

that inactivates L1 for an unknown reason, and R261A/R262A is an ORF1 mutant that does not bind RNA.

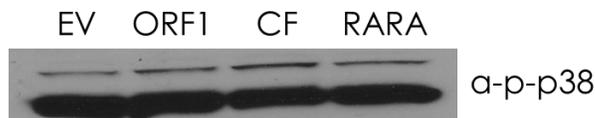


Figure 6. Mutations in ORF1 do not affect its ability to activate p38 phosphorylation.

C111F is an ORF1 mutant that inactivates L1 for an unknown reason, and R261A/R262A is an ORF1 mutant that does not bind RNA. HeLa cells were plated in 6-well plates and transfected with 3 ug ORF1p using Mirus TransIT HeLaMONSTER transfection reagent. Puromycin was applied at 10 ug/ml 24 hours after transfection, then lowered to 2.5 ug/ml at 48 hours post transfection and maintained until cell lysate harvest at 120 hours post transfection.

The effects of transiently transfected ORF1 on p38 phosphorylation in HeLa cells over time.

Due to the inconsistency of results regarding ORF1 effect on p38 phosphorylation, we felt it was necessary to determine if p38 phosphorylation varied in a pattern with time. ORF1 expression is at its peak around 48 hours post transfection and progressively diminishes and becomes undetectable by Western blot after 120-144 hours. During this decline p-p38 increases and then swiftly increases at 120 hours post transfection. This inverse correlation suggest ORF1 may be inhibiting p-p38 in some way. Subsequent experiments also showed that ORF1p inhibited phosphorylation of p38 at 24 hour post transfection (data not shown), indicating that this is an early effect of ORF1p expression.

Significant cell death was observed in the well with ORF1p at the 120 hour time point. In a subsequent time course experiment, it was observed that p38 was highly activated in the EV well also when the experiment was carried out to the point of cell death in the EV well.

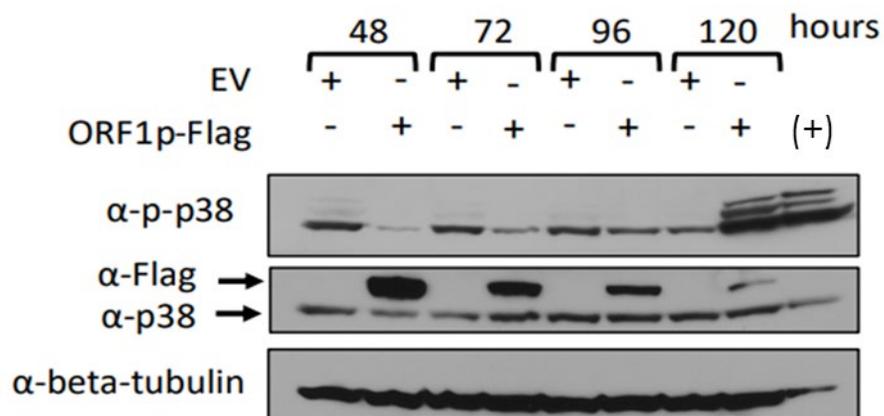


Figure 7. ORF1 inhibits p-p38, but p-p38 rebounds once ORF1 is degraded. HeLa cells were plated in 6-well plates and transfected with 3 ug ORF1p using Fugene-6 transfection reagent. Puromycin was applied at 10 ug/ml 24 hours after transfection, then lowered to 2.5 ug/ml at 48 hours post transfection and maintained until all cell lysate harvests were completed. Harvests were done at 48h, 72h, 96h, and 120h. Western blots were performed and X-ray film developed.

DISCUSSION

Our results show that ORF1p does not significantly affect p38 mRNA, or reproducibly affect p38 phosphorylation levels, in HeLa cells.

With respect to our results regarding the effect of ORF1p on p38 mRNA, our results appear to be inconsistent with those reported in Kuchen et al. However, several factors should be considered. Kuchen et al used rheumatoid arthritis synovial fibroblasts whereas this study used HeLa cells. Kuchen et al reported that despite a mutation (D702Y), knocking out reverse transcriptase activity in ORF2, the LINE-1 element still induced p38-delta expression. They attributed the increase in p38-delta mRNA and protein expression to ORF1, but may not have considered ORF2 endonuclease activity. Studies have reported that ORF2 endonuclease activity contributes to double-strand break formation in DNA (Gasior, Wakeman, Xu, & Deininger, 2006). DNA damage has been reported to activate p38 (Wood, Thornton, Sabio, Davis, & Rincon, 2009). Knocking out ORF2 endonuclease activity would require a D205A mutation. This suggests ORF2 endonuclease activity may have actually been what activated p38-delta mRNA and protein expression. If ORF2 does in fact activate p38, a protein that phosphorylates ORF1 on amino acids required for retrotransposition, then a positive feedback loop of p38 activation may nevertheless exist in the context of the LINE-1 element and warrants future investigation.

p38 activation coincides with the commencement of dramatic cell death, most likely due to plasmid loss and attendant loss of puromycin resistance. Thus, the activation of

p38 at that time is most likely due to processes unrelated to ORF1p per se, and this interpretation is strongly supported by our findings, in a subsequent time course experiment, that cells transfected only with the empty vector control, which also confers puromycin resistance, also show an increase in p38 activation upon commencement of cell death, albeit at approximately 1 – 2 days later.

Apart from a correlation with p38 activation and cell death, the most consistent results appear to be that ORF1p somewhat represses p38 phosphorylation at early time points. The potential biological significance of this remains to be elucidated

CONCLUSION

A previous report suggested ORF1 induced p38-delta expression in rheumatoid arthritis synovial fibroblasts (Kuchen et al., 2004). However, ORF1 did not induce an increase in p38-delta expression in HeLa cells. In conclusion, the effect of ORF1p on p38 mRNA expression may depend on the type of tissue. Regarding the effect of ORF1p on p38 phosphorylation, Western blots consistently suggested a link between ORF1p and p38 activation that warrants future investigation, perhaps using more sensitive techniques, different cell lines, or human samples.

FUTURE DIRECTIONS

Because p38 deregulation is such an influential factor in disease, further research should be done regarding ORF1p effect on p38 phosphorylation. There may be other cell lines more suitable for these experiments and that should be looked into. The transfection of commonly expressed proteins such as alpha tubulin and beta actin should be done to determine whether the effects of ORF1p on p38 were specific or simply the result of overexpressing a protein in cells. P38 inhibitors have been characterized and may prove useful in assessing ORF1p effects on p38. Transfecting siRNA against ORF1 in cell lines that express high levels of ORF1 such as an epithelial cancer cell line or 2102EP could have an impact on p38 phosphorylation and may be another path to elucidating the relationship.

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