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Osmotic and Heat Stress-dependent Regulation of MLK4β and MLK3 by the CHIP E3 Ligase in Ovarian Cancer Cells

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Abstract

Mixed Lineage Kinase 3 (MLK3), a member of the MLK subfamily of protein kinases, is a mitogen-activated protein (MAP) kinase kinase kinase (MAP3K) that activates MAPK signalling pathways and regulates cellular responses such as proliferation, invasion and apoptosis. MLK4β, another member of the MLK subfamily, is less extensively studied, and the regulation of MLK4β by stress stimuli is not known. In this study, the regulation of MLK4β and MLK3 by osmotic stress, thermostress and heat shock protein 90 (Hsp90) inhibition was investigated in ovarian cancer cells. MLK3 and MLK4β protein levels declined under conditions of prolonged osmotic stress, heat stress or exposure to the Hsp90 inhibitor geldanamycin (GA); and MLK3 protein declined faster than MLK4β.–Similar to MLK3, the reduction in MLK4β protein in cells exposed to heat or osmotic stresses occurred via a mechanism that involves the E3 ligase, carboxy-terminus of Hsc70-interacting protein (CHIP). Both heat shock protein 70 (Hsp70) and CHIP overexpression led to polyubiquitination and a decrease in endogenous MLK4β protein, and MLK4β was ubiquitinated by CHIP *in vitro*. In untreated cells and cells exposed to osmotic and heat stresses for short time periods, small interfering RNA (siRNA) knockdown of MLK4β elevated the levels of activated MLK3, c-Jun N-terminal kinase (JNK) and p38 MAPKs. Furthermore, MLK3 binds to MLK4β, and this association is regulated by osmotic stress. These results suggest that in the early response to stressful stimuli, MLK4β-MLK3 binding is important for regulating MLK3 activity and MAPK signalling, and after prolonged periods of stress exposure, MLK4β and MLK3 proteins decline via CHIP-dependent degradation. These findings provide insight into how heat and osmotic stresses regulate MLK4β and MLK3, and reveal an important function for MLK4β in modulating MLK3 activity in stress responses.

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Conflict of Interest

The authors declare no conflict of interest.

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Keywords

Mixed Lineage Kinase 3 (MLK3); Mixed Lineage Kinase 4 (MLK4); mitogen activated protein kinase (MAPK); carboxyl-terminus of Hsc70-interacting protein (CHIP); ubiquitination; stress signalling

1. Introduction

The mixed lineage kinases (MLKs) are a family of serine and threonine mitogen-activated protein (MAP) kinase kinase kinases (MAP3Ks) that regulate MAP kinase (MAPK) signalling in response to mitogenic and stressful stimuli [1–6]. MAPK signalling pathways transduce signals from the plasma membrane and mediate specific transcriptional responses to a wide range of stimuli [7]. Activated MAP3Ks promote activation of MAPK kinases (MAP2Ks), which in turn phosphorylate and activate MAPKs in a phosphorelay module [7]. Activated MAPKs translocate to the nucleus and induce transcription of genes implicated in various cellular responses including proliferation, migration, motility, and differentiation.

The MLK family consists of three sub-families: the MLKs, the dual leucine zipper-bearing kinases (DLKs) and the zipper sterile α-motif kinases (ZAKs) [8]. Members of the MLK subfamily include MLKs 1–3 and 4α and 4β, which all possess an N-terminal SH3 domain, a Cdc42-Rac interactive binding (CRIB) domain, a leucine zipper region, a catalytic kinase domain and a proline rich C-terminal region [8]. MLK3, the most extensively studied member of the MLK subfamily, has critical functions in migration and invasion in breast, gastric, and ovarian cancer cells; and MLK3 regulates neuronal cell apoptosis [9–12]. *MLK3* has more than 70% sequence identity to the *MLK4* catalytic domain; however, recent studies indicate non-redundant functions of these enzymes in normal and neoplastic cells [3, 4, 13, 14].

MLK4 is expressed in the liver, lung, brain, kidney and pancreas; and has two isoforms, *MLK4α* and *MLK4β*, generated by alternative splicing [13]. MLK4β negatively regulates basal as well as stimulus-induced MAPK signalling, ovarian cancer cell invasion, and matrix metalloproteinase 9 activity [3, 4]. In response to TNFα and osmotic stress, MLK4β down-regulates p38 signalling, which was proposed to be mediated through binding and inhibition of MLK3 kinase activity [4]. MLK4 interacts with toll-like receptor 4 and suppresses lipopolysaccharide-induced c-Jun N-terminal kinase (JNK) and extracellular stimulus-regulated kinase (ERK) signalling [3]. Mutated MLK4 kinase has been detected in microsatellite stable colorectal tumours, and these mutant alleles have been characterized as being either loss-of-function or tumorigenic [14, 15]. Thus, MLK3 and MLK4 are important regulators of cellular transformation, and effective targeting of these enzymes in tumor cells requires a thorough understanding of MLK protein level and kinase activity modulation.

Recent findings have shed light on the stress-induced degradation of MLK3 protein. MLK3 is associated with heat-shock protein 90 (Hsp90) in breast cancer cells [16]. Hsp90 functions as a stabilising partner that preserves the native conformation and ligand binding sites of the client protein [17]. Geldanamycin (GA), an Hsp90 inhibitor, binds the ATP pocket on Hsp90 and blocks the Hsp90 ATPase activity [18]. The inhibition of ATPase activity causes Hsp90

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to lose association with client proteins [19]. These proteins are then ubiquitinated and targeted for proteasomal degradation by an Hsp70-dependent mechanism [20]. Hsp70 recruits an E3 ubiquitin ligase, carboxy-terminus of Hsc70-interacting protein (CHIP), that ubiquitinates and marks target proteins for degradation by the 26S proteasome [17]. We have previously shown that MLK3 undergoes CHIP-mediated polyubiquitination and proteasomal degradation in response to GA, heat shock and osmotic shock [21]. In light of recent findings that MLK4 influences cellular responses important for tumor development, we sought to gain insight into the modulation of MLK4β protein in ovarian cancer cells. This study is focused on the regulation MLK3 and MLK4β proteins, and MAPK signalling in response to heat, osmotic stress and Hsp90 inhibition in ovarian cancer cells.

2. Materials and Methods

2.1. Cell culture

Human SKOV3 and TOV112D ovarian cancer, and human embryonic kidney 293 (HEK293) cells were purchased from the American Type Culture Collection (ATCC), Manassas, VA, USA. SKOV3, TOV112D and HEK293 cells were cultured in Dulbecco's modified Eagle's medium (Mediatech, Herndon, VA, USA) supplemented with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA), 2 mM L-glutamine, 25 μg/ml streptomycin and 25 I.U. penicillin (Mediatech, Inc.). Cells were cultured in a humidified atmosphere with 5% CO_2 at 37 °C.

2.2. Plasmid and siRNA transfections

The following mammalian expression plasmids for expression of human CHIP, MLK3, MLK4β and Hsp70 were used in this study: pCMV-FLAG-CHIP, pCMV-FLAG-MLK3, pCMV-His-MLK3 and pCMV-GST-MLK4β, pEBG-GST. pCMV-EGFP-Hsp70 was obtained from Addgene, Cambridge, MA, USA [22]. The plasmids that were used for the expression of human MLK3 and CHIP in *E. coli* were: pCMV-His-MLK3, pCMV-GST-MLK4β and pCMV-GST-CHIP. Transient transfections were performed using PolyJet™ (SignaGen Laboratories, Rockville, MD, USA), and siRNA knockdown was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) or GeneMute™ (SignaGen Laboratories) as described by the manufacturer. MLK4 siRNA oligos (Ambion, Life Technologies, Grand Island, NY, USA) have the following sequence: 5′-GGGCAGTGATGACTGAGAT-3′ which corresponds to nucleotides 1208–1227. The MLK3 siRNA oligo sequence was described previously [6]. The human CHIP and non-target siRNA oligos were from Santa Cruz Biotechnology, Dallas, TX, USA.

2.3. Cell treatments

Cells were either left untreated, treated with vehicle, or treated with a final concentration of 10 μM GA (InvivoGen, San Diego, CA, USA), 50 μM cycloheximide (CHX) (Thermo Fisher Scientific, Rockford, IL, USA), 250 mM sorbitol, or cultured at 42 °C for the indicated time periods, as previously described [21]. The cells were harvested immediately after treatments and immunoprecipitations were performed or whole cell extracts were prepared.

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2.4. Protein purification from bacterial and mammalian cells

Mammalian FLAG-CHIP, GST-MLK4β and His-MLK3 proteins were expressed in BL21 *E. coli* cells and purified as performed previously [21].

2.5. In vitro binding assay and immunoprecipitation

For binding assays, 1–2 μg of His-tagged or GST fusion proteins (purified from BL21 *E. coli* or or expressed and purified from HEK293 cells) were incubated together in lysis buffer (50 mM Tris-HCl pH 7.5, 1% Triton X-100, 2 mM EGTA, 0.1% βMe, 50 mM NaF, 1 mM Na_3VO_4, 1 μM leupeptin, 1 μM aprotinin, 1 mM PMSF and 1 mM benzamidine) and rotated with 20 μl Ni-NTA-agarose (for His-pulldown) or GSH-agarose beads (for GST-pulldown) at 4 °C for 2 h. After incubation, the beads were washed with high stringency wash buffer (0.1% Triton X-100, 50 mM Tris-HCl, pH 7.5, 2 mM EGTA, 0.1% βMe, 0.5 M LiCl, 50 mM NaF, 1 mM Na_3VO_4, 1 μM leupeptin, 1 μM aprotinin, 1 mM PMSF, and 1 mM benzamidine) and then with wash buffer (same as high stringency wash without LiCl). His-tagged and GST fusion protein levels were assessed by immunoblotting with the appropriate antibodies.

For immunoprecipitations, HEK293 cells were transfected with appropriate mammalian expression plasmids. 24 h post transfection the cells were lysed in 1 ml IP lysis buffer (supplemented with 10 mM N-ethylmaleimide when detecting ubiquitinated proteins). The lysates were incubated with FLAG (Agilent, Santa Clara, CA, USA), green fluorescent protein (GFP) (Novus Biologicals, Littleton, CO, USA), MLK3 (D-11, Santa Cruz Biotechnology, Santa Cruz, CA), MLK4 or ubiquitin (Novus Biologicals) antibodies and 25 μl Protein-G sepharose beads (Pierce, Thermo Fisher Scientific Inc.) at 4 °C for 2 h with rotation. The beads were collected and washed with high-stringency wash buffer (as described above) and then with wash buffer (as described above). The samples were boiled in SDS sample buffer and analyzed by immunoblotting.

2.6. Immunoblotting

Immunoblotting was carried out with the following primary antibodies: MLK3 (C-20), GST (Z-5), JNK1 (C-17), Hsp90 (H-114), β-Actin (C-4), and CHIP (H-231) (Santa Cruz Biotechnology). The activation-state phospho-JNK (p-JNK; Thr183/Tyr185), phospho-MLK3 (p-MLK3; Thr277/Ser281), phospho-ERK (p-ERK; Thr202/Tyr204), and phospho-p38 (p-p38; Thr180/Tyr182) were obtained from Cell Signaling Technology, Beverly, MA, USA. Other antibodies that were used for immunoblotting were GFP and MLK4 (Novus Biologicals), FLAG (Agilent), Ubiquitin (BD Biosciences, San Jose, CA) and Hsp70 (W-27) (Thermo Scientific, Freemont, CA, USA).

2.7. In vitro ubiquitination assay

Mammalian His-CHIP and GST-MLK4β proteins were purified from BL21 *E. coli*. A Customized E2 Screening Kit (UBPBio, Aurora, CO, USA) provided the purified E2 enzymes, UBE1 enzyme, human ubiquitin, and these components were mixed with His-CHIP and GST-MLK4β to carry out the ubiquitination reactions according to the manufacturer's instructions [21].

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Quantification and statistical analysis

Immunoblots are representative of three independent experiments (N=3), and quantification and statistical analysis was performed on three independent biological replicates. Densitometric analysis of immunoblots was performed using Image J software (National Institutes of Health). Statistical analysis of two sample assays was performed with Student's *t* test, error bars represent the standard error of the mean, while asterisk indicates statistical significance (p-value < 0.05).

3. Results

3.1. Osmotic and heat stresses reduce endogenous MLK4β

Excessive or prolonged exposure of cells to stress stimuli can drive protein degradation and misfolding. We previously observed that MLK3 protein is degraded in response to heat and osmotic stresses via CHIP-dependent ubiquitination in ovarian cancer cells. To determine if MLK4β protein is also regulated by osmotic and heat stresses, SKOV3 ovarian cancer cells were treated with 250 mM sorbitol (osmotic stress) or cultured at 42 °C over an 8 h time course (Fig. 1A) [21]. In sorbitol-treated SKOV3 cells, endogenous MLK4β was significantly reduced at 2, 4, 6 and 8 h in comparison to untreated cells, whereas endogenous MLK3 was significantly reduced at 0.3, 1, 2, 4, 6 and 8 h, in comparison to untreated cells (Figs. 1A and 1C). Cells treated with sorbitol had elevated, active, phosphorylated JNK (p-JNK) observed at 2 and 4 h, and elevated active, phosphorylated p38 (p-p38) observed at all time points between 2 h and 8 h (Fig 1A). An increase in activated ERK (p-ERK) was observed at 2, 4, 6 and 8 h in comparison to untreated cells (Fig. 1A). For cells exposed to heat stress, both MLK4β and MLK3 protein levels were significantly reduced in comparison to untreated cells at all time points tested from 0.3 to 8 h (Figs. 1B and 1C). p-JNK and p-p38 were increased at 2, 4, and 6 h, and p-ERK was increased at all time points between 2 h and 8 h in comparison to untreated cells (Fig. 1B). Heat and sorbitol caused a similar reduction in MLK4β and MLK3 proteins in ovarian cancer TOV112D cells (Supplemental Fig. 1).

3.2. MLK4β suppresses MAPK signalling pathways in response to stress stimuli

Stress exposure for short time periods (2 h) resulted in increased p-JNK, p-p38, and pERK (sorbitol); increased p-JNK (1h heat) and increased p-ERK and p-p38 (2 h heat), and reduced MLK4β (Figs. 1A and B). As MLK4β suppresses MAPK signalling in certain cellular contexts, we postulated that the reduction in MLK4β might contribute to elevation inMAPK signalling in response to these stress stimuli [4]. To examine the role of MLK4β in ERK, p38 and JNK activation in response to short-term exposure to heat and osmotic stresses, RNA interference-mediated knockdown of MLK4β was performed in SKOV3 cells that were subsequently exposed to heat or treated with sorbitol for 2 h or 4 h. Cell extracts were immunoblotted for activated forms of MLK3, ERK, JNK and p38. MLK4 siRNA knockdown cells treated with sorbitol had increased p-p38 and p-ERK at 0 h, 2 h and 4 h, and increased p-JNK at 2 h in comparison to control siRNA-transfected cells (Fig. 2A). In response to heat exposure for 2 h, MLK4 siRNA knockdown cells had enhanced JNK and p38 activation, but not ERK activation in comparison to cells transfected with control siRNA (Fig. 2B). These results indicate that MLK4 has a suppressive effect on JNK and p38

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activation in response to thermostress, and ERK, JNK and p38 activation in response to osmotic stress. Notably, phospho-MLK3 levels were increased in MLK4 siRNA knockdown cells that were untreated or exposed to osmotic or heat stresses, which supports previous findings that MLK4β suppresses MLK3 activation [4]. Interestingly, we observed that MLK3 is required for sorbitol, but not heat-induced activation of ERK, JNK and p-38 (Supplemental Figure 2).

3.3. MLK4β interacts with MLK3 and the interaction is regulated by osmotic stress

To investigate the effect of osmotic stress on MLK4β-dependent regulation of MLK3, the association between MLK3 and MLK4β was analyzed in the absence or presence of sorbitol treatment. A direct association between MLK3 and MLK4β was tested in an *in vitro* binding assay with recombinant human His-MLK3 and GST-MLK4β proteins expressed in *E. coli.*. His-MLK3, GST-MLK4β, and GST proteins purified from E.coli (Supplemental Figure 3) were combined, and His-MLK3 pull-downs were performed. GST-MLK4β was detected in the His-MLK3 pulldowns, which suggests a direct interaction between GST-MLK4β and His-MLK3 *in vitro* (Fig. 3A). To verify that these two proteins are associated in cells, endogenous MLK3 was immunoprecipitated from SKOV3 ovarian cancer cells exposed to osmotic stress over a 6 h time course, and endogenous MLK4β was detected in the MLK3 immunoprecipitates by immunoblotting. The association between between MLK3 and MLK4β was observed in the cells exposed to osmotic stress for 0, 0.3 and 1 h; after 1h the MLK3-MLK4β association was minimal (Fig. 3B).

3.4. Hsp90 inhibition reduces MLK4β levels in ovarian cancer cells

Hsp90 is associated with MLK3 in breast and ovarian cancer cells, and Hsp90 inhibition with GA triggers a decline in MLK3 protein level [16, 21]. We previously observed that in response to GA, MLK3 is ubiquitinated and undergoes proteasome-dependent degradation by a mechanism that requires the CHIP E3 ubiquitin ligase [21]. We speculated that MLK4β may be regulated by a similar CHIP-dependent process in SKOV3 cells. SKOV3 cells were treated with 10 μM GA for 4–12 h, and endogenous MLK4β protein levels were assessed. Hsp90 inhibition resulted in a decline in endogenous MLK4β and MLK3, and increased expression of Hsp70 (Fig. 4). Furthermore, reduction in endogenous MLK4β and MLK3 in cells treated with 50 μM of the translational inhibitor CHX was accelerated at 10 and 12 h when cells were treated with both CHX and GA, which suggests that MLK4β stability is dependent on Hsp90 (Fig. 4). In addition, MLK4β did not decline as rapidly as MLK3 in CHX-treated cells (Fig. 4). CHX treatment also caused a decrease in pERK and p-JNK (at 10 and 12 h) and a transient increase in p-p38 (compared to untreated cells); but did not affect total ERK, JNK and p38 protein levels (Supplemental Figure 4).

3.5. MLK4β binds to CHIP and Hsp70

MAPK proteins such as DLK1, ASK1 and MLK3 undergo proteasomal-dependent degradation by a mechanism that involves Hsp70 and the stress-regulated E3 ligase CHIP [21, 23–25]. The observed reduction in MLK4β protein in response to GA, heat and osmotic stresses led us to speculate that, like MLK3, MLK4β might be regulated by CHIP and Hsp70. To asses the interaction between MLK4β and Hsp70, GFP-Hsp70 was transiently expressed with GST-MLK4β in HEK293 cells. GST-MLK4β pulldowns were assessed by

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immunoblotting with GST and GFP antibodies. In cells expressing both GFP-Hsp70 and GST-MLK4β, GFP-Hsp70 was detected in the GST-MLK4β pulldowns (Fig 5A). To analyze the binding between MLK4β and CHIP, recombinant FLAG-CHIP and GST-MLK4β were combined in an *in vitro* binding assay and GST-MLK4β pulldowns were immunoblotted with FLAG and GST antibodies (Fig 5B). FLAG-CHIP was pulled down with GST-MLK4β only in samples that contained both proteins, which indicates that GST-MLK4β binds directly to FLAG-CHIP. These findings are consistent with the notion that MLK4β could be regulated by the chaperone-coupled ubiquitin proteasome system.

To determine if CHIP is important for the observed reduction in MLK4β protein in response to osmotic and heat stresses, HEK293 cells were transiently transfected with CHIP siRNA or non-specific siRNA and subjected to either heat or sorbitol treatments. Heat or sorbitol treatment resulted in a decline in MLK4β in cells transfected with non-target siRNA, but not in CHIP siRNA knockdown cells (Fig. 5C). These results suggest that CHIP is an important mediator of stress-stimulated degradation of MLK4β.

3.6. CHIP ubiquitinates MLK4β

To investigate the possibility that CHIP ubiquitinates MLK4β, an *in vitro* ubiquitination assay with purified UBE1, one of eight purified E2 enzymes, purified His-CHIP, ubiquitin, and GST-MLK4β substrate was performed. The eight different E2s are known to function with CHIP or known to participate in stress-related degradation of proteins [26]. MLK4β ubiquitinated in the samples that contained an E2 from the UBCH5 family (UBCH5a, b c, or d) (Fig. 6A, upper panel). CHIP autoubiquitination was also observed which verified CHIP function in the assay (Fig. 6A, lower panel). These results indicate that CHIP can function together with UBCH5 family E2s to ubiquitinate MLK4β. Next, we tested if CHIP or Hsp70 could mediate ubiquitination of MLK4β in cells. HEK293 cells were transiently transfected with pCMV vector, FLAG-CHIP, GFP-Hsp70, or both FLAG-CHIP and GFP-Hsp70. Immunoprecipitations were performed from cell lysates with ubiquitin antibody, and the immunoprecipitates were immunoblotted with MLK4β antibody. An induction of MLK4β ubiquitination was evident in the cells that overexpressed both FLAG-CHIP and GFP-Hsp70 in comparison to cells that expressed the empty vector, FLAG-CHIP or GFP-Hsp70 alone (Fig. 6B). Furthermore, in the cells with FLAG-CHIP and GFP-Hsp70 overexpression, total MLK4β protein level was substantially reduced, which is consistent with the hypothesis that CHIP- and Hsp70-dependent MLK4β ubiquitination facilitates its degradation (Fig. 6B).

4. Discussion

In this study, the regulation of MLK4β by stress stimuli and the CHIP E3 ligase was investigated. We observed a CHIP-dependent decline of MLK4β protein in response to prolonged treatments with GA, osmotic stress and thermostress. MLK4β binds to Hsp70 and CHIP, and CHIP ubiquitinates MLK4β *in vitro*. Therefore, in ovarian cancer cells exposed to these stresses, MLK4β protein levels are controlled by the chaperone-ubiquitin system.

In untreated cells, siRNA knockdown of MLK4β expression elevated the levels of p-JNK and p-p38, which indicates a suppressive function for MLK4β in JNK and p38 activation. These results are consistent with previous reports that MLK4 functions as a negative

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regulator of p38 and JNK signalling in HCT116 colon cancer cells, and negatively regulates Toll receptor signalling and LPS-induced ERK and JNK activation in murine macrophages [3, 4]. However, the results are contrary to findings that MLK4 is an activator of JNK in HCT15 colon cancer cells [14]. These studies employed different cell types that were cultured under different adherence conditions, and possibly the variation in these critical parameters influenced the effect of MLK4β expression on MAPK activation.

We previously observed that MLK4β suppresses the activation of MLK3 and tumor cell invasion [4]. We report here that MLK4β directly interacts with MLK3; and osmotic stress regulates this association in SKOV3 cells. Osmotic stress promoted a dissociation of MLK3 and MLK4β, enhanced MLK3 activation, and increased JNK and p38 activities, which we propose is due to loss of MLK4-dependent inhibition of MLK3, and is a critical component of the initial cellular response to osmotic stress (Fig. 7) [11, 27].

After prolonged exposure to osmotic or heat stress, MLK4β protein levels declined via a CHIP-dependent mechanism. Furthermore, functional Hsp90 is required for maintenance of both MLK3 and MLK4β protein levels. Other signalling proteins involved in growth control are also stabilized by Hsp90 such as Raf-1, mutant B-Raf, mutant p53, and pp60^{vsrc} [28–31]. Other important signalling proteins regulated by CHIP include tumor necrosis factor receptor-associated factor 2 (TRAF2), c-ErbB2, and dual leucine zipper kinase (DLK) [23, 32, 33]. Therefore, the CHIP-Hsp70 protein quality control pathway influences MAPK signalling through multiple protein targets.

5. Conclusions

These results indicate that endogenous MLK4β protein is regulated by Hsp90 inhibition, heat and osmotic stress in ovarian cancer cells. In untreated cells, the association between MLK4β and MLK3 limits MLK3 activity (Fig. 7). Exposure to osmotic stress for short time periods disrupts the MLK4β-MLK3 interaction and elevates the level of active MLK3 and MLK3-dependent MAPK signalling. Depending on the cellular context, elevated levels of active MLK3 and MAPK signalling could promote apoptosis or cellular transformation. These results also show that after prolonged exposure to osmotic stress, thermo stress, or Hsp90 inhibition, CHIP-dependent ubiquitination mediates a decline in MLK4β and MLK3 protein levels. This study provides insight into how specific stress stimuli regulate MLK4 and MLK3 protein levels and influence MAPK signalling in ovarian cancer cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

MLK3	Mixed Lineage Kinase 3

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MLK4	Mixed Lineage Kinase 4
MAPK	mitogen-activated protein kinase
MAP3K	MAPK kinase kinase
MAP2K	MAPK kinase
JNK	c-Jun N-terminal kinase
siRNA	short interfering RNA
ERK	extracellular signal-regulated kinase
HEK293	human embryonic kidney 293
DLK	dual leucine zipper-bearing kinase
ZAKs	zipper sterile α-motif kinases
CRIB	Cdc42-Rac interactive binding
Hsp90	heat-shock protein 90
Hsp70	heat shock protein 70
CHIP	carboxy-terminus of Hsc70-interacting protein
CHX	cycloheximide
GA	geldanamycin
GFP	green fluorescent protein

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Highlights

- Osmotic stress, heat, and Hsp90 inhibition reduce MLK4β and MLK3 protein levels
- CHIP ubiquitinates MLK4β
- CHIP mediates osmotic and heat stress-induced decline of MLK4β
- Osmotic stress regulates MLK4β-MLK3 association
- MLK4β negatively regulates MLK3 activity and MAPK signalling pathways

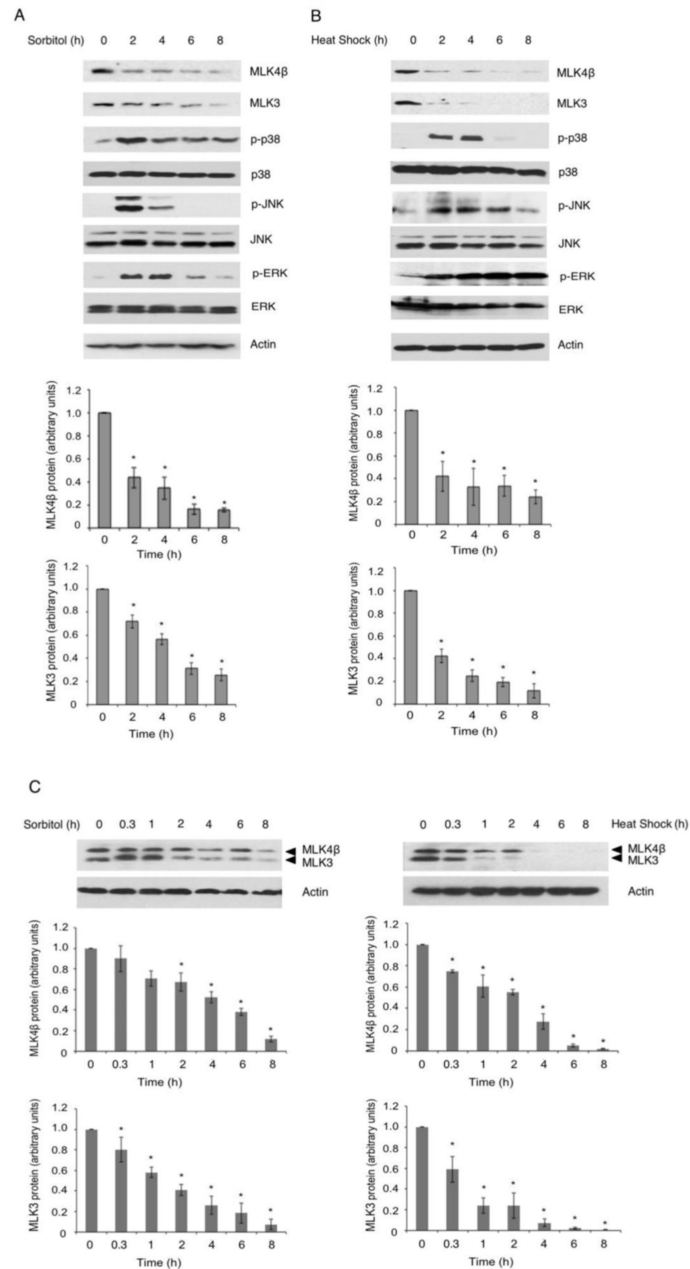

Fig. 1. Endogenous MLK4β declines in response to thermo and osmotic stresses in SKOV3 cells

SKOV3 cells were treated with (A) 250 mM sorbitol or (B) 42 °C heat shock for the 0, 2, 4, 6, and 8 h time points, and whole cell extracts were immunoblotted (upper panels) with the indicated antibodies. (C) SKOV3 cells were treated with (A) 250 mM sorbitol or (B) 42 °C heat shock for 0, 0.3, 1, 2, 4, 6, and 8 h time points, and whole cell extracts were immunoblotted with the indicated antibodies (upper panels). Quantitation of immunoblots (lower panels), N=3, * p-value < 0.05 compared to the untreated cells.

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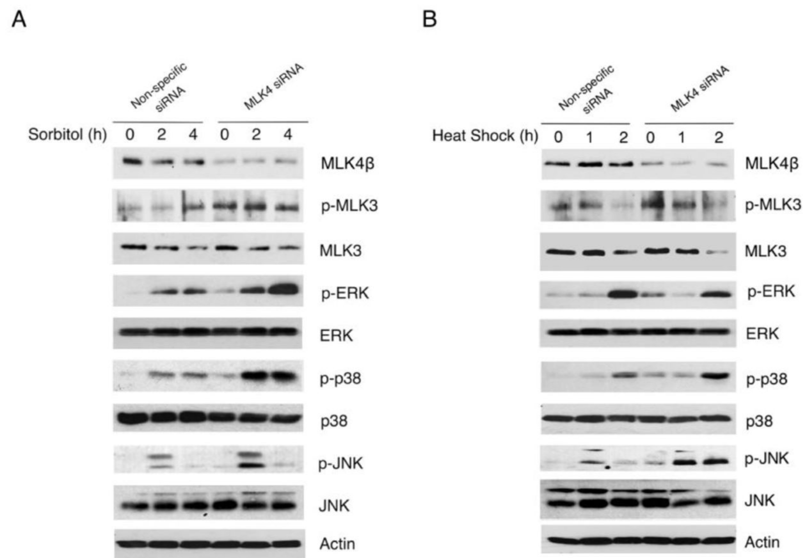

Fig. 2. MLK4β suppresses heat- and osmotic stress-induced MAPK signalling

SKOV3 cells were transfected with either MLK4 or non-specific siRNA and exposed to (A) 250 mM sorbitol for 0, 2, or 4 h, or (B) 42 °C for 0, 1, or 2 h. Whole cell lysates were immunoblotted with the indicated antibodies.

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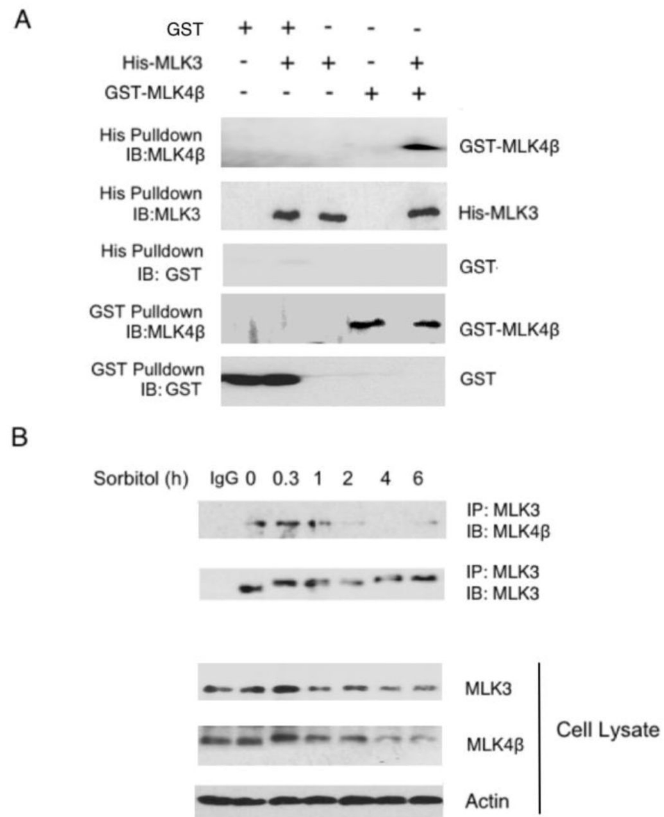

Fig. 3. MLK4β interacts with MLK3

(A) Recombinant His-MLK3, GST-MLK4β, and GST proteins were combined in a binding assay. His and GST pulldowns were performed and the samples were immunoblotted with MLK3, MLK4β, or GST antibodies. GST pulldowns were immunoblotted with MLK4β or GST antibodies. (B) SKOV3 cells were treated with 250 mM sorbitol and endogenous MLK3 was immunoprecipitated. The interaction between endogenous MLK3 and MLK4β was assessed at 0, 0.3, 1, 2, 4, and 6 h by immunoblotting with the indicated antibodies. Cell lysates were immunoblotted with the indicated antibodies.

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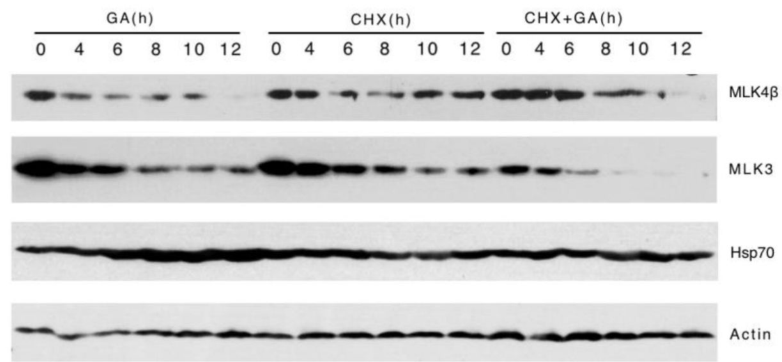

Fig. 4. GA reduces the level of endogenous MLK4β in SKOV3 cells

SKOV3 cells were treated with GA (10 μM), (50 μM) cycloheximide (CHX), or CHX (50 μM) and GA (10 μM) for 0, 4, 6, or 8, 10 or 12 h, and whole cell extracts were immunoblotted with antibodies to detect MLK4β, MLK3, Hsp70 and β-Actin.

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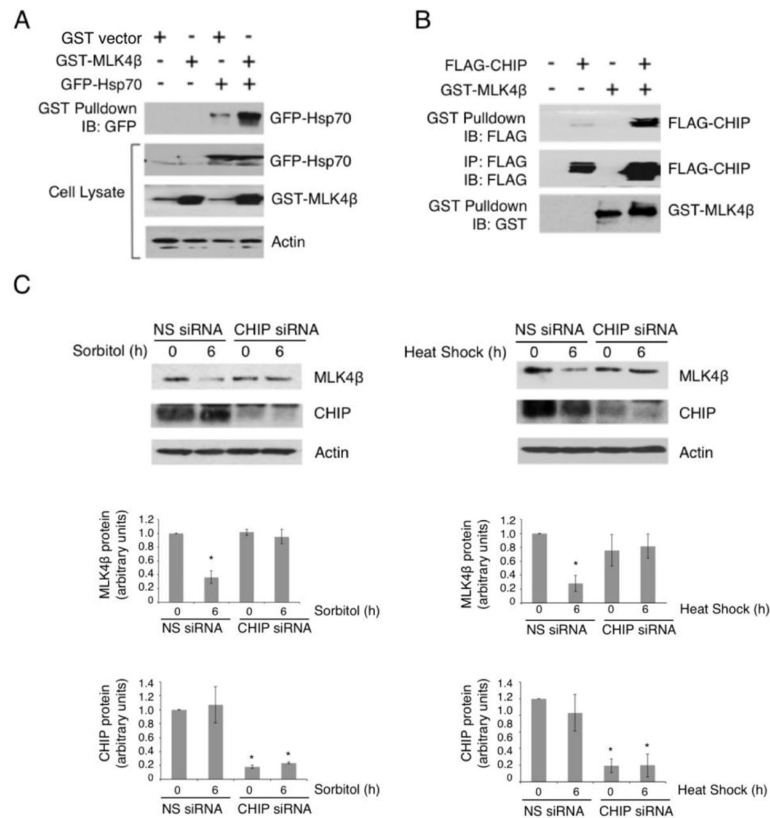

Fig. 5. MLK4β interacts with CHIP and Hsp70

(A) HEK293 cells were transfected with combinations of GST-MLK4β and GFP-Hsp70 plasmids as indicated. GST pulldowns were immunoblotted with anti-GFP antibody. Whole cell extracts were immunblotted with MLK4β, GFP and β-Actin antibodies. (B) Purified, recombinant FLAG-CHIP and GST-MLK4β proteins were combined in an *in vitro* binding assay. GST-MLK4β pulldowns and FLAG-CHIP immunoprecipitates were immunoblotted with GST and FLAG antibodies. (C) HEK293 cells were transfected with non-specific or CHIP siRNA and treated with 250 mM sorbitol or exposed to thermostress at 42 °C for 0 and 6 h. Cell extracts were immunoblotted with the indicated antibodies (upper panels). Quantitation of immunoblots, N=3, * p-value < 0.05 compared to the NS siRNA transfected, untreated cells (lower panels).

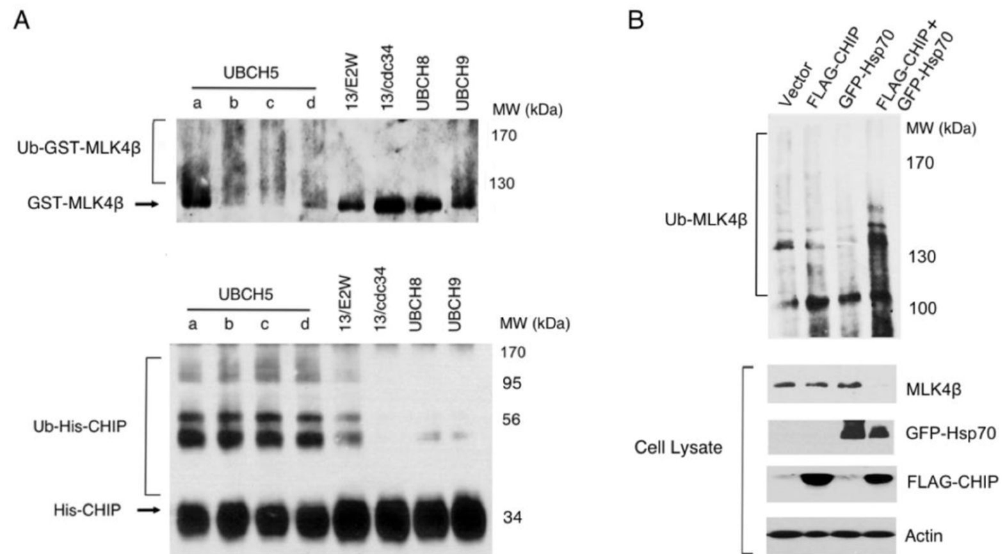

Fig. 6. CHIP ubiquitinates MLK4β

(A) *In vitro* ubiquitination reactions were performed with purified E1, 1 of 8 screened E2 enzymes, ubiquitin, FLAG-CHIP, and GST-MLK4β proteins. The reactions were carried out with the following E2 enzymes: UbcH5(a, b, c, d), Ubc13/Uev1a + Ube2W (13/2W in the figure), Ubc13/Uev1a + Cdc34 (13/Cdc34 in the figure), UbcH8, and UbcH9. The samples were immunoblotted with MLK4β (upper panel) and CHIP (lower panel) antibodies. (B) HEK293 cells were transiently transfected with pCMV vector, FLAG-CHIP or GFP-Hsp70. Immunoprecipitations were performed with ubiquitin antibody and the immunoprecipitates were immunoblotted with MLK4β antibody to detect ubiquitinated MLK4β. Cell lysates were probed with MLK4β, GFP, FLAG and β-Actin antibodies.

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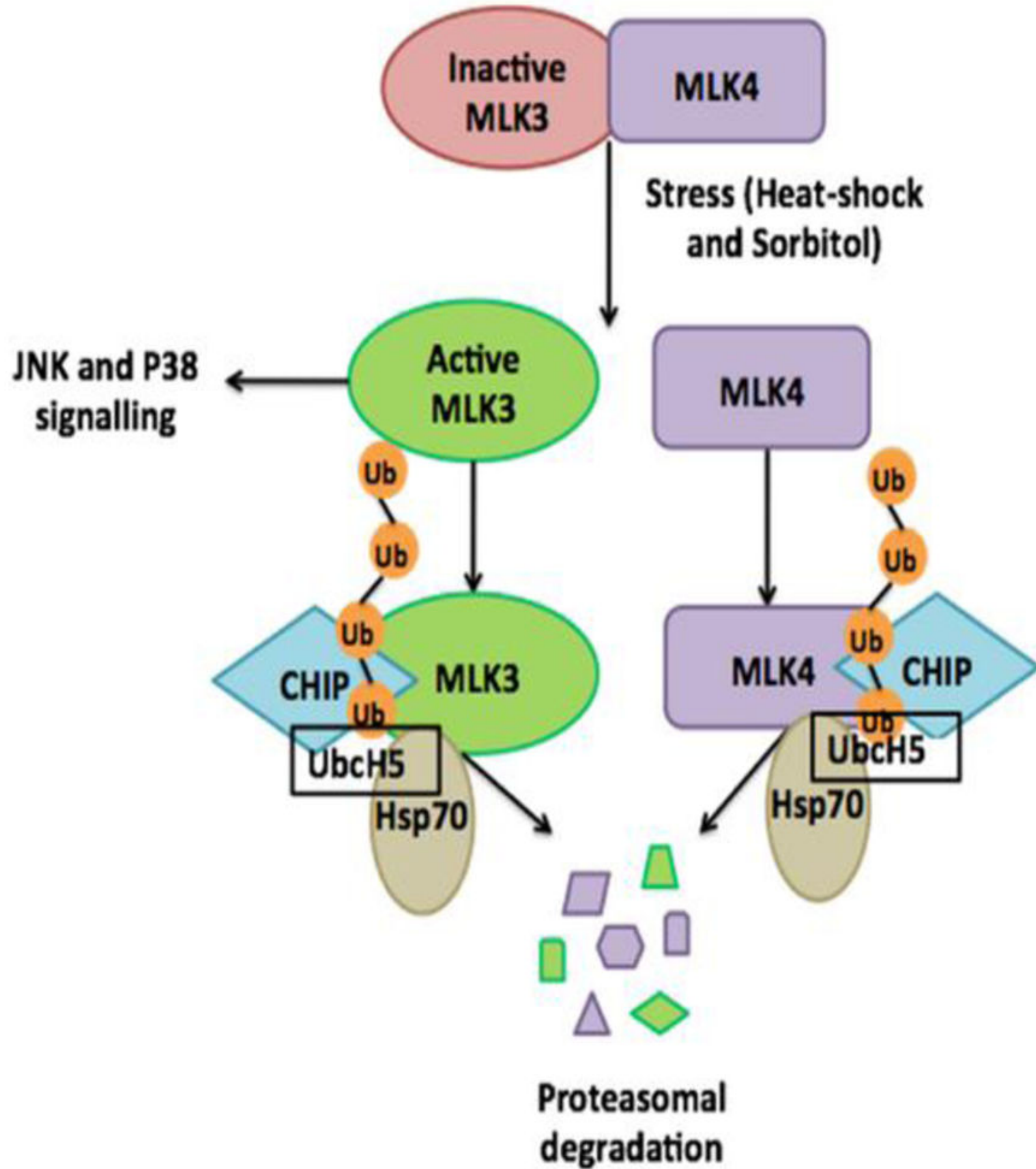

Fig. 7. Proposed model of MLK4β and MLK3 regulation by osmotic and heat stresses

Stress stimuli promote MLK3/MLK4β dissociation, which leads to an increase in MLK3, JNK and p38 activities in ovarian cancer cells. Prolonged stress exposure promotes CHIP and Hsp70-mediated MLK4β and MLK3 proteasomal degradation.